THE INFLUENCE OF PHOSPHODIESTERASE INHIBITOR, ROLIPRAM, ON PLASMA TUMOR NECROSIS FACTOR-a LEVELS AND HAEMODYNAMICS IN LIPOPOLYSACCHARIDE-TREATED RATS

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The influence of phosphodiesterase inhibitor, rolipram, on plasma tumor necrosis factor-α levels and haemodynamics in lipopolysaccharide-treated rats

by

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ABSTRACT

Septic shock is the thirteenth most common cause of death in the United States and the leading cause of death of individuals in intensive care units once it progresses to multiple organ dysfunction syndrome (MODS) (Parrillo et al., 1993). Mortility ranges from 20% to 95% (Eidleman et al., 1995; Parrillo et al., 1993; Wiessner et al., 1995). Septic shock is caused most often by gram-negative bacteria and it has increased dramatically in the past 10 years. Even when properly treated with available therapies, it carries a 60% mortality (Wiessner et al., 1995).

The administration of lipopolysaccharide (LPS) has been reported to produce hypotension and reduced cardiac output. The aim of the present investigation was to (a) examine the influence of type IV phosphodiesterase inhibitor rolipram on haemodynamics, plasma levels of tumor necrosis factor-α (TNF-α) levels, and production of inducible nitric oxide synthase (iNOS) in the lungs, *ex vivo*, in LPS-treated rats, and (b) determine the cardiovascular effects of a selective α_1-adrenoceptor agonist, methoxamine, in the absence or presence of rolipram in rats treated with LPS.

Blood pressure, cardiac index, heart rate and arterial resistance were assessed in Long-Evans rats anaesthetized with thiobutabarbital. Cardiac output was measured using radioactive labeled microspheres and arterial blood pressure was measured *via* an intra-arterial catheter. Plasma levels of TNF- α were measured by an immunoassay technique,

and nitric oxide synthase (inducible & constitutive) activity in lung homogenate was assessed by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline.

Administration of LPS (0.8 mg/kg i.v.) to animals resulted in a significant reduction in cardiac index over time. Changes in arterial resistance, heart rate and blood pressure were insignificant over time in LPS-treated animals. The administration of LPS to rats resulted in a substantial rise in the plasma levels of TNF-α. Furthermore, the injection of LPS resulted in a significant increase in the iNOS activity in lungs. Pre-treatment with rolipram (10 mg/kg) or dexamethasone (5 mg/kg) prevented the decline in cardiac index in animals that received LPS. Infusion of methoxamine into animals injected with rolipram and pre-treated with LPS did not result in significant changes in cardiac index. In contrast, in animals treated with dexamethasone and subsequently LPS, infusion of methoxamine significantly reduced cardiac index and increased blood pressure and arterial resistance. Pre-treatment with rolipram (10 mg/kg) or dexamethasone in animals injected with LPS significantly prevented the rise in TNF-α when compared to respective values in vehicle treated animals. However, pre-treatment with dexamethasone but not rolipram was found to significantly reduce iNOS activity in the lungs of animals injected with LPS.

The present observations support the view that cardiac index can be maintained in animals treated with LPS independent of iNOS inhibition. Furthermore, our findings seem to support the idea that induction of NOS may occur independently of TNF-α in LPS-treated rats.

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1.0 INTRODUCTION

The cardiovascular system serves the body by supplying blood to the tissues in sufficient volume to meet metabolic needs. Blood carried in the vasculature provides oxygen and nutrients to different parts of the body and removes carbon dioxide and metabolic waste products. These transport functions are made possible by the cardiovascular system (Guyton et al., 1973; Sagawa, 1973; Levy, 1979; Greenway, 1982).

It is recognised that the main determinants of cardiac output are heart rate, cardiac contractility, blood volume, preload and afterload (Greenway, 1982). Under certain pathophysiological conditions, alteration in the function of the cardiovascular system play a critical role in the demise of circulation (Forfia et al., 1998). For instances, in endotoxaemia, hypotension and reduction of cardiac output are evident. Recently it has been suggested that changes that result from endotoxaemia are due to an over-production of nitric oxide (NO) (Forfia et al., 1998). Clearly, there is evidence in the current literature to indicate that the administration of lipopolysaccharide (LPS) results in the induction of inducible nitric oxide synthase (iNOS) (Salter et al., 1991; Thiemermann et al., 1993). Evidently, the administration of LPS has been reported to result in reduction in cardiac output and associated with this is a fall in blood pressure (Poon et al., 1997; Cheng et al., 1998). The prevailing view seems to support the idea that LPS mediated

reduction in cardiac output is the result of an over-production of NO, which is to be the key mediator responsible for the collapse of the cardiovascular system.

1.1 Nitric oxide production

The production of NO in mammalian cells is dependent on the enzyme nitric oxide synthase (NOS) which produces NO from the amino acid L-arginine (Palmer et al., 1988). NO is generated by oxidation from the terminal guanidinium nitrogen of L-arginine and the reaction is both oxygen and nicotinamide adenine dinucleotide phosphate (NADP) dependent, and produces L-citrulline in addition to NO (Tayeh, 1989; Bush et al., 1992). To date, three different isoforms of NOS have been identified by protein purification and molecular cloning approaches. These isoforms are: (1) a constitutive form (NOS-I or nNOS), which is dependent on Ca^{2+} and calmodulin for its enzymatic action and is mainly present in neural tissue, both central as well as peripheral (Mayer et al., 1990; Forstermann et al., 1991; Schmidt et al., 1991). (2) A constitutive (NOS-III or eNOS) form which also requires Ca^{2+} and calmodulin for its enzymatic action and is present to a major extent in vascular endothelial cells, (Pollock et al., 1991). (3) A Ca^{2+} independent form (iNOS or NOS-II) which was originally isolated from macrophages (Hevel et al., 1991), and subsequently found to be present in vascular smooth muscle cells and hepatic cells and can be induced by the action of cytokines (Stuehr et al., 1991; Evans et al., 1992; Wood et al., 1990).

NOS activity requires a particular cofactor, NADPH, as a source of electrons for oxygen activation and substrate oxidation (Watanabe et al., 1992). The heme moiety of NOS resembles cytochrome P-450. Due to this similarity, carbon monoxide (CO) and other heme binding agents inhibit NOS activity. It is believed that the heme component of NOS represents the catalytic center, responsible for binding and reducing molecular oxygen and subsequent oxidation (Havel et al., 1991). Calmodulin is also important for the regulation of NOS activity but different quantities of calmodulin are required by different isoenzymes (Abu-Soud et al., 1993).

There are two reactions by which NO is produced from L-arginine. The initial reaction involves N-hydroxylation of the guanidium nitrogen to form N-hydroxy-L-arginine. This reaction utilizes one equivalent of NADPH and oxygen to conduct a simple two-electron oxidation of nitrogen (Marletta et al., 1988). However, the subsequent steps in the conversion of N-hydroxy-L-arginine to NO and L-citrulline remain unclear. Recent studies supported the view that there are two mechanisms responsible for the production of NO. First, nitroxyl (HNO) has been shown to possess biological activity indistinguishable from NO, which seems attributable to the rapid conversion of HNO to NO, by a variety of physiologically relevant oxidants including superoxide dismutase (SOD), oxygen and hemoproteins (Fukuto et al., 1992, 1993). Second, SOD has been demonstrated to directly enhance the formation of free NO from L-arginine by NOS (Hobbs et al., 1994). Thus, SOD appears to accelerate the conversion of an intermediate in the L-arginine and NO pathway. In a similar manner to cytochrome

P-450, NOS also appears to be able to uncouple from its substrate, L-arginine, and generate superoxide anion and hydrogen peroxide via the NADPH-dependent reduction of molecular oxygen (Klatt et al., 1992; Heinzel et al., 1992; Pou et al., 1992). Therefore, it can be concluded that oxygen and NADPH are essential for the production of NO from L-arginine and SOD plays an intermediate role.

1.2 Regulation of NO synthesis

Regulation of NOS activity and NO synthesis is different for constitutive and inducible isoforms. For both nNOS and eNOS, the main mechanism of regulation is provided by the Ca^{2+}/ calmodulin system. At resting intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$=100 nM), cNOS does not interact with calmodulin and therefore it is inactive. But when $[Ca^{2+}]_i$ increases, calmodulin binds to NOS and stimulates NO formation (Schmidt et al., 1991). In endothelial cells, the presence of specific receptors for bradykinin, thrombin, or adenosine-5`-triphosphate or simply shear stress of blood flow increases $[Ca^{2+}]_i$ and therefore activate eNOS activity (Buga et al., 1991). Thus, due to cellular regulation of $[Ca^{2+}]_i$ the production of NO by cNOS can be controlled. It has been demonstrated that calmodulin antagonists such as trifluperazine and calmidazolium block $[Ca^{2+}]_i$ dependent activation of cNOS (Mayer et al., 1992; Schini et al., 1992).

