SALT SENSITIVITY IN C57 AND eNOS KNOCKOUT MICE

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SALT SENSITIVITY IN C57 AND eNOS KNOCKOUT MICE

by

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Abstract

Objectives: The present study examined the basal blood pressure (BP) and salt sensitivity of endothelial nitric oxide synthase (eNOS) knockout (KO) mice and their control strain (C57) mice.

Methods: Mice, age 12-18 wks, were fed a regular (0.7% NaCl) or a high (8% NaCl) salt diet for 6 weeks. BP, heart rate, pulse pressure and activity levels were recorded by telemetry. Results were calculated for the 12h light, 12h dark and total 24h periods.

Results: eNOS-KO mice exhibited an augmented basal BP and a heightened level of BP salt sensitivity. C57 mice displayed a slight degree of BP salt sensitivity. The effects of salt were more pronounced in the dark phase of the day perhaps reflecting the nocturnal nature of mice

Conclusion: eNOS-KO mice have a higher basal BP and BP salt sensitivity than the control strain.

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Chapter 1: Introduction

1.1 Hypertension:

1.1.1 Definition

Hypertension is defined as a chronic rise in arterial blood pressure (BP) (Goldman, 1999; Safar, 1996). Hypertension is a risk factor for many forms of cardiovascular disease such as myocardial infarctions, angina, stroke, and retinal and kidney damage, and is a leading cause of death in the Western world (Lilly, 1998; Swales, 1994).

1.1.2 Classification

The stage and severity of hypertension is normally classified based on the person's systolic and diastolic BP. Normal blood pressures are considered to be those less than 120/80 mmHg. For every increase in BP of 20/10 mmHg greater than 115/75 mmHg the risk of cardiovascular disease doubles (Chobanian et al, 2003). For classification on the levels of pre-hypertension and hypertension see table 1 (American Heart Association, 2004a). Hypertension affects about 1 billion people world wide thereby making it a serious problem in today's culture (American Heart Association, 2004).

Both genetic and environmental factors play a role in the development of primary hypertension. Environmental factors include a stressful lifestyle, a high salt diet, excessive alcohol intake, and obesity (Goldman, 1999). A combination of several genetic

Table 1: Classification of Hypertension

Classification of blood pressure based on the American Heart Association recommendations (American Heart Association, 2004a).

Category	*Systolic Blood Pressure* (mmHg)	*Diastolic Blood Pressure* (mmHg)
Normotensive	<120	<80
Prehypertensive	120-139	80-89
Hypertension (stage 1)	140-159	90-99
Hypertension (stage 2)	160 +	100+

factors involving alterations in the sympathetic nervous system, the renin-angiotension system, endothelial function, and abnormal sodium management by the kidney may also contribute to the development of hypertension (Antman, 2002; Genazzani, 2001; Kaplan & Lieberman, 1998; Robertson & Ball, 1994). While salt is an example of an environmental factor affecting hypertension, it also interacts strongly with the genetic determinants of hypertension. High salt consumption is prevalent in the modern diet. The combination of a high salt intake and an impaired ability to excrete a salt load may contribute to the development of hypertension (Roman, 1986).

1.2 Salt Sensitivity and Hypertension

1.2.1 Definition

Salt sensitivity refers to a change in BP that is directly related to a change in salt intake. A significant rise is considered to be about 10 mmHg or 10% increase in BP when the daily intake of sodium increased from normal (109 mmol or 2.5g Na/day) levels to a high salt diet (e.g. 249 mmol or 5.7g Na/day) (Weinberger, 1996; Kimura & Brenner, 1995). Individuals who are salt sensitive respond differently to a high salt diet than do those who are termed salt resistant. Salt resistant people experience little or no change in BP when the dietary salt intake is increased. In 1989, Hollenberg and Williams reported that up to 50-60% of people with hypertension are salt sensitive. Several mechanisms have been proposed to explain the different BP responses to salt among the two groups and epidemiological studies have been conducted to correlate BP with the level of dietary salt intake.

1.2.2 Salt Consumption

The risk of cardiovascular disease is augmented in individuals that exhibit salt sensitivity. The high level of salt in a typical North American diet places people at risk because of the potential salt-induced augmentation of BP. BP's above 115/75 mmHg have been shown to increase the risk of death and illness (Chobanian et al, 2003). A high salt diet also increases the risk of cardiovascular mortality in hypertensive individuals independent of an additional rise in BP (Tobian, 1991). The high prevalence of salt sensitive hypertension arises in part from the difficulty of eating a low salt diet in a developed country. The high salt content of processed food and the majority of restaurant food, regardless of whether or not it is "fast food", as well as the "continual presence of the salt shaker" all contribute to a dietary salt intake that is much higher than in historical hunter gatherer societies. Thus, a salt sensitive person can develop an increased BP from merely eating a typical diet in today's society. That is, salt sensitive hypertension does not necessarily arise from a person eating a diet that is higher in salt than his/her counterparts. It simply requires a 'normal' salt intake.

Today, typical North American salt intakes exceed those previously consumed by earlier generations and greatly exceed those of some current hunter gather societies. In Paleolithic times, the population consumed as little as 1g NaCl (0.4g Na) per day (1g of NaCl is about 38% Na by weight) (MacGregor & Wardener, 1998). In societies in which the daily salt intake is less than 50 to 100 mmol (1.15-2.3g of NaCl) the presence of hypertension and resulting illnesses are rare (Weinberger, 1996). Epidemiological studies have suggested that as the average salt intake intensified so did the prevalence of cardiovascular disease (Weinberger, 1996).

Today an average American has a daily salt intake of about 10g NaCl (~170mmol) (Watanabe et al, 2002; Mancilha-Carvalho et al, 1989) but the recommended adequate daily intake is only 1.5g of Na per day (65mmol) (National Academy of Sciences, 2004). The recommended tolerable upper limit (UL) is 5.8g of NaCl per day (National Academy of Sciences, 2004). Despite this, 90% of Canadian men and 50% of Canadian women consume more than this level (National Academy of Sciences, 2004). If the typical person consumed the recommended amount of salt they would meet their nutritional requirement but also greatly reduce their risk of salt-induced hypertension.

1.2.3 Epidemiological studies

In an attempt to provide a correlation between dietary salt levels, BP and cardiovascular mortality, numerous epidemiological studies have been conducted including the Solomon Islands study, the Intersalt study and the DASH (Dietary Approaches to Stop Hypertension) study.

An early epidemiological study that focused on the effects of salt on BP was conducted by Page et al (1974). It specifically examined the BP and salt intakes of the people living on the six Solomon Islands. Of the six groups examined the general results indicated that the more acculturated the society, the greater the salt intake and the higher the BP (Page et al, 1974). Of particular interest is the Lau subgroup who had an elevated salt intake (150-220mEq/day versus a range from <20mEq to 130mEq/day among the other groups) and higher systolic and diastolic BP's across both sexes and most age

groups (Page et al, 1974). The groups that ingested salt at lower levels showed no increase in BP with age independent of factors such as weight (Page et al, 1974). The results from this study clearly indicated that the rise in BP with age is not a predetermined event in the time course of a human lifespan. The process of acculturation, and specifically the change in diet to a higher salt intake, was suggested to be the underlying cause of the current age related upward BP trend (Page et al, 1974).

The Intersalt study was conducted in the 1980's in response to a lack of standardized data on BP in different countries (Intersalt Cooperative Research Group, 1988). The Intersalt study examined a total of 52 groups across 32 countries. Four hundred people, half male and half female, were recruited at random at each test center. Each person provided urine samples, body mass information and alcohol consumption levels and had their BP measured by trained personnel. Only 4 of the 52 test centers had average sodium intake levels that were low enough to eliminate hypertension or undesired increases in BP with age. The other 48 groups had an average sodium intake of approximately 9 grams of NaCl per day (154mmol NaCl/day). Among them, the increased salt intake was related to a higher incidence of hypertension and cardiovascular related death (Stamler, 1997; Mancilha-Carvalho et al, 1989; Intersalt Cooperative Research Group, 1988). The Intersalt study estimated that if the sodium intake was reduced by 100mmol/day (e.g. from 150mmol to 50mmol) then the mean BP would drop by 2-3 mmHg and the age related increase in BP would drop by about 9 mmHg (Stamler, 1997; Intersalt Cooperative Research Group, 1988). A drop of 2-3 mmHg is predicted to correspond with a decrease in cardiovascular mortality and morbidity of about 3-14%,

and a drop of 9 mmHg is predicted to correspond with a 13-23% reduction (Stamler, 1989).

Urinary sodium excretion (used to estimate the salt intake levels) ranged in varying populations from 0.2 mmol/d in the Yanomamo Indians (Brazil) to 242 mmol/d in northern China (Intersalt Cooperative Research Group, 1988). The inhabitants of St. John's, Canada excreted about 184 mmol sodium/d (Intersalt Cooperative Research Group, 1988). The Yanomamo Indians (0.2mmol NaCl/d or <0.01g NaCl), Xingu Indians (5.8 mmol NaCl/d or 0.3g NaCl), Papua New Guinean (26.8 mmol NaCl/d or 1.5g NaCl) and the Kenyans (51.3 mmol NaCl/d or 3g NaCl) had no negative salt related changes in BP (Mancilha-Carvalho et al, 1989; Intersalt Cooperative Research Group, 1988). The average systolic BP of these four groups was 103 mmHg (Mancilha-Carvalho et al, 1989). In comparison, the average systolic BP of the remaining 48 groups was 120 mmHg (Mancilha-Carvalho et al, 1989). Hypertension and related diseases were absent in the four low salt groups and increased to 33.5% in the 48 high salt groups studied (Stamler, 1997; Intersalt Cooperative Research Group, 1988).

The Dietary Approaches to Stop Hypertension (DASH) study also assessed the effect of salt on BP. The original DASH study examined the effect of a control diet (typical western diet), a diet high in fruits and vegetables or a combination diet that was high in fruits and vegetables and low in dietary fats (Svetkey et al, 1999). The findings indicated that the combination diet was most effective in lowering BP with reductions in BP of 11mmHg (systolic) and 5.5 mmHg (diastolic) without any salt intake restrictions or weight loss (Svetkey et al, 1999). The DASH-sodium study examined the effects of

sodium intake with either a combination diet or a typical western diet. This study confirmed the benefits of the DASH combination diet but, more importantly, it provided concrete evidence that a moderate reduction in salt down to <1.15g NaCl /day significantly lowers BP (Fleet, 2001). In participants consuming the western type diet, a change from high (3.45g NaCl/day) to low (1.15g NaCl/day) sodium intake was associated with a decrease in systolic BP of 6.7 mmHg. In comparison, a person consuming the combination DASH diet showed a decrease in systolic BP of 3.0 mmHg when their dietary intake was changed from a high to low dietary salt intake (Fleet, 2001). These results show that although the DASH combination diet is effective in controlling BP, the restriction of salt plays an equally significant role in BP control. The expansion of the original DASH study to include varying levels of salt emphasized the importance of sodium restriction as a method to control hypertension. The DASH-sodium study provided evidence to support the concept that sodium restriction was more effective in controlling BP than the individual effects of the tested diets (Fleet, 2001). Taken together, the epidemiological studies provide evidence for the important role of salt in controlling and elevating BP. The exact mechanism and time course through which salt raises BP is unknown but several theories have been proposed.

1.3 Studies of Salt Sensitivity in Humans

Salt sensitivity in man is evident as a change in BP when the dietary salt intake is altered. In humans, for ethical reasons, a decrease in salt intake is typically used to test for salt sensitivity. Epidemiological studies (described above) have demonstrated that

increases in BP observed with age (generally considered 'normal' in Western societies) do not exist in societies with a low dietary salt intake (Stamler, 1997; Mancilha-Carvalho et al, 1989; Intersalt Cooperative Research Group, 1988). This suggests that a slow, progressive salt sensitivity of BP is common in developed cultures consuming high levels of dietary salt. However, rapid changes in BP can also be observed with acute changes in salt intake.

Short term studies have examined the effect of salt on humans over periods ranging from 4 hours to about 1.5 weeks. Luft et al (1979) looked at the effect of varying levels of high salt intake over 3 day periods and found that increased salt intake (from 300mmol/d to 1500mmol/d) elevated BP. The responses to the high salt diet showed an increase in BP ranging from 1.5-34%, demonstrating variability in individual responses to salt. Over a shorter time span, Weinberger et al (1986) examined the effects of salt and volume infusion over a 4 hour period on the first day and the effects of induced salt and volume depletion the following day. They classified salt sensitive people as having a change in BP of 10 mmHg or greater and salt resistant people as having a 5 mmHg change or less in response to salt infusion and depletion (Weinberger et al, 1986). These studies provide evidence for a relatively rapid BP response to a high salt intake. The epidemiological data and the results of the short term studies demonstrate that humans have both a short term and long term BP response to a high salt diet.

1.4 Salt Sensitivity in Animals

Animal studies have demonstrated salt sensitivity over a wide range of time courses and in a number of species. The long term effects of high salt intakes on BP have been examined in several studies.

In 1948, Lenel et al examined the BP of chickens exposed to control (tap water), 0.9% and 1.2% NaCl in the drinking water. Systolic and diastolic pressures progressively increased and were 51 and 37 mmHg respectively above the control BP levels after 70 days of salt intake (Lenel et al, 1948). Meneely et al (1953) examined the effect of 2.8, 5.6, 7.0, 8.4 and 9.8% NaCl feed on BP in Sprague-Dawley (SD) rats over the course of 9 months. Increases in dietary NaCl content elevated BP with the largest increase being ~25mmHg above control levels (Meneely et al, 1953). However, the BP recordings provided excellent long term data but no information on the time course of the response. Dahl et al (1968) examined the effect of salt on salt-sensitive rats that had been selectively bred from SD rats. The rats were placed on 0.4, 1, 2, 4 or 8% NaCl feed for a period of 1 year. BP measurements were taken every month starting from 4 weeks of age. BP increased with elevated salt intake. An 8% NaCl diet caused the average systolic BP to rise from 147 to 210 mmHg (Dahl et al, 1968).

Cherchovich et al (1976) studied the effect of dietary salt in Papio hamadryas (monkeys) over 1-1.5 years. BP increased in response to a high salt (4% NaCl) diet. The earlier the age of first exposure to high salt, the greater the overall rise in BP (Cherchovich et al, 1976). Denton et al (1995) studied the BP of chimpanzees that were fed a high salt diet for 20 months (5 g/day for 19 wks, 10 g/day for 3 wks and 15 g/day

for 67 wks). At the end of the experiment the average systolic BP of salt sensitive animals had risen by 33 mmHg and the diastolic BP by 10 mmHg

A long term study was conducted on mice by Ebihara and Martz in 1971. The Cox/Swiss strain of mice was provided with 1% NaCl drinking water for 10 weeks and BP measurements were taken once a week using the tail-cuff method (Ebihara & Martz, 1971). BP gradually increased to about 30 mmHg above control levels after 10 weeks (Ebihara & Martz, 1971). Gros et al (2002) used tail-cuff to measure BP in order to determine the effect of either a normal or 8% salt diet on BP in C57Bl/6 mice. BP measurements began after 2 weeks of exposure and the experiment showed that male mice experienced an 18% average increase in BP when exposed to a high salt (8% NaCl) diet for 3 weeks (Gros et al, 2002). These studies support the idea that an augmented dietary salt intake has a long term, progressive effect on BP.

Short term studies have examined the rapid response of BP to a high salt intake in dogs subjected to a 2/3 renal mass reduction in order to induce salt-sensitivity (Manning et al, 1979; Coleman et al, 1969). Coleman et al (1969) had a 7 day control period after which the dogs were either allowed to drink saline ad lib or were infused intravenously with saline at a constant rate for 2 weeks. Both group showed significant increases in BP over the 2 week period with the elevations in BP being higher in animals infused with saline (Coleman et al, 1969). Both groups of dogs showed an initial abrupt rise in BP over the first week followed by slower progressive elevations in BP during the second week. Manning et al (1979) used an 8 day control period followed by a 13 day exposure to saline infusion. An initial and rapid elevation in BP of about 20 mmHg in response to

salt loading was observed on day 1 of exposure (Manning et al, 1979). This increase persisted during the course of the saline infusion and was followed by a further rise of about 10 mmHg over the next 12 days (Manning et al, 1979).

Gross et al (2000) examined short term changes in BP in mice of a mixed genetic background (mostly C57Bl/6) using abdominal telemeter implants over 7 weeks. The high salt diet (4% NaCl) was given for about 1 week before the animals were exposed to a deoxycorticosterone (DOCA) tablet placed under their skin (Gross et al, 2000). In the 1 week time frame there was no effect on BP in the mice (Gross et al, 2000). In contrast, Carlson and Wyss (2000) examined BP in C57 mice using telemetry. The mice were given 1 week to recover from the telemeter implantation, followed by 4 days of control BP recordings. The diet was then switched to 8% NaCl and the mice were allowed to adapt to this salt level for 1 week after which BP was recorded for 4 days. The high salt diet caused a significant increase in BP of about 9 mmHg (Carlson & Wyss, 2000). This is relevant to the objectives of the present study because it uses telemetry to measure BP in C57 mice. However, the study did not document the initial salt response (week 1) or the time course of the BP changes to a high salt diet.

Although fast and slow BP responses have been described, there is still very little data available on the chronology of BP changes in response to salt intake. Van Vliet et al. (2005) suggested that the time course of the BP response to a high salt intake in Dahl salt sensitive rats has two distinct phases. The first response is a significant rapid rise in BP that is then followed by a slow gradual rise in BP. Since the two phases can be separated in crossbred rats, they may have different underlying mechanisms. Thus, it is

important to consider the time course when evaluating salt sensitivity in an animal model, including the knockout mice of our present study.

1.5 Mechanisms of Salt Sensitivity

Many physiological systems have been implicated in salt sensitive hypertension. The central nervous system as well as the kidney and nitric oxide system have been shown to play important roles in the development of high BP as a result of a high salt diet. There is evidence indicating that each of these systems plays a role in altering BP in response to salt intake and that alterations in multiple systems may contribute to the development of salt sensitive hypertension.

1.5.1 The Kidney

Salt sensitivity is at least partly dependent on the kidney and its function. Dahl et al (1972) and Morgan et al (1990) demonstrated the importance of the kidney in salt sensitivity by transplanting kidneys from Dahl salt-sensitive to salt-resistant rats. Their results showed that the mechanisms promoting a large part of the hypertensive response to salt ingestion were intrinsic to the kidney itself (Morgan et al, 1990; Dahl et al, 1972). Morgan et al (1990) demonstrated the involvement of both renal and non-renal factors by transplanting kidneys between Dahl salt sensitive and salt resistant rats and then feeding the rats high salt (8% NaCl) for 2 weeks. Both salt resistant rats with salt sensitive kidneys (DRS) as well as salt sensitive rats with salt resistant kidneys (DSR) showed augmented BP in response to salt (Morgan et al, 1990). Dahl salt resistant rats with two

salt resistant kidneys had an average BP of 103 mmHg, where as DRS rats had a BP of approximately 145 mmHg. DSR rats had a BP of about 151 mmHg while Dahl salt sensitive rats with two salt sensitive kidneys had an average BP of 160 mmHg (Morgan et al, 1990). In this experiment the kidney's role was demonstrated in the DRS rats whereas the importance of non-renal factors was shown in the DSR rats.

The intrinsic ability of the kidneys to regulate sodium excretion may play an important role in the development of salt sensitive hypertension. Several studies have shown that Dahl salt sensitive rats excrete lower levels of sodium than their salt resistant counterparts (Vaneckova, 2002; Maude & Kao-Lo, 1982; Roman, 1986; Tobian et al, 1978). Vaneckova (2002) examined isolated kidneys from 12 week old Dahl salt sensitive and salt resistant rats fed a low salt diet. This study showed that Dahl salt sensitive rats exhibited a lower sodium excretion than the salt resistant rats which was possibly due to differences in tubular re-absorption (Vaneckova, 2002). Maude and Kao-Lo (1982) also showed that Dahl salt sensitive rats excreted less sodium than salt resistant rats when fed a high salt (8% NaCl) diet. The pressure natriuresis and pressure glomerular filtration rate curves of the Dahl salt-sensitive rats were shifted to the right indicating a decreased filtered sodium load (Maude & Kao-Lo, 1982). Roman (1986) examined the changes in the pressure natriuresis relationship in Dahl salt-sensitive and salt- resistant rats fed either a control (0.3% NaCl) or high salt (8% NaCl) diet for 4 weeks. This study found that Dahl salt-sensitive rats on high salt required a higher perfusion pressure to maintain normal renal blood flow and had a lower sodium excretory capacity than the Dahl salt-sensitive rats on control salt or Dahl salt-resistant rats

(Roman, 1986). Consistent with the above results, Tobian et al (1978) demonstrated that the isolated kidneys of Dahl salt sensitive rats exhibited a respective 52% and 47% decrease in sodium excretion at 130 and 160 mmHg when compared to Dahl salt resistant rats. The above studies support the idea that salt sensitive hypertension is associated with a reduction in the ability of the kidney to excrete sodium.

In the presence of a reduced ability to excrete sodium, a high salt diet could lead to hypertension by way of the renal body fluid feedback mechanism. In salt sensitive individuals, a high salt diet is presumed to cause an increase in extra cellular fluid volume (ECFV), cardiac output (CO) and BP through volume expansion (Coleman & Guyton, 1969). An increase in CO causes the mean arterial pressure and the amount of blood flow to the tissues to rise. Through whole body autoregulation, the total peripheral resistance (TPR) increases which helps return tissue perfusion and CO to normal and also greatly limits volume loading (VanVliet & Montani, 2005; Muntzel & Drueke, 1992; Coleman & Guyton, 1969). As the CO returns toward control levels, the BP remains elevated due to the sustained increase in TPR (VanVliet & Montani, 2005). The result of this is a long term elevation in BP referred to as volume loading hypertension.

1.5.2 The Central Nervous System

The central nervous system (CNS) is presumed to play a role in promoting salt sensitive hypertension because a number of treatments affecting the CNS have been shown to attenuate or prevent salt induced hypertension: blockade of the brain's ouabain-

like substance (OLS), the brain's renin angiotensin system, and sympathetic system activation, and brain lesions.

The OLS is involved in the brain's activation of the CNS's renin-angiotensin system and the subsequent excitation of the sympathetic system. The OLS levels in the brain are increased in response to a high salt diet. If the brain's endothelial Na+ channels (ENaC) or the OLS are blocked, the expected salt induced rise in BP is eliminated in salt sensitive Dahl rats (Wang & Leenen, 2002). Wang and Leenen (2002) determined that the blockade of brain ENaCs only affected the CNS, and not the peripheral, production of OLS.

Huang and Leenen (1994) found that by blocking the OLS, salt sensitive hypertension and sympathetic activation were reduced in salt sensitive Dahl rats fed a high salt diet. Further studies showed that blocking the OLS (Zhao et al, 2001), the OLS and brain angiotensin II (Ang II) (Huang & Leenen, 1998; Huang & Leenen, 1996), or only the brain's renin-angiotensin system (Leenen & Yuan, 2001) abolished salt induced hypertension in Dahl salt-sensitive rats. The final effect of the ouabain-Ang II cascade is sympathetic hyperactivity which then leads to increased BP (Huang & Leenen, 1999; Huang & Leenen, 1998). Huang et al (2001) compared the level of responsiveness to different sodium concentrations in the cerebral spinal fluid and showed that Dahl salt sensitive rats have larger increase in BP, HR and renal sympathetic nerve activity as well as a greater ouabain response to a given level of sodium than Dahl salt resistant rats. It was concluded that salt sensitive rats appear to be more sensitive to changes in levels of salt intake and the amount of salt that enters the brain and therefore salt sensitive rats

experience a much greater hypertensive effect when fed a high salt diet than salt resistant rats.

Although both the brain and the kidney have been suggested to be key players in hypertension induced by a high salt diet, a study by Huang et al (2004) showed that the brain registered an increase in salt concentration in the cerebral spinal fluid (CSF) prior to an increase in BP or heart rate (HR). This suggests that the brain may be responsible for initiating the development of hypertension in response to salt ingestion (Huang et al, 2004; Gavras, 1986). This is supported by the finding that sympathetic inhibition eliminates the salt induced rise in BP observed in salt sensitive animals (Oparil et al, 1988; Friedman et al, 1979; Takeshita et al, 1979). This demonstrates that an intact sympathetic nervous system (SNS) is necessary for the development of salt sensitive hypertension in the Dahl salt-sensitive rat model (Leenen et al, 2002; Haung & Leenen, 1999). That is, blocking the brain's connection with the kidney appears to limit the development of salt sensitive hypertension.

1.5.3 The Nitric Oxide System

Nitric oxide (NO) is thought to play a role in the body's normal response to salt (Manning et al, 2001; Tolins & Shultz, 1994). The results of several studies suggest NO plays a role in the development of salt sensitive hypertension.

First, a rise in NO production normally occurs in response to an increase in dietary salt intake (Kiraku et al, 1999). In normal mice fed a high salt diet the urinary excretion of NO metabolites increased along with the BP (Kiraku et al, 1999). Tolins and

Shultz (1994) similarly demonstrated that when SD rats were placed on a high salt (4% NaCl) diet their excretion of NO metabolites was enhanced.

Second, the increase in NO production with high salt appears to be attenuated in salt sensitive hypertension. Manning et al (2001) demonstrated that Dahl salt sensitive rats had a lower urinary excretion of NO metabolites than their salt resistant counterparts. Ni et al (1999) and Ni & Vaziri (2001) showed that Dahl salt sensitive rats consuming a high salt diet had a lowered expression of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) and a higher expression of neuronal nitric oxide synthase (nNOS) than salt resistant rats. These studies suggest that there is attenuated production of NO by eNOS with increased salt intake in salt sensitive rats (Manning et al, 2001; Kiraku et al, 1999).

Third, inhibition of NO production leads to salt sensitive hypertension. Several studies have shown that the administration of a nitric oxide synthase (NOS) inhibitor to rats results in salt sensitivity: i.e. a rise in BP when placed on a high salt diet (Tan et al, 2000; Rudd et al, 1999; Yamada et al, 1996; Tolins & Shultz, 1994). A study by Tolins and Shultz (1994) showed the significance of NO in the regulation of sodium and BP. By comparing SD rats fed a high salt diet (4% NaCl) or a high salt diet and L-NAME, it was shown that BP control was possible as long as the NO system remained intact (Tolins and Shultz, 1994). The control SD rats on a salt diet had an increased urinary excretion of NO metabolites while BP remained normal (Tolins and Shultz, 1994). In contrast, rats fed both salt and L-NAME showed a rise in BP with no increase in urinary excretion of NO metabolites (Tolins and Shultz, 1994). Rudd et al (1999) also concluded that NO is

required for normal BP control. When Dahl salt sensitive rats were placed on a high salt (8% NaCl) diet and given a NOS inhibitor (AMT), their BP rose significantly above the levels seen on the high salt diet alone (Rudd et al, 1999). Obst et al (2004) used mice implanted with telemeters to monitor BP to show that NOS inhibition via L-NAME combined with a high salt intake led to a rise in mean arterial pressure (MAP) that was directly related to an increase in TPR. TPR was taken to be the quotient of MAP and CO, with CO being measured using a Transonic flow probe (Obst et al, 2004). Irrespective of the exact mechanism involved, the results of these studies suggest that NO is important in BP regulation and salt sensitive hypertension.

A fourth and final argument for the importance of NO is that increases in NO production can lead to the correction of salt sensitivity (Manning et al, 2001; Miyata & Cowley, 1999). In Dahl salt-sensitive rats, the administration of L-arginine can restore the amount of urinary NO metabolites to the level seen in salt-resistant rats (Manning et al, 2001). At the same time, it eliminates the effect of salt on BP in this model (Manning et al, 2001). Miyata & Cowley (1999) demonstrated that intra-renal medullary administration of L-arginine was sufficient to eradicate salt induced hypertension in Dahl salt-sensitive rats, suggesting a critical NO deficiency may be present within the renal medulla. The ability of L-arginine to reduce salt sensitivity in Dahl salt sensitive rats likely stems from the presence of endogenous NOS-inhibitors that compete with L-arginine, such as ADMA (N^G, N^G-dimethyl-L-arginine) (Matsuoka et al, 1997). Since L-arginine is a substrate for NOS, increasing its concentration in the body would help

overcome this competitive inhibition and thus help increase the production of NO (Boger, 2004; Masuda et al, 2003; Leiper & Vallance, 1999).

1.6 Nitric Oxide Synthase

NOS is the enzyme that catalyzes the production of NO in the body. There are three different isoforms of NOS: eNOS or NOS III, iNOS or NOS II and nNOS or NOS I. In addition to the regulation of BP, NO influences many other physiological processes such as inflammatory responses, immune control, blood volume control and pressure natriuresis, and neurotransmission in the brain and peripheral nervous system (Kone, 2004; Lirk et al, 2002; Torreilles, 2001; Kubes, 2000; Person et al, 2000; Yun et al, 1997).

1.6.1 Inducible Nitric Oxide Synthase

The main roles of iNOS involve inflammatory responses and immune functions. iNOS, the gene of which is located on chromosome 17 of the human genome, is necessary for normal healing of the skin, removal of certain bacteria, viruses, fungi and parasites, and for T cell proliferation and leukocyte recruitment in immune responses (Kone, 2004; Lirk et al, 2002; Torreilles, 2001; Kubes, 2000).

iNOS has also been shown to be also expressed in the medullary region of the kidney where it appears to play a role in the development of salt sensitive hypertension (Rudd et al, 1999; Morrissey et al, 1994). Increasing its expression in the renal medulla causes a decrease in BP (Kurihara et al, 1998). In Dahl salt resistant rats, the

administration of selective iNOS inhibitors (e.g. aminoguanidine) caused an increase in BP when the rats were consuming a high salt (8% NaCl) diet (Tan et al, 2000; Rudd et al, 1999; Chen & Sanders, 1993). The chronic inhibition of iNOS with aminoguanidine in SD rats on a high salt diet produced an increase in BP, a decrease in urinary flow and increase in body weight due to water retention (Mattson et al, 1998). This data suggests that iNOS may play a role in maintaining a normal BP when on a high salt diet. Induction of iNOS production has the opposite effect of iNOS inhibition. Ishimitsu et al (1994) used interleukin-2 to increase the expression of iNOS and discovered that a decrease in BP was produced even when the rats consumed a high salt (4% NaCl) diet.

Cowley et al (1995) suggested that a reduction in renal medullary blood flow was partly responsible for the development of hypertension during NOS inhibition. L-NAME was administered directly into the medullary region causing an increased re-absorption of sodium and water and a rise in BP (Cowley et al, 1995). Since iNOS is the main form of NOS located in the renal medulla (Mattson et al, 1998), it was proposed that this isoform was at least partly responsible for the effect of NOS inhibitors administered to this region. It was suggested that the inhibition of iNOS produced a greater renal medullary re-absorption of salt and water, leading to a rise in ECF volume and BP. If the expression of iNOS was enhanced then a greater amount of salt and water would be excreted via the kidney and the BP would decrease. These findings suggest that iNOS has an important role in salt and water re-absorption/excretion and impairment of its normal functioning may contribute to salt sensitive hypertension.

