Nitric oxide impairs baroreflex gain during acute psychological stress

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ABSTRACT

Psychological stress can suppress baroreflex function, but the mechanism has not been fully elucidated. Nitric oxide in brain and the adrenal cortex, as well as plasma glucocorticoids, increase during stress and have been shown to suppress reflex gain in unstressed animals. Therefore, the purpose of this study was to test the hypothesis that stress, caused by exposure to a novel environment, decreases baroreflex gain in rabbits through the actions of nitric oxide to increase corticosterone release. Baroreflex control of heart rate and plasma corticosterone levels were quantified before and after blockade of nitric oxide synthase (NOS) with Nω-nitro-L-arginine (L-NNA, 20mg/kg, iv) in conscious rabbits exposed to a novel environment and the same rabbits once they had been conditioned to the environment. Stress significantly reduced baroreflex gain from −23.4 ± 2 to −12.2 ± 1.6 beats per min (bpm)/mmHg (p<0.05) and increased plasma corticosterone levels from 5.4 ± 0.7 to 15.5 ± 5.0 ng/ml (p<0.05). NOS blockade increased gain in stressed animals (to -27.2 ± 5.4 bpm/mmHg, p<0.05) but did not alter gain in unstressed rabbits (-26.8 ± 4.9 bpm/mmHg), such that gain was equalized between the two states. NOS blockade increased plasma corticosterone levels in unstressed animals (to 14.3 ± 2.1 ng/ml, p<0.05) but failed to significantly alter levels in stressed rabbits (14.0 ± 3.9 ng/ml). In conclusion, psychological stress may act via nitric oxide, independently of increases in corticosterone, to decrease baroreflex gain.

Keywords: Conscious rabbits, corticosterone, novel environment, Nω-nitro-L-arginine.
INTRODUCTION

Psychological stress can exert profound effects on the cardiovascular system, causing increases in sympathetic activity, blood pressure, and heart rate [for review see (42)] and decreases in the sensitivity or gain of the baroreflex (17; 37; 55; 56). Persistent psychological stress may be a contributing factor to hypertension and heart failure (16); acute stress is linked with myocardial infarction (22; 54). Interestingly, these diseases are also associated with decreases in baroreflex gain (2; 11), and, in the case of myocardial infarction, a decrease in baroreflex gain is linked to an increased death rate (3; 52). Importantly, improving baroreflex gain leads to decreased mortality in these patients (32). Despite the potential deleterious effects of stress-induced decreases in baroreflex gain, relatively little is known about the mechanism.

Indirect evidence suggests that nitric oxide, a gaseous neurotransmitter, may be involved. Endogenous nitric oxide can act in the brain to modulate central control of the cardiovascular system [see (29) for review] and can decrease baroreflex gain (33; 40; 41). Acute stress increases the activity of neurons containing nitric oxide synthase (NOS) activity in brain regions involved in cardiovascular regulation (23; 30). In addition, in rats, acute stress increases nitric oxide production in the brain stem and hypothalamus, in particular the paraventricular nucleus (PVN) (13; 27). A positive correlation exists between the increase in nitric oxide in the PVN and the increase in blood pressure during stress (27).

In addition to central actions, stress-induced nitric oxide may also act peripherally to alter the cardiovascular system. Nitric oxide production is increased in the adrenal cortex during stress (58) and can stimulate the release of corticosterone from isolated rat adrenals (39; 44). Since glucocorticoids can decrease baroreflex gain (50), nitric oxide may exert its effects in part through...
increased glucocorticoid production. Despite these data, whether nitric oxide contributes to the decrease in reflex gain caused by stress has not been previously investigated.

Therefore, we tested the hypothesis in conscious rabbits that acute stress caused by exposure to a novel environment decreases baroreflex gain via a nitric oxide-mediated mechanism, and that part of this mechanism involves increased plasma corticosterone levels. To test this hypothesis, we determined if rabbits exhibited a lower baroreflex gain on the first day they were placed in a testing box (stressed), compared to their baroreflex gain after conditioning to the box (unstressed). We also determined if systemic blockade of NOS in stressed rabbits decreased plasma corticosterone levels as it restored baroreflex gain.