Inducible form of NOS contains highly bound calmodulin and therefore it is not controlled by Ca^{2+} (Xie et al., 1994). For this isoform, NO synthesis is regulated by expression of protein that are not constitutively expressed in tissues, but that require

induction by specific cytokines such as tumour necrosis factor alpha (TNF-α), interleukin-1, and interferon-gama (Hevel et al., 1991; Stuehr et al., 1991). The actions of these cytokines cause an increase in the transcription of appropriate NOS gene thus resulting in production of iNOS (Stuehr et al., 1991). Cytokines not only induce NOS but also increase the availability of co-factors, to increase NO synthesis (Hattori et al., 1993). Evans and associates (1992) have reported that cytokines such as TNF-α can regulate the synthesis of iNOS from immune cells (Evans et al., 1992).

NOS activity also appears to be regulated by a negative feedback mechanism that is mediated by NO. Recent studies have shown that NO generated by nNOS and iNOS is capable of inhibiting subsequent enzymatic activity (Griscavage et al., 1993). It appears that the enzymatic activity of nNOS and eNOS may also be regulated by phosphorylation (Bredt et al., 1991). In addition, it seems that cyclic adenosine 5`-monophosphate (cAMP)-dependent protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase have been found to modulate the activity of NOS (Bredt et al., 1991; Nakane et al., 1991). These reports indicate that the activity of NOS is under the regulation of a number of intracellular mediators.

1.3 Gram negative endotoxin and NOS

It has been found that during septic shock induced by endotoxin from gram-negative bacteria, the left ventricular ejection fraction is decreased (Parker et al., 1990). This reduction of ejection fraction indicates that there is a decrease in left ventricular

contraction due to reduction of the left ventricular end-systolic pressure to volume ratio (Parker et al., 1990). A number of studies suggest that NO may play a pivotal role in decreasing myocardial contractility. It has been suggested that TNF-α, interleukin-2 (IL-2), and interleukin-6 (IL-6), are mainly responsible for the induction of NO (Finkel et al., 1992; Walley et al., 1994). As mentioned earlier these cytokines can induce a Ca^{2+} independent form of NOS, iNOS (Gross et al., 1990). As result of induction of NOS, there is enhanced production of NO within myocytes, and nearby endothelial and macrophages which can cause a reduction of myocardial contractility (Finkel et al., 1992). A number of potential pathways have been implicated by which NO may produce depression of the myocardium. First, there could be an increase in turn-over of cyclic guanosine monophosphate (cGMP) which can result in a decrease in cytosolic availability of Ca^{2+} leading to depression of the myocardium (Stuehr et al., 1991). Second, NO may decrease myocardial contractility by forming toxic peroxynitrites in the presence of oxygen free radicals (Schulz et al., 1992). Finally, NO binds to heme related proteins thereby inhibiting the cytosolic and mitochondrial proteins (Estrada et al., 1992). Furthermore, NOS inhibition during endotoxaemia has been found to increase capillary leak, and this may cause edema and thus "indirectly" impair ventricular systolic and diastolic function (Hutcheson et al., 1990).

Previously, it has been demonstrated that NOS inhibitor, N^{ω}-nitro-L-arginine (L-NNA), prevents the decrease in left ventricular contractility during endotoxaemia in intact animals (Stuehr et al., 1991). Moreover, based on the measurement of the end-systolic

pressure volume relationship, it has been demonstrated that L-NNA partially prevents the depression in left ventricular contractility in anesthetised pig with endotoxaemia (Kaszaki et al., 1996). Therefore, it would appear that NO plays a critical role in the depression of the myocardium in the latter stages of sepsis.

Although NO has been suggested to be an important mediator in reducing myocardial contractility *in vitro* (Finkel et al., 1992), it would appear that it has a minor protective role in the early stage of myocardial depression in endotoxaemia *in vivo* (Kaszaki et al., 1996). This is a paradoxical effect of NO which appears to occur at the initial stages of sepsis. This difference between the *in vitro* and *in vivo* effects of NO initially could be attributed to various protective effects of NO in the intact animal. Certainly, NO may play a direct role in the maintenance and control of microvascular blood flow by virtue of it dilator effect (Parker et al., 1990). In addition, NO may have an indirect effect on microvascular blood flow by inhibiting platelet aggregation and leukocyte adhesion (Radomski et al., 1990; Kubes et al., 1991). It is evident that a comparison between the impact of inhibiting NOS *in vivo* and *in vitro* is quite complex as different variables play different roles in each paradigm.

1.4 Septic shock and TNF-α

There is evidence in the current literature which indicates that administration of LPS results in the release of cytokines such as TNF-α (Thiemermann et al., 1993; Liebeman et al., 1989). In addition, it is believed that TNF-α is one of the factors that activates the

process responsible for iNOS, ultimately leading to an over-production of NO in the body (Ruetten et al., 1997). Multiple organ and system failure is the main pathology associated with septic shock which occurs after a serious bacterial, viral,or parasitic infection initiate a series of immunological, metabolic, and haemodynamic reactions in the host (Solorzano et al., 1987). Over the past ten years, it has been recognized that one class of endogenous host mediators, the cytokines (TNF-α, and interleukin-1), contributes significantly to the pathophysiology of septic shock (Lieberman et al., 1989). Cytokines are capable of mediating a wide range of biological effects.

TNF-α was first purified and characterised by Aggarwal and colleagues (1985). They established that this polypeptide has a molecular weight of 17 kDa. Subsequently, Beutler and associates (1987) reported that it contains 157 amino acids. It is secreted by a variety of myeloid cells, such as monocytes, lymphocyte, Kupfer cells (Hesse et al., 1988), and peritoneal macrophages (Halme et al., 1989). Mast cells and endothelial cells also synthesize TNF-α. Expression of TNF-α is very strictly controlled on a transcriptional as well as translational level (Beutler et al., 1988). Unstimulated monocytes express low levels of TNF-α messenger ribonucleic acid (mRNA), and stimulation causes both increased translation and transcription of the mature protein within minutes (Beutler et al., 1988). Numerous infections and inflammatory stimuli elicit TNF-α synthesis, including bacterial cell wall-derived LPS bacterial exotoxins, protozoa, fungi and viral particles (Wong et al., 1986). Bacterial infections after injecting bacterial endotoxin in rats, rabbits, and baboons cause an increase in circulating levels of

TNF-α which reaches a peak within 90 to 120 minutes post injection (Beutler et al., 1985; Hesse et al., 1988). Further studies demonstrated that bolus infusion of endotoxin in animals and humans induces a similar monophasic peak 1.5 hours after infusion (Michie et al., 1988). Experimental studies have further demonstrated a causal relationship between TNF-α and sepsis. Beutler and associates (1985) have reported that circulating TNF-α levels appear in rabbits within 15 minutes after a sublethal intravenous dose of endotoxin, where TNF-α levels peaked within 2 hours, and returned to baseline within 5 hours (Beutler et al., 1985). Hesse and coworkers (1988) demonstrated the same findings in humans. They have shown that TNF-α is detectable in the human plasma within 30 minutes and reaches the peak level at 1.5 hours after infusion of endotoxin, and these responses occurred temporally correlated with the appearance of symptoms (Michie et al., 1988; Ding et al., 1989). Similar observations have also been reported in rabbits (Ulevitch et al., 1989) and rats (He et al., 1992).

More specific effects of TNF-α were identified following administration of TNF-α. For example, high doses of TNF-α in animals have been reported to precipitate a syndrome similar to that seen in human septic shock (Tracey et al., 1987). Further, acute infusion of TNF-α in rats has been shown to produce hypotension , lactic acidosis, and subsequently death (Tracey et al., 1986). Pathological findings following a lethal dose of TNF-α have shown adrenal necrosis, pulmonary congestion, and intestinal congestion and intestinal necrosis consistent with those found in septic shock (Tracey et al., 1987). Furthermore, administration of TNF-α to humans elicits similar metabolic and

hemodynamic changes, including an increase in glucose and free fatty acid turnover, amino acid efflux and energy expenditure (Warren et al., 1987; Michie et al., 1988; Van der Poll et al., 1991). Also TNF-α stimulated expression of a cell surface tissue factor initiates coagulation via generation of thrombin (Van der Poll et al., 1990).