1.6.2 Neuronal Nitric Oxide Synthase

nNOS, the gene of which is located on the 12th human chromosome, is widely expressed in the brain, peripheral nervous system, renal efferent arterioles and the macula densa (Boron & Boulpaep, 2003; Torreilles, 2001; Braam et al., 2000; Kubes, 2000; Rudd et al, 1999; Kurtz & Wagner, 1998; Yun et al, 1997). In the brain, it is thought to help regulate sympathetic output to the periphery (Krukoff, 1998). In neurons, NO is thought to act as a messenger molecule or neurotransmitter, aid in neuronal development and help regulate synaptic plasticity and gene expression (Yun et al, 1997). In the macula densa, it is thought to play a role in renin release and possibly tubuloglomerular feedback (TGF) (Braam et al, 2000; Braam, 1999; Rudd et al, 1999).

Tan et al (1999) described that salt resistant Dahl rats treated with the selective nNOS inhibitor 7-nitroindazole (7NI) exhibited a significant increase in BP when placed on a high salt diet. A decrease in nNOS could affect the BP in many ways, including an effect on the CNS to increase sympathetic activity or a direct effect on the macula densa to alter the release of renin by the kidney, or influence the TGF (Tandai-Hiruma et al, 2005; Tan et al, 1999).

While the nNOS system has been suggested to contribute to the regulation of renin secretion and synthesis (Braam et al, 2000; Braam, 1999; Beierwaltes, 1997), the exact role of nNOS in the process of renin secretion is a matter of controversy (Castrop et al, 2004; Ollerstam et al, 2001; Wagner et al, 2000). Several studies support the idea that nNOS is mandatory for the secretion of renin. Reviews by Braam (1999 & 2000), support the concept that nNOS system is linked to long term changes in the delivery of

sodium to the macula densa and salt excretion. It was suggested that increases in salt delivery to the macula densa promoted a decrease in nNOS activity and renin release (Braam, 1999). Since nNOS and renin levels change simultaneously it has been suggested that nNOS may be involved in both the production and secretion of renin. The above views were based on the results of Beierwaltes (1997) who showed that chronic inhibition of nNOS with 7NI in SD rats fed a low sodium (0.03% NaCl) diet resulted in decreased renal venous renin concentration and secretion. In contrast, rats fed a normal (0.2% NaCl) or low sodium diet under conditions of nNOS inhibition showed no changes in renin levels or production (Beierwaltes, 1997). These findings support the idea that nNOS may directly influence the production or excretion of renin.

Several studies suggest that NO, irrespective of which NOS isoform it is produced by, must be present for the stimulation of renin secretion (Castrop et al, 2004; Ollerstam et al, 2001; Wagner et al, 2000). Wagner et al (2000) suggested that NO derived from L-arginine by the actions of nNOS was not ultimately responsible for renin secretion. Evidence from the latter study showed that nNOS knockout mice did not have a reduced basal level of renal renin compared to controls (Wagner et al, 2000). However, during acute treatment with L-NAME, renin mRNA decreased in both nNOS knockout mice and their controls (Wagner et al, 2000). Ollerstam et al (2001) also reported that nNOS had acute, but not chronic effects, on renin secretion. SD rats were fed a low salt (0.012% NaCl), control (0.7% NaCl) or high salt (4% NaCl) diet and were treated with either a placebo or 7NI for an acute (4 day) or chronic (4 week) time period. Acute treatment with 7NI lead to elevated renin levels in rats fed low salt. No differences were observed

between the control or high salt diet rats in either the acute or chronic time periods and no differences were observed in the low salt diet rats in the chronic administration of 7NI. Castrop et al (2004) reported that the stimulation of renin secretion by loop diuretics was not markedly altered in either nNOS or eNOS knockout mice. This supports the idea that NO may be required for renin secretion, but renin secretion is unaffected by the specific NOS isoform producing the NO. To further support this idea, L-NAME, a non-specific inhibitor of NO, was injected into isolated kidneys along with exogenous NO (S-nitroso-N-acetyl-penicillamine) (Castrop et al, 2004). While L-NAME decreased the response of renin in the presence of loop diuretics, the addition of exogenous NO restored the normal response (Castrop et al, 2004). Thus, NO may simply have a permissive role in renin secretion.

The NO system also plays a role in TGF (Braam et al, 2000). NO appears to be important in the resetting of the TGF to lower sensitivity levels in the face of volume expansion (Brown et al, 2004). By resetting the TGF to lower sensitivity levels, more fluid is able to flow past the macula densa cells before the feedback loop is activated and decreases glomerular filtration rate (GFR). This increase in distal fluid delivery helps increase salt excretion and therefore helps re-establish salt balance to maintain the desired ECFV. Although it is unknown *exactly* which NOS isoform is responsible, nNOS is likely involved. nNOS, present in the cells of the macula densa, may be stimulated by an increase in delivery of fluid and sodium to the macula densa (Brown et al, 2004; Braam et al, 2000). Brown et al (2004) examined the effect of either L-NAME or 7NI in volume expanded SD rats and found that the effect on TGF was similar with both the specific and

non-specific NOS inhibitor. The study reported that in the presence of volume expansion and a nNOS inhibitor, there was an increase in the sensitivity and reactivity of the TGF system rather than the decrease in sensitivity seen in the absence of an NO inhibitor (Brown et al, 2004). This resulted in the TGF system being more easily activated (i.e. by a lower fluid flow past the macula densa) enabling it to produce a decrease in GFR. This increase in sensitivity would promote fluid retention and volume loading and suggests that NO (produced by nNOS activity) is necessary for the control of TGF during volume expansion (Brown et al, 2004).

The results of several studies suggest that it is the altered sensitivity or responsiveness of the TGF system that causes volume expansion to occur and BP to rise in animals that are developing hypertension (Persson et al, 2000). NOS was suggested to play an important role in keeping the sensitivity of the TGF constant, therefore helping to control BP (Brown et al, 2004; Braam et al, 2000; Braam, 1999). The non-selective NOS inhibitor L-NAME has been suggested to cause an increased TGF sensitivity and BP (Persson et al, 2000; Thorup et al, 1996). In order to determine if a specific isoform of NOS affects the TGF, the selective nNOS inhibitor 7NI was used to determine the specific contribution of nNOS (Persson et al, 2000; Thorup et al, 1996). Through the use of 7NI, it was suggested that nNOS was likely responsible in governing the sensitivity of the TGF system and the resulting changes in BP (Persson et al, 2000; Thorup et al, 1996).

Other studies suggest that nNOS is only vital in the regulation of the TGF in acute conditions (Ollerstam & Persson, 2002). Ollerstam et al (1997) looked at the effect of nNOS inhibition with 7NI over acute and chronic time frames in SD rats. Initially, the

TGF sensitivity was increased and there was a decrease in GFR, but after 1 week the effect of the nNOS inhibitor was lessened and the sensitivity of the TGF was returning toward normal levels. After 4 weeks of exposure to 7NI, the TGF sensitivity was similar to control rats not exposed to nNOS inhibitors. Ren et al (2001) also provided evidence for the short term influence of nNOS on TGF using nNOS knockout mice. nNOS knockout mice showed similar TGF sensitivity levels to their control wild type mice (Ren et al, 2001). Whereas, when the control mice were given 7NI to inhibit nNOS they showed an increased sensitivity of the TGF (Ren et al, 2001). These findings suggest that although nNOS plays an important role in resetting the TGF sensitivity, the influence may only be acute and temporary.

nNOS may also affect BP through its interaction with the sympathetic nervous system. As previously discussed, sympathetic nerve hyperactivity may contribute to salt-induced increases in BP. Spontaneously hypertensive rats (SHR) have been shown to have increased renal sympathetic nerve activity and increased sympathetic reactivity when compared to control normotensive rats (Li et al, 1997; Lundin et al 1984). A review by Krukoff (1998) suggested that NO produced via nNOS in the brain is involved in limiting the amount of sympathetic output reaching the periphery. By decreasing the sympathetic nerve output, nNOS may play a role in regulating the amount of sympathetic hyperactivity and therefore BP. Plochocka-Zulinska & Krukoff (1997) demonstrated an increase in nNOS gene expression in the brains of SHR's compared to Wistar-Kyoto normotensive rats at 14 weeks of age. They postulated that the increase in nNOS

expression was required to return the increased sympathetic hyperactivity back to normal levels thus lowering the BP (Plochocka-Zulinska & Krukoff, 1997).

Other studies emphasize the importance of brain nNOS. Togashi et al (1992) administered L-NAME into the CNS of Wistar rats and reported an increase in BP and sympathetic nerve activity. These results suggest that by eliminating the ability of nNOS to regulate the sympathetic outflow (via L-NAME), a resultant increase in sympathetic activity and BP occurred. Tandai-hiruma et al (2005) examined the effect of S-methyl-L-thiocitrulline (a selective nNOS inhibitor) on the renal sympathetic nerve activity (RSNA) in Dahl salt sensitive rats fed either a high salt (8% NaCl) or regular salt (0.4% NaCl) diet for 4 weeks. A significant increase in RSNA occurred in both groups but was significantly greater in the high salt versus the regular salt diet rats (Tandai-hiruma et al, 2005). In both the regular and high salt diet rats, the administration of L-arginine reversed the effects of the nNOS inhibitor (Tandai-hiruma et al, 2005). These studies show that nNOS plays an important role in controlling sympathetic nerve activity and therefore BP.

1.6.3 Endothelial Nitric Oxide Synthase

NO is produced from L-arginine by the actions of eNOS in the endothelial cells lining blood vessels. The eNOS gene, which is located on chromosome 7 in humans, provides an important contribution to blood vessel tone, systemic and renal hemodynamics, NaCl re-absorption in the renal tubules and endothelial mediated vasodilation (Kone, 2004; Ortiz et al, 2003; Torreilles, 2001; Braam et al., 2000).

A study by Van Vliet et al (2003) showed that eNOS knockout mice had higher 24 hour BP's when compared to the control C57 mice. Eliminating eNOS limits the body's ability to elicit vasodilation. BP can be increased by elevating vascular tone and decreasing fluid excretion, which will augment ECFV, peripheral resistance and BP. eNOS appears to be activated by vasoconstrictors such as angiotensin II as well as by shear stress on the blood vessels (Parzak et al, 2001). NO may act as a counterbalancing mechanism to ensure that excessive levels of vasoconstrictors do not result in the production of extreme elevations in BP (Parzak et al, 2001).

More specific roles of eNOS include regulation of renal vascular tone, including that of the afferent arterioles of the glomerulus (Parzak et al, 2001; Gabbai & Blantz, 1999: Kurtz & Wagner, 1998). In the absence of NO, with no counter balancing effect in place, angiotensin II causes unopposed renal vasoconstriction (Parzak et al, 2001). Constriction of the afferent arterioles limits the amount of fluid (salt and water) that can be filtered at the glomerulus and reduces the pressure within the peritubular capillaries. This promotes an increase in the re-absorption of the tubular fluid and a decrease in salt and water excretion as well as an increase in renin release. These alterations ultimately can increase ECFV and BP.

NO has been speculated to play a role in salt and water absorption in the tubules of the kidney including the proximal tubule, thick ascending limb and cortical collecting ducts (Herrera & Garvin, 2005; Ortiz et al, 2003; Ortiz et al, 2003a; Ortiz & Garvin, 2002). eNOS is responsible for the inhibition of salt and water re-absorption in the thick ascending loop of Henle (THALs), therefore suggesting it plays an important role in fluid

balance (Herrera & Garvin, 2005). In the presence of a high salt diet, the expression of eNOS in the THALs is increased allowing a greater amount of salt and water to be excreted (Herrera & Garvin, 2005; Ortiz et al, 2003a). This could help to reduce the total body fluid volume and BP. Ortiz et al (2003a) examined the effect of a high salt diet on the eNOS expression in the THALs of SD rats. After 7-10 days of consuming a high salt intake (1% NaCl added to the drinking water), eNOS expression in the THALs was increased about 3.9-fold over the regular salt rats (Ortiz et al, 2003a). It was noted that although the expression of eNOS increased, the level of NO produced in response to a 1mmol/L injection of L-arginine was the same in isolated THALs from both the regular salt and high salt diet mice (Ortiz et al, 2003a). To compensate for the lack of enhanced NO production in the high salt diet mice, it was suggested that the sensitivity of the THALS to NO was augmented in the presence of a high salt diet (Ortiz et al, 2003a). Another study that supports the contribution of eNOS to the regulation of salt and water uptake examined eNOS knockout mice and gene transfer of eNOS into the THALs (Ortiz et al, 2003). Gene transfer of eNOS caused an elevated production of NO and an inhibitory effect on salt re-absorption after about 7-10 days (Ortiz et al, 2003). These studies indicate that eNOS in the THALs plays an important role in fluid balance and possibly BP control.

As previously discussed, NO plays an important role in governing renin release. Shesely et al (1996) showed that, although the renal renin mRNA levels were lower, eNOS knockout mice had higher circulating renin levels than control C57 mice. In contrast, Beierwaltes et al (2002) reported that while the BP and HR were higher in

eNOS knockout mice than the control mice, the plasma renin levels were similar, suggesting that eNOS was not involved in controlling plasma renin levels (Beierwaltes et al, 2002). In the latter study, L-NAME caused an increase in BP and renal vascular resistance and a decrease in renal blood flow in C57 mice. However, no effect was seen in the eNOS knockout mice (Beierwaltes et al, 2002). L-NAME inhibited the increase in renin levels in response to decreased perfusion pressure in C57 mice (Beierwaltes et al, 2002). However, the observation that this response was maintained in eNOS knockout mice suggested that NO formed by a non-eNOS isoform was likely involved in controlling renin release. Consistent with the above view, Castrop et al (2004) also found no difference in the plasma renin concentration in eNOS knockout mice and their controls. Although these studies do not support a role of eNOS in mediating renin secretion, NO likely plays a permissive role allowing renin secretion to take place (Castrop et al, 2004; Kurtz and Wagner, 1998).

1.7 Rationale

Salt sensitivity affects a large portion of the developed world where daily salt intakes are higher than physiologically required. The modern North American diet is characterized by extremely high intake of sodium which consequently increases the risk of hypertension and cardiovascular disease. The NO system plays an important role in governing salt and water balance. An impaired ability of the body to produce NO may be an important contributor to salt induced hypertension in salt sensitive individuals. The contribution of each isoform of NOS to salt sensitivity is unclear. Several studies have

attempted to use specific NOS inhibitors to study the physiological effect of NO on BP and salt and water balance. Although these studies emphasize the importance of NO in governing these processes, the lack of specificity of NOS inhibitors limits definite conclusions as to the specific role that various NOS isoforms play in BP control and salt induced hypertension. In particular, the role of altered eNOS function in the production of salt induced hypertension remains unclear as specific eNOS inhibitors are not available. In view of this, we attempted to study the effects of salt on BP in eNOS knockout mice.

1.8 Objectives of the study

In order to investigate salt sensitivity in eNOS knockout mice, we first need to understand the characteristics of salt sensitivity in the control C57 strain of mice. Salt sensitive hypertension has been shown to develop over both short and long term time scales. To assess the time course of salt sensitivity, telemetry experiments were conducted in both C57 mice and eNOS knockout mice.

The main objectives of this study were to determine the effect of a high salt diet on the BP, HR, pulse pressure and activity of C57 mice and eNOS knockout mouse. In order for these objectives to be achieved, implanted BP telemeters were used to provide a detailed description of the time course of physiological changes in response to an increase in dietary salt.

1.9 Hypotheses

Several hypotheses were assessed in this thesis project. The first hypothesis is that the addition of a high salt diet will cause an increase in BP in C57 mice. Several studies have suggested varying levels of salt sensitive in C57 mice (Ni et al, 2003; Gros et al, 2002; Sugiyama et al, 2001; Carlson & Wyss, 2000). Our goal is to determine the exact level of salt sensitivity exhibited in C57 mice being fed a high salt (8% NaCl) diet as well as its time course. The second hypothesis of this thesis is that eNOS knockout mice will demonstrate a larger BP response to a high salt diet than seen in the C57 mice. An enhanced salt sensitivity in eNOS knockout mice will suggest that eNOS plays a role in the body's normal response to an increase in salt intake.

Chapter 2: Methodology

2.1 Materials and Methods:

2.1.1 Experimental animals

The experiments were approved by the Memorial University of Newfoundland Animal Care Committee. C57 control mice (eNOS +/+, C57Bl/6J, stock no. 000664) and eNOS-deficient mice (eNOS -/-, B6.129P2-NOS^{tm1Unc}, stock no. 002684) were bred locally or purchased from Jackson Laboratories. The functionality of the eNOS gene was removed by using a targeting construct (pENOSX) to alter the calmodulin binding site by placing a premature stop codon along the translation command (Shesely et al, 1996). The eNOS (-/-) mice showed an absence of the eNOS protein when immunohistochemical staining was performed (Shesely et al, 1996). The eNOS knockout mice also had decreased body weights compared to their control but no significant differences were seen between the heart, aorta, liver, kidney, brain, spleen or adrenal glands of the two strains (Shesely et al, 1996). Jackson laboratories cross bred the eNOS (-/-) strain produced by Shesley et al (1996) to the C57Bl/6J strain eight times, which should result in a 99.6% homogeneity in the genetic background (Van Vliet et al, 2003).

Males and females of both strains were housed at the Memorial University Animal Care unit from 5 weeks of age. Mice were allowed 3 weeks to adapt to the environment during which time they were ear tagged. At 8 weeks of age the animals were mated with animals of the same strain (eNOS(-/-) x eNOS(-/-), eNOS(+/+) x eNOS(+/+)). The offspring were weaned at 4 weeks of age and ear tagged at

approximately 5-7 weeks of age. Mice used in the experiments underwent telemeter implantation between 11 and 18 weeks of age. The mice were housed separately about 2 weeks prior to implantation.

2.1.2 General husbandry

Both strains of mice were housed in the same room (breeding room). Following the implantation of a telemeter, animals involved in an experiment were housed in a separate recording room. The temperature of the breeding room was 25 ±1 °C to help assist with breeding, whereas the temperature of the recording room was slightly lower (23 ±0.5 °C). Both rooms were maintained on a 12hr light-dark cycle with lights turning on at approximately 7:00am and off at 7:00pm. All mice were fed a standard feed containing 0.7% NaCl, 1.0% calcium, 0.91% potassium and 0.75% phosphorus (Lab diet 5P00, Prolab). Experimental animals, chosen at random to ingest a high salt diet, were fed the same feed with extra salt added to bring the level to 8% NaCl. This level of salt has previously been shown to cause changes in blood pressure in mice (Gros et al, 2002; Carlson & Wyss, 2000). All animals were provided with new cages on a weekly basis and no more than 5 mice (usually 2-3 mice) were housed per cage. Each cage contained standard rodent bedding material, a plastic tube and a sheet of paper towel to be used for bedding. Food and water intake was calculated from the change in the food and water weights from week to week. Mice were checked periodically throughout the week to ensure comfort and well being.

2.2 Experimental Protocol

Males 11-18 weeks of age were weighed and given a single injection of approximately 0.03ml (0.03ml per 25-30g mouse) of ketamine/xylazine (90/10 mg/ml) intramuscularly. The necks of anesthetized animals were shaved and the remaining hair was removed with a depilatory cream (Nair for Men, Cream Hair Remover for Body, Carter-Horner Corp, Canada) that was left on for approximately 4 minutes. Lubrication (lubricating jelly, Healthcare Plus, Jedmon Products Limited, Canada) was placed over the eyes of each mouse to ensure that they did not experience unnecessary drying. The mice were placed on a heated pad (37°C) and their forelimbs were secured to the pad with tape. The tails were also secured on the heating pad to help maintain their body temperature. The mice were given approximately 0.03ml of atropine (0.5mg/ml) subcutaneously to dry up respiratory secretions and to attenuate the xylazine induced drop in HR, and approximately a 0.01ml dose of penicillin (300 000 mg/ml) intramuscularly to decrease the risk of infection. If necessary, the anesthetic was topped up with approximately 0.01ml of ketamine (33mg/ml) to allow the procedure to be completed.

Once the depilatory cream was removed and the pre-medications administered, the mice were covered in sterile gauze to help maintain their body heat and maintain a clean environment. An incision was made from the mandible to the sternum, the submaxillary glands were separated and the left carotid artery exposed using a combination of fine and gross dissection. The carotid artery was separated from the vagus nerve and three silk ligatures (6.0) were passed under it. The caudal ligature (5.0

or 6.0 silk) was not tied but used to retract the vessel to stop blood flow. The second ligature was tied loosely and placed near the caudal ligature. The rostral ligature was tied tightly twice to permanently occlude blood flow.

Using gentle tension, the artery was occluded and prepared for the insertion of the telemeter's catheter tip. The catheter tip (with the protective cap removed) was grasped using specialized forceps and the artery was incised with a bent # 27 or 25 gauge needle. Once the catheter tip was inserted, the caudal ligature was loosened and the loosely tied ligature was secured around the artery over the catheter. The catheter ridge (an indentation in the catheter tubing) was advanced until the ridge was approximately 1 mm caudal to the bifurcation of the carotid artery. This location was found to be appropriate to position the catheter tip just past the junction of the carotid artery with the aorta. The rostral ligature was then tied around the catheter to further secure it. Despite the potential limitations of occluding a carotid artery, the carotid implant technique has been widely used because it is a successful technique and generally less stressful on the animal compared to the abdominal implant technique (Carlson & Wyss, 2000).

Using large hemostats, a subcutaneous pocket was made in the right side of the mouse from the shoulder down to the hip. Sterile saline was used to lubricate the pocket to help facilitate the insertion of the telemeter body. Once the telemeter body was positioned in the subcutaneous pocket, the incision was closed with suture (6.0 chromic gut). Following surgery, each mouse was placed in their original cage that was positioned on a heating pad to help mice maintain their body temperature and reduce thermogenic energy expenditure. The mice remained on the heating pad for at least 48

hours following surgery to ensure optimal recovery. After approximately 72 hours the mice were moved to the recording room where they were placed on the telemeter receiver plates. For each recording period, the telemeters were turned on at least 12 hours prior to the data collection. After 10 to 14 days of recovery the animals telemeters were turned on to collect the first 24 hours of control data (Thursday AM- Friday AM). Four days later the telemeters were again turned on and allowed to record for 3 more days before the experimental mice were switched to 8% salt feed (Tuesday AM- Friday AM). Once the change in feed occurred, data was recorded for another 6 days before the telemeters were switched off (Saturday AM- Friday AM). Following this, the telemeters recorded data for 36 hours per week for another 5 weeks (Wednesday AM- Friday AM). Following the last day of recording, the animals were anesthetized using ketamine/xylazine (90/10 mg/ml), and the telemeters were removed.

2.2.1 Telemetry

All telemeter equipment was purchased from D.S.I. The mice were implanted with D.S.I model TA11PA-20 telemeters. Data was collected using model RLA 1020 receiver plates on a 20 channel data exchange matrix with Dataquest ART Silver 2.1 or Gold 2.3 acquisition system. The data was recorded at 500 Hz for 3 seconds in 30 second intervals. Every 3 second recording period provided an average BP, HR, pulse pressure and activity reading. The activity signal of the telemetry system was counted over each 30 second sampling period (Van Vliet et al, 2003). The percentage of time spent active was calculated as the number of sample periods with activity counts

greater than zero. The level of activity was calculated as the mean of log (activity) when activity >0.

2.2.2 Catheter Tip Placement

Prior to the removal of the catheter from the carotid artery, but after the telemeter body was removed from the subcutaneous pocket, the location of the catheter tip in the aorta was determined. The chest cavity was opened and the aorta exposed. A ruler was placed near the aorta so that the distance of the tip from the junction of the carotid artery and aorta could be calculated.

2.2.3 Tail and Blood Samples

Once the telemeters were removed from each mouse a blood sample and a segment of the tail were taken. The blood was centrifuged and the plasma was removed. The samples were clearly labeled and stored in the freezer.

2.2.4 Calibration of Telemeters

Telemeters were calibrated both before and after implantation. The pressure readings were taken at atmospheric pressure at room temperature and atmospheric pressure and +200 mmHg at 37° C. Two recordings were taken at each pressure and temperature. The averages of the recordings taken at 37° and atmospheric pressure both before and after implant were used to determine the offset value which was used to correct the recorded data. This offset value was inserted into the data analysis program

HD stats, an Excel spreadsheet developed by the Van Vliet laboratory for the routine analysis of 24 hour telemeter data. The program then automatically applied the offset value to the recorded data.

2.2.5 Aorta Removal and Sheath Examination

During the course of the experiment it was noticed that one reason why telemetry signals occasionally deteriorated with time was the development of a reactive growth over the catheter tip. To determine the extent of the growth, upon removal of the catheter and telemeter the carotid artery and aorta were removed. A cut was made along the length of the aortic arch. The aorta was then opened so that the junction of the two blood vessels was exposed allowing any growth that may have been present to be seen. Only the data from mice that showed a catheter tip clear of growth and a 24h pulse pressure greater than 20 mmHg were used in this study.

2.3 Statistical Analysis

Daily telemetry data was inserted in the data analysis program HD stats, an Excel spreadsheet developed by the Van Vliet laboratory. From this program, information was provided on 24 hr and 12 hr means, standard deviations and ranges for MAP, pulse pressure, HR and activity. Minitab 13 (statistical program) was used to test for significant differences and relationships between MAP, HR, pulse pressure and activity using Analysis of Variance (ANOVA). A general linear model was used to test for significant effects of diet (control or high salt), time and genotype. A Dunnet's test was

used to compare data at individual time points to the baseline control day 4 (the last day of the control period). A Tukey's test was used to determine differences between groups during each phase. An ANOVA was used to compare both the absolute values on day 4 of the control period and the food and water intakes over the course of the experiment.

Chapter 3: Results

The first part of the Results section will describe the hemodynamic values recorded during the control period in the C57 and eNOS knockout mice. Following this, the response to a high salt diet will be discussed within the individual strains. Finally, differences between the strains will be discussed. The initial control period included 4 days on a normal diet of 0.7% salt after which the diet was increased to 8% salt for 6 weeks for the experimental animals while the control animals remained at normal salt.

For ease of understanding, the group of C57 mice on the regular salt diet will be called C57-RS, the group of C57 mice on the high salt diet will be called C57-HS, the group of eNOS knockout mice on the regular salt diet will be called eNOS-KO-RS and the group of eNOS-KO mice on the high salt diet will be referred to as eNOS-KO-HS.

3.1 Experimental Numbers

In total, 28 mice were used to conduct statistical analysis. Both the C57-RS and C57-HS groups contained 7 mice, whereas the eNOS-KO-RS group contained 8 mice and the eNOS-KO-HS group contained 6 mice. Due to a mistake in the recording schedule, data for 2 mice from the eNOS-KO-HS group and one mouse from the eNOS-KO-RS group were not available for the first and second day of the high salt diet. As well, due to a clot at the catheter tip and loss of signal, data for one eNOS-KO-RS mouse is missing on the final day of the experiment and data for one C57-RS mouse is missing on the final two days of the experiment. The fact that 54 mice started the experiment and only 28

completed the full protocol demonstrates the difficulty maintaining a good signal for 8 weeks of recording.

3.2 Control Period Hemodynamics

Control period hemodynamics are summarized in tables 2, 3 and 4. With one exception, no differences were observed in the hemodynamic parameters of the regular and high salt groups during the control period (table 3). There were no major differences in HR, pulse pressure or activity levels between the two strains (table 4) or between the experimental groups in each strain (table 2 and 3).

When the data of the two experimental groups within each strain were combined (table 4) significant differences were observed in the 24h MAP, 12h light period and 12h dark period MAP, as well as the MAP range over 24h and the daily minimum and maximum MAP (table 4). In each case, the values in eNOS-KO mice were greater than those of the C57 control mice.

3.3 Response to a High Salt Diet

3.3.1 Food and Water Intakes

Water intake data are summarized in table 5. Water intakes of both C57 and eNOS knockout mice were significantly increased when fed a high salt diet. eNOS-KO-HS showed a greater water intake in the experimental period relative to the corresponding C57 group. No significant differences were seen in the water intake of the C57-RS or the eNOS-KO-RS mice over the course of the experiment. As well, no

Table 2: Comparison of control period hemodynamics in the four experimental groups. The mean (± SE) for the absolute values of each variable is reported.

	C57 Mice		eNOS Mice	
	Control Diet (n=7)	Experimental Diet (n=7)	Control Diet (n=8)	Experimental Diet (n=6)
Mean Arterial Pressure (mmHg)				
24h MAP	108 ± 1	105 ± 1	123 ± 2	121 ± 2
Light Period MAP	100 ± 2	97 ± 1	113 ± 2	113 ± 1
Dark Period MAP	116 ± 1	112 ± 1	131 ± 3	130 ± 3
Range of 24h MAP	54 ± 2	54 ± 2	79 ± 8	71 ± 5
24h Minimum MAP	83 ± 1	81 ± 1	89 ± 1	90 ± 2
24h Maximum MAP	137 ± 1	135 ± 1	168 ± 7	161 ± 3
Heart Rate (bpm)				
24h Heart Rate	578 ± 10	542 ± 11	547 ± 9	571 ± 10
Light Period Heart Rate	551 ± 15	499 ± 11*	508 ± 9	531 ± 9
Dark Period Heart Rate	605 ± 9	585 ± 18	586 ± 11	612 ± 14
Pulse Pressure (mmHg)				
24h Pulse Pressure	30 ± 1	31 ± 2	33 ± 2	34 ± 2.4
Light Period Pulse Pressure	29 ± 1	31 ± 2	32 ± 2	33 ± 2
Dark period Pulse Pressure	30 ± 1	31 ± 2	33 ± 2	35 ± 3
Active Time (% time active)				
24h Active Time	37 ± 2	36 ± 3	43 ± 5	40 ± 1.5
Light Period Active Time	19 ± 2	19 ± 2	19 ± 1	22 ± 3
Dark Period Active Time	56 ± 3	53 ± 6	54 ±3	60 ± 2
Intensity of Activity **				
24h Intensity of Activity	1.2 ± 0.1	1.2 ± 0.0	1.2 ± 0.1	1.2 ± 0.1
Light Period Intensity of Activity	0.98 ± 0.0	0.96 ± 0.1	0.94 ± 0.0	1.0 ± 0.0
Dark Period Intensity of Activity	1.24 ± 0.1	1.25 ± 0.1	1.3 ± 0.0	1.3 ± 0.1

*$p<0.05$ between diet groups, within strain (see table 3).

** -Dimensionless (no units) because it represents the mean of log (activity) when activity >0.

Table 3: P-values for the analysis of variance for the control period hemodynamics in C57 and eNOS knockout mice. Statistical analysis was conducted using a general linear model analysis of variance to test for differences in the experimental groups within the strains of mice during the control period.

