

REGULATION ON NITRIC OXIDE SYNTHESIS AND
SUPEROXIDE GENERATION BY
C-REACTIVE PROTEIN

CENTRE FOR NEWFOUNDLAND STUDIES

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Regulation of Nitric Oxide Synthesis and Superoxide Generation by C-Reactive Protein

Shobhitha Ratnam

**Thesis submitted to the School of Graduate Studies in partial fulfillment of the
requirements for the degree of Doctor of Philosophy**

**Department of Biochemistry
Memorial University of Newfoundland
St John's, Newfoundland
Canada**

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Abstract

Activated macrophages utilize both nitric oxide (NO) and superoxide ($O_2^{\cdot-}$) for defense against microbes. During periods of inflammation, cells of the immune system are activated by microbial products and/or cytokines, triggering the induction of immune/inflammatory nitric oxide synthase (iNOS) and the assembling of NADPH oxidase complex resulting in the generation of NO and $O_2^{\cdot-}$. However, simultaneous generation of NO and $O_2^{\cdot-}$ could be harmful to host cells since this may lead to the production potentially toxic molecules. Therefore, the regulation of the production of NO and $O_2^{\cdot-}$ is critical to host survival. Biological response to tissue injury or infection is characterized by an acute phase response that includes the amplified synthesis of C-reactive protein (CRP). Previous reports have indicated that CRP modulates $O_2^{\cdot-}$ generation. Therefore, this thesis has examined the role of CRP in the regulation of NO synthesis and $O_2^{\cdot-}$ generation in a macrophage model.

CRP stimulated NO synthesis in rat peritoneal macrophages and in the murine 264.7 cell line alone or in synergy with interferon- γ . The CRP-induced increase in NO production was due to an increased synthesis of the inducible isoform of iNOS preceded by an increase in steady state iNOS mRNA level.

CRP decreased the production of $O_2^{\cdot-}$ by stimulated macrophages, and activated phosphatidylcholine-directed phospholipase C (PC-PLC). Decreased production of $O_2^{\cdot-}$ corresponded with the activation of PC-PLC. In contrast, PC-

PLC activation was found to be necessary in CRP-mediated signaling leading to iNOS transcription. Increased PC hydrolysis may be one mechanism cells use to avoid simultaneous generation of NO and $O_2^{\cdot-}$.

CRP also activated extracellular signal-regulated kinase (ERK) signaling cascade and increased the phosphorylation of I- κ B. Studies with various cell-permeable inhibitors of PC hydrolysis and tyrosine kinases suggested that PC-PLC activation and tyrosine phosphorylation are essential for CRP-mediated phosphorylation of ERK and I- κ B. Furthermore, the induction of iNOS by CRP was shown to be at least partly mediated by the activation of ERK signaling pathway resulting in I- κ B phosphorylation and the ensuing iNOS transcription.

The specific role of CRP in host defense was examined by using *Chlamydia trachomatis*, an intracellular pathogen. CRP decreased the viability of *C. trachomatis*, and this effect was reversed by blocking NO production with NG-monomethyl L-arginine (NMMA), a competitive inhibitor of iNOS. This suggested that the CRP-induced increase in NO production during inflammatory periods plays an important role in the protection against microbial pathogens.

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Publications

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Ratnam S. and Mookerjee S. 1996. Induction of nitric oxide synthase by C-reactive protein. American Society of Biological Chemists. New Orleans. FASEB J 10, Abstr. 2301, p A1398.

Ratnam S. and Mookerjee S. 1995. Inhibition of respiratory burst and phosphatidylcholine hydrolysis by rat C-reactive protein. American Society of Biological Chemists. San Francisco. FASEB J 9, Abstr. 305. p. A 1308.

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List of Abbreviations

APP	Acute phase proteins
BME	β -mercaptoethanol
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cAMP	Cyclic Adenosine monophosphate
CPs	C polysaccharide
CRP	C-reactive protein
CVD	Cardiovascular diseases
D609	Tricyclodecan-9-yl-xanthogenate
DAG	1,2, Diacylglycerol
dBcAMP	Dibutyl cAMP
DPBS	Dulbecco's phosphate buffered saline
EGF	Epidermal growth factor
ERK1/ERK2	Extracellular signal regulated kinase1 and 2, p42 and p44 kDa MAPK
FCS	Fetal calf serum
fMLP	Formyl methionyl leucyl phenylalanine
γ -IRE	Gamma interferon regulatory factor
G-proteins	Guanine nucleotide binding proteins
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

HOG1	High osmolarity glycerol kinase, p38 kinase
HPLC	High pressure liquid chromatography
IFN- γ	Interferon gamma
I κ -B	Inhibitory kappa B
IL-1	Interleukin 1
IL-6	Interleukin 6
L-NAME	N ^ω nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEK	MAP kinase kinase, ERK kinase
MEKK	MAP kinase kinase kinase
NF- κ B	Nuclear factor kappa B
NMMA	L-N ^G -monomethyl L-arginine
NO	Nitric oxide
NOS	Nitric Oxide synthase
nNOS, ncNOS, NOSI	Neuronal nitric oxide synthase
eNOS, ecNOS, NOSIII ...	Endothelial nitric oxide synthase
iNOS, NOS II	Inducible nitric oxide synthase
O ₂ ⁻	Superoxide
PAGE	Polyacrylamide gel electrophoresis
PC-PLC	Phosphatidylcholine-specific phospholipase C
PC-PLD	Phosphatidylcholine-specific phospholipase D
PI-PLC	Phosphatidylinositol-specific phospholipase D
PKA	Protein kinase A
PKC	Protein kinase C

PLA ₂	Phospholipase A ₂
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PnC	Pneumococci
RHD	Rel homology domain
ROI	Reactive oxygen intermediates
RONI.....	Reactive oxynitrogen intermediates
SAPK.....	Stress-activated protein kinase
	c-Jun terminal kinase, JNK
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siCAM-1	Soluble intercellular adhesion molecule 1
STAT.....	Signal transducers and activators of transcription
TCF	Ternary complex factors
TLC	Thin layer chromatography
TNF- α	Tumor necrosis factor-alpha

List of Inhibitors Used

Inhibitor	Target Enzyme	Comments
D609	Phosphatidylcholine-specific Phospholipase-C	Competitive inhibitor of PC-PLC. Does not inhibit PI-PLC or PC-PLD. $K_i = 5 - 10 \mu\text{M}$
U-73122	Phosphatidylinositol-specific Phospholipase	Inhibits agonist induced PI-PLC activation in human neutrophils and platelets. $IC_{50} = 1-2.1 \mu\text{M}$
Staurosporin	Broad spectrum protein kinases	IC_{50} for PKC = 0.7 nM IC_{50} for PKA = 7 nM IC_{50} for PKG = 8.5 nM
Bisindolylmaleimide	Protein kinase C	Selective PKC inhibitor PKC, $K_i = 0.8 - 0.2 \text{ nM}$ PKA, $K_i = 2 \text{ nM}$
Genistein	Tyrosine kinase	Broad tyrosine kinase inhibitor IC_{50} for 2.5 - 25 μM IC_{50} for PKA, PKC = >100 μM
Tyrphostin AG 126	Tyrosine kinase	Inhibits p42 phosphorylation and iNOS activity in mouse peritoneal macrophages. $IC_{50} = >100 \mu\text{M}$
PD 98059	Extracellular signal-regulated kinase kinase (MEK, ERKK)	Non-competitive inhibitor of MEK. Binds to a site other than ATP binding site or an allosteric activator. $IC_{50} = 2 - 10 \mu\text{M}$
SB 202190	p38 kinase	Selective inhibitor of p38 kinase. $IC_{50} = 280 - 350 \text{ nM}$
Pyrrolidine dithiocarbamate (PDTTC)	NF- κ B complex	Inhibits NF- κ B activation and Nitric oxide synthesis

INTRODUCTION- A REVIEW

During inflammatory states, macrophages and other phagocytes are activated to produce various effector molecules that aid in the killing of invading organisms, removal of debris and restoration of homeostasis. Some of these responses include the generation of superoxide and nitric oxide. A variety of extracellular molecules, on ligation to receptors present on the surface of these cells, initiate pathways that mediate the production of these highly reactive molecules. C-reactive protein (CRP) is a prototypical acute phase protein whose synthesis increases dramatically during inflammation. This thesis has examined the interactions of this protein with macrophages with particular reference to its role in superoxide and nitric oxide synthesis. The aim of this review is to briefly introduce to the reader, the acute phase response with emphasis on the structure and properties of human and rat CRPs. In addition, this review includes some information on macrophage secretory products such as superoxide and nitric oxide. Some of the molecular signal transduction mechanisms involved in the activation of macrophages and relevant to CRP stimulation are also presented.

Section 1. Acute Phase Response

A. Introduction

Infection or trauma can cause significant damage to the integrity of living organisms. The tissue damage that results from such injury must be repaired in order to restore the homeostatic balance. This is generally achieved by triggering a local inflammatory response which involves many reactions directed at preventing and containing tissue damage, isolating and destroying infectious organisms and repairing and removing damaged tissue. This response to injury is manifested by inflammation and characterized by a series of reactions that includes both local and systemic responses (Kushner, 1981; Baumann and Gaudie, 1994). Local responses include platelet aggregation, blood clotting, vasodilation, proteinase activation as well as cytokine and chemokine production (Koj et al., 1991). These activate leukocytes/macrophages, fibroblasts and endothelial cells at the site of inflammation to produce more cytokines (Koj et al., 1993). Cytokines are soluble proteins that mediate communication among cells, both locally and systemically through binding to specific receptors. Their biological effects vary widely depending on the type of target tissue. In addition to the local responses, a general systemic reaction commonly known as the acute phase response is set in motion. This event is marked by an increase in hepatic synthesis of a number of plasma

proteins and is triggered by the injury itself as well as by cellular and biochemical components that participate in the inflammatory process such as cytokines. Cytokines such as Interleukin 1 (IL-1), Interleukin 6 (IL-6) and tumor necrosis factor (TNF- α) are known to regulate the synthesis of acute phase proteins following tissue injury, trauma, inflammation or sepsis (Baumann et al., 1994; Kushner et al. 1988). The main mediator of the acute phase reaction is IL-6, which in turn is regulated by IL-1. The acute phase protein patterns also vary from species to species. While some of the acute phase proteins are strongly induced, others are only increased marginally or are even repressed.

B. Acute Phase Proteins (APP)

Acute phase proteins (APP) or acute phase reactants (APR) is the generic name given to a group of approximately 30 different biochemically and functionally unrelated proteins (Koj, 1985). The levels of acute phase proteins in the serum are either increased (positive APPs) or reduced (negative APPs) after the onset of an inflammatory reaction. Acute phase proteins are synthesized predominantly in the liver (Kushner et al., 1981, Kushner, 1988., Heinrich et al, 1990). The various acute phase proteins differ markedly in their plasma levels. Acute phase proteins regulate immune responses, function as mediators and inhibitors of inflammatory processes, act as transport proteins for products generated during the inflammatory process and play an active role in tissue repair and remodeling (Raynes, 1994). Some of the acute phase proteins function as cytokines. CRP, for example, activates monocytes and macrophages to synthesize a variety of cytokines, while some other acute

phase proteins influence the chemotactic behavior of cells (Barna et al., 1993; Ballou et al., 1992; Tilg et al., 1993; Pue et al., 1996; Galve-de Rochemonteix et al., 1993; Cermak et al., 1993). Some acute phase proteins possess antiproteolytic activity and presumably block the migration of cells into the lumen of blood vessels thus helping to prevent the establishment of a generalized systemic inflammation (Raynes, 1994). A failure to control these processes, i. e., an uncontrolled acute phase reaction, may eventually result in severe pathological consequences.

The elevated serum concentrations of certain acute phase proteins are of diagnostic relevance and also of prognostic value. Their measurement, for example, allows inflammatory processes to be distinguished from functional disturbances with similar or identical clinical manifestations. In chronic disorders such as rheumatoid arthritis or malignant diseases a rise in the synthesis of acute phase proteins, parallels the degree and progression of the inflammatory processes (Hind et al., 1984).

C. Biosynthesis of Acute Phase Proteins

The liver is the major organ for the synthesis of APP. APP synthesis occurs in the hepatic parenchymal cells (Hurlimann et al., 1966). The periportal cells are the first to respond to an inflammatory stimulus but in severe inflammation all cells respond by synthesizing a plethora of APP (Mackiewicz et al., 1991; Raynes, 1994). Richie et al described and partially characterized a 23 to 30 kDa polypeptide from monocytes which they called hepatocyte stimulating

factor (Ritchie, 1983) . This factor was eventually found to be IL-6. In addition to IL-1 and IL-6, leukemia inhibitory factor also stimulates hepatoma cells and hepatocytes (Gauldie et al., 1987; Baumann and Schendel, 1991; Baumann and Wong, 1989). In addition, corticosteroids and insulin have been shown to influence the synthesis of APPs (Campos and Baumann, 1992). An overview of acute phase protein synthesis is shown in Fig.1

D. Pentraxins

Many of the acute phase proteins belong to a distinct family of proteins collectively known as the pentraxins. Members of this family of proteins include CRP, serum amyloid P component (SAP), and TNF-stimulated gene (TSG 14 or PTX3), the most recently discovered homologous molecule. Pentraxins are characterized by a distinct ultrastructural arrangement (Gewurz et al., 1995). Five or ten identical subunits are arranged in a pentameric radial symmetry. Pentraxins are found in horseshoe crab and in all vertebrate species studied so far. Although their conservation through evolution signifies an important role for these group of proteins, their major function is yet to be defined (Gewurz et al., 1995). The most well known of this group is CRP which can be classified as a pentraxin as well as an acute phase protein.

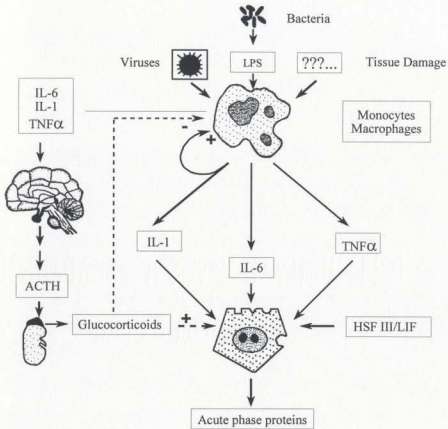


Fig. 1 Overview of hepatic acute phase protein synthesis. Adapted from Henrich et al., 1990

Section 2. C-Reactive Proteins

A. Human CRP

1. Historical Background

CRP, a prototypical acute phase protein in humans, was first detected by Tillet and Francis in the serum of patients infected with *Pneumococcus pneumoniae* (Tillet and Francis, 1930). This substance was shown to complex with the C polysaccharide derived from the pneumococcal cell wall in the presence of Ca^{++} (Abernathy and Avery, 1941) and was called C precipitin. C precipitin was found to occur in the serum of individuals suffering from a variety of infections and in conditions that resulted in trauma such as myocardial infarction and surgery. Abernathy and Avery found this factor to be a protein and this led to its designation as C-reactive protein. Avery based this on the property of forming a complex with C polysaccharide (CPS) in the presence of Ca^{++} ions (Abernathy and Avery, 1941). The significant residue in CPS that reacts with CRP was shown to be phosphocholine (Volanakis and Kaplan, 1971). This phosphocholine ligand binding property has been conserved through evolution for at least half a billion years, from the horseshoe crab (*Limulus polyphemus*) to the modern human (Kushner, 1988). *Limulus* CRP is presumed to play a role in defense mechanisms (Pistole and Graf, 1984). Although higher organisms have acquired sophisticated defense and repair mechanisms, descendants of

limulus CRP have survived and acquired the ability to interact with newer defense mechanisms in vertebrates. Variations in the blood concentration and response to inflammatory stimuli as well as physiologic behavior of CRP has changed substantially over time (Pistole and Graf, 1984). Nevertheless, CRP and proteins analogous to CRP continue to be expressed and have been demonstrated in many species (Anderson and McCarty, 1951; Baldo and Fletcher, 1973; Robey et al., 1983; Patterson, 1965). The stable conservation of structure and binding specificity through an extremely long evolutionary time of over 500 million years suggests that CRP structure/function may be of importance to the survival of the organism (Kilpatrick and Volanakis, 1991).

2. Biosynthesis of Human CRP

Like most other acute phase proteins, CRP is synthesized by hepatocytes. Cytokines and glucocorticoids are known to regulate CRP synthesis (Kushner, 1993). The single copy of the CRP gene located on the proximal arm of chromosome 1 (more precisely between bands q12 and q23) spans approximately 2.5 kbp of DNA and is located 7.7 kbp upstream of a CRP pseudogene (Floyd-Smith et al., 1986; Goldman et al., 1987). The gene consists of two exons separated by a single 278 bp long intron. The first exon encodes a putative signal peptide consisting of 18 amino acids and the first two amino acids of the mature protein. The remaining 204 amino acids and a 1.2 kbp 3' untranslated region are encoded by a second exon (Lei et al., 1985; Woo et al., 1985). Primer extension analysis indicated that the mRNA cap site is located 104 nucleotides upstream of the initiation codon. A promoter region

consisting of a TATA box and CAAT box located 29 and 81 nucleotides, respectively, upstream of the cap site has also been identified (Lei et al., 1985; Woo et al., 1985). Northern blotting showed a length of 2.2 kbp for CRP mRNA (Tucci et al., 1983).

Activation of CRP gene transcription is regulated by cytokines (Floyd-Smith et al., 1986). IL-6 has been shown to activate members of the C/EBP family of transcription factors in hepatoma cell lines (Rezzonicka et al., 1995). The binding of IL-6 to its receptor complex can lead to the activation of Janus kinases with subsequent phosphorylation, dimerization and translocation of signal transducers and activators of transcription 3 (STAT3) to the nucleus. STAT3 then binds to specific response elements in the promoter regions of cytokine responsive genes. The promoter regions of a number of human and rat acute phase genes contain TT(N)AA sequences capable of binding STAT proteins (Zhang et al., 1996). STAT1 is also activated by IL-6 and binds to a similar consensus motif called the gamma interferon activation site (GAS) (Hill and Treisman, 1995; Poli and Ciliberto, 1994). A STAT3 response element in the human CRP promoter with sequence TTCCCGAA is also shown to be necessary for optimal IL-6 induced transcription of CRP (Zhang et al., 1996).

3. Structure of Human CRP

Human CRP contains five noncovalently-associated identical subunits (Osmand, 1977). Sedimentation studies have indicated a molecular weight of 118,000 for the native pentameric CRP molecule (Volanakis et al., 1978). Each

of the five protomers consists of 206 amino acids with a calculated molecular weight of 23,017 and is arranged in a cyclic pentameric fashion (Lei et al., 1985; Oliveira et al., 1979). There are no potential glycosylation sites in the amino acid sequence and no carbohydrate is present in purified human CRP. Each monomer also contains an intrachain disulphide link. The tertiary fold consists of a core of 2 sheets of antiparallel β strands with a single long α helix folded on top of the β sheet. Two calcium ions are bound to each of the subunits. On the pentameric face opposite to the calcium there is a cleft which may be important in non-calcium-dependent interactions (Shrive et al., 1996).

4. Biological Actions of CRP

i. Binding to Phosphocholine and Activation of the Complement System

The first recognized binding specificity was to CPS of the cell wall of pneumococci (PnC). CPS is a ribitol teichoic acid to which phosphocholine is attached by ester linkage to galactosamine. The calcium-dependent binding of CRP was found to be largely dependent on the specific reactivity with phosphocholine groups (Volanakis, 1971). Equilibrium dialysis studies have shown that in the presence of Ca^{++} , human and rabbit CRP bind to 1 molecule of phosphocholine per subunit with an association constant of $1.6 \times 10^5 \text{ M}^{-1}$ and a valence of one per noncovalent subunit (Anderson et al., 1978; Rassouli et al., 1992). The binding specificity of human CRP to phosphocholine has an absolute requirement for both choline and phosphate moieties. Substitution of

phosphate with sulphate or sulphonates decreases or abolishes the binding to human CRP (Young and Williams, 1978). The biological importance of the phosphocholine-binding specificity of CRP may be very significant since this group is widely distributed in microbial and mammalian cell surfaces and products.

CRP was shown to activate the complement system when complexed with PnC and with phospholipids (Kaplan and Volanakis, 1974; Volanakis and Kaplan, 1974). It is believed that complement activation proceeded through the classical pathway. CRP complexed to polycations, positively charged liposomes, PC:LPC liposomes and nuclear DNA has been shown to activate the classical complement pathway (Siegel et al., 1975; Richards et al., 1979; Volanakis and Narkates, 1981; Robey, et al., 1985). It has also been reported that insoluble CRP-PnC precipitates can be solubilized by complement. C3 has also been shown to bind to CRP and PnC covalently during the solubilization reaction (Volanakis, 1982). It was shown by site-directed mutagenesis studies that negatively charged Asp 112 along with positively charged Lys 114 and Arg 116 of the Clq binding site of CRP played a major role in the binding of Clq to CRP (Agarwal and Volanakis, 1994). The three-dimensional structures of pentraxins indicates that these residues are involved in protomer-protomer interactions (Srinivasan et al., 1994). A conformational change of the native molecule may be necessary to expose the residues involved before complement activation can be initiated. The pentraxin-binding site on Clq has also been identified. They bind to distinct amino-terminal collagen-like regions of Clq (Jiang et al., 1991; Jiang et al., 1992). Activation of the complement

pathway initiated by CRP leads to the assembly of an effective C3-convertase and possibly results in the generation of host defense-related complement fragments such as anaphylatoxins C3a and C4a and opsonins C4b, C3b and iC3b (Kilpatrick and Volanakis, 1991).

Binding of CRP has been shown with several polycations including poly-L-lysine and poly-L-arginine polymers, lysine and arginine rich histones, myelin basic protein and leukocyte cationic proteins (DiCamelli 1980; Siegel, 1975). Calcium was inhibitory to the binding of CRP to polycations but this was facilitated in the presence of phosphocholine (Dicamelli et al., 1980; Pontempa et al., 1981). Interaction of CRP with phosphocholine and polycations has been shown to result in complement activation (Siegel et al., 1975; Volanakis et al., 1974; Osmand et al., 1975).

ii Binding to Ca^{++}

CRP binds to two Ca^{++} molecules with equal affinity (Gotschlich and Edelman, 1965). These two Ca^{++} binding sites regulate phosphocholine-binding activity of CRP and its biological activities (Mullenix and Mortensen, 1994). Ca^{++} binding is believed to mediate the binding to phosphocholine by inducing a conformational change in the CRP molecule (Young and Williams, 1978). The conformational changes initiated at the two Ca^{++} binding sites may be propagated to the adjoining regions which may allosterically change CRP to accommodate phosphocholine bearing substrates (Mullinix et al., 1994).

iii Binding to Sites of Injury

It has been proposed that the function of CRP relates to its ability to recognize foreign pathogens and to bind to damaged host cell membranes and initiate their elimination. CRP was also shown to bind to cells with altered or damaged membranes. Disturbance to normal membrane structure by the addition of LPC was required for the binding of CRP to PC liposomes. (Volanakis and Wirtz, 1979; Volanakis, and Narkates, 1981). CRP has also been found to bind to complement damaged cell membranes in a calcium and phosphocholine-dependent manner. The binding co-localized with the terminal complement attack complex, and was directed to membrane phospholipids. Disruption of the lipid bilayer by complement attack was required for CRP binding to occur to both liposomes and cells (Li et al., 1994). This is consistent with the hypothesis that CRP binding at sites of inflammation may be mediated by exposed phospholipids on damaged cell membranes.

iv. Binding to Fibronectin

Many other binding specificities have been described for CRP. It was shown that CRP bound to fibronectin in a phosphocholine inhibitable manner (Salonen et al., 1984). Both of these specificities have potentially important biological roles. A CRP molecule has been reported to bind to nine fibronectin molecules with a K_d of 1.47×10^{-7} M via phosphocholine-binding site (Tseng and Mortensen, 1988). Binding of CRP to fibronectin inhibits its attachment to normal rat kidney fibroblasts (Tseng and Mortensen, 1989). CRP was also shown to bind to

immobilized fibronectin in a dose and calcium-dependent manner (Mori et al., 1991). A peptide derived from CRP corresponding to 27-38 amino acids of the CRP peptide chain was found to have cell attachment properties (Fernandez et al., 1992). This cell-binding motif required for cell adhesion (FTVCL) was not found in any other proteins (Mullenix et al., 1994). This suggests that CRP may play a role in the formation of extracellular matrix needed for tissue repair.

v. Opsonic Properties of CRP

Initial evidence indicating that CRP may be an opsonin was provided by Löfström, who demonstrated that CRP induced agglutination and capsular swelling of certain types of *Streptococcus pneumoniae* (Löfström, 1943; Löfström, 1944.; Hokama et al., 1962). Ganrot and Kindmark demonstrated that CRP enhanced phagocytosis of a variety of Gram-positive and Gram-negative pathogens (Ganrot and Kindmark, 1969; Kindmark, 1971). Human monocytes ingested sheep blood cells when CRP and complement fragments were localized on the red cell membranes. CRP alone had very little effect, and red cells from which CRP has been removed by treatment with EDTA were found attached to the monocytes but were not ingested. Thus CRP was shown to provide additional signals necessary for phagocytosis to proceed (Mortensen et al., 1976). Opsonic properties of CRP were shown to depend on its ability to activate complement (Edwards et al., 1982). Some reports have shown that phagocytosis of CRP-opsonized sheep blood cells by neutrophils required prior stimulation by either phorbol esters or a less than 10-kDa product of stimulated mononuclear cells (Kilpatrick and Volanakis, 1985). This interaction was shown

to be independent of complement activation and was not mediated by Fc III receptors of neutrophils. CRP complexed to PnC was shown to cause a significant increase in degranulation of neutrophils as compared to PnC alone. This degranulation could be potentiated by treatment of neutrophils by small molecular weight products from mononuclear cells (Kilpatrick and Volanakis, 1985; Kilpatrick et al., 1987). Additional studies using a murine pneumococcal infection model have shown that CRP protects mice against fatal infection by type 3 and type 4 *S. pneumoniae* (Mold et al., 1981; Yother et al., 1982). Human and Rabbit CRP were also shown to increase the blood clearance of *S. pneumoniae* (Horowitz et al., 1987). Furthermore, transgenic mice expressing human CRP had extended life spans compared to their non-transgenic litter mates, when infected with *S. pneumoniae* (Szalai et al., 1995). CRP may provide protection from the development of fatal levels of PnC in the blood. Optimal activity requires a functioning complement system. The opsonic properties of CRP may thus be related to protection against microbial infection.

vi. Binding to Cells of the Immune System

The interactions of human CRP with cells of the immune system have been studied extensively (Kolb-bachofen, 1991). CRP has been known to bind specifically to mononuclear phagocytes and modulate their functions (Hokama et al., 1986; Zahedi et al., 1986; Barna et al., 1987; Buchta et al., 1987; Dobrinich and Spagnuolo, 1991). The nature of the effects appears to depend on the state of CRP conformation, such as native protein, aggregated form or in proteolytically cleaved form (Shields, 1993). CRP conformationally altered by

mild acidic conditions was shown to enhance neutrophil motility, phagocytosis and tumoricidal activity (Hokama et al., 1962; Ganrot and Kindmark, 1969; Miyazawa and Inoue, 1990; Shields, 1993; Barna et al., 1984). In contrast, native CRP has a negative effect on superoxide generation, chemotaxis, enzyme secretion and protein phosphorylation (Buchta et al., 1987, Buchta et al., 1988). Peptides generated from CRP by neutrophil proteases also modulate phagocytic functions of the mononuclear phagocytes (Robey et al., 1987; Buchta et al., 1986; Shephard et al., 1989). CRP has also been shown to activate the transcription of a variety of cytokines. A number of studies have shown that CRP induced higher levels of mRNA expression of IL-1, TNF- α , and IL-6 in monocytes and tissue macrophages (Ballou et al., 1992; Tilg et al., 1993; Pue et al., 1996, Galve-de Rochemonteix et al., 1993). In addition, CRP has been shown to induce the synthesis of tissue factor (TF) by monocytes (Cermak et al., 1993).

Modulation of neutrophil/monocyte/macrophage functions by CRP is believed to be mediated by the binding of CRP to specific receptors on these cells. Specific receptors have been shown to be present on neutrophils, monocytes macrophages and U937 cells (Dobrinich and Spagnuolo, 1991; Zeller, 1989; Zahedi et al., 1989; Tebo and Mortensen, 1990). CRP also binds to lymphocytes (Kuta and Baum, 1986). This binding was shown to be independent of the phosphocholine-binding site (Tebo and Mortensen, 1990). Although binding of CRP to Fc receptors has been suggested, the nature of this binding is not yet clear (Kilpatrick and Volanakis, 1991). A subpopulation of lymphocytes with natural killer activity may synthesize CRP and retain it on the surface (Kuta and

Baum, 1986). These data collectively show that CRP binds to phagocytic cells in a specific and reversible manner, and that upon binding a biological response is elicited.

5. C-Reactive Protein as a Marker for Cardiovascular Disease

Many athero-thrombotic events that mark cardiovascular diseases (CVD) occur among individuals without any apparent classical risk factors (Ross, 1993). Several lines of research indicate that there may be an association between chronic inflammation and the initiation and progression of atherosclerosis (Ross, 1993). Pathological studies have demonstrated that atherosclerotic lesions are heavily infiltrated with cellular components associated with inflammation. In addition to neutrophils, macrophages, pro-inflammatory cytokines and adhesion molecules, CRP has been shown to localize in atherosclerotic lesions (Kloner et al., 1991; Moreno et al., 1994; Poston et al., 1992; Cybulsky and Gimbrone, 1991; Reynolds and Vance, 1987; Hatanaka et al., 1995; Torzewski et al., 1998). CRP was also suggested to be involved in the immobilization and concentration of LDL within the arterial wall and was shown to participate in the Ca^{++} -dependent binding to, and aggregation of, LDL and VLDL (Pepys et al., 1985; DeBeer et al., 1982). In addition, there is also a growing body of evidence showing that CRP is an important risk factor for acute manifestations of coronary artery disease (Berk et al., 1990; Liuzzo et al., 1994; Haverkate et al., 1997).

While the role of CRP in inflammation is well documented, its role as a marker of cardiovascular diseases (CVD) is still emerging. Two studies using separate assays have established the prognostic value of CRP in patients with angina. The European Concerted Action On Thrombosis (ECAT) and Angina Pectoris Study Group have shown that on average, CRP levels were 33% higher in angina patients who went on to have an acute coronary event within two years (Haverkate et al., 1997; Thompson et al., 1995). It was also identified as an independent risk factor in apparently healthy middle aged men of Physicians Health Study (PH), at risk for first-ever myocardial infarction or stroke (Kuller et al., 1996; Ridker et al., 1998; 1998a; Tracy et al., 1997; Ridker, et al., 1997). Data from the PH is especially informative because this study evaluated a group of low risk men with no prior history of CVD and low rate of cigarette smoking. The data from PH indicate that initially healthy men with baseline levels of CRP in the highest quartile had a three-fold increase in the risk of developing future myocardial infarction and twice the risk of developing stroke compared with men in the lower quartile (Ridker et al., 1997). These risk estimates were stable over an 8-10 -year follow up period. This reported risk was independent of lipid-related and non-lipid-related cardiovascular risk factors including smoking status, total and HDL cholesterol, triglycerides, lipoprotein (a), and fibrinogen and was reduced by treatment with aspirin in direct proportion to the base-line CRP value. Moreover, high baseline levels of CRP and total cholesterol increased the risk of developing CVD to more than five fold, a risk estimate greater than the product of the risk associated with either CRP or cholesterol alone (Ridker et al., 1998). Furthermore, elevated baseline levels of CRP are also associated with a four-fold increase in developing severe peripheral

arterial disease independent of risk factors (Kuller et al., 1996, Ridker et al., 1998).

Elevated CRP levels are considered to be nonspecific but sensitive markers of the acute phase response to infectious agents, immunologic stimuli and tissue damage. Whether CRP has direct vascular effects or is merely a marker for inflammation remains uncertain. Other acute phase proteins such as serum amyloid A and fibrinogen also appear to be associated with vascular risk (Ridker et al., 1998b). Interestingly, plasma concentrations of soluble intercellular adhesion molecule 1 (sICAM-1), which are known to be elevated many years in advance of a first ever myocardial infarction, have been shown to correlate with the level of CRP (Ridker et al., 1998a; 1998b). Since cellular adhesion molecules such as ICAM-1 are critical in the adhesion of circulating leukocytes to the endothelial cell and subsequent transmigration, this may provide further evidence that cellular mediators of inflammation have a critical role in atherogenesis (Poston et al., 1992; Cybulsky and Gimbrone, 1991; Ridker et al., 1998). However, whether an elevated CRP level reflects the underlying endothelial dysfunction due to prevalent atherosclerosis, contributes to lipid peroxidation or is a marker for environmental and/or infectious agents remains to be clarified.

B. Rat CRP

1. Historical Background

In 1978, Mookerjea recognized a factor in rat serum that inhibited the heparin-lipoprotein precipitation reaction. This factor was identified as a protein and was found to exert its effect through binding to a phosphocholine ligand (Mookerjea, 1978). This protein was later characterized as a glycoprotein with an apparent molecular weight of 125000 Da. Due to its specific binding property to phosphocholine, this protein was named phosphocholine-binding protein (Nagpurkar and Mookerjea, 1981). Rat serum contains two distinct pentraxins, one binding to C-polysaccharide and the other to agarose. The former protein, designated as rat CRP (De Beer, et al., 1982b), was found to be identical to phosphocholine-binding protein isolated earlier from rat serum in 1981 by Nagpurkar and Mookerjea (Nagpurkar and Mookerjea, 1981). Eventually cDNA cloning and amino acid analysis revealed the close homology of this protein to other mammalian CRPs and the protein was renamed "rat CRP" (Rassouli et al., 1992). Rat CRP is distinct from many mammalian CRPs in that it is normally present in substantial quantities in rat serum (0.5-0.6 mg/ml) and increases 2 to 3-fold during inflammation and injury (Nagpurkar and Mookerjea, 1981; De Beer, et al., 1982b).

2. Structure

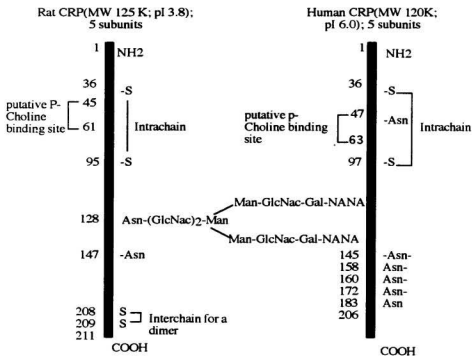
Rat CRP has been shown to have 71.7% identity with human CRP. Rat CRP is

a polypeptide of 211 amino acids and shares extensive homology with human, rabbit and mouse CRP. Rat CRP is longer than human CRP by 7 amino acid residues. Rat CRP consists of 5 identical monomers, of which two form a dimer linked by inter-chain disulphide bonds between Cys-208 and Cys-209. The extra heptapeptide contains two cys (residues 208 and 209) at the C terminus of the CRP. Cys 208 and Cys 209 form two interchain disulphide bonds between two monomers to create a dimeric component (Rassouli et al., 1992). Unlike human CRP, rat CRP is a glycoprotein. Rat CRP contains 18% carbohydrate, composed mainly of complex-type bi-antennary chains and possibly a small amount of tri-antennary chains. There are two potential sites for N-glycosylation, Asn-128 and Asn-147. One of these residues, Asn-128, is the site of attachment of complex oligosaccharide chains. These glycosylation sites are present on each of the subunits of rat CRP (Rassouli et al, 1992; Sambasivam et al, 1992). Rat CRP is more acidic than either human or rabbit CRP possibly due to the terminal N-acetyl-neuraminic acid in the covalently bound carbohydrate on this protein. A diagrammatic comparison of the primary structure of rat CRP and human CRP is shown in Fig. 2.

3. Biological Properties

i. Binding to Phosphocholine

While human and rabbit CRP binds to five molecules of phosphocholine, rat CRP binds only to three molecules of this ligand, but with an affinity similar to that of human CRP (Rassouli et al, 1992; Anderson et al., 1978). It is possible



Asn 128 & Asn 147 potential N-glycosylation sites (Asn-X-Ser) unique to rat CRP. Asn 128 is glycosylated with a complex type bi-antennary structure.

No potential glycosylation sites.

Fig. 2. Comparison of primary structure of rat CRP with human CRP.
Figure constructed based on results of Rassouli et al. (1992), Sambasivam et al. (1992) for rat CRP and Lei et al. (1985) for human CRP.

that the three non-covalently held subunits are responsible for the binding to phosphocholine.

ii. Binding to Lipoproteins

The physiological relevance of the binding of rat CRP to apo B- and apo E-containing lipoproteins was explored by Saxena et al., by using membranes from estradiol-treated rats. Rat CRP was shown to bind to LDL forming a CRP-LDL complex. CRP did not bind to the LDL receptor but binding of LDL to its receptor was inhibited by the formation of rat CRP-LDL complexes (Saxena et al., 1986). Rat CRP also binds to multilamellar liposomes in a Ca^{++} -dependent manner. This binding required the incorporation of 25% LPC into the liposomes and was inhibited by phosphocholine showing that the binding is specific towards the phosphocholine head group (Nagpurkar et al., 1983). Rat CRP immobilized to sepharose selectively bound to apo B and apo E containing lipoproteins (Saxena et al. 1987). Rat CRP was also shown to bind to synthetic lipid emulsions consisting of egg lecithin with triacylglycerol or trioleoylglycerol in the presence of divalent cations. These findings are similar to the data obtained with human CRP (De Beer et al. 1982; Rowe et al., 1984). Complexing of CRP to LDL is suggested to result in charge modification of LDL and increased degradation of this molecule by macrophages through the scavenger receptor (Mookerjee et al., 1994).

iii. Binding to Macrophages

Rat CRP also bound to macrophages and was internalized and degraded into small peptides by these cells (Nagpurkar et al., 1993). This property was independent of phosphocholine-binding and was found not to utilize Fc receptors, suggesting the presence of specific receptors for CRP on macrophages (Nagpurkar et al., 1993). Whether the degraded peptides from rat CRP have any biological role in modulating macrophage function is not yet known.

iv. Binding to Platelets

Rat CRP was also shown to bind specifically to rat platelets in a Ca^{++} - and phosphocholine-dependent manner and to prevent ADP and platelet activating factor (PAF)-induced platelet aggregation (Nagpurkar et al., 1988; Randell et al., 1990). It was suggested that both rabbit and rat CRP may bind to the same receptor sites on platelets (Randell et al., 1990).

Section 3. Secretory Products of Macrophages

Mononuclear phagocytes constitute a major host regulatory effector system of cells which populate all tissues of the body, and are not only phagocytic but are also avid secretory cells, and possess potent destructive potential against microbes and neoplastic cells. They are active phagocytic cells, and serve as antigen-presenting cells for T-lymphocytes. On activation, profound changes occur in their morphology such as an increase in size, and appearance of numerous complex surface folds, large number of vacuoles, lysosomes, phagosomes and endoplasmic reticular elements. They become more sensitive (primed) to agents that trigger the production of oxygen radicals and secrete a wide range of products including enzymes, coagulation factors, proteins of the complement system, bioactive lipids, adhesion molecules, superoxide ($O_2^{\cdot-}$) and nitric oxide (NO) (Papadimitriou et al., 1989). Macrophage activation is mediated by microbial pathogens and their products as well as lipid mediators and cytokines which include lipopolysaccharide (LPS) phorbol myristate acetate (PMA) and interferon- γ (IFN- γ). The secretion of NO and $O_2^{\cdot-}$, both of which are associated with macrophage cytotoxicity, are potentially toxic to the host and the regulation of the production of these molecules is critical to host survival (Bastian and Hibbs, 1994). Since the scope of this thesis is limited to NO and $O_2^{\cdot-}$, this review deals only with these two macrophage secretory products.