It has been reported that prophylactic administration of a polyclonal anti TNF-α antiserum protected mice from the lethal effects of endotoxin (Beutler et al., 1985). Tracey and associates (1987) also demonstrated that the septic shock due to lethal *Escherichia coli* infusion in primates can be prevented with a monoclonal murine anti-human TNF-α antibody. These results clearly identified the proximal role of TNF-α in the inflammatory cascade of sepsis. Although TNF-α has the capacity to elicit deleterious responses in the host, it has also been found that TNF-α possesses significant beneficial properties, including the capacity to elicit an endogenous anti-viral and anti-bacterial response. It serves as an endogenous pyrogen with immuno-stimulatory activity (Dinarello et al., 1986). TNF-α promotes the release of neutrophil from the bone marrow, as well as enhance neutrophil function. It initiates neutrophils margination, transendothelial passage (Moser et al., 1989) and activation (Ulich et al., 1987), including degranulation, production of super oxide radicals, and release of lysozymes (Shalaby et al., 1985), which enhance antibody dependent cellular cytotoxic and neutrophil mediated inhibition of functional growth (Djeu et al., 1986). Furthermore, it promotes differentiation of myelogenous cells to monocytes and macrophages, as well as activation

of these cells. TNF-α also participates in inhibition of intracellular replication of viral and parasitic organisms (Beutler et al., 1985).

1.5 The role of cGMP/cAMP in TNF-α production

Most of the effects of NO are mediated through a unique cGMP signaling pathway. NO activates the enzyme guanylate cyclase, and thereby elevating intracellular cGMP concentration (Ignarro et al., 1982; Craven et al., 1978; Ignarro, 1990). This increase in cGMP subsequently activates certain protein kinase, which phosphorylate target proteins involved in regulation of cell function (Ignarro, 1990; Stewart et al., 1994; Kuo et al., 1995). Although the role of cGMP as a NO second messenger is undisputed, some findings have led to the speculation regarding the existence of cGMP-independed signal transduction pathways for NO. Studies have shown that some effects of NO cannot be reproduced with cell permeable cGMP analogs. For example, the synthesis of TNF-α is increased in human peripheral blood mononuclear cells (Van Dervort et al., 1994), as well as LPS-stimulated neutrophil preparations by exogenous NO (Lander et al., 1995). Although NO increases cGMP concentrations in these cells, cGMP analogs have no effect on TNF-α production (Van Dervort et al., 1994; Lander et al., 1995). Collectively, these investigations suggest that NO might use cGMP-independent signaling pathways for some of its cellular functions.

Studies have found that enzyme adenylate cyclase can be modified by NO (Duhe et al., 1994). Treatment of cell membranes with NO decreases cAMP production by

inhibiting calmodulin activation of type I adenyl cyclase, presumably through thiol nitrosylation at the calmodulin-binding site (Duhe et al., 1994; Vorherr et al., 1993). Notably, increase in cAMP in leukocyte activate cAMP-depended protein kinase. This cAMP-depended protein kinase phosphorylate transcription factors that bind to the cAMP-response element on the TNF-α promoter, thereby inhibiting TNF-α mRNA transcription (Zhong et al., 1995). The effect of NO on type I adenyl cyclase suggest that NO might up-regulate TNF-α systhesis in human monocytes by decreasing cAMP concentrations.

1.6 The relationship between TNF-α and phosphodiesterase

A part of the physiological responses to both cGMP and cAMP are governed by a family of phosphodiesterase (PDE) that specifically hydrolyse the cyclic nucleotide to biologically inert 5'-nucleotide. There are five sub-types of PDE that have been isolated and characterised. The Ca^{2+}/calmodulin dependent or type I PDEs catalyze both cGMP and cAMP hydrolysis and there are at least six isoforms of this type (Beavo et al., 1990; Wu et al., 1992). This form of PDE is found in high concentrations in the brain, heart, lung and testis, and to a lesser extent in the kidney, adrenal glands, pancreas, and thyroid. One of its isoforms, a 63-kDa protein, catalyses cGMP hydrolysis several times more efficiently than cAMP hydrolysis (Sharma et al., 1986). Furthermore, a 75-kDa form that is expressed in the central nervous system appears to specifically degrade cGMP (Yamamoto et al., 1983). The Types II and III are both cAMP selective PDEs, but they

have significant relevance to the NO-cGMP transduction system since the rate of hydrolysis by these two enzymes is stimulated and inhibited respectively, by cGMP (Martins et al., 1982; Yamamoto et al., 1983). Type IV is a cGMP specific PDE, but its activity is not affected by cGMP (Li et al., 1990; Charbonneau et al., 1990). Type V PDE is an important regulator of cGMP function. This enzyme was first identified and partially purified from rat lung and platelets (Francis et al., 1980; Coquil et al., 1983). It has been reported that the intracellular concentration of cAMP plays an important role during inflammation (Brandt et al., 1992). TNF-α production inside the monocyte and macrophages increases during inflammation (Semmler et al., 1993; Evan et al., 1992; Torphy et al., 1992; Trophy et al., 1993). cAMP analogs such as dibutyryl cAMP (dbcAMP) can also increase the production of TNF-α. PDE inhibitors, attenuate the catabolism of cAMP and cGMP and regulation of inflammatory function in many cells, including monocytes, mast cells, basophils and neutrophils (Torphy et al., 1992; Giembycz et al., 1992; Torphy et al., 1993). Evidently a specific putative selective inhibitor of type IV PDE rolipram, (Nemoz et al., 1985) reportedly was able to reduce TNF-α production *in vitro* (Navarro et al., 1998; Navikas et al., 1998) and *in vivo* (Griswold et al., 1998). It has been suggested from *in vitro* studies that PDE inhibitors can suppress LPS induced TNF-α production, and the type-IV PDE is mainly involved in this process. This type of agent which increases the intracellular concentration of cAMP can inhibit the TNF-α production inside the macrophages.

1.7 Nature of problem and Experimental Objectives

The administration of LPS has been reported to produce hypotension (Thiemermann, 1994). It has also been reported that a reduction in cardiac output is associated with the fall in blood pressure (Poon et al., 1997; Cheng et al., 1998). It has also been suggested that changes in haemodynamics resulting from the administration of LPS are the result of an over-production of NO (Forfia et al., 1998). There is evidence in the literature that indicates that the administration of LPS does result in the induction of inducible iNOS (Salter et al., 1991; Thiemermann et al., 1993). It has been reported that LPS is responsible for the over-production of NO within the system (Thiemermann, 1994). The increase in NO production has been suggested to result in vascular hyporeactivity ultimately producing loss of vascular tone and cardiovascular collapse (Fleming et al., 1991; Gray et al., 1991; Kengatharan et al., 1996).

There is evidence in the current literature which indicates that administration of LPS results in the release of cytokines such as TNF-α (Thiemermann et al., 1993; Lieberman et al., 1989). In addition, it is believed that TNF-α is one of the factors that activates the process responsible for iNOS induction, ultimately leading to an over-production of NO in the body (Ruetten et al., 1997), and this over production of NO can be inhibited by rolipram, a putative selective inhibitor of PDE type IV (Nemoz et al., 1985).
Collectively, published data in the literature seem to support the view that in septic shock cardiovascular collapse results from an increase in plasma TNF-α which eventually results in from an increase in over-production of NO (Kengatharan et al., 1996; Avontuur

et al., 1990). The over-production of NO has been implicated in the development of vascular hypo-reactivity treated vasoconstrictor such as noadrenaline. Thus, the purpose of the present investigation was twofold: (a) to examine the influence of rolipram on hemodynamics, plasma TNF-α levels and production of iNOS in the lungs, *ex vivo*, in LPS-treated rats, and (b) to determine the effects of administration of a vasoconstrictor selective α_1-adrenoreceptor agonist, methoxamine to observe the vascular hypo-reactivity and haemodynamics in LPS-treated rats in the absence or presence of rolipram. Furthermore, in an additional series of experiments a parallel comparison was made between the effects of dexamethasone, an inhibitor of iNOS, and rolipram on the cardiovascular system in rats treated with LPS.

2. METHODS

2.1. Surgical preparation of animals

Male Long-Evans rats (330 - 360 g) were anaesthetized with thiobutabarbital (100 mg/kg) i.p.. Catheters (polyethylene tubing; I.D. 0.58 mm, O.D. 0.965 mm) were inserted into the left and right iliac arteries and veins. The catheters inserted into left iliac artery and vein were used for the measurement of blood pressure, and drug/vehicle administrations, respectively, while the catheters inserted into right iliac artery and vein were used for blood withdrawal of radiolabeled microspheres and return of blood samples after each cardiac output measurements, respectively. An additional catheter was inserted into the left ventricle via the right carotid artery for injection of radiolabeled microspheres. The animals were tracheotomized and allowed to stabilize for a period of 1 h while arterial pressure and heart rate were monitored continuously.

All catheters were filled with heparinized saline (25 iu/ml). Body temperature was maintained at 37 ± 1°C using a heating lamp and monitored using a rectal thermometer. Arterial blood pressure was recorded with a pressure transducer (Gould Statham, USA; Model PD23B) connected to a Gould Universal amplifier and recorder (Gould, France, Model 8188-2202-00). Heart rate was calculated from the blood pressure tracing.