	C57-RS vs. C57-HS	C57-HS vs. eNOS-KO-HS	C57-RS vs. eNOS-KO-RS	eNOS-KO-RS vs. eNOS-KO-HS
Mean Arterial Pressure				
24h Mean Arterial Pressure	0.4347	**<0.0001**	**<0.0001**	0.7840
Light Period Mean Arterial Pressure	0.5227	**<0.0001**	**<0.0001**	0.9877
Dark Period Mean Arterial Pressure	0.8474	**<0.0001**	**<0.0001**	0.9896
Range of Mean Arterial Pressure	0.5979	0.0732	**0.0028**	0.9998
24h Minimum Arterial Pressure	0.2948	0.0837	**<0.0001**	0.6733
24h Maximum Arterial Pressure	0.9990	**0.0007**	**0.0002**	0.9732
Heart Rate				
24h Heart Rate	0.0543	0.1686	0.0974	0.2760
Light Period Heart Rate	**0.0080**	0.1749	**0.0256**	0.3990
Dark Period Heart Rate	0.6462	0.4536	0.6573	0.4590
Pulse Pressure				
24h Pulse Pressure	0.9162	0.7852	0.6420	0.9600
Light Period Pulse Pressure	0.8269	0.9505	0.5411	0.9996
Dark period Pulse Pressure	0.9746	0.5671	0.7582	0.8460
Active Time				
24h Active Time	0.9882	0.7011	0.6131	0.9809
Light Period Active Time	1.0000	0.4870	0.9973	0.6010
Dark Period Active Time	0.9488	0.5668	0.9608	0.5817
Intensity of Activity				
24h Intensity of Activity	1.0000	0.9586	0.9816	0.9994
Light Period Intensity of Activity	0.9643	0.7644	0.8356	0.5471
Dark Period Intensity of Activity	0.9986	0.9877	0.8891	0.9972

Table 4: Comparison of control period hemodynamics in C57 and eNOS knockout mice. Analysis of variance revealed only one significant difference between control and experimental groups during the control period (table 2) so the groups were combined as is shown below.

	C57 Mice (n=14)	eNOS Mice (n=14)
Mean Arterial Pressure (mmHg)		
24h MAP	106 ± 0.7	123 ± 1.3 *
Light Period MAP	98 ± 1.2	113 ± 1.0 *
Dark Period MAP	114 ± 0.9	131 ± 2.0 *
Range of MAP	54 ± 1.2	76 ± 4.5 *
24h Minimum MAP	82 ± 0.7	89 ± 1.2 *
24h Maximum MAP	136 ± 0.9	165 ± 4.2 *
Heart Rate (bpm)		
24h Heart Rate	560 ± 8.6	557 ± 7.2
Light Period Heart Rate	525 ± 11	518 ± 7
Dark Period Heart Rate	595 ± 10	597 ± 9
Pulse Pressure (mmHg)		
24h Pulse Pressure	30 ± 1.0	33 ± 1.5
Light Period Pulse Pressure	30 ± 1	33 ± 2
Dark period Pulse Pressure	31 ± 1	34 ± 2
Active Time (% time active)		
24h Active Time	37 ± 1.6	42 ± 2.7
Light Period Active Time	19 ± 1	20 ± 1
Dark Period Active Time	55 ± 3	56 ± 2
Intensity of Activity **		
24h Intensity of Activity	1.2 ± 0.0	1.2 ± 0.0
Light Period Intensity of Activity	0.97 ± 0.0	0.97 ± 0.0
Dark Period Intensity of Activity	1.2 ± 0.0	1.3 ± 0.0

*-$p<0.05$ between groups, unpaired student's T-test.

** -Dimensionless (no units) because it represents the mean of log (activity) when activity >0.

Table 5: Comparison of daily water intake values in C57 and eNOS knockout mice. Average water intakes are reported in g/kg body weight/d. Statistical analysis was conducted using an analysis of variance general linear model.

	C57 Mice		eNOS Knockout Mice	
	Control Group	Experimental Group	Control Group	Experimental Group
Control diet	183 ± 15.5	185 ± 24.3	205 ± 10.0	214 ± 9.0
6 wk Experimental period	160 ± 9.8	540 ± 33.1 *	195 ± 9.4	814 ± 48.9 *

*-$p<0.05$ within strain difference between control and experimental *period*
-$p<0.05$ within strain difference between the control and experimental *group*
-$p<0.05$ between strain difference

difference was seen in the water intake of the regular and high salt groups during the control period.

Food intake data are summarized in table 6. Within the eNOS-KO strain, food consumption was significantly ($p<0.001$) greater in the eNOS-KO-HS mice versus the eNOS-KO-RS mice in the experimental period. The food intake of the eNOS-KO-HS mice was also greater than that of the corresponding C57 group. The food intake of the C57-RS, C57-HS and eNOS-KO-RS did not change between the control and experimental periods. As well, no difference was observed in the food intake during the control period between the control or experimental diet mice in either strain.

3.3.2 Implantation Data

Data concerning the age and body weight of animals at the time of telemeter implantation are summarized in table 7. The average age of C57 and eNOS knockout mice at telemeter implantation was about 14 wks. The average body weight at the time of implantation was significantly lower in eNOS knockout mice. This was expected because the body weight of knockout mice is generally lower than that of the control strain (Van Vliet et al, 2003).

3.4 Time Course of Responses to Dietary Salt in C57 Mice

Statistical analysis was conducted using Dunnet's test to determine at what time points significant differences in hemodynamic values occurred relative to the last day of

Table 6: Comparison of daily food intake values in C57 and eNOS knockout mice. Average intakes are reported in g/kg body weight/d. Statistical analysis was conducted using an analysis of variance general linear model.

	C57 Mice		eNOS Knockout Mice	
	Control Group	Experimental Group	Control Group	Experimental Group
Control Diet	148 ± 7.2	141 ± 6.9	152 ± 7.4	159 ± 5.7
6 wk Experimental period	136 ± 6.4	140 ± 7.0	139 ± 4.8	183 ± 11.2

-$p<0.05$ within strain difference between the control and experimental group
-$p<0.05$ between strain difference

Table 7: Implant data for C57 and eNOS knockout mice.

	C57 Mice	eNOS Knockout Mice
Average Implant Age (wks)	13.6 ± 0.5	14.3 ± 0.4
Implant Age Range (wks)	11-18	13-17
Average Implant Weight (g)	26.9 ± 0.6	24.4 ± 0.4 *
Implant Weight Range (g)	24-30	24-27

*-$p<0.05$ between strains, Students t-test.

the control period. No significant differences were observed within the control period.

3.4.1 Mean Arterial Pressure

Mean arterial pressure data is summarized in figures 1, 2, 3 and 4. C57 mice experienced a significant rise in 24h MAP when consuming a high salt diet. The salt induced rise in BP appeared to follow a two part time course (figure 1). The first part consisted of a significant initial transient rise in BP seen immediately following initiation of the high salt diet. The second phase consisted of a slow continual rise in BP which reached significance at the end of the experiment. C57-RS mice did not exhibit a change in MAP from control levels.

The effect of salt on MAP was observed in both the light and dark phases of the day (figure 1). The light phase showed a biphasic response with statistical significance seen only at the start of week 1 and during week 6. In the dark phase the increase of MAP was more sustained with significance seen during week 1, 3, 4 and 6 on the high salt diet. By week 5 on the high salt diet most of the increase in 24h MAP appeared to occur during the dark phase of the day (figure 1). A similar tendency was apparent in the circadian variation in MAP (figures 2 & 3).

Although the Dunnett's test did not reveal any significant changes in the range of the MAP or the daily minimum or maximum MAP, a tendency was observed for the range and the daily maximum to increase initially when the C57 mice were placed on a high salt diet (figure 4).

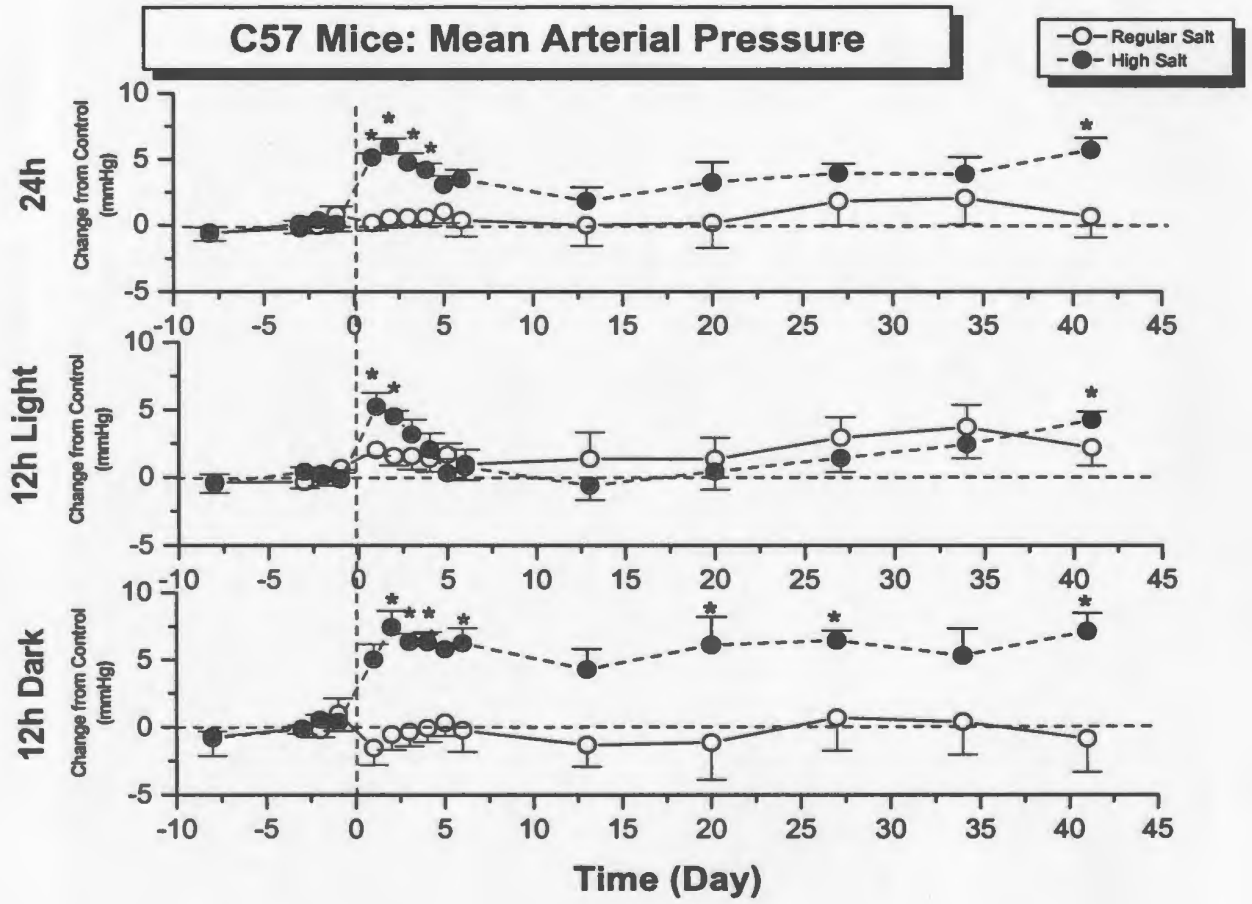

Figure 1: The effect of a high salt diet on mean arterial pressure (MAP) in control (C57) mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h MAP, the middle graph shows the effect on MAP during the 12h light phase of the day and the bottom graph shows the effect on MAP during the 12h dark phase of the day. Significant differences from the final day of the control period based on Dunnet's test are indicated by '*' located above the data point.

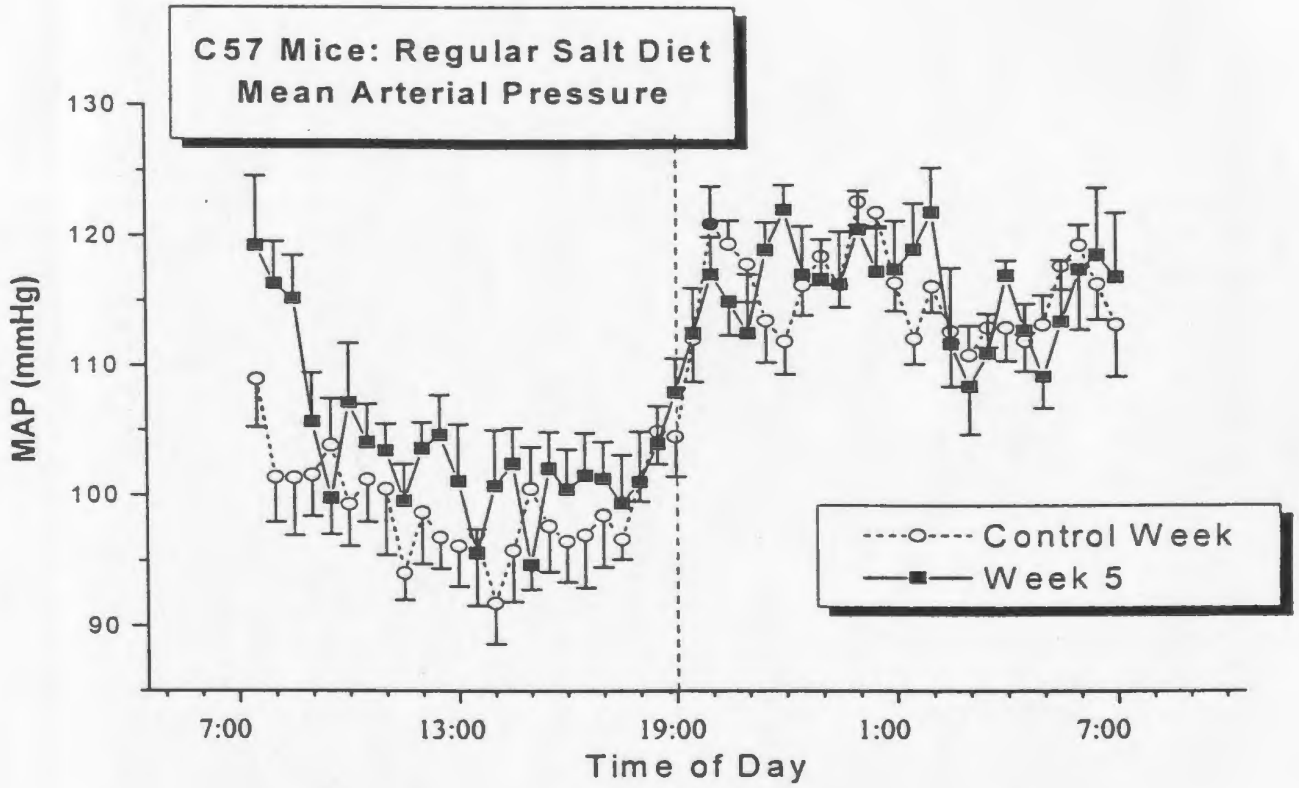

Figure 2: Comparison of circadian rhythm during the control week and during experimental week 5 for control (C57) mice fed the regular salt diet. The mean arterial pressures (MAP) shown here are half hour averages over a 24h period. The first half of the time scale represents the light period of the day and the second half, to the right of the vertical line, represents the dark phase of the day.

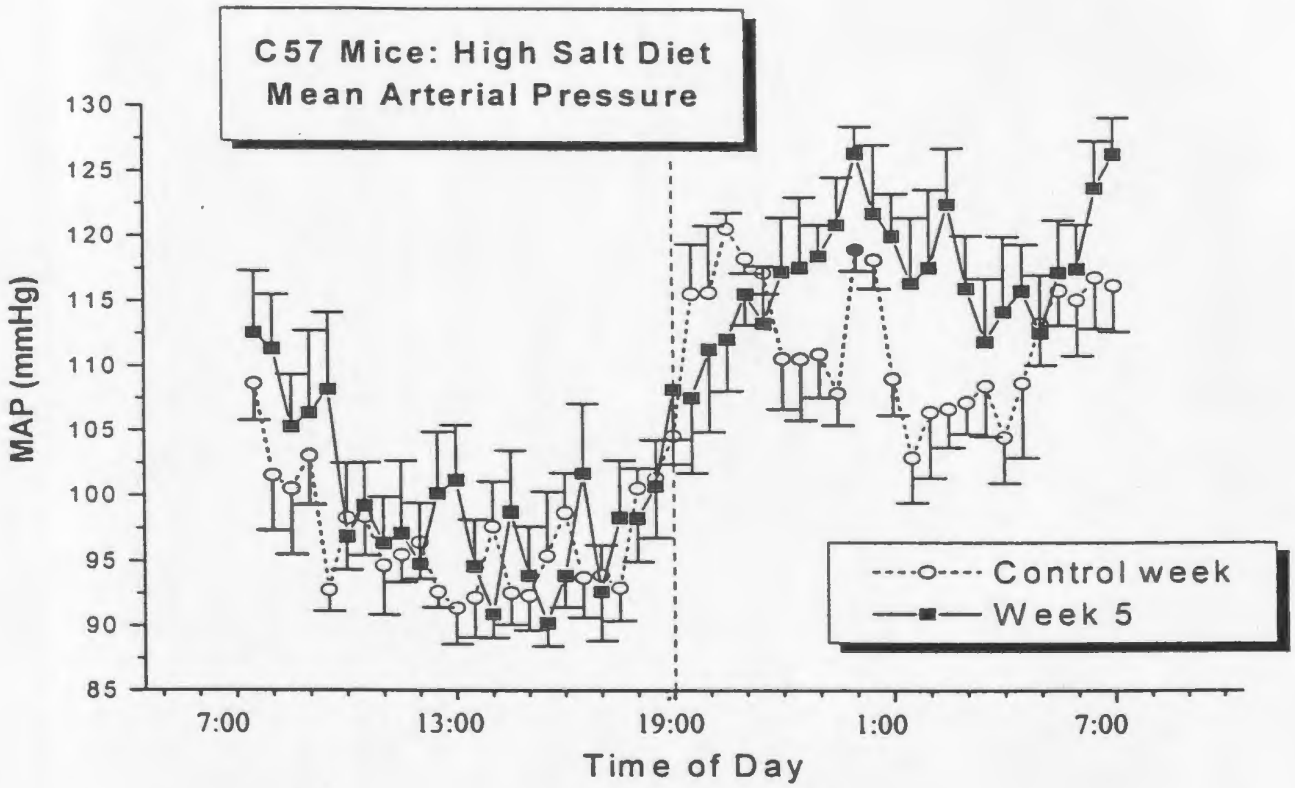

Figure 3: Comparison of circadian rhythm during the control week and during experimental week 5 for control (C57) mice fed the high salt diet. The mean arterial pressures (MAP) shown here are half hour averages over a 24h period. The first half of the time scale represents the light period of the day and the second half, to the right of the vertical line, represents the dark phase of the day.

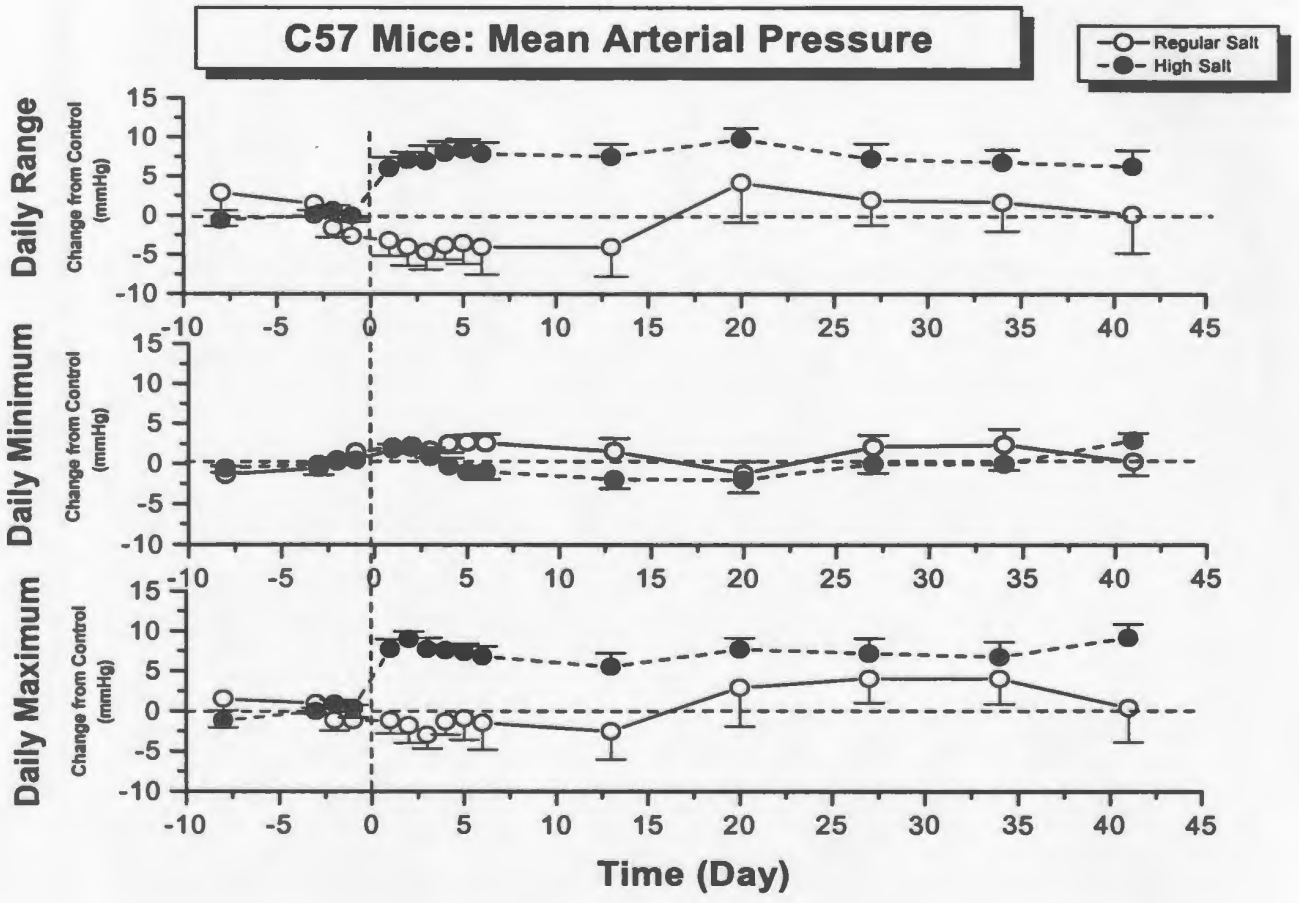

Figure 4: The effect of a high salt diet on the 24h range, minimum and maximum mean arterial pressure (MAP) in control (C57) mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h range of the MAP, the middle graph shows the effect on the daily minimum MAP and the bottom graph shows the effect on the daily maximum MAP. A Dunnet's test indicated no significant differences from the final day of the control period were present.

3.4.2 Heart Rate

HR data are summarized in figure 5. In general, the HR tended to decrease over the course of the experiment with greater effects tending to be seen in the C57-RS group (figure 5). The decrease in 24h HR was significant during weeks 3, 4 and 6 on regular salt (figure 5). C57-HS mice showed a tendency towards a small (not significant) increase in HR during the first few days on a high salt diet followed by a gradual decrease (also not significant) until week 6 (figure 5). This initial tendency for HR to increase corresponds with an initial increase in activity at the onset of the high salt diet (see below).

3.4.3 Pulse Pressure

Pulse pressure data is summarized in figure 6. The pulse pressure of the C57-HS mice increased gradually during the course of the experiment (figure 6). A significant increase was observed during the final week of the high salt diet. Significant increases in pulse pressure were also observed during week 5 and 6 of the high salt diet in the dark phase of the day whereas no significant changes were observed in the light phase of the day. In C57-RS mice there was a downward trend in pulse pressure but no significant changes were seen in either the 24h, light or dark phase data (figure 6).

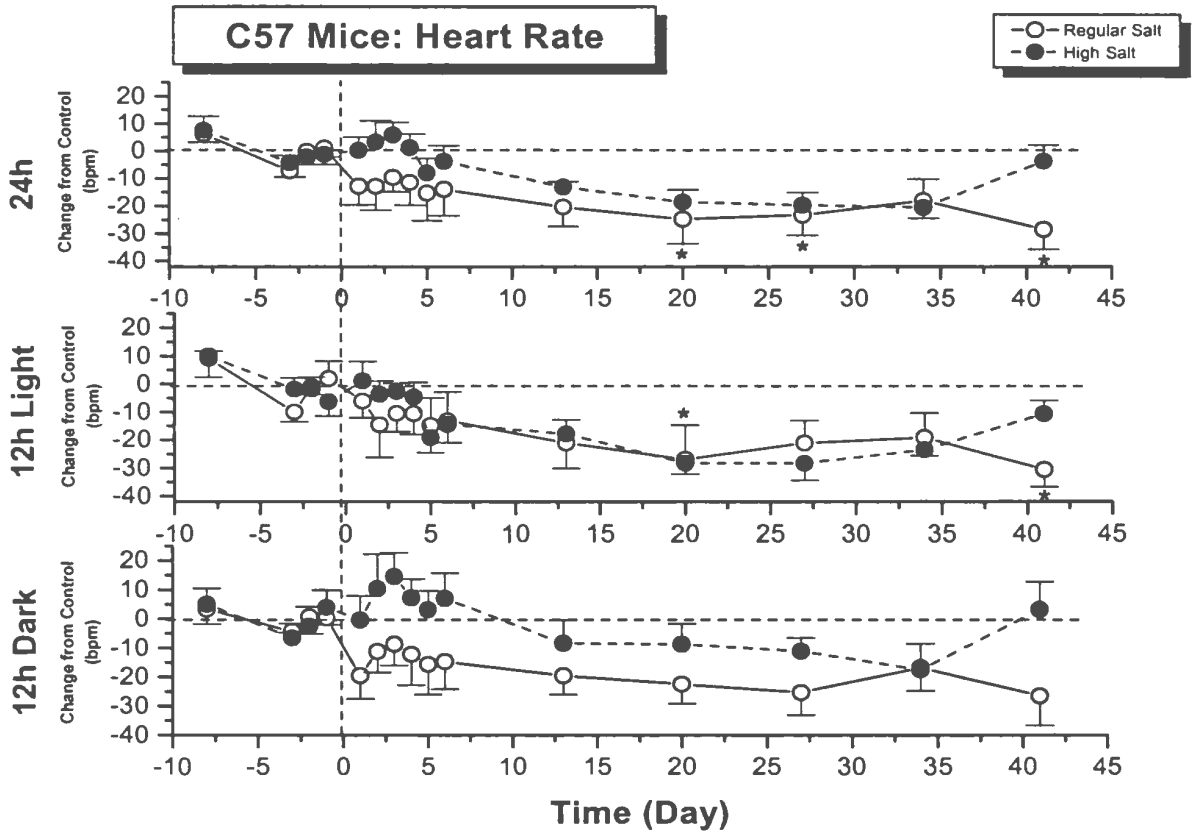

Figure 5: The effect of a high salt diet on the heart rate (HR) in control (C57) mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h HR, the middle graph shows the effect on HR during the 12h light phase of the day and the bottom graph shows the effect on HR during the 12h dark phase of the day. Significant differences from the final day of the control period based on Dunnet's test are indicated by '*' located adjacent to the data point.

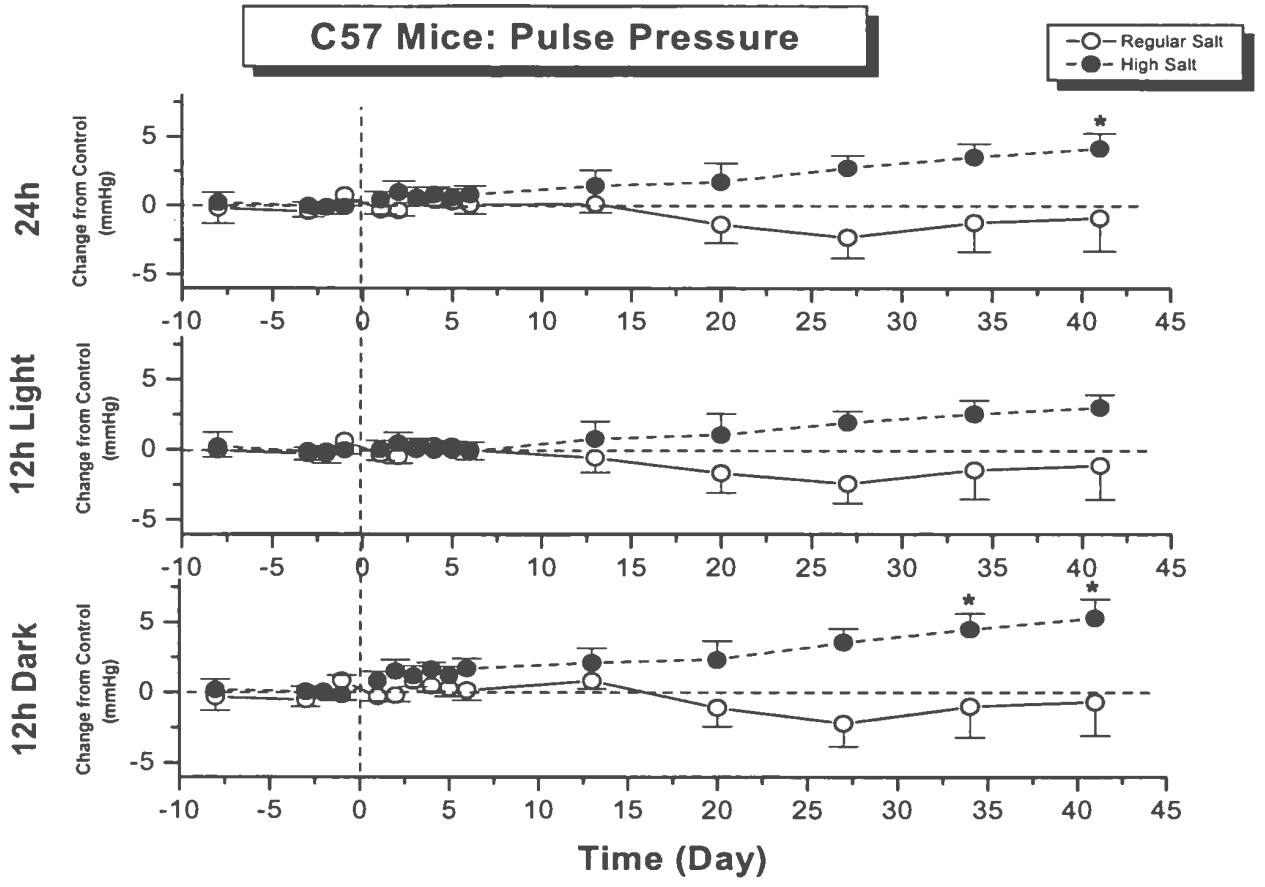

Figure 6: The effect of a high salt diet on the pulse pressure in control (C57) mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h pulse pressure, the middle graph shows the effect on pulse pressure during the 12h light phase of the day and the bottom graph shows the effect on pulse pressure during the 12h dark phase of the day. Significant differences from the final day of the control period based on Dunnet's test are indicated by '*' located above the data point.

3.4.4 Activity

Activity data is summarized in figures 7 and 8. No changes from the control values were observed in either the C57-RS or C57-HS mice in the 24hr active time or 24h intensity of activity (figures 7 & 8). In the C57-HS mice, active time and the intensity of activity tended to increase, similar to the initial increase seen in HR. However, this tendency only reached significance in the case of the active time on day 1 of salt exposure in the light phase (figure 7).

3.5 Time Course of Responses to Dietary Salt in eNOS Knockout Mice

As with the C57 mice, statistical analysis was conducted using Dunnet's test to determine at what time points significant differences occurred relative to the last day of the control period. No significant differences occurred within the control period.