A. Nitric Oxide (NO)

Nitric oxide (NO), more than any biological messenger discovered so far, has become an example for the way a biological molecule can regulate numerous critical cell functions. To understand NO and its diverse functions, it is necessary to understand the physical characteristics of this molecule. The enzymatic combination of a single atom of oxygen with a single atom of nitrogen forms the smallest product of mammalian biosynthesis (Billiar., 1995). It is a molecule with an unpaired electron, which makes it highly reactive. Target molecules include oxygen, other radicals, thiol groups and metals. This highly reactive molecule has a very short half life, typically in the range of a few seconds. NO interacts with oxygen, producing nitrite and nitrate (NO_2^- and NO_3^-), inactivating the molecule. The combination of NO with superoxide forms peroxynitrate, nitrogen dioxide or hydroxyl radicals (Stamler et al., 1992), which can be potentially harmful to target cells. NO also interacts with prosthetic iron groups and thiol groups forming complexes that may activate or inactivate target enzymes. For example, heme-dependent activation of guanylate cyclase results in increased vasorelaxation and neurotransmission. A number of enzymes are also inhibited by NO (Feldman., et al; 1993, Nathan, 1992). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is inhibited by iron nitrosylation of an active site thiol group (Molina et al., 1992) whereas, aconitase, NADH: ubiquinone oxidoreductase and succinate: ubiquinone oxidoreductase of the electron transport chain are inhibited due to attacks on the iron of the iron sulfur clusters essential for the function of these enzymes (Drapier et al., 1986). The inhibition

of these and other enzymes by NO results in cytostasis and cytolysis of invading microorganisms and tumour cells (Hibbs et al., 1987; Stuehr and Nathan, 1989). Unlike many secretory products, NO is uniquely qualified to traverse the cell membrane due to its lipophilic nature. Since NO is rapidly consumed and inactivated within the cell or when released from the cell, or in circulation when it interacts with the heme group of hemoglobin, its activity was thought to be confined to the local environment. Stamler et al., have now shown that NO may form stable adducts with albumin (Stamler et al., 1992) or glutathione in the form of stable nitrosothiols which may circulate or diffuse to remote sites and release bioactive NO (Billiar, 1995). It has been suggested that high levels of GAPDH found in platelets may serve as an intracellular storage and transport system for NO (McDonald et al., 1993). If large amounts of NO can indeed be stored and transported to remote sites as stable adducts, NO can possibly exert hormone-like properties.

1. Nitric Oxide Synthase Isozymes

NO can be generated by almost all nucleated cells by three distinct isoforms of nitric oxide synthase. NO synthases (NOS; L-arginine, NADPH: oxidoreductases; EC 1.14.13.39) constitute a family of at least three distinct isoforms which include low output neuronal (nNOS, ncNOS NOSI), endothelial isoforms (eNOS, ecNOS, NOSIII) and an inducible high output isoform (iNOS, NOSII) (Moncada and Palmer, 1991; Wang and Marsden, 1995). Both nNOS and eNOS are constitutively expressed, whereas expression of iNOS requires induction by cytokines and bacterial products such as lipopolysaccharide. All

NOS isoenzymes have been purified, characterized and cloned.

i. NOS I (bNOS, ncNOS)

The NOS I isoform was first identified in brain cells of various species (Bredt et al., 1990; Mayer, 1990). Although NOS I is referred to as the neuronal enzyme, it has been found in other tissues, notably skeletal muscle, pancreatic islet cells, kidney macula densa cells and certain epithelial cells (Bredt, et al., 1994). NOS I activity in the brain is associated with synaptic plasticity while in the peripheral nervous system, NOS I activity is associated with smooth muscle relaxation (Kerwin et al., 1995). NO has also been shown to be a neurotransmitter in a class of peripheral autonomic neurons referred to as non-adrenergic, non-cholinergic nerves (Gillespie et al., 1989; Ramgopal et al., 1989). Deficiency in ncNOS activity has also been associated with pyloric stenosis (Vanderwinden et al., 1992). Mice lacking ncNOS also exhibited inappropriate sexual and aggressive behavior (Nelson, et al., 1995). The human ncNOS gene is assigned to the 12q24.2 region of chromosome 12 (Kishimoto et al., 1993, Xu et al., 1992). Although ncNOS is considered to be constitutively expressed, the NOS gene may also be transcriptionally regulated. The 5' flanking regions of the human neuronal ncNOS exon 1 contain potential binding sites for transcription factors such as AP-2, TEF-1/MCBF, CREB/ATF, c-fos, NRF-1, NF-1 and NF- κ B like sequences (Hall et al., 1994). A major start site 28 nucleotides downstream from a TATA box has also been shown.

ii. NOS III (ecNOS)

The human ecNOS gene was assigned to 7q35-7q36 region of chromosome 7 (Marsden et al., 1993). This gene contains 26 exons and 25 introns spanning approximately 21 kb of human genomic DNA. It encodes an mRNA of 4052 nucleotides and is present as a single copy in the haploid human genome (Marsden et al., 1993). Translation initiation and termination sites are in exon 1 and exon 26, respectively, leaving a 5'-untranslated region of 22 nucleotides. The full-length open reading frame is 3609 base pairs encoding a protein of 1203 amino acids. Analysis of the 5'-flanking regions indicates that the ecNOS promoter is devoid of a TATA box. The 5'-flanking regions also contain putative AP-1, AP-2, NF-1, heavy metal, acute-phase response, sheer stress and sterol regulatory cis-acting DNA elements (Marsden et al., 1993; Robinson et al., 1994). NO produced by the constitutive NOS III or ecNOS is involved in regulation of blood pressure, organ blood flow distribution, inhibition of adhesion and activation of platelets. Endothelium-derived NO has now been shown to be identical to endothelium-derived relaxation factor (EDRF) (Furchgott et al., 1980; Palmer et al., 1987; Ignarro et al., 1987). It is now known that endothelium-derived NO is synthesized by ecNOS. This enzyme is thought to exist bound to plasma and microsomal membrane by a myristoylation site on the enzyme. ecNOS is myristoylated and palmitoylated, which explains its membrane localization (Busconi and Michel, 1993). Phosphorylation of the enzyme results in its detachment from the membrane and translocation to the cytosol, and phosphorylation of this enzyme has been proposed to regulate its activity (Michel et al., 1993). Physical factors such as sheer stress increase the

gene expression of this enzyme. The promoter region of the eNOS contains a sheer stress response element, suggesting that increased gene expression and greater capacity for vasodilation may follow increased sheer stress (Marsden et al., 1993). Besides its role in vasodilation, NO prevents platelet adherence and aggregation (Radomski et al., 1987), neutrophil aggregation (Kubes et al., 1991) and regulates endothelial permeability changes (Kubes et al., 1992). Impaired NO biosynthesis by the endothelium leads to adverse consequences. NO synthesis has been shown to be impaired in spontaneously hypertensive rats and in humans with essential hypertension (Panza et al., 1990; Durante et al., 1988). NO also inhibits smooth cell proliferation. Impairment of platelet and smooth-muscle proliferation may contribute to progressive vascular dysfunction and injury seen in diseases such as diabetes mellitus and atherosclerosis. Advanced glycosylation products prominent in vessel walls in diabetes, and oxidized low-density lipoproteins which are elevated in atherosclerosis, have been shown to reduce either the availability or production of NO (Bucala et al., 1991; Chin et al., 1992). It is likely that NO plays an important role in vascular function since alterations in NO synthesis contributes to vascular disease or be a consequence of vascular pathological process (Billiar, 1995).

iii. NOS II (iNOS)

NOS II (iNOS) is induced by various inflammatory stimuli, and unique among nitric oxide synthases in that its expression and activity is independent of intracellular Ca^{++} levels. Binding affinity to calmodulin by iNOS is sufficiently great at low levels of Ca^{++} that its activity is not regulated by Ca^{++} fluxing

(Stuehr et al., 1991; Cho et al., 1992). iNOS can be induced in almost all nucleated cells. These include macrophages, lymphocytes, hepatocytes, chondrocytes, glia and neurons, tumor cells, pancreatic islets, vascular smooth muscle cells, mesangial cells, and renal tubular epithelium (Wang and Marsden, 1995). This isoform is expressed in response to pro-inflammatory agents such as LPS, IL-1 β , TNF- α , and IFN- γ . A distinct feature of nearly all cell types that express iNOS is the strong synergy exerted by multiple simultaneous agents (Billiar., 1995). The human iNOS gene is localized to 17q11.2-q12 region of chromosome 17 (Marsden et al., 1994; Chartrain et al., 1994). The human gene consists of 26 exons and 25 introns spanning approximately 37 kb of human genomic DNA. Translation initiation and termination sites are in exon 2 and exon 26, respectively, leaving a 5'-untranslated region of 22 nucleotides. The full-length open reading frame is 3459 base pairs encoding a protein of 1153 amino acids. A transcription initiation site 30 nucleotides downstream of a TATA box has been demonstrated in genomic DNA from hepatocytes stimulated with IFN- γ and LPS. Induction of iNOS in murine macrophages has been shown to include transcriptional activation through binding of specific transcriptional factors to the 5' flanking region of the gene (Xie et al., 1993; Lowenstein et al., 1993). Analyses of the cloned iNOS promoter have revealed the presence of numerous consensus sequences for the binding of transcription factors, namely nuclear factor kappa B (NF- κ B), gamma interferon regulatory factor (γ -IRE), NF-IL6, a palindromic TNF-RE like-site, and a liver-specific factor consensus sequence (Chartrain et al., 1994). Of these potentially relevant transcription factors, NF- κ B and IRE have been shown to be functionally important to iNOS induction (Martin et al., 1994, Xie et al., 1994). A proximal

region of the promoter (-85/-76) interacts with the NF- κ B trans activating factor. This DNA binding protein resides in the cytosol of quiescent cells in association with an inhibitory complex I- κ B. When cells are activated I- κ B dissociates from the complex and the DNA-binding complex translocates to the nucleus where it binds to the *cis*-regulatory regions of the iNOS promoter (Xie et al., 1994, Lowenstein et al., 1993). p50/c-rel and p50/Rel A heterodimers represent at least a component of the *trans*-acting NF- κ B complex that participates in transcriptional regulation (Xie et al., 1994). Activation through different second messenger pathways have been shown to converge on the activation transcription factor NF- κ B (Kleinert et al., 1996). Analysis of the promoter-reporter constructs have shown that the more distal regions (-901/-913) are involved in IFN- γ stimulated changes in iNOS mRNA expression (Xie et al., 1994; Lowenstein et al., 1993; Goldring et al., 1996). The importance of IRF elements has been shown in reporter assays using deletion constructs, and in mice which have a targeted disruption of the IRF-1 gene (Martin et al., 1994; Kamijo et al., 1994).

Although transcriptional activation of iNOS is well characterized, little is known about the intracellular signaling pathway leading to iNOS induction. Modulation by protein kinase A (PKA), protein kinase C (PKC) or tyrosine kinase signaling pathways have been described. LPS, TNF- α , IL-1- β and IFN- γ are presumed to bind to their respective receptors and initiate the activation of a pathway that includes the activation of NF- κ B complex and subsequent stimulation of iNOS transcription (Xie et al, 1994, Kleinert et al., 1996). However, the signaling pathway involved was not only dependent on the

agonist but also on the cell type. For example, in rat mesangial cells and rat peritoneal macrophages, cyclic AMP (cAMP) elevating agents have been shown to stimulate iNOS expression (Kunz et al., 1994; Alonso et al., 1995), while in rat primary astrocytes and RAW 264.7 cells, an increase in intracellular cAMP results in a decrease in NO synthesis (Greten et al., 1995; Pahan et al., 1997). In 3T3 fibroblasts, the protein kinase A (PKA) pathway, the protein kinase C (PKC) pathway and the receptor tyrosine kinase pathway were all able to activate iNOS synthesis, but in murine macrophage RAW 264.7 cells neither PKA nor PKC pathways are able to stimulate NF- κ B activation and iNOS induction (Vincenti et al., 1992). However, upregulation of PKC- ϵ has been shown to promote the expression of iNOS in the same cell line (Diaz-Guerra et al., 1996; Paul et al., 1995). In J774 cells activators of PKA-stimulated iNOS synthesis whereas, activators of PKC were without effect (Muroi et al., 1993). Activation of tyrosine kinases, and more specifically p42/p44 mitogen-activated protein kinases (ERK1/ERK2), have been correlated to the activation of iNOS in LPS stimulated murine peritoneal macrophages as well as in cytokine treated cardiac myocytes and microvascular endothelial cells (Novogrodsky et al., 1994; Singh et al., 1996). Moreover, nerve growth factor which stimulates ERKs in PC12 cells, activates several isoforms of NOS including iNOS in this cell line (Traverse et al., 1992; Poluha et al., 1997). Involvement of p38 kinase in the regulation of iNOS synthesis in mouse mesangial cells and mouse primary astrocytes is also described (Da Silva et al., 1997; Guan et al., 1997). It seems that individual cell type and inducing agents may dictate the second messenger pathway from the cell surface to the nucleus leading to iNOS synthesis.

2. Structure of NOS Enzymes

NOS enzymes can be characterized as cytochrome P450-like heme proteins (Bredt et al., 1991; White and Marletta., 1992). They can be broadly divided into a reductase domain at the COOH terminus and an oxidative domain at the NH₂ terminus with the central portion of the protein containing a consensus sequence for Ca⁺⁺/calmodulin binding. The C-terminal or reductase domain shares homology with mammalian cytochrome P450 reductase and binds to NADPH, FAD and FMN (Bredt et al., 1991). There is a highly conserved 320 amino acid region in the oxygenase (NH₂) domain in all NOS enzymes that may represent binding sites for (6R)-5,6,7,8-tetrahydrobiopterin (BH₄), heme and Arginine (Kerwin et al., 1995). Generally, ncNOS and ecNOS exist as non-interactive monomers in unstimulated cells. On binding to heme, BH₄, and L-arginine, a conformational change is presumed to take place (Nathan and Xie, 1994) and this permits dimerization of the enzyme (Baek et al., 1993). A head to tail orientation is envisioned whereby the oxygenase domain approximates the reductase domain. This would permit interdomain contact without violating any constraints, and this also may imply that the molecule is elongated (Schmidt et al., 1991). In contrast, the basic hydrophobic domain in iNOS monomers can bind calmodulin even at trace levels of Ca⁺⁺ in the cytoplasm of resting cells (Cho et al., 1992). Since these positive allosteric regulators are plentiful in cells transcribing iNOS, neither monomers of iNOS nor calmodulin-deficient iNOS dimers are likely to accumulate under normal circumstances. Structural domains and binding specificities of NOS isozymes are represented schematically in Fig. 3. Calmodulin is thought to bind to iNOS tightly enough to

ensure sustained enzymatic activity even in the absence of intracellular Ca^{++} . This property ensures that iNOS is tonically active in the presence of NADPH, oxygen and L-arginine (Abu-Soud and Stuehr., 1993). Thus the major control for ncNOS and ecNOS is Ca^{++} levels in the cytosol whereas the major controlling event for iNOS is the rate of iNOS mRNA transcription.

3. Mechanism of NOS Mediated NO Biosynthesis

All three NOS isoenzymes catalyze a five-electron oxidation of L-arginine (Arg) to L-citrulline and NO, using NADPH as electron source and BH_4 , FAD, FMN, and iron protoporphyrin IX (heme) as co-factors, for monomer assembly, or catalytic activity or both (Baek et al., 1993). Using murine iNOS and [^{15}N]guanidino-labeled Arg, it was shown that the enzyme oxidizes one of the two equivalent guanidino nitrogens (Iyengar et al., 1987). The first step in the reaction involves the synthesis of an intermediate, hydroxy L-arginine (Stuehr et al., 1991a). Oxidation of Arg to NOHArg was shown to require one NADPH, and the conversion of NOHArg to citrulline and NO to require an additional 0.5 NADPH. It was also shown that hydroxylated nitrogen of NOHArg is the same nitrogen that comprises part of the product, NO (Leone et al., 1991). NOHArg is very electrophilic and is readily converted to citrulline (Stuehr et al., 1991a). The overall reactions involved in the synthesis of NO from arginine is represented in Fig. 4.

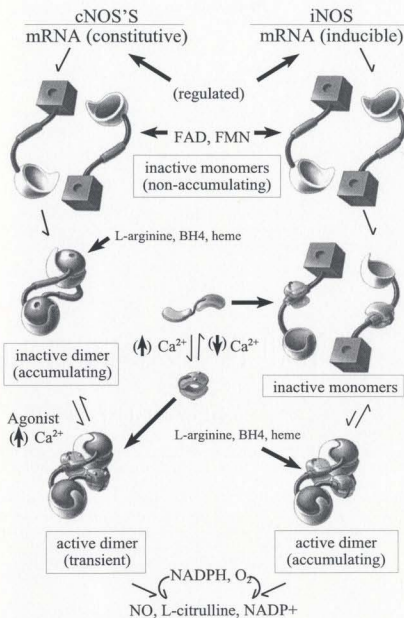


Fig. 3. **Essential differences between constitutive and inducible NOS isoforms.** Schematic representation of the binding of cofactors and sequence of events leading to the formation of active homodimers of NOS isoforms. Adapted from Nathan and Xie, 1994.

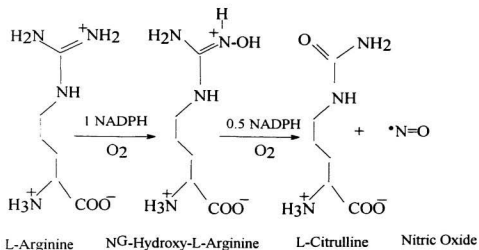


Fig. 4. **NO synthase catalyzed oxidation of L-arginine.** NO synthases generate NO by catalysing NADPH-dependent 5-electron oxidation of the guanidino group of L-arginine. L-arginine is N^ω-hydroxylated to N^ω-hydroxy-L-arginine, first. The intermediate remains bound to the enzyme and is oxidatively cleaved to L-citrulline and NO. The overall reaction involves reduction of two oxygen molecules requiring a total of eight electrons. Five reducing equivalents are derived from guanidino nitrogen oxidation and oxidation of NADPH provides three additional electrons. (Adapted from Feldman et al., 1993; Marletta, 1993; Mayer, 1995)

4. Regulation of NO Synthesis.

i. Calcium-Calmodulin

The enzymatic activity of nNOS and eNOS depends upon the level of intracellular Ca^{++} , which promotes the binding of calmodulin to the enzymes. Binding of calmodulin to an exposed basic hydrophobic site is presumed to bring about the conformational change. Calmodulin binding serves as a switch which allows the flow of electrons to the heme group of the enzyme (Abu-Soud and Stuehr, 1993). Calmodulin binding is also required for iNOS enzymatic activity, although elevated intracellular Ca^{++} levels are not necessary. Calmodulin is thought to bind to iNOS tightly enough to sustain enzymatic activity at resting Ca^{++} levels.

ii. L-Arginine

Cellular production of iNOS is dependent on L-arginine availability. This amino acid may be obtained from exogenous sources via blood circulation, from intracellular protein degradation or endogenous synthesis of arginine. The regeneration of L-arginine from L-citrulline through the urea cycle enzymes arginosuccinate synthase and arginosuccinate lyase constitutes the endogenous pathway (Morris and Billiar, 1994). Tissues and cells other than liver and kidney also contain low levels of arginosuccinate synthase and arginosuccinate lyase, which together can synthesize arginine from citrulline. Arginine can be regenerated from citrulline, which is a co-product of NOS

reaction. NO-producing vascular cells and cytokine activated macrophages have been shown to convert citrulline to arginine (Mitchel et al., 1990; Wu and Brosnan, 1992). While arginosuccinate lyase protein and mRNA were shown to be basally expressed, inflammatory stimuli such as lipopolysaccharide and IFN- γ induced arginosuccinate synthase along with iNOS in macrophages and vascular smooth muscle cells (Nussler et al., 1994; Hattori et al., 1994). More recently, both arginosuccinate lyase and arginosuccinate synthase protein, and mRNA were shown to increase significantly from basal levels in the lungs and spleen whereas these enzymes remained unchanged in the liver of lipopolysacchaide-treated rats (Nagasaki et al., 1996). The cell uptake of arginine is also a regulated process. Arginine transport into the cell is dependent upon plasma membrane transport system termed "system y⁺" (White., 1985). Arginine transport activity and mRNA of cationic amino acid transporter -1 (CAT-1), 2A (CAT-2A) and 2B (CAT-2B) which exhibit system y⁺ properties have been reported to increase following exposure to inflammatory mediators (Simmons et al., 1996). Glucocorticoids which inhibit NO synthesis have been shown to suppress the expression of CAT-1 and CAT-2B and CAT-2A mRNA in response to cytokines in cardiac microvascular cells (Simmons et al., 1996a). Thus the transport and resynthesis of arginine may be important points of control in the regulation of NO synthesis.

iii. Tetrahydrobiopterin (BH₄)

(6R)-5, 6, 7, 8-Tetrahydrobiopterin (BH₄) is essential for the bioactivity of all isoforms of NOS. Pathways mediating pteridine and NOS biosynthesis is tightly

coupled (Mayer and Werner., 1995). Besides being a cofactor in the enzymatic conversion of arginine to citrulline and NO, BH₄ may aid in maintaining NOS in an active dimeric conformation. BH₄ may also participate in the presentation of electrons to the catalytic center and act as an allosteric activator or both. Another potential role of BH₄ may be to prevent the inactivation of NOS by nitrogen oxides (Nathan and Xie., 1994). New findings indicate that synthesis of BH₄ may be regulated within the cells expressing NOS. GTP cyclohydrolase-1 is the rate limiting enzyme in the synthesis of BH₄. The steady state level of mRNA for this enzyme is co-induced by pro-inflammatory cytokines and lipopolysaccharide in a variety of cell types (Mayer., 1995; Gross and Levi, 1992). Glucocorticoids also suppress the cytokine-stimulated induction of GTP cyclohydrolase 1. This shows that BH₄ synthesis may be important in the regulation NOS activity (Simmons et al., 1996a).

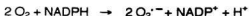
iv. Phosphorylation

All three NOS isoforms contain consensus sequence sites for phosphorylation by diverse protein kinases. Protein kinase C (PKC), cyclic AMP-dependent protein kinase (PKA) and calmodulin-dependent protein kinase II can phosphorylate NOS in vitro (Bredt et al., 1992). iNOS has been shown to be phosphorylated on tyrosine residues, and tyrosine kinases and phosphatases are shown to be involved in the regulation of iNOS enzyme activity (Pan et al., 1996). In response to bradykinin and ionomycin, ecNOS has been shown to be phosphorylated on serine residues (Michel et al., 1993). Phosphorylation of ecNOS results in its translocation back to cytosol. Although the physiological

consequences of phosphorylation are not yet clear, this suggests a potential role for kinases in the localization of eNOS within the cell.

B. Superoxide

The principal function of macrophages and other phagocytic cells is the destruction of invading microbial pathogens and removal of inflammatory debris. Phagocytes mediate their innate immunological response by releasing many cytotoxic products that aid in the destruction of invading pathogens. These include proteins such as lysozyme, elastase, peroxidases and reactive oxygen species (ROI) such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO^{\cdot}). $O_2^{\cdot-}$ is the precursor of ROIs including H_2O_2 and HO^{\cdot} (Fridovich, 1986), and is produced when NADPH oxidase is activated. The substrate for this oxidase was identified to be NADPH, a continuous supply of which is generated by the pentose phosphate pathway (Rossi, 1986). The NADPH oxidase is a membrane-associated enzyme that catalyzes the one electron reduction of oxygen to $O_2^{\cdot-}$ at the expense of NADPH (Babior, 1995).



Oxidation of cellular molecules by ROI contributes to killing of phagocytized microbes. In addition, the pumping of electrons unaccompanied by protons results in an increase in vacuolar pH. This increase in pH activates neutral proteases, which also aid in the killing and digestion of pathogens. The generation of superoxide by the NADPH oxidase of neutrophils is accompanied

by the efflux of H^+ ions through a H^+ channel (Henderson et al., 1988). Impairment in any of the components of the NADPH oxidase causes chronic granulomatous disease which is characterized by a predisposition to infection by many extracellular pathogens (Bastian and Hibbs, 1994; Segal, 1989).

1. NADPH (Respiratory Burst) Oxidase

NADPH oxidase is a complex enzyme consisting of both membrane and cytosolic components. Of these, an unusual cytochrome b (cytochrome b_{558} or cytochrome b_{246}) is located in the plasma membrane and is composed of two subunits, an α -subunit with a molecular weight of 21 kDa (21 kDa phagocyte oxidase factor, $p21^{phox}$) and a β -subunit with a molecular weight of 91 kDa ($gp91^{phox}$). $gp91$ protein is heavily glycosylated and contains binding sites for FAD and NADPH (Green and Pratt, 1988; Segal et al., 1992). The $p21^{phox}$ is a transmembrane protein with portions exposed on both sides of the membrane. $p21^{phox}$ is necessary for the stability of $gp91^{phox}$ (Chanock et al., 1994). Cytosolic components of the NADPH oxidase include 47 kDa ($p47^{phox}$) and 67 kDa ($p67^{phox}$) proteins. $p47^{phox}$ is a highly basic protein and contains two SH3 domains through which it interacts with other proteins. The C-terminal quarter of this protein contains phosphorylation target motifs with many serines and basic residues. Expression of $p47^{phox}$ is restricted to cells of the phagocytic or lymphocytic lineage. The $p67^{phox}$ is a protein of 526 amino acids and contains SH3 domains (Chanock et al., 1994). NADPH activation is also dependent on small GTP binding proteins, Rac2, Rap1A or both (Bokoch et al., 1993).

2. Activation of NADPH Oxidase

NADPH oxidase is normally dormant in resting cells and is only activated when cells are stimulated by appropriate stimuli such as microbial pathogens, N-formyl methionyl leucyl phenylalanine (fMLP), and protein kinase C (PKC) agonists such as PMA (Babior, 1995; Carnutte et al., 1994). Different stimuli activate different signaling pathways. Response to PMA is slower and more prolonged while response to fMLP is of lower intensity and shorter duration, Ca^{++} dependent. Cells can be 'primed' by sub-activating levels of various stimuli. This 'priming' reduces the lag and amplifies the response to a subsequent stimulus (Segal and Abo, 1993). Activation of NADPH oxidase involves the phosphorylation of p47^{phox} and translocation of both p47^{phox} and p67^{phox} to the plasma membrane (Cross and Jones, 1991). It is believed that in unstimulated cells, p21rac protein is present largely as the GDP bound form in a complex with RhoGDI (GDP-dissociation factor), an association that requires modification by isoprenylation near its carboxy terminus which could be necessary for attachment to the membrane (Morel et al., 1991). Activation is initiated by the separation of p21rac from GDI and movement to the membrane along with p47^{phox} and p67^{phox} where it forms a complex with flavocytochrome (Clark et al., 1990; Quinn et al., 1993; Abo et al., 1994). This process is also accompanied by the exchange of GDP for GTP (Rossi, 1986). Hydrolysis of GTP-bound Rac leads to inactivation and dissociation of the oxidase complex (Bokoch, 1993). The components of NADPH oxidase and its mode of activation are shown in Fig. 5.

3. Electron Transport

The electron transport pathway is very unusual in the respiratory oxidase system. It contains cytochrome b with a very low mid-point potential of -245mV , the lowest seen in any such molecule in the mammalian system. It is also known as cytochrome b_{558} because of the absorption maxima of its alpha band (Segal et al., 1978). This cytochrome b_{558} is abundant in neutrophils, monocytes and macrophages (Segal, 1989). It is located on the plasma membrane and is distributed in specific granules, which fuse with the plasma membranes. NADPH is considered to be the physiological electron donor, $\text{O}_2^{\cdot-}$ is the product, and FAD is the obligatory electron carrier. Both NADH and NADPH can serve as substrates, but NADPH is the electron donor *in vivo*. The K_m for NADPH ($0.03\text{--}0.08\ \mu\text{M}$) is one tenth of that of NADH (Babior et al., 1976; Babior, 1992). The activation of this oxidase is associated with an increase in NADPH production via the hexose monophosphate shunt pathway. The NADPH binding site is considered to be $\text{gp91}^{\text{phox}}$ (Segal et al., 1992). Oxidase also requires FAD ($K_m\ 60\ \text{nM}$) (Babior, 1992). The FAD is probably associated with $\text{gp91}^{\text{phox}}$ (Segal et al., 1992; Rotrosen et al., 1992). It is generally believed that Cytochrome b_{558} is the terminal electron carrier of the oxidase (Babior, 1992). Transfer of electrons from FADH_2 to the electron acceptor cytochrome b occurs with the release of the protons to the cytosolic side of the membrane (Fig. 5a). Translocation of protons into the phagosome or to the cell-exterior is thought to occur through a proton Channel (Henderson et al., 1988). The NADPH complex formation may induce conformational changes in the flavocytochrome favourable for NADPH binding or for electron transport (Abo et al., 1992).

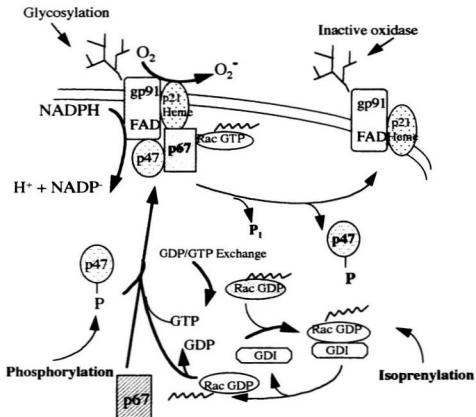


Fig. 5. Structural features of NADPH oxidase. Schematic representation of the components and pathways involved in the activation of NADPH oxidase resulting in the formation of O_2^- as a result of stimulation by agonists. (Adapted from Bastian and Hibbs, 1994)

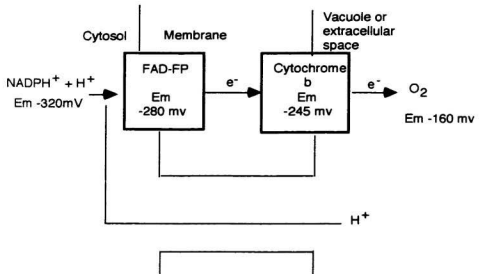


Fig. 5a. Electrogenic superoxide generating NADPH oxidase in the plasma membrane of human phagocytes. Adapted from Henderson et al., 1988

4. Regulation of NADPH Oxidase

Activation of NADPH oxidase is triggered by a number of molecules including complement factor C5a, phorbol ester PMA and fatty acids (Grinstein et al., 1993). Activation by PMA is thought to be mediated through PKC-catalyzed phosphorylation of p47^{phox} and possibly other oxidase subunits. PKC inhibitors such as H7 and protein phosphatase 1 and 2A inhibitors such as calyculin C and okadaic acid were shown to modulate $O_2^{\cdot-}$ production by activated neutrophils (Curnutte et al., 1994). Receptor-mediated activation of the oxidase appears to involve both tyrosine and serine-threonine phosphorylation. Tyrosine phosphorylation was shown to occur during cell activation by fMLP, interleukin-8 and platelet activating factor (Richard et al., 1994). fMLP was also shown to activate four neutrophil kinases, all of which were able to phosphorylate p47^{phox} (Grinstein et al., 1993). Phosphatidylinositol (PI) and PI 3,4,5 phosphate have also been shown to be involved in regulation of $O_2^{\cdot-}$ generation. (Arcard and Wyman, 1993). Inhibitors of phospholipase A₂ (PLA₂) also blocked $O_2^{\cdot-}$ synthesis which could be reversed by the addition of arachidonic acid (Dana et al., 1994). Also, phosphatidylcholine-specific phospholipase C (PC-PLC) reversibly inhibited $O_2^{\cdot-}$ generation (Traynor et al., 1993). Moreover, Rac activation and translocation were also shown to be regulated by tyrosine kinase activation (Dorseuil et al., 1995). All these findings collectively suggest that depending upon the stimulus, phospholipases, PKC, and tyrosine phosphorylation have a role in regulating $O_2^{\cdot-}$ generation.

Section 5. Signal Transduction Mechanisms in Macrophages

The cellular signaling events in activated monocytes/macrophages leading to complex responses related to host defense have been well elucidated. Intracellular events in response to agents such as LPS are mediated by phospholipases and protein kinases. Activation of phosphatidylinositol directed PLC (PI-PLC) by LPS results in the release of diacylglycerol and inositol 1,4,5-triphosphate. The former mediates the stimulation of protein kinase C, and the latter induces an increase in intracellular calcium concentration (Hamilton and Adams, 1987). Agonists that stimulate PI breakdown activate PI-PLC isoforms (β , γ , δ) and these play a major role in the physiological responses exerted by various agonists (Berridge and Irvine, 1984). Although most attention has been focused on signaling through PI breakdown, it is now clear that many agonists induce rapid hydrolysis of phosphatidylcholine (PC) by phospholipase C (PC-PLC) and phospholipase D (PC-PLD) generating 1,2-Diacylglycerol (DAG) and phosphatidic acid (PA), respectively (Billah and Anthes, 1990; Exton, 1994). Stimulation of monocytes and macrophages also results in the phosphorylation and activation of several protein kinases including protein tyrosine kinases (PTK), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK), all of which play a role in cellular proliferation and differentiation (Chow et al., 1995).

A. Role of PC Hydrolysis in Cell Signaling

PC is the principal phospholipid class found in mammalian tissues and accounts for more than 50% of the total cell phospholipid content. Novel signaling pathways involving PC-PLC activity that produce DAG and phosphocholine have been implicated in cell activation by many agonists provoking mitogenic and nonmitogenic responses that may function through the activation of guanine nucleotide binding proteins (G-proteins) (Cook, and Wakelam, 1991; Larrodera et al., 1990; McKenzie et al., 1992). Ligands such as epidermal growth factor (EGF) that exert their effects through receptors with intrinsic tyrosine kinase activity have also been shown to activate PC hydrolysis by a PC-PLC catalyzed pathway (Pettitt et al., 1994).

1. PC-PLC

The PC-PLC enzymes are characterized by the cleavage of the phosphodiester linkage yielding phosphocholine and DAG from PC (Fig. 6.). Although PC-PLC enzymes from bacterial origin are well characterized, there are very few studies on purified mammalian PC-PLC (Wolf and Gross, 1985; Clark et al., 1986). A lysosomal PC-PLC with acidic pH maximum and divalent cation dependency is known but is unlikely to be involved in signal transduction (Exton, 1994). Mammalian PC-PLCs have been partially purified from heart cytosol, seminal plasma and U937 promyelocytic cells. These enzymes have neutral pH optima and hydrolyze PC preferentially (Wolf and Gross, 1985; Clark et al., 1986; Sheikhnejad and Shrivastava, 1986). The seminal enzyme has an apparent

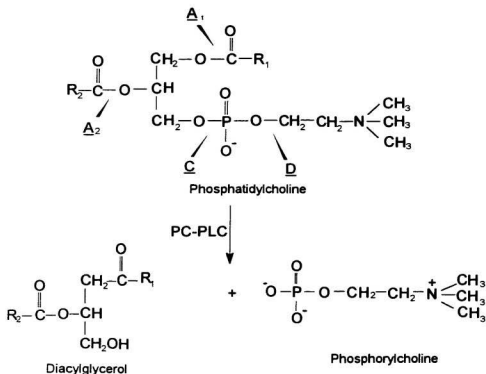


Fig. 6. **Phospholipases involved in Phosphatidylcholine hydrolysis.** Arrow denotes the site of cleavage catalyzed by these enzymes. Phospholipases A₂, D and C catalyzes the formation of bio-active lipids; arachidonic acid and lysophosphatidylcholine by PLA₂, Choline and phosphatidic acid by PLD and phosphocholine and diacylglycerol by PLC.

molecular weight of 125,000 and is comprised of two subunits of 69000 and 55000 (Sheikhnejad and Shrivastava, 1986). The canine heart enzyme was slightly stimulated by Ca^{++} and Mg^{++} (30%) while Cd^{++} and Zn^{++} significantly inhibited the enzyme activity (Wolf, and Gross, 1985). The seminal enzyme was shown to be little affected by Mg^{++} and did not respond to Ca^{++} (Sheikhnejad and Shrivastava, 1986).

i. Regulation of PC-PLC by G-proteins

PC hydrolysis by PLD or PLC is regulated by G-proteins. Addition of guanosine 5'-O-(3-thiotriphosphate) ($\text{GTP}\gamma\text{S}$) to permeabilized cells and isolated rat liver plasma membranes resulted in the release of choline or phosphocholine (Exton, 1994). Although Mg^{++} was required for the activation of these enzymes by G-proteins, the role of Ca^{++} has not been conclusive. Prior stimulation of liver plasma membranes with cholera toxin or injection of islet activating protein did not affect PC hydrolysis. PC-PLC was also shown to be coupled to purinergic receptors in these membranes by a GTP binding protein. Hydrolysis of PC-PLC was thought to contribute to the formation of DAG and influence PKC activity (Irving and Exton, 1987).

ii. Regulation of PC-PLC by Protein kinase C

Extensive work has shown that tumor-promoting phorbol esters stimulate the breakdown of PC (Billah and Anthes, 1990; Shukla and Halenda 1991). In addition, PKC antagonists block the effects of agonists and phorbol esters on

PLC activity (Exton, 1990; Billah and Anthes, 1990). However, it is not known whether PKC is involved in the stimulation of PC lipases by those agonists which do not stimulate PI hydrolysis (Exton, 1994).

iii. Role of Tyrosine Phosphorylation in the Activation of PC-PLC

A large number of growth factors promote PC hydrolysis, and in many instances this effect is unrelated to PI hydrolysis (Rosoff et al., 1988; Halstead et al., 1995). Activation by many growth factors is inhibited or abolished by down-regulation of PKC, indicating a role for PKC in the activation of phospholipases (Exton, 1994). However, the activation of PC phospholipases may also involve a PKC independent mechanism involving receptor tyrosine kinase activity and the signal cascades resulting from their activation (Exton, 1994). PC-PLC activity was associated with phosphotyrosine in NIH3T3 cells and the increased enzyme activity was abolished by treatment with a phosphatase. This activation was blocked by pertussis toxin indicating that it is regulated by a G-protein coupled pathway (Choudhury et al., 1991).

iv. PC Hydrolysis and Ras Activation

Preliminary reports suggested that an accumulation of DAG seen in cells transformed with Ha-ras oncogene was independent of PI hydrolysis, but was associated with an increase in phosphocholine and phosphoethanolamine (Wolfman and Macara, 1987; Lacal et al., 1987). Swiss 3T3 cells transformed by p21^{ras}, or fibroblasts transfected with a temperature sensitive mutant of Ki-ras

were also shown to exhibit increased PC hydrolysis (Price et al., 1989; Lopez-Barahona et al., 1990). Moreover, in NIH3T3 cells transformed by K-ras, stimulation with bradykinin caused a greater production of phosphocholine and DAG with a concomitant decrease in PC than seen in wild type cells (Fu et al., 1992). Studies using a dominant negative mutant of ras (Ha-ras Asn 17) has also shown that hydrolysis of phosphatidylcholine by a PC-PLC couples Ras to activation of Raf protein kinase during mitogenic signal transduction. Possible sites of Ras-Raf coupling by PC-PLC are shown in Fig. 7. These data also indicated that second messengers derived from PC-PLC mediated PC hydrolysis function downstream from Ras in mitogenic signal transduction (Cai et al., 1992). In addition, hydrolysis of PC-PLC was shown to bypass the block caused by the expression of mutant Ras, Ha-Ras Asn 17 (Cai et al., 1992a). Dominant negative mutants of Raf-1 or PKC- ζ revert NIH3T3 fibroblasts, stably transfected with PC-PLC gene, to normal non-transformed cells (Bjorkoy et al., 1995). Receptor-stimulated PC hydrolysis has also been shown to be necessary in platelet derived growth factor (PDGF)-stimulated activation of MAPK in Rat-1 fibroblasts (van Dijk et al., 1997). Inhibition of PC-PLC activation also resulted in profound inhibition of MEK and MAPK activation in LPS-treated BAC-1.2F5 macrophages (Buscher et al., 1995). DAG derived from PC-PLC catalyzed PC hydrolysis is presumed to have a role in Raf-1 activation, either by binding to its amino-terminal regulatory domain or by activating an upstream signal transduction component distinct from PKC (Buscher et al., 1995).

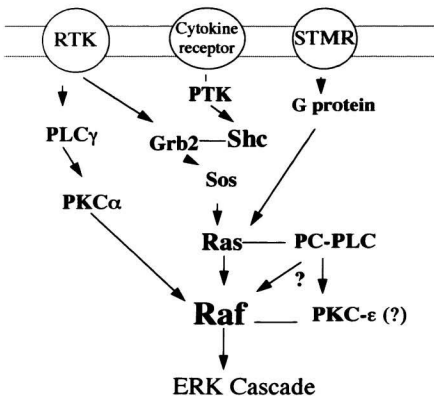


Fig. 7. **Role of PC hydrolysis in Raf dependent signal transduction.** Diagram illustrates the coupling of Ras to activation of Raf protein kinase by PC -PLC and PKC- ϵ during mitogenic signaling. (Adapted from Cai et al., 1993; Daum et al., 1994). RTK, receptor tyrosine kinase; STMR, seven transmembrane receptor; Sos, son of sevenless; Grb and Shc, adaptor proteins

v. Physiological Significance of Agonist-Stimulated PC Hydrolysis

The physiological significance of agonist-stimulated PC hydrolysis is not yet totally elucidated. DAG production from agonist-stimulated PC hydrolysis is prolonged and does not alter cellular Ca^{++} , unlike that of PI hydrolysis. PC hydrolysis provides a positive feed back signal to PKC and does not cause down regulation of PKC- α and PKC- β (Exton, 1994). Although the products of PC hydrolysis such as DAG and PA may be important in signaling mechanisms, alteration of lipid composition of membranes may also be important in regulating membrane-mediated functions such as protein trafficking, secretion and neurotransmitter release. DAG derived from PC was considered to be ineffective in activating PKC. However, Ha and Exton have shown that PC hydrolysis stimulated by PDGF can activate PKC- ϵ but not PKC- α (Ha and Exton, 1993). It is assumed that while DAG derived from PC can activate Ca^{++} independent PKC isozymes except those that may not respond to DAG, PI-derived DAG can only activate enzymes that respond to Ca^{++} and DAG.