2.2. Measurement of cardiac output

This technique has been described in detail elsewhere (Pang, 1983). Briefly, suspensions of microspheres (Mandel Canada; 15 μm diameter) labeled with ^{57}Co (20,000-22,000 in 150 μL) were injected into the left ventricle over a period of 10 s. Blood was withdrawn from the right femoral artery at the rate of 0.35 ml/min starting 15 s. before microsphere injection using an infusion/withdrawal pump (Kd Scientific USA; Model 120) for 1 min. The blood sample and syringes used for injection of microspheres or withdrawal of blood were counted for radioactivity at 80-160 Kev using a dual channel automatic gamma counter (LKB Wallac, Clinic Gamma Counter, Canada; Model 1272). The withdrawn blood sample was slowly injected back into the animals immediately after counting of radioactivity.

2.3. Experimental Protocol

Series I: Animals were randomly assigned to two groups (n = 5): saline-treated (0.8 ml/kg bolus; Group I) and LPS (0.8 mg/kg; Group II). After the completion of surgery, blood pressure and heart rate were continuously monitored for 60 min, after which each animal received either saline or LPS. Five blood samples (120 μl each) were collected into a pre-chilled syringe containing EDTA to yield a final concentration of 1 mg/ml. After centrifugation, the plasma was frozen and stored at -80°C until it was assayed for TNF-α. The first blood sample was taken just before the administration of saline or LPS and the other four samples were collected at 30, 60, 120 and 180 min post saline or LPS

administration. Cardiac output was also measured five times in these groups of animals, the first measurement being just before the administration of saline or LPS and four other measurements thereafter every hr. At the end of each experiment, the lungs were quickly excised, placed in liquid nitrogen and stored at -80°C.

Series II: Animals were randomly assigned to four groups (n = 5): vehicle-treated (2-hydroxypropyl-β-cyclodextrin; 2.0 ml/kg; Group III), rolipram-treated (3 mg/kg; Group IV and 10 mg/kg; Group V), and dexamethasone-treated (5 mg/kg; Group VI). After the stabilization period each animal was treated with vehicle or drugs. 15-20 min post treatment with vehicle/drugs a blood sample (120 μl) was collected for plasma TNF-α measurements as previously described, and cardiac output was measured. Each animal (Groups III-VI) was then treated with a bolus dose of LPS (0.8 mg/kg). Two more blood samples (120 μl each time) were subsequently collected for plasma TNF-α assessment at 60 and 120 min post-LPS treatment. Four hr after the administration of LPS a second cardiac output measurement was made. Subsequently, methoxamine (100 or 300 μg/kg/min) was infused and cardiac output was measured 14-16 min after the start of infusion. In each animal, repeated cardiac output measurements were made during the infusion of each dose of methoxamine. The time allowed between each dose of methoxamine was 15-16 min. At the end of each experiment, the lungs were quickly excised, placed in liquid nitrogen and stored at -80°C.

2.4. TNF-α assay in plasma

The total TNF-α in plasma was determined by a commercially available colorimetric enzyme linked immunosorbent assay kit (R&D Systems, MN, USA) for rat TNF-α. The sensitivity of the assay was 6 pg/ml.

2.5. Nitric oxide synthase assay in lungs

Nitric oxide synthase was assessed by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline as described by Thiemermann et al., (1993), with slight modifications. Frozen lungs were homogenized on ice in buffer composed of (in mM): Tris-HCl, 50; EDTA, 0.1; EGTA, 0.1; 2-mercaptoethanol, 12; and phenylmethylsulfonyl fluoride, 1 (pH 7.4). 50 µl of homogenates were incubated in the presence of [^{3}H]L-arginine/L-arginine (10 µM), NADPH (1.0 mM), calmodulin (10 µg/ml), tetrahydrobiopterin (5.0 µM) and Ca^{2+} (2.0 mM) (total volume of 200 µl) at 37°C for 30 min. The reaction was stopped using stop buffer (1.0 ml) of the following composition (in mM): HEPES, 20; EDTA, 2.0; and EGTA, 2.0 (pH 5.5). Each sample was applied to a 2-ml column of Dowex AG 50W-X8 (sodium form) (Bio-Rad Laboratory, Canada) and eluted four times with 1.0 ml of stop buffer. Radioactivity in each sample was measured using a scintillation counter (Beckman, USA; Model LS 3801). Assays were performed in duplicate in the presence of NADPH to determine constitutive NOS activity, the absence of NADPH to determine the extent of [^{3}H]L-citrulline formation independent of NOS, and in a Ca^{2+}-free buffer containing NADPH and EGTA (5 mM) to determine Ca^{2+}-

independent iNOS activity. Protein concentration was measured using Bradford's method (Bradford, 1976).

2.6. Chemicals

Rolipram, thiobutabarbital, L-arginine and 2-hydroxypropyl-β-cyclodextrin were purchased from Research Biochemical International (Natick, MA, USA). All other fine chemicals were purchased from Sigma Chemical Company (Ontario, Canada). Rolipram was dissolved in 2-hydroxypropyl-β-cyclodextrin and this was the vehicle used in the experiments.

2.7. Analysis of data

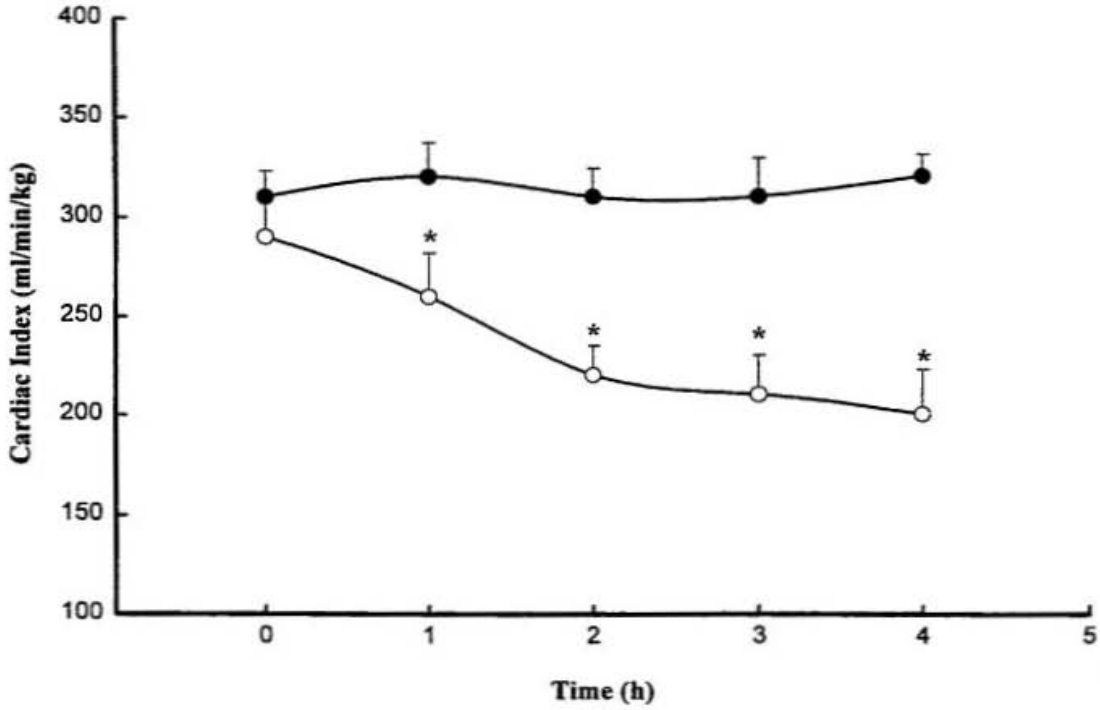
Mean arterial blood pressure (mmHg) is reported as diastolic blood pressure plus one third of the difference between systolic and diastolic blood pressures. Cardiac output (ml/min) was calculated as the rate of withdrawal of blood multiplied by injected c.p.m. divided by c.p.m. in withdrawn blood. Cardiac index is cardiac output divided by body weight. Arterial resistance (mmHg min/ml/kg) was obtained by dividing mean blood pressure by cardiac index.

The data were analyzed by one-way analysis of variance with repeated measures for comparison. Duncans multiple range test was used for comparison between means. In all cases, a probability of error of less than 0.05 was selected as the criterion for statistical significance.

3. RESULTS

There were no significant changes in haemodynamic values (cardiac index, mean blood pressure, arterial resistance and heart rate) over time after the administration of saline (Figure 1 & 2). In addition, plasma levels of TNF-α did not change following the administration of saline (Figure 3).