3.5.1 Mean Arterial Pressure

The MAP data is summarized in figures 9, 10, 11 and 12. eNOS knockout mice exhibited a significant rise in MAP when consuming a high salt diet (figure 9). The 24h MAP was significantly increased throughout the entire experimental period and did not exhibit the pronounced biphasic time course seen in the C57 mice (figure 1). The eNOS-KO-RS mice did not show any significant changes in MAP from the control level.

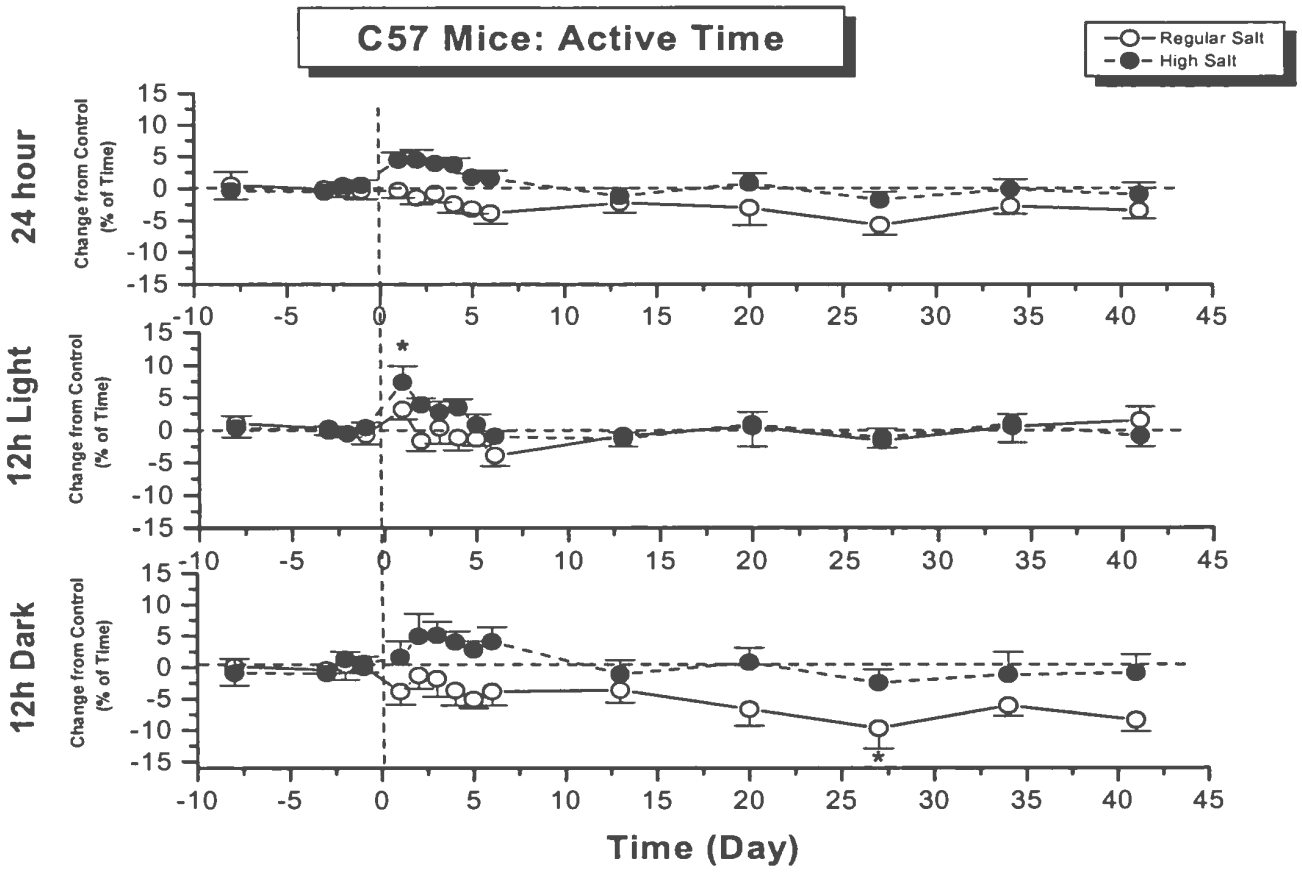

Figure 7: The effect of a high salt diet on the active time (percent of time spent active) in control (C57) mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h active time, the middle graph shows the effect on active time during the 12h light phase of the day and the bottom graph shows the effect on active time during the 12h dark phase of the day. Significant differences from the final day of the control period based on Dunnet's test are indicated by '*' located above the data point.

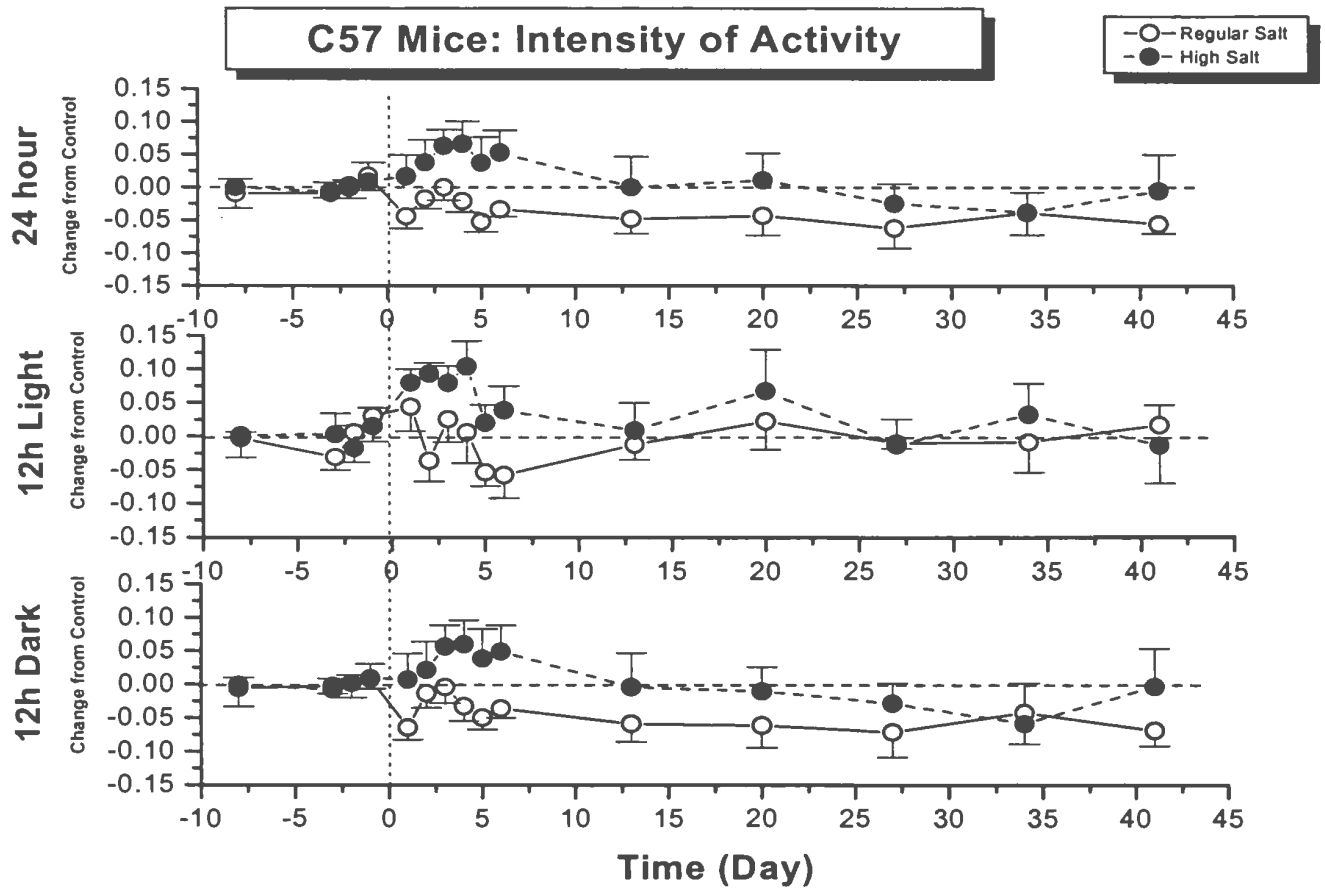

Figure 8: The effect of a high salt diet on the intensity of activity in control (C57) mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h intensity of activity, the middle graph shows the effect on intensity of activity during the 12h light phase of the day and the bottom graph shows the effect on intensity of activity during the 12h dark phase of the day. A Dunnet's test indicated no significant differences were present. The plotted values are dimensionless (have no units) because it represents the intensity of activity as the mean of log (activity) when activity >0.

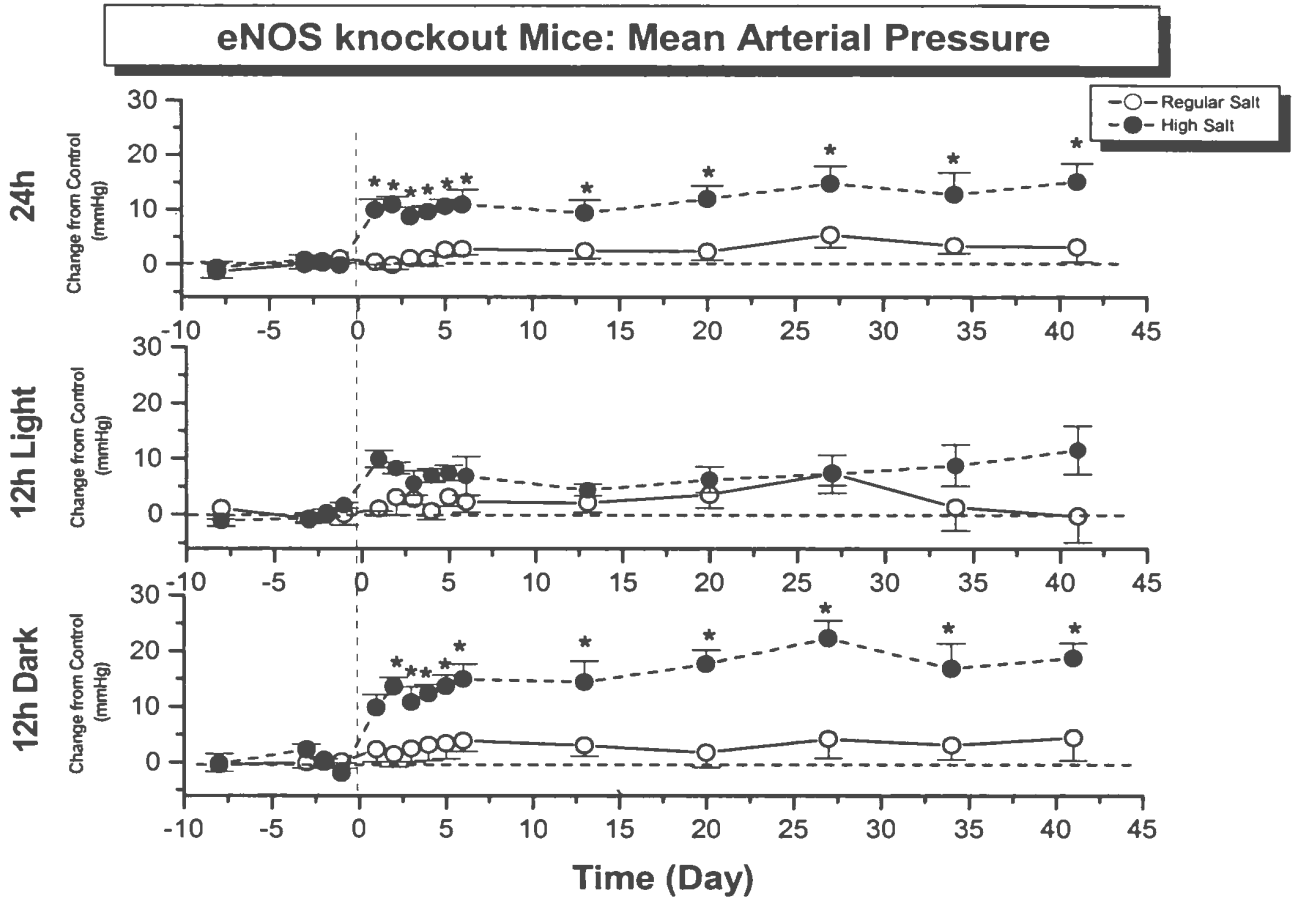

Figure 9: The effect of a high salt diet on the mean arterial pressure (MAP) in eNOS knockout mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h MAP, the middle graph shows the effect on MAP during the 12h light phase of the day and the bottom graph shows the effect on MAP during the 12h dark phase of the day. Significant differences from the final day of the control period based on Dunnet's test are indicated by '*' located above the data point.

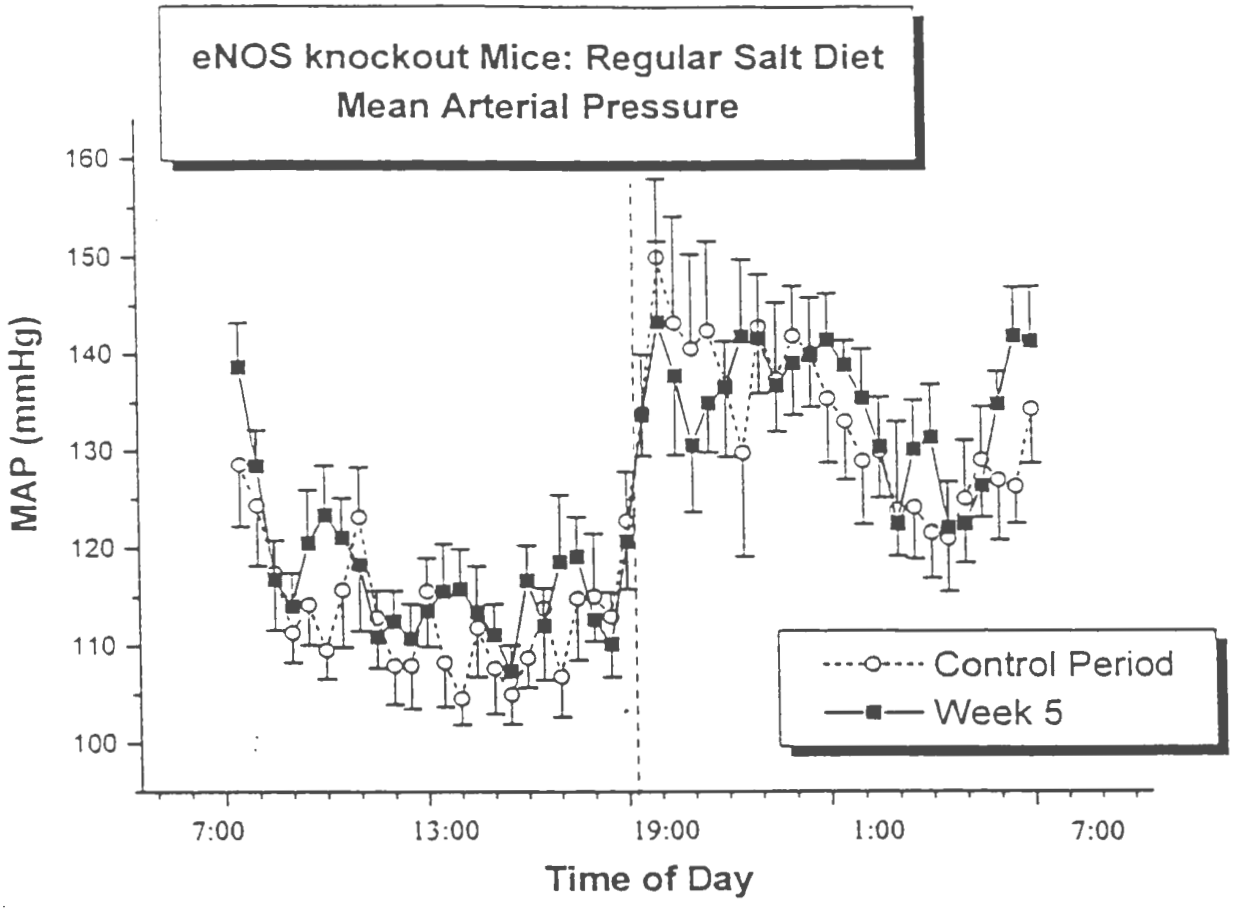

Figure 10: Comparison of circadian rhythm during the control week and during experimental week 5 for eNOS knockout mice fed the regular salt diet. The mean arterial pressures shown here are half hour averages over a 24h period. The first half of the time scale represents the light period of the day and the second half, to the right of the vertical line, represents the dark phase of the day.

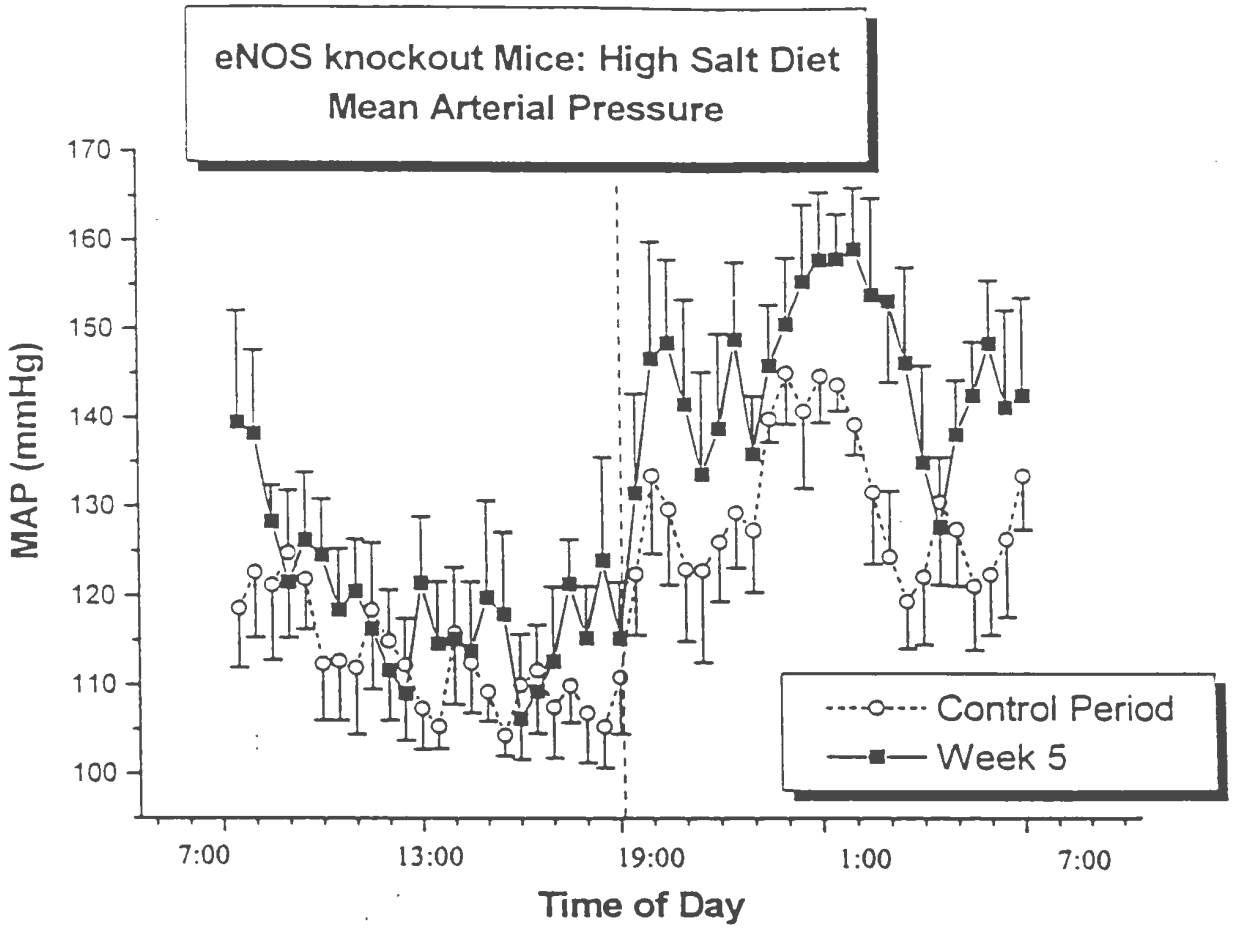

Figure 11: Comparison of circadian rhythm during the control week and during experimental week 5 for eNOS knockout mice fed the high salt diet. The mean arterial pressures shown here are half hour averages over a 24h period. The first half of the time scale represents the light period of the day and the second half, to the right of the vertical line, represents the dark phase of the day.

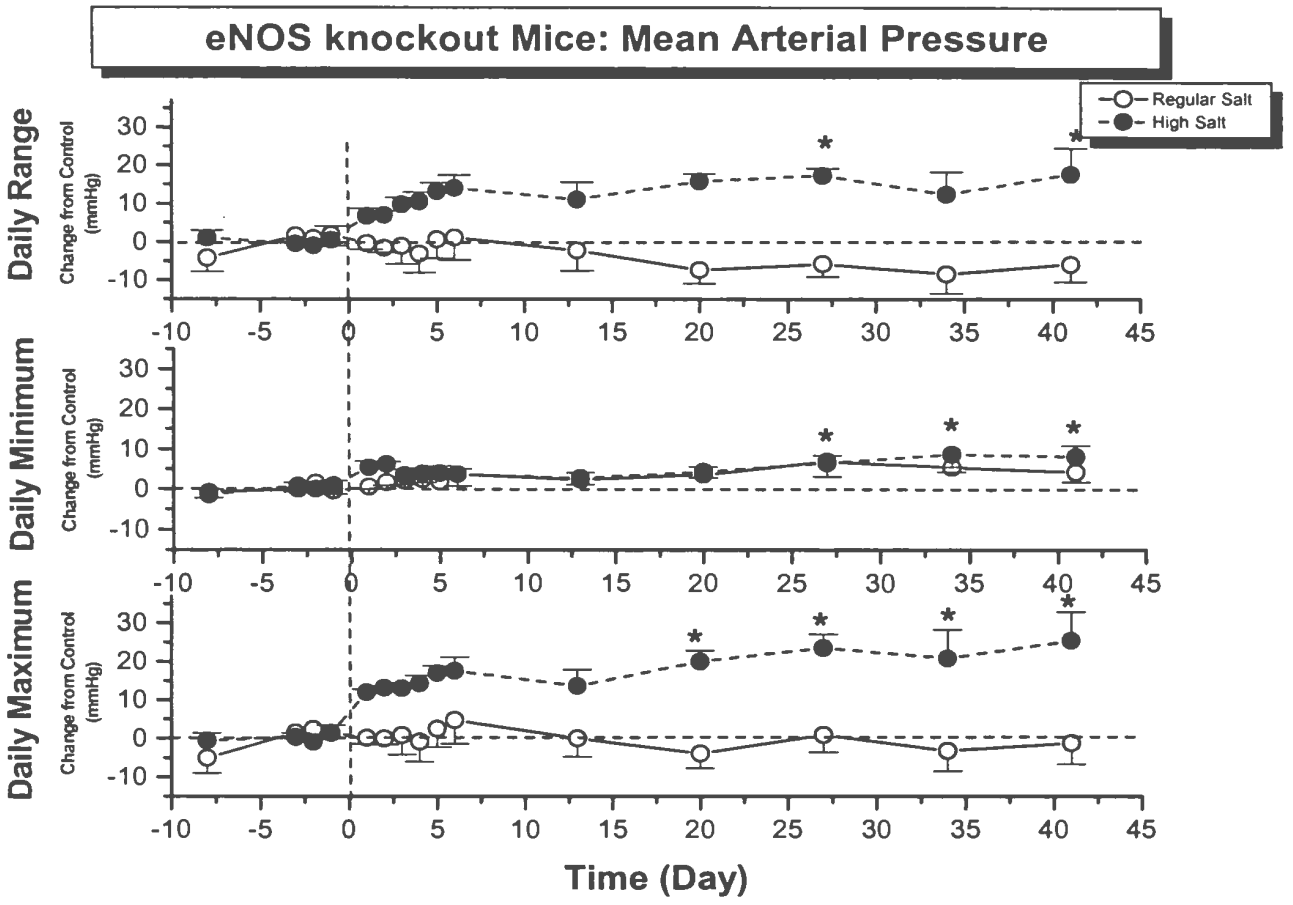

Figure 12: The effect of a high salt diet on the 24h range, minimum and maximum mean arterial pressure (MAP) in eNOS knockout mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h range of the MAP, the middle graph shows the effect on the daily minimum MAP and the bottom graph shows the effect on the daily maximum MAP. Significant differences from the final day of the control period based on Dunnet's test are indicated by '*' located above the data point.

The high salt diet caused an increase in MAP that was mainly confined to the dark phase in the eNOS-KO mice (figure 9). Although a tendency for the MAP to increase was seen in the light phase, no significant differences were observed. During the dark phase, the increase in MAP was well sustained during the experimental period. A similar tendency was apparent in the circadian variation in MAP (figures 10 & 11).

The daily range, minimum and maximum MAP showed a tendency to increase that reached significance by the end of the experiment (figure 12). The daily maximum MAP appeared to increase considerably more than the daily minimum MAP, thereby expanding the 24h range.

3.5.2 Heart Rate

HR data is summarized in figure 13. No significant differences were observed in the 24h, light or dark HR values in either the control or high salt diet groups. The HR of the eNOS-KO-HS mice showed a tendency to decrease below that of the control mice (figure 13). This tendency was most pronounced in the case of the data for the 24h and light period.

3.5.3 Pulse Pressure

The pulse pressure data is summarized in figure 14. In general, the pulse pressure of eNOS mice on the high salt diet showed a tendency to gradually increase over the course of the experiment but no significant differences were observed (figure 14). This is similar to the effect seen in C57-HS mice. However, the eNOS-KO-RS mice did not

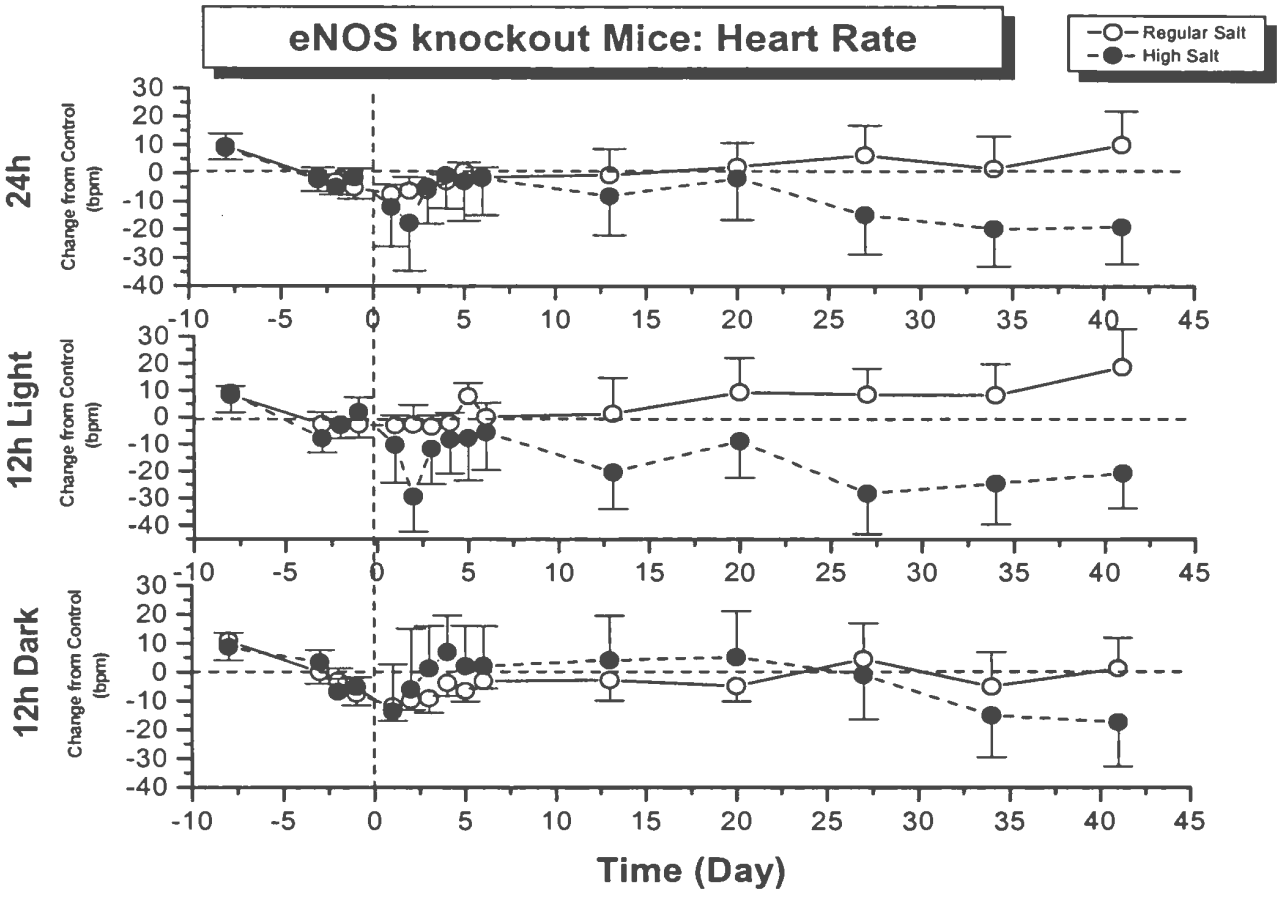

Figure 13: The effect of a high salt diet on the heart rate (HR) in eNOS knockout mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h HR, the middle graph shows the effect on HR during the 12h light phase of the day and the bottom graph shows the effect on HR during the 12h dark phase of the day. A Dunnet's test indicated no significant differences from the final day of the control period were present.