Besides its role in the activation of the MAP kinase cascade, agonist-stimulated PC-PLC activation is pivotal in the rapid induction of NF- κ B activity. Activation of the U937 promonocyte cell line by TNF stimulated PC-PLC producing 1,2 DAG, which in turn activated PKC and an acidic sphingomyelinase. While PKC induction delayed NF- κ B activation, ceramide generated by the acidic sphingomyelinase caused rapid NF- κ B activation (Schutz et al., 1992). Inhibition of PC-PLC also inhibited NF- κ B activation and cytokine production in alveolar macrophages stimulated by LPS (Carter et al., 1998). In a murine

model, lethal shock induced either by TNF, LPS, or staphylococcal enterotoxin was prevented by blocking PC-PLC activity suggesting that PC-PLC may serve as a novel target for anti-inflammatory drugs (Machleidt et al., 1996). LPS-induced septic shock has been attributed to uncontrolled NO production, and it is interesting to note that both NO production and NF- κ B activation are inhibited by tricyclodecan-9-yl-xanthogenate (D609), an inhibitor of PC-PLC (Carter et al., 1998; Tschaikovsky et al., 1994; Muller-Decker, 1989; Schutz et al., 1992).

B. Mitogen-activated Protein Kinases

The mitogen-activated protein kinases (MAPK) are important mediators of signal transduction from the cell surface to the nucleus and play a crucial role in cell growth and differentiation (Davis, 1994). They are ubiquitously expressed serine/threonine kinases activated by a wide array of stimuli that include cytokines, growth factors, and hormones (Davis, 1994). Multiple pathways activated by distinct signaling pathways have been described. In mammals, the mitogen-activated protein kinase superfamily includes p42 and p44 kDa MAPK (extracellular signal-regulated kinase 1 and 2, ERK1/ERK2), stress-activated protein kinase (SAPK, c-Jun terminal kinase, JNK), and p38 kinase (high osmolarity glycerol kinase, HOG1) subfamilies which comprise three interwoven signal transduction cascades. ERK, JNK and p38 kinases are the terminal enzymes in a three kinase cascade where each kinase phosphorylates the down stream member in the sequence. A three kinase module consists of MAP kinase kinase kinase (MEKK) that activates MAP kinase kinase (MEK) which in turn activates the terminal MAP kinase (Fig. 8).

1. The ERK Subgroup of MAPKs

The ERKs were initially identified by Sturgill et al (Sturgill and Wu, 1991). Activation of ERK occurs through dual phosphorylation of threonine and tyrosine (202 and 204 of human ERK) in the subdomain VIII by ERK1/ERK2 kinase (MEK). It was also shown that the activating phosphorylation occurred within the motif Thr-Glu-Tyr (Payne et al., 1991). MEK, in turn, is phosphorylated and activated by several MAPKK kinases (MAPKKK, MEKK) including Raf isoforms, c-MOS and MEKK1 (Johnson et al., 1994). These upstream regulators are controlled by receptor tyrosine kinases, seven-transmembrane receptors and cytokine receptors etc. ERKs are proline-directed kinases that phosphorylate Ser/Thr-Pro motifs. Optimal phosphorylation requires the extended consensus sequence Pro-Xaa_n-Ser/Thr-Pro, where Xaa is any basic or neutral amino acid and n = 1 or 2 (Gonzalez et al., 1991). The best characterized ERK substrates are cytoplasmic phospholipase A2 (cPLA₂) and the transcription factor Elk-1. Phosphorylation of cPLA₂ by ERKs at Ser 505 results in an increase in enzymatic activity of cPLA₂ with increased release of arachidonic acid and the formation of lysophospholipids from membrane phospholipids (Lin et al., 1993). MAPKs also directly phosphorylate Elk-1, one of the ternary complex factors (TCF) (Janknecht et al., 1993). Phosphorylation of Elk by ERKs results in binding to the serum response factor with serum response element and causes an increase in the expression of many genes (Marais et al., 1993).

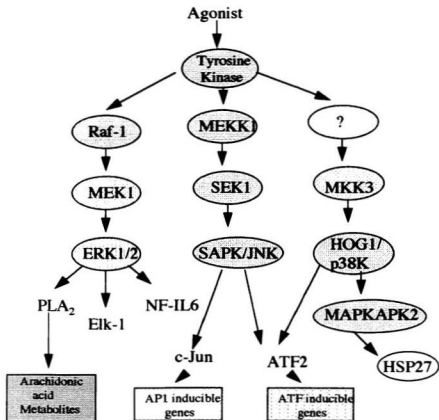


Fig. 8. Signal transduction cascades in macrophages (Adapted from Sanghera et al., 1996).

2. The SAPK (JNK) Subgroup of MAPKs

The stress-activated protein kinases (SAPK) also referred to as c-Jun N terminal kinase (JNK) protein kinases, were first identified as a protein kinase activity that phosphorylates the amino terminal of c-Jun transcription factor at Ser 63 and Ser 73 in UV-treated cells. In contrast, ERK phosphorylates the inhibitory C-terminal site at Ser-243 of c-Jun (Kyriakis and Avruch, 1996; Minden et al., 1994). N-terminal phosphorylation of c-Jun induces the formation of c-Jun homodimers and c-Jun/c-fos heterodimers. Human and mouse JNKs have been purified and cloned and found to be 46 kDa and 55 kDa proteins (Derijard et al., 1994; Kyriakis et al., 1994). Analysis of the primary sequence of JNKs indicates that they are related to ERKs. The phosphorylation motif for these enzymes has been identified as Thr-Pro-Tyr. JNKs are activated by UV radiation, proinflammatory cytokines, and environmental stress. Members of the Rho family, a sub group of the Ras superfamily of small GTP binding proteins such as Rac and Cdc42 have been shown to be important for the activation of JNK/SAPK cascade (Coso et al., 1995; Coso et al., 1995a; Minden et al., 1995). However, JNK/SAPK activation by the protein synthesis inhibitor anisomycin was shown to be independent of these small GTP-binding proteins (Coso et al., 1995).

3. The p38 Subgroup of MAPKs

A p38 protein kinase that is phosphorylated on tyrosine in response to lipopolysaccharide (LPS) is the most recent member of the MAPK family (Han,

et al., 1994; Rouse et al., 1994). p38 kinase is also activated by hyperosmolar medium and can substitute for HOG1 in *Saccharomyces cerevisiae*. It is possible that JNK and p38 kinase are activated by parallel stress-activated signal transduction pathways. p38 kinase has been cloned and shown to be similar to the yeast high osmolarity glycerol response 1 (HOG1) kinase (Han, et al., 1994). p38 HOG1 kinase is defined by the regulatory dual phosphorylation motif on the tripeptide of TGY in place of TEY in ERKs and TPY in JNK/SAPKs. An upstream dual-specificity kinase of p38 HOG1 kinase, MAP kinase kinase 3 (MKK3) has also been identified (Derrijard et al., 1995). Recent studies have suggested that p38 MAPK activates MAPK-activated protein kinase-2 (MAPKAP2), which in turn, phosphorylates the small heat shock protein 27 (HSP27). The physiological role of this event is controversial but it may help the cell to resist thermal stress (Rouse et al., 1994). Several other investigators have provided evidence that p38 MAPK may also be involved in the regulation of cytokine production (Lee, et al., 1994).

4. Physiological Relevance of MAP Kinase Signaling Cascades

Several lines of evidence indicate a pivotal role for ERK cascade in cell growth and proliferation. Mitogenic stimulation by various extracellular agonists correlates with ERK activation. Dominant negative mutants of Ras or Raf-1 kinase were shown to inhibit growth factor-induced proliferation, while constitutively active Raf-1 induced cell proliferation (Miltnerberger et al., 1993; Pronk, et al., 1994). Dominant negative or constitutively active MEK mutants

also inhibit or accelerate cell proliferation of NIH3T3 cells, respectively (Seger et al., 1994; Brunet et al., 1994; Pages et al., 1993). Transfection with antisense cDNA (or antisense oligodeoxynucleotides) directed against ERKs or ERK mutants was also shown to inhibit DNA synthesis and cell proliferation (Robinson, et al., 1996). These data indicate that the ERK cascade plays an important role in cell proliferation and oncogenesis.

The JNK/SAPK signaling pathway seems to be involved in mediating cell growth inhibition and cell death. Expression of a constitutively active mutant of MEKK, an upstream kinase of JNK/SAPK has been shown to inhibit cell death (Yan et al., 1994). More recent data suggest that activation of p38 kinase and JNK/SAPK kinase stimulates apoptosis, while activation of the ERK cascade prevents apoptosis (Xia et al., 1995). Cellular differentiation is another physiological response linked to ERK cascade. ERK activation has been linked to differentiation in monocytes, PC12 cells and T cells (Cowley et al., 1994; Alberola-Ila et al., 1995). It is suggested that the prolonged activation of ERKs leads to differentiation, while transient activation results in proliferation (Marshall, 1995). Based on the information available so far, both cellular proliferation and differentiation seem to be controlled by the ERK pathway and the duration of activation may be critical for cell growth and development.

C. Nuclear Factor kappa B

Nuclear factor kappa B (NF- κ B) is a ubiquitous multiunit transcription factor that can rapidly activate genes involved in immune and acute phase response,

growth control and apoptosis (Baeuerle, 1991; Beg and Baltimore, 1996). It is activated by a wide variety of pathogenic signals and functions as a potent and pleiotropic transcriptional activator (Baeuerle and Henkel, 1994; Sen and Baltimore, 1986). This factor was first discovered in the nuclei of mature B cells where it was bound to a 10 base pair DNA motif (GGGACTTCC) in the kappa immunoglobulin light chain enhancer (Sen and Baltimore, 1986). It is now shown to be involved in the regulation of many genes, including that encoding iNOS (Baeuerle, 1991; Nathan and Xie, 1994; Kleinert et al., 1996).

1. Components of NF- κ B Complex

In vertebrates, at least five distinct DNA binding proteins are known to heterodimerize forming complexes with distinct transcriptional activity. The DNA-binding protein complex is composed mainly of proteins with molecular weights of 50 kDa and 65 kDa and belongs to a larger group of proteins, generally referred to as the family of Rel-related proteins. On the basis of their structure, they are divided into two subclasses. The first consists of p65-Rel A, relB and c-Rel which are synthesized as mature proteins and contain the so called Rel homology domain (RHD) within the N-terminal 300 amino acids (Verma et al., 1995). The RHD is responsible for DNA binding, dimerization and nuclear localization of the dimers. In addition, these proteins also contain non-conserved transactivation domains. The second class of Rel-related proteins is composed of p50 NF- κ B1 and p52 NF- κ B2 (Verma et al., 1995). These proteins are produced as precursor proteins p105-NF- κ B1 and p100-NF- κ B2, and have a C-terminal ankyrin repeat domain (ARD) in addition to the highly homologous

RHD. This ARD is removed by ubiquitin-dependent proteolytic processing in the 26S proteasome generating mature p50-NF- κ B1 and p52-NF- κ B2 (Blank et al., 1992; Fan and Maniatis, 1991). While rel-B forms only heterodimers with p50 or p52 NF- κ B, other members of both subclasses can form all possible homo- and heterodimers, generally referred to as Rel-NF- κ B. The active form of NF- κ B dimers is frequently composed of p50 and RelA (p65). This heterodimer binds with extremely high affinity to the decameric DNA sequence of GGGPuNNPyPyCC-3' (Grimm and Baeuerle 1993).

2. Inhibitory kappa B (I κ -B)

Two types of inactive Rel-NF- κ B are found in the cytoplasm of unstimulated cells. The first is formed by Rel dimers that are bound to inhibitory proteins, I- κ B α , I- κ B β , I- κ B γ and Bcl-3 (Verma et al., 1995). These inhibitory proteins have ARDs consisting of five to seven ankyrin repeats, which are required for the binding to the RHD of Rel-NF- κ B. Binding of I- κ B to Rel-NF- κ B masks the nuclear localization signal (NLS) of Rel-NF- κ B and thus causes the retention of the complex in the cytoplasm (Beg et al., 1992; Naumann et al., 1993). A second type of inactive complex is formed by precursor proteins p105-NF- κ B1 and p100-NF- κ B2 (Mercurio et al., 1993; Dobrzanski et al., 1994; Naumann et al., 1993). These proteins dimerize with mature Rel-related proteins giving rise to inactive heterodimers. The ARD in the C-terminal of p105NF- κ B1 and p105NF- κ B2 is functionally homologous to I κ -B. It masks the NLS of the dimer and retains it in the cytoplasm.

3. Activation of NF- κ B

Activation by a number of agents including mitogens, lipopolysaccharide and cytokines such as TNF- α leads to phosphorylation of I κ -B (Baeuerle and Henkel, 1994; Grilli et al., 1993; Israel, 1995) followed by ubiquitin-dependent degradation in the 26S proteasome (Henkel et al., 1993; Brown et al., 1995; Finco and Baldwin, 1995). In addition, p105-NF- κ B and p100-NF- κ B are phosphorylated and processed (Mercurio et al., 1993). Degradation of I κ -B α and processing of p105-NF- κ B1 results in the unmasking of NLS which allows translocation of Rel-NF- κ B to the nucleus and the activation of specific gene transcription. Activation of NF- κ B (p50-NF- κ B1-p65-RelA dimer) is initiated by the phosphorylation of Ser32 and Ser36 of I κ -B α , the inhibitory protein bound to NF- κ B. This phosphorylated form of I κ -B α has reduced mobility as compared to non-activated I κ -B α . (Brown et al., 1995; DiDonato et al., 1996). Mutation of both Ser32 and Ser36 completely inhibits the ligand-induced I κ -B α phosphorylation, whereas mutation of only one of these residues yields a partially phosphorylated form of I κ -B α (Traenckner et al., 1995). Moreover, mutation of one or both of these serines renders I κ -B α resistant to ubiquitination and subsequent degradation (DiDonato et al., 1996; Roff et al., 1996), showing that phosphorylation of both serines is an absolute requirement for the induction of I κ -B α degradation. I κ -B phosphorylation and subsequent degradation and translocation of activated NF- κ B is shown in Fig. 9. The kinases responsible for the phosphorylation of I κ -B α were found to occur as a 700 kDa complex (Chen et al., 1996). Fractionation of this complex yielded three polypeptides of 85, 87, and 64 kDa which exhibited the highest phosphorylating activity towards Ser32

and Ser 36 of I κ -B (DiDonato, et al., 1997). cDNA cloning and analysis showed that the 85 kDa protein is similar to the serine/threonine kinase, CHUK (conserved helix-loop-helix kinase) (DiDonato, et al., 1997). Two subunits (85 and 87 kDa proteins) have been since designated as IKK α and IKK β . Both IKK α and IKK β show 52% identity, can phosphorylate I κ -B- α . Another kinase involved in NF- κ B signaling was termed NIK (NF- κ B inducible kinase) and was found to share sequence homology with several MAPKKs (Malinin, et al., 1997). In addition, a 90 kDa S6 kinase has also been shown to phosphorylate I κ -B- α (Schouten et al., 1997)

4. NF- κ B and iNOS transcription

Phosphorylation of I κ -B at serine 32 and ser 36 results in dissociation of the NF- κ B complex and translocation of the DNA binding complex to the nucleus, where it binds to the cis-regulatory regions of the iNOS promoter leading to iNOS transcription (Xie et al., 1994; Lowenstein et al., 1993). p50/c-Rel and p50/RelA heterodimers represent at least a component of the trans-acting NF- κ B complex that participates in the iNOS transcriptional regulation (Xie et al., 1994). The binding of these heterodimers in association with additional nuclear proteins to the proximal region of the promoter comprised of nucleotides -85 to -76 was shown to be critical in iNOS transcription (Xie et al., 1994).

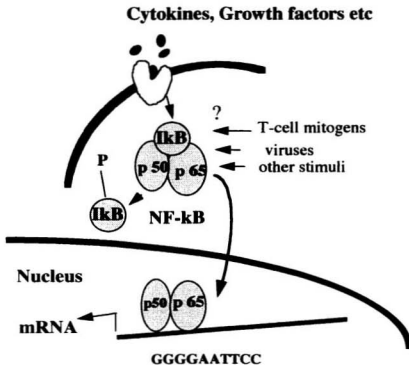


Fig. 9. **Schematic representation of NF-κB activation pathway.** Intracellular events leading to phosphorylation of I-κB and dissociation and translocation of the active of NF-κB complex to the nucleus in response to cytokines, mitogens and other stimuli are shown.

Section 6. Purpose of the Present Research

Elevated serum levels of CRP are considered to be sensitive markers of the acute phase response to infectious agents, immunological stimuli and tissue damage. The conservation of CRP over millions of years of evolution and the magnitude of the CRP response to various stimuli provide compelling reasons for a potentially important biological role for this protein. Over the years, numerous biological actions of CRP or peptides derived from CRP have been described including the activation of T-cells and macrophages, stimulation of cytokine production, killing of cancer cells, modulation of superoxide generation and protection from microbial pathogens. So far, many of these actions of CRP have been attributed mainly to its properties of antibody-like opsonisation and complement activation. Notwithstanding, the knowledge of the functions and mechanism of action of CRP has not been significantly advanced.

In recent years, data have accumulated to show that during periods of infection and inflammation, activated macrophages express NO and this contributes to their cytotoxic and tumoricidal activity. This provided the basis for the hypothesis that CRP may exert these actions through the stimulation of macrophage NO production. Therefore, a study was undertaken to determine whether CRP could induce iNOS enzyme using rat peritoneal macrophages and the murine macrophage cell line, RAW 264.7. This led to the novel observation that CRP indeed induced iNOS and ensuing NO synthesis in this model. This potentially important finding suggested an alternative mechanism by which CRP may induce tumoricidal and cytotoxic activity in macrophages, in addition to its role

in complement activation. Hence, a further study was carried out using a *Chlamydia trachomatis* model to test the hypothesis that CRP enhances the microbicidal activity of macrophages through the activation of NO production.

Most reports published to date support the finding that human CRP inhibits respiratory burst activity in neutrophils and macrophages. However, there has been no study on the effect of rat CRP on superoxide production. Therefore, a study was undertaken to elucidate the effect of rat CRP on superoxide generation and its role as an antioxidant. An attempt was also made to explain the differential regulation of superoxide production and nitric oxide generation by CRP in macrophages.

Several reports have described the binding of human and rat CRP to macrophages possibly through specific receptors, while others have described the cellular effector responses to CRP stimulation. However, virtually no information is available on the second messengers generated by the ligation of CRP to its putative receptor, nor on the communication of these signals to the cell nucleus. Hence, a study was carried out to elucidate some of the signaling pathways activated in response to CRP, and to examine whether these would lead to the stimulation of iNOS transcription. Diverse second messenger pathways have been shown to converge in the activation of NF- κ B complex, an event that is crucial for the induction of iNOS. Therefore, various cellular mechanisms that lead to the phosphorylation and translocation of this transcription factor to the nucleus were also studied in order to dissect the mechanism by which CRP may stimulate iNOS induction in macrophages.

MATERIALS AND METHODS

Section 1. Materials

A. Animals

1. Rats

Male Sprague-Dawley rats (175-200g) were obtained from Charles River Canada (La Prairie, PQ), or Animal care facilities, Memorial University of Newfoundland (St John's, NF), and were fed purina rat chow *ad libitum* (Ralston-Purina of Canada, Don mills, ON) and had access to water and chow till the time of sacrifice. Rats were exsanguinated under general anesthesia with ketamine-xylazine (ketamine 40 mg/Kg, xylazine 10 mg/kg) by cardiac puncture or from the abdominal aorta after laparotomy, and were killed by cervical dislocation after the procedure.

B. Cell Lines

1. RAW 264.7 Cells

RAW 264.7 cells were obtained from American Tissue Type Culture Collection (Rockville, MD). This line was established from a tumor induced by Abelson murine leukemia virus in BALB/c mouse. These are negative for surface immunoglobulin (slg-), Ia (Ia-) and Thy-1.2 (Thy-1.2-), can pinocytose neutral red and can phagocytose latex beads and zymosan. They are also capable of antibody-dependent lysis of sheep erythrocytes and tumor cell targets. These cells, treated with LPS for two days acquire the ability to lyse erythrocytes but not tumor cells (Raschke et al., 1978).

2. McCoy cells

McCoy cells (ATCC CRL 1696) were obtained from American Type Culture Collection (Rockville, MD). These are fibroblast like cells, susceptible to chlamydia strains and are commonly used to propagate chlamydia.

C. Chemicals and Reagents

All chemicals and reagents were of analytical grade and were obtained commercially. Arginine analog N^ωnitro-L-arginine methyl ester (L-NAME) dibutyl cAMP (dB cAMP), choline chloride, phosphocholine chloride (Calcium

salt), α -1-acid glycoprotein, bovine serum albumin (BSA), guanidium isothiocyanate, Interferon- γ , xanthine oxidase, N-(1-naphthyl)ethylenediamine dihydrochloride, sulphanilamide, cytochrome c and phorbol myristate acetate were from Sigma Chemical Company (St. Louis, MO). Dulbecco's phosphate buffered saline (DPBS), RPMI 1640 medium (with L-glutamine, 1mM L-arginine but no phenol red), penicillin, streptomycin and trypan blue were from Gibco BRL (Burlington, ON). Endotoxin-tested fetal calf serum (FCS) was obtained from Immuno Corp. (Montreal, PQ). Thioglycollate broth was obtained from Difco (Detroit, MI). L-N^G-Monomethylarginine (NMMA), staurosporin, tricyclodecan-9-yl-xanthogenate (D609), {1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1-H-pyrrole-2,5-dione} (U73122), [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole; FHPI] (SB 202190), 2'-Amino-3'-methoxyflavone (PD 98059), 4', 5,7-trihydroxy isoflavone (genistein), [α -cyano-(3-hydroxy-4-nitro)cinnamonitrile] (tyrphostin AG 126), Biotin-NH labeling kit and {2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide, HCl} (bisindolylmaleimide) were obtained from Cal Biochem (La Jolla, CA). All other reagents and chemicals were of analytical grade. Nitrate reductase (*Aspergillus* species) and lactate dehydrogenase were from Boehringer Mannheim (Laval, PQ). p-Aminophenyl phosphocholine-conjugated agarose affinity absorbent and bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce Chemical Co. (Rockford, IL).

Megaprime labeling kit (RPN 1606) and Hybond N+ positively charged nylon membranes were obtained from Amersham Life Science (Oakville, ON). Micro Biospin chromatography columns, Tween 20, PVDF blotting membranes and

ethidium bromide solution were from Bio-Rad laboratories (Hercules, CA). RNA molecular weight markers (1.6 - 7.4 kb) were from Boehringer Mannheim, Laval, PQ and RNA markers (0.7 - 4.6) and prestained protein molecular weight markers were from New England Biolabs (Beverly, MA) or Bio-Rad laboratories. Quickprep micro mRNA purification kit, Phastgel buffer strips, precasted gradient and homogeneous Phastgels (native and SDS) were obtained from Pharmacia Biotech (Baie d'urfé, PQ).

D. Radioisotopes

[Methyl- ^{14}C]choline chloride (55 mCi/mmol) and [α - ^{32}P]dCTP (3000 Ci/mmol) were from Dupont NEN (Boston, MA). 1,2-Dipalmitoyl-sn-glycerol-3-[^3H -methyl]phosphocholine ([^3H -choline]DPPC; 37 Ci/mmol) were from Amersham, Canada.

E. Antibodies

Anti-mouse iNOS polyclonal antibody raised in rabbits was from Transduction Laboratories (Lexington, KY). Goat anti-rabbit IgG conjugated to alkaline phosphatase was from Bio-Rad laboratories. Polyclonal antibodies against phosphorylated and phosphorylation state-independent p42/p44MAPK, MAP kinase kinase (MEK), SAPK, p38 kinase and $\text{I}\kappa\text{-B}\alpha$ were from New England Biolabs. A nonradioactive kit for measuring MAP kinase activity including monoclonal antibodies against phosphorylated p42/p44 (ERK1/ERK2) and anti-

phosphorylated Elk was obtained from New England Biolabs. Anti-rabbit IgG conjugated to horseradish peroxidase and phototope®-HRP Western blot detection kit were from New England Biolabs (Beverly, MA). Antibiotin antibodies were obtained from New England Biolabs and from Jackson ImmunoResearch labs (West Grove, PA). Protein A agarose was obtained from Transduction Laboratories or Cal Biochem.

Antiserum to rat CRP was raised in rabbits as described by Nagpurkar and Mookerjee (Nagpurkar and Mookerjee, 1981). Antibodies were affinity purified from rabbit antiserum using protein A agarose.

F. cDNA Probes

The cDNA probe specific for murine iNOS (1.8 kb fragment of mouse macrophage iNOS gene) was from Cayman Chemical Company (Ann Arbor, MI). The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (1.1 kb fragment) attached to a T7 RNA polymerase promoter was obtained from Clontech Laboratories (Palo Alto, CA). This probe covers the region from nucleotide 71 to 1053. Both probes were labeled by random primer labeling.

Section 2. Methods

A. Preparative Procedures

1. Preparation of Sepharose-Phenylphosphocholine Affinity Adsorbent

Sepharose-phenylphosphocholine affinity adsorbent was prepared as described by Nagpurkar and Mookerjee (1981). Briefly, this involved the reduction of p-nitrophenylphosphocholine with H_2 and coupling the product to CNBr-activated sepharose 4B. The resulting affinity adsorbent was stored in 10 mM Tris-HCl buffer (pH 7.4), containing 0.01% sodium azide at 4°C.

2 Isolation and Purification Of CRP

i. Rat CRP

Rat CRP was isolated from normal serum of Sprague-Dawley rats (300-450g) using a Sepharose phenylphosphocholine affinity adsorbent column as previously described (Nagpurkar and Mookerjee., 1981). In some instances, p-aminophenyl phosphocholine-conjugated agarose affinity adsorbent (Pierce

Chemical Co., Rockford, IL) was also used. Briefly, 10-15 ml of normal rat serum was passed through a column containing p-aminophenyl phosphocholine conjugated agarose beads (10 ml gel) equilibrated with 10 mM Tris (pH 7.4), 150 mM NaCl and 2 mM Ca^{++} . The column was washed extensively and the bound protein was eluted with 8 mM phosphocholine. Eluted protein fractions were dialyzed against buffer containing 10 mM Tris (pH 7.4) and 150 mM NaCl. Dialyzed fractions were reappplied to a fresh column of p-aminophenyl phosphocholine adsorbent and the process was repeated. Purity of the protein was routinely checked by SDS-PAGE using the Phast system (Pharmacia) and by high pressure liquid chromatography (HPLC) (Perkin Elmer series 4 or Beckman System Gold) using a gel filtration column (Beckman, UltraSphrogel 2000SEC) as described (Nagpurkar et al, 1993). Purified rat CRP was tested (E-toxate, Sigma) according to manufactures instructions and was found to be negative for endotoxin. Random batches of rat CRP was also passed through a polymyxin agarose column to ensure complete removal of any trace of endotoxin. Protein concentration was determined by the method of Lowry et al., using bovine serum albumin as standard (Lowry et al., 1951). Purified protein with purity greater than 95 %, as determined by the HPLC profile, was stored at -20 °C until use.

ii. Human CRP

Human CRP was purchased from Cal Biochem. The buffer diluent of CRP containing azide supplied by the manufacturer was exchanged for 10 mM Tris, 150 mM NaCl, 2 mM Ca^{++} using centricon-10 concentrators just before use.

3. Preparation of Macrophages

i. Isolation of Rat Peritoneal Macrophages

Rat peritoneal macrophages were obtained by injecting rats intraperitoneally with 5 ml of sterile 4% thioglycollate broth. Rats were killed by cervical dislocation 72 h later and macrophages were harvested by peritoneal lavage with DPBS without Ca^{2+} and Mg^{2+} as previously described (Edelson and Cohn, 1976). The cells were collected by centrifugation (10 min at 400 x g) of the lavage, and washed once with DPBS and suspended in RPMI 1640 medium containing 10% v/v FCS, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin solution. Macrophage number was determined using an improved Neubauer counting chamber (Hausser Scientific, PA). Cell viability was monitored by trypan blue exclusion.

4. Cell Culture

i. Rat Peritoneal Macrophages

Rat peritoneal macrophages (1×10^6 cells) were plated in 24-well plates (Falcon) and incubated for 2 h at 37°C under 5% CO_2 in a humidified incubator. The plates were then washed with RPMI 1640 three times and fresh

medium containing 10% FCS and antibiotics was added and were incubated for a further 24 h. The plates were then washed to remove non-adherent cells, the medium was replaced and CRP and/or other reagents were added.

ii RAW 264.7 Cells

RAW 264.7 cells were seeded at a concentration of 0.5×10^6 cells/well in RPMI 1640 medium containing 10% v/v FCS along with penicillin (100 units/ml) and streptomycin (100 $\mu\text{g/ml}$) and cultured in 24-well Falcon plates (Becton and Dickinson, NJ) for 24h under 5% CO_2 . The plates were then washed to remove non-adherent cells, the medium was replaced and CRP, IFN- γ and/or other reagents were added. The plates were then incubated for various time periods ranging from 0 to 72 h. Cell viability was monitored by the trypan blue exclusion test (Freshney, 1983). IFN- γ stock obtained from the manufacturer (100,000 units/0.5 ml) was diluted in sterile PBS containing 1% BSA and frozen in aliquots at -70°C . Throughout the study, IFN- γ was used at concentrations ranging from 10 to 50 units/ml, and CRP was used at concentrations ranging from 20 to 500 $\mu\text{g/ml}$ as specified.

5. Preparation and Fractionation of Macrophage Sonicates

Cell monolayers were washed with ice cold DPBS, scraped into DPBS and centrifuged at $900 \times g$ for 15 min. The pellet was suspended in 20 mM Tris buffer (pH 8.0) containing 137 mM NaCl, 5 $\mu\text{g/ml}$ pepstatin A, 1 $\mu\text{g/ml}$

chymostatin, 5 $\mu\text{g/ml}$ aprotinin and 100 μM PMSF, and sonicated (3x10 s) at 60 watts using a Branson Sonifier fitted with a microtip to disrupt cells. Sonicates were centrifuged at 100,000 x g for 1h at 4°C using a Beckman model L3-50 ultracentrifuge and supernatants were stored at -70°C.

B. Analytical Procedures

1. Polyacrylamide Gel Electrophoresis

Isolated CRP samples were routinely analyzed by SDS-PAGE on Phastsystem (Pharmacia). Typically, CRP samples (1-2 $\mu\text{g/ml}$) were separated on 12% homogeneous or 8-25% gradient precast Phast Gels. Gels were stained with Coomassie R 250 (Pharmacia Blue R) by the development unit of the phast system. A typical SDS PAGE profile is shown in Fig. 1

Macrophage lysates or cytosol were separated on 7.5% or 12% SDS gels unless otherwise specified using a Mini-Protein II cell (BioRad) in electrode (running) buffer (25 mM Tris, 200 mM glycine, 1% SDS, pH 8.3). Gels were usually stained with Coomassie blue stain. For Western blot analysis, samples were separated on SDS PAGE and transferred to PVDF membranes (BioRad) using Transblot cell (BioRad) in transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol) (Towbin et al., 1979)



Fig. 1. SDS gel profile of purified rat and human CRP. 1-2 μ g CRP was separated in the presence or absence of β -mercaptoethanol (BME) on 8 to 25% gradient SDS gels on the Phast gel separation system (Pharmacia) and stained and destained using the Phast gel development unit. Lanes 1 and 6, molecular weight standards; lane 2, human CRP + BME; lane 3, human CRP; lane 4, rat CRP + BME; lane 5, rat CRP

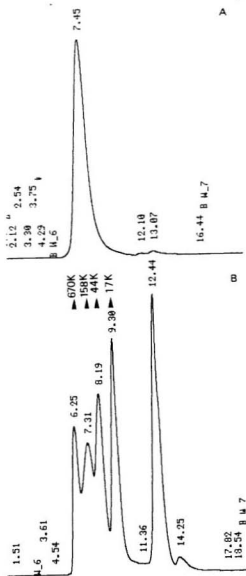
2. High Pressure Liquid Chromatographic (HPLC) Analysis.

The purity of rat and rabbit CRP was also determined by HPLC analysis using a Perkin Elmer series 4 or Beckman System Gold HPLC unit using a gel filtration column (UltraSpherogel 2000SEC, 7.5 x 300 mm, Beckman). Protein samples (10-50 μ g) were filtered through 0.45 μ m filters and injected into the column equilibrated with 10 bed volumes of 0.05 M Na₂SO₄, 0.02 M NaH₂PO₄ buffer (pH 6.8) and eluted at a flow rate of 1 ml/min. The eluent from the column was continuously monitored at 280 nm using an LC-95 UV/visible spectrophotometer detector (Perkin-Elmer) or System Gold detection module 166. The area under the absorbance peak from the eluted protein was determined using an LCI-100 Laboratory Computing Integrator (Perkin-Elmer) or Hewlett-Packard 3394A integrator and % area was reported as % purity of the protein. Typical HPLC profiles are shown in Fig. 2.

3. Thin Layer Chromatography (TLC)

TLC was used to separate choline metabolites and various phospholipids. Samples were applied to Whatman K5 silica gel 150A TLC plates (200 μ m thick layer) and chromatographed in a mobile phase-saturated TLC development chamber.

Fig. 2. HPLC profile of purified CRP. Rat CRP (20 μg) in a final volume of 20 μl was applied to a HPLC gel filtration column. Trace represents absorbance recorded at 280 nm by LC-95 UV/Visible spectrophotometer detector while CRP was eluting from the column. **A.** rat CRP (20 μg); **B.** MW standards.



4. Separation of Choline Metabolites

Samples were extracted with chloroform:methanol/ 2% acetic acid:water (100:100:50). To measure the incorporation of radioactivity in water soluble products, the upper phase was evaporated under nitrogen and resuspended in methanol. 50 μ l aliquots were applied to Whatman K5 silica gel 150A TLC plates and developed in a solvent system containing methanol/0.9% NaCl/ ammonium hydroxide (100:100:2). Authentic standards consisting of phosphatidylcholine (PC), lysophosphatidylcholine (LPC) glyceryl-3-phosphocholine (GPC), phosphorylcholine (phosphocholine) and choline were also chromatographed. After development, plates were air dried and stained with iodine vapour to localize the products. The silica gel from the plates was then scraped into vials containing Ready Safe liquid scintillation cocktail (Beckman) or Scintisafe Gel (Fisher Scientific) and associated radioactivity was measured by liquid scintillation counting using a Wallac 1209 Rackbeta Scintillation counter.

5. Separation of Phospholipids

To measure the incorporation of [methyl- ^{14}C]choline into phosphatidylcholine (PC), the lower phase from the cell extracts were dried under nitrogen, resuspended in chloroform:methanol (1:2) and applied to TLC plates and developed in a solvent system consisting of CHCl_3 : CH_3OH : H_2O (100:60:5). Authentic standards consisting of PC (5 μg), LPC (5 μg), PE (5 μg) and sphingomyelin (5 μg) were also chromatographed. Plates were air dried and

the products were identified by staining with iodine vapour, and radioactivity associated with lipids was quantitated by liquid scintillation counting.

6. Cell Labeling and Estimation of PC Hydrolysis

Rat peritoneal cells were grown overnight in 6 well culture plates (Falcon). Non adherent cells were removed by washing the cells twice with RPMI 1640 medium. Cell labeling and phospholipase assays were performed essentially as described by Schutz et al., (Schutz et al., 1992) with slight modifications. Cells were radiolabeled for 24 h with methyl[^{14}C] choline (0.5 to 1 $\mu\text{Ci/ml}$) in RPMI 1640 containing 10% FCS and antibiotics. The medium was then replaced with RPMI containing 0.5% FCS and incubation was continued for another 24 h. Cells were washed three times with DPBS and incubated for a further 3 h in medium containing 1% BSA. Cells were then stimulated with CRP in medium containing 1% BSA. Control cells were treated with an equal volume of PBS. The reaction was stopped by adding methanol cooled to 4°C followed by lipid extraction (Bligh and Dyer, 1959) and phase separation. Water soluble choline metabolites were resolved by drying the sample under nitrogen and performing thin layer chromatography in a solvent system containing methanol /0.9% NaCl/ ammonium hydroxide (100:100:2). RAW 264.7 cells grown to 50-60% confluency, and radiolabeled for 48 h with methyl [^{14}C] choline (0.5 to 1 $\mu\text{Ci/ml}$) were also stimulated by IFN- γ and/or CRP and PC hydrolysis was measured as described above.

Section 3. Studies on Macrophage Function

A. Generation of Superoxide ($O_2^{\cdot-}$)

Adherent rat peritoneal macrophages were washed twice with DPBS. Cells were incubated at 37°C with 80 μ M cytochrome c, CRP (50-300 μ g/ml) and other agents in Hanks balanced salt solution (HBSS) to a final volume of 1 ml. PMA (1 μ g/ml) was added to trigger $O_2^{\cdot-}$ generation. Reaction was stopped by the addition of 2 μ g/ml of superoxide dismutase and placing the plates on ice. An aliquot of the medium was transferred to ice-cold PBS, and superoxide dismutase cytochrome c reduction was measured in the supernatants by measuring the optical density at 550 nm. Cytochrome c reduction was determined using a molar extinction coefficient of 21.1 $M^{-1} cm^{-1}$. The rate of cytochrome c reduction was taken as an indicator of NADPH oxidase activity and $O_2^{\cdot-}$ generation.

B. $O_2^{\cdot-}$ Generation in a Cell Free System

$O_2^{\cdot-}$ was also generated in a cell-free system containing xanthine oxidase (10 mUnits/ml) and acetaldehyde (20 mM) in the presence of cytochrome c (80 μ M) and various concentrations of CRP for 5 min at 37°C as described (McCord and Fridovich, 1968; Dobrinich and Spagnuolo, 1991). $O_2^{\cdot-}$ generation was calculated by using an extinction coefficient of 21.1 $M^{-1} cm^{-1}$ for cytochrome c.

C. Studies on the Induction of Nitric Oxide Synthase in Macrophages by CRP

1. Nitrite Determination

Accumulation of nitrites in the culture medium was used as an indicator of NOS activity. Nitrite levels in the culture medium were determined by mixing 200 μ l of the cell culture medium with an equal volume of Griess reagent and incubating the mixture for 10 min at room temperature (Stuehr and Marletta 1985). Absorbance was read at 543 nm against a standard curve using sodium nitrite. Griess reagent was prepared by mixing equal volumes of sulfanilamide (2%) in H_3PO_4 (5%) with naphthylethylenediamine (0.2%) dihydrochloride in H_2O just before use to give a final working concentration of 1% sulfanilamide /0.1% naphthylethylenediamine dichloride /2.5% H_3PO_4 .

2. NOS Enzyme Assay

Cell monolayers were washed with ice cold DPBS, scraped into DPBS and centrifuged at 900 x g for 15 min. The pellet was suspended in 20 mM Tris buffer (pH 8.0) containing 137 mM NaCl, 5 μ g/ml pepstatin A, 1 μ g/ml chymostatin, 5 μ g/ml aprotinin and 100 μ M PMSF, and sonicated (3x10 s) to disrupt cells. Sonicates were centrifuged at 100,000 x g for 1h. Supernatants and total lysates containing approximately 200 μ g of protein were assayed for NOS in a assay system containing Tris-HCl (pH 7.9), tetrahydrobiopterin (4

mM), DTT (3 mM), FAD (4 mM), L-arginine (2 mM) and NADPH (2 mM) and protease inhibitors for 16 h at 30°C (). The nitrate formed was reduced to nitrite with 2 units/ml nitrate reductase, and residual NADPH oxidized by the addition of sodium pyruvate (5 μ mol) and lactate dehydrogenase (5 units). The nitrite formed was analyzed by Griess reaction as described earlier (Stuehr and Marletta 1985).