The administration of LPS to animals resulted in a significant reduction in cardiac index over time (Figure 1A). At 4 hr post-LPS treatment, cardiac index was reduced by over 27% when compared to cardiac index measured prior to the administration of LPS. Although arterial resistance and heart rate did increase over time in LPS-treated animals, these changes were found to be insignificant (Figure 1B & 2B). There were no appreciable changes in mean blood pressure in animals treated with LPS over time (Figure 2A). The administration of LPS to rats resulted in a substantial rise in the plasma levels of TNF-α (Figure 3). The time course for the peak and subsequent decline in plasma concentrations of TNF-α was within the 180 min time frame after the injection of LPS (Figure 3). In the present investigation, the peak concentration of TNF-α detected in the plasma at 120 min post-LPS injection was 190 times that of control levels prior to LPS administration. Furthermore, the injection of LPS resulted in a significant increase in the iNOS activity in the lungs of animals *ex vivo* (Table 1).


Cardiac Index (ml/min/kg)
400
350
300
250
200
150
100
0
1
2
3
4
5
Time (h)
*
*
*

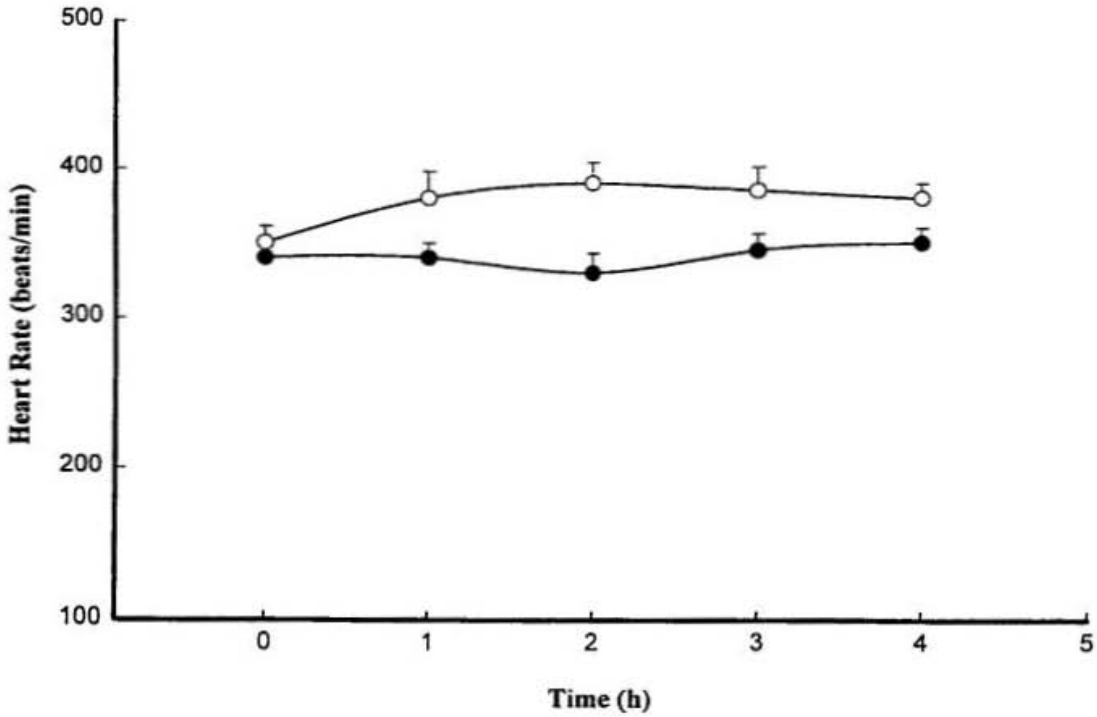
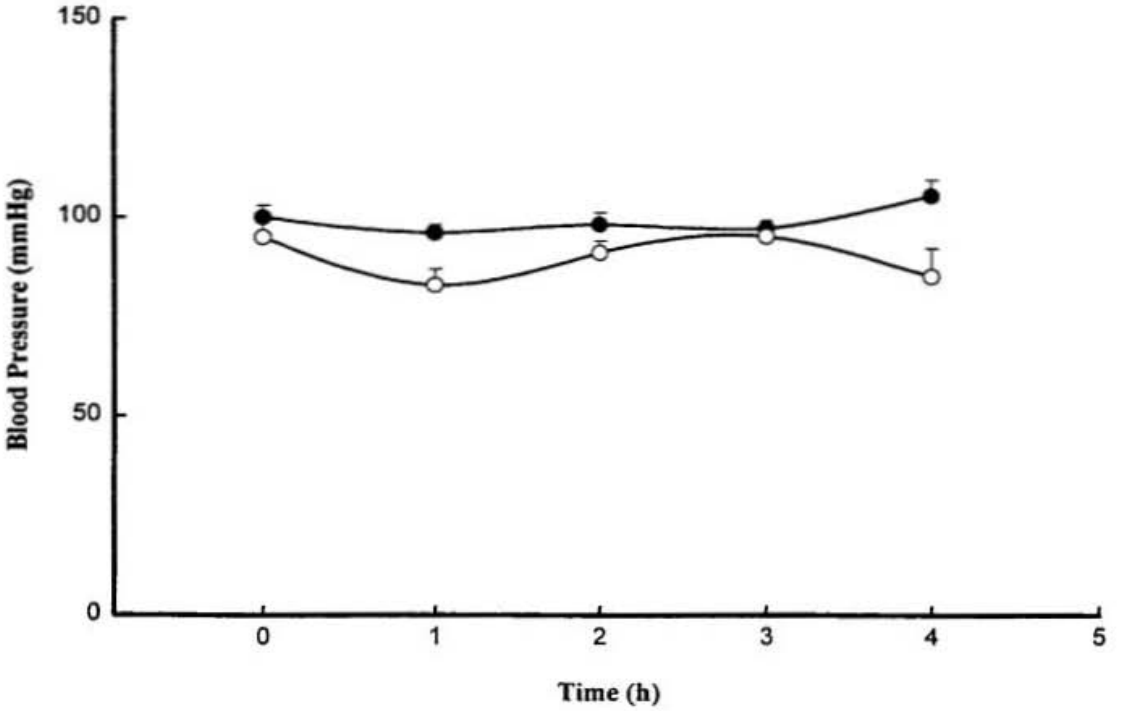

Heart Rate (beats/min)
500
400
300
200
100
0
1
2
3
4
5
Time (h)

Figure 1. The effects of treatment of anaesthetized rats with saline (0.8 ml/kg) (closed circles) or LPS (0.8 mg/kg) (opened circles) on (A) cardiac index and (B) heart rate over time. Data represents the mean of five experiments ± SEM. *Significantly different from respective values in saline-treated animals, $P< 0.05$.


150
100
50
0
Blood Pressure (mmHg)
0
1
2
3
4
5
Time (h)

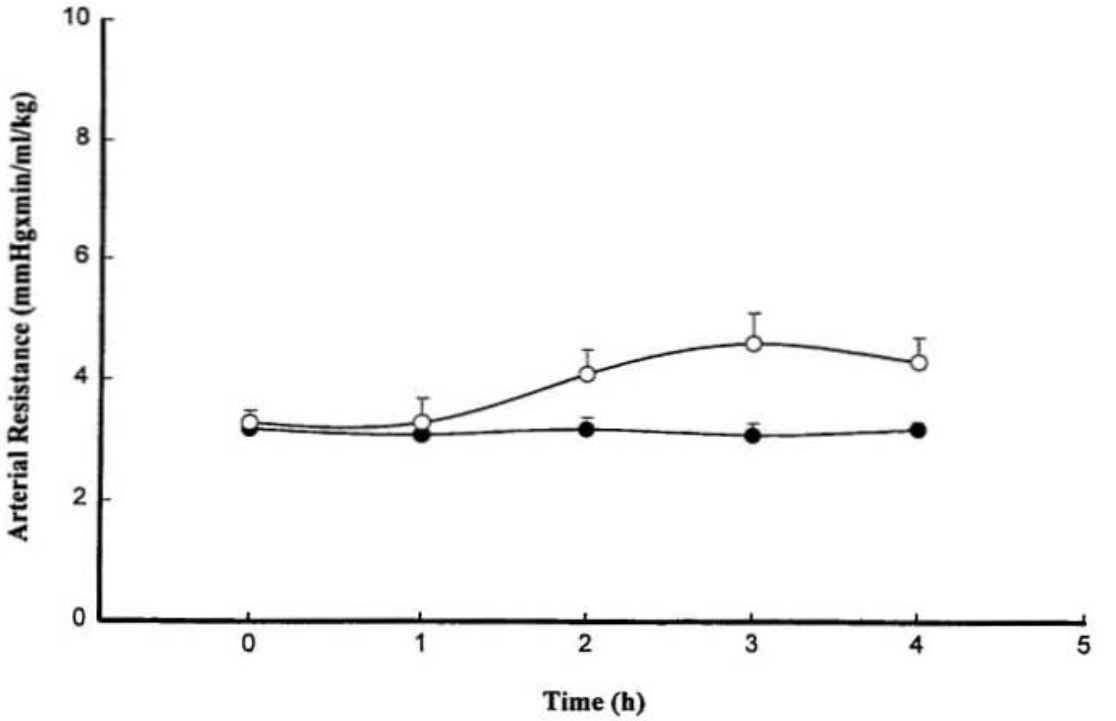

10
8
6
4
2
0
Arterial Resistance (mmHgxmin/ml/kg)
0
1
2
3
4
5
Time (h)

Figure 2. The effects of treatment of anaesthetized rats with saline (0.8 ml/kg) (closed circles) or LPS (0.8 mg/kg) (opened circles) on (A) blood pressure and (B) arterial resistance over time. Data represents the mean of five experiments ± SEM.