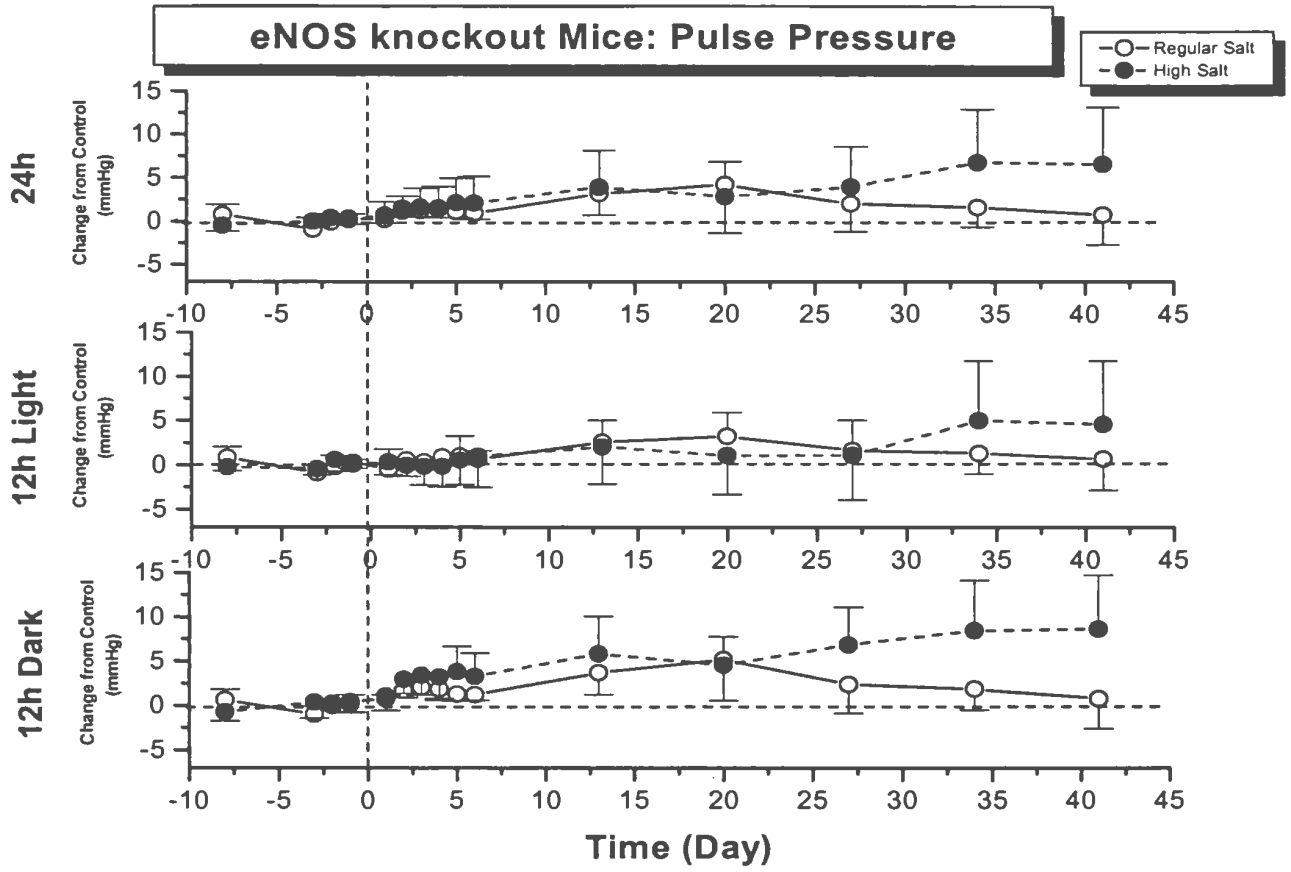

Figure 14: The effect of a high salt diet on the pulse pressure in eNOS knockout mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h pulse pressure, the middle graph shows the effect on pulse pressure during the 12h light phase of the day and the bottom graph shows the effect on pulse pressure during the 12h dark phase of the day. A Dunnet's test indicated no significant differences from the final day of the control period were present.

show a similar downward trend in pulse pressure as was seen in the case of C57-RS mice (figure 14).

3.5.4 Activity

Activity data is summarized in figure 15 and 16. The active time showed a tendency to increase when the mice were placed on a high salt diet (figure 15). However, the increase was only significant on day 1 of the high salt group in the light phase. No significant changes were observed in the control group.

In general, no significant changes or specific trends were evident in the intensity of activity during the 24hr light or dark phases (figure 16).

3.6 Responses to Dietary Salt: Comparison between C57 and eNOS Knockout Mice

Statistical analysis was conducted using ANOVA and Tukey's test to determine differences between the four experimental groups: C57-RS, C57-HS, eNOS-KO-RS and eNOS-KO-HS. When comparing the C57 mice to the eNOS knockout mice, the response to salt was broken into four phases (control, early, middle and late) in order to ensure that the power of the statistical test was not diminished. These divisions were arbitrarily assigned to help simplify the interpretation of the statistical comparisons.

Analysis of variance showed that the effects of strain, diet and time were all highly significant in all variables except intensity of activity (tables 8 & 9). Statistical outcomes between the interaction of strain, day and time varied between variables (tables 8 & 9).

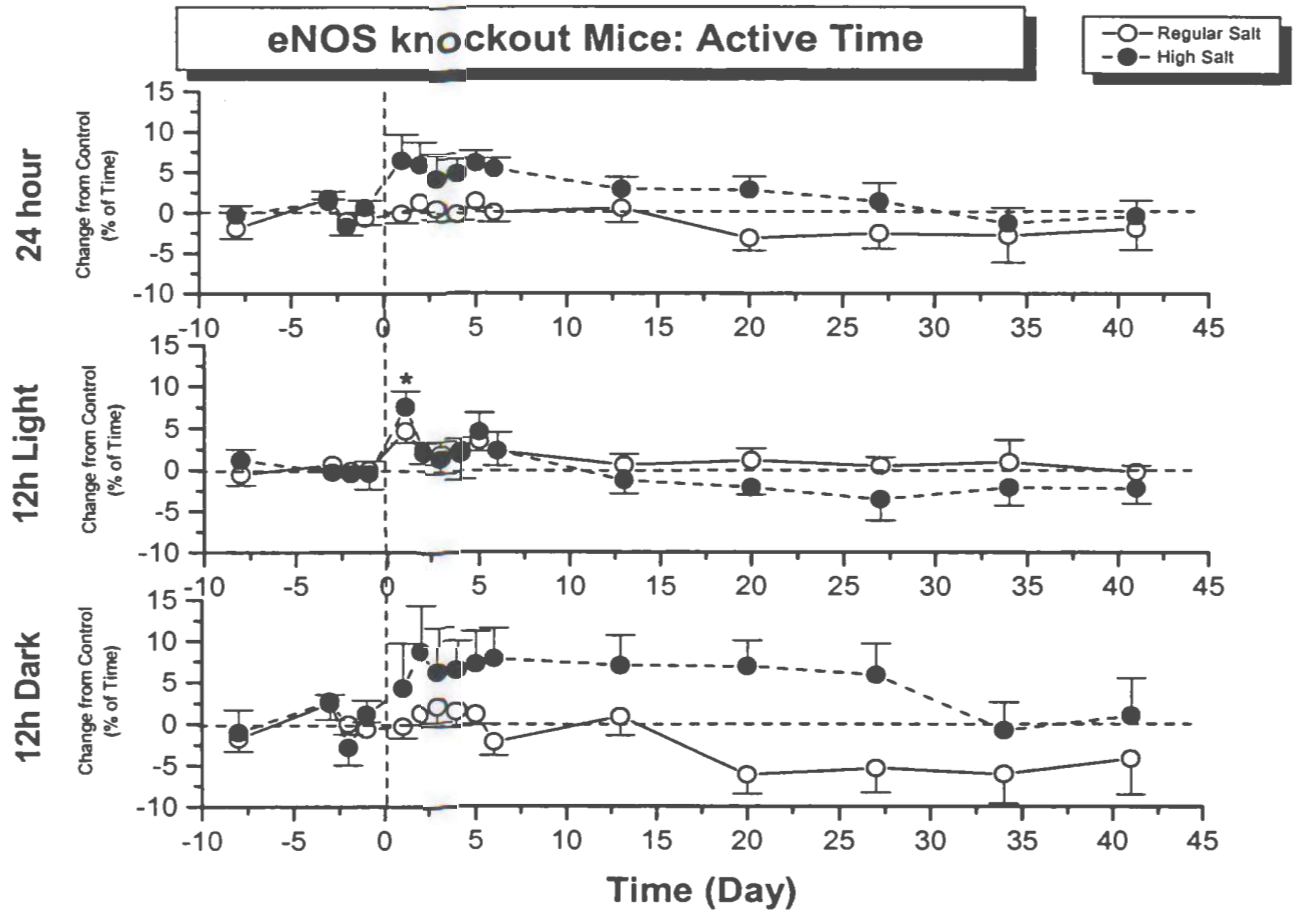

Figure 15: The effect of a high salt diet on the active time (percent of time spent active) in eNOS knockout mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h active time, the middle graph shows the effect on active time during the 12h light phase of the day and the bottom graph shows the effect on active time during the 12h dark phase of the day. Significant differences from the final day of the control period based on Dunnet's test are indicated by '*' located above the data point.

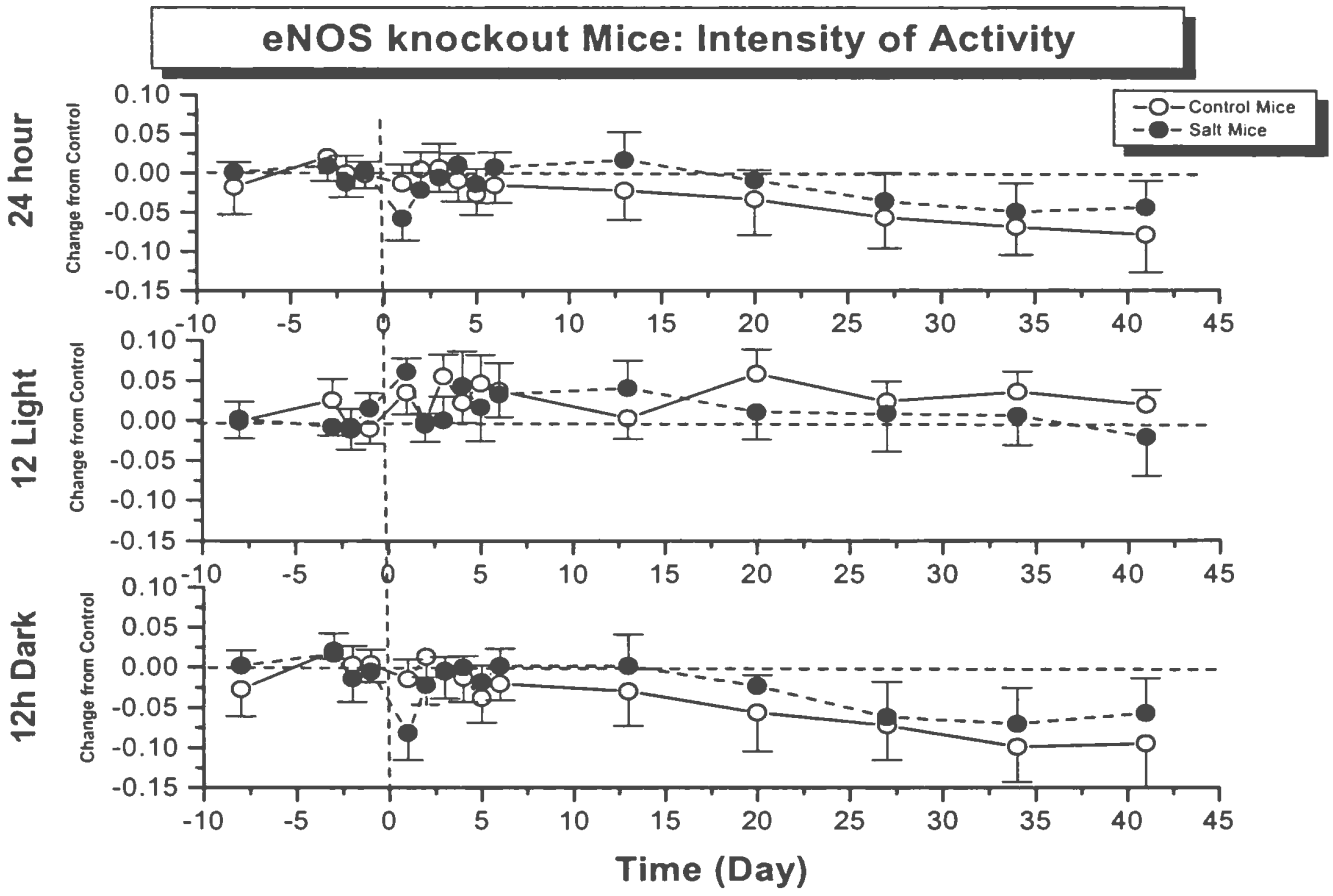

Figure 16: The effect of a high salt diet on the intensity of activity in eNOS knockout mice. The experimental high salt diet was started at time 0, indicated by the vertical dashed line. The top graph shows the effect of salt on the 24h intensity of activity, the middle graph shows the effect on intensity of activity during the 12h light phase of the day and the bottom graph shows the effect on intensity of activity during the 12h dark phase of the day. A Dunnet's test indicated no significant differences were present. The plotted values are dimensionless (have no units) because it represents the intensity of activity as the mean of log (activity) when activity >0.

Table 8: P-values for the analysis of variance of hemodynamic data in the four experimental groups. Statistical analysis using analysis of variance compared strain, diet, time and the interaction between these points across several variables.

Mean Arterial Pressure	24h	Light Period	Dark Period
Strain	**<0.0001**	**<0.0001**	**<0.0001**
Diet	**<0.0001**	**<0.0001**	**<0.0001**
Time	**<0.0001**	**<0.0001**	**<0.0001**
Strain*Diet	**<0.0001**	**<0.0001**	**<0.0001**
Strain*Time	**<0.0001**	**0.023**	**<0.0001**
Diet*Time	**<0.0001**	**0.012**	**<0.0001**
Strain*Diet*Time	**<0.001**	0.078	**<0.01**
Heart Rate			
Strain	**<0.0001**	**<0.0001**	**0.004**
Diet	0.829	**0.001**	**0.003**
Time	**<0.0001**	**<0.0001**	**0.003**
Strain*Diet	**<0.0001**	**<0.0001**	**0.013**
Strain*Time	**0.004**	**0.005**	**0.029**
Diet*Time	0.075	**0.026**	**0.023**
Strain*Diet*Time	**0.024**	**0.012**	0.15
Pulse Pressure			
Strain	**<0.0001**	**0.017**	**<0.0001**
Diet	**0.001**	0.075	**<0.0001**
Time	**0.001**	0.108	**<0.0001**
Strain*Diet	0.396	0.153	0.844
Strain*Time	0.076	0.245	**0.015**
Diet*Time	**0.002**	**0.043**	**<0.0001**
Strain*Diet*Time	0.799	0.642	0.879
Active Time			
Strain	**0.001**	0.705	**<0.0001**
Diet	**<0.0001**	0.536	**<0.0001**
Time	**<0.0001**	**<0.0001**	**<0.0001**
Strain*Diet	0.734	**0.015**	0.372
Strain*Time	**0.041**	**0.031**	**0.051**
Diet*Time	**<0.0001**	**0.002**	**<0.0001**
Strain*Diet*Time	0.779	0.413	0.556
Intensity of Activity			
Strain	0.333	0.575	0.206
Diet	**<0.0001**	0.256	**0.001**
Time	**<0.0001**	**0.013**	**<0.0001**
Strain*Diet	0.084	**0.015**	0.121
Strain*Time	0.475	0.945	0.398
Diet*Time	0.121	0.077	0.224
Strain*Diet*Time	0.09	0.112	0.161

Table 9: P-values for the analysis of variance for the daily range, minimum and maximum MAP for the four experimental groups. Statistical analysis of variance compared strain, diet, time and the interaction between these points across range of MAP, daily minimum MAP and daily maximum MAP.

Comparison	Range of MAP	24h Daily Minimum	24h Daily Maximum
Strain	0.573	**<0.0001**	**<0.0001**
Diet	**<0.0001**	0.908	**<0.0001**
Time	**0.014**	**<0.0001**	**<0.0001**
Strain*Diet	0.06	**0.001**	**<0.0001**
Strain*Time	0.578	**<0.0001**	**0.034**
Diet*Time	**<0.0001**	0.556	**<0.0001**
Strain*Diet*Time	**0.003**	0.091	**<0.0001**

3.6.1 Mean Arterial Pressure

Between group comparisons for MAP are summarized in figures 17, 18, 19, 20, 21 and 22 and in tables 10, 11, 12, 13, 14 and 15. In both C57 and eNOS knockout mice, MAP was significantly increased by the high salt diet (figures 17, 18 & 19 and tables 10, 11 & 12). The response of eNOS knockout mice was significantly greater than that of C57 mice (tables 10, 11 & 12). eNOS knockout mice showed a sustained increase in MAP whereas the response of the C57 mice was mostly transient.

In general, the MAP response to high salt appeared to be most pronounced during the dark phase of the day (figure 19). In C57 mice, the MAP response to high salt was largely confined to the dark phase of the day. Although the most predominant effect of the high salt diet was also observed in the dark phase of the day in eNOS-KO, statistical differences were also observed during the light phase of the day (figures 18 & 19 and tables 11 & 12).

During the control period, the eNOS-KO mice had significantly higher absolute values for the range, daily minimum and maximum (table 2 & 4). The eNOS-KO-HS mice showed a salt-induced increase in the daily range and minimum by the end of the experiment and a sustained increase in the daily maximum throughout the entire experimental period (figures 20, 21 & 22; tables 13, 14 & 15). In contrast, the C57-HS mice showed an initial increase in the daily range and maximum and no significant change in the daily minimum (figures 20, 21 & 22; tables 13, 14 & 15).

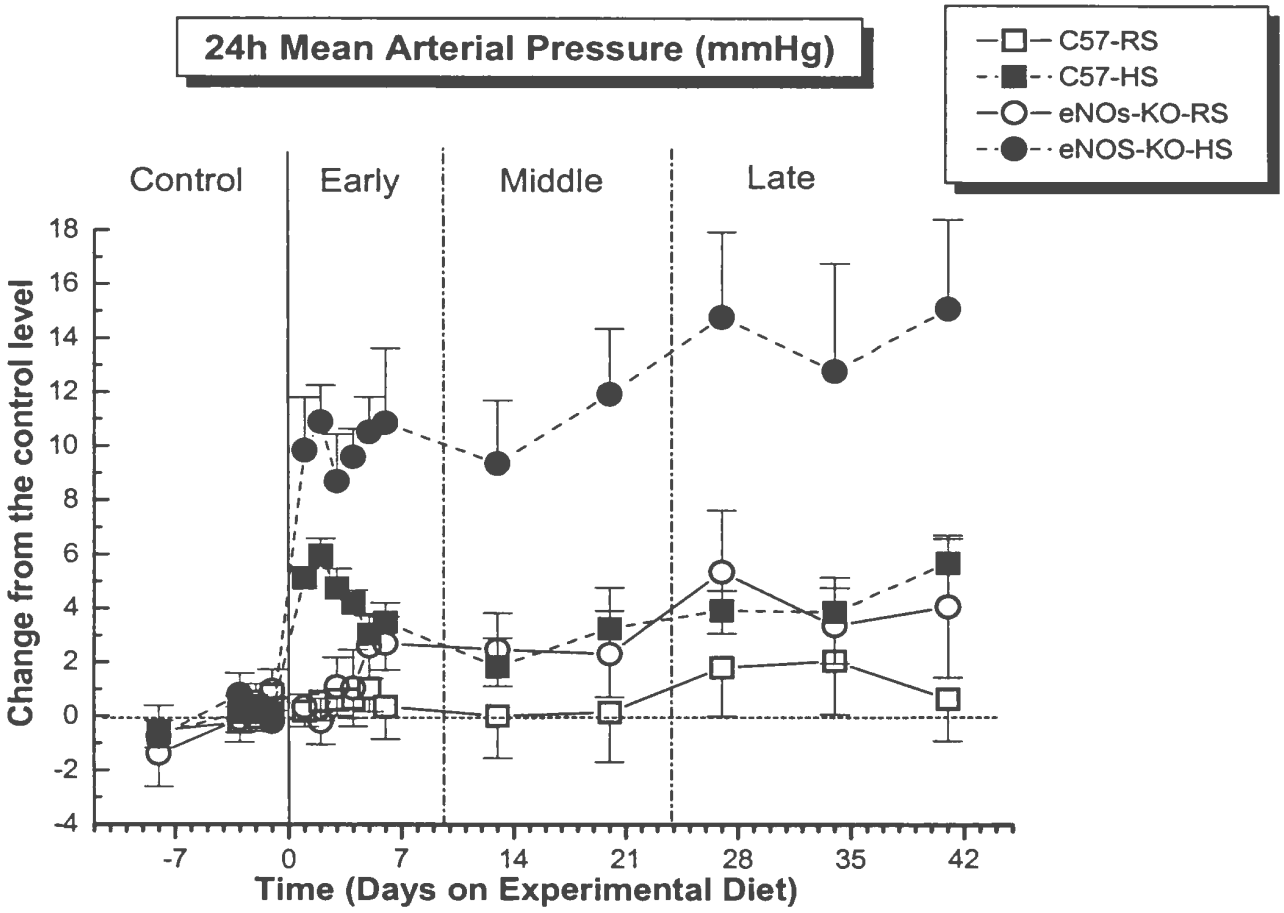

Figure 17: Comparison of the effect of a high salt diet on 24h mean arterial pressure in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 10.

Table 10: The effect of a high salt diet on 24h mean arterial pressure in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	**<0.0001**	**<0.0001**	**<0.0001**
eNOS-KO-HS vs. C57-HS	1.0000	**<0.0001**	**<0.0001**	**<0.0001**
eNOS-KO-RS vs. C57-RS	1.0000	0.9960	0.8717	0.3380
C57-RS vs. C57-HS	1.0000	**<0.0001**	0.8390	0.2420

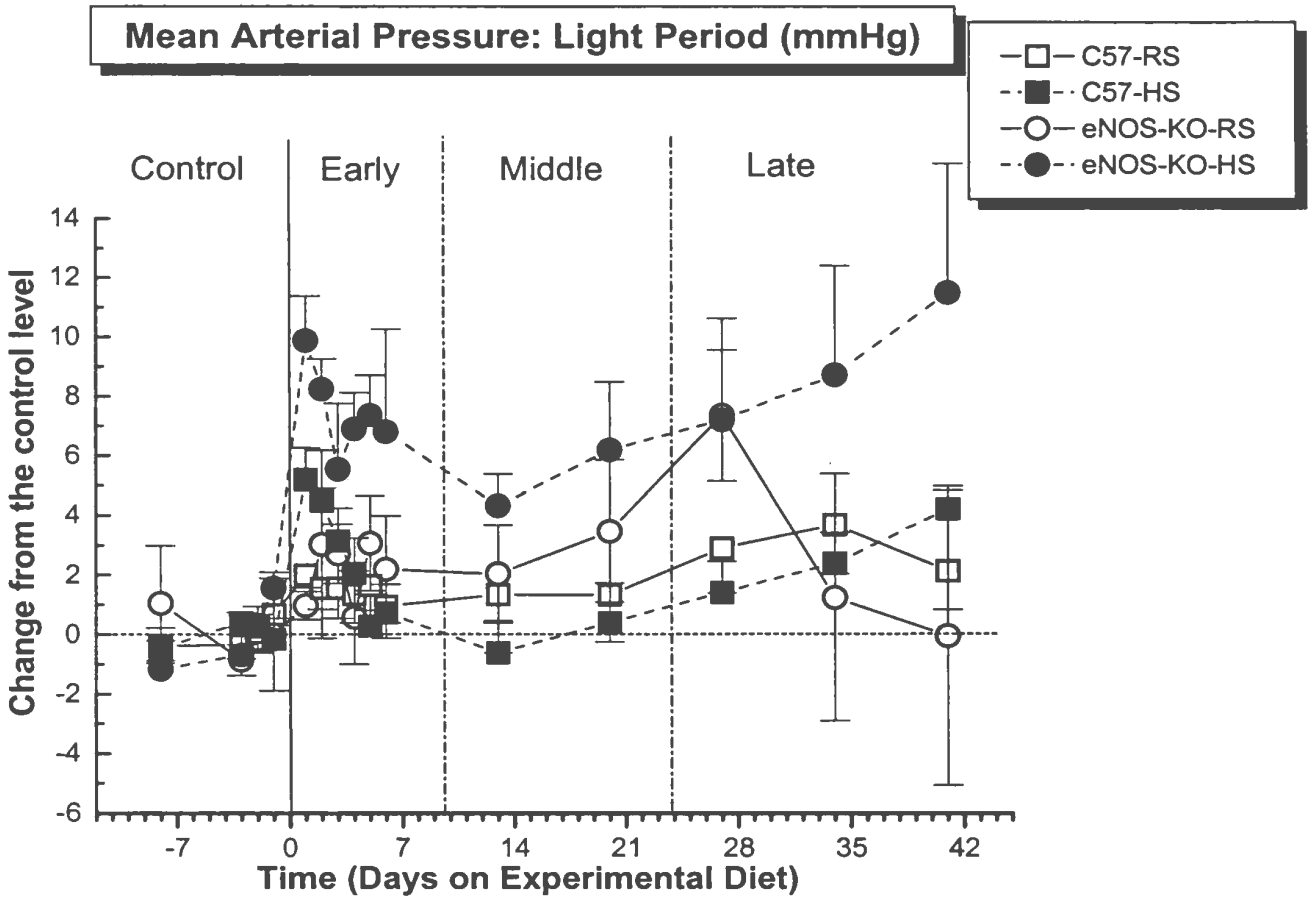

Figure 18: Comparison of the effect of a high salt diet on 12h light period mean arterial pressure in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 11.

Table 11: The effect of a high salt diet on 12h light period mean arterial pressure in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	**0.0001**	0.9819	**0.0008**
eNOS-KO-HS vs. C57-HS	1.0000	**0.0008**	0.1169	**0.0004**
eNOS-KO-RS vs. C57-RS	1.0000	1.0000	1.0000	1.0000
C57-RS vs. C57-HS	1.0000	0.9980	1.0000	1.0000

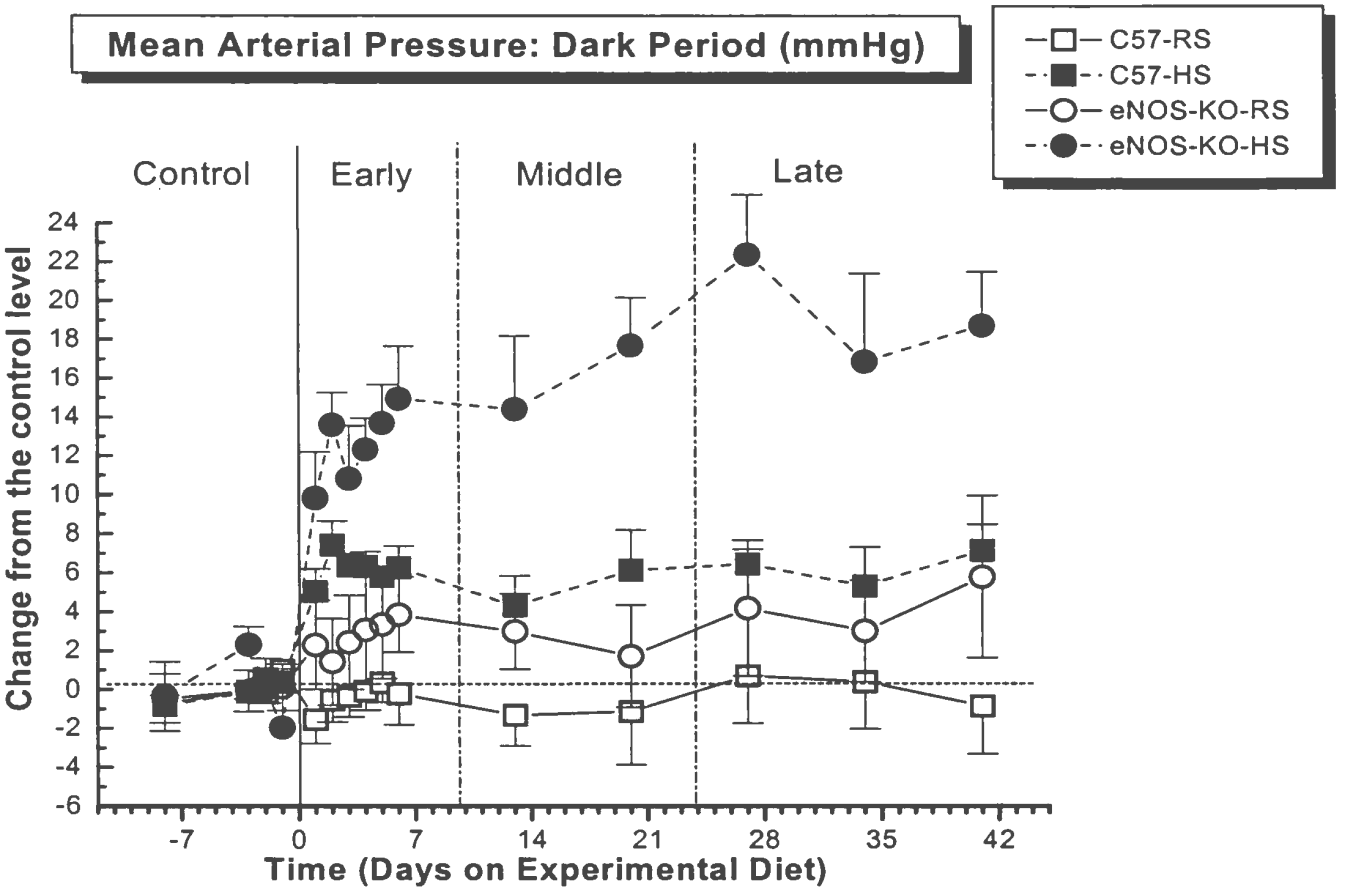

Figure 19: Comparison of the effect of a high salt diet on 12h dark period mean arterial pressure in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 12.

Table 12: The effect of a high salt diet on 12h dark period mean arterial pressure in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	**<0.0001**	**<0.0001**	**<0.0001**
eNOS-KO-HS vs. C57-HS	1.0000	**<0.0001**	**<0.0001**	**<0.0001**
eNOS-KO-RS vs. C57-RS	1.0000	0.1510	0.8230	0.3000
C57-RS vs. C57-HS	1.0000	**<0.0001**	0.4000	**0.0060**

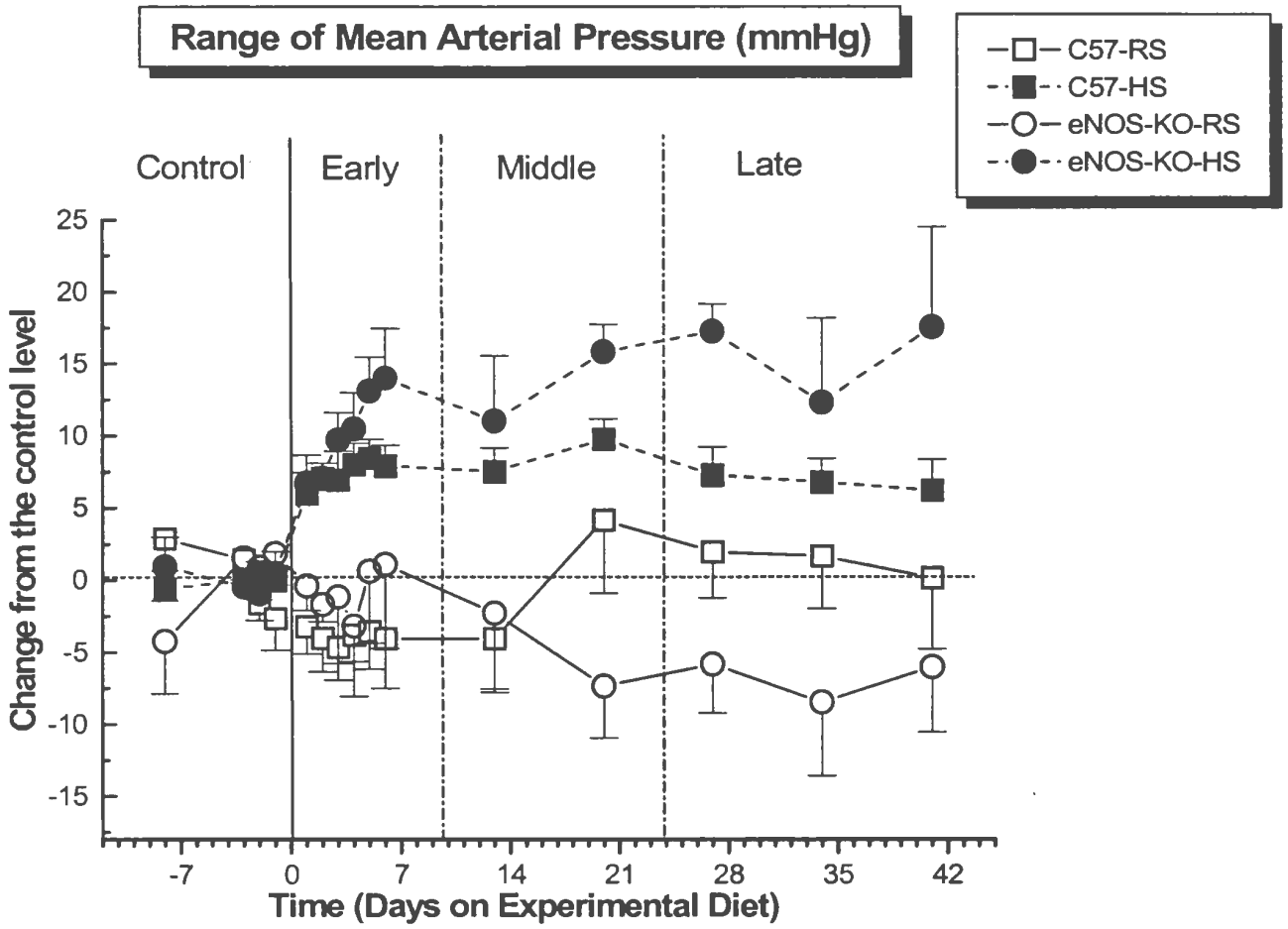

Figure 20: Comparison of the effect of a high salt diet on 24h range of mean arterial pressure in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 13.