3. Western Blot of iNOS Protein

Cell monolayers were grown in 6 well plates or 25 ml flasks to near confluency and washed with DPBS once. Fresh medium was added and cells were treated with various agents for the required duration as described. The monolayers were rinsed rapidly with ice-cold DPBS. Boiling lysis buffer (125 mM Tris pH 6.8, 2% SDS, 5% glycerol, 0.003 % bromophenol blue and 1% mercaptoethanol) was added (1 ml/flask) and cells were scraped, transferred into microfuge tubes and boiled for an additional 5 min. The viscosity of the sample was reduced by several passages through a 26 gauge needle, and the samples were centrifuged to remove any insoluble material. To 10 μ l boiled lysate (in Laemmli sample buffer) 40 μ l water and 50 μ l of 50% TCA were added and proteins were precipitated for 10 min on ice. This was then centrifuged in a microfuge for 5 min and washed once with 10% TCA. Pellet was then resuspended in 20 μ l of 1M NaOH, 180 μ l of water was added and protein concentration was measured in 100 μ l by the bicinchoninic acid (BCA) method (Pierce, Rockford, Ill). Macrophage lysate was separated on 7.5% reducing SDS-PAGE and transferred in 20% methanol, 25 mM Tris, 192 mM glycine (pH

8.3) to 0.2 μ pore size PVDF membranes (Bio-Rad, CA) (Towbin et al., 1979). The membranes were blocked with 3% BSA in Tris buffered saline (20 mM Tris, pH 7.5, 500 mM NaCl) and incubated overnight with rabbit anti-iNOS polyclonal antibody (Transduction Laboratories, Lexington, KY) (1:3000 in Tween TBS; 20 mM Tris, pH 7.5, 500 mM NaCl, 1% BSA, 0.05% Tween), then washed and re-incubated for 2h with goat anti-rabbit IgG (1:3000 dilution in blocking buffer) conjugated to alkaline phosphatase (Bio-Rad). The membranes were then washed and developed in carbonate buffer (0.1M NaHCO₃, 10 mM MgCl₂, pH 9.8) containing 5-bromo-4-chloro-3-indoyl phosphate (0.15%) and nitroblue tetrazolium (0.3%). In some experiments primary iNOS antibody was diluted to 1:10,000 in 5% non-fat dry milk in Tween TBS and anti rabbit IgG (1:2000 dilution in 5% non-fat dry milk in Tween TBS) conjugated to horseradish peroxidase (New England Biolabs) was used as the second antibody. iNOS protein was then detected by using a chemiluminescent detection system (Phototope®-HRP western blot detection kit, New England Biolabs) and exposing the blot to Kodak Biomax ML film.

4. Northern Blot

Total RNA was isolated by a rapid guanidinium isothiocyanate method as previously described (Chomczynski, and Sacchi, 1987). In some cases mRNA was isolated using the quickPrep micro mRNA purification kit (Pharmacia). RNA was denatured in formamide sample buffer (50%/ formaldehyde (6.5%) at 65°C for 15 min separated on formaldehyde (1.8%)/agarose (0.8%) and transferred to nylon (S&S Nytran) membranes (Schleicher & Schuell, Keene, NH) or Hybond

N+ (Amersham, Ont.) using the turbo blotter system (Schleicher & Schuell, Keene, NH). RNA was fixed by drying the membranes and baking for 2 h at 80°C in a vacuum oven. The membranes were then prehybridized, and hybridized with a cDNA probe specific for murine iNOS (1.8 kb fragment of mouse macrophage iNOS gene, Cayman Chemical Company, Ann Arbor, MI), which was random-primer labeled with [α -³²P]dCTP (3000 Ci/mMol, 10 mCi/ml; Dupont) using the MegaPrime labeling kit (Amersham) according to the manufacturer's instructions. The membranes were either simultaneously hybridized or stripped and reprobed with a random primer radiolabeled GAPDH cDNA probe (Clontech Laboratories, Palo Alto, CA) to control for equal loading of RNA. The membranes were washed twice with 2 x SSC (1 x SSC = 0.3 M sodium citrate and 0.03 M sodium chloride), 0.1% SDS at room temperature, followed by two washes at low stringency (0.2 x SSC, 0.1% SDS at room temperature), two washes at medium stringency (0.2 x SSC, 0.1% SDS at 42°C) and finally two washes at high stringency (final wash, 0.1 x SSC, 0.1% SDS at 50°C). Blots were then rinsed once in 2 x SSC and autoradiography was done by exposing the membrane to Kodak XAR or Biomax film. (Sambrook et al., 1989). Autoradiograph were scanned on LKB 2222-020 enhanced Ultrascan XL laser densitometer and mRNA levels were quantitated by Gelscan XL software (Pharmacia LKB Biotechnology).

Section 4. Studies On The Signaling Mechanism In Macrophages

A. Role of Phospholipases and Protein Kinases on Macrophage Activation

1. Effect of Phospholipase Inhibitors

RAW 264.7 cells were grown in 6 well or 24-well plates to 60-80% confluency. Cells were then washed and fresh medium was added. U73122, an inhibitor of PI-PLC, (Smith et al., 1996) was dissolved in DMSO as a 10 mM stock and diluted in the medium at concentrations ranging from 0 to 50 μ M before adding to culture plates. DMSO diluted in medium to similar concentrations was used as control. D609, a specific inhibitor of PC-PLC (Schutz et al., 1992) was dissolved in water as a 5 mg/ml solution and diluted in medium to the desired concentration. At the start of the experiment, cells were washed and fresh medium was added. Cells were preincubated with various concentrations of inhibitors for 30 min before adding CRP or CRP/IFN- γ . Incubation was continued for 6 to 24 h and medium was aspirated and analyzed for the presence of nitrites. Cells treated similarly were also washed with PBS and SDS sample buffer was added to the culture plates. Cells were scraped off the plates, sonicated briefly (10 s), centrifuged briefly to remove insoluble material and were subjected to SDS PAGE and Western blot analysis to detect iNOS protein.

2. Studies on the Role of Protein Kinase C on Macrophage Activation

Macrophages were grown in 24-well plates to 60-80% confluency. Phorbol-12-myristate13-acetate was dissolved in DMSO at a concentration of 1 mg/ml and was diluted to concentrations ranging from 100 ng to 1 μ g/ml in cell culture medium just before use. Staurosporine, a potent broad spectrum protein kinase inhibitor, was dissolved in DMSO at a concentration of 10 mM and diluted to concentrations ranging from 0 to 20 nM in cell culture medium before use. Bisindolylmaleimide III HCl was dissolved in sterile PBS just before use and diluted in cell culture medium at concentrations ranging from 0.5 to 2 μ M. Just before commencing the experiment, cells were washed and fresh medium was added. Cells were then preincubated with various concentrations of these agents for 10 to 30 min before adding CRP or CRP/IFN- γ . Incubation was continued for 6 to 24 h and the medium was aspirated and analyzed for the presence of nitrites. In some experiments, cells were lysed in SDS sample buffer and stored at -70 $^{\circ}$ C until ready to use.

3. Studies on the Effect of Cyclic AMP Modulating Agents on Macrophage Activation

Dibutyryl cyclic AMP (100 μ M), a cell permeable cAMP analogue that preferentially activates cAMP dependent protein kinases, cholera toxin (1 μ g/ml), which catalyses ADP ribosylation of a subunit of G protein (Gs) and

increases the cellular level of cAMP and pertussis toxin ($1 \mu\text{U/ml}$), that catalyzes the ADP-ribosylation of guanine nucleotide-binding regulatory protein, G_i were added to the culture medium before cell stimulation. Cells were preincubated with various concentrations of these agents as indicated 10-30 min before adding CRP or CRP/IFN- γ . Incubation was continued for 18 to 24 h and medium was aspirated and analyzed for the presence of nitrites.

4. Studies on the Role of Tyrosine Phosphorylation on Macrophage Activation

RAW 264.7 cells were grown in 24-well plates to 60-80% confluency. Cells were then washed and fresh medium was added. Tyrosine kinase inhibitors genistein and tyrphostin AG 126 were dissolved in DMSO at a concentration of 10 mM and diluted in the culture medium at the appropriate concentration (0 to 100 μM) just before adding to the culture plates. DMSO diluted in the culture medium to similar concentrations was used as control. Cells were preincubated with various concentrations of inhibitors for 30 min before adding CRP, IFN- γ or a combination of CRP and IFN- γ . Incubation was continued for 18 to 24 h and medium was aspirated and analyzed for the presence of nitrites.

B. Assays for Mitogen Activated Protein Kinases

1. p42/p44MAPK (ERK) Activation

RAW 264.7 cells (2×10^6 cells per well) were cultured for 2 days in 6 well plates in medium containing 0.5% FCS. Medium was aspirated and fresh medium containing 0.5% FCS was added and cultured for a further two hours before adding CRP or CRP/IFN- γ in fresh medium containing 0.5% FCS, and incubated for various times ranging from 0 to 240 min. At the end of the incubation, media were aspirated and cells were washed once with PBS. Cells were then lysed in SDS sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM DTT and 0.1% bromophenol blue), scraped and sonicated for 10-15 seconds to shear DNA and reduce viscosity. Samples were then heated to 95-100°C for 5 min. 50 to 60 μ g of protein was separated on 12% polyacrylamide gels along with biotinylated protein MW markers and transferred to PVDF membranes. Membranes were then blocked with 5% non-fat dry milk in Tween TBS overnight and probed with a polyclonal antibody against either phosphorylated or phosphorylation-independent p42/p44 MAPK (ERK1/ERK2). Blots were washed and incubated with anti-rabbit IgG (1:2000 dilution in blocking buffer) conjugated to horseradish peroxidase and detected by using the phototope®. HRP Western blot detection kit (New England Biolabs, MA) and exposing to Kodak Biomax ML film. Protein bands were quantitated by using an LKB 2202 Ultrascan laser densitometer (Pharmacia Biotechnology).

ERK and iNOS activation was also assayed in the presence of PD 98059, a

specific inhibitor of MEK activation by Raf kinase (Alessi et al., 1995). PD 98059 was dissolved in DMSO and diluted to concentrations ranging from 0 to 100 μ M in the culture medium just before use. Cells were preincubated with this agent for 60 min before the addition of CRP and CRP/IFN- γ and incubation was continued for periods ranging from 6 to 24 h. Medium was analyzed for the presence of nitrites and cells were lysed and stored at -70 $^{\circ}$ C until use.

2. Assessment Of Phosphotransferase Activity in Macrophages

I Immune Complex Kinase Assay

RAW264.7 cells were grown to near confluency in 6 well plates in RPMI 1640 containing penicillin/streptomycin and 0.5% fetal calf serum . Cells were treated with CRP (50 μ g) for various times as mentioned earlier. At the end of the incubation, culture supernatants were aspirated and cells were washed once with ice cold PBS. 500 μ l of ice cold lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerol phosphate, 1mM sodium metavanadate, 1 μ g/ml Leupeptin and 1 mM PMSF) was added and plates were incubated on ice for 15 min. Cells were scraped off the plate, transferred to microfuge tubes and sonicated 3 x 5 s on ice. Sonicates were centrifuged for 10 min at 4 $^{\circ}$ C at 12000 x g. 200 μ l of the supernatant (containing approximately 200 μ g protein) was precleared with 20 μ l of 50% protein A agarose and immunoprecipitated with phosphospecific p42/p44 MAPK mAb (1:100 dilution) overnight at 4 $^{\circ}$ C with

gentle rotation. Immune complexes were incubated with 30 μ l of 50% protein A agarose beads for 3 h at 4°C and micro centrifuged for 2 min at 12000 x g. The pellet was washed twice with lysis buffer and once with kinase buffer (25 mM Tris (pH 7.5), 5 mM β glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄ and 10 mM MgCl₂). The pellet was suspended in 50 μ l of kinase buffer containing 200 μ M ATP and 2 μ g Elk fusion protein and was incubated for 30 min at 37 °C. The reaction was terminated by the addition of 25 μ l of 3X SDS sample buffer (187 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 150 mM DTT, 0.3% bromophenol blue). An aliquot of the reaction mixture (25 μ l) was separated on 10 or 12% SDS PAGE, transferred to a PVDF membrane, probed with a polyclonal antibody specific to phosphorylated Elk and detected by chemiluminescence reagents (New England Biolabs) and quantitated by densitometric analysis using LKB 2202-02 laser densitometer (Pharmacia LKB).

3. MAP Kinase Kinase (MEK, MAPKK) Activation

MEK activation was assessed by a method similar to that described above except that an antibody that detects MAP kinase kinase (MEK) only when phosphorylated at ser 217/221 was used to analyze MEK activation by CRP, and was detected by chemiluminescent reagents.

4. Stress-Activated Protein Kinase (SAPK) Activation

SAPK activation was assessed by a method similar to that described above except that a polyclonal antibody that detects SAPK only when phosphorylated

at Thr183/Tyr185 was used to analyze SAPK activation by CRP.

5. Inhibitor kappa B (I- κ B) Phosphorylation.

Phosphorylation of I- κ B was determined as described for p42/p44 MAPK except that a specific antibody raised against a synthetic phospho-Ser32 peptide corresponding to residues 26 to 39 (DDRHDS*GLDSMKDC) of I- κ B- α (New England Biolabs). I- κ B- α antibody (phosphorylation-state independent) was also used as a control for immunoblotting.

In some experiments, pyrrolidinedithiocarbamate (PDTC) an inhibitor of NF- κ B activation (Xie et al., 1994; Kleinert et al., 1996), was used to inhibit NF- κ B processing. For this, PDTC was dissolved in PBS and used at concentrations ranging from 0 to 100 μ M. Medium was analyzed for the presence of nitrites and cells were lysed and stored at -70 $^{\circ}$ C until use.

Section 5. Studies on the Antimicrobial Properties of CRP

1. Preparation of *Chlamydia trachomatis* Stock Culture.

C. trachomatis type H (ATCC 879) and McCoy cells (ATCC CRL 1696) were obtained from American Type Culture Collection. McCoy cells were grown in RPMI 1640 medium (Gibco, 11835-030) containing glutamine but not phenol red and supplemented with 10% heat-inactivated fetal bovine serum (FBS). The growth medium also contained vancomycin (100 µg/ml), gentamicin (10 µg/ml) and amphotericin B (2 µg/ml). 24-well cell (12 mm) culture plates (Falcon 3047) were seeded with 1 ml cell suspension containing approximately 300,000 cells per well and incubated at 35°C under 5% CO₂ until complete monolayers were formed. The cell culture was ascertained to be free of contamination at all times. Frozen ampoules of *C. trachomatis* were quick thawed in a water bath at 35°C. Growth medium was removed from the monolayers and each well was inoculated with 100 µl of the thawed *C. trachomatis* suspension. The culture plates were covered with a plate sealer and cover and centrifuged at 700 x g at 35°C for 90 min. The inoculum was then aspirated and replaced with 1 ml of fresh chlamydia growth medium. The composition of the growth medium was the same as above except that it was supplemented with 2 % heat-inactivated fetal bovine serum (FBS) and contained 2 µg/ml cycloheximide. The plates were incubated as before for 48 hr

and harvested for blind passaging of *C. trachomatis*. For this, the infected monolayers were disrupted by a quick freeze-thaw cycle and the cells scraped and sonicated for 10 min to obtain the *C. trachomatis* culture suspension. The above procedure was repeated until the culture showed 80-90% infectivity as demonstrated by *C. trachomatis* inclusions. The monolayers were finally harvested by one cycle of freeze-thaw and the cell suspension was placed in a conical centrifuge tube with beads and vortexed for 1 min, dispensed in 5 ml volume and sonicated for 10 min to disrupt the infected cells. The suspensions were combined in conical tubes and centrifuged at 400 x g for 7 min to pellet cellular debris. The supernatant served as the *C. trachomatis* inoculum which was dispensed into 5 ml aliquots and stored frozen at -70°C until ready to use.

2. Preparation of Cell Culture, Priming of McCoy cells with IFN- γ and CRP and Inoculation of *C. trachomatis*.

McCoy cell monolayers were grown in 75 cm² cell culture flasks with growth medium containing 10% FBS, trypsinized, and a cell count was done. Each well of four sets of the 24-well cell culture plates were seeded with 1 ml cell suspension containing 500,000 cells in growth medium with 10% FBS. Two sets of the plates contained 12 mm cover slips to facilitate staining of monolayers and quantitation of inclusion bodies. The remaining two sets were utilized to determine the number of infectious particles by titration. All plates were incubated at 35°C under 5% CO₂ until complete monolayers were formed.

At 10 h prior to inoculation with *C. trachomatis*, the growth medium was aspirated and 1 ml maintenance medium with 2% FBS was added to each well. To this, the following signals were added at the indicated concentrations: IFN- γ (50 units), CRP (50 μ g) or IFN- γ (50 units + CRP 50 μ g), with and without NMMA (0.4 mM). Ten hours after the signals were added, the growth medium containing the signals was aspirated and saved.

The stock *C. trachomatis* suspension was quick thawed at 35°C and a 1:100 dilution was prepared in maintenance medium. This was mixed well by vortexing, and 100 μ l of the suspension was then added to all cell culture wells except the cell culture control wells. The plates were centrifuged at 700 x g for 90 min at 35 °C. The saved growth medium containing the signals was returned to the respective wells, and the plates were incubated at 35°C for 48 h under 5% CO₂.

3. Direct Quantitation of *C. trachomatis* Inclusions.

For this purpose, those culture plates containing cover slips were used. The culture supernatant was utilized to measure NO production by McCoy cells. For the latter purpose, 200 μ l of the culture supernatant was aspirated into glass vials at 24 and 48 h incubation and analyzed for NO as described below. The remaining medium was aspirated and the monolayers on the cover slips were fixed with 1 ml methanol for 10 min. Methanol was aspirated and the fixed monolayers were briefly rinsed with PBS. Each cover slip was carefully covered with 50 μ l of FITC-conjugated antibody to *C. trachomatis* major outer

membrane protein (anti MOMP) (Syva Micotrak-Behring, Cupertino, CA), and the stain was allowed to react for 15 min at room temperature. The cover slips were then carefully removed from the cell culture wells with forceps, the edges gently touched to blotting paper to remove excess stain and then placed face down in a drop of mounting medium containing glycerol on a microscope slide. The cover slips were read under an epifluorescence microscope and the number of inclusions on each cover slip was counted and photograph of the representative areas of the monolayers were taken.

4. Quantitation of Chlamydial Infectivity.

For this purpose, the second set of culture plates containing no cover slips was used. The monolayers were disrupted by a quick freeze-thaw cycle, the cells adhering to the bottom of the wells scraped and sonicated for 1 min in a water bath sonicator. Cell suspensions were then aspirated from individual wells into test tubes with glass beads and vortexed to release *C. trachomatis* inclusion bodies. Ten-fold serial dilutions (10^{-1} - 10^{-6}) of each of the suspensions were prepared in maintenance medium containing 2 % FBS.

Fresh monolayers of McCoy cells were prepared in growth medium with 10% FBS in 24-well cell culture plates containing cover slips. Prior to inoculation, the growth medium was removed and 100 μ l of each dilution of *C. trachomatis* suspension was inoculated onto the monolayer, plates then centrifuged, fresh growth medium containing cycloheximide was added and the plates incubated as before for 48 h. The monolayers on the cover slips were fixed, stained and

examined under the microscope as described before and the number of inclusions were quantitated.

Statistical Analysis

The data are expressed as mean \pm SD. Student's unpaired t test was used when comparing two groups and one way analysis of variance (ANOVA) was used when making multiple comparisons. All statistical calculations were performed using 'Graph Pad InStat' software. Data were considered significant when $p < 0.05$

INDUCTION OF NITRIC OXIDE SYNTHASE IN MACROPHAGES BY CRP

Section 1. Introduction

The biological response to tissue injury or infection is characterized by an acute phase response that includes the amplified synthesis of CRP leading to as much as a 1000-fold increase over normal serum levels (Morley and Kushner, 1982). It is also remarkable that CRP has been conserved throughout vertebrate evolution (Pepys, 1981). Although the phylogenetic conservation of CRP points to its important role during periods of immunological challenge, no precise function of this protein has yet been elucidated. Interactions of CRP and cells of the immune system are well documented (Tebo and Mortensen, 1990; Tebo and Mortensen, 1991; Zahedi et al., 1989; Buchta et al., 1987; Nagpurkar et al., 1993). Several reported biological activities have also indicated a pro-inflammatory role for CRP (Zahedi et al., 1989; Barna et al., 1993; Ballou et al., 1992; Tilg et al., 1993; Pue et al., 1996; Galve-de Rochemonteix et al., 1993). Various studies have shown that human CRP modulates the generation of reactive oxygen intermediates (ROI) in neutrophils, monocytes and macrophages (Buchta et al., 1987; Shephard et al., 1990; Dobrinich and Spagnuolo, 1991; Tebo and Mortensen, 1991). Biochemical mechanisms that

can explain the activated macrophage cytotoxicity include the synthesis ROI such as superoxide ($O_2^{\cdot-}$) by NADPH oxidase, as well as the production of reactive nitrogen intermediates (RNI) such as nitric oxide (NO) by nitric oxide synthase (iNOS) (Bastien and Hibbs, 1994). CRP also has been reported to induce tumoricidal activity (Tebo and Mortensen, 1991), and since tumoricidal activity in macrophages has been linked to their capacity to generate NO (Xie et al., 1995), it seemed likely that CRP may also be involved in the regulation of macrophage functions relating to the generation of cytotoxicity. A study was undertaken to test this hypothesis using a macrophage model and the results showed that CRP did indeed activate the synthesis of iNOS protein, and thus may actively participate in the control of inflammatory response.

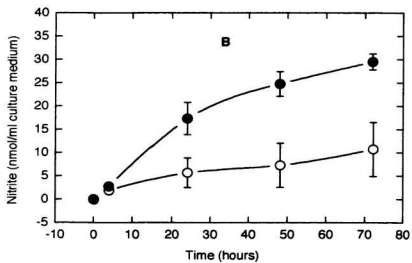
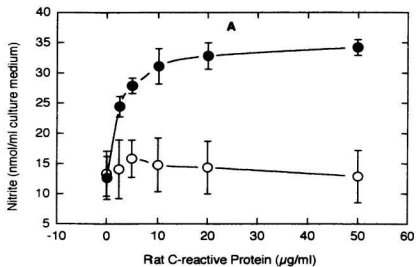
Section 2. Results

A. Induction of iNOS by CRP

To determine whether CRP could induce NO synthesis, rat peritoneal macrophages were stimulated with CRP or an equal volume of Tris buffer, the diluent of CRP. Control rat peritoneal macrophages exhibited a small but significant baseline NOS activity as measured by nitrite production in the culture medium (Fig. 1A). Basal NO release by rat peritoneal macrophages was variable. However, an increase in nitrite production was detectable when cells were stimulated with a minimum CRP concentration of 2.5 μ g/ml, and this

Fig. 1. Effect of CRP on generation of NO in rat macrophages. Rat peritoneal macrophages were incubated for 18 h with CRP (●) or an equal volume of buffer (○) and aliquots of the medium were analyzed for nitrite by Griess reaction. **A.** Effect of increasing concentrations of CRP on NO synthesis by rat peritoneal macrophages. **B.** Rat peritoneal macrophages were incubated with (●) or without (○) rat CRP (20 μ g/ml) for varying times. Error bars represent mean \pm SD of six experiments.

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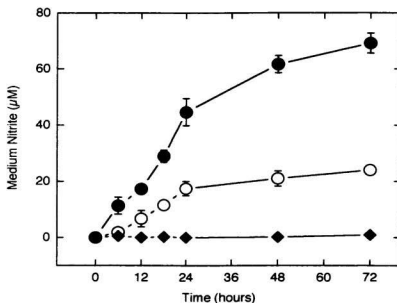


Fig. 2. **Stimulation of NO generation in RAW 264.7 cells by CRP.**

RAW 264.7 cells were incubated with rat CRP (50 µg/ml) in the presence (●) or absence (○) of IFN-γ (50 units/ml) for various time intervals. Control cells were incubated with an equal volume of buffer (◆). Aliquots of the medium were analyzed for nitrite. Values are expressed as mean ± S.D. of three to six separate experiments.

reached a maximum level of more than two-fold at a concentration of 20 $\mu\text{g/ml}$. The stimulation by CRP also resulted in a time-dependent accumulation of nitrite in the medium which reached a maximum level at 48 h and was sustained for at least 72 h (Fig. 1B).

Stimulation of RAW 264.7 cells with CRP also resulted in the release of modest, but significant, amounts of NO, measured as nitrite in the medium. Untreated control RAW 264.7 cells did not generate any NO (Fig. 2A). There were detectable levels of nitrite accumulation by 8 h after CRP treatment which progressively increased over 72 h. Although the production of nitrite by RAW 264.7 cells stimulated by CRP alone was modest, addition of IFN- γ (50 units/ml) along with CRP in the incubation medium resulted in a substantial increase in the range of 3-4 fold in nitrite production (Fig. 2A). Presence of nitrite was detectable in the medium within 4 h, when macrophages were stimulated by CRP in the presence of IFN- γ . The synergistic activation of NOS in RAW 264.7 cells by CRP and IFN- γ is shown in Fig. 3. Combinations of CRP (0-100 $\mu\text{g/ml}$) and IFN- γ (0 to 100 units/ml) in a checker board layout assay showed that, while there was a concentration-dependent increase in NO generation when cells were stimulated with either CRP or IFN- γ , the combination of these agents produced an impressive synergistic effect resulting in a dramatic increase in NO synthesis. A CRP concentration as low as 10 $\mu\text{g/ml}$ was sufficient to produce near maximum increase of NO generation in the presence of 10 units/ml of IFN- γ .

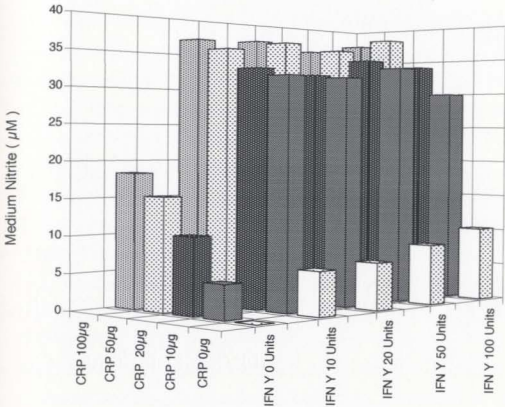


Fig. 3. **Synergistic effect of increasing concentrations of CRP and IFN- γ on generation of NO in RAW 264.7 cells.** RAW 264.7 cells were incubated for 24 h with increasing concentrations of rat CRP and IFN- γ in a checker board layout and aliquots of the medium were analyzed for nitrite as in Fig.1. Bar graph shown represent the mean of three to six separate experiments.

B. NOS Enzyme Activity

Arginine analogs such as N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (NMMA), which are potent inhibitors of NOS activity, inhibited nitrite synthesis by rat peritoneal macrophages stimulated by CRP. Addition of L-NAME at concentrations ranging from 0-2 mM to these cells progressively inhibited nitrite production (Fig. 4A). In RAW 264.7 cells, L-NMMA was found to be the more potent inhibitor of iNOS. Nitrite production by CRP/IFN- γ stimulated RAW cells was completely abolished at a concentration of 2 mM NMMA. In contrast, a concentration of 10 mM of L-NAME was required to inhibit 85% of nitrite synthesis by CRP/IFN- γ treated RAW cells (Fig 4B). This indicated that the NO generated by macrophages on CRP stimulation was derived from L-arginine.

To determine that the accumulation of nitrite was indeed due to the oxidation of arginine to NO and citrulline catalyzed by iNOS, iNOS enzyme activity was assessed in RAW 264.7 cell extracts. Catalytic activity of iNOS was assayed in cell lysates and 100,000 x g supernatant (cytosol) fractions prepared from control cells and from RAW 264.7 cells treated with CRP and CRP/IFN- γ . An increase in NOS activity was seen in both cell lysates and cytosol obtained after stimulation with either CRP or CRP/IFN- γ . In this study, CRP (50 μ g/ml) or IFN- γ (50 units/ml) independently stimulated NOS activity but their combination resulted in a dramatic increase in NOS activity in both cell lysates and cytosol of RAW 264.7 cells (Fig 5A and 5B).

Fig. 4. Effect of L-arginine analogues on the CRP-induced NO generation. **A.** Rat peritoneal macrophages were stimulated with rat CRP (20 $\mu\text{g/ml}$) in the presence of increasing concentrations of L-NAME for 18 h and medium nitrite were analyzed by Griess reaction. Control cells (○) CRP treated cells (●). **B.** RAW 264.7 cells were incubated for 24 h with rat CRP (50 $\mu\text{g/ml}$) and IFN- γ (50 units/ml) in presence of increasing doses of arginine analogues, L-NAME (○) or NMMA (●) and aliquots of the medium were analyzed for nitrite production as in **A**. Values are expressed as mean \pm S.D. of three to six separate experiments.

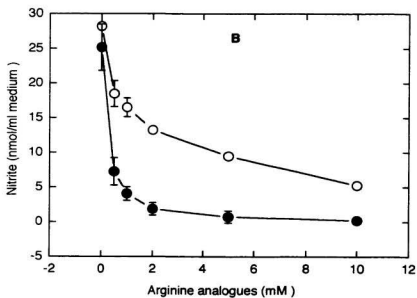
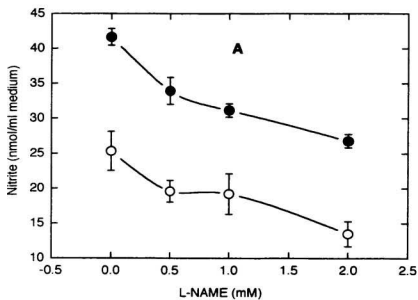
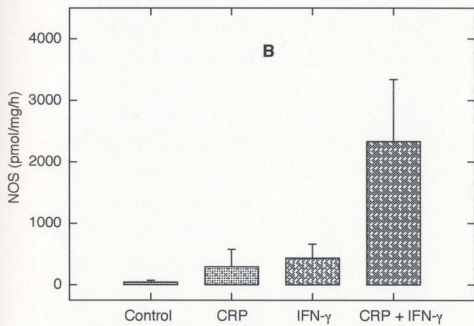
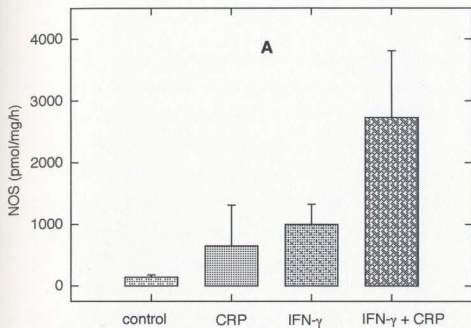


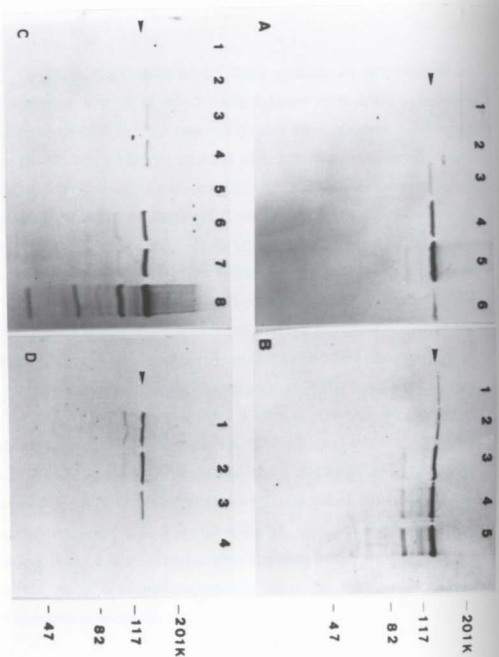
Fig. 5. Effect of CRP on nitric oxide synthase activity in the cell lysates and cytosol of RAW 264.7 cells. RAW 264.7 cells were incubated with rat CRP (50 μ g/ml), IFN- γ (50 units/ml) or combination of CRP + IFN- γ for 16 h. Cells were then lysed (lysate) and 100,000 x g supernatants (cytosol) were prepared. Both cell lysates (**A**) and cytosol (**B**) were assayed for iNOS activity as described in "Materials and Methods". Values are expressed as mean \pm S.D. of three to six separate experiments.



C. Western Blot Analysis of iNOS Protein

Western blot analysis was carried out using a polyclonal antibody specific to iNOS to detect NOS protein in the rat peritoneal macrophage cell and RAW 264.7 cell lysates obtained after stimulation with CRP. The Western blot results provided further confirmation of NOS induction by CRP. The induction of iNOS was found to be concentration-dependent by Western blot analysis as well. iNOS protein was detectable by Western blot when rat peritoneal macrophages were treated with as little as 5 $\mu\text{g/ml}$ of CRP and RAW 264.7 cells treated with 10 $\mu\text{g/ml}$ (Fig. 6A and B). Western immunoblot analysis was also carried out in RAW 264.7 cell lysates treated with CRP and/or IFN- γ . The results again confirmed that CRP did indeed induce an increase in iNOS protein levels (Figs. 6A, 6C). Human CRP (20, 50 and 100 $\mu\text{g/ml}$) and IFN- γ (50 units/ml) by themselves increased iNOS protein levels in RAW 264.7 cells, but when CRP (20 $\mu\text{g/ml}$) was used in combination with IFN- γ (50 units/ml), there was a dramatic synergistic effect on iNOS concentration (Fig. 6A). Similarly rat CRP increased iNOS protein synthesis in RAW 264.7 cells. Rat CRP (10, 20 and 50 $\mu\text{g/ml}$) induced a concentration dependent increase in iNOS protein levels and together with IFN- γ (50 units/ml) showed synergistic increase in iNOS protein concentration confirming the results shown in figures 2 and 3. Fig. 6D shows the time course of iNOS induction in RAW 264.7 cells stimulated with CRP (20 $\mu\text{g/ml}$) and IFN- γ (50 units/ml). In these cells, iNOS protein was clearly detectable at 6 h post stimulation. This seemed to reach a maximum at 12 h, and although there was no net increase in iNOS protein level, more degradative products seemed to appear at 24 h after stimulation (Fig. 6D).

Fig.6. Western blot analysis of iNOS protein in rat peritoneal macrophages and RAW 264.7 cells treated with CRP. Cells were stimulated with CRP for 18 h and cell lysates were prepared. 10-20 μ g lysate protein was separated on 7.5% SDS gels and immunoblotted and probed with an antibody to murine iNOS. Bound antibodies were detected by alkaline phosphatase conjugated second antibody as described in materials and methods. Method of stimulation is indicated. **A.** RAW 264.7 cells treated with human CRP; lane 1, untreated; lanes 2, 3 and 4, human CRP 10, 20 and 50 μ g/ml respectively; lane 5, human CRP 20 μ g and IFN- γ 50 units per ml; lane 6, IFN- γ 50 units/ml. **B.** Rat peritoneal macrophages treated for 18 h with different doses of rat CRP; lane 1, untreated; lanes 2, 3, 4, 5 rat CRP 5, 10, 20, 50 μ g/ml, respectively. **C.** RAW 264.7 cells treated with rat CRP; lane 1, untreated; lanes 2, 3, 4, rat CRP 10, 20, 50, μ g/ml respectively; lane 5, empty lane; lane 6, CRP 10 μ g/ml and IFN- γ 50 units; lane, 7. CRP 20 μ g and IFN- γ 50 units/ml; lane 8, rat CRP 50 μ g and IFN- γ 50 units/ml. **D.** RAW 264.7 treated with IFN- γ 50 units and rat CRP 50 μ g/ml for different times; lanes 1, 2, 3, 4 treated for 24, 12, 6 and 0 h respectively. Arrow (\blacktriangleright) designates iNOS protein (MW 130 kDa). Lower molecular weight bands denote iNOS protein degradation bands. Blots represent at least three separate experiments.



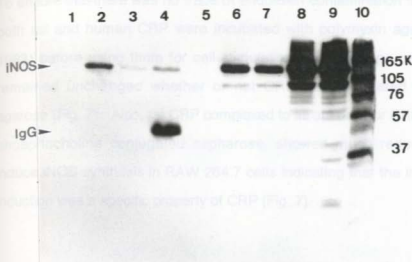


Fig. 7. Specificity of CRP in stimulating iNOS induction. RAW 264.7 cells were stimulated with CRP for 18 h and cell lysates were prepared. 10-20 μ g lysate protein was separated on 7.5% SDS gels, immunoblotted and probed with an antibody to murine iNOS. Method of stimulation is indicated. Lanes: 1, Control; 2, rat CRP (50 μ g/ml); 3, rat CRP incubated with phosphocholine conjugated sepharose. 4, rat CRP incubated with antibody to rat CRP; 5, IFN- γ (20 units/ml); 6, human CRP (50 μ g/ml); 7, human CRP incubated with polymyxin agarose; 8, IFN- γ (20 units/ml) and rat CRP (50 μ g/ml); 9, IFN- γ (20 units/ml) and rat CRP incubated with polymyxin agarose (50 μ g/ml); Lane 10, Biotinylated MW standards. Blot is representative one of three experiments with similar results.

To ensure that there was no trace of endotoxin contamination in CRP samples, both rat and human CRP were incubated with polymyxin agarose (Issekutz, 1983) before using them for cell stimulation. Macrophage response to CRP remained unchanged whether or not CRP was incubated with polymyxin agarose (Fig. 7). Also, rat CRP complexed to its antibody or CRP incubated with phosphocholine conjugated sepharose, showed much reduced capacity to induce iNOS synthesis in RAW 264.7 cells indicating that the increase in iNOS induction was a specific property of CRP (Fig. 7).

D. Northern Blot Analysis of iNOS Induction by CRP

Northern blot analysis was carried out to determine whether CRP influenced iNOS mRNA transcription. Treatment of rat peritoneal macrophages with rat CRP (10-50 $\mu\text{g/ml}$) increased iNOS mRNA levels in these cells (Fig. 8). Laser densitometric analysis showed that when normalized for GAPDH mRNA levels, there was a two-fold increase in iNOS levels in cells treated with 50 $\mu\text{g/ml}$ CRP as compared to control cells. The iNOS mRNA appeared as a single band of 4.2 kilobases.

In RAW 264.7 cells, iNOS mRNA was not apparent in control cells (Fig. 9A). However, iNOS mRNA was clearly detectable when cells were stimulated for 12 h by human CRP alone (10-50 $\mu\text{g/ml}$). At this time iNOS mRNA induced by IFN- γ (20 units/ml) alone was also detectable. Stimulation with both CRP and IFN- γ together resulted in substantial iNOS mRNA synthesis (Fig. 9A).

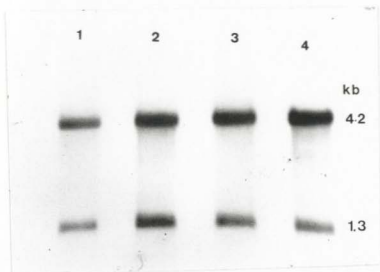
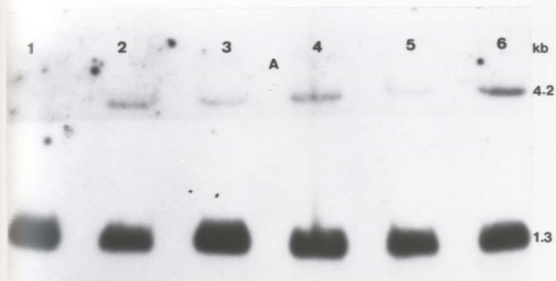


Fig. 8. Northern blot analysis of iNOS mRNA in rat peritoneal macrophages. Rat peritoneal cells were stimulated for 12 h with varying concentrations of rat CRP. Total RNA isolated from control and CRP stimulated cells was separated and iNOS mRNA (4.2 kb) and phosphoglycerinaldehyde dehydrogenase mRNA (1.3 kb) were analysed as described in materials and methods. lane 1, untreated. Lanes 2, 3, 4, rat CRP 10, 20, or 50 μ g per ml, respectively. Blot represents typical results from one of 3 separate experiments.

Fig. 9. Effect of CRP or CRP and IFN- γ on iNOS mRNA expression in RAW 264.7 cells. RAW 264.7 cells treated with human or rat CRP in the presence or absence of IFN- γ for indicated periods and total RNA was isolated. 15-20 μ g of total RNA was separated, blotted and iNOS mRNA (4.2 kb) and GAPDH mRNA (1.3 kb) were analyzed by northern blot hybridization. **A. Effect of human CRP.** RAW 264.7 cells treated with human CRP for 12 h; lane 1, control; lanes, 2, 3, 4, human CRP 10, 20, 50, μ g/ml, lane 5, IFN- γ (20 units /ml); lane 6, human CRP 20 μ g and IFN- γ 20 units/ml). **B. Effect of rat CRP.** RAW 264.7 cells treated with rat CRP for 8 h; lane 1, control; lane 2, rat CRP 50 μ g/ml; lane 3, IFN- γ (50 units/ml); lane 4, rat CRP 50 μ g/ml and IFN- γ (50 units/ml). Blots represent results of one of three experiments with similar results.



In these experiments, the housekeeping gene GAPDH mRNA was expressed equally in both control and CRP treated cells confirming that iNOS mRNA was induced only when stimulated by CRP, IFN- γ or a combination of both. Although iNOS mRNA was barely visible at 8 h post-stimulation when treated with rat CRP (20 μ g/ml) or IFN- γ (50 units/ml) individually, this was also substantially increased when cells were co-stimulated with 50 μ g/ml rat CRP and 50 units of IFN- γ . (Fig. 9B).