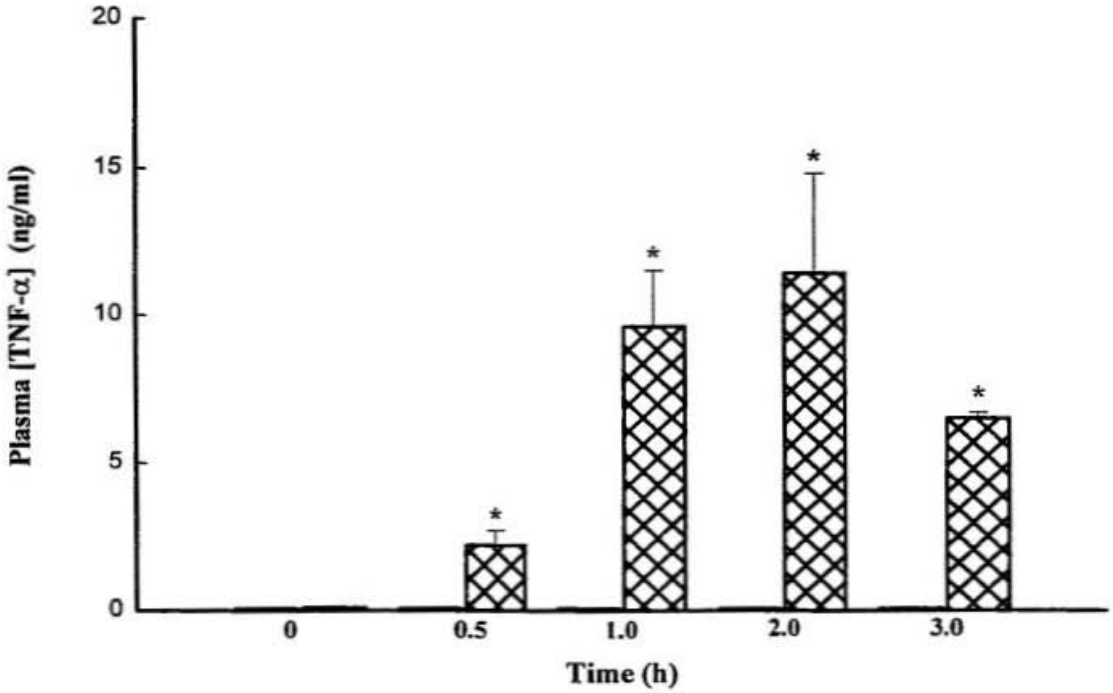

20
15
10
5
0
Plasma [TNF-α] (ng/ml)
*
*
*
*
0
0.5
1.0
2.0
3.0
Time (h)

Figure 3. The effects of treatment of anaesthetized rats with saline (0.8 ml/kg) (closed bars) or LPS (0.8 mg/kg) (cross-hatched bars) on plasma concentration of TNF-α over time. Data represents the mean of five experiments ± SEM. *Significantly different from respective values in saline-treated animals, $P < 0.05$.

Table 1. Values of enzymatic activity of inducible nitric oxide synthase (iNOS) and constitutive (cNOS) forms of nitric oxide synthase (pmol/mg protein/min) in lungs of various groups of animals treated with saline (0.8 ml/kg; Group I), LPS (0.8 mg/kg; Group II), and vehicle (2 ml/kg; Group III), rolipram (3 mg/kg; Group IV or 10 mg/kg; Group V) and dexamethasone (5 mg/kg; Group VI) prior to treatment with LPS (0.8 mg/kg). Each value represents mean of five experiments ± S.E.M.

Groups	iNOS	cNOS
Saline (I)	0.3 ± 0.04	0.4 ± 0.1
LPS (II)	15.0 ± 3.0[ab]	2.8 ± 0.8[a]
Vehicle+LPS (III)	13.2 ± 1.6[ab]	1.8 ± 0.6[a]
Rolipram3+LPS (IV)	19.0 ± 3.0[ab]	1.8 ± 0.6[a]
Rolipram 10+LPS (V)	17.0 ± 4.0[ab]	2.6 ± 0.8[a]
Dexamethasone (VI)	4.30 ± 0.7[a]	0.80 ± 0.2

[a]Significantly different from group I; $P < 0.05$
[b]Significantly different from group VI; $P < 0.05$

3.1. Effects of rolipram and dexamethasone on haemodynamics in LPS-treated rats

Prior to the administration of LPS, pre-treatment of animals with rolipram (3 & 10 mg/kg) or dexamethasone (5 mg/kg) did not result in significant changes to cardiac index, mean blood pressure, arterial resistance or heart rate when compared to animals that were treated with vehicle (Table 2 & 3). There were no significant changes detected in mean blood pressure, arterial resistance or heart rate in animals that were pretreated with vehicle, rolipram or dexamethasone after LPS injection (Table 2 & 3). In animals that were pre-treated with vehicle, LPS-treatment reduced cardiac index by over 25% when compared to cardiac index measured prior to the administration of LPS (Table 2). In contrast, pre-treatment with rolipram (10 mg/kg) and dexamethasone prevented the decline in cardiac index in animals that received LPS (Table 2). Cardiac index was significantly higher in rolipram (10 mg/kg) and dexamethasone treated animals when compared to vehicle-treated animals injected with LPS (Table 2).

3.2. Effects of α_1-adrenoceptor stimulation on haemodynamics in LPS-treated rats

Infusion of methoxamine (100 & 300 μg/kg/min) did not appear to have any significant effects on cardiac index in either vehicle-treated or rolipram-treated rats when compared to the respective values within each group prior to infusion of the α_1-adrenoceptor agonist 4 hr post-LPS treatment (Table 2). In contrast, the administration of methoxamine (100 & 300 μg/kg/min) to dexamethasone-treated rats produced a

Table 2. Haemodynamic changes in various groups of animals treated with LPS (0.8 mg/kg) in the absence or presence of vehicle (2 ml/kg), rolipram (3 or 10 mg/kg) or dexamethasone (Dexa; 5 mg/kg). The values represents the mean of five experiments ± S.E.M.

	Pre-LPS	Post-LPS (+4hr)	Methoxamine	Methoxamine
(Groups)	Cardiac Index (ml/min/kg)		(100 μg/kg/min)	(300 μg/kg/min)
Vehicle (III)	295 ± 22.0	220 ± 10.0[a]	236 ± 11.0	200 ± 8.0
Rolipram (IV)	334 ± 17.0	258 ± 12.0	258 ± 20.0	226 ± 7.0
Rolipram (V)	342 ± 15.0	280 ± 21.0[c]	288 ± 29.0[d]	290 ± 27.0[cd]
Dexa (VI)	302 ± 27.0	318 ± 36.0[c]	192 ± 10.0[b]	216 ± 20.0[b]
	Mean Blood Pressure (mmHg)			
Vehicle (III)	102 ± 2.0	89 ± 6.0	99 ± 4.0[d]	107 ± 3.0[b]
Rolipram (IV)	96 ± 3.0	81 ± 6.0	84 ± 6.0[d]	85 ± 6.0[d]
Rolipram (V)	103 ± 5.0	83 ± 6.0	88 ± 5.0[d]	92 ± 4.0[d]
Dexa (VI)	117 ± 5.0	107 ± 7.0	114 ± 7.0[b]	116 ± 6.0[b]

[a]Significantly different from Pre-LPS within the same group; $P < 0.05$
[b]Significantly different from Post- LPS-treatment (+4 hr) within the same group; $P < 0.05$
[c]Significantly different from respective values in vehicle-treated group; $P < 0.05$
[d]Significantly different from respective values in dexamethasone-treated group; $P < 0.05$

Table 3. Haemodynamic changes in various groups of animals treated with LPS (0.8 mg/kg) in the absence or presence of vehicle (2 ml/kg), rolipram (3 or 10 mg/kg) or dexamethasone (Dexa; 5 mg/kg). The values represents the mean of five experiments ± S.E.M.