Table 13: The effect of a high salt diet on 24h range of mean arterial pressure in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	0.9620	**0.0111**	**<0.0001**
eNOS-KO-HS vs. C57-HS	1.0000	0.5502	0.9999	0.6681
eNOS-KO-RS vs. C57-RS	1.0000	1.0000	0.9995	0.7136
C57-RS vs. C57-HS	1.0000	**0.0032**	0.9058	0.9883

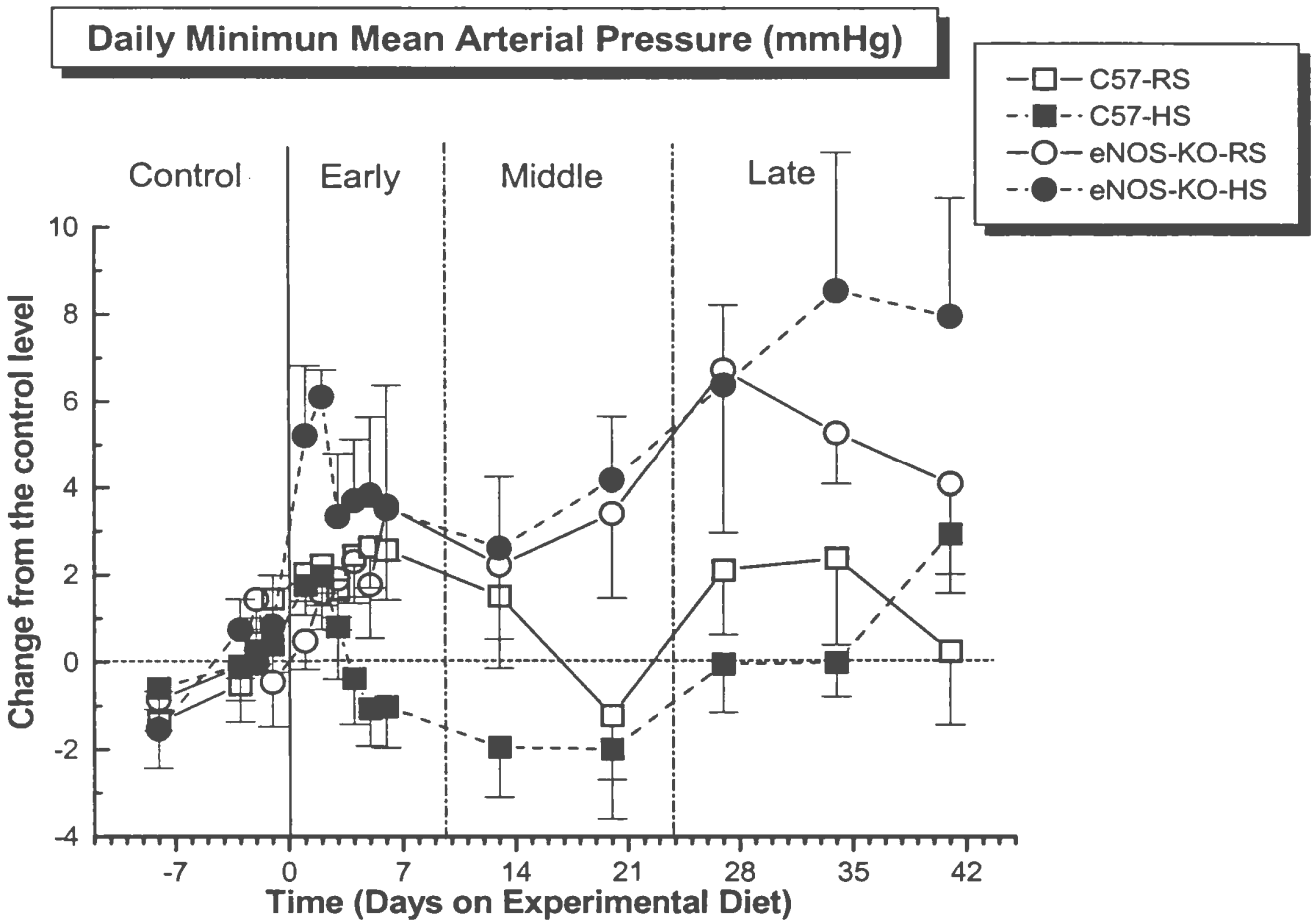

Figure 21: Comparison of the effect of a high salt diet on daily minimum mean arterial pressure in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 14.

Table 14: The effect of a high salt diet on daily minimum mean arterial pressure in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	0.2339	1.0000	**0.662**
eNOS-KO-HS vs. C57-HS	1.0000	**0.0001**	**0.0026**	**<0.0001**
eNOS-KO-RS vs. C57-RS	1.0000	1.0000	0.6594	**0.0094**
C57-RS vs. C57-HS	1.0000	0.3206	0.9367	1.0000

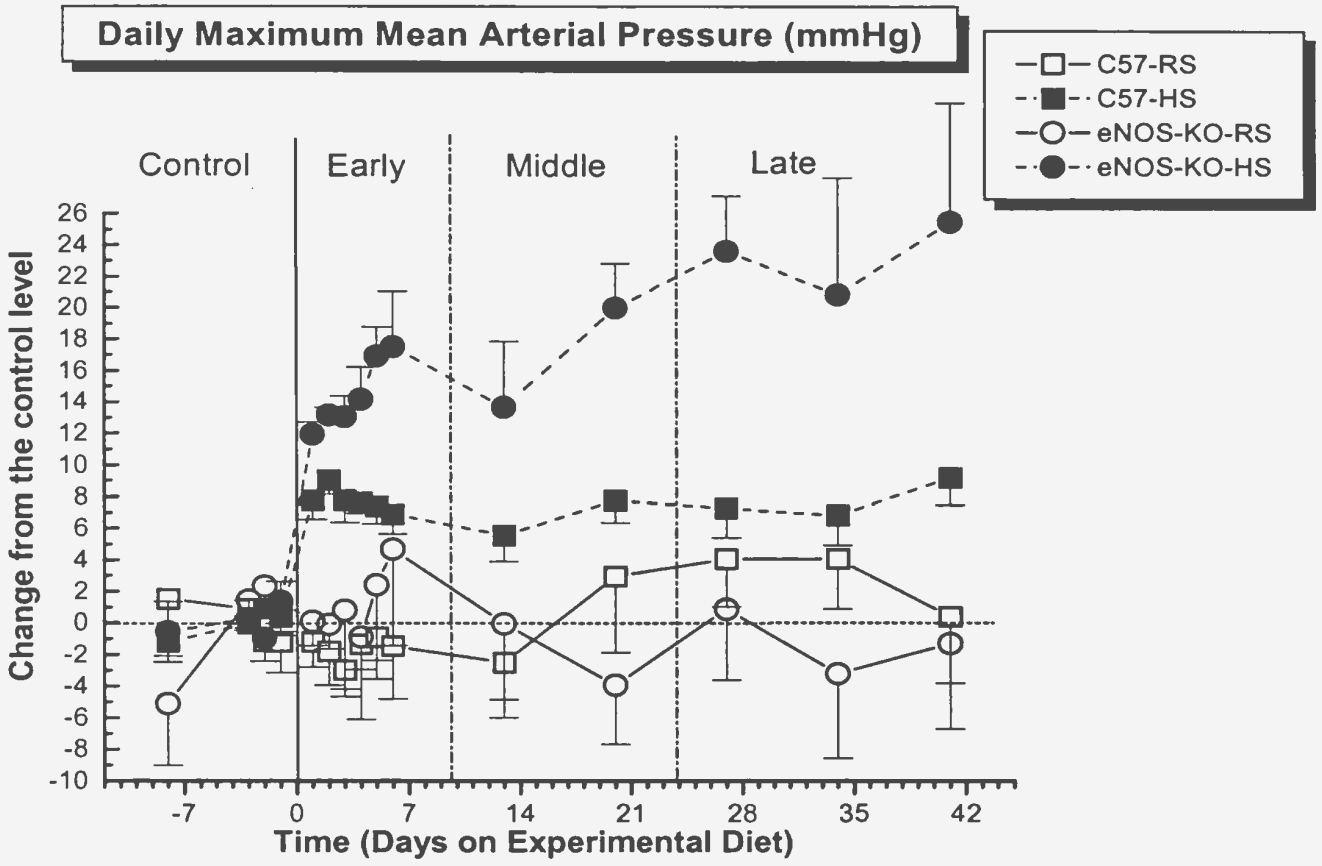

Figure 22: Comparison of the effect of a high salt diet on daily maximum mean arterial pressure in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 15.

Table 15: The effect of a high salt diet on daily maximum mean arterial pressure in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	**<0.0001**	**<0.0001**	**<0.0001**
eNOS-KO-HS vs. C57-HS	1.0000	**0.0121**	0.0646	**<0.0001**
eNOS-KO-RS vs. C57-RS	1.0000	0.9434	1.0000	0.9283
C57-RS vs. C57-HS	1.0000	**<0.0001**	0.6918	0.7940

3.6.2 Heart Rate

The HR data is summarized in figures 23, 24 and 25 and in tables 16, 17 and 18. In C57 mice, a high salt diet caused a tendency for the HR to increase above that of the control group but only reached significance during the early phase of the dark period (figures 23, 24 & 25 and tables 16, 17 & 18). A high salt diet in eNOS knockout mice caused a tendency for the HR to decrease from that of the control group, reaching significance in the late phase of the 24h and the light period (tables 16, 17 & 18). In general, salt-induced changes in HR were modest relative to the high absolute values of HR in mice (table 2).

3.6.3 Pulse Pressure

The pulse pressure data is summarized in figures 26, 27 and 28 and in tables 19, 20 and 21. In both C57 and eNOS knockout mice, pulse pressure tended to increase on the high salt diet (figure 26). In the C57 mice, the change in pulse pressure reached significance in the late phase of both the 24h and the dark periods (figures 26 & 28 and tables 19 & 21). The eNOS knockout mice only exhibited significant differences in the late phase of the dark period (table 21). Overall, the pulse pressure response of the two strains of mice was not significantly different (tables 19, 20 & 21).

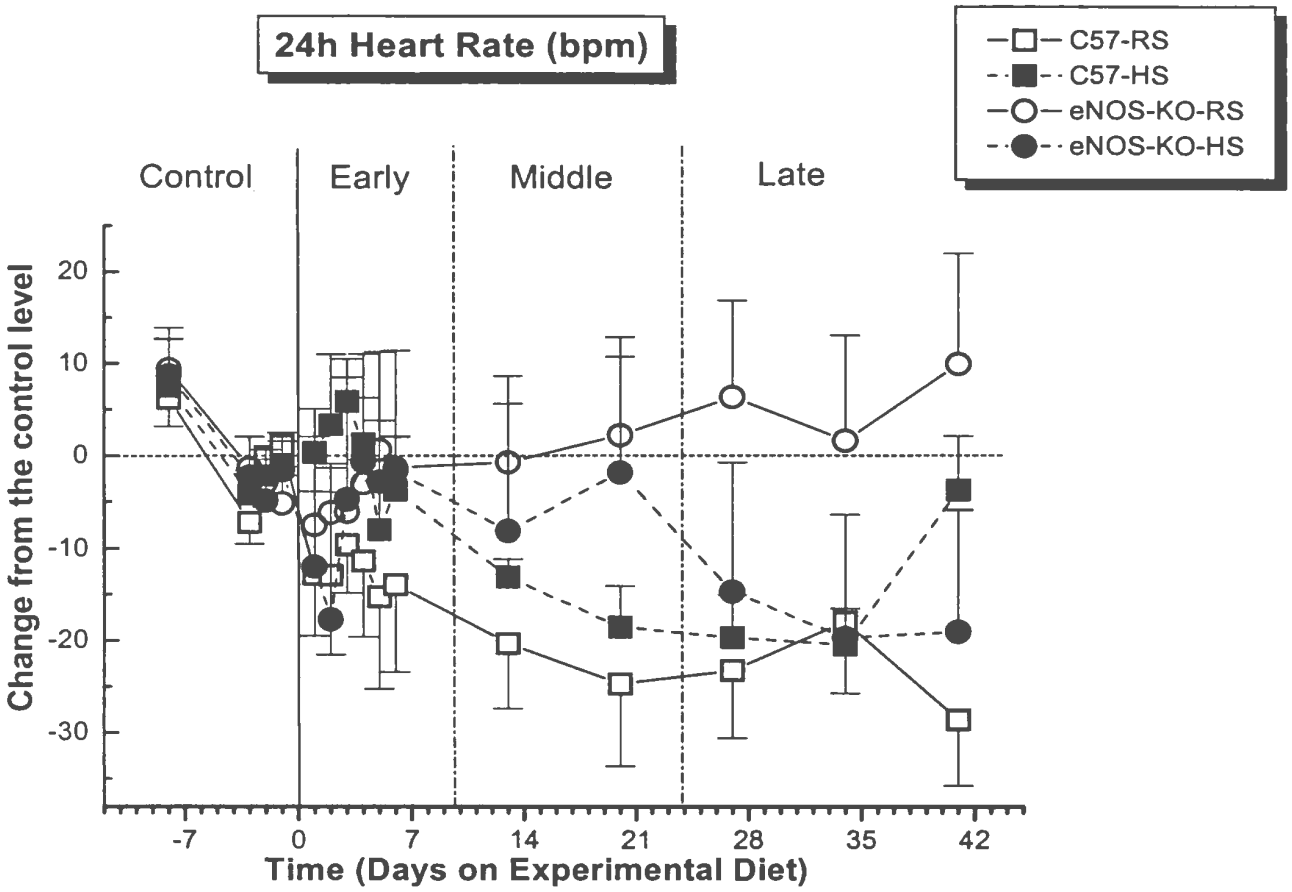

Figure 23: Comparison of the effect of a high salt diet on 24h heart rate in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 16.

Table 16: The effect of a high salt diet on 24h heart rate in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	1.0000	1.0000	**0.0028**
eNOS-KO-HS vs. C57-HS	1.0000	0.9962	0.9970	**<0.0001**
eNOS-KO-RS vs. C57-RS	1.0000	0.6045	**0.0325**	**0.0001**
C57-RS vs. C57-HS	1.0000	0.0960	0.9990	0.9797

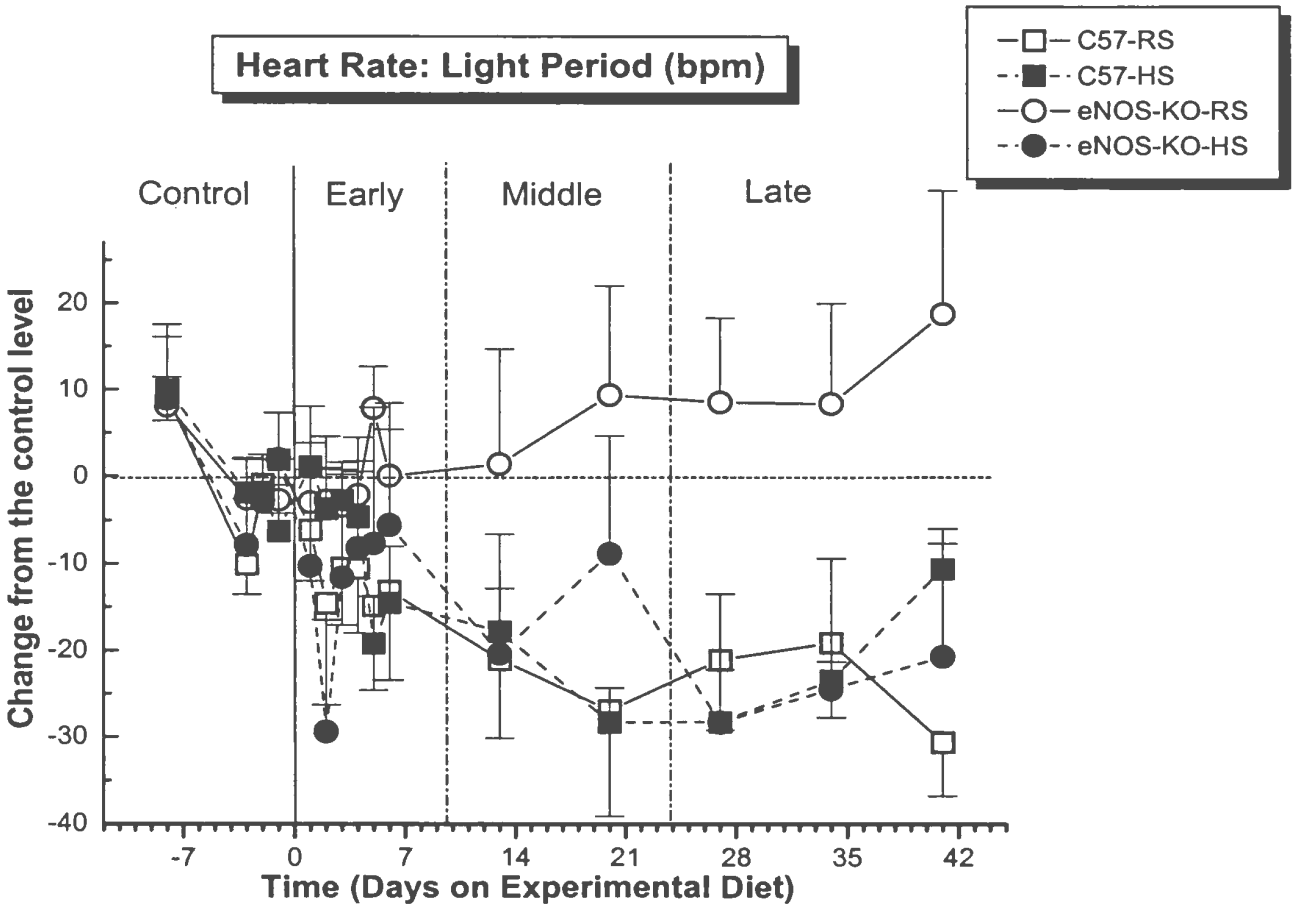

Figure 24: Comparison of the effect of a high salt diet on 12h light period heart rate in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 17.

Table 17: The effect of a high salt diet on 12h light period heart rate in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	0.6184	0.3945	**<0.0001**
eNOS-KO-HS vs. C57-HS	1.0000	1.0000	0.9990	1.0000
eNOS-KO-RS vs. C57-RS	1.0000	0.4022	**0.0065**	**<0.0001**
C57-RS vs. C57-HS	1.0000	0.9998	1.0000	1.0000

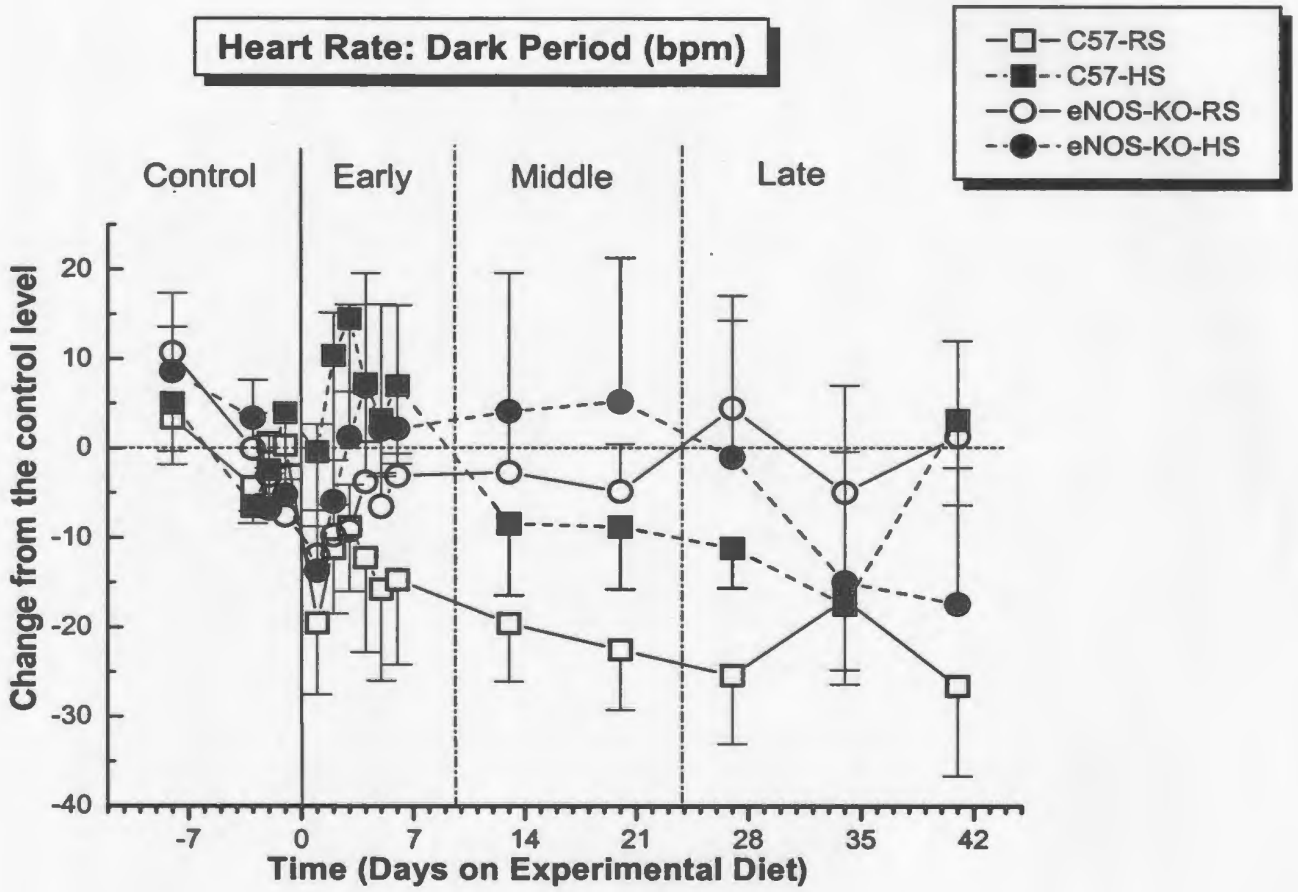

Figure 25: Comparison of the effect of a high salt diet on 12h dark period heart rate in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 18.

Table 18: The effect of a high salt diet on 12h dark period heart rate in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	0.9811	0.9994	0.9241
eNOS-KO-HS vs. C57-HS	1.0000	0.9830	0.9568	1.0000
eNOS-KO-RS vs. C57-RS	1.0000	0.9834	0.6059	**0.0195**
C57-RS vs. C57-HS	1.0000	**0.0004**	0.9644	0.6447

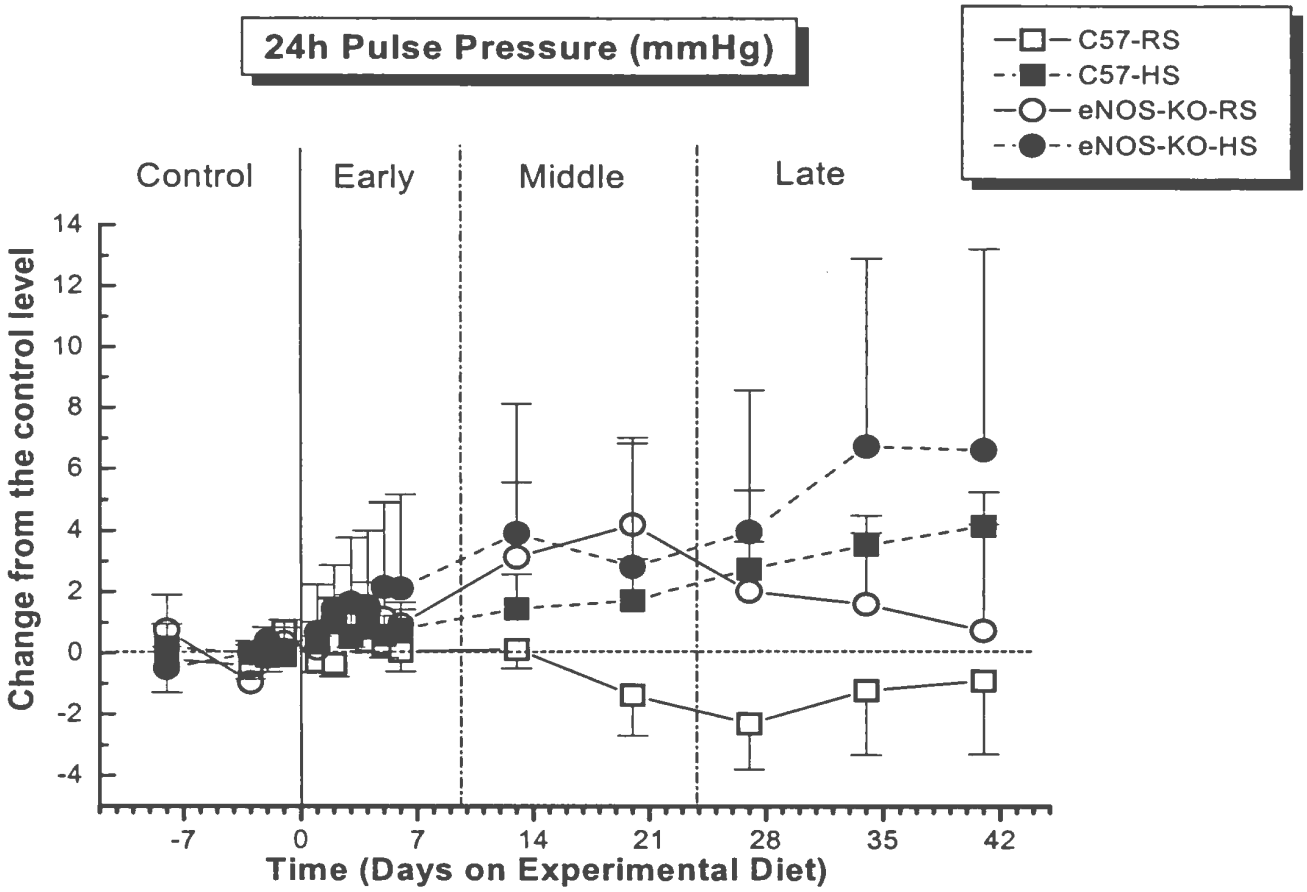

Figure 26: Comparison of the effect of a high salt diet on 24h pulse pressure in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 19.

Table 19: The effect of a high salt diet on 24h pulse pressure in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	1.0000	1.0000	0.1179
eNOS-KO-HS vs. C57-HS	1.0000	0.9990	0.9990	0.9603
eNOS-KO-RS vs. C57-RS	1.0000	0.9990	0.3287	0.6726
C57-RS vs. C57-HS	1.0000	1.0000	0.9943	**0.0247**

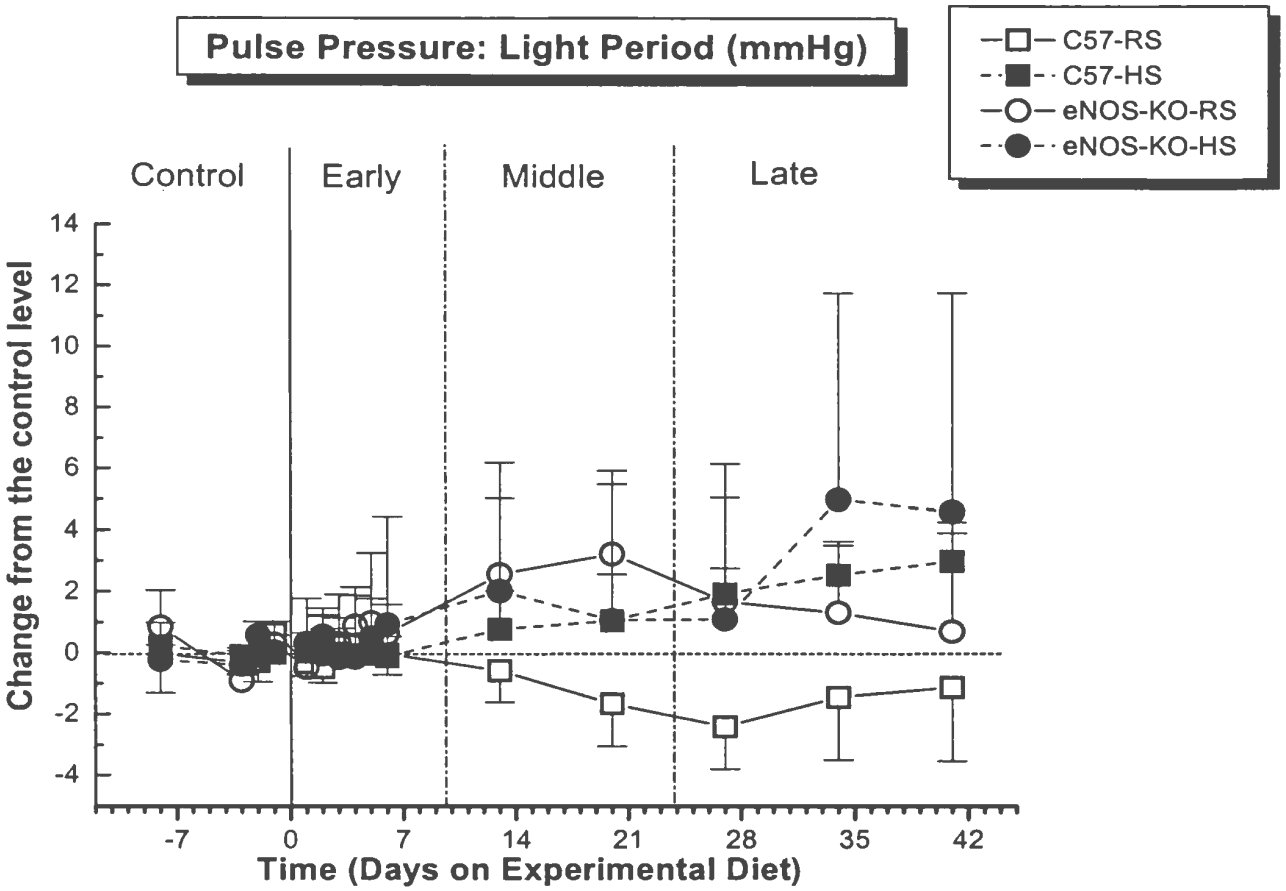

Figure 27: Comparison of the effect of a high salt diet on 12h light period pulse pressure in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 20.