E. Effect of Phosphocholine and Bivalent Cations on CRP Induced Macrophage NO Synthesis

Many of the biological actions of CRP have been attributed to its property of binding to phosphocholine. To test whether the activation of iNOS in macrophages by CRP may involve its binding to this ligand, RAW 264.7 cells were incubated with CRP in the presence of phosphocholine. Addition of phosphocholine up to 1000 X molar excess had no effect on iNOS stimulation by rat CRP (Fig. 10A). This suggested that iNOS induction, and presumably the binding to or uptake of CRP by macrophages, was not mediated through the phosphocholine-binding site of rat CRP. In contrast, EDTA inhibited NOS activation in these cells in a dose-dependent manner (Fig. 10B). Since iNOS enzyme activity does not depend on Ca^{++} levels, this may indicate that the binding of CRP to macrophages may depend on the presence of bivalent cations as shown previously (Tebo and Mortensen, 1991; Nagpurkar et al., 1993)

F. Effect of Conditioned Medium on Macrophage Activation by CRP

IFN- γ stimulated macrophage-conditioned medium was prepared by incubating RAW 264.7 cells at 37 °C with IFN- γ (50 units/ml) or an equal volume of PBS in RPMI 1640 containing FCS and antibiotics for 60 min and aspirating the medium (IFN-CM-1 and PBS-CM-1). Cells were then washed twice with PBS, fresh medium was added and incubation continued for another 60 min. This second lot of medium (IFN-CM-2 and PBS-CM-2) was aspirated and cells were incubated in fresh medium for another 24 h. Fresh cells were then incubated with IFN-CM-1 and IFN-CM-2 as well as PBS-CM-1 and 2 with CRP (50 μ g) for 48 h and accumulated nitrite was measured using Griess reagent. The results showed that there was no significant difference in nitrite generation between cells treated with IFN-CM-1 and IFN-CM-2 (p value = >0.05). However, the difference in nitrite production between cells treated with PBS-CM-1 and cells treated with IFN-CM-1 or IFN-CM-2 was highly significant (p value = <0.001 , Table 1). Raw 264.7 cells were also stimulated for periods ranging from 10 min to 6 h with CRP, IFN- γ or a combination of both. Medium containing agents was removed at the end of the prescribed period and cells were incubated for 24 h in fresh medium. Fig. 11 shows that a significant increase was seen with 1 h of stimulation with CRP, CRP/IFN and near maximum responses were obtained when cells were treated for 4 h with these agents.

Fig. 10. Effect of phosphocholine and EDTA on CRP stimulated NO synthesis. **A.** 50 $\mu\text{g/ml}$ CRP (0.4 μM) was mixed with various concentrations of phosphocholine for 15 min before adding to macrophage cultures. RAW 264.7 cells were incubated for 24 h and medium nitrites were assayed. Phosphocholine concentrations are as indicated. **B.** RAW 264.7 cells were incubated with buffer, CRP (50 $\mu\text{g/ml}$) and CRP (50 $\mu\text{g/ml}$) and IFN- γ (50 units/ml) for 24h in the presence of EDTA concentrations ranging from 0 to 5 mM and medium nitrites are analyzed as described. Control (\circ). CRP (\blacktriangledown). CRP/IFN- γ (\bullet). Data represent mean \pm S.D. of three separate experiments.

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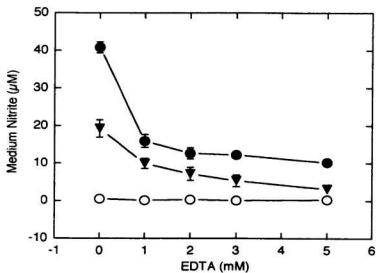
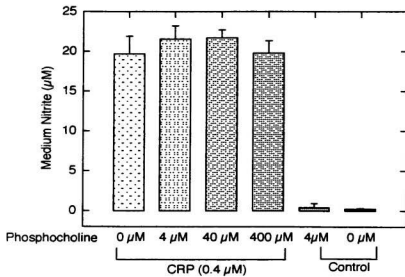


Table 1. Effect of IFN- γ pretreatment of macrophages on stimulation by CRP.

Stimulus	PBS-CM-1	PBS-CM-2	IFN-CM-1	IFN-CM-2
Control	0.00 μ M	0.01 μ M	26.16 \pm 0.67 μ M	26.81 \pm 3.6 μ M
CRP (50 μ g/ml)	N.D	24.85 \pm 1.95 μ M	71.54 \pm 3.13 μ M	56.47 \pm 6.24 μ M

RAW 264.7 cells were incubated in PBS (control) or IFN- γ conditioned medium and stimulated with rat CRP (50 μ g/ml) for 48 h and medium nitrite was analyzed by Griess reaction as described. Data represent mean \pm SD of net nitrite (μ M) produced in 6 experiments. N.D, not determined

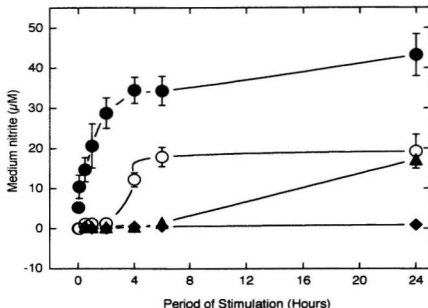


Fig. 11. **Time of stimulation required for iNOS induction.** RAW 264.7 cells were exposed to 50 $\mu\text{g/ml}$ of CRP (○) 50 units/ml of IFN- γ (▲) and a combination of both (●) for periods ranging from 10 min to 6 h. Control cells (◆) were treated with equal volume of PBS. Culture supernatants were aspirated at the end of the prescribed period and incubation was continued for 24 h. Cells were also incubated for 24 h with medium containing these agents. Aliquot of the medium was analyzed for nitrite by Griess reaction. Data represent mean \pm SD of net nitrite (μM) produced in 4 to 6 experiments.

Section 3. Discussion

Data obtained from the above studies show that CRP induces a significant increase in steady state iNOS protein levels with a concomitant increase in the production of NO in rat peritoneal macrophages and established murine continuous cell line RAW 264.7. Rat peritoneal macrophages exhibited a small but significant basal NOS activity. While this baseline NOS activity was variable, stimulation by CRP consistently increased NO production by two to three-fold depending on the duration of incubation. Baseline NOS activity, iNOS protein and mRNA in rat peritoneal macrophages could be due to partial activation during the isolation of the cells or due to thioglycollate treatment of the rats. Similar observations were previously noted in thioglycollate-elicited mouse macrophages (Novogrodsky et al., 1994). The induction of NOS activity by CRP was time- and dose-dependent, and was demonstrated not only in rat peritoneal macrophages but also in RAW 264.7 cells. The RAW 264.7 cell line was specifically chosen because the biochemical and molecular regulation of iNOS in these cells has been well characterized (Xie et al., 1992). In this cell line, CRP induced NOS activity at a minimal concentration of 5 $\mu\text{g/ml}$, and the synergistic effect of CRP with IFN- γ was found to be quite dramatic. The synergy was more pronounced when concentrations lower than 20 $\mu\text{g/ml}$ of CRP were used. CRP induced iNOS enzyme is also catalytically active as demonstrated by its ability to catalyze the conversion of L-arginine to citrulline and NO. This enzyme activity was present in cell lysates and cytosol obtained from CRP-

stimulated macrophages. The L-arginine analogues, L-NAME and NMMA are potent inhibitors of NOS (Southan and Szabo, 1996), and the observation that they inhibit CRP or CRP/IFN- γ -induced NO production provides evidence that this stimulation is indeed mediated through an increase in iNOS enzyme activity. Western blotting provided further evidence that there was an increase in iNOS protein levels following the addition of CRP alone or CRP/IFN- γ in combination. These data also showed that the observed increase in medium nitrite levels reflected an increase in enzyme protein levels and not an increase in the enzymatic activity. This effect of CRP is preceded by a proportional increase in iNOS mRNA level showing that CRP was able to trigger an increase in gene transcription in macrophages.

Many of the biological activities of CRP have been attributed to its ability to bind to phosphocholine. The phosphocholine-binding sequence on CRP polypeptide has been conserved through evolution and remains a distinct property of these proteins. This study has shown that the presence of 1000 x molar excess of phosphocholine in the medium did not prevent the activation of iNOS by CRP in macrophages. This may be because phosphocholine does not impede the binding of CRP to macrophages. This inference is consistent with the reports which showed that the binding of human CRP to monocytes (U-937 cells) or rat CRP to rat peritoneal macrophages was not inhibited by phosphocholine (Tebo and Mortensen, 1990, Nagpurkar et al., 1993). Binding of CRP was proposed to involve a site other than the phosphocholine binding site on CRP. This also indicates that binding of CRP to macrophages may involve specific receptors as described in monocytes (Tebo and Mortensen,

1990, 1991). However, the activation of iNOS was inhibited by EDTA showing that bivalent cations are important in CRP-mediated macrophage activation. Since iNOS activity (unlike the constituent isoforms) is not dependent on the alterations of Ca^{++} concentrations, and because EDTA was added to the extracellular medium, these results may also be interpreted to mean that EDTA interfered with the binding of CRP to macrophages. This again is consistent with the previous reports showing Ca^{++} dependent binding of CRP to its receptor (Tebo and Mortensen, 1990, Nagpurkar et al., 1993).

The results presented here also indicate that the induction of iNOS protein in macrophages is a specific property of CRP. Incubation of rat CRP with protein A agarose coupled to a specific antibody against it or phosphocholine-conjugated sepharose, resulted in the loss of much of its iNOS inducing property. This indicates that specific removal of CRP from the medium reduced its macrophage activating property. To address the possibility that the response of RAW 264.7 cells to CRP might be due to contamination with endotoxin, CRP was incubated with polymyxin agarose for at least two hours before it was used to stimulate RAW 264.7 cells. Polymyxin binds to endotoxin avidly (Issekutz, 1983). CRP exposed to polymyxin agarose retained the ability to activate iNOS in RAW 264.7 cells. In addition, random batches of CRP were also tested for the presence of endotoxin. CRP samples had no detectable endotoxin contamination as judged by limulus amoebocyte assay.

Unlike endothelium and neurons which produce NO through a calcium and calmodulin-dependent constitutive form of NOS, the production of NO in

macrophages and many other types of cells is mediated through calcium-independent isoform of NOS (Nathan et al., 1994; Xie et al., 1992). iNOS is known to be induced during inflammation by IFN- γ , LPS and various combinations of cytokines (Nathan and Hibbs, 1991). Macrophages can also be primed by IFN- γ to produce an abundance of NO when these cells are exposed to a second signal such as LPS or TNF. A distinctive feature of nearly all cell types that express iNOS is the strong synergy exerted by multiple agents (Nathan and Hibbs, 1991). Periods of inflammation or trauma are also marked by a dramatic increase in CRP, whose level increases from trace amounts to as much as 100-300 $\mu\text{g/ml}$ in humans (Gewurz, 1982; Kushner, 1982; Zahedi et al., 1989). In the rat however, CRP is normally present in serum (500-600 $\mu\text{g/ml}$) and increases two-to three-fold during acute phase (Sambasivam et al., 1993). Rat, rabbit and human CRP induced iNOS activity in rat peritoneal macrophages (Ratnam and Mookerjee, 1998). This is not surprising considering the high degree of homology between CRP from these species. Both human and rat CRP have been shown to bind and interact with neutrophils (Dobrinich, and Spagnuolo, 1991), monocytes (Tebo and Mortensen, 1990, 1991) and macrophages (Zahedi et al., 1989). Human CRP also acts as an opsonin and takes part in complement activation (Kilpatrick and Volanakis, 1985). CRP and peptides generated from CRP have been shown to induce tumoricidal activity in macrophages (Barna et al., 1987, Tebo and Mortensen, 1991). It is conceivable that such a molecule may assume the role of a second signal in the induction of iNOS enzyme in partially-activated or primed macrophages. Although two bovine pentraxins whose concentrations ranged from 5 to 40 $\mu\text{g/ml}$ have been identified neither of them behave like an acute phase reactant nor do they bind

to phosphocholine (Maudsley et al., 1987). Hence it is not surprising that the presence of 10% FBS in the culture medium had no effect on iNOS induction.

The experiments with conditioned medium show that once the cells are primed by IFN- γ , they continue to amplify the response to CRP in RAW 264.7 cells despite the absence of IFN- γ in the medium. Moreover, these cells may secrete substances into the medium that may either generate active peptides or may alter the macrophage cell surface membranes to increase its capacity to bind and internalize CRP. CRP and peptides generated from CRP by neutrophil membrane proteases were shown to modulate macrophage functions including chemotaxis and superoxide generation (Shephard et al., 1990, 1992). Retro-inverso peptide analogues derived from CRP and resembling the amino acid sequence of tuftsin have been shown to induce NO synthesis (Arcoleo et al., 1997). It is also of interest to note that both rat and human CRP contain tuftsin-like peptide sequences (Buchta et al., 1986). Tuftsin, a tetrapeptide (TKPR) derived from the C_H2 domain of IgG by proteases, is a known activator of phagocytic cells and has been implicated as an inducer of NO synthesis (Cillari et al., 1994, Khare et al., 1997). Previous work from this laboratory (Nagpurkar et al., 1993) has shown that rat CRP not only binds to rat macrophages but also is internalized and degraded into small peptides. Degradation of CRP into bio-active peptides at inflammatory sites may be necessary to induce the synthesis of pro-inflammatory molecules by cells of the immune system. The magnitude and rapidity of iNOS synthesis in IFN- γ primed cells by CRP may be due to the ability of these cells to secrete more proteases that may cleave CRP to bio-active peptides. Alternatively, these cells may be primed to present an

increased number of CRP receptors. Based on the information cited above and this study data, it may be speculated that during periods of trauma, enzymatically or conformationally altered CRP (and possibly other acute phase proteins), may act as immunomodulators and augment the immune response and accelerate the resolution of the inflammatory event. Furthermore, this process may be aided by T-Cell or other immune cell products such as interferons and interleukins.

Sustained production of NO catalyzed by iNOS appears to be important in defense mechanisms against intracellular pathogens. NO generated by activated macrophages contributes to the cytotoxic activity against microbes and tumour cells (Nathan and Hibbs, 1991; Stuehr, and Nathan, 1989; Xie, et al., 1995). It is possible that the tumoricidal activity seen in CRP-treated monocytes (Tebo and Mortenson, 1991) and the protection rendered by CRP against pathogens (Mold et al., 1981; Szalai, et al., 1995) are at least partly mediated through the production of NO. Thus the expression of iNOS mRNA and subsequent generation of NO could be a major immunomodulatory and anti-inflammatory function exerted by CRP. In conclusion, this study presents evidence that CRP by itself, or synergistically with IFN- γ , activates macrophages and induces the synthesis of iNOS. This provides a basis for the role of CRP in the inflammatory process, and its influence on antimicrobial and tumoricidal properties of activated macrophages. Furthermore, this study data strongly suggest that the induction of iNOS is one of the mechanisms by which CRP may exert its role in immunomodulation.

STUDIES ON MACROPHAGE SIGNALING MECHANISMS ACTIVATED BY CRP

Section 1. Introduction

In the previous chapter, iNOS induction and subsequent production of NO in rat peritoneal macrophages and in a murine macrophage cell line, RAW 264.7 were shown to be stimulated by CRP alone or synergistically with IFN- γ . Although macrophage iNOS has been cloned and characterized (Xie et al., 1992), little is known about the intracellular signal transduction pathway leading to the induction of iNOS. Activation of PC-PLC has been implicated in the pathway leading to iNOS activation in a variety of cell types (Sands et al., 1994; Kengatharan, et al., 1996; Tschaikowsky et al., 1994). Agonist-stimulated PC-PLC activity has been demonstrated in monocytes and macrophages (Choudhury, et al., 1991a; Carter et al, 1998, Buscher et al., 1995). Pathways involving PC-PLC activation have presented an alternative to the classical signaling cascades involving inositol lipids and an alternate source for DAG production (Billah and Anthes, 1990). PC-PLC has also been shown to control NF- κ B (Schutze et al., 1992, Carter et al., 1998), a key transcription factor involved in the regulation of iNOS transcription (Xie et al., 1994). Moreover, one of the salient features of CRP is its ability to bind to phosphocholine, a product of PLC-mediated PC hydrolysis. Therefore, a study was carried out to

examine the possibility that PC-PLC may be activated in response to CRP stimulation and to evaluate its role in the signaling pathway leading to iNOS synthesis. Activation of PC-PLC has been linked to tyrosine phosphorylation (Choudhury, et al., 1991). Furthermore, inhibitors of tyrosine kinase phosphorylation have been shown to interfere with iNOS transcription (Dong et al., 1993; Novogrodsky et al., 1994) and post-translational modification of iNOS (Pan et al., 1996). Therefore, the effect of tyrosine kinase activation on iNOS induction by CRP was also studied. Since protein kinase C was also reported to influence iNOS synthesis in many cell types (Severn et al., 1992; Paul et al., 1995), the role of protein kinases in the pathway leading to iNOS transcription in response to CRP stimulation was also examined.

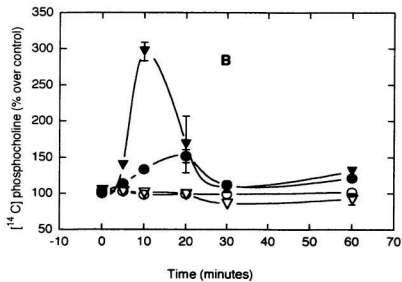
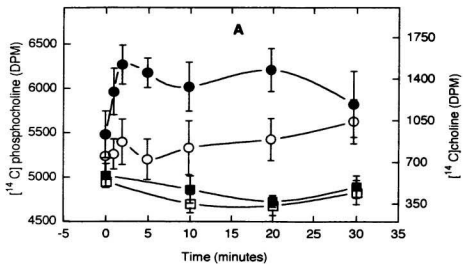
Section 2. Results

A. Phosphatidylcholine Hydrolysis in Macrophages

To study the effect of CRP on PC-PLC activation in intact macrophages, thioglycollate-elicited rat macrophages were metabolically labeled with 0.5 μ Ci of methyl [14 C]choline for 24 h. These cells were made quiescent by incubating them in a medium containing 0.5% FCS for 2 h. Cells were then washed three times with PBS and incubated with CRP (50 μ g/ml) or an equal volume of buffer (Tris 10 mM, NaCl 150 mM, pH 7.4) for up to 30 min. The products were extracted (Bligh and Dyer, 1959; Yavin, 1976) and analyzed by

Fig. 1. PC hydrolysis in macrophages stimulated with CRP. **A.** Rat peritoneal cells were labeled with ^{14}C choline for 24h. Cells were washed and stimulated with CRP (50 $\mu\text{g/ml}$) for various times. Hydrolysis of PC was monitored by determining water-soluble phosphocholine counts. Circles represent phosphocholine counts in the cell extracts from control (○) or CRP-treated (●) cells. Squares represent choline counts in cell extracts obtained from control (□) and CRP stimulated cells (■). Data represent mean \pm SD of three experiments. **B.** RAW 264.7 cells were similarly labeled with ^{14}C choline for 48 h, washed and stimulated with CRP (50 $\mu\text{g/ml}$) or CRP (50 $\mu\text{g/ml}$) and IFN- γ (50 units/ml) for 0 to 60 min. PC hydrolysis was also assayed in the presence of D609 (20 $\mu\text{g/ml}$). Hydrolysis of PC was monitored by measuring the release of water soluble phosphocholine counts by TLC as described. Data represent mean \pm SD of three experiments. Control (○); CRP (●); CRP/IFN- γ (▼); CRP/IFN- γ + D609 (▽).

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thin layer chromatography (TLC). Analysis of the water soluble products on TLC showed an increase in [^{14}C]phosphocholine levels indicating an increase in PC-PLC activity. Production of [^{14}C]phosphocholine was rapidly increased following stimulation in cells treated with CRP, as compared to cells treated with buffer. This increase in PC hydrolysis in the presence of CRP fell to normal levels in about 30 min after stimulation (Figure 1A). These results showed that CRP activated PC-PLC activity in rat macrophages. Also, there was no detectable increase in [^{14}C]choline levels in CRP-treated cells above that of control cellular levels, indicating that PC-PLD activity was not stimulated by CRP.

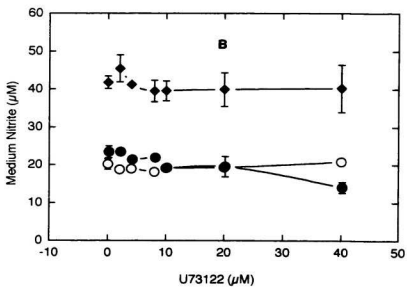
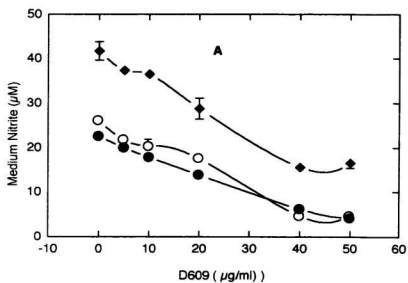
RAW macrophages were also labeled with [^{14}C]choline for 48 h and made quiescent by incubating them in medium containing 0.5% FCS. Cells were washed and exposed to CRP (50 $\mu\text{g/ml}$) for various times and the products were extracted (Bligh and Dyer, 1959). Results obtained were very similar to those of rat peritoneal cells (Figure 1B). However, in the presence of IFN- γ , the rate of PC hydrolysis was more rapid and pronounced than that with CRP alone. Tricyclodecan-9-yl-xanthogenate (D609), a specific inhibitor of PC-PLC, abrogated the increase in PC hydrolysis in response to IFN- γ and CRP. These results showed that CRP activated PC-PLC activity in RAW 264.7 cells as well.

B. Inhibition of NOS Induction by PC-PLC Inhibition

Addition D609 to the culture medium decreased NO production by RAW 264.7

Fig. 2. Nitrite production by RAW 264.7 cells in the presence of phospholipase enzyme inhibitors. **A.** RAW 264 7 cells were stimulated by 50 μ g of CRP (●), 50 units of IFN- γ (○) or a combination of CRP and IFN- γ (◆) for 24 h in the presence of increasing concentrations of D609. **B.** Cells were treated similarly as A in the presence of increasing concentrations of U73122 as indicated. CRP (●), IFN- γ (○), CRP and IFN- γ (◆). Data represent mean \pm SD of three experiments.

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cells stimulated with CRP or CRP/IFN- γ in a dose-dependent manner (Fig 2A). In contrast, U73122, an inhibitor of agonist-stimulated PI-PLC (Smith et al., 1996), had no effect on NO synthesis by macrophages in a parallel experiment (Fig. 2B). To verify that D609, and therefore PC-PLC, acted proximally to the site of iNOS protein synthesis, cells stimulated with CRP and IFN- γ for 18 h in the presence of D609 or U73122 were lysed and immunoblotted with a polyclonal antibody specific to iNOS. D609 treatment resulted in a dose-dependent decrease of iNOS protein level in IFN/CRP-treated cells. In contrast, U73122 treatment had no effect on iNOS protein concentration in activated RAW 264.7 cells (Fig. 3). To ensure that D609 did not interfere with the catalytic activity of NOS enzyme, NOS activity was also assayed in RAW cell lysates and cytosol in the presence of D609. No significant reduction in the rate of synthesis of NO from L-arginine was observed when D609 was added directly to CRP/IFN treated RAW 264.7 cell lysates and cytosol and assayed for NOS activity (Fig 4). These results showed that, while there was a dose-dependent inhibition of iNOS protein synthesis when cells were treated with D609, it had no direct effect on the enzymatic synthesis of NO from L-arginine. This also indicated that the role of PC-PLC is proximal to the initiation of iNOS transcription. CRP-stimulated nitrite synthesis was also inhibited by D609 in rat peritoneal macrophages. In these cells, D609 at a concentration of 20 $\mu\text{g/ml}$ abolished CRP-stimulated NO synthesis ($p < 0.001$; unpaired two tailed t test). However, D609 had no effect on the basal NOS levels (Fig. 5). In these cells, U73122 also had no significant inhibitory effect on NO synthesis stimulated by CRP.

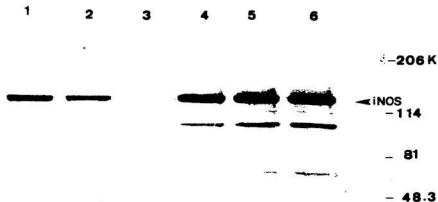


Fig. 3. **Western blot of RAW 264.7 cell lysates treated with D609 and U73122.** RAW 264.7 cells stimulated for 18 h with a combination of CRP (50 μ g/ml) and IFN- γ (50 units/ml) in the presence of increasing concentrations of D609 or U73122. Cells were lysed and cell lysates were separated on 7.5% denaturing SDS PAGE, immunoblotted and probed with an antibody to murine iNOS. Bound antibodies were detected by alkaline phosphatase conjugated second antibody as described in materials and methods. Lower molecular weight bands represent degradation products of iNOS protein. Lane. 1 D609 (10 μ g) 2. D609 (20 μ g) 3. D609 (50 μ g). 4. U 73122 (20 μ M). 5. U 73122 (50 μ M) 6. No inhibitor. Blot represents three experiments with similar results.

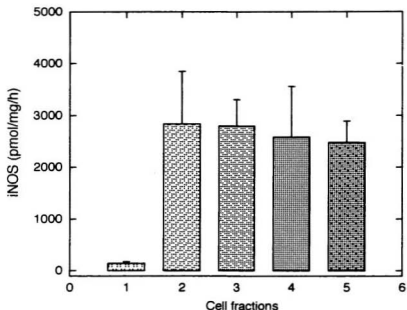


Fig. 4. Effect of D609 on iNOS enzyme activity in RAW 264.7 cell lysates and cytosol. Cells were stimulated with CRP (50 $\mu\text{g/ml}$) and IFN- γ (50 units/ml) for 16 h. Cells were then lysed and fractionated. Cell lysates and cytosol (100000 X g supernatant) were assayed for iNOS activity in the presence and absence of D609 (20 $\mu\text{g/ml}$) as described in materials and methods. 1, control lysates. 2, lysates from stimulated cells. 3, lysates from stimulated cells + D609. 4, cytosol from stimulated cells. 5, cytosol from stimulated cells + D609. Data represent mean \pm SD of three experiments.

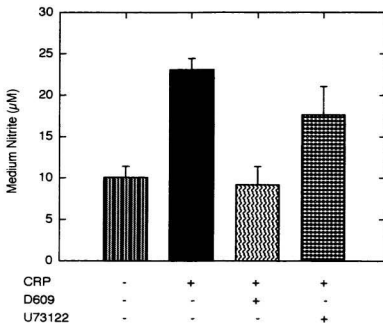


Fig. 5. **Effect of phospholipase inhibitors on nitrite generation by rat peritoneal macrophages stimulated by CRP.** Rat peritoneal macrophages were stimulated with CRP (20 $\mu\text{g/ml}$) in the presence of D609 (10 $\mu\text{g/ml}$) or U73122 (20 μM) for 18 h and accumulated medium nitrites were assayed. Method of stimulation is indicated by the presence (+) or absence (-) of agents. Data represent mean \pm SD of three experiments.

C. Tyrosine Phosphorylation and iNOS Induction

To evaluate the role of tyrosine kinases in the mediation of iNOS induction by CRP, RAW 264.7 cells were pre-treated for 60 min with tyrosine kinase inhibitors, genistein and tyrphostin AG 126, before the addition of CRP (50 $\mu\text{g/ml}$), IFN- γ (50 units/ml) or CRP/IFN- γ . Incubation was continued for 18 h and accumulated nitrites in the culture medium were analyzed. Tyrosine kinase inhibitors caused a concentration-dependent inhibition of CRP-induced NO synthesis in RAW 264.7 cells (Fig. 6A and 6B). There was a significant reduction of NOS activity with 20 μM genistein, and NOS activation was almost completely abrogated by 100 μM tyrphostin AG 126. This indicated PTK activity was involved in the triggering of iNOS synthesis by CRP in RAW 264.7 cells.

D. Role of cAMP in CRP-mediated NOS Induction

In rat peritoneal macrophages phorbol myristate acetate (PMA), a DAG analogue and a potent activator of PKC, activated the induction of iNOS. Cholera toxin, an activator of adenylyl cyclase, also increased the synthesis of NO in these cells. Additionally, dibutyryl cAMP, a cell permeable cAMP analogue and activator of protein kinase A, also increased the synthesis of NO. Both PKC and PKA activation resulted in increased NO generation in these cells (Fig. 7A). In contrast, neither of these agents was capable of stimulating NO synthesis in RAW 264.7 cells. PMA, a DAG analogue and protein kinase C activator, did not have any effect on iNOS activation in RAW 264.7 cells by itself, nor did it have any effect when added along with CRP.

Fig. 6. Effect of tyrosine kinase inhibitors on nitrite generation by RAW 264.7 cells stimulated by CRP and IFN- γ . RAW 264.7 cells were stimulated by 50 μ g of CRP (●), 50 units of IFN- γ (○) or a combination of 50 μ g of CRP and 50 units of IFN- γ (▽) for 24 h in the presence of increasing concentrations of genistein (**A**) and tyrphostin AG 126 (**B**). Data points represent mean \pm SD of three experiments.

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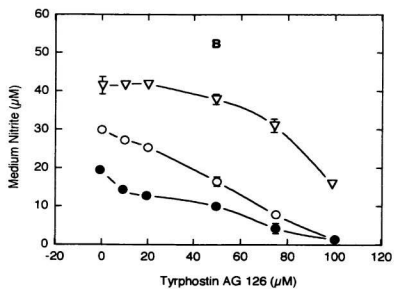
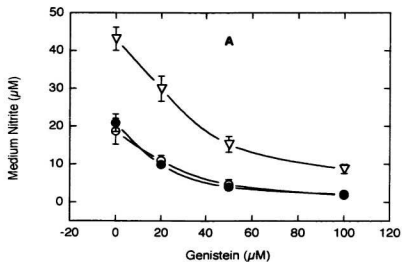
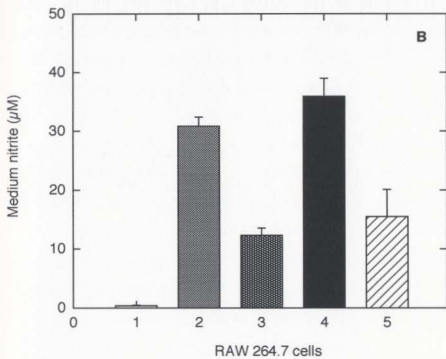
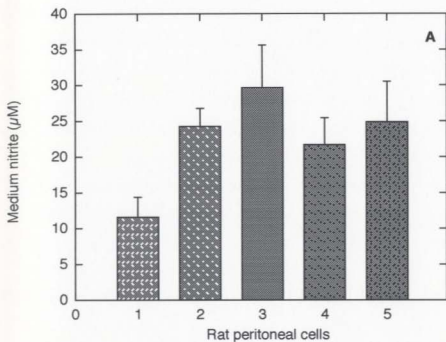


Fig.7. Effect of protein kinase modulators on nitrite generation in macrophages. A. Rat peritoneal cells were stimulated by CRP (50 μ g), PMA (1 μ g/ml), dibutyryl cAMP (100 μ M) cholera toxin (1 μ g/ml) for 18 h and nitrites in the culture medium were assayed. 1, control. 2, CRP. 3, PMA. 4. dibutyryl cAMP. 5. Cholera toxin. **B. Effect of cyclic AMP modulating agents on NO synthesis by RAW 264.7 cells stimulated by CRP and IFN- γ .** RAW 264.7 cells were left unstimulated or stimulated with CRP (50 μ g/ml) and IFN- γ (50 units/ml) for 18 h with agents as indicated. 1. Control. 2. CRP /IFN- γ . 3. CRP /IFN- γ + dibutyryl cAMP (100 μ M) 4. CRP /IFN- γ + pertussis toxin (1 μ U/ml). 5. CRP /IFN- γ + cholera toxin (1 μ g/ml). Data represent mean \pm SD of three experiments.

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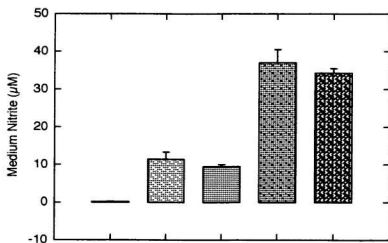
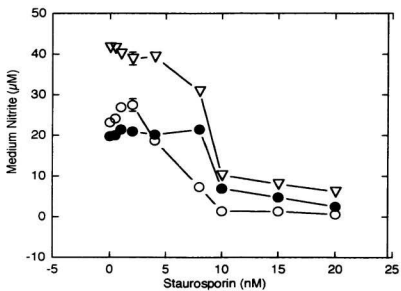
In addition, cyclic AMP modulating agents such as dibutyryl cyclic AMP (100 μ M), a cell-permeable cAMP analogue that preferentially activates cAMP-dependent protein kinases, inhibited iNOS induction by CRP and IFN- γ by 60%. Cholera toxin (1 μ g/ml), which catalyses ADP ribosylation of a subunit of G protein and increases the cellular level of cAMP, also inhibited iNOS induction by CRP/IFN- γ . These results suggested that while adenylyl cyclase, and possibly cyclic AMP activated protein kinases, may be inhibitory to iNOS synthesis in RAW 264.7 cells, these second messengers upregulate iNOS expression in rat peritoneal cells (Fig. 7B).

E. Role of Protein Kinase C in iNOS Induction

Staurosporin, an inhibitor of broad serine/threonine kinases, inhibited iNOS activation by CRP in a dose-dependent manner. There was significant reduction of nitrite synthesis at a concentration of 4 nM when NOS was induced by CRP alone while 8 nM of staurosporin was necessary to cause definitive reduction when RAW 264.7 cells were stimulated with CRP and IFN- γ (Fig. 8A). In contrast, bisindolylmaleimide, a more specific inhibitor of PKC (K_i = 10 nM for PKC), was unable to block CRP or CRP/IFN- γ -induced iNOS synthesis at concentration up to 2 μ M (Fig. 8B). Many of the inhibitory compounds used in this study were dissolved in DMSO. To ensure that DMSO by itself did not influence NO production, RAW 264.7 cells were stimulated with CRP and/or IFN- γ for 24 h with various concentrations of DMSO diluted 1:100 in PBS. At the concentrations used, DMSO did not affect iNOS induction by CRP (Fig. 9).

Fig. 8. Effect of protein kinase C inhibitors on nitrite generation by RAW 264.7 cells stimulated by CRP and IFN- γ . **A.** RAW 264.7 cells were stimulated by 50 μ g of CRP (○), 50 units of IFN- γ (●) or a combination of 50 μ g CRP and 50 units of IFN- γ (▽) for 24 h in the presence of increasing concentrations of staurosporin . **B.** RAW 264 7 cells were stimulated by 50 μ g of CRP, 50 μ g CRP and 50 units of IFN- γ for 18h in the presence of bisindolylmaleimide (2 μ M). Presence (+) or absence (-) of agents used is indicated. Data represents mean \pm SD of three to six experiments.

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CRP(50 $\mu\text{g/ml}$)
 IFN (50 U/ml)
 BIM (2 μM)

-	+	+	+	+
-	-	-	+	+
-	-	+	-	+

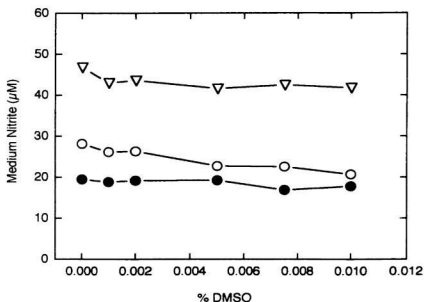


Fig. 9. Effect of DMSO on nitrite generation by RAW 264.7 cells stimulated by CRP and IFN- γ . RAW 264.7 cells were stimulated by 50 μ g of CRP (●), 50 units of IFN- γ (○) or a combination of 50 μ g CRP and 50 units of IFN- γ (▽) for 24 h in the presence of increasing concentrations of DMSO for 24 h and accumulated nitrites in the culture medium were assayed by Griess reaction. Data represent mean of three experiments.

Section 3. Discussion

A number of recent reports have suggested that PC hydrolysis is critically involved in signal transduction pathways in response to stimulation by polypeptide growth factors and oncogenic Ras proteins (Larrodera et al., 1990; Cai et al., 1992; 1993; Exton, 1994). PC-PLC rather than PC-PLD seems to be responsible for a sustained increase in cellular diacylglycerol (DAG) preceding the induction of DNA synthesis in fibroblasts and xenopus oocytes (Larrodera et al., 1990; Garcia de Herreros et al, 1991). One of the striking properties of CRP is its affinity for the phosphocholine ligand, which is also the product of phosphatidylcholine hydrolysis, catalyzed by PC-PLC. In this study, a rapid increase in the activation of PC-PLC was shown to occur in RAW 264.7 cells treated with CRP. Product analysis revealed that in response to CRP, only phosphocholine levels increased among the labeled metabolites recovered from the aqueous phase. Significant increase in the generation of phosphocholine was also observed in [^{14}C]choline-labeled rat peritoneal macrophages when treated with CRP indicating phospholipase C activity. This rapid increase in the labeled phosphocholine fraction fell to near normal levels within 30 min. However, the results were often variable with regard to the time dependence and the extent of phosphocholine formation. The difficulties in assessing PC-PLC activity in cells have been comprehensively described (Cook and Wakelam, 1992). It is possible that CRP, like growth factors, not only evokes hydrolysis of PC but also activates (re)synthesis of PC resulting in a PC cycle (Van Dijk, et al., 1996). The role of CRP may be to enhance PC turnover,

rather than the production of sustained levels of DAG. Previous work from this laboratory has shown that CRP enhances the hydrolysis of dipalmitoyl phosphatidylcholine (DPPC) releasing DAG and phosphocholine in a cell-free assay (Mookerjee and Hunt 1995). To rule out the possibility that CRP may be contaminated with PC-PLC, or that PC-PLC may be co-purified along with CRP, PC-PLC from *Bacillus cereus* was applied to a column containing sepharose-phosphocholine affinity adsorbent. Although bacterial PC-PLC bound weakly to the column, PC-PLC activity was not detected in column fractions when eluted with 8 mM phosphocholine as described in the methods for purification of CRP (Hunt and Mookerjee, unpublished observations). Furthermore, PC-PLC activity has not been demonstrated in normal rat serum from which rat CRP was isolated. Therefore, it is quite unlikely that CRP was contaminated with PC-PLC. Catalysis by PC-PLC also results in the generation of DAG. It has been established that distinct molecular species of DAG are generated by PC-PLC and PI-PLC, and that PI hydrolysis is neither necessary nor required for DNA synthesis (Cuadrado et al., 1990; Margolis et al., 1990; Downing et al., 1989; Hill et al., 1990; and Peters et al., 1992). While PI hydrolysis results in a rapid and transient increase in DAG levels, and a rise in cellular Ca^{++} levels, PC hydrolysis by PC-PLC or a combined action of PC-PLD and PC-PLC results in a prolonged and sustained increase in DAG levels, whereas Ca^{++} levels remain unchanged. It has been proposed that, while PI hydrolysis would activate both Ca^{++} dependent and independent PKCs, PC hydrolysis may only activate atypical, Ca^{++} independent PKC isozymes (Exton, 1994).

D609, a specific inhibitor of PC-PLC, was shown to inhibit NO synthesis in J774

cells stimulated by LPS and IFN- γ (Tschaikovsky et al., 1994). The antiviral xanthate compound D609 has also been shown to be an effective and specific inhibitor of PC-PLC (Muller-Decker, 1989; Schutze et al., 1992). Data presented here show that PC-PLC activation is a necessary event in the pathway mediating the induction of iNOS by CRP. This conclusion was drawn since D609, a specific inhibitor to PC-PLC, was able to block the de novo iNOS protein synthesis as evident from Western blot analysis. Assuming that D609 specifically inhibited PC-PLC activation as described previously (Muller-Decker, 1989; Schutze et al., 1992; Tschaikovsky et al., 1994), it can be inferred that activation of PC-PLC may be an early event in the pathway leading to the induction of iNOS, and that inhibition of PC-PLC by D609 resulted in impaired iNOS protein synthesis. It was also necessary to add D609 before the addition of CRP to attain complete inhibition of CRP-induced iNOS synthesis. Moreover, D609 had no direct effect on the enzymatic conversion of L-arginine to L-citrulline and NO by activated RAW 264.7 cell lysates and cytosol. This also proved that once iNOS enzyme protein synthesis has occurred, D609 had no effect on the catalytic activity of iNOS enzyme in activated RAW 264.7 cells. Furthermore, it is unlikely that activation of PI-PLC is involved in the mediation of iNOS induction, since U73122, a potent inhibitor of PI-PLC, with an IC₅₀ of 1 to 2.1 μ M in human neutrophils and platelets (Smith et al., 1996), failed to block the induction of iNOS in RAW 264.7 cells stimulated by CRP and IFN- γ , when it was added at concentrations up to 20 μ M. This compound also had no significant inhibitory effect on iNOS synthesis in rat peritoneal cells. It is possible that PI-derived DAG does not play a role in signaling events resulting in iNOS expression in these cells. PMA sensitive PKC isozymes also seem to

activate iNOS induction in these cells. However, the results presented here show a clear-cut role for PC-PLC in the activation of iNOS synthesis in rat macrophages as well as in RAW 264.7 cells. Activation of an unusual PC-PLC has also been shown to directly influence NO synthesis in LPS/IFN- γ treated murine macrophages (Sands et al., 1994). PC-PLC activation has also been directly linked to increased DNA binding of NF- κ B, a key factor involved in the transcription of iNOS and various inflammatory cytokines (Yamamoto et al., 1997; Spitsin et al., 1997).