	Pre-LPS	Post-LPS (+4hr)	Methoxamine	Methoxamine
(Groups)	A_R (mmHg min/ml/kg)		(100 μg/kg/min)	(300 μg/kg/min)
Vehicle (III)	0.38 ± 0.03	0.39 ± 0.03	0.42 ±0.02[d]	0.51 ± 0.02[bd]
Rolipram (IV)	0.29 ± 0.01	0.31 ± 0.02	0.33 ± 0.03[d]	0.38 ± 0.04[d]
Rolipram (V)	0.30 ± 0.02	0.30 ± 0.03	0.31 ± 0.02[d]	0.33 ± 0.02[d]
Dexa (VI)	0.40 ± 0.03	0.35 ± 0.02	0.61 ± 0.07[b]	0.56 ± 0.05[b]
	Heart Rate (beats/min)			
Vehicle (III)	344 ± 11	430 ± 28	392 ± 23	382 ± 19
Rolipram (IV)	424 ± 13	462 ± 13	460 ± 12	456 ± 17
Rolipram (V)	398 ± 19	445 ± 16	428 ± 16	430 ± 24
Dexa (VI)	332 ± 14	380 ± 12	388 ± 24	372 ± 20

[b]Significantly different from Post- LPS-treatment (+4 hr) within the same group; $P < 0.05$
[d]Significantly different from respective values in dexamethasone-treated group; $P < 0.05$

significant reduction in cardiac index when compared to respective values prior to methoxamine infusion in dexamethasone-treated rats 4 hr post-LPS treatment (Table 2). Administration of methoxamine did not significantly affect mean blood pressure and arterial resistance in rolipram treated animals when compared to respective values prior to infusion of methoxamine 4 hr post-LPS treatment. However, in vehicle-treated animals, infusion of methoxamine significantly increased blood pressure and arterial resistance at the higher but not lower dose when compared to respective values prior to the administration of α_1-adrenoceptor agonist 4 hr post-LPS treatment (Table 2 & 3). Moreover, administration of methoxamine also significantly increased mean blood pressure and arterial resistance at both dose levels in dexamethasone-treated rats when compared to respective values prior to the infusion of methoxamine 4 hr post-LPS treatment (Table 2 & 3). Stimulation of α_1-adrenoceptors did not result in any significant changes in heart rate in any group of animals (Table 3).

3.3. Effects of rolipram and dexamethasone on plasma levels of TNF-α

Plasma concentrations of the cytokine TNF-α were significantly elevated in all groups subsequent to injection of LPS (Table 4). In animals that had received rolipram (10 mg/kg) or dexamethasone, significantly lower plasma levels of TNF-α were detected at both 60 and 120 min post LPS-treatment when compared to respective values in vehicle-treated animals that had also received LPS (Table 4). However, even though plasma levels of TNF-α were lower in LPS-treated rats administered the lower dose of

Table 4. Plasma TNF-α values in various groups of animals treated with LPS (0.8 mg/kg) in the absence or presence of vehicle (2 ml/kg), rolipram (3 or 10 mg/kg) or dexamethasone (Dexa; 5 mg/kg). The values represents the mean of five experiments ± S.E.M.

	TNF-α (ng/ml)		
(Groups)	Pre-LPS Treatment	1-hr Post-LPS	2-hr Post-LPS
Vehicle (III)	0.004 ± 0.001	6.0 ± 2.1[a]	14.8 ± 3.8[a]
Rolipram (IV)	0.008 ± 0.004	3.4 ± 0.9[a]	9.6 ± 3.5[a]
Rolipram (V)	0.003 ± 0.001	3.1 ± 1.0[ab]	4.0 ± 1.2[ab]
Dexa (VI)	0.008 ± 0.005	0.8 ± 0.2[ab]	0.9 ± 0.3[ab]

[a]Significantly different from respective values pre-LPS treatment; $P < 0.05$
[b]Significantly different from respective values in group III; $P < 0.05$

rolipram (3 mg/kg) when compared to vehicle-treated animals, differences in plasma levels of TNF-α were not found to be significant (Table 4).

3.4. Effects of rolipram and dexamethasone on NOS activity in lungs in LPS-treated animals

The activity of NOS was elevated significantly following the treatment of animals with LPS (Table 1). Pre-treatment of animals with rolipram or vehicle did not affect NOS activity in animals that had received LPS. However, dexamethasone pre-treatment significantly reduced iNOS activity in the lungs of animals that had received LPS when compared to vehicle-treated animals that had also received LPS (Table 1).

4.0. DISCUSSION

Cytokines, such as TNF-α and interleukin-1β (IL-1β) play an important role in the cardiovascular sequelae of endotoxaemia (Cavaillon et al., 1992; Blackwell and Chritsman, 1996). In a number of pathopysiological states such as septic shock (Kumar et al., 1996), acute viral myocarditis (Smith et al., 1992), cardiac allograft rejection (Arbustini et al., 1991), myocardial infarction (Maury et al., 1990), and congestive heart failure (Levine et al., 1990) the concentration of TNF-α in blood increases. Recently, it has been reported that the administration of TNF-α alone or in combination with a low dose of endotoxin causes several cardiovascular effects including peripheral vasodilatation, hypotension, circulatory shock and organ damage (Billiau and Vanderkerckhove, 1991). It has been established that during endotoxaemia, there is a rise in TNF-α concentration in blood (Beutler et al., 1985). However, it is not known if co-infusion of TNF-α and IL-Iβ produces cardiovascular changes similar to those that have observed with LPS administration. Like LPS, TNF-α also induces the Ca^{2+} independent isoform of NOS *in vitro* (Drapier et al., 1988; Kilbourne and Belloni, 1990). Systemic administration of TNF-α increases NO production (Kosaka et al., 1992). An increase in NO production results in systemic vasodilatation (Kilbourne et al., 1990) and vascular hyporeactivity to vasoconstrictors (Vicaut, 1992).

Gardiner and associates (1998) have reported that TNF-α evokes significant hypotension accompanied by tachycardia, and dilatation in the renal and hindquarters

vascular beds. But this effect is not seen in mesenteric vessels due to endothelin production by the TNF-α (Goto et al., 1996; Gardiner et al.,1996). It has been also reported that TNF-α induces rapid (minutes) as well as slow (hours) effects on heart muscle (Tracy et al., 1993). It has been established that TNF-α is responsible for many of the cardiovascular sequelae of septic shock (Tracey and Cerami 1994).

Current evidence in the literature suggests that phosphodiesterase type-IV inhibitors are potential tools that can inhibit the production of TNF-α as a result of the action in elevating the intracellular cAMP level (Strieter et al., 1988; Endres et al., 1991). Rolipram is a specific type IV PDE inhibitor, the type IV PDE being the predominant isoenzyme present in monocytes and it is the enzyme responsible for controlling the cellular production of TNF-α (Beavo and Reifsnyder, 1990; Nicholson et al., 1991; Torphy et al., 1991). Semmler and associates (1993) have found that PDE inhibitors like rolipram, methylxanthine and pentoxifylline, markedly suppress the TNF-α production in human mononuclear cells. They also have established that the inhibitory action of rolipram is very selective for decreasing TNF-α level rather than IL-1β level. The substantial effectiveness of rolipram in suppressing TNF-α synthesis may be explained by its effect in inhibiting type IV PDE (Torphy et al., 1991).

Evidence from the present study indicates that treatment with LPS results in a progressive decline in cardiac index over time. There was also an increase in circulating levels of TNF-α in plasma, as well as an induction of NOS activity in lungs *ex vivo*. Pretreatment of animals with the putative selective phosphodiesterase type IV inhibitor,

rolipram, or synthetic glucocorticoid, dexamethasone, prevented the decline in cardiac output due to LPS administration. Rolipram and dexamethasone also significantly reduced the rise in plasma levels of TNF-α that had resulted from injection of LPS. However, only dexamethasone but not rolipram was able to significantly inhibit iNOS activity in lungs of animals that had received LPS.

Administration of LPS to rats leads to hypotension (Thiemermann, 1994). More recently, it was reported that a single bolus injection of LPS to the rat resulted in a progressive reduction of cardiac output over time. Associated with this reduction in cardiac output there was a reduction in mean circulatory filling pressure, an index of total venous tone (Poon et al., 1997). Treatment of rats with LPS did not appear to significantly affect vascular resistance to venous return (Poon et al., 1997). However, endotoxic shock has been reported to lead to an impairment of portal venous flow with portal venous resistance causing an increase in splanchnic blood pooling and subsequent decrease in venous return and thus cardiac output in anaesthetized pigs (Ayuse et al., 1995). Taken together, the evidence would suggest that the reduction in cardiac output in endotoxic shock is, in part, due to a reduction in venomotor tone. In the present investigation, data indicate that pretreatment with LPS resulted in a reduction in cardiac output without any significant change in arterial resistance. In addition there were no significant changes in heart rate. Thus it is possible that the reduction in cardiac output observed in animals treated with LPS in the present investigation was the result of a reduction in venous return (Poon et al., 1997; Ayus et al., 1995). It is possible that

treatment with rolipram and dexamethasone augmented venous return thereby resulting in the maintenance of cardiac output in LPS-treated rats.