Table 20: The effect of a high salt diet on 12h light period pulse pressure in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	1.0000	1.0000	0.9672
eNOS-KO-HS vs. C57-HS	1.0000	1.0000	1.0000	1.0000
eNOS-KO-RS vs. C57-RS	1.0000	1.0000	0.5450	0.7736
C57-RS vs. C57-HS	1.0000	1.0000	0.9980	0.2139

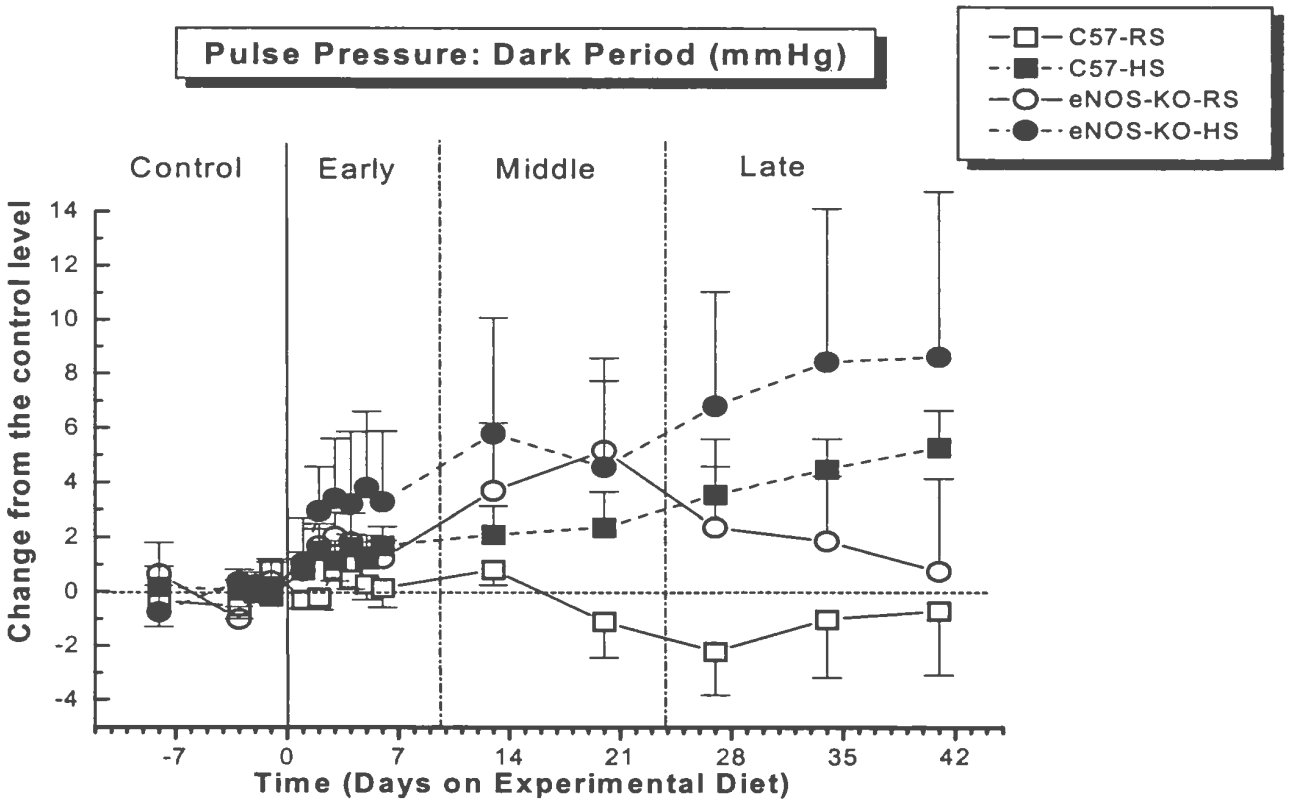

Figure 28: Comparison of the effect of a high salt diet on 12h dark period pulse pressure in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 21.

Table 21: The effect of a high salt diet on 12h dark period pulse pressure in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	0.9630	1.0000	**0.0004**
eNOS-KO-HS vs. C57-HS	1.0000	0.9350	0.9304	0.4180
eNOS-KO-RS vs. C57-RS	1.0000	0.9903	0.1963	0.6091
C57-RS vs. C57-HS	1.0000	0.9979	0.9850	**0.0018**

3.6.4 Active Time

The active time data is summarized in figures 29, 30 and 31 and tables 22, 23 and 24. In both the C57 and eNOS knockout mice, the active time was initially statistically increased when the mice were placed on the high salt diet (figures 29, 30 & 31). In general, the change in active time in response to a high dietary salt intake was most pronounced during the dark phase of the day (figure 31; tables 22, 23 & 24). In the eNOS-KO-HS mice, the increase in active time seen in the dark phase was sustained throughout the experiment (figure 31). However, the C57-HS mice only showed a significant increase in the active time in the dark period during the early phase and late phase (tables 22, 23 & 24).

3.6.5 Intensity of Activity

The intensity of activity data is summarized in figures 32, 33 and 34 and in tables 25, 26 and 27. C57-HS mice showed a greater intensity of activity during the early phase when placed on a high salt diet (tables 25, 26 & 27). C57-HS mice, unlike the eNOS-KO-HS mice, had an initial increase in the level of activity in the 24h, light and dark periods of the day (figures 32, 33 & 34). No significant differences were seen in the intensity of active in the eNOS knockout mice (tables 25, 26 & 27).

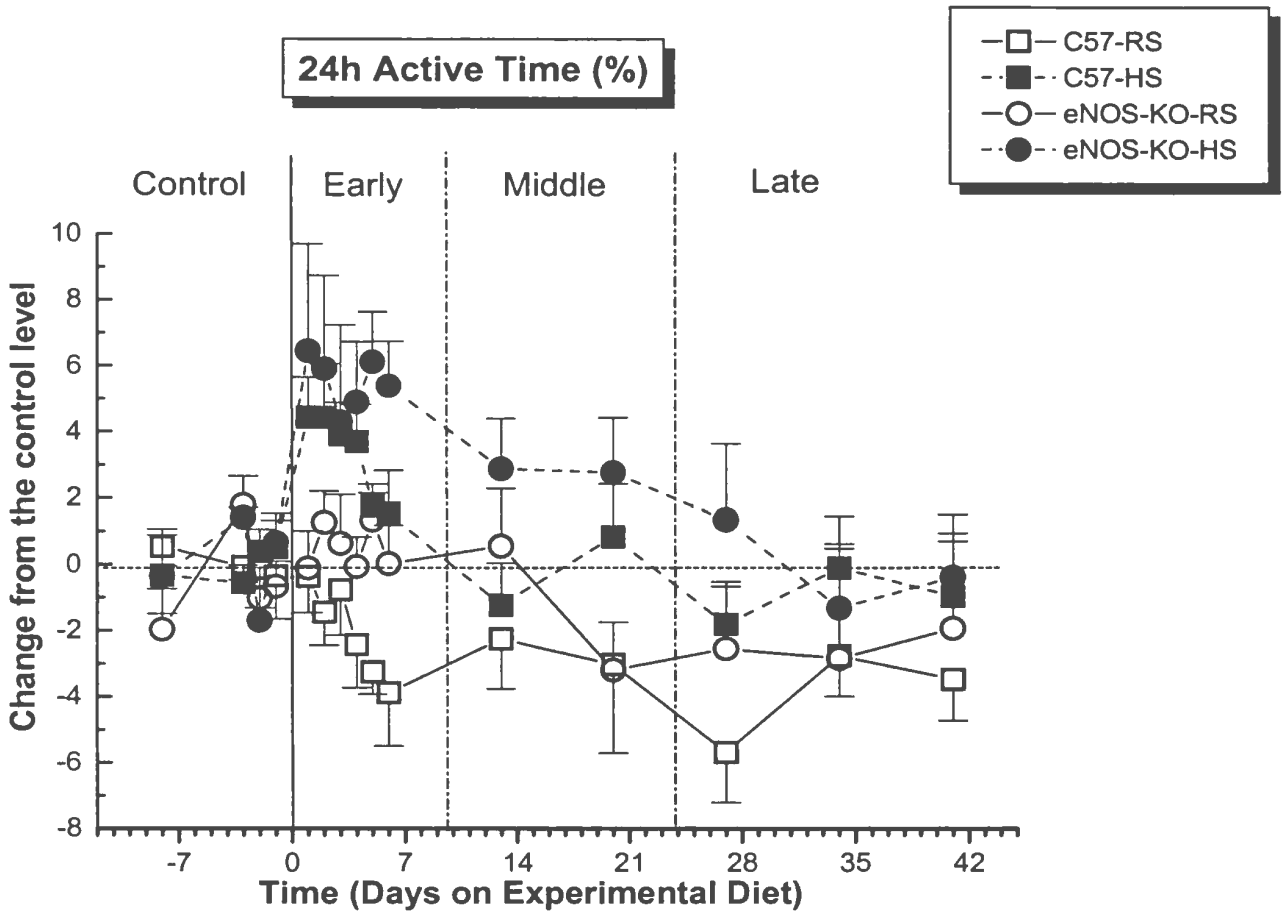

Figure 29: Comparison of the effect of a high salt diet on 24h active time (percent of time spent active) in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 22.

Table 22: The effect of a high salt diet on 24h active time in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	**<0.0001**	0.2209	0.8339
eNOS-KO-HS vs. C57-HS	1.0000	0.5360	0.8017	1.0000
eNOS-KO-RS vs. C57-RS	1.0000	0.1106	0.9990	0.9948
C57-RS vs. C57-HS	1.0000	**<0.0001**	0.9419	0.3939

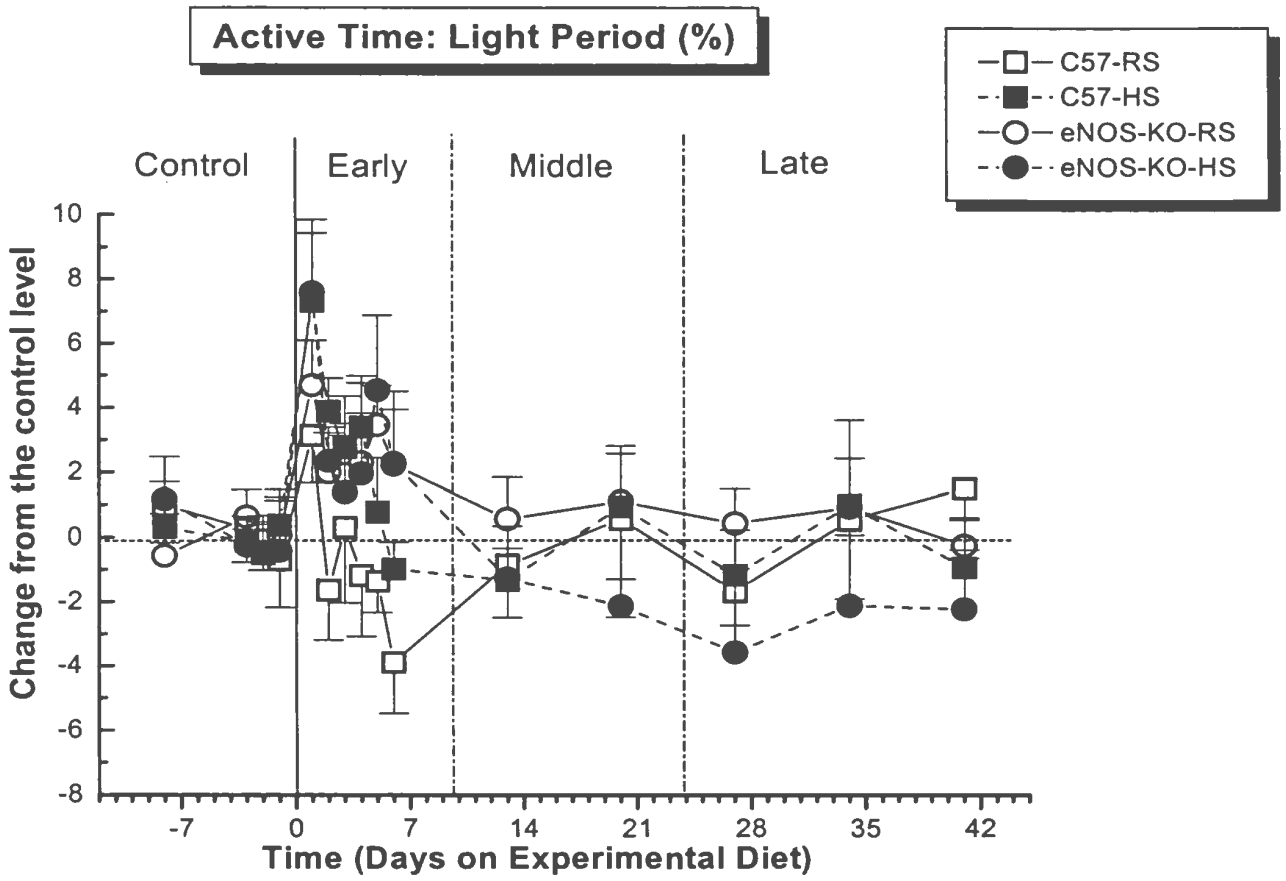

Figure 30: Comparison of the effect of a high salt diet on 12h light period active time (percent of time spent active) in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 23.

Table 23: The effect of a high salt diet on 12h light period active time in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	1.0000	0.9511	0.5131
eNOS-KO-HS vs. C57-HS	1.0000	1.0000	0.9990	0.9207
eNOS-KO-RS vs. C57-RS	1.0000	**0.0032**	1.0000	1.0000
C57-RS vs. C57-HS	1.0000	**0.0027**	1.0000	1.0000

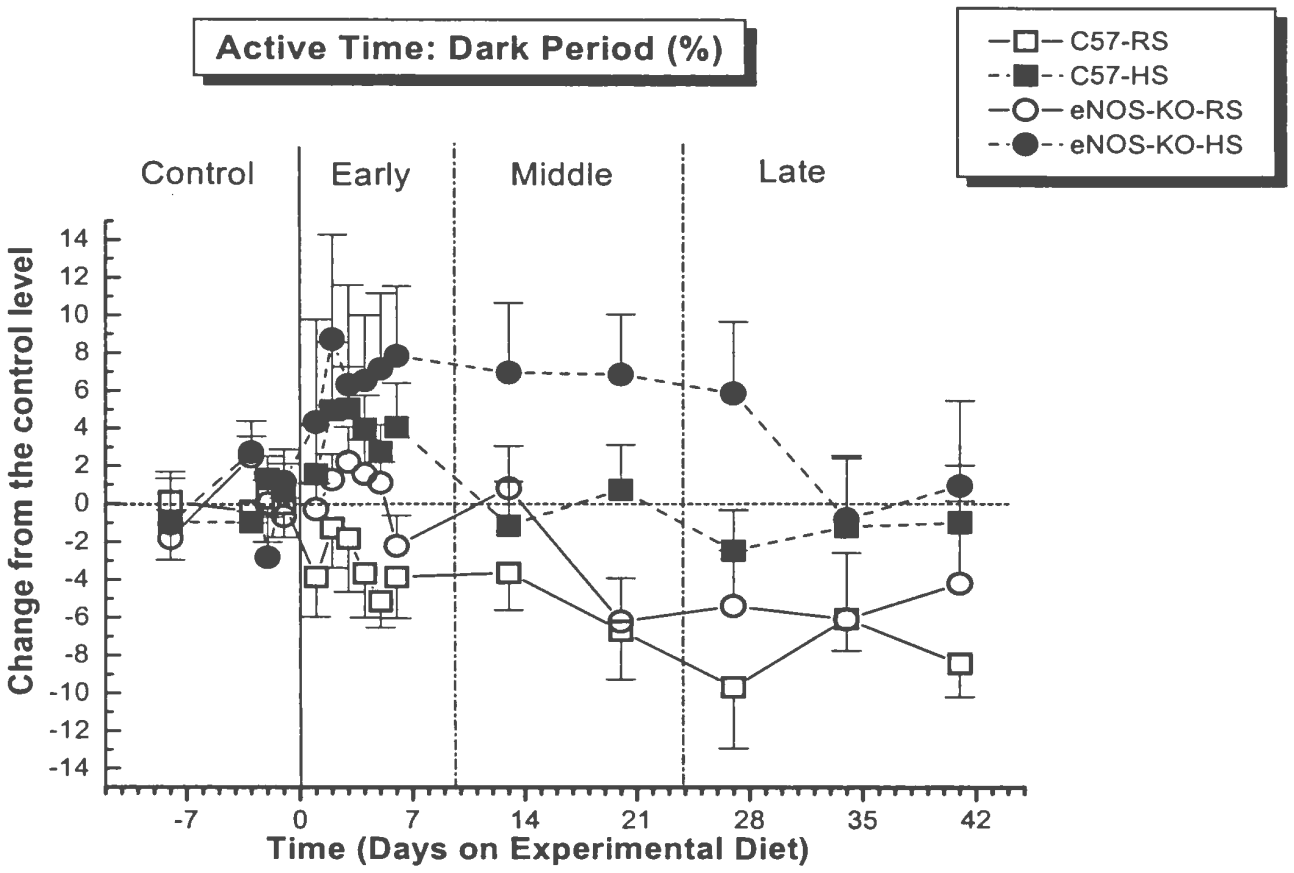

Figure 31: Comparison of the effect of a high salt diet on 12h dark period active time (percent of time spent active) in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The statistical results are summarized in table 24.

Table 24: The effect of a high salt diet on 12h dark period active time in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	**0.0010**	**0.0039**	**0.0146**
eNOS-KO-HS vs. C57-HS	1.0000	0.7120	0.1843	0.9163
eNOS-KO-RS vs. C57-RS	1.0000	0.1805	0.9990	0.9813
C57-RS vs. C57-HS	1.0000	**0.0001**	0.7344	**0.0440**

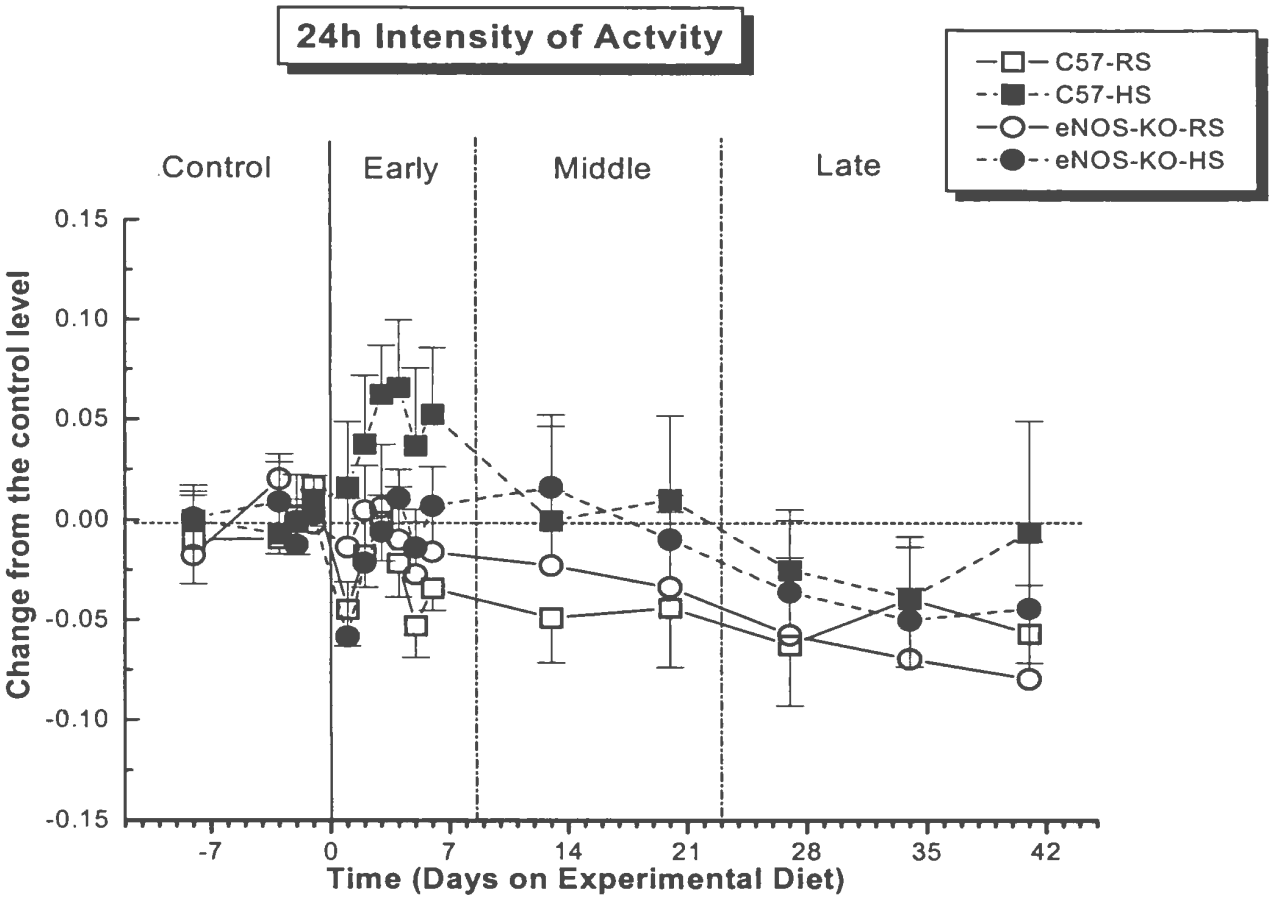

Figure 32: Comparison of the effect of a high salt diet on 24h intensity of activity in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The plotted values in this graph are dimensionless (no units) because it represents the intensity of activity as the mean of log (activity) when activity >0. The statistical results are summarized in table 25.

Table 25: The effect of a high salt diet on 24h intensity of activity in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	1.0000	0.9979	0.9986
eNOS-KO-HS vs. C57-HS	1.0000	**0.0362**	1.0000	0.9990
eNOS-KO-RS vs. C57-RS	1.0000	0.9948	1.0000	1.0000
C57-RS vs. C57-HS	1.0000	**0.0001**	0.8140	0.9892

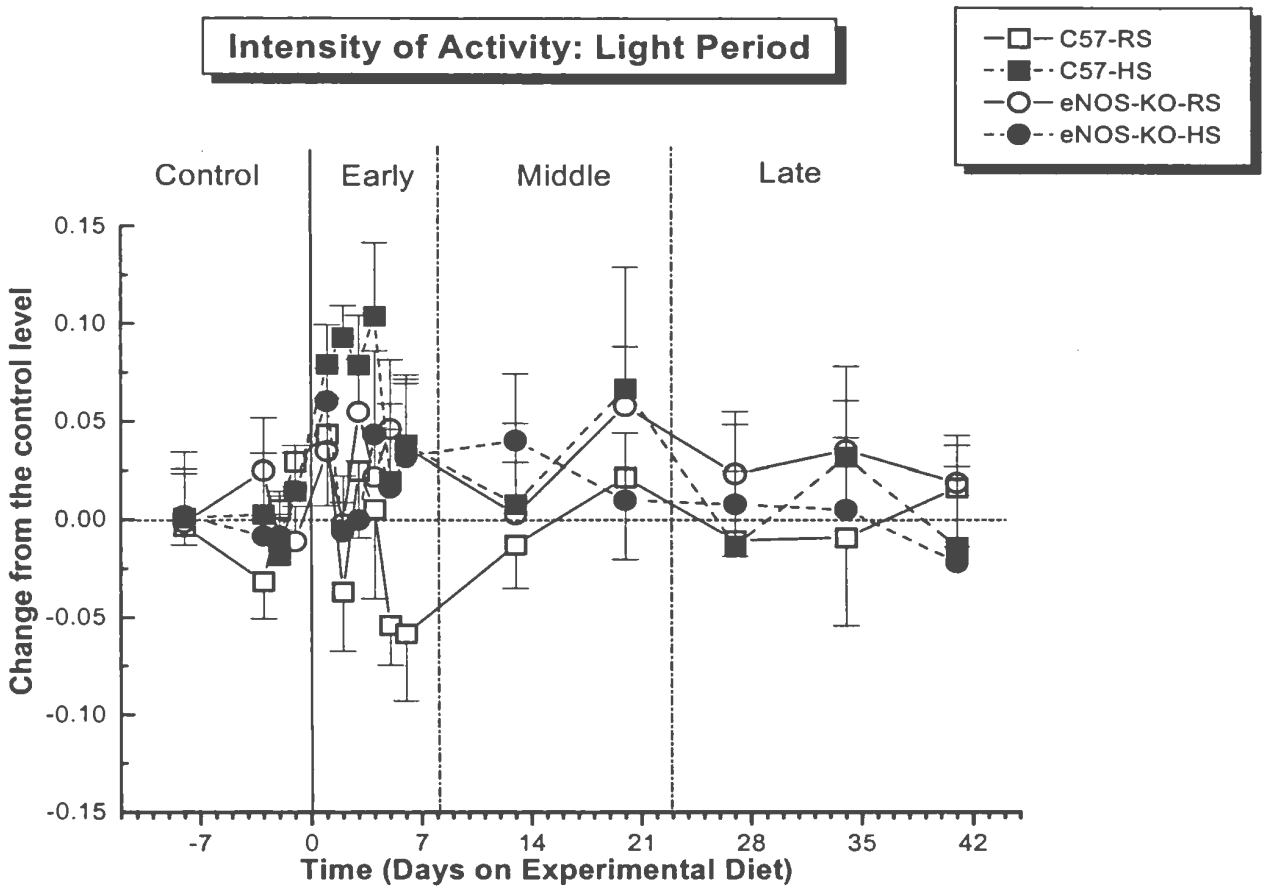

Figure 33: Comparison of the effect of a high salt diet on 12h light period intensity of activity in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The plotted values in this graph are dimensionless (no units) because it represents the intensity of activity as the mean of log (activity) when activity >0. The statistical results are summarized in table 26.

Table 26: The effect of a high salt diet on 12h light period intensity of activity in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	1.0000	1.0000	0.9983
eNOS-KO-HS vs. C57-HS	1.0000	0.4717	1.0000	1.0000
eNOS-KO-RS vs. C57-RS	1.0000	0.2869	0.9990	0.9987
C57-RS vs. C57-HS	1.0000	**0.0002**	0.9910	1.0000

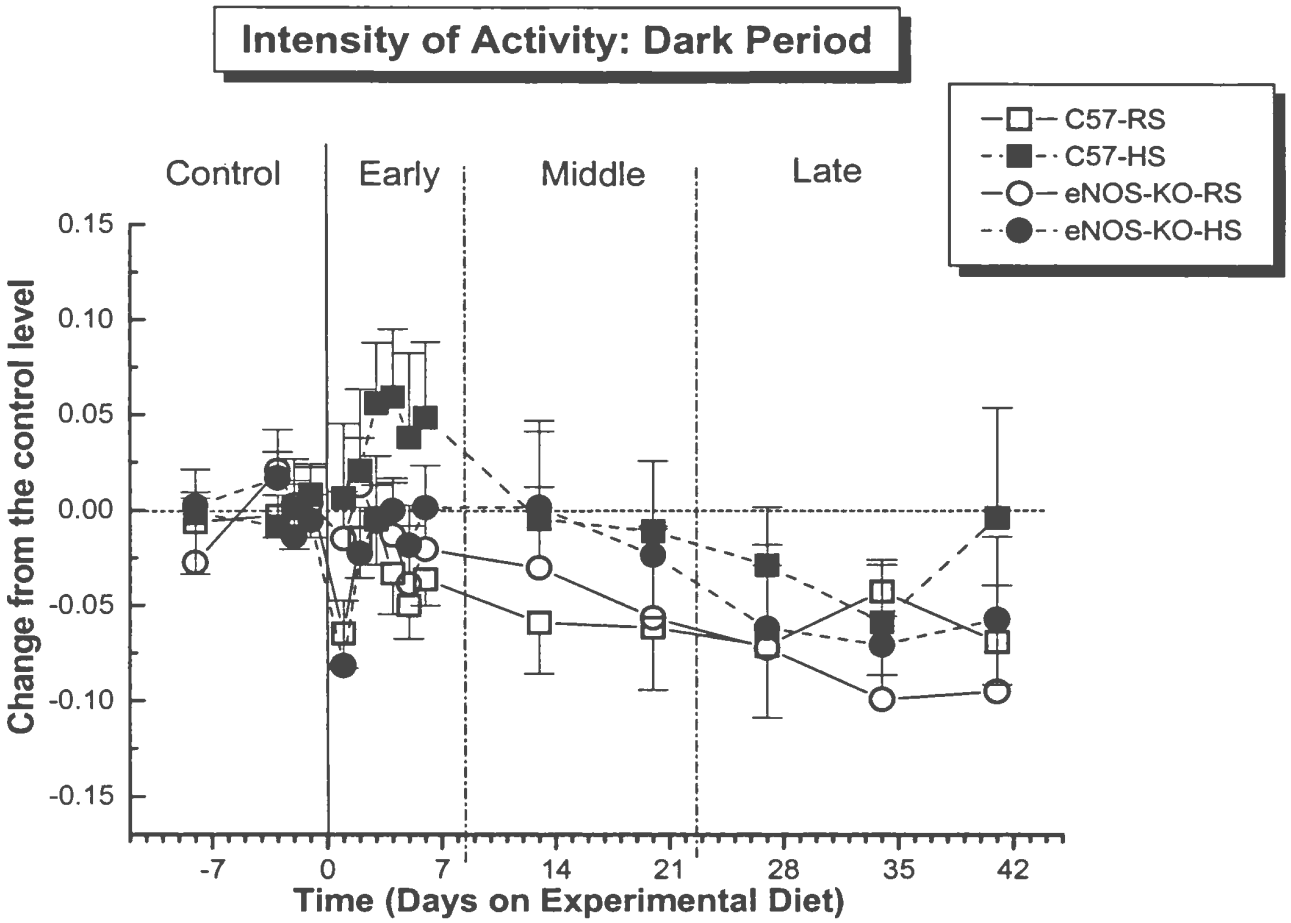

Figure 34: Comparison of the effect of a high salt diet on 12h dark period intensity of activity in C57 and eNOS knockout mice. The experimental diet is started at time 0, indicated by the solid vertical line. The experimental period is broken into four phases (control, early, middle and late) to ensure the power of the statistical comparisons were not diminished. The plotted values in this graph are dimensionless (no units) because it represents the intensity of activity as the mean of log (activity) when activity >0. The statistical results are summarized in table 27.