Activation of PC-PLC has also been linked to the activation of tyrosine phosphorylation (Choudhury et al., 1991). The results presented here showed that NOS activity is inhibited by genistein, a broad range tyrosine kinase inhibitor, and Tyrphostin AG 126, a much more selective inhibitor of tyrosine kinases. While genistein inhibited iNOS synthesis at an IC₅₀ of approximately 50 μ M in CRP and CRP/IFN- γ treated cells, a much higher concentration of tyrphostin AG 126 was required to completely inhibit CRP/IFN- γ stimulated cells. These results are in agreement with previously reported data obtained from murine peritoneal macrophages (Dong et al., 1993; Novogrodsky, et al., 1994). These inhibitory effects were specific to the compound used and not a nonspecific effect of the diluent such as DMSO. Many of the inhibitory compounds used in this study were dissolved in DMSO at 1000 X concentration and were further diluted in medium before adding to cell culture. Although DMSO itself may exhibit some anti-inflammatory properties, the DMSO vehicle by itself showed no effect on NOS induction in RAW cells even at concentrations much higher than that used with test compounds.

Staurosporin, which potently inhibits PKC with a reported IC_{50} of 0.7 nM (Hoffman and Newlands, 1991; Nishimura and Simpson, 1994), inhibited the induction of iNOS activity indicating that the pathway that relays the signal from CRP stimulation may entail the activation of protein kinase(s). However, it should be noted that although staurosporin is a potent inhibitor of PKC, it is also a broad serine/threonine kinase inhibitor and can inhibit other protein kinases at an IC_{50} of 10 nM or less. A more specific PKC inhibitor, bisindolylmaleimide with an IC_{50} of 5 to 70 nM (Toullec et al., 1991), had no effect on CRP/IFN stimulated iNOS activity at a concentration of 2 μ M. Moreover, the PKC activator PMA failed to activate iNOS synthesis in RAW 264.7 cells. It is entirely possible that PMA sensitive protein kinase C (PKC) isoforms are not involved in the activation of iNOS transcription in RAW cells. However, this does not rule out the possible involvement of atypical PKCs, which may be poorly inhibited by bisindolylmaleimide, in the signal mediating NOS synthesis. Transfection of PKC- ϵ in RAW 264.7 cells was shown to result in the expression of iNOS (Diaz-Guerra et al., 1996). Overexpression of PKC- ζ has also been shown to increase the expression of iNOS in mesangial cells (Miller et al, 1997). In contrast, PMA-sensitive PKC seems to be involved in the stimulation of iNOS synthesis in rat peritoneal macrophages since treatment with PMA resulted in the production of substantial quantities of NO. It is likely that PMA sensitive PKC isoform(s) may be regulated by PC derived diacylglycerol (DAG) or other products of PC hydrolysis in rat peritoneal cells.

The possibility that cyclic AMP-regulated protein kinase A may be involved in

the mediation of signals generated by CRP was also explored. Elevation of cellular cAMP levels by treating rat peritoneal macrophages with cholera toxin or by dibutyryl cAMP increased the production of NO by these cells. On the contrary, RAW 264.7 cells failed to produce NO when stimulated by either of these agents. Moreover, cholera toxin and the cell-permeable cAMP analogue, dibutyryl cAMP, reduced the level of CRP-induced nitrite synthesis. In contrast, pertussis toxin had a slight stimulatory effect on NO synthesis induced by CRP/IFN- γ . These data led to the conclusion that increased cAMP levels and activation of adenylyl cyclase may be inhibitory to iNOS induction in RAW 264.7 cells. This is in agreement with previous work showing that accumulation of cAMP inhibited NO generation (Hasko et al., 1998) as well as NF- κ B activation in RAW 264.7 cells (Vincenti et al., 1993). The slight enhancement of NO synthesis in pertussis toxin treated cells may also indicate that adenylyl cyclase may have a regulatory role on the induction of iNOS in these cells.

In conclusion, the data shown here together with previous findings as noted above, suggest that phosphatidylcholine hydrolysis and protein tyrosine phosphorylation may be important in the signaling pathway leading to iNOS induction in macrophages. However, there seems to be some important differences between rat peritoneal macrophages and RAW 264.7 cells regarding the role of protein kinases activation in mediating iNOS induction. Furthermore, these results demonstrate a definite involvement of PC-PLC activation and tyrosine phosphorylation in the signaling pathway leading to iNOS induction triggered by CRP.

THE REGULATION OF SUPEROXIDE GENERATION BY CRP IN MACROPHAGES

Section 1. Introduction

The principal function of macrophages is the destruction of invading microbial pathogens and removal of inflammatory debris. Macrophages and other phagocytes utilize various effector responses to achieve this goal. This includes the generation of reactive oxygen intermediates (ROI) and reactive oxynitrogen intermediates (RONI). However, both ROI and RONI are potentially toxic to the host, and the regulation of the generation of these molecules is critical to the host survival (Bastien and Hibbs, 1994). While superoxide anion ($O_2^{\cdot -}$) is the precursor of ROI such as hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO^{\cdot}) (Fridovich, (1986), biosynthesis of NO may lead to the production of RONIs such as nitrosonium (NO^+) ions, nitroxyl (NO^-) ions, nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$) and S-nitrosothiols (Stamler et al., 1992). $O_2^{\cdot -}$ is produced when the respiratory burst oxidase (NADPH oxidase) is activated by stimuli such as microbial pathogens, N-formyl methional leucyl phenylalanine (fMLP) or phorbol myristate acetate (PMA). $O_2^{\cdot -}$ is generated mainly by macrophages, monocytes and PMNs which are

expendable cells involved in host defense function (Bastien and Hibbs, 1994). In contrast, NO is generated by almost all nucleated cells from L-arginine by three distinct isoforms of nitric oxide synthase (NOS) which include constitutive low output endothelial and neuronal isoforms (eNOS and nNOS) and an inducible high output isoform (iNOS) (Nathan and Xie, 1994). iNOS is induced when cells are stimulated by combinations of cytokines and bacterial products like lipopolysaccharide (Nathan, 1992). Macrophages are capable of producing copious amounts of $O_2^{\cdot-}$ and NO when triggered by appropriate stimuli. During the period of immunological challenge, pathways leading to NO as well as $O_2^{\cdot-}$ can potentially be activated. However, simultaneous generation of $O_2^{\cdot-}$ and NO can lead to the production of $ONOO^-$ and HO^\cdot which can cause considerable damage to microbial pathogens as well as host cells (Rosen, et al., 1995; Beckman, et al., 1990; Zhu, et al., 1992). Therefore, the regulation of the production of these molecules is critical to host survival. During periods of inflammation or infection, the level of serum CRP increases in many species. Human CRP has also been reported to influence the generation of $O_2^{\cdot-}$ (Tebo and Mortensen, 1991; Dobrinich and Spagnuolo, 1991; Buchta et al., 1987). However, the underlying mechanism by which CRP may regulate $O_2^{\cdot-}$ generation by these cells remains unclear. Moreover, the effects of rat CRP on $O_2^{\cdot-}$ generation by macrophages has not been examined to date. The results presented in previous chapter showed a significant role for PC-PLC in mediating macrophage responses to CRP. Therefore, the role of rat CRP and PC-PLC in PMA-stimulated $O_2^{\cdot-}$ production was examined.

Section 2. Results

A. Activation of $O_2^{\cdot-}$ -Generation in Rat Macrophages

Resident rat peritoneal macrophages were incapable of $O_2^{\cdot-}$ production and released minimal amounts of $O_2^{\cdot-}$ when stimulated with PMA. In contrast, rat peritoneal macrophages elicited either with 4% thioglycollate or with 1% caesin, produced copious amounts of $O_2^{\cdot-}$ when triggered with PMA (1 $\mu\text{g/ml}$). There was no significant difference between thioglycollate- and caesin- elicited macrophages (Table 1). Hence all subsequent experiments were done only with thioglycollate- elicited macrophages.

B. Effect of CRP on $O_2^{\cdot-}$ Production

The generation of $O_2^{\cdot-}$ by peritoneal macrophages in response to PMA was prolonged and sustained for long periods. This increase of $O_2^{\cdot-}$ production was linear up to 120 min. A significant decrease in $O_2^{\cdot-}$ production was seen when CRP was included in the assay. Inhibition of $O_2^{\cdot-}$ generation by CRP was also linear up to 120 min. Cytochrome c reduction by PMA-stimulated macrophages was decreased to almost 50% at a concentration of 150 $\mu\text{g/ml}$ of CRP (Fig. 1). This inhibition by CRP was also concentration dependent. A 20% reduction was seen with 50 $\mu\text{g/ml}$ of CRP while more than 80% inhibition was noticed when 500 $\mu\text{g/ml}$ was used. In the absence of the stimulus, CRP

Table 1. $O_2^{\cdot-}$ generation by rat peritoneal macrophages.

	Resident Cells	4%Thioglycollate broth-elicited	1% Caseinate- elicited
Unstimulated	0.003 μ M	0.35 \pm 1.33 μ M	1.49 \pm 1.25 μ M
PMA- stimulated	1.05 \pm 0.555 μ M	21.89 \pm 1.06 μ M	26.43 \pm 4.08 μ M

Sprague Dawley rats were injected intraperitoneally with either 5 ml of 1% sodium caseinate, 4% thioglycollate broth or an equal volume of sterile phosphate buffered saline (PBS). Macrophages were harvested after 72 h and $O_2^{\cdot-}$ generation (μ M cytochrome c reduction) was determined as described in materials and methods. The results shown are means of six experiments \pm SD.

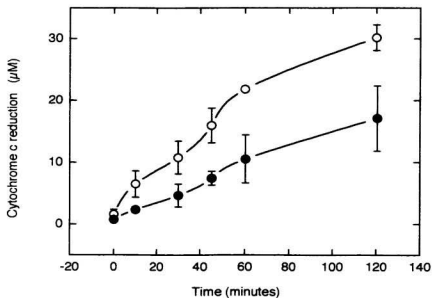


Fig. 1. **Effect of CRP on $\text{O}_2^{\cdot-}$ generation by PMA-stimulated rat peritoneal macrophages.** Cells were incubated with (●) or without (○) CRP (150 $\mu\text{g}/\text{ml}$) for 5 min and then stimulated with PMA (1 $\mu\text{g}/\text{ml}$) to trigger superoxide production for various time intervals. Error bars represent mean \pm SD of three to five experiments.

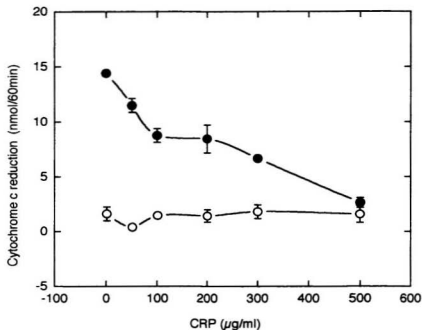


Fig. 2. **Inhibition of $O_2^{\cdot-}$ by CRP as a function of concentration.** Cells were preincubated with increasing concentrations of CRP for 5 min before triggering $O_2^{\cdot-}$ production by PMA (●) or left unstimulated (○) and were further incubated for 60 min. Reduction of cytochrome c was used to monitor $O_2^{\cdot-}$ generation. Error bars represent mean \pm SD of four to eight experiments.

had no effect on the basal cytochrome c reduction (Fig. 2).

C. Comparison of CRP with Superoxide Dismutase

To test whether CRP acted strictly as a scavenger of $O_2^{\cdot-}$, the action of superoxide dismutase (SOD) was compared to that of CRP by adding CRP or SOD at various intervals ranging from 0 to 60 min after stimulating cells with PMA and continuing the incubation for up to 90 min. Cells incubated with PMA alone for 90 minutes served as control. It was necessary to add CRP within 30 min of PMA stimulation to cause any significant inhibition of $O_2^{\cdot-}$ synthesis. In contrast, addition of SOD even after 60 min of stimulation resulted in a significant decrease of $O_2^{\cdot-}$ release. A lag period was observed with CRP induced reduction of cytochrome c as compared to the action of SOD (Fig. 3).

D. Effect of CRP on $O_2^{\cdot-}$ in a Cell Free System.

To determine that the inhibition of cytochrome c reduction by rat CRP in stimulated macrophages was due to an effect on cellular effector mechanisms and not due to scavenging of $O_2^{\cdot-}$, the effect of rat CRP was assessed in a cell free system. In this system, $O_2^{\cdot-}$ was chemically generated by xanthine oxidase using acetaldehyde as the substrate. CRP, up to a concentration of 300 $\mu\text{g/ml}$, had no effect in $O_2^{\cdot-}$ in this system. However, small but significant inhibition of cytochrome c reduction in this system was observed when CRP was present in excess of 300 $\mu\text{g/ml}$. This indicated that CRP may also exert some scavenging activity when present in high concentration (Table 2).

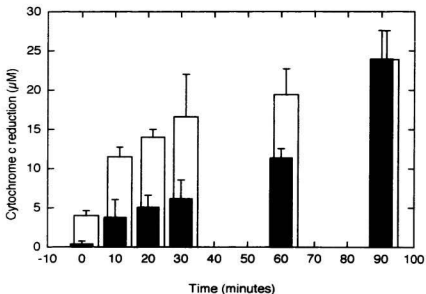


Fig. 3. **Comparison of the effect of CRP and SOD on PMA-triggered $O_2^{\cdot-}$ generation in rat peritoneal macrophages.** Rat macrophages were stimulated with PMA. CRP (300 $\mu g/ml$) (□) or superoxide dismutase (2 $\mu g/ml$) (■) was then added at the time points (0 - 60 min) indicated. Incubation was continued for 90 min and $O_2^{\cdot-}$ was measured by the reduction of cytochrome c. Cells incubated with PMA alone for 90 minutes were used as control. Data represent mean \pm SD of four to six experiments.

Table. 2 **Effect of CRP on $O_2^{\cdot-}$ production in a cell free system.**

Treatment	Cytochrome c reduction (μM)
CRP (0 μg)	14.77 \pm 0.663
CRP (75 μg)	14.78 \pm 0.7366
CRP (150 μg)	14.23 \pm 0.19
CRP (300 μg)	13.48 \pm 0.775
CRP (450 μg)	12.43 \pm 0.792*

$O_2^{\cdot-}$ was produced in a cell-free system consisting of xanthine oxidase and acetaldehyde in the presence of various concentrations of CRP as described in materials and methods. Values represent mean \pm SD of net $O_2^{\cdot-}$ produced in 6 experiments.

* $P < 0.05$ versus assay without CRP by students t-test

E. Effect Of Non-specific Proteins on $O_2^{\bullet-}$ Production by Macrophages

To verify whether the decrease in $O_2^{\bullet-}$ generation by CRP is a nonspecific effect due the presence of increased protein levels in the medium, cytochrome c reduction by activated macrophages was also estimated in the presence of various proteins. Cells were pretreated with equal concentrations of BSA, human IgG, CRP, and α -1 acid glycoprotein, another acute phase protein (200 μ g/ml), for 5 min and then stimulated with PMA (1 μ g/ml). The results showed that significant inhibition of cytochrome c reduction occurred only in the presence of CRP (Fig. 4).

F. Effect of PC-PLC Inhibition on $O_2^{\bullet-}$ Generation and NO Synthesis

In chapter 4, CRP was shown to activate PC-PLC, and this correlated with NO synthesis in macrophages. To discern the effect of PC-PLC in $O_2^{\bullet-}$ generation by macrophages, the effect of D609, a specific inhibitor of PC-PLC, was studied. In contrast to the effect seen on NO synthesis, D609 increased PMA triggered $O_2^{\bullet-}$ generation in macrophages. D609 had no effect on $O_2^{\bullet-}$ generation on its own in the absence of $O_2^{\bullet-}$ triggering agents such as PMA (Fig. 5a). However, D609 completely inhibited the stimulation of NO synthesis by CRP activated rat macrophages at a concentration of 10 μ g/ml (Figure 5B).

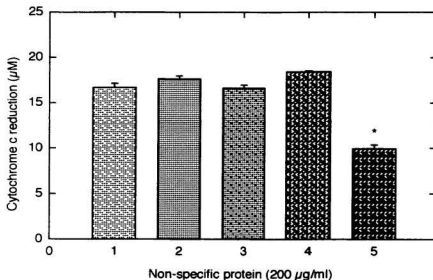
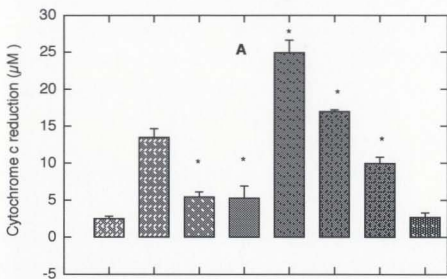


Fig. 4 Effect of non-specific proteins on $O_2^{\bullet -}$ generation in PMA-stimulated rat macrophages. Cells were preincubated with 200 μ g of the indicated protein for 5 min before triggering $O_2^{\bullet -}$ production by PMA and were further incubated for 60 min. Reduction of cytochrome c was used to monitor $O_2^{\bullet -}$ generation. 1 control. 2. BSA. 3. Human IgG. 4. α -1 acid glycoprotein. 5. CRP. Error bars represent mean \pm SD of three to six experiments.

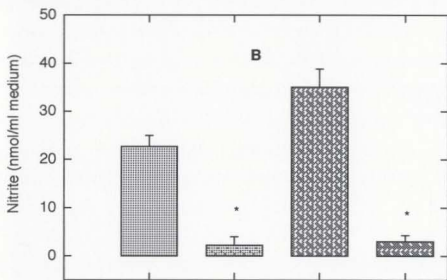
* $P < 0.05$ versus assay with PMA alone by students t-test

Fig. 5. **Stimulation of $O_2^{\cdot-}$ generation by rat peritoneal macrophages by D609.** **A.** Rat macrophages were stimulated by PMA ($1\mu\text{g/ml}$) and $O_2^{\cdot-}$ production was measured in the presence of CRP ($150\mu\text{g/ml}$), PC-PLC (0.05 units/ml), or D609 ($20\mu\text{g/ml}$). Presence (+) or absence (-) of agents used is shown as indicated. Data represent mean \pm SD of four experiments. * $P < 0.05$ versus assay with only PMA by students t-test. **B. Inhibition of NO synthesis by D609.** Rat macrophages were pretreated with D609 ($20\mu\text{g/ml}$) for 20 min before the addition of CRP ($20\mu\text{g/ml}$). Presence (+) or absence (-) of agents used is shown as indicated. Data represent mean \pm SD of four experiments.

* $P < 0.05$ versus assay without D609 by students t-test



PMA	-	+	+	+	+	+	+	-
CRP	-	-	+	-	-	+	-	-
PC-PLC	-	-	-	+	-	-	+	-
D609	-	-	-	-	+	+	+	+



CRP	-	-	+	-
D609	-	+	-	+

Interestingly, addition of PC-PLC derived from *Bacillus cereus* (0.05 units/ml) also decreased PMA-triggered $O_2^{\cdot-}$ generation. This decrease in $O_2^{\cdot-}$ generation by PC-PLC was reversed by D609 (Fig. 5A). For comparison, a bar graph showing the inhibition of CRP-induced NO is shown in Fig. 5B.

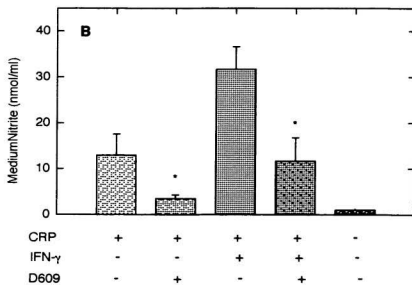
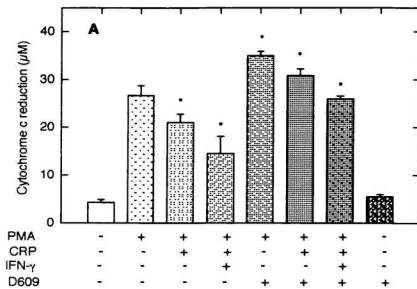
G. $O_2^{\cdot-}$ Generation by RAW 264.7 Cells.

Increased superoxide production in response to PMA was also assayed in RAW 264.7 cells by estimating the rate of cytochrome c reduction. Results were similar to that obtained with rat peritoneal cells. In RAW 264.7 cells, treatment with CRP (100 μ g/ml) also decreased PMA-stimulated cytochrome c reduction. There was a further decrease in $O_2^{\cdot-}$ synthesis when cells were exposed to IFN- γ (50 units/ml) and CRP (100 μ g/ml). Similar to the effect seen in rat peritoneal cells, D609 (20 μ g/ml) significantly enhanced the synthesis of PMA-stimulated $O_2^{\cdot-}$ generation. D609 also reversed the inhibitory effect of CRP and CRP/IFN- γ mediated inhibition of $O_2^{\cdot-}$ synthesis by RAW 264.7 cells (Fig. 6). Inhibition of NO synthesis by D609 has been described in detail in the previous chapter. Comparison with Fig 6B shows that while D609, an inhibitor of PC-PLC resulted in a decrease in NO synthesis, it increased $O_2^{\cdot-}$ synthesis in RAW 264.7 cells and rat peritoneal macrophages.

H. Effect of Phosphocholine on $O_2^{\cdot-}$ Synthesis

Many of the biological actions of CRP are attributed to its phosphocholine

Fig. 6. **A. Stimulation of PMA induced superoxide generation in RAW 264.7 cells by D609.** RAW 264 cells were primed with 10 units/ml of IFN- γ for 16 h. Cells were then washed and stimulated by PMA (1 μ g/ml) and $O_2^{\cdot-}$ production was measured in the presence of CRP (100 μ g/ml), CRP (100 μ g/ml) IFN- γ (50 units/ml), and D609 (20 μ g/ml). Presence (+) or absence (-) of agents used is shown as indicated. Data represent mean \pm SD of four experiments. * P < 0.05 versus assay with only PMA by students t-test. **B. Inhibition of NO synthesis by D609.** RAW 264.7 cells were pretreated with D609 (20 μ g/ml) or equal volume of PBS for 20 min before the addition of CRP (50 μ g/ml) or CRP (50 μ g/ml) and IFN- γ (50 units/ml). Presence (+) or absence (-) of agents used is shown as indicated. Data represent mean \pm SD of four to six experiments. * P < 0.05 versus assay without D609 by students t-test



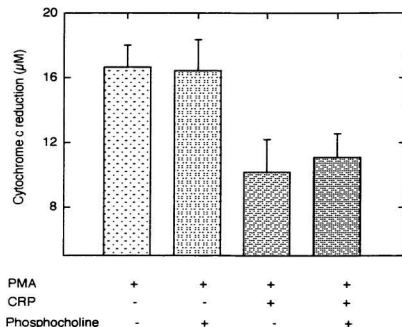


Fig. 7. Effect of phosphocholine on inhibition of $O_2^{\cdot -}$ production by CRP. CRP ($125 \mu\text{g} \approx 1 \mu\text{M}$) was incubated with $10 \mu\text{M}$ phosphocholine for 10 min before adding to the incubation medium. Rat peritoneal macrophages were then stimulated with PMA ($1 \mu\text{g}/\text{ml}$) for 60 min and cytochrome c reduction was determined as described in materials and methods. Presence (+) or absence (-) of agents used are indicated. Data represent mean \pm SD of four to six experiments.

binding property. To test whether the suppression of $O_2^{\cdot-}$ synthesis by CRP is mediated through its phosphocholine binding property, 125 μ g of CRP ($\sim 1 \mu$ M) was incubated with 10 μ M phosphocholine for 10 min before adding to the incubation medium, and subsequent stimulation of rat peritoneal macrophages by PMA. Binding to phosphocholine had no significant effect on the inhibition of $O_2^{\cdot-}$ generation by CRP indicating that this property was not mediated through its phosphocholine binding site (Fig. 7).

Section 3. Discussion

This study has shown that unlike its effect on the stimulation of NO generation, rat CRP suppresses $O_2^{\cdot-}$ production in macrophages. There have been conflicting reports on the effect of human CRP on $O_2^{\cdot-}$ generation. While there have been reports that human CRP decreases the production of $O_2^{\cdot-}$ by activated neutrophils and monocyte/macrophages (Dobrinich and Spagnuolo, 1991; Buchta, et al., 1987), there are also reports of increased $O_2^{\cdot-}$ and H_2O_2 production after prolonged exposure to CRP (Barna et al., 1984; Tebo and Mortensen, 1991). Peptides generated from activated macrophages have also been shown to suppress $O_2^{\cdot-}$ (Shephard et al., 1990). The results presented here show that rat CRP, which shares 65% homology with human CRP, inhibits $O_2^{\cdot-}$ generation by PMA-stimulated macrophages in a time and dose dependent manner. This inhibition was specific to CRP since similar

concentrations of various proteins such as BSA, IgG or another acute phase protein, α -1 acid glycoprotein, failed to inhibit cytochrome c reduction in PMA-stimulated rat macrophages. It is unlikely that this inhibition was due to a change in Ca^{++} levels, since CRP used in these assays contained no chelating agents. Although some investigators use EDTA for eluting CRP from the affinity absorbent, this laboratory routinely employs an excess of phosphocholine for elution of CRP from the phosphocholine affinity absorbent column, and extensive dialysis to remove the bound phosphocholine. Divalent cations are also necessary for optimal binding of CRP to macrophages presumably through a specific receptor (Nagpurkar et al., 1993).

It has been suggested that CRP at high concentrations may act as a scavenger of $\text{O}_2^{\cdot-}$ radicals (Dobrinich and Spagunolo, 1991). These data show that when CRP was added at various intervals after PMA stimulation, significant reduction of $\text{O}_2^{\cdot-}$ occurred only when CRP was added within 30 min after the addition of PMA. SOD inhibited cytochrome c reduction significantly at all time points after the addition of PMA. Moreover, there was only a slight inhibition of chemically induced $\text{O}_2^{\cdot-}$ generation which was statistically significant only when CRP was present in excess of 300 $\mu\text{g/ml}$. Buchta et al. have shown that human CRP at concentrations as high as 100 $\mu\text{g/ml}$ did not scavenge $\text{O}_2^{\cdot-}$ in a cell-free $\text{O}_2^{\cdot-}$ generation system (Buchta et al., 1987). This indicates that CRP may decrease the level of $\text{O}_2^{\cdot-}$ produced by stimulated macrophages by direct cell inhibition. However, CRP may also be involved in scavenging free radicals when it is present in very high concentrations as seen during periods of inflammation and tissue injury, when it may demonstrate some antioxidant

properties and thus limit the effects of free radicals. CRP has also been shown to decrease PMA-induced phosphorylation of several proteins including those of 85, 66, 54, 47 and 43 kDa (Buchta et al., 1988). These data are consistent with the view that activation by CRP would influence events in the intracellular compartment of neutrophils and macrophages where the components of signal transduction and metabolic pathways are fully accessible (Buchta et al., 1987; Nagpurkar et al., 1993).

The data presented here also suggest that the modulation of macrophage function by CRP may involve PC hydrolysis. A significant increase in the generation of phosphocholine was demonstrated (chapter 4), when [^{14}C]choline-labeled macrophages were stimulated with CRP, indicating a rise in phospholipase C activity. To delineate the role of PC-PLC in $\text{O}_2^{\cdot-}$ generation, D609, a specific inhibitor of PC-PLC (Muller-Decker, 1989; Schutze, et al., 1992), was used to block the activity of this enzyme. While the recapitulated results from previous chapters show that D609 inhibits NO synthesis, the data presented in this study show that treatment with this compound increases the secretion of $\text{O}_2^{\cdot-}$ significantly in PMA-stimulated macrophages while it had no effect on $\text{O}_2^{\cdot-}$ generation in unstimulated macrophages. Moreover, addition of bacterial PC-PLC to the cell culture did indeed decrease $\text{O}_2^{\cdot-}$ generation and D609 was able to offset this inhibition. Bacterial PC-PLC was previously shown to inhibit $\text{O}_2^{\cdot-}$ production by reversibly inhibiting NADPH oxidase in human polymorphonuclear leukocytes (Traynor et al., 1993). These data indicate a role for PC-PLC in the modulation of $\text{O}_2^{\cdot-}$ generation as well as NO synthesis. Assuming that D609 causes

specific inhibition of PC-PLC as previously reported (Muller-Decker, 1989; Schutze, et al., 1992), the inhibition of $O_2^{\cdot-}$ secretion and the stimulation of NO synthesis by CRP is possibly mediated through the activation of PC-PLC. It is also possible that the intact membrane PC may act as a steric facilitator in the assembly of the components of NADPH oxidase (Traynor et al., 1993).

Few studies have examined the binding properties of CRP to phagocytic cells, and limited data are available on the structure of the putative CRP receptor which is thought to be distinct from Fc receptors (Tebo and Mortensen, 1990; Crowell et al., 1991; Dobrinich and Spagunolo, 1991; Zahedi et al., 1989). While opsonic properties and complement activation are dependent on the binding of CRP to phosphocholine (Kaplan and Volanakis, 1974; Volanakis and Kaplan, 1974a; Kilpatrick and Volanakis, 1991), binding to monocytes and macrophages possibly via its receptor, was shown to be independent of its phosphocholine binding property (Tebo and Mortensen, 1990; Nagpurkar et al., 1993). Suppression of macrophage $O_2^{\cdot-}$ production by CRP also seems to be independent of phosphocholine binding. Similar conclusions were made in chapter 3 where iNOS induction in macrophages by CRP was shown to be independent of its phosphocholine binding property. This was explained by the assumption that the phosphocholine binding site on the surface of each CRP subunit is distinct from the binding site involved in ligation with its receptor.

Although 5-20 $\mu\text{g/ml}$ CRP is sufficient to stimulate iNOS synthesis in rat peritoneal macrophages, concentrations of 50 $\mu\text{g/ml}$ or more are required to cause significant inhibition of PMA-stimulated superoxide production. PMA

activates the phosphorylation of $p47^{\text{phox}}$ and subsequent translocation and assembly of the NADPH complex (Bastien and Hibbs, 1994), and this involves a relatively rapid series of events. Preincubation with CRP may initiate events that may prevent the activation of NADPH complex. Higher concentrations of CRP may be required not only to initiate PC hydrolysis and hitherto unknown events, but also for some free radical scavenging activity. Activation of iNOS induction by CRP may also involve the initiation of a signaling pathway that may include PC hydrolysis and subsequent events leading to iNOS transcription. Partially activated macrophages are known to express iNOS mRNA on stimulation by a second signal (Bastien and Hibbs, 1994; Nathan, 1992). Thioglycollate-elicited macrophages may require far less concentrations of a second signal such as CRP to initiate the cascade of events leading to the prolonged activation of iNOS than that required for the inhibition of $O_2^{\cdot-}$ production triggered by an extremely potent agonist such as PMA.

During the period of bacterial invasion, macrophages are deployed to destroy and remove invading microorganisms or inflammatory debris. The level of CRP also increases dramatically during periods of immunologic challenge (Gewurz et al., 1982), and has been shown to localize at inflammatory sites (Kushner, et al, 1963). Macrophages and other phagocytes generate $O_2^{\cdot-}$ as a result of phagocytic triggering. $O_2^{\cdot-}$ is the precursor of other ROIs including H_2O_2 and hydroxyl radical (Fridovich, 1986). Pumping of large concentrations of electrons as $O_2^{\cdot-}$ unaccompanied by protons results in an increase in vacuolar pH, and subsequent activation of neutral proteases which aid in the

killing and digestion of bacteria (Segal and Abo; 1993). Although the production of ROI contributes to the bactericidal property of macrophages, oxidation of cellular molecules by ROI can result in severe damage to the host tissue (Rosen et al., 1995). Macrophages also produce an abundance of NO when activated by an appropriate stimulus. Although both NADPH oxidase and iNOS activity can be induced in macrophages, the secretion of $O_2^{\cdot-}$ and NO does not occur simultaneously and is shown to be independently regulated (Martin and Edward, 1993). Ding et al. reported that of the 12 cytokines studied, only IFN- γ primed macrophages for increased ROI and RONI production (Ding et al., 1984). Monocytes produce $O_2^{\cdot-}$, but as they mature into macrophages, NO is employed to mediate cytotoxicity (Martin and Edward, 1993). NO has been reported to neutralize the cytotoxicity of $O_2^{\cdot-}$ under experimental conditions (Harbrecht et al., 1992). Independent regulation averts the potential reaction between $O_2^{\cdot-}$ and NO to form ONOO $^-$ (Beckman et al., 1990) which can produce irreversible damage to both microbes as well as host cells (Beckman et al., 1990; Zhu et al., 1992). In general, cells seem to avoid simultaneous generation of $O_2^{\cdot-}$ and NO. In summary, these results show that CRP differentially regulates NADPH oxidase and iNOS activity in macrophages. Increased hepatic synthesis and elevation of plasma CRP concentration during periods of inflammatory response may contribute to the mechanism by which immunologically activated cells avoid simultaneous $O_2^{\cdot-}$ and NO biosynthesis.

ROLE OF EXTRACELLULAR SIGNAL- REGULATED KINASE 1 AND 2 (ERK1/ERK2) AND I κ -B- α ACTIVATION BY CRP IN THE INDUCTION OF NITRIC OXIDE SYNTHASE IN RAW 264.7 CELLS

Section 1. Introduction

The results from chapter 4 demonstrated that activation of PI-PLC and/or PMA sensitive PKC isoenzymes is not likely to be involved in the pathway leading to iNOS induction by CRP in RAW 264.7 cells. Furthermore, these data also led to the conclusion that an increase in cellular cAMP, and presumably the activation of adenylyl cyclase and PKA, may even be inhibitory to NO synthesis in this cell line. In contrast, activation of PC-PLC and tyrosine kinases is found to be important in pathways leading to iNOS induction in CRP-stimulated RAW 264.7 cells. Cai et al. have shown that activation of the small GTP binding protein Ras-stimulated the hydrolysis of PC by PC-PLC. Hydrolysis of PC by PLC also couples Ras to Raf protein kinase leading to the subsequent activation of MAP kinases (Cai et al., 1993). Tyrosine phosphorylation and activation of p42 and p44 kDa mitogen-activated protein kinases (p42/p44 MAPK, ERK1/ERK2) have been shown to influence iNOS synthesis in LPS-stimulated mouse peritoneal macrophages, as well as in cytokine-stimulated cardiac muscle cells

(Novogrodski et al., 1994; Singh et al., 1996). In view of these facts, an investigation was carried out to see whether CRP activated ERK1/ERK2, and whether these ubiquitously expressed serine/threonine kinases are the mediators of signal generated by CRP stimulation that led to iNOS induction.

The predominant mechanism underlying the induction of iNOS in varied cell types is transcriptional regulation. Analysis of the cloned murine iNOS promoter has revealed the presence of numerous consensus sequences for the binding of various transcription factors (Lowenstein et al., 1993; Xie et al., 1993; Martin et al., 1994; Goldring et al., 1996). Of all the relevant transcription factors, nuclear factor- κ B (NF- κ B) and interferon regulatory factor have been shown to be important for NOS induction (Xie et al., 1994; Martin et al., 1994). NF- κ B is a multiunit transcription factor that can rapidly activate genes involved in immune response (Baeuerle and Henkel, 1994). The DNA-binding protein complex is composed mainly of proteins with molecular weights of 50 kDa and 65 kDa and shares considerable homology with the proto-oncogene c-rel (Finco and Baldwin, 1995). In the unstimulated state, this complex is present in the cytosol bound to the inhibitory protein I- κ B- α . Phosphorylation of I- κ B- α leads to the polyubiquitination and degradation of I- κ B- α (Henkel et al., 1993; Israel, 1995), resulting in dissociation of the NF- κ B complex and translocation of the DNA binding complex to the nucleus. This complex binds to specific promoter/enhancer regions including that of iNOS promoter leading to iNOS transcription (Xie et al., 1994). Both NF- κ B activation and iNOS transcription were shown to be influenced by PC-PLC activation in many cell types (Tschaikowsky et al., 1994; Schutz et al., 1992; Kengatharan et al., 1996). PC-PLC has also been

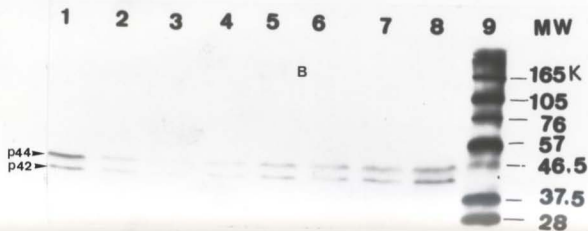
linked to the activation of Raf protein kinase by Ras and subsequent activation of mitogen-activated ERK1/ERK2 (Cai et al., 1993). Several lines of evidence suggest that the Ras \rightarrow MAPK cascade may mediate NF- κ B activation (Li and Sedivy, 1993; Finco and Baldwin, 1993; Bertrand et al., 1995; Blenis, 1993; Schouten et al., 1997). These data implied that the activation of PC-PLC and ERKs are likely to influence NF- κ B activation. Since NF- κ B activation and translocation was shown to be a critical event in the stimulation of iNOS transcription, the role of CRP induced I- κ B phosphorylation in the regulation of iNOS synthesis was studied to define a possible mechanism by which CRP may activate macrophages.

Section 2. Results

A. Kinetics of ERK1/ERK2 Activation

To determine whether CRP may stimulate the phosphorylation of ERK1/ERK2 in intact macrophages, RAW 264.7 cells were exposed to CRP for various times ranging from 0 to 360 min. Cells were immediately lysed, and cellular proteins were separated by SDS-PAGE and immunoblotted against an antibody that detects ERK1/ERK2 only when catalytically activated by phosphorylation at Thr 202 and Tyr 204. A significant increase in the phosphorylated form of ERK1/ERK2 was seen in lysates from CRP treated cells as compared to control cell lysates. The increase in phosphorylation was detectable at 30 min after rat CRP treatment with a maximum response at 60 min.

Fig. 1. Kinetics of ERK1/ERK2 activation by rat CRP. RAW 264.7 cells were stimulated with rat CRP or Tris buffer for 0 to 360 min. 40-50 μ g of cell lysate protein was separated on 12% SDS-PAGE and immunoblotted against an antibody specific to phosphorylated ERK1/ERK2. Bound antibodies were detected by horseradish peroxidase conjugated anti-rabbit second antibody and chemiluminescent detection method. **A.** Cells treated with rat CRP (50 μ g/ml). lanes 1- 8; 0, 15, 30, 60, 90, 120, 240, and 360 min respectively. Lane 9, Biotinylated MW standards **B.** control cells. lanes 1-8, 0, 15, 30, 60, 90, 120, 240, and 360 min respectively. lane 9, Biotinylated MW standards. Blots represent at least three experiments with similar results.



This stimulation was sustained for up to 90 min and started to wane at about 120 min (Fig. 1A). In contrast, no increase in ERK1/ERK2 phosphorylation was observed in control cells (Fig. 1B). Activation of ERK1/ERK2 by human CRP was readily detectable after 30 min of stimulation which reached a maximum at 60 min (Fig. 2A). The extent of ERK1/ERK2 phosphorylation was also dependent on the concentration of CRP. Fig. 2B shows the phosphorylation of ERK1/ERK2 in RAW 264.7 cells when treated with increasing concentrations of human CRP for 60 min.

Addition of IFN- γ (20 units/ml) along with rat CRP had no significant effect on the extent of phosphorylation of ERK1/ERK2, but it was detectable at a much earlier time and was sustained for longer periods. When lysates were probed with an antibody to phosphorylated ERK1/ERK2, phosphorylation was clearly discernible at 20 min post stimulation, and was sustained for at least up to 240 min (Fig. 3A). When the same blots were probed with an antibody to ERK1/ERK2 independent of its phosphorylation status, increasing retardation in its mobility was evident at 20 min which returned to basal levels in 240 min (Fig. 3B). . When RAW 264.7 cells were incubated with IFN- γ (20 units/ml) alone, no increase in the phosphorylation of ERK1/ERK2 over unstimulated control cells was detected (Fig. 3C).

B. Inhibition of CRP Induced ERK1/ERK2 Activation

Various inhibitor compounds were used to discern the role of the ERK cascade in stimulating iNOS gene expression in macrophages in response to CRP.