However, it is also possible that reduction of cardiac output may be due to reduction of cardiac contractility. Finkel and associates (1992) have reported that TNF-α causes a reduction in the contractile force in the isolated hamster papillary muscle and this effect can be blunted by NOS inhibitor (Finkel et al., 1992). A similar NO dependent effect of TNF-α described in isolated rabbit ventricular tissues (Goldhaber et al., 1996). In contrast, Yokoyama and associates (1993) showed that the reduction of contractile force of feline myocytes induced by TNF-α was not affected by pretreatment with either N^G-nitro-L-arginine or N^G monomethyl-L-arginine (Yokoyama et al., 1993). The impact of TNF-α on isolated hamster papillary muscle, was immediate, and this effect of TNF-α on the myocardium is unlikely to be due to over-production of NO by the activation of iNOS (Finkel et al,. 1992; Goldhaber et al., 1996; Yokoyama et al., 1993). In the present investigation, the reduction of cardiac output in LPS treated rats may have been due to a reduction in contractile function of the myocardium.

It is evident from the present investigation that pre-treatment with rolipram in LPS-treated rats did not result in inhibition of iNOS but that cardiac index in these animals was maintained. This may imply that the reduction in cardiac index following the administration of LPS is not necessarily due to an over-production of NO. There is evidence to indicate that treatment of patients in a state of septic shock with a non selective inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methyl ester, results in

increase mean arterial pressure and arterial resistance with concomitant reduction in cardiac output (Avontuur et al., 1998). Furthermore, under experimental conditions, treatment of rats with methylene blue in endotoxaemia results in increased arterial resistance and reduction in cardiac output (Cheng et al., 1998). The evidence in the literature seems to suggest that inhibition of nitric oxide synthase and/or NO pathways does not improve cardiac output in endotoxaemia, and that cardiac output can be maintained independently of iNOS inhibition in endotoxaemia.

In the present investigation, we have found that pretreatment of animals with dexamethasone attenuated the rise in TNF-α concentration in plasma following treatment with LPS. This finding is consistent with the evidence in the literature which also indicates that glucocorticoids are capable of suppressing the rise in TNF-α plasma concentration (Stosic-Grujicic et al., 1982; Smith et al., 1980; Oppenheim et al., 1982). It is also apparent from the present investigation that pretreatment with dexamethasone also inhibited the activity of iNOS. Such a finding is consistent with reports presented in the literature (Beutler et al., 1990). However, in our view it is unlikely that the inhibitory effect of this substance in maintaining the cardiac output is the result of inhibition of NOS. It is perhaps most likely that the effects of dexamethasone in maintaining cardiac output in LPS treated rats was in part, due to its effect in reducing levels of plasma TNF-α. It is evident from the literature that inhibition of NOS does not result in improvment in cardiac output during endotoxaemia (Avontuur et al., 1998; Cheng et al., 1998).

NO is an important mediator for regulation of vascular tone as well as blood pressure (Huang et al., 1995; Takahashi et al., 1995; Kassab et al., 1998). However evidence suggest that there is an over-production of NO in endotoxic and hemorrhagic shock (Szabo and Thiemermann, 1994). It has also been established that the acute vasodilatation, sustained hypotension, and hyporeactivity to adrenergic agonists, which characterise the circulatory failure in endotoxic shock *in vivo*, are mediated by increased release of NO (Thiemermann and Vane, 1990; Kilbourn et al., 1990; Wright et al., 1992). Under physiological conditions, production of NO from L-arginine by the constitutive NO synthase (NOS) present in vascular cells keeps the vasculature in a state of active vasodilatation (Rees et al., 1990). An enhanced formation of NO in response to LPS is an important mediator of hypotension, peripheral vasodilatation and vascular hyporeactivity to vasoconstrictor agents in endotoxaemia. In addition, LPS and a number of cytokines induce NOS in phagocytic cells (Stuehr et al., 1989).

The current result indicate that infusion of methoxamine into animals injected with rolipram and pre-treated with LPS did not result in a significant increase or decrease in either cardiac index or heart rate. However, in animals treated with dexamethasone and pre-treated with LPS, infusion of methoxamine significantly reduced cardiac index. This was probably due to a substantial increase in arterial resistance. Since, dexamethasone did inhibit iNOS, vascular reactivity to methoxamine was not reduced and thus an increase in arterial resistance occurred following infusion with methoxamine.

It appears that the reduction in cardiac output in dexamethasone treated rats was not the result of significant changes in the heart rate. Similar pattern was observed with infusion of noradrenaline in animals that were hemorrhaged but were pre-treated with the same dose of dexamethasone (Tabrizchi, 1998). Under normal circumstances an increase in arterial resistance (afterload) can result in a reduction in cardiac output which occurs due to increased impedance to flow (Nekooeian et al., 1996). Moreover, a reduction in arterial resistance can result in the opposite effect and thus increase cardiac output under normal, as well as pathophysiological conditions (Tabrizchi, 1997; Nekooeian et al., 1998).

Treatment of animals with LPS resulting in endotoxaemia leads to an induction of iNOS secondary to an elevation of circulating levels of TNF-α. However, the decline in cardiac output following administration of LPS does not correlate well with induction of iNOS. At this point, it is evident that cardiac index is reduced within an hour following injection of LPS. Similar observation were made by other investigators (Poon et al., 1997; Forfia et al., 1998). It seems that iNOS begins to have a substantial impact on the cardiovascular system within approximately 3 hr post-LPS injection (Thiemermann et al., 1994). It is also evident from the present investigation that the peak concentration of TNF-α in plasma does not occur until at least 120 min post-LPS injections. Such an observation is supported by other reports (Michie et al., 1998; Ruetten et al., 1997). However, it is also apparent that the concentration of TNF-α is significantly elevated one hr post-LPS, and certainly this could trigger other processes that may have an immediate

impact on cardiac output and circulatory system. It has been demonstrated that anti-TNF-α antibodies prevent endotoxin-induced depression of myocardiac contractility (Tracey et al., 1987). Tracey and associates also hypothesized that myocardiac depression occurs in response to TNF-α because they found that infusion of TNF-α produces hypotension and decreased cardiac output whereas pretreatment with TNF-α monoclonal antibody attenuates these effects (1987). Myocardial contractility however, was not measured in their studies. Collectively, evidence in the literature and present findings indicate that the initial negative impact of LPS on the cardiovascular system, and especially on cardiac output, may be independent of iNOS activity.

The possibility that LPS can produce induction of NOS independent of TNF-α also needs to considered. Recently, it was reported that *in vivo* exposure of human colon epithelial cells to *Escherichia coli* results in induction of NOS (Witthoft et al., 1998). In addition, there is evidence in the literature which suggests that LPS is able to induce NOS in cytokine receptor-deficient mice (Le Roy et al., 1998). Moreover, in a recent report, it was demonstrated that endogenous TNF-α is not required for LPS-mediated induction of NO in rats (Xie et al., 1997). However, in contrast to these observations, earlier studies had indicated that pre-treatment with TNF-α monoclonal antibodies prevented symptoms associated with shock in anaesthetized baboons (Tracey et al., 1987). Recently, Thiemermann and associates (1993) had reported that pretreatment of rats with monoclonal antibody for TNF-α prevented the induction of NOS activity in the lung of animals that were subsequently treated with LPS (Thiemermann et al., 1993). However,

in the present investigation, the data indicate that a significant reduction in the circulating levels of TNF-α in animals treated with rolipram did not prevent induction of NOS in the lungs of animals treated with LPS. The present findings support the view that perhaps mediators other than TNF-α may contribute to induction of NOS in animals treated with LPS (Ruetten et al., 1997). However, it is possible that the effect of LPS may directly activate pathways that result in induction of NOS.

To summarise, the present findings indicate that treatment of rats with LPS results in a reduction in cardiac index, elevated plasma levels of TNF-α, as well as, induction of NOS in lungs. Pre-treatment with rolipram and dexamethasone prevents the decline in cardiac index and significantly reduces the rise in plasma levels of TNF-α as a result of LPS. Our findings show that dexamethasone inhibits induction of NOS where as rolipram does not. The results suggests that the induction of NOS may occur, in part, independent of TNF-α. Furthermore, circulating TNF-α may affect cardiac output independent of iNOS.

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