Table 27: The effect of salt on 12h dark period intensity of activity in C57 and eNOS knockout mice. This table provides p-values for the statistical comparisons between groups (Tukey's test). The response is broken down into four phases.

Comparison	Control Week	Early Week 1	Middle Week 2-3	Late Week 4-6
eNOS-KO-RS vs. eNOS-KO-HS	1.0000	1.0000	0.9993	0.9996
eNOS-KO-HS vs. C57-HS	1.0000	0.1308	1.0000	0.9939
eNOS-KO-RS vs. C57-RS	1.0000	0.9976	1.0000	0.9981
C57-RS vs. C57-HS	1.0000	**0.0015**	0.9040	0.9958

3.7 Mean Arterial Pressure Vs Activity: C57 and eNOS knockout mice.

In order to clarify the cause of the salt-induced increase in the MAP, analysis was conducted to look at the influence of changes in activity on the MAP. To gain a greater understanding of the cause of the increase in MAP, we examined 1) the level of activity (see previous sections), 2) the mean MAP level when active (MAP_{ACTIVE}) and 3) the mean MAP level when inactive ($MAP_{INACTIVE}$). Table 28 shows the mean changes in MAP_{ACTIVE} and $MAP_{INACTIVE}$ during week 1 and week 5 relative to the control period.

Overall, both C57 and eNOS knockout mice showed an increase in MAP_{ACTIVE} when consuming a high salt diet (table 28). During the first and fifth week of salt exposure, the C57-HS mice only exhibited a significant increase in MAP_{ACTIVE} in the 24h and light period whereas the eNOS-KO-HS mice showed a significant increase in the 24h, light and dark periods. The eNOS-KO-HS mice also had an increase in $MAP_{INACTIVE}$ in the dark period of week 1 and in the 24h, light and dark periods of week 5. No significant differences were exhibited in the $MAP_{INACTIVE}$ of the C57 mice. No significant differences were observed in C57-RS or eNOS-KO-RS mice in either week 1 or 5. The elevation of the MAP_{ACTIVE} suggests that not all the MAP increases seen with the high salt diet can be explained by the coinciding increases in activity.

Table 28: Comparison between mean arterial pressure (MAP) during periods of activity and inactivity in C57 and eNOS knockout mice. This table shows the effect of a control or high salt diet on the change in MAP from control values in periods of activity and inactivity during the 24h, light and dark periods. Data is presented for weeks 1 and 5 of the experimental diet.

		24h MAP		12h Light MAP		12h Dark MAP	
Week 1		***Act=0***	***Act>0***	***Act=0***	***Act>0***	***Act=0***	***Act>0***
C57	**Control**	0 ±1.0	-0.7 ±0.8	0.5 ±1.2	1.4 ±1.0	-1.1 ±0.96	-1.3 ±0.9
	Salt	2.3 ±1.1	6.6 ±0.9 *	2.1 ±1.0	6.0 ±1.3 *	2.5 ±1.1	7.0 ±0.9
eNOS	**Control**	-0.2 ± 1.1	-0.8 ±2.2	-0.4 ±1.0	1.0 ±1.5	0.4 ±1.7	-1.2 ±3.0
	Salt	4.8 ±1.3	11.4 ±0.6 *	3.4 ±1.7	7.4 ±2.7 *	12.2 ±4.6 *	13 ±0.9 *
Week 5							
C57	**Control**	3.1 ±1.9	0.9 ±1.6	2.9 ±1.3	2.5 ±1.5	1.0 ±2.5	0.2 ±1.4
	Salt	2.7 ±0.9	6.3 ±1.2 *	1.5 ±1.0	6.5 ±1.0 *	3.8 ±1.6	6.4 ±1.5
eNOS	**Control**	4.6 ±1.8	0 ±2.7	3 ±1.3	3.2 ±1.0	5.4 ±3.4	-0.6 ±3.4
	Salt	11 ±3.3 *	17.3 ±4.1 *	6.8 ±3.3 *	11 ±5.4 *	18 ±3.3 *	19 ±4.3 *

*-$p<0.05$, significant difference from control period values (Dunnet's test).

Chapter 4: Discussion and Conclusion

A main objective of this study was to investigate the effect of a high salt diet on the MAP, HR and activity levels in eNOS knockout mice. A second objective was to describe the effect of a high salt diet on hemodynamic values in C57 mice. A final objective was to describe differences in basal MAP in eNOS-KO and C57 control mice. Our data show that eNOS knockout mice have a heightened salt sensitivity compared to the control C57 mice although the C57 mice display a slight degree of salt sensitivity. eNOS knockout mice also have a higher basal MAP when compared to C57 mice.

Relative to the observed changes in MAP and activity levels, changes in the other measured variables were less dramatic. The high salt diet had only a slight effect on the HR and pulse pressure. Salt-induced changes in HR were limited relative to the high absolute values of HR in mice. The C57-HS mice experienced a slight (not significant) increase in HR when the high salt diet was initially started whereas the eNOS-KO-HS mice exhibited a slight (not significant) decrease in HR at the onset of the high salt diet. In both C57 and eNOS-KO mice, pulse pressure exhibited a progressive increase on the high salt diet, reaching significance by the end of the experiment. It is possible that with a longer experimental protocol more dramatic changes might have been observed in the pulse pressure.

4.1 Salt Sensitivity in C57 Mice

The C57 mice exhibited a significant increase in MAP in response to the high salt

diet during the 6 weeks of exposure. Our results suggest that C57 mice are moderately salt sensitive. The maximum increase observed in the average 24h MAP was approximately 6 mmHg above control values. This extent of increase was observed during both the initial transient response to the high salt diet and also at the end of the progressive MAP increase seen in the last 3 weeks of the experiment. The modest extent of salt-induced changes in MAP in the C57 group (a.k.a. not entirely resistant to changes induced by high salt) suggests that they are an appropriate background strain with which to investigate the salt sensitivity caused by a knocked-out gene. If the C57 mice were highly salt resistant then it would be less likely that a knockout strain made with their genetic background would show salt-induced changes in BP even if the deleted gene played a role in controlling salt sensitivity. In other words, a highly salt resistant genetic background would have the potential to mask an effect of a gene on salt sensitivity. At the same time, the modest level of salt sensitivity of C57 mice would not make them an appropriate choice to investigate experimental treatments for salt sensitive hypertension. Because the salt-induced increases in MAP were only 6 mmHg above control levels, investigations of the effect of experimental treatments for salt sensitivity would need to be able to resolve changes in BP of only a few mmHg.

The salt induced increase in MAP appeared to have a two phase time course. Van Vliet et al (2005) suggested that there are two distinct phases of salt sensitivity in Dahl salt sensitive rats. Although the mechanisms behind each are unknown, the two phases appear to have a different genetic basis (Van Vliet et al, 2005). The initial increase in MAP in our C57 mice may be due to volume loading (Manning et al, 1979; Douglas et al,

1964). The subsequent decrease of MAP after approximately one week of salt exposure to almost normal levels suggests that the body adapts to the elevated salt and water intake. It is unclear exactly what physiological mechanism is responsible for returning the MAP back to normal levels. Osborn and Hornfeldt (1998) showed a similar decrease in MAP values in SD rats. When fed a high salt diet (4% NaCl for 3 weeks and then 8% NaCl for 3 weeks), an initial increase in MAP was gradually reduced to near normal levels after 3 weeks. In contrast, the return of MAP to normal levels did not occur in baroreceptor denervated rats (Osborn & Hornfeldt, 1998). This suggests that the baroreceptors are important in the regulation of MAP in the presence of a high salt diet. Because mice have a faster life cycle and metabolism than rats, it is plausible that such an effect of the baroreceptors may be evident by the end of the first week of exposure to high salt.

The second phase of the MAP response to high salt was a gradual and progressive increase in BP. Although the MAP observed in our C57 mice rose over the course of the experiment, the increase was modest but may have progressed further if the mice continued consuming a high salt diet. In Dahl salt sensitive rats this slow and progressive rise in BP appears to be largely irreversible (Van Vliet, unpublished data). Long term changes such as remodeling of the arteries, kidney damage and re-setting of the renal function curve may all contribute to the slow rise in BP (Lilly, 1998).

Previous studies have also suggested that C57 mice display a slight degree of salt sensitivity (Gros et al, 2002; Sugiyama et al, 2001; Carlson & Wyss, 2000). Carlson and Wyss (2000) examined the MAP in female C57 mice (n= 5, age unspecified) using

telemeters to monitor BP. The mice were originally fed a regular salt (0.6% NaCl) diet and then switched to a high salt (8% NaCl) diet. After one week on the high salt diet, telemeter recordings of MAP were conducted for a period of 4 days (Carlson & Wyss, 2000). In the present investigation, the male C57 mice experienced an increase of MAP of about 6%. Similarly, Carlson and Wyss (2000) found an increase of about 8%. Data from the latter study supports the idea that C57 mice are slightly salt sensitive but does not provide any concrete information regarding the time course of the salt induced augmentation of MAP. In our study the major increase in MAP occurred in the first week of salt exposure, whereas Carlson and Wyss (2000) did not begin to monitor MAP until 8 days after the mice were placed on the high salt diet. Carlson and Wyss (2000) found a significant increase in MAP during days 8-11 on high salt. In contrast, we found significant elevations in MAP on days 1-4 and at week 6 on high salt while no major changes were observed from day 5 to week 5. The difference in the gender of the mice may have contributed to these differences in results. Male mice appear to show a higher predisposition to salt sensitive hypertension (Gros et al, 2002). Gros et al (2002) demonstrated that 8 week old C57 male mice experienced a significant increase in systolic BP after 3 weeks on a high salt (8% NaCl) diet which was associated with a major loss of myogenic tone. In contrast, the equivalent female mice did not show an increase in systolic BP nor a decrease in myogenic tone after 7 weeks on the high salt diet (Gros et al, 2002).

Gros et al (2002) used the tail-cuff method to measure BP in order to determine the effect of either a normal (n=5) or 8% salt (n=5) diet on BP in male C57 mice (8 weeks of age). At week 3 of salt exposure, the mice on the high salt diet showed an average 18% increase in MAP (Gros et al, 2002). Sugiyama et al (2001) also examined the effect of salt on the MAP in male C57 mice (n=8). The latter study also used the tail cuff method for the measurements of MAP in C57 mice, 8 ± 1 week of age, given high salt water (1% NaCl) for 2 weeks. A significant increase of 14% in MAP at the end of the 2 week exposure to the high salt water was reported (Sugiyama et al, 2001). One reason for the differences in the results of the above studies and those of the present study may have been due to the methods used to measure BP (tail cuff compression versus telemetry). The results of the present investigation concur with the findings of the other studies (Gros et al, 2002; Sugiyama et al, 2001; Carlson and Wyss, 2000) indicating that C57 mice are modestly salt sensitivity.

At least one other study used C57 mice as a model of salt sensitivity (Lantelme et al, 2002). However, in the study by Lantelme et al (2002), salt sensitivity was not actually measured. The major problem with assuming that C57 mice are salt sensitive and using them as a comparison group against salt resistant mice is the risk of finding negative results simply because the overall salt effect on the MAP of C57 mice is modest.

Another study has suggested that C57 mice are salt resistant (Ni et al, 2003). Ni et al (2003) fed mice (n=8) a high salt diet (8% NaCl) for 7-10 days before measuring their MAP for 30 minutes using catheters. The mice, 7-8 weeks of age, showed an increase in MAP of about 11 mmHg which was reported as statistically insignificant. In

our present study, the total elevation of MAP was only 6 mmHg but this modest change was statistically significant. It has been reported by Van Vliet et al (2003) that the calculation of mean BP over extended periods of time (e.g. 12 or 24 hours) allows for less variation in reported values, smaller confidence limits and therefore an increased likelihood of detecting significant differences where they exist. Alternatively, extended sampling periods can allow smaller sample sizes to be used to test a hypothesis while maintaining adequate statistical resolution. Thus, the study by Ni et al (2003) may represent an example of the impact of the method of BP measurement on the overall results

Based on the data from our study, it can be suggested that telemetry allows for detailed characterization of BP in mice. Telemetry allows for the continuous measurement of hemodynamic and activity data in a free moving and relatively stress free animal. It is the recommended technique for accurate assessment of BP in experimental animals (Kurtz et al, 2005). If measurement techniques other than telemetry are to be employed, the timing of the measurements may be vital to the results. According to the present data, the most likely time period to observe the initial salt-induced elevations in MAP is within the first 5 days of exposure while long term notable increases in MAP may require 5-6 weeks of salt exposure to manifest.

In addition to the 24h MAP, the MAP during the light and dark periods of the day were also independently examined. Monitoring of the MAP during the light period indicated a biphasic response with statistically significant changes in the response seen at the start of week 1 and during week 6. In the dark period the increase in MAP was more

sustained with statistical significance occurring during week 1, 3, 4 and 6. The more consistently significant effect of salt in the dark period may be due to the nocturnal lifestyle of the mice. C57 mice are more active during dark periods and less active during light periods (Van Vliet et al, 2003). Van Vliet et al (2003) have suggested that activity has a pronounced effect on the MAP with higher MAP values being observed in the active periods than in the inactive periods. Other studies also suggest that mice are nocturnal animals with higher average MAP values in the dark phase of the day when the animals are presumably more active, have high metabolic needs and are consuming more feed (Mattson, 2001; Carlson & Wyss, 2000; Menton et al, 2000; Li et al, 1999). By extension, if mice are more active and consume more salt during the dark phase, this could in part explain the greater effect of the high salt diet on the dark period MAP.

Activity levels in the C57 mice also showed a tendency to be affected by the high salt diet. In the C57-HS mice, there was a tendency for an initial transient increase in the active time and the intensity of activity. This increase in activity may have reflected the mice searching for a more desirable food source than the newly started high salt diet. These initial increases in activity may have contributed to the initial BP response to salt. By increasing the time spent active, the mice would increase the time spent at the higher levels of MAP that are associated with activity, thus raising the overall 24h MAP.

4.2 Basal BP in eNOS knockout Mice

eNOS-KO mice had significantly greater basal MAP values than C57 mice. This was true for the dark and light periods as well as the 24h daily MAP values.

Furthermore, the eNOS-KO mice had significantly higher values for the daily range, minimum and maximum MAP which help explain why the basal BP was higher in eNOS-KO mice. These findings confirm previous studies showing that eNOS knockout mice have higher basal MAP values than C57 mice (Van Vliet et al, 2003).

As stated earlier, eNOS provides an important contribution to endothelial mediated vasodilation, blood vessel tone, systemic and renal hemodynamics, and NaCl re-absorption in the renal tubules (Kone, 2004; Ortiz et al, 2003; Torreilles, 2001; Braam et al., 2000). Knocking out the eNOS gene will likely impair each of these functions. Consequently, eNOS knockout mice are expected to have increased vascular tone and reduced renal excretory capacity. Together, these may augment ECFV, peripheral resistance and BP.

The elevated nature of the basal MAP in eNOS-KO mice in comparison to C57 mice has previously been shown in several studies (Labonte & D'Orleans-Juste, 2004; Van Vliet, 2003; Beierwaltes et al, 2002). The only other study to date that has examined basal hemodynamics in eNOS knockout mice using telemetry data was conducted by Van Vliet et al (2003). In the latter study, an increase in 24h MAP, daily range, maximum and minimum MAP and 24h pulse pressure were demonstrated (Van Vliet, 2003). In contrast to the data presented by Van Vliet et al (2003), the evidence from the present study did not show significant differences in 24h pulse pressure.

4.3 Salt Sensitivity in eNOS knockout Mice

eNOS-KO mice exhibited a significant rise in MAP when consuming a high salt diet. The 24h MAP of the eNOS-KO-HS mice was significantly increased throughout the experimental period and did not exhibit the decrease in MAP back to near normal values during the second week of the high salt diet as was observed in the C57 mice. Instead, the eNOS-KO-HS mice showed a sustained increase in MAP once the high salt diet was initiated. Overall, the two phases of salt sensitivity observed in the C57 control mice were not as evident in the eNOS-KO mice. It appeared that the mechanisms (i.e. ability of the C57 mice to effectively deal with salt induced volume loading) responsible for reversing the initial spike in MAP at about week 2 of high salt were not effective in the knockout strain.

Although both the C57 and eNOS-KO mice exhibited statistically significant increases in MAP with consumption of a high salt diet, the response of eNOS-KO mice was about 2-2.5 times greater than that of C57 mice. The augmented response of the eNOS-KO mice suggests that NO may normally play an important role in the control of BP and salt balance. The removal of the eNOS gene could affect the MAP and salt sensitivity by altering several mechanisms including blood vessel tone, endothelial mediated vasodilation, systemic and intra-renal hemodynamics, and NaCl re-absorption within the renal tubules and collecting ducts (Kone, 2004; Ortiz et al, 2003; Torreilles, 2001; Braam et al., 2000).

The MAP during both the light and dark periods of the day were independently examined in the eNOS-KO mice. Although a tendency for the MAP to increase was seen

in the light phase, the effect of salt on the MAP in the eNOS-KO mice was mainly confined to the dark phase. Overall, in both the C57 and eNOS-KO mice, the MAP response to high salt appeared most pronounced during the dark phase of the day. As mentioned earlier, the nocturnal lifestyle of the mice, as well as the increased activity and food intake during the dark phase of the day may explain the increases in MAP seen in the dark period.

The eNOS-KO mice also exhibited an increase in the range and daily minimum and maximum MAP values. At the onset of the high salt diet there was a tendency for the range and maximum MAP values to increase and by the end of the experimental period all three variables were significantly elevated above control levels. In contrast, the daily range, maximum and minimum MAP in the C57 mice showed a tendency to increase but not reach statistical significance. Because the daily maximum increased more than the daily minimum in both the C57 and eNOS-KO mice, there was a resultant increase in the daily range of the MAP. The increase in the daily maximum MAP may have reflected an increased reactivity or variability of BP in response to the high salt diet.

4.4 The Contribution of Activity to Changes in MAP

The activity levels of the C57 and eNOS-KO mice were both elevated by the high salt diet. The change in active time in response to a high dietary salt intake was most pronounced during the dark phase of the day. The increase in active time was more sustained in the eNOS-KO mice than in the C57 mice during the dark phase. In contrast

to the C57 mice, no significant differences were observed in the effect of high salt on the intensity of activity in the eNOS-KO mice. By spending a greater proportion of time active, the overall 24h MAP would be expected to increase because more time would be spent at the higher MAP level (rather than at the lower MAP levels associated with inactivity). Thus, at least part of the increase in MAP was due to the increases in the proportion of time spent active. However, as discussed below, much of the increase in MAP appears to have been independent of activity.

The MAP levels associated with periods of activity and inactivity are important components that affect the overall 24h MAP. "MAP_{ACTIVE}" refers to the average mean arterial pressure of the animal during periods of locomotor activity (activity signal > 0). The "$MAP_{INACTIVE}$" refers to the average mean arterial pressure when the animal is inactive (activity signal = 0). Through out the day, the mice frequently alternate between periods of activity and inactivity with greater periods of activity and higher average MAP values being observed during the dark phase of the day. Both strains of mice exhibited an increase in MAP_{ACTIVE} following both short (wk 1) and long (wk 5) term exposure to the high salt diet. As well, the $MAP_{INACTIVE}$ (basal) of the eNOS-KO mice was augmented by exposure to salt with greater effects seen during the latter phase of the experiment.

The eNOS-KO mice showed a significant increase of the MAP_{ACTIVE} in the 24h, light and dark periods, whereas the C57 mice only exhibited significance differences in the 24h and light periods with a tendency seen in the dark phase. The elevation of the MAP_{ACTIVE} suggests that not all the MAP increases observed with the high salt diet can

be explained by an increase in the amount of time spent active. The increase in MAP_{ACTIVE} is independent of an increase in active time because for the MAP_{ACTIVE} to increase the actual MAP when the animal is active has to be augmented. That is, an increase in MAP_{ACTIVE} will increase the overall 24h MAP, independent of the increases in active time.

Both the C57 and eNOS-KO mice increased their active time and MAP_{ACTIVE} once the high salt diet was initiated, suggesting that these occurrences contribute to the initial increase in 24h MAP seen in the early phase of exposure to salt. As the experiment progressed, MAP remained elevated even though active time returned to near normal values. Thus, the increase in MAP was independent of any change in activity. In the C57 mice, the initial salt induced increases seen in MAP may be related to both the initial increases in activity and also the increase in MAP_{ACTIVE} seen when the animals were exposed to a high salt diet. The latter elevation of the C57-HS mice MAP is likely due to the change in the MAP_{ACTIVE}. The increased activity as well as the elevated MAP_{ACTIVE} in the eNOS knockout mice may account for the initial increase seen in MAP within the first week of salt exposure. By week 5 of dietary salt exposure, increases are exhibited in both MAP_{ACTIVE} and $MAP_{INACTIVE}$ suggesting that the animals were hypertensive irrespective of activity.

To conclude, it appears that the initial salt-induced increases in MAP are a result of the augmented active time in combination with increases in the average MAP level during periods of activity and inactivity. By the end of the experiment, the active time and intensity of activity had returned to near normal levels, suggesting that the final

increase in 24h MAP must be largely independent of the time spent active. C57 mice showed less of an increase in MAP when exposed to salt. This may explain why they also showed less elevation in both MAP_{ACTIVE} and $MAP_{INACTIVE}$ throughout the experiment. In eNOS-KO mice, the salt-induced increases in the 24h MAP were more affected by variables other than activity (e.g. cardiovascular consequences of eliminating eNOS), giving further support to the important role eNOS plays in controlling the MAP when salt is high in the diet.

4.5 Role of eNOS in Salt Sensitivity

Salt sensitive hypertension has, in several studies, been shown to be affected by the presence or absence of NO. A rise in NO production normally occurs in response to an increase in dietary salt intake in normal rats whereas the increase in NO production with high salt appears to be attenuated in salt sensitivity hypertension (Kiraku et al, 1999). Manning et al (2001) demonstrated that Dahl salt sensitive rats had a lower production of NO than their salt resistant counterparts. Furthermore, inhibition of NO production leads to salt sensitive hypertension. Several studies have shown that the administration of a NOS inhibitor to rats results in salt sensitivity (Tan et al, 2000; Rudd et al, 1999; Yamada et al, 1996; Tolins & Shultz, 1994). A final piece of evidence to support the importance of NO in salt sensitive hypertension in that increases in NO production can lead to the correction of salt sensitivity (Manning et al, 2001; Miyata & Cowley, 1999). In Dahl salt-sensitive rats, the administration of L-arginine can restore

the amount of NO excreted/produced to the level seen in salt-resistant rats eliminating the effect of salt on the MAP in the salt sensitive animals (Manning et al, 2001).

In the present study, removal of the ability of NO to be produced by eNOS caused salt sensitive increases in BP to develop with a greater intensity than seen in control C57 mice. While other investigators have examined the role of nNOS and iNOS, the role of eNOS has remained relatively unclear because selective eNOS inhibitors are not available. By using eNOS-KO mice to examine salt sensitive hypertension we were able to look closely at the influence of this NOS isoform on MAP and salt sensitivity.

As discussed above, eNOS-KO mice demonstrated a higher basal MAP level in the control period. This signifies the importance of NO produced from eNOS on the normal control of BP. Once the high salt diet was initiated, the eNOS-KO mice exhibited a further increase in MAP that was 2-fold greater than the C57 mice. This affords evidence to the importance of eNOS in providing a normal response to a high salt diet. Both eNOS-KO mice as well as rats treated with L-NAME experience an increase in MAP when exposed to a high salt diet (Tan et al, 2000; Rudd et al, 1999; Yamada et al, 1996; Tolins & Shultz, 1994). Since an increase in MAP was observed in the absence of eNOS as well as when all isoforms of NOS were inhibited, this suggests that NO produced from eNOS is at least partially responsible for the control of BP and salt balance.

Recent studies have demonstrated that eNOS gene polymorphisms (variations in the alleles of the gene) are present in a number of people with hypertension and other cardiovascular diseases (Wattanapitayakul et al, 2001; Hingorani, 2000). My results

suggest that the presence of an eNOS gene polymorphism associated with reduced eNOS function may in fact increase the susceptibility of these individuals to salt sensitive hypertension.

4.6 The "Renal Function Curve" in C57 and eNOS knockout Mice

Historically, the relationship between salt intake/output and the MAP has been graphically depicted as the 'Renal Function Curve' (Norman et al, 1980). This curve represents the steady state balance between salt intake/output and MAP. Because it represents the steady state, it reflects the influence of every physiological mechanism in the body that effects salt excretion and MAP, not just the influence of the kidney.

Using data from the present study, renal function curves were created to provide a graphical summary of the basal MAP and the BP response to salt in both the C57 and eNOS-KO mice (figure 35). Salt intakes for the renal function curve were estimated in terms of the number of mEq/kg body weight/day consumed during the control period and during the first week of the experimental diet. These intakes were then plotted against the MAP level at the end of the control period and first week of experimental diet. The points on the graph represent the MAP at a given level of sodium intake under steady state conditions (i.e. after 1 week on a given level of salt intake). The increased basal MAP of the eNOS-KO mice on the regular salt diet is apparent as the rightward shift of the curve (point C in comparison to point A). As has been described by Van Vliet and Montani (2005), a rightward shift of the renal function curve will result in a higher equilibrium BP level and hypertension. Once the sodium content of the diet was

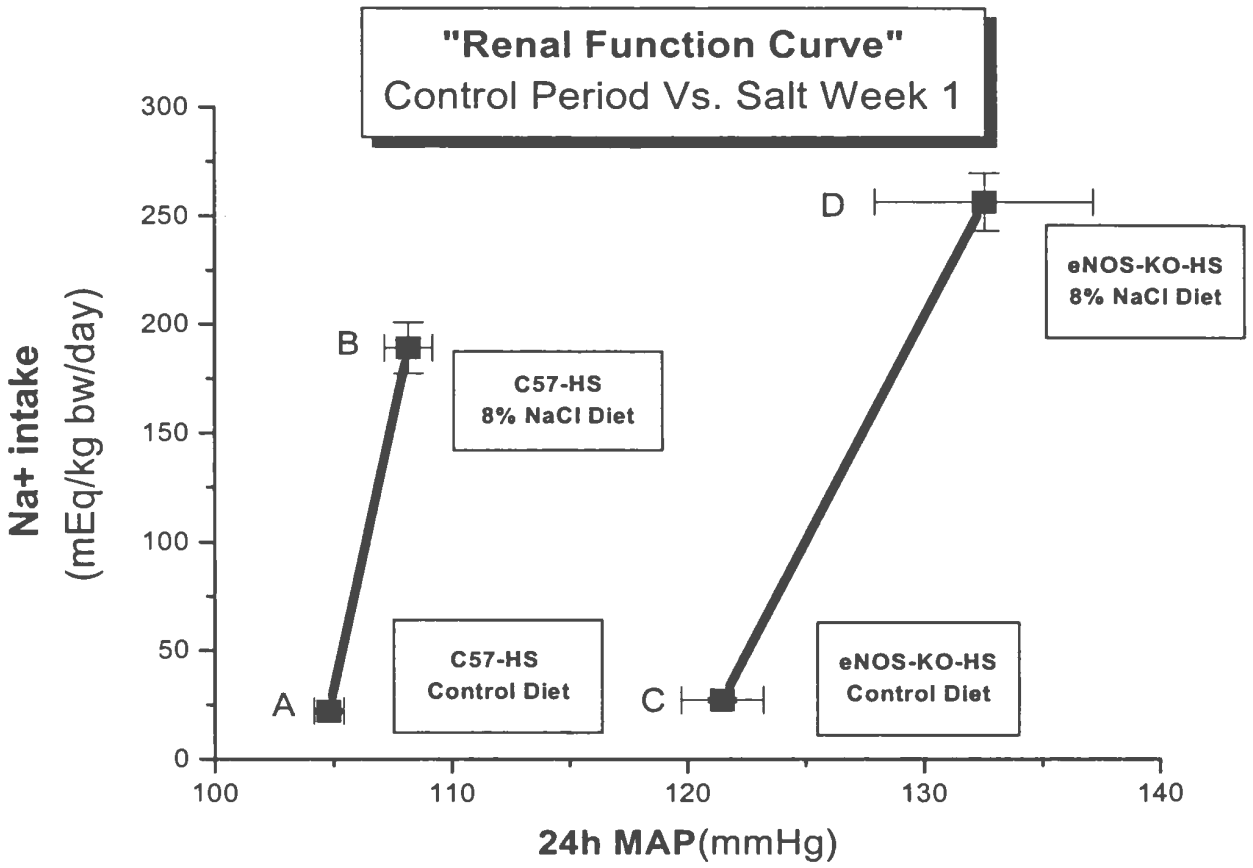

Figure 35: Renal function curve of C57 and eNOS knockout mice. The lines on the graph represent the MAP at a given sodium intake level. The near vertical line in the case of the C57 mice indicates a modest salt sensitivity whereas the shallow slope of the eNOS-KO group represents an enhanced effect of salt on the MAP. The rightward shift of the renal function curve of the eNOS-KO mice represents an elevated basal MAP when compared to C57 mice.

increased, the MAP of the eNOS-KO mice increased along the slope of the renal function curve from point C to point D. The renal function curve of the eNOS-KO mice has a slope of 20.7 ± 7.9 which is significantly different than the slope of the renal function curve of the C57 mice (49.5 ± 5.2). The relatively steep line in the case of the C57 mice indicates a modest level of salt sensitivity, as a vertical renal function curve indicates complete salt insensitivity (Van Vliet & Montani, 2005). In contrast, the eNOS-KO mice have a renal function curve with a shallower slope, indicative of their greater salt sensitivity. In summary, eNOS-KO mice have an elevated basal MAP as is evident by the rightward shift of their renal function curve and an increased BP response to salt which is seen in the shallow slope of their renal function curve.

Although it has now been shown that eNOS-KO mice are salt sensitive and it can be assumed that it is the absence of the eNOS gene that accounts for the augmented MAP response to the high salt diet, the exact mechanisms that cause this increase to occur are unknown. It can be speculated that one or more of the many physiological systems affected by eNOS could play a role in the elevated basal MAP and the augmented salt sensitivity. For example, increases in MAP could be due to an effect on blood vessel tone and or reactivity, renin secretion or tubular re-absorption (Ortiz et al, 2003; Parzak et al, 2001).

4.7 Conclusion

eNOS-KO mice exhibited an augmented basal MAP and also a heightened level of salt sensitivity compared to the control C57 mice. The short term increase in the

proportion of time spent active likely contributed to the initial rise in MAP observed once the high salt diet was initiated. The long term increase in the MAP appeared to be highly independent of the amount of time spent active.

Previous studies have shown that NO plays a role in the body's normal response to salt and impairment of this action is a factor in salt sensitivity. These studies have shown that the MAP increased in the presence of a high salt diet when a non selective NOS inhibitor was administered. Studies on eNOS gene polymorphisms also suggest that the presence of an irregular eNOS gene increases the occurrence or risk of hypertension and cardiovascular disease (Wattanapitayakul et al, 2001; Hingorani, 2000), though an effect on salt sensitivity has not been investigated. Our study demonstrated that the selective removal of eNOS caused an increase in basal MAP and salt sensitivity. Our results suggest that eNOS may play an important role in salt sensitive hypertension.

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