Fig. 2. **ERK1/ERK2 activation by human CRP.** RAW 264.7 cells treated with human CRP (50 $\mu\text{g/ml}$) or buffer for 0 to 120 min. 50-60 μg of cell lysate protein was separated on 12% SDS-PAGE and immunoblotted against an antibody specific to phosphorylated ERK1/ERK2. Bound antibodies were detected by horseradish peroxidase conjugated anti-rabbit second antibody and chemiluminescent detection method. **A.** Lanes 1-6, 0, 15, 30, 45, 60, and 120 min respectively. lane 7, MW standards. **B.** Cells treated with increasing concentrations of human CRP. lanes, 0-5; CRP 0, 5, 10, 20, 50 and 100 $\mu\text{g/ml}$ for 60 minutes respectively; lane 6, Phospho ERK control protein supplied by the manufacturer; lane 7, Biotinylated MW standards. Blots represent at least three experiments with similar results.

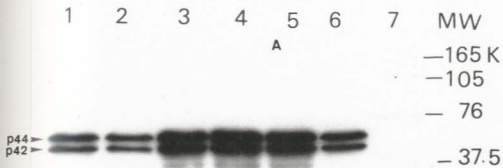


Fig. 3. Activation of ERK1/ERK2 by rat CRP and IFN- γ . RAW 264.7 cells were stimulated with CRP (50 μ g) and IFN- γ (20 units) for 0 to 240 min as described. 40-50 μ g of cell lysate protein was separated on 12% SDS-PAGE and immunoblotted. **A.** Cell lysates immunoblotted against antibody specific to phosphorylated ERK1/ERK2. lanes, 1 to 8, 0, 10, 20, 30, 60, 90, 120 and 240 min respectively; lane 9, MW standards. **B.** Same blot stripped and reprobed with an antibody to ERK1/ERK2. **C.** Lysates from cells treated with CRP (50 μ g), IFN- γ (20 Units), or CRP + IFN- γ for 60 min and immunoblotted against an antibody specific to phosphorylated ERK1/ERK2. lane 1, control; lane 2, CRP; lane 3, IFN- γ ; lane 4, CRP + IFN- γ ; lane 5, MW standards. Blots represent at least three experiments with similar results.

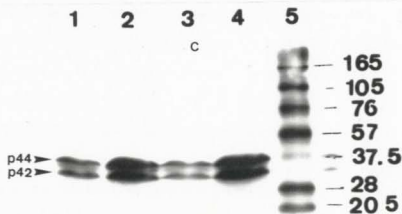
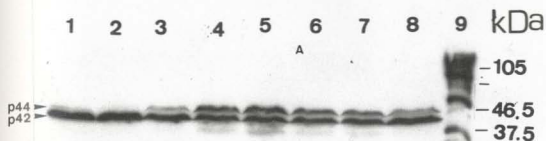
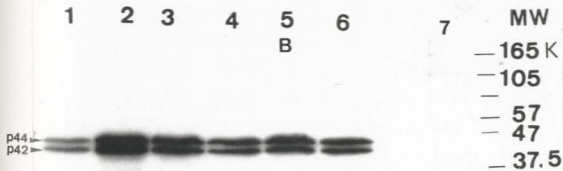
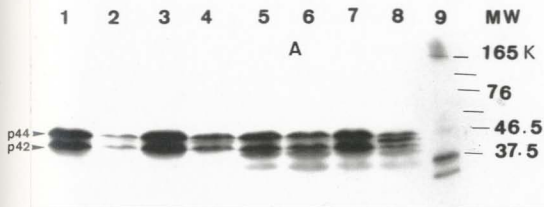


Fig. 4A shows the inhibition of CRP-mediated activation of ERK1/ERK2 by various compounds. Hydrolysis of PC has been linked to the activation of Raf kinases by RAS (Cai et al., 1993). Since MAPK Kinase (MEK) is a downstream substrate of Raf kinase, activation of Raf would lead to the activation of ERK. To verify that PC hydrolysis, and possibly the RAS-Raf pathway is involved in CRP-mediated ERK activation, D609, a specific inhibitor of PC-PLC, was used to block CRP induced PC-PLC activation. RAW cells were pretreated with D609 (20 μ g/ml) for 60 min before the addition of CRP. D609 strongly inhibited CRP-mediated MAPK activation. Laser densitometric analysis showed that there was a 90% reduction of ERK1/ERK2 activation by 20 μ g/ml D609 (Fig. 4A, lane 2). In contrast, U73122 (20 μ M), an inhibitor of PI-PLC had no significant effect on MAPK phosphorylation (lane 3). Recent studies have shown that a synthetic compound, PD 98059 was able to specifically block the activation of MAP Kinase kinase (MEK), the upstream kinase of ERK. Consequently it also inhibited the activation of ERK1/ERK2, both in vitro and in vivo, with no effect on parallel kinases such as stress activated protein kinase or p38 kinase (Alessi et al., 1995). PD 98059 (50 μ M) decreased activation ERK1/ERK2 by CRP by 45% and 55%, respectively (lane 4). In contrast, SB 202190 (20 μ M), a specific inhibitor of the parallel p38 kinase (Lee et al., 1994; Jiang et al., 1996), had little effect on ERK1/ERK2 activation (lane 5). Since ERK1/ERK2 require phosphorylation on both Ser and Tyr for complete activation, effect of the tyrosine kinase inhibitor tyrphostin AG 126 was studied. This compound at a concentration of 100 μ M also reduced the activation of ERK1/ERK2 to CRP to 47% and 72% of CRP-stimulated samples (lane 6). Effect of two other agents, pyrrolidine dithiocarbamate (PDTc), and sodium salicylate, that were previously

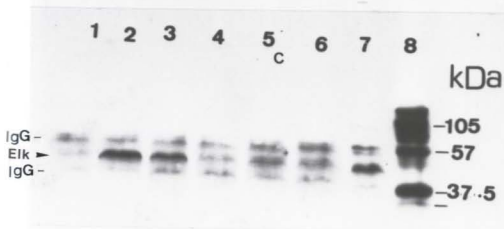
Fig. 4. **Effect of inhibitors on ERK1/ ERK2 activation.** **A.** RAW 264.7 cells were pre-incubated with various agents for 60 min and stimulated with CRP (50 μ g/ml) and IFN- γ (20 units/ml) for 60 min. Cells were lysed and 40-50 μ g of cell lysate protein was separated on 12% SDS-PAGE and immunoblotted against an antibody to phosphorylated ERK1/ERK2. lane 1, IFN/CRP; lane 2, IFN/CRP + D609 (20 μ g/ml); lane 3, IFN/CRP + U-73122 (20 μ M); lane 4, IFN/CRP + PD 98059 (50 μ M); lane 5, IFN/CRP + SB 202190 (20 μ M); lane 6, IFN/CRP + Tyrphostin AG 126 (100 μ M); lane 7, IFN/CRP + PDTC (50 μ M); lane 8, IFN/CRP + sodium salicylate (20 mM); lane 9, MW standards. **B.** Immunoblots of cell lysates pre-treated with increasing concentrations of PD 98059 and then stimulated with rat CRP. lane 1, Control ; lane 2, CRP (50 μ g/ml); lane 3. CRP + PD98059 (10 μ M); lane 4, CRP (50 μ g/ml) + PD 98059 (20 μ M); lane 5, CRP (50 μ g/ml) + PD 98059 (50 μ M); lane 6. CRP (50 μ g/ml) + PD 98059 (100 μ M); ; lane 7, MW standards. Blots represent at least three experiments with similar results.



shown to decrease iNOS production in macrophages (Xie et al., 1994, Kepka-Lenhaart et al., 1996) was also examined. PDTC had no effect on MAPK activation in RAW 264.7 cells (lane 7), but pretreatment of RAW 264.7 cells with salicylate (20 mM), an anti-inflammatory agent resulted in considerable reduction in ERK1/ERK2 phosphorylation triggered by CRP (lane 8). Considerable reduction in ERK1/ERK2 activation was also observed in RAW 264.7 cells pretreated with increasing concentrations of PD 98059 and subsequently stimulated with rat CRP alone or a combination of CRP and IFN- γ (Fig. 4B).

To verify that the phosphorylated ERK1/ERK2 protein detected by Western blot was catalytically active, activation of ERK1/ERK2 by CRP was further studied by an immune complex kinase assay. Cell lysates prepared after appropriate stimulation were selectively immunoprecipitated with a monoclonal antibody that recognizes activated ERK1/ERK2 enzyme, and immune complexes were tested for their ability to phosphorylate Elk-1 GST fusion protein, a preferred substrate of ERK1/ERK2 (Marais et al., 1993). Immune complex kinase assays showed an increase in phosphotransferase activity towards Elk-1 in human CRP treated RAW 264.7 cells. Cells stimulated with CRP (50 $\mu\text{g/ml}$) showed similar kinetics of activation as seen in the Western blot (Fig. 5A). Treatment with increasing doses of PD 98059 (10-100 μM) also resulted in decreased phosphotransferase activity in cells treated with rat CRP (50 $\mu\text{g/ml}$) or human CRP (20 $\mu\text{g/ml}$) for 60 min. The inhibition by PD 98059 was much more robust when lower concentrations of CRP was used (Fig. 5B and 5C).

Fig. 5. Phosphotransferase activity in CRP-stimulated RAW 264.7 cells. Cell lysates were immunoprecipitated with an antibody against phosphorylated ERK1/ ERK2 and phosphotransferase activity was assessed by analyzing the phosphorylation of Elk-1. **A.** Immunoprecipitated lysates from cells treated with IFN γ (20 units/ml) + human CRP (50 μ g/ml). lanes 1, 2, 3, 4, 5, 6, 7, 8; 0, 10, 30, 60, 90, 120, 180 and 240 min respectively; lane 9, MW standards. **B.** Effect of PD 98059 on phosphotransferase activity in RAW 264.7 cells. Cells were pre-treated with PD 98059 for 60 min and then stimulated for 60 min by CRP (50 μ g) and IFN γ (20 units/ml). lane 1, control; lane 2, Rat CRP (50 μ g); lane 3, IFN (20 units/ml) + CRP (50 μ g/ml); lane 4, IFN/CRP + PD 98059 (10 μ M); lane 5, IFN/ CRP + PD 98059 (20 μ M). lane 6, IFN/CRP + PD 98059 (50 μ M); lane 7, MW standards. **C.** Cells were prepared as described above except that they were stimulated with human CRP (20 μ g/ml). lane 1, control; lane 2, human CRP (20 μ g/ml); lane 3, human CRP + PD 98059 (10 μ M); lane 4, human CRP + PD 98059 (20 μ M); lane 5, human CRP + PD 98059 (50 μ M); lane 6, human CRP + PD 98059 (100 μ M); lane 7, Activated ERK positive control; Lane 8, MW standards. Arrow indicates phosphorylated Elk. Blots represent three experiments with similar results.



C. Activation of MAPK by CRP is Independent of Protein kinase C Activation

To determine whether protein kinase C (PKC) is involved in the activation of ERK1/ERK2 by CRP, RAW 264.7 cells were pretreated with bisindolylmaleimide (100 nM), a specific PKC inhibitor, before stimulation with CRP (Kuchera et al., 1993; Toullec et al., 1991). Cells were also stimulated with phorbol myristate acetate (PMA) which served as a positive control. Fig. 6 shows that bisindolylmaleimide had no effect on ERK1/ERK2 activation by CRP (50 μ g/ml) but decreased PMA (100 ng/ml) induced phosphorylation of ERK1/ERK2 significantly. These observations suggest that the activation of ERK1/ERK2 in RAW 264.7 cells by CRP or CRP/IFN occurred independent of PKC activation.

D. MAP kinase kinase 1 (MEK) Activation by CRP

Inhibition of ERK1/ERK2 by D609 and PD 98059 indicated the possibility that Raf1 \rightarrow MEK \rightarrow ERK1/ERK2 pathway may be utilized by CRP for signaling to the nucleus to initiate iNOS synthesis. To evaluate CRP-mediated MAP kinase kinase 1 (MEK) activation, CRP treated RAW 264.7 cell lysates were immunoblotted against an antibody that recognized MEK1 only when phosphorylated at Ser217 and Ser221. These blots revealed an increase in the phosphorylated form of MEK in CRP treated cells. The time-dependent increase in MEK phosphorylation by CRP was detectable at 30 min after exposing cells to CRP, reaching a maximum at 60 min and starting to diminish

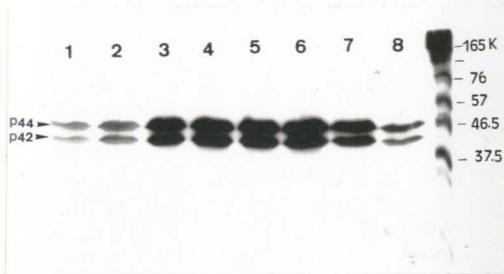


Fig. 6. ERK1/ERK2 activity in RAW 264.7 cells pretreated with PKC inhibitor, bisindolmaleimide. Cells were cultured in medium and pretreated with bisindolmaleimide ($0.1 \mu\text{M}$) for 60 min and stimulated with CRP ($50 \mu\text{g/ml}$) or CRP/IFN- γ (20 units/ml) for 60 min. As a positive control, cells were also treated with PMA (100 ng/ml) for 20 min. lane 1, control; lane 2, control + bisindolmaleimide; lane 3, rat CRP ($50 \mu\text{g/ml}$); lane 4, rat CRP + bisindolmaleimide ($0.1 \mu\text{M}$); lane 5, rat CRP + IFN- γ (20 units/ml); lane 6, rat CRP + IFN- γ + bisindolmaleimide; lane 7, PMA (100 ng/ml); lane 8, PMA + bisindolmaleimide. Blots represent of three experiments with similar results.

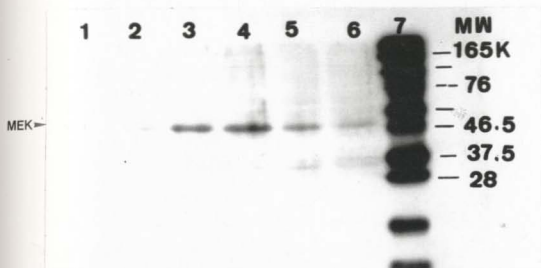


Fig. 7. **ERK1/ERK2 kinase (MEK) activation by CRP.** RAW 264 7 cells were stimulated with human CRP (20 μ g) for various periods ranging from 0 to 120 min. 50-60 μ g of cell lysate protein was separated on 12% SDS-PAGE and immunoblotted against antibody specific to phosphorylated MEK. Lanes, 1 to 6; 0, 15, 30, 60, 90 and 120 min; lane 7. MW. Standards. Blots represent three experiments with similar results.

thereafter (Fig. 7). Since MEK1 and MEK2 are the only known downstream physiological substrates for Raf-1 kinase (Daum et al., 1994), these data indicate the possibility that activation of Raf-1 kinase may precede MEK activation by CRP.

E. Effect of CRP on p46/p54 Stress Activated Protein Kinase (SAPK)

The notion that CRP may also stimulate parallel MAP kinases such as p46/p54 MAPK (SAPK) was also explored. Although non-phosphorylated SAPK was detected in control and CRP-treated RAW 264.7 cell lysates, these cells failed to show a positive reaction when immunoblotted against an antibody to phosphorylated SAPK. However, increased phosphorylation of SAPK was observed when cells were pre-incubated with D609 and sodium salicylate, both of which were capable of inhibiting CRP-mediated ERK1/ERK2 phosphorylation. Neither PD 98059, which clearly inhibited ERK1/ERK2 activation, nor other inhibitors tested, including SB 202190, a p38 kinase inhibitor, had any effect on SAPK phosphorylation (Fig. 8). These observations indicated that SAPK activation may not be involved in mediating cell signals initiated by CRP.

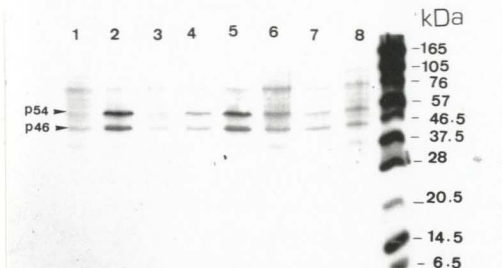


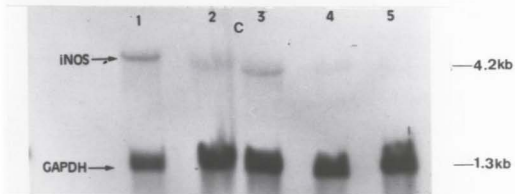
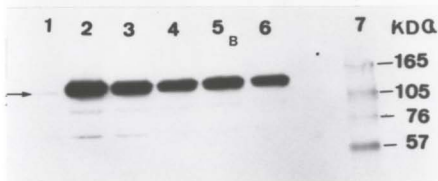
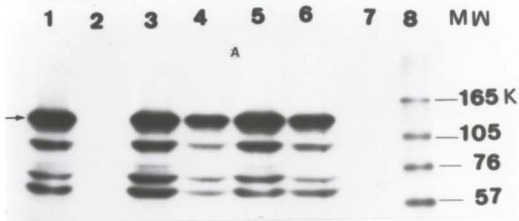
Fig. 8. Effect of CRP on the activation of SAPK in RAW 264.7 cells.

Cells were pre-incubated with various agents for 60 min and stimulated with CRP (50 μ g) and IFN- γ (20 Units/ml) for 60 min. Cells were lysed and 40-50 μ g cell lysate protein was separated on 12% SDS-PAGE and immunoblotted against an antibody to phosphorylated SAPK (p46/p54). Lane 1, IFN/CRP; lane 2, IFN/CRP + D609 (20 μ g/ml); lane 3, IFN/CRP + U-73122 (20 μ M); lane 4, IFN/CRP + sodium salicylate (10 mM); lane 5, IFN/CRP + sodium salicylate (20 mM); lane 6, IFN/CRP + SB 202190 (50 μ M); lane 7, IFN/CRP + PD 98059 (50 μ M); lane 8, IFN/CRP + tyrphostin AG 126 (100 μ M); lane 9, MW standards. Blots represent at least three experiments with similar results.

F. Correlation of ERK1/ERK2 Activation and iNOS Synthesis

The next objective was to determine whether there was any correlation between MAP kinase activation and iNOS induction in RAW 264.7 cells stimulated with CRP. Cells were pretreated for 60 min with substances that inhibited ERK1/ERK2 before stimulating with CRP and IFN- γ . Cells treated with D609, PD 98059, and Tyrphostin AG 126 had reduced levels of iNOS as seen on Western blots (Fig. 9). In contrast, compounds such as U73122 and SB 202190 that did not inhibit ERK1/ERK2 activation had no significant effect on iNOS induction by CRP/IFN- γ . Pre-treatment of cells with increasing concentrations of PD 98059 also resulted in an inhibition of iNOS protein synthesis induced by CRP (Fig. 9B). Cells stimulated with human CRP (20 μ g/ml) in the presence of D609 or PD 98059 also had reduced levels of iNOS mRNA (Fig. 9C). These findings collectively showed that PC hydrolysis, tyrosine phosphorylation and ERK1/ERK2 pathway may be important in the pathway leading to CRP-stimulated iNOS transcription.

Fig. 9. Correlation of ERK1/ERK2 activation with iNOS induction stimulated by CRP and IFN- γ . RAW 264.7 cells were pre-incubated with various agents for 60 min and stimulated with CRP (50 μ g) and IFN- γ (20 Units/ml) for 12 h. Cells were lysed and 10 μ g cell lysate protein was separated on 7.5 % SDS-PAGE and immunoblotted against an antibody to iNOS and detected by chemiluminescent reagents. Arrow denotes iNOS protein (130 kDa). Lower molecular weight bands represent degradation products of iNOS protein. **A.** lane 1, IFN/CRP; lane 2, IFN/CRP + D609 (20 μ g/ml); Lane 3, IFN/CRP + U-73122 (20 μ M); lane 4, IFN/CRP + PD 98059 (50 μ M); lane 5, IFN/CRP + SB 202190 (20 μ M); lane 6, IFN/CRP + Tyrphostin AG 126 (100 μ M); lane 7, empty lane; lane 8, MW standards. **B.** Immunoblots of cell lysates pre-treated with increasing concentrations of PD 98059 and stimulated with rat CRP for 12 h. lane 1. Control. lane 2, CRP (50 μ g/ml); lane 3. CRP + PD 98059 (10 μ M); lane 4, CRP + PD 98059 (20 μ M); lane 5. CRP + PD 98059 (50 μ M); lane 6. CRP + PD 98059 (100 μ M); lane 7, MW Standards. Arrow denotes iNOS protein. **C.** Northern blot analysis of iNOS mRNA from RAW 264.7 cells pre-treated with inhibitor compounds and stimulated with human CRP for 12 h. lane 1, CRP (20 μ g/ml); lane 2, CRP + D609 (20 μ g/ml); Lane 3, CRP + U-73122 (20 μ M); lane 4, CRP + PD 98059 (50 μ M); lane 5, control. Blots represent at least three experiments with similar results.



G. Activation of I- κ B by CRP

Activation of the NF- κ B complex is initiated by phosphorylation and subsequent proteolytic degradation of I- κ B protein (Finco and Baldwin, 1995; Henkel et al., 1993). Phosphorylation of I- κ B in RAW 264.7 cell extracts was assessed by immunoblotting against an antibody that specifically recognized phosphorylated I- κ B- α . Stimulation of RAW 264.7 cells by CRP resulted in increased phosphorylation of I- κ B. This was detectable in about 60 min after rat CRP treatment and was sustained for at least up to 240 min (Fig. 10A). In the presence of IFN- γ and CRP, I- κ B phosphorylation could be detected as early as 10-20 min and was sustained over a longer period of time (Fig. 10B). Extracts from cells stimulated with CRP were also immunoblotted against an antibody that recognizes phosphorylation state-independent I- κ B. Comparison of these blots with that recognized the phosphorylated isoform of I- κ B showed that CRP activated I- κ B over a period of time, and that this activation reached a maximum at 120 min and decreased thereafter. A low level of activation was sustained for at least 360 min (Fig 11A and 11B). Inhibitors of ERK1/ERK2 also diminished I- κ B- α phosphorylation induced by CRP. Sodium salicylate that reduced the activation of ERK1/ERK2 also inhibited I- κ B phosphorylation. In contrast, agents such as U73122, or SB 202190 that had no effect on ERK1/ERK2 activation, did not affect the phosphorylation of I- κ B (Fig. 12). This indicated that I- κ B phosphorylation and ERK1/ERK2 activation may follow a similar or parallel pathway in CRP-stimulated 264.7 cells.

Fig. 10. Activation of I- κ B α by CRP. RAW 264.7 cells were stimulated with rat CRP (50 μ g) alone or in combination with IFN- γ (20 units/ml) for periods ranging from 0 to 240 min as described. 50-60 μ g of cell lysate protein was separated on 12% SDS-PAGE and immunoblotted with an antibody specific to phosphorylated I- κ B α . **A.** Cells stimulated with CRP (50 μ g/ml) and probed with an antibody to phosphorylated I- κ B α . Lanes, 1 to 7; 0, 10, 30, 60, 90, 120 and 240 min respectively; lane 9. MW. Standards; **B.** Cells stimulated with CRP (50 μ g/ml) and IFN- γ (20 units/ml)) and probed with an antibody to phosphorylated I- κ B α . Lanes, 1 to 8; 0, 15, 30, 45, 60, 90, 120 and 240 min respectively; lane 8. MW. Standards. Blots represent at least two experiments with similar results.

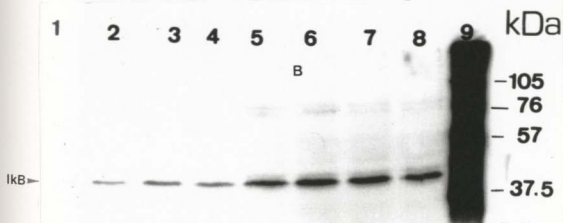
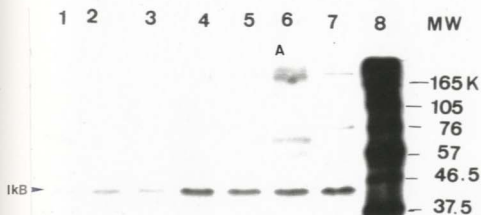
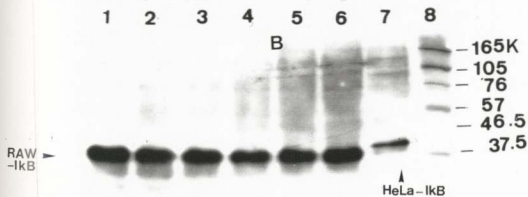
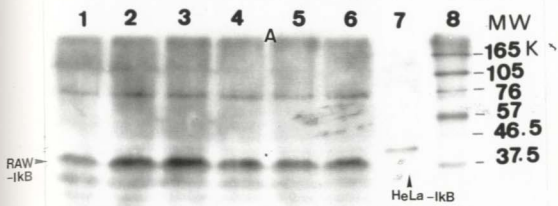


Fig. 11. Comparison of phosphorylated and non-phosphorylated isoforms of I- κ B α in macrophages stimulated by CRP. RAW 264.7 cells were stimulated with rat CRP (50 μ g) and IFN- γ (20 units/ml). **A.** Cell lysates immunoblotted with an antibody to phosphorylated I- κ B α . Lanes, 1 to 6; 0, 60, 120, 180, 240 and 360 min respectively; lane 7, positive control supplied by the manufacturer (TNF-stimulated HeLa cell extract); lane 8. MW. Standards. **B.** Same lysates immunoblotted with an antibody to non-phosphorylated I- κ B α . Lanes, 1 to 6; 0, 60, 120, 180, 240 and 360 min, respectively; lane 7; positive control supplied by the manufacturer (unstimulated HeLa cell extract); lane 8. MW. Standards. Blots represent at least two experiments with similar results.



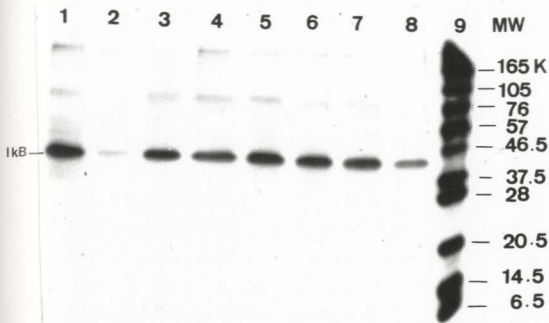
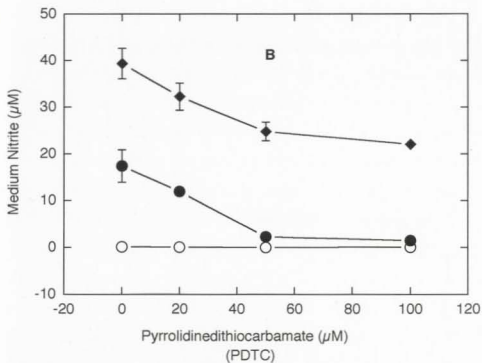
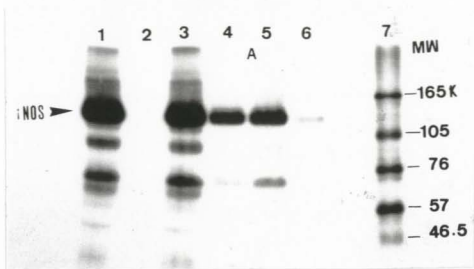


Fig.12. Effect of inhibitors on I- κ B α activation by CRP. Cells were pre-incubated with various agents for 60 min and stimulated with CRP (50 μ g) and IFN- γ (20 units/ml) for 60 min. Cells were lysed, separated on 12% SDS-PAGE and immunoblotted against an antibody to phosphorylated I- κ B α . Lane 1, IFN/CRP; lane 2, IFN/CRP + D609 (20 μ g/ml); lane 3, IFN/CRP + U-73122 (20 μ M); lane 4, IFN/CRP + PD 98059 (50 μ M); lane 5, IFN/CRP + SB 202190 (20 μ M); lane 6, IFN/CRP + tyrphostin AG 126 (100 μ M); lane 7, IFN/CRP + PDC (50 μ M); lane 8, IFN/CRP + sodium salicylate (20 mM); lane 9, MW standards. Blots represent at least three experiments with similar results.

H. Correlation of NF- κ B Activation and iNOS Synthesis

To establish a correlation between I κ B activation and iNOS induction in RAW 264.7 cells stimulated with CRP, cells were pretreated for 60 minutes with agents that were previously shown to inhibit ERK, PC-PLC and NF- κ B before stimulating with CRP. Cells treated with D609, PD 98059, and Tyrphostin AG 126 had reduced levels of phospho-I κ B and iNOS as seen on Western blots (Fig. 13A). In contrast, U73122 which did not inhibit ERK1/ERK2 or I κ B activation had no significant effect on iNOS induction by CRP. Activation of the NF- κ B complex can be blocked by thiol compounds such as pyrrolidine dithiocarbamate (PDTC). 50 μ M PDTC almost completely inhibited iNOS induction by CRP. Figure 13B shows the dose-dependent inhibition of iNOS activation by PDTC in RAW cell lysates stimulated with CRP.

Fig. 13. Effect of ERK and I- κ B inhibitors on iNOS synthesis stimulated by CRP. RAW 264.7 cells were pre-incubated with various agents for 60 minutes and stimulated with CRP (50 μ g) for 18 h. **A.** Cells were lysed and 20 μ g cell lysate protein was separated on 7.5 % SDS-PAGE and immunoblotted against an antibody to iNOS. lane 1, CRP; lane 2, CRP + D609 (50 μ g/ml); lane 3, CRP + U-73122 (20 μ M); lane 4, IFN/CRP + PD 98059 (50 μ M); lane 5, CRP + Tyrphostin AG 126 (100 μ M) lane 6, CRP + PDTC (50 μ M); Lane 8, MW standards. Blots represent at least three experiments with similar results. **B.** RAW 264.7 cells were pre-incubated with increasing concentrations of PDTC for 30 min and stimulated with CRP (50 μ g) or CRP (50 μ g) and IFN- γ (20 Units/ml) for 18 h. Accumulated nitrites in the medium were assayed with Griess reaction. Control (○). CRP (●). CRP and IFN- γ (◆). Data represent mean \pm SD of three experiments.



Section 3. Discussion

The results presented in this chapter have shown that iNOS synthesis induced by rat and human CRP in murine macrophage RAW 264.7 cell line is at least partly dependent on ERK1/ERK2 activation. RAW 264.7 cells were chosen to define the molecular signaling mechanisms in response to CRP. Besides the fact that biochemical and molecular regulation of iNOS in RAW 264.7 cells is well characterized (Xie et al., 1992), human CRP has also been shown to activate these cells and induce significant tumoricidal activity independently of lymphokines and LPS (Zahedi and Mortensen, 1986). Moreover, activation of ERK1/ERK2 in response to a variety of stimuli has been demonstrated in this cell line (Sanghera et al., 1996). These factors made this cell line an attractive model to study the molecular mechanisms triggered by CRP. The data presented here show that stimulation of RAW 264.7 cells by CRP resulted in a marked increase in the activation of ERK1/ERK2. These data also show a strong correlation between ERK phosphorylation and iNOS activation. Activation of ERK1/ERK2 by CRP may be a necessary early event in the signaling pathway mediated by CRP. This conclusion is based on the following observations: i) Both rat and human CRP activated ERK1/ERK2 and induced iNOS in RAW 264.7 cells; ii) Substances that inhibited ERK1/ERK2 activation in RAW cells by CRP also inhibited iNOS induction in these cells; iii) The reduction in iNOS protein synthesis in response to CRP was proportional to the extent of ERK1/ERK2 inhibition; iv) PD 98059, a specific inhibitor of MEK activation by

upstream kinases, inhibited ERK1/ERK2 activation and also suppressed iNOS induction by CRP. However, complete inhibition of CRP-induced ERK activation and iNOS induction by PD 98059 was not achieved, even when the cells were preincubated with this agent for as long as 6 h. Alessi et al. have shown that while 50 μ M PD 98059 prevented the activation of p42 MAPK by insulin or low levels of EGF (0.01-0.1 ng/ml) almost completely, only 8% inhibition was achieved when a strong dose of EGF (100 ng/ml) was used (Alessi et al., 1995). It is likely that CRP is a potent agonist for ERK1/ERK2 stimulation in RAW 264.7 cells, or the concentration of PD 98059 is not sufficient to inhibit total MEK activation. This may also be due to the enormous amplification potential of this kinase cascade. The concentration of PD 98059 could not be increased beyond 100 μ M due to the extreme insolubility of this compound in aqueous medium. However, the inhibition of CRP-induced iNOS protein synthesis by PD 98059 strongly suggests a role for ERK activation in mediating the signal leading to iNOS gene transcription.

Unlike serum and growth factors which increase ERK phosphorylation very rapidly, activation of ERK1/ERK2 by CRP is slower and sustained for longer periods of time. This may reflect the kinetics of CRP binding to cells. Tebo and Mortensen have shown that monomer human CRP was internalized by U-937 cells in about 30 min at 37°C and reached a maximum at 90 min, accompanied by its degradation into smaller peptides (Tebo and Mortensen, 1991). Activation of ERK1/ERK2 by human CRP was clearly evident at 30 min of CRP treatment, which was sustained for over 60 min and started to decline in about 120 min. It is possible that the peptides generated from CRP may be

responsible for MAPK activation. Activation of ERK1/ERK2 by rat CRP followed slower time kinetics, and CRP maximally activated ERK1/ERK2 at 60-90 min after stimulation. It is also possible that glycosylated rat CRP may be less susceptible to proteolysis. Degradation of rat CRP by macrophages has been shown to increase linearly in a time and concentration dependent manner (Nagpurkar et al., 1993). A membrane protease from activated neutrophils has also been shown to degrade CRP into biologically active peptides (Robey et al., 1987; Shephard et al., 1990).

Addition of IFN- γ to the RAW cell culture medium along with CRP resulted in a dramatic increase in iNOS synthesis. However, IFN- γ and CRP together did not cause an increase in the extent of ERK1/ERK2 phosphorylation, but phosphorylation of these kinases occurred at a much earlier time (20 min) and was sustained for longer periods. IFN- γ alone (up to 50 units/ml) did not increase ERK1/ERK2 phosphorylation above baseline levels. This can be explained by different ways. i) IFN- γ treatment may upregulate and increase CRP receptor concentration; ii) Macrophages primed with IFN- γ may secrete increased levels of proteases that may degrade native CRP protein into bioactive peptides; iii) It is also likely that macrophages primed with IFN- γ may bind and/or internalize CRP more rapidly. It has been reported that RAW 264.7 cells exhibited increased Fc receptor levels after IFN- γ stimulation (Weinshank et al., 1988). Although receptors for CRP on U-937 and PU5 1.8 cells were shown to be distinct from IgG Fc receptors (Zahedi et al., 1989), there have also been reports indicating that CRP also bound to Fc gamma RI to some extent (Crowell et al., 1991). iv) IFN- γ and CRP may also activate macrophages via

separate and distinct signaling pathways, which may converge at the level of iNOS promoter activation. Alternatively, IFN- γ may also enhance CRP induced iNOS mRNA stability.

In order to test the potential role for lipid second messengers in CRP-mediated cell activation, the effect of phospholipase inhibitors on the phosphorylation of ERK1/ERK2 and on iNOS induction was examined. These data show that a specific inhibitor to PC-PLC decreased the phosphorylation of ERK1/ERK2 in response to CRP, while inhibition of PI-PLC had no appreciable effect on ERK1/ERK2 activation. Activation of Ras proteins by mitogens was shown to result in increased PC hydrolysis, and overexpression of PC-PLC was able to bypass the effect of dominant negative Ras mutants (Cai et al., 1993, Cai et al., 1992). From these data, and the observation that inhibition of PC-PLC negatively influenced ERK1/ERK2 activation, it was inferred that activation ERK1/ERK2 by CRP may involve a mechanism that may stimulate Raf and PC-PLC. CRP was previously shown to stimulate the hydrolysis of PC in rat peritoneal cells as well as in a cell free system (Ratnam and Mookerjee, 1998; Mookerjee and Hunt, 1995). In the present study, the activation of ERK1/ERK2 was shown to be independent of PKC activation in cells activated by CRP. Bisindolylmaleimide, a specific inhibitor of PKC, had no effect on ERK1/ERK2 activation by CRP but had significant inhibitory effect in phorbol myristate acetate (PMA)-stimulated cells. Interestingly, the protein kinase C (PKC) activator PMA failed to activate iNOS synthesis in RAW 264.7 cells, although this agent activated ERK1/ERK2 in RAW 264.7 cells. It is entirely possible that PMA sensitive PKC isoforms are not involved in the activation of iNOS

transcription. However, this does not rule out the possible involvement of non typical PKC such as PKC- ϵ or PKC- ζ , which are poorly inhibited by bisindolylmaleimide. Both of these isoforms have been implicated in the signal transduction pathway mediating iNOS induction (Diaz-Guerra et al., 1996., Miller et al., 1997).

CRP also activates MEK1/2, the major upstream kinase of ERK1/ERK2. MEK can be activated by Raf or MEK Kinase (MEKK). Although the involvement of other MEK kinases cannot be ruled out based on the data provided here, it seems likely that CRP activates Ras \rightarrow Raf \rightarrow MEK pathway, provided that D609 by virtue of inhibiting PC-PLC enzyme activity, blocked the activation of Raf by Ras (Schutze et al., 1992, Cai et al., 1993). Moreover, these data also showed no activation of parallel p46/p54 MAPK (SAPK) in CRP treated RAW 264.7 cells. Since SAPK and p38 kinases are activated MEK kinases other than Raf (Yan et al., 1996) it is likely that these upstream kinases are not activated by CRP stimulation. Furthermore, D609 and salicylate which inhibited ERK1/ERK2 and iNOS, activated SAPK. Whether SAPK activation may lead to suppression of iNOS, preceded by an inhibition of I- κ B kinases, will be an interesting avenue to explore. Taken together, these results indicate that CRP may preferentially activate ERK1/ERK2 in a PKC independent manner.

This study has shown that CRP treatment results in a substantial increase in the phosphorylation of I- κ B. The time course for activation I- κ B was very similar to that of ERK1/ERK2 activation. CRP alone was capable of stimulating I- κ B which was clearly detectable at 60 min post stimulation and was sustained for up to

120 min. In the presence of IFN- γ , the phosphorylation was detectable as early as 15 min. These time kinetics of I- κ B activation seem to correlate with that of ERK activation. Phosphorylation of I- κ B has been reported to be pivotal for the ubiquitination and subsequent degradation of I- κ B. Although phosphorylation of I- κ B in response to CRP was obvious, the degradation of I- κ B was not clear. Immunoblotting with an antibody that recognized native non-phosphorylated I- κ B was expected to show the degradation represented by a decrease in the quantity of I- κ B protein or the appearance of small molecular weight bands. Nevertheless, a reduction of protein concentration is seen at 180 min which returned to normal levels by 240 min.

These data also show that agents that inhibited ERK1/ERK2 also suppressed I- κ B activation. In addition, inhibition of I- κ B phosphorylation by PD 98059 indicates a distinct role for ERK1/ERK2 in the activation of I- κ B in CRP-stimulated RAW 264.7 cells. These studies with various cell-permeable inhibitors of PC hydrolysis and tyrosine phosphorylation suggest that PC-PLC activation and tyrosine phosphorylation are essential for CRP-mediated phosphorylation of I- κ B and ensuing dissociation of NF- κ B trans-acting factor for the induction of iNOS transcription. PC hydrolysis has also been linked to the activation of NF- κ B complex and induction of iNOS (Schutz et al., 1992, Spitsin et al., 1997).

Several lines of evidence indicate that Ras \rightarrow MAP kinase cascade mediates ligand-induced activation of NF- κ B. Ha Ras and Raf-1 were shown to be required for agonist-induced NF- κ B activation (Finco and Baldwin, 1993). Li

and Sedivy showed that Raf-1 kinase phosphorylated I- κ B- α in vitro. Using a yeast two hybrid system, they also showed that Raf-1 kinase is associated with I- κ B- α (Li and Sedivy, 1993). The data presented in this thesis suggest that CRP-mediated activation may involve Raf-1 activation and subsequent I- κ B phosphorylation. Although the evidence presented here has shown a definite role for ERK1/ERK2 kinases in the phosphorylation of I- κ B, it is also possible that CRP may activate specific I- κ B kinases. A specific I- κ B kinase has now been purified. A 900 kDa complex containing at least two proteins, a 85 kDa and 87 kDa, with close homology to conserved helix-loop-helix ubiquitous kinase (CHUK) has been described in He La cells (DiDonato, et al., 1997). A diagrammatic interpretation of the possible pathways by which CRP may activate gene transcription is illustrated in Fig. 14.

Although growth promoting factors, such as fibroblast growth factor and phorbol esters, have been known to activate ERK1/ERK2, they were shown to inhibit iNOS mRNA levels in some cell types (Heck et al., 1992). However, factors like nerve growth factor activated ERK1/ERK2 and induced iNOS in other cell types such as PC12 cells (Peunova et al., 1995). Weisz et al have shown that EGF (and possibly MAPK activation) potentiated the increase of iNOS mRNA significantly in LPS treated RAW 264.7 cells but had no effect on IFN- γ treated cells (Weisz et al., 1994). In contrast, CRP was found to stimulate iNOS production in IFN- γ treated cells. This may be attributed to the fact that CRP is involved in the differentiation and not the proliferation of cells. Prolonged exposure of promonocytes (U-937 cells) and macrophages to CRP has been

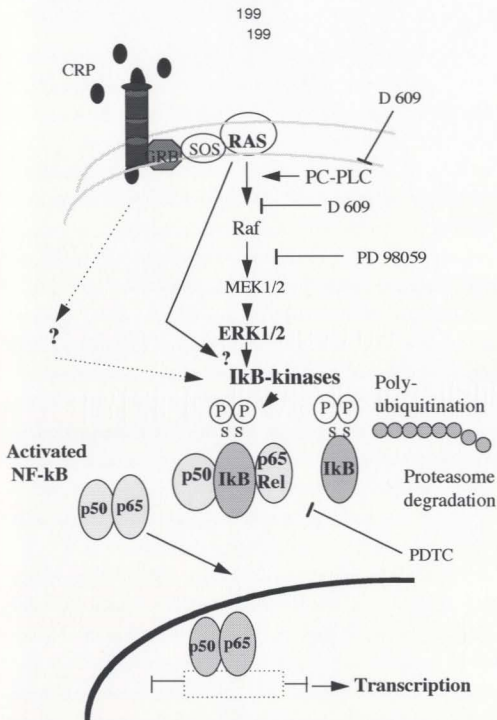


Fig. 14. A schematic representation of possible transduction pathways activated by CRP in RAW 264.7 cells.

shown to result in the differentiation into more mature cytotoxic and tumoricidal cells (Tebo and Mortensen, 1991, Barna et al., 1984). It remains to be seen if the prolonged activation of ERK1/ERK2 by CRP especially in the presence of IFN- γ may have a role in the differentiation of RAW 264.7 cells. Although growth factors and PMA activate ERK1/ERK2 in RAW 264.7 cells, they are ineffective in NF- κ B stimulation and iNOS induction in this cell line (Sanghera et al., 1996, Vincenti et al., 1992). It is possible that sustained activation of ERK1/ERK2 in response to CRP may lead to cell differentiation and result in iNOS transcription. It has been argued that the duration of ERK1/ERK2 activation determines whether cells undergo proliferation or differentiation (Marshall, 1995). Co-stimulation of factors yet to be identified may also be necessary to initiate iNOS protein synthesis. In many cell types, a combination of cytokines such as IL-1 β , TNF- α and IFN- γ or lipopolysaccharide and cytokines is necessary to induce iNOS expression. Since CRP has been shown to induce the rapid synthesis of IL-1 α , or IL-6 (Ballou et al., 1992; Tilg et al., 1993; Pue et al., 1996; Galve-de Rochemonteix et al., 1993), there is a possibility that CRP may be inducing iNOS secondary to the production of these cytokines.

Activation of ERK1/ERK2 was not due to a possible contamination of CRP samples with endotoxin. As stated in chapter 3, CRP samples had no detectable endotoxin contamination as judged by the limulus amoebocyte assay. CRP incubated with polymyxin agarose for at least two hours, retained the ability to activate ERK and iNOS in RAW 264.7 cells. Moreover, the kinetics of ERK1/ERK2 activation by CRP are very different from that of LPS. Sanghera et al. have reported that ERK1/ERK2 in RAW 264.7 cells underwent increased

tyrosine phosphorylation in response to LPS as early as 5 min, was maximally activated at 10 min and decreased to near normal levels in 30 min (Sanghera et al., 1996). In contrast, CRP did not activate ERK appreciably for up to 30 min and reached a maximum at 60 min. Furthermore, CRP did not activate the parallel MAP kinases, SAPK/JNK or p38 kinase, whereas all three MAPKs have been shown to be activated by LPS (Sanghera et al., 1996). In addition, D609 and salicylate, which inhibited CRP induced ERK1/ERK2 activation, increased the phosphorylation of SAPK/JNK. Also, contrary to CRP induced NOS synthesis, LPS and IFN- γ induced iNOS protein was shown to be unaffected by PD 98059 (Caivano, 1998). Regardless, it is interesting to note that both CRP and LPS have also been shown to increase the transcription of IL-1, TNF- α and tissue factor (Ballou et al., 1992; Tilg et al., 1993; Pue et al., 1996; Galve-de Rochemonteix et al., 1993; Cermak et al., 1993). It is possible that CRP and LPS stimulation may follow similar or distinct signaling pathways but achieve some of the same target response. There is also the possibility that CRP-stimulated iNOS synthesis may be secondary to the synthesis of cytokines such as TNF- α or IL-1.

In order to dissect MAPK pathways and explain its physiological roles, one approach has been the generation of dominant negative mutants and overexpression of these in cells. Dominant negative mutant forms of p21 Ras, c-Raf, and MEK have been shown to inhibit the activation of ERK1/ERK2 and growth factor-dependent cell proliferation or differentiation (Cai et al, 1993). However, besides the fact that generation of cell lines that stably express them is time consuming, their expression may also lead to erroneous conclusions.

For example, over-expression of inactive MEK that can be phosphorylated by Raf may not only prevent the activation of endogenous wild type MEK, but also the activation of other cellular substrates of Raf. Similarly dominant negative mutants of Raf may effect Ras-dependent processes that are independent of Raf. In addition, the need for many hours of treatment with transfection agents may also result in unwanted secondary effects. Moreover, some cell types may be more resistant to transfection. In the absence of genetic analyses such as gene "knock out" or dominant negative mutants, signal transduction pathways are often dissected through small cell permeant molecules that are specific inhibitors of a particular protein kinase. The advantage in this approach is that the effects of these inhibitors can be investigated in any cell type. Therefore, such an approach was utilized in this study to dissect the signaling pathway generated by CRP-stimulated cells leading to iNOS gene transcription.

This study data provide support for the hypothesis that activation of mononuclear phagocytes may constitute an important biological role for CRP during periods of immunological challenge. In conclusion, these results indicate that one of the mechanisms by which CRP may exert its role in immunomodulation may be through the activation of ERK1/ERK2 cascade. Furthermore, evidence has been provided that show that the induction of iNOS by CRP may be at least partly mediated by the activation of MAPK cascade.

NITRIC OXIDE INDUCTION BY CRP: ANTIMICROBIAL MECHANISM FOR INHIBITION OF *CHLAMYDIA* *TRACHOMATIS* REPLICATION

Section 1. Introduction

The role of CRP in host defense mechanisms is thought to be related to its ability to recognize foreign pathogens as well as damaged host cells and to initiate their elimination by interacting with humoral and cellular effector systems (Kilpatrick and Volanakis, 1991). Many of the activities of CRP observed in vitro involve its binding to various microbial pathogens. CRP has been known to enhance the phagocytosis of a variety of Gram-positive and Gram-negative bacterial pathogens (Ganrot and Kindmark, 1969; Kindmark, 1971). Studies using infected murine models have shown that CRP protects mice against fatal infection by type 3 and type 4 *Streptococcus pneumoniae*. (Mold et al., 1981; Yother et al., 1982). Human and Rabbit CRP were also shown to increase the blood clearance of *S. pneumoniae*, and there is evidence that CRP may provide protection from the development of fatal levels of pneumococci in the blood (Horowitz, et al., 1987). Also, transgenic mice expressing human CRP have been shown to be protected against *S. pneumoniae* infection (Szalai et al., 1995). So far these actions of CRP have been attributed mainly to its antibody-

like opsonisation and complement activation. However, recently CRP or peptides derived from CRP have been shown to stimulate the induction of immune/inflammatory nitric oxide synthase (iNOS) and subsequent production of NO in macrophages (Arcoleo et al., 1997; Ratnam et al., 1998). NO has been identified as a major regulatory molecule of the immune system. It is a principal cytotoxic mediator of activated immune effector cells and known to be produced by a variety of cells and tissues (Hibbs, et al., 1987). Consequently, NO has been shown to inhibit the growth and function of a diverse array of facultative intracellular microorganisms (Hibbs et al., 1987; Karupiah et al., 1993). This provided the basis for the hypothesis that, in addition to opsonisation and complement activation, CRP may also induce the production of NO, which in turn could play the role of effector molecule in host resistance to infection. To test this hypothesis, a study was carried out to determine whether CRP inhibits microbial growth through the production of NO. *Chlamydia trachomatis*, an intracellular pathogen well studied in this respect, was utilized as a model for this study (Zhong et al., 1989). NO has been shown to be an effective molecule involved in the destruction of *chlamydiae* and NO production was shown to be one mechanism utilized in IFN- γ activated murine macrophages (Chen et al., 1996; Mayer et al., 1993). The L-arginine analog, N-guanidino-monomethyl L- arginine (NMMA), has been shown to competitively inhibit NO synthesis in murine cell culture without affecting the cells (Chen et al., 1996; Mayer et al., 1993). In delineating the mechanism through which IFN- γ and/or LPS dependent antichlamydial activity is mediated, Chen et al. and Mayer et al. utilized NMMA to confirm the role of NO as the effector molecule in IFN- γ induced antichlamydial effect (Mayer et al., 1993; Chen et al., 1996).

Therefore, NO production and the recovery rate of *C. trachomatis* in unstimulated and IFN- γ activated McCoy cells were measured in the presence and absence of CRP. NMMA, an inhibitor of iNOS enzymatic activity, was used to block the activity of iNOS enzyme to demonstrate whether the immunological response modulated by CRP is mediated through the production of NO.

Section 2. Results

A. Stimulation of NOS Activity in Control McCoy Cells

In the first series of experiments, baseline data on the effects of CRP and IFN- γ alone and their combination on uninfected McCoy cells were obtained to determine whether CRP and IFN- γ could induce NO synthesis in the absence of chlamydia-derived products such as LPS. The results indicated that uninfected control McCoy cells treated with PBS alone showed no detectable increase in NO generation as measured by nitrite levels in the culture supernatant. The uninfected McCoy cells incubated with CRP (50 μ g/ml) alone also showed little or no increase in NO generation (Fig. 1) but the cells incubated with IFN- γ (50 units/ml) alone showed a small increase in nitrite levels. However, when 50 μ g CRP was added along with IFN- γ , the NO production increased nearly 10 fold above the levels generated by IFN- γ alone.

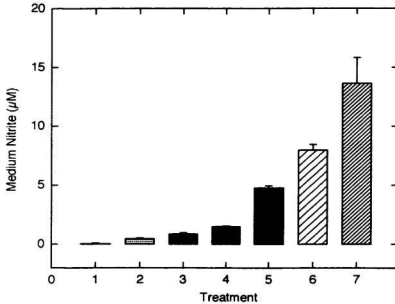


Fig.1. Activation of NO synthesis in McCoy cells by CRP. McCoy cells were incubated with rat CRP (50 µg/ml) or IFN-γ (50 units/ml) for 24 h, or with increasing CRP concentrations ranging from 10 to 100 µg/ml in the presence of IFN-γ (50 units/ml). Control cells were incubated with an equal volume of buffer. Aliquots of the culture supernatant were assayed for accumulated nitrites. 1. control. 2. CRP (50 µg/ml). 3. IFN-γ (50 units/ml). 4. IFN-γ + CRP (10 µg/ml). 5. IFN-γ + CRP (20 µg/ml). 6. IFN-γ + CRP (50 µg/ml). 7. IFN-γ + CRP (100 µg/ml). Values are expressed as mean ± S.D. of three to six separate experiments.

This synergistic activation of NO was detectable when as little as 10 μg CRP was used along with IFN- γ . Addition of CRP (10 $\mu\text{g}/\text{ml}$) increased NO production by IFN- γ primed McCoy cells more than 1.5-fold and this activation increased linearly with increasing concentration of CRP reaching over 15-fold when 100 $\mu\text{g}/\text{ml}$ CRP was used (Fig. 1).

B. Stimulation of NOS Activity in *C. trachomatis* Infected McCoy Cells

In these series of experiments, McCoy cells infected with *C. trachomatis* but not stimulated with CRP or IFN- γ served as a baseline control for NO threshold levels as molecules derived from *C. trachomatis* were expected to induce NO in McCoy cells. The assay results indicated no significant elevation in NO activity in the *C. trachomatis* infected culture supernatant as compared with uninfected control culture supernatant. Therefore, the level of NO induction attributable to LPS or other molecules derived from possible cell wall lysis of *C. trachomatis* was considered to be negligible (Fig. 2). Addition of NMMA to the above infected cultures did not have any significant effect on the production of NO by McCoy cells (Fig. 2). The addition of CRP alone to *C. trachomatis* infected cell culture induced NO only marginally beyond the baseline threshold levels observed in control cultures. Consequently, the addition of NMMA in combination with CRP also did not show any significant effect. In contrast, IFN- γ induced an 8-fold increase in NO levels, and the level of NO induction was reversed by approximately 50% when NMMA was added along with IFN- γ .

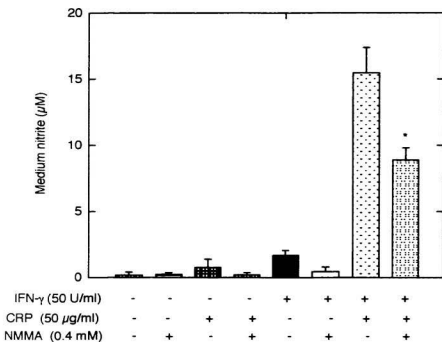


Fig. 2. **Effect of CRP, IFN- γ and NMMA on NO generation by *C. trachomatis* infected McCoy cells.** McCoy cells infected with *C. trachomatis* were stimulated with CRP (50 µg/ml) and/or IFN- γ (50 units/ml) in the presence or absence of NMMA (0.4 mM). Presence (+) or absence (-) of agents used is shown as indicated. Data represent mean \pm SD of four experiments. * $P < 0.05$ versus assay without NMMA by students t-test

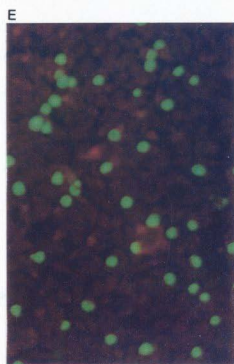
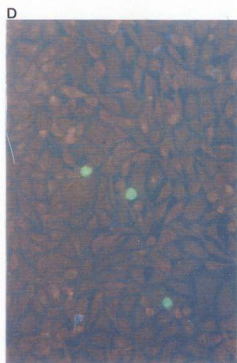
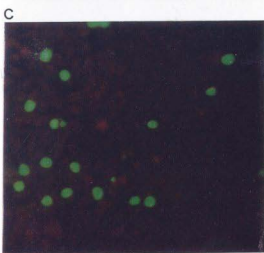
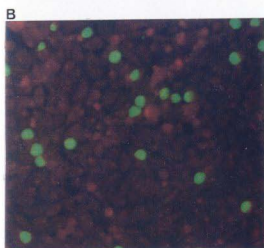
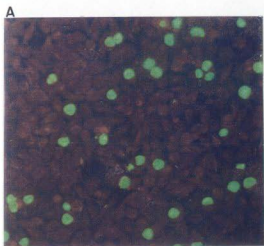
There was a dramatic increase in NO levels in *C. trachomatis* infected McCoy cells when CRP was added along with IFN- γ . The level of NO reached over 75-fold over the levels observed in infected control cultures and 8.5 fold over the levels in infected cells stimulated with IFN- γ alone. The level of NO production by the combination CRP/IFN- γ treatment was reduced to nearly one half when the inhibitor NMMA (0.4 mM) was included with the CRP-IFN- γ signals (Fig. 2).

C. Correlation of NO production with *C. trachomatis* replication

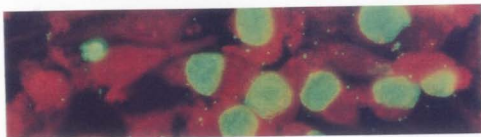
Microscopic examination of cell cultures stained with *C. trachomatis* immunofluorescence antibody provided visual evidence on the effect of the signals on *C. trachomatis* replication and to correlate the extent of inhibition of *C. trachomatis* replication with observed NO levels (Fig. 3). There was a significant reduction in the number of *C. trachomatis* inclusions in cultures treated with a combination of CRP and IFN- γ . When NMMA was added along with CRP and IFN- γ , a significant reversal in the inhibition of *C. trachomatis* replication was observed. This effect was not obvious in cultures treated either with CRP or IFN- γ alone. The above effect correlated with NO levels observed in respective culture supernatants.

To determine the effect of NO on the viability of *C. trachomatis*, the culture suspensions of *C. trachomatis* were serially diluted and cultured and colony forming unit (CFU) counts were obtained. The cultures treated with CRP alone

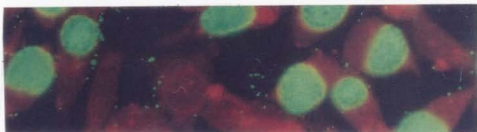
Fig. 3. Correlation between nitrite production and inhibition of *C. trachomatis* replication in McCoy cells. McCoy cell monolayers infected with *C. trachomatis* were grown on cover slips and stimulated with CRP (50 μ g/ml), IFN- γ (50 units/ml), or a combination of CRP (50 μ g/ml) and IFN- γ (50 units/ml) in the presence or absence of NMMA (0.4 mM). The monolayers were fixed in methanol, rinsed with PBS and probed with FITC conjugated antibody to *C. trachomatis* MOMP, and read under an epifluorescence microscope and the monolayers were photographed. **A.** control. **B.** CRP (50 μ g/ml). **C.** IFN- γ (50 units/ml). **D.** IFN- γ (50 units/ml) + CRP (50 μ g/ml). **E.** IFN- γ (50 units/ml) + CRP (50 μ g/ml) + NMMA (0.4 mM). Magnification, 400 x. Page 210b shows similar experiment at 1000 x magnification.



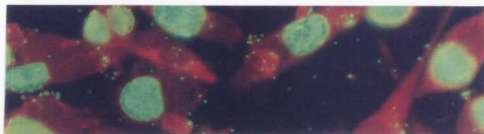
A



B



C



D



E

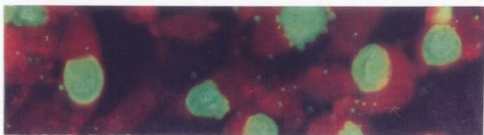


Table 1. **Correlation between *Chlamydia trachomatis* viability and NO levels in McCoy cells.**

Treatment	<i>Chlamydia trachomatis</i> colony forming units	Culture medium nitrites (μM)
Cell control	$67 \times 10^4 \pm 15 \times 10^4$	0.184 ± 0.23
Cell control + NMMA	$61 \times 10^4 \pm 12 \times 10^4$	0.229 ± 0.13
IFN- γ	$46.3 \times 10^4 \pm 10.2 \times 10^4$	1.667 ± 0.385
IFN- γ + NMMA	$68 \times 10^4 \pm 5.57 \times 10^4$	$0.445 \pm .333$
CRP	$53 \times 10^4 \pm 12.8 \times 10^4$	0.75 ± 0.64
CRP + NMMA	$73.7 \times 10^4 \pm 7.37 \times 10^4$	0.186 ± 1.62
IFN- γ + CRP	$7.37 \times 10^2 \pm 1.4 \times 10^2$	14.12 ± 3.71
IFN- γ + CRP + NMMA	$6.63 \times 10^3 \pm 1.52 \times 10^3$	7.4 ± 3.32

McCoy cells were pre-treated with CRP (50 $\mu\text{g/ml}$), IFN- γ (50 units/ml), or a combination of CRP (50 $\mu\text{g/ml}$) and IFN- γ (50 units/ml) in the presence or absence of NMMA (0.4 mM) for 10 h and the cells were infected with *C. trachomatis*. NO synthesis was measured by assessing the nitrite levels in the culture medium and CFUs were quantitated as described in materials and methods.

showed a marginal decrease in CFUs when compared to control *C. trachomatis* infected cells. The reversal of this inhibitory effect by NMMA was marginally significant. IFN- γ by itself did not show any appreciable effect on *C. trachomatis* viability. In contrast, the combination CRP-IFN- γ treatment showed a 100-fold decrease in CFUs. When NMMA was added to CRP-IFN- γ stimulated cells there was a 10-fold increase in the number of CFUs. These data are summarized in Table 1.

Section 3. Discussion

Inflammatory stimuli such as IFN- γ and LPS have been shown to induce the production of NO in murine macrophages (Stuehr and Marletta, 1985). Subsequently Hibbs et al. made a seminal discovery that NO produced by the oxidation of L-arginine by activated macrophages is associated with cytotoxicity against tumor cells suggesting that NO might play a role in host defense against infection (Hibbs et al., 1987). Numerous studies carried out since, have established that NO has potent broad spectrum microbiocidal activity against wide array of microbial pathogens (De Groote and Fang, 1995). *C. trachomatis*, an intracellular pathogen previously shown to be susceptible to NO (Chen et al., 1996; Mayer et al., 1993). was chosen as a model for this study. Moreover, an immunofluorescent monoclonal antibody is available against surface antigens (anti MOMP) of this organism and is routinely used in clinical evaluations making the detection and quantitation of inclusion bodies of this pathogen highly specific and accurate.

Although most of the experiments reported in this thesis utilized RAW 264.7 cells as a model, McCoy cells were used for this series of experiments. Chen et al. reported that RAW 264.7 cells were a good model to grow *C. trachomatis* (Chen et al., 1996). However, propagation of chlamydiae in RAW 264.7 cells proved to be quite difficult since these cells phagocytosed chlamydiae very effectively, and therefore establishing infected controls was found to be difficult with this cell line. However, McCoy cells, the cell culture of choice for chlamydiae in routine clinical practice, were found to be efficient NO producers when stimulated by CRP and IFN- γ . Moreover, it has been previously shown that McCoy cells are efficient producers of NO when stimulated with IFN- γ in synergy with TNF- α , IL-1, *E. coli* LPS or molecules derived from the microorganism itself (Mayer et al., 1993). The same report also indicated that for optimal production of NO in McCoy cells, IFN- γ priming must precede or be coincident with chlamydial infection. Mayer et al. also found a dramatic increase in NO production by infected cells that were primed with IFN- γ , and this was attributed to chlamydial products themselves acting as second signals (Mayer et al., 1993). In contrast, in the present study, although IFN- γ alone increased NO synthesis in infected and uninfected McCoy cells over control levels, the magnitude of the increase was not significantly different between infected and uninfected cells. It is possible that cell wall lysis and release of microbial products were minimal in the *Chlamydia trachomatis* infected culture used in this study.

This study showed that CRP induced NO production in both *C. trachomatis*

infected and uninfected McCoy cells in synergy with IFN- γ . In these series of experiments, the signals including IFN- γ were added to the cell culture ten hours prior to infection with *C. trachomatis*. As stated earlier, McCoy cells are efficient producers of NO when primed with IFN- γ and exposed to a second signal such as IL-1, TNF- α or LPS (Mayer et al., 1993). These results show that CRP also acted as a second signal in this cell line. The combination CRP-IFN- γ treatment resulted in very high levels of NO production by McCoy cells with a corresponding inhibition of *C. trachomatis* replication. These data also showed that the magnitude of this inhibitory effect was related to the level of NO produced by the cells detected as nitrite in the culture supernatant. However, the mechanism through which NO affects chlamydial growth remains unknown. NO is known to interact with prosthetic iron groups and thiol groups forming complexes that may activate or inactivate many target enzymes. For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is inhibited by iron nitrosylation of an active site thiol group (Molina., et al., 1992); whereas, aconitase and NADH:ubiquinone oxidoreductase and succinate: ubiquinone oxidoreductase of the electron transport chain are inhibited due to attacks on the iron of the iron sulfur clusters resulting in Fe²⁺ release or Fe²⁺ nitrosylation (Drapier et al., 1986; 1988). The inhibition of these and other essential enzymes by NO results in cytoysis and cytolysis of invading microorganisms and tumor cells (Hibbs et al., 1987, Stuehr and Nathan, 1989). This also may increase the initial availability of Fe²⁺ for the potentiation of oxidative damage and can eventually deplete cellular iron stores. Reactions with thiols can also alter protein function and catalyze disulfide bonds and other modifications. NO may also react directly with DNA resulting in deamination and/or cross-linking

(Wink et al., 1991). NO has been also been shown to decrease ATP production, and this in turn could affect chlamydial viability as *C. trachomatis* is an ATP-dependent intracellular parasite. These data show that CRP acting as a second signal to IFN- γ in *C. trachomatis* infected McCoy cells may limit the growth of this intracellular pathogen. iNOS is a cytoplasmic enzyme that may serve as a primary defense against intracellular pathogens that have invaded the intracellular environment of not only cells of the immune system but also many other somatic cells including those not specialized in host defense (Nathan, 1992; Amber et al., 1988). NO produced by these cells may cause stasis/lysis of the intracellular pathogen and inhibit host enzymes. This potential inhibition of host enzymes are deemed reversible (Drapier and Hibbs, 1988; Amber et al., 1988). This reversibility of NO-induced effects may explain why NO was selected as a primary defense against intracellular pathogens during the evolutionary process (Bastian and Hibbs, 1994).

The microbicidal activity exhibited by cells treated with IFN- γ and CRP can be significantly inhibited by using a competitive inhibitor of iNOS enzyme activity, providing evidence that this activity is mediated through the generation of NO. Although many inhibitors of iNOS have been described, NMMA was utilized in this study to verify NO production activated by CRP did indeed contribute to the destruction of *C. trachomatis*. NMMA has been shown to competitively inhibit iNOS activity (Southam and Szabo, 1996; Mayer, 1993). Moreover, NMMA has been shown to be equally potent in inhibiting constitutive and inducible NO synthases (Southam and Szabo, 1996). At the concentrations used, NMMA inhibited NOS activity to approximately 50% of total NO production of IFN- γ and

CRP stimulated cells. However, this inhibition of NO translated into a 100% increase in the viability of *Chlamydiae* showing that the microbicidal property exerted by CRP/IFN- γ stimulated McCoy cells may at least be partly due to the production of NO. In addition to NO production, other mechanisms may also exist in activated McCoy cells. IFN- γ and CRP are capable of inducing the production of cytokines such as IL-1 and TNF (Ballou et al., 1992; Tilg et al., 1993; Pue et al., 1996; Galve-de Rochemonteix et al., 1993). Moreover, CRP has been known to activate the complement pathway and this has been shown to be involved in CRP mediated protection against *S. pneumoniae* (Mold et al., 1981; Szalai et al., 1995). Nevertheless, the reversal of microbicidal activity by NMMA in CRP/IFN- γ stimulated cells shows that NO production, along with CRP mediated opsonisation and complement activation, is an important means by which CRP may provide protection against microbial pathogens.

It is well documented that activation of the complement pathway initiated by CRP results in the generation of host defense related complement fragments and opsonins and these provide protection against microbial infection. (Kilpatrick, and Volanakis, 1991). The data presented in this report show that additional mechanisms such as enhanced iNOS induction by CRP in immunologically activated cells may also play a role in antimicrobial activity during periods of infection. In conclusion, as NO production is one of the principal mechanisms of macrophage cytotoxicity to microorganisms, increased hepatic synthesis of CRP during periods of infection may result in elevated levels of NO synthesis and this may be one of the mechanisms by which microbial growth is curbed in vivo.

Conclusions and Future Directions

This thesis has examined the role of CRP in modulating macrophage activation. Rat and human CRP were shown to stimulate nitric oxide (NO) synthesis in rat peritoneal macrophages and in the murine 264.7 cell line. In the latter cell line, CRP also synergized with IFN- γ resulting in a dramatic increase in NO production. This increase in NO production measured as accumulated nitrite in the cell culture medium was shown to result from increased enzymatic oxidation of the terminal guanidino nitrogen of L-arginine catalyzed by NOS enzymes. This activity was inhibited by substrate analogues of arginine further substantiating the above observation. There was also increased NOS enzyme activity in the cellular fractions of macrophages treated with CRP alone or in combination with IFN- γ . This increase in NOS activity was shown to result from an increased synthesis of the inducible isoform of NOS enzyme by immunoblot analysis using an antibody that specifically recognized the 130 kDa iNOS protein. An increase in steady state iNOS mRNA level in CRP or CRP/IFN- γ stimulated cells was evident from Northern blot analysis using a cDNA probe derived from mouse macrophage iNOS gene. The above findings led to the conclusion that during periods of infection or trauma, CRP may assume a proinflammatory role and activate macrophages leading to the production of NO in conjunction with molecules produced by cells of the immune system especially those derived from T-cells. Although iNOS expression has been demonstrated in many human cells including keratinocytes, chondrocytes and hepatocytes, expression of this enzyme in human macrophages has been

disputed. Human mononuclear phagocytes do not seem to produce significant quantities of NO when stimulated with LPS and IFN- γ and it may appear that a study in rodent macrophage cell culture models with CRP, a major acute phase protein in humans, may not have much relevance in human disease states. However, crosslinking of CD69, a member of the natural killer family of signal transducing receptors and infection by HIV have been shown to result in NO production. HIV infected macrophages also respond to LPS and TNF- α to produce NO. It is possible that CRP also will activate NO in human cells during a variety of pathogenic processes.

This study has also shown that rat CRP decreased the production of $O_2^{\cdot-}$ by PMA-stimulated macrophages. The data obtained using D609 showed that activation of PC-PLC may be one of the mechanisms by which cells may control the overproduction of free radicals during infectious states when NADPH oxidase is activated. CRP may protect the host cells from harmful effects of free radicals by activating PC-PLC or other cellular mechanisms, and at very high concentrations, it may also possess some scavenging activity. CRP activates nitric oxide synthesis while suppressing $O_2^{\cdot-}$ generation. Hepatic synthesis of CRP during periods of inflammation and trauma may be one mechanism by which these two pathways are regulated differentially to avoid the potential production of ONOO $^{\cdot-}$, NO $_2$ and HO $^{\cdot}$. Moreover, activation of PC-PLC and generation of second messengers from PC hydrolysis may be a mechanism cells may use to avoid simultaneous generation of NO and $O_2^{\cdot-}$.

Interaction of rat CRP with macrophages was very similar to that of human CRP

and neither was mediated through its phosphocholine binding site. Although rat CRP differs from human CRP in many aspects such as glycosylation, pI and the normal blood concentration, there is substantial amino acid homology between these two proteins, and these results were not entirely surprising. It can be speculated that a region of close amino acid sequence homology of the CRP peptide may be responsible for binding to CRP receptor. However, normal rat CRP concentration of 0.5 mg/ml or more may mean that this protein can exert considerable antioxidant properties by inhibiting cellular production of superoxide and by scavenging free radicals while this may only be available to humans during periods of inflammation or trauma.

The finding that the stimulation of macrophages by CRP results in a rapid increase in PC hydrolysis and that this increase in PC hydrolysis as well as iNOS induction in CRP stimulated cells can be inhibited by D609, showed a crucial role for PC-PLC in the signaling pathway initiated by CRP. In addition, tyrosine phosphorylation was found to be necessary in the pathway leading to iNOS induction by CRP. Both PKC activators and cyclic AMP elevating agents increased iNOS synthesis in rat peritoneal macrophages. It was concluded that DAG derived from PC hydrolysis may activate PKC(s) in rat peritoneal cells. In contrast, neither PKC activation nor cAMP elevation in RAW cells stimulated iNOS synthesis. Elevation of cAMP was in fact inhibitory to CRP-stimulated increase in iNOS activity in RAW 264.7 cells. This may mean that the pathways activated by CRP may differ downstream of PC-PLC. Nevertheless, PC-PLC activation may be pivotal in CRP mediated macrophage activation.

Stimulation with CRP resulted in a time and concentration-dependent increase in ERK1 and ERK2 phosphorylation in RAW 264.7 cells indicating that ligation of CRP, possibly to its receptor, initiated a cascade of events that resulted in ERK phosphorylation. The finding that D609 inhibited ERK activation showed that PC-PLC acted proximally to ERK activation. This also indicated that the signaling cascade Ras→Raf→MEK→ERK may be activated by CRP since activation of Raf by Ras requires the activation of PC-PLC. Raf has also been shown to be the target of inhibitory signals of PKA. It is possible that cAMP elevating agents decreased NOS induction in RAW 264.7 cells presumably through the inhibition of Raf→MEK→ERK pathway. At present, MEK1 and MEK2 are the only known physiological substrates for Raf. It can be deduced from these observations that the Raf→MEK→ERK pathway is involved in cell activation by CRP leading to iNOS synthesis in RAW 264.7 cells. Furthermore, inhibition of CRP mediated iNOS synthesis by the MEK inhibitor PD 98059 indicated that activation of the ERK cascade by CRP is at least partly responsible for iNOS induction by CRP. Interestingly, parallel MAPKs such as SAPK which are primarily activated by cellular stress and inflammatory cytokines leading to growth arrest and apoptosis were not activated by CRP. The finding that certain inhibitors of iNOS such as D609 and salicylate activated SAPK may indicate that activation of this kinase may be inhibitory to iNOS induction and a reciprocal control may exist in the regulation of iNOS by ERK and SAPK pathways.

Studies showing that inhibition of I κ B phosphorylation in RAW 264.7 cells resulted in the suppression of iNOS synthesis in response to CRP led to the

conclusion that iNOS activation by CRP is dependent on activation of I κ B. CRP, on binding to macrophages would increase the phosphorylation and subsequent dissociation of I κ B from the NF- κ B complex accompanied by the translocation of the NF- κ B heterodimers to the nucleus, where they may bind to the iNOS promoter. Significant inhibition of I κ B phosphorylation caused by agents such as D609, tyrphostin AG 126 and PD 98059 indicated that PC-PLC, tyrosine phosphorylation and ERK activation may contribute to the signaling pathway leading to I κ B phosphorylation. Moreover, inhibition of ERK1/ERK2 and I κ B phosphorylation by these agents correlated well with the reduction in iNOS synthesis. From these results, it was inferred that ligation of CRP to its receptor or cellular uptake of CRP initiated a signaling cascade that may involve ERK and I κ B phosphorylation leading to the activation of iNOS transcription.

The decreased viability of *C. trachomatis* in IFN- γ /CRP treated McCoy cells has shown that i) CRP may decrease the viability of intracellular pathogens through the activation of NO production; ii) non-macrophage cell lines can also be activated by CRP provided that these cells are primed with IFN- γ . During infection/inflammation, synthesis of CRP by the liver is enhanced. It is conceivable that during infectious periods, T-cells may be activated to produce IFN- γ and this in turn would enhance the binding or uptake of CRP or CRP peptides by immune and non-immune cells and result in their activation. This would also indicate that CRP may play an active role in the resolution of inflammation. Furthermore, in addition to its well known role in opsonisation and complement activation, microbicidal activity attributed to CRP may also be mediated through the production of NO.

Future Directions

1. Characterization of Cellular Receptor for CRP

The results presented in this thesis strongly support the notion that CRP modulates macrophage functions. To enable this, human and rat CRP must bind to rat peritoneal macrophages and RAW 264.7 cells possibly through specific receptors. Binding of human and rat CRP to monocytes/macrophages via a specific receptor has been demonstrated previously. However, the nature of the receptor as well as binding/uptake of this protein is yet to be fully characterized. Presence of such CRP receptors on rat macrophages and RAW 264.7 cells needs to be confirmed. This can be accomplished by the isolation and characterization of the receptor protein. Specific proteases and lipases may be used to identify the binding sites that may involve receptor proteins or lipids. Such studies will also elucidate a relationship between binding sites of CRP on macrophages and the mechanism of activation of PC-PLC. These studies may be extended to examining the role of G-proteins, Ca^{++} mobilization and protein kinases in 'CRP receptor' mediated activation. This may also provide useful clues to the nature and mechanism of PC-PLC activation. Another interesting aspect would be to examine the different DAG species produced during CRP/PC-PLC activation and determining their relative importance in activation of down stream protein kinases and eventual iNOS gene transcription.

2. Role of IFN- γ and Mechanism of Synergy

IFN- γ as well as IFN- γ conditioned medium were shown to facilitate an increased cellular response to CRP. It has been speculated in this thesis that cells activated with IFN- γ may present or release increased levels of proteases, leading to the degradation of CRP resulting in the formation of bio-active peptides. This hypothesis needs to be examined. The formation of small peptides from CRP when incubated with rat macrophages has been previously observed. If an increase in the rate and magnitude of the peptide formation is noted, characterization, amino acid analysis and sequence identification of the CRP peptides that may stimulate iNOS synthesis (or other biological responses) have to be performed. Such analysis would greatly increase the knowledge on the role of CRP and the mechanism of its synergy with IFN- γ in cell activation. Alternatively, activation by IFN- γ and CRP may synergise at the level of iNOS gene transcription. Analyzing the promoter/enhancer-reporter constructs would clarify the mechanism of synergy at this level.

3. Role of Ras/Small GTP Binding Proteins

This thesis has shown a critical role for MAP kinase in mediating CRP-initiated signal to the nucleus. Although these data indicate the activation of Ras and Raf prior to ERK phosphorylation, this needs to be verified. Activation of small GTP binding proteins such as p21 Ras can be assessed by analyzing the fraction that are bound to GTP or GDP. For this, cells labeled with carrier-free

[^{32}P]orthophosphate can be stimulated with CRP and immunoprecipitated with an antibody specific to p21Ras and the bound GTP or GDP can be analyzed using polyethylamine-cellulose thin layer chromatography. The role of Ras proteins in iNOS induction by CRP or other stimuli can then be analyzed by transfecting dominant negative or constitutively active Ras mutants in macrophages. Similarly, expression of constitutively active or dominant negative mutants of kinases down stream of Ras such as Raf, MEK and ERKs may be transfected into cells to confirm the necessity of these pathways in mediating the signal to the nucleus for activation of iNOS gene transcription by CRP. Another approach would be the use of antisense phosphorothioate-modified oligodeoxynucleotides (ODN) directed against ERK1/ERK2 isoforms. Transfection of this antisense ODN would result in specific depletion of ERK expression and abolition of ERK activity. Some preliminary experiments have been done using antisense ODN in RAW 264.7 cells and the results showed an inhibition of CRP stimulated NOS activity in these cells. However, these results have to be confirmed using appropriate controls.

4. Role of Transcription Factors

The predominant mechanism underlying the induction of iNOS is transcriptional regulation. DNA footprinting of the promoter/enhancer regions of the iNOS gene has revealed consensus sequences for many transcription factors such as NF-IL6, TNF-RE and Oct in addition to the well known NF- κ B and IRF binding sequences. It will be interesting to see if CRP can activate the transactivation or/and DNA binding of these transcription factors. Involvement of transcription

factors in iNOS induction by CRP can be verified by gel shift and supershift assays of these DNA binding proteins and by transfecting plasmids containing multiple repeats of the consensus sequences linked to a reporter gene and measuring the reporter activity.

5. Role of Cytokine Mediators

CRP has also been shown to induce the expression of higher levels of mRNA of IL-1, TNF- α , and IL-6 in monocytes and tissue macrophages. It may be important to assess the role played by these cytokines in mediating the stimulatory effect on iNOS synthesis by CRP. Neutralizing antibodies to these cytokines may be used to delineate the involvement of these molecules in the regulation of iNOS activation by CRP.

6. Production of Inflammatory Molecules

Since results presented in this thesis indicate that CRP is a proinflammatory molecule, it will be interesting study the role of CRP in the regulation of other molecules involved in immune regulation such as cytosolic phospholipase A₂ (PLA₂) and the induction of cyclooxygenase 2 (COX2) by macrophages stimulated by CRP. There is evidence that activation of PLA₂ is controlled by ERK, which has been shown to be phosphorylated on CRP treatment of macrophages. If COX2 is indeed activated by CRP, this would provide valuable information on the inflammatory process and possible means for drug therapy and selective inhibition of COX2.

7. Role of CRP in Atherosclerosis

There is a growing body of evidence showing that even slight elevations of CRP is an important risk factor for acute manifestations of coronary artery disease. Recent reports have also suggested that bacterial infection by *C. pneumoniae* or *H. pylori* may precipitate atherosclerosis. Whether an elevated CRP level reflects the underlying endothelial dysfunction due to prevalent atherosclerosis, or is a passive marker for environmental and/or infectious agents, needs to be clarified. The critical question is whether CRP would also actively engage in the formation of atherosclerotic plaques and contribute to the progression of arterial diseases. There is evidence showing that unstable angina is associated with leukocyte activation. This thesis has also shown that CRP modulates macrophage function. MAP kinases, involved in cellular proliferation and differentiation, are also activated by CRP. It is, therefore, important to study whether CRP may directly influence macrophage or endothelial cell proliferation and contribute to plaque formation at sites of vascular injury.

Although CRP was discovered nearly seventy years ago, no consensus has yet emerged on its precise physiological role. Nevertheless, the results presented in this thesis have provided some fundamental information on the molecular basis for inflammatory and immune responses to CRP. Further investigations including the research proposals suggested in this section should clarify the role of this protein during inflammatory responses.

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