METHODS

All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Female (\(n = 10\)) New Zealand White rabbits (Western Oregon Rabbit; Philomath, OR) weighing 3.7 ± 0.1 kg on the first experimental day were used for these experiments. The rabbits were received when they were 14 weeks old, and allowed a minimum of 6 days to acclimate to the laboratory environment before surgery.

Surgical preparation. Surgery was performed to implant non-occluding abdominal aortic and vena caval catheters as previously described (20). Briefly, the animals were initially anesthetized with a cocktail containing ketamine (58.8 mg/kg), xylazine (5.9 mg/kg), and acepromazine (1.2 mg/kg) administered subcutaneously. They were intubated, placed on a respirator, and ventilated with 100% oxygen throughout the surgery. A surgical plane of
anesthesia was maintained with ketamine (10mg/ml) administered intravenously as needed. A midline abdominal incision was made, and polyethylene catheters with Silastic tips were implanted in the abdominal aorta (one) and vena cava (two). The catheters were tunneled subcutaneously from the abdominal cavity, and exited at the nape of the neck.

After a minimum 2-wk recovery period, a second surgery was performed to implant the vena caval occluder used to decrease mean arterial pressure in the baroreflex protocols. As we were interested in the effects of nitric oxide on the baroreflex, we avoided using the nitric oxide donor, sodium nitroprusside, to lower pressure. We have found that baroreflex curves generated in pregnant and non-pregnant rabbits exhibit similar parameters whether using an occluder (present results) or sodium nitroprusside infusion (6) to decrease pressure. Rabbits were initially anesthetized with ketamine (250mg, sc), intubated, placed on a respirator and ventilated with 100% oxygen; a surgical plane of anesthesia was maintained with isoflurane (2%). An occluder was implanted around the thoracic inferior vena cava via a right thoracotomy through the fifth intercostal space. The distal end of the occluder again exited at the nape of the neck, and the incision was closed in layers.

The ends of the occluder and catheters were protected in a plastic pillbox, which was sutured to the rabbits' skin. The rabbits were given an intramuscular injection of enrofloxacin (22.7 mg) just before each surgery and intravenous injections of this antibiotic for the four days following surgery. The animals also received buprenorphine hydrochloride (0.09 mg sc) 2-3 h after surgery, and again the next day to minimize pain. The catheters were flushed immediately after surgery and then 3 times weekly with the use of sterile 0.9% saline, and filled with heparin (1,000 U/ml) to maintain patency.
Baroreflex curve generation. On the experimental day, the rabbits were placed in a 37x17x21 cm black Plexiglas box, with screened openings to prevent overheating, and were allowed approximately 30 min to settle. Mean arterial pressure (MAP) and heart rate (HR) were measured continuously via the aortic catheter using a Statham pressure transducer, a Grass tachometer, Grass polygraph and a Biopac (Santa Barbara, CA) MP100 data acquisition and analysis system. To determine the baroreflex relationship between MAP and HR, and to ensure steady-state changes in both the sympathetic and parasympathetic nervous system (14), a slow ramp method of altering MAP was performed. Arterial pressure was lowered via inflation of the occluder around the vena cava until HR reached maximum values. MAP was lowered at an average rate of 0.51 ± 0.07 mmHg/s, and this part of the baroreflex curve was generated in 57 ± 5 s (Figure 1). MAP was raised by infusing increasing doses of phenylephrine (0.5, 1, 2, 4, and 8 μg · kg⁻¹ · min⁻¹), increasing the dose every 15 s until MAP was increased by 20 mmHg over basal (6) (Figure1). This took 59 ± 4 s. Therefore, the entire curve was generated in 116 ± 7 s. More than one occluder inflation was performed in each experiment, and at least 10 minutes was allowed between each inflation to allow MAP and HR to return to basal conditions.

Protocol. In order to eliminate the physical stress of surgery as a contributing factor, two weeks were allowed after the final surgery before first exposure to the experimental Plexiglas box. In most rabbits, this first exposure to a novel environment was stressful as indicated by a HR higher than 170 bpm. Only rabbits that exhibited this high HR were considered stressed. Baroreflex curves were then generated in five rabbits before and 105-120 min after bolus intravenous administration of the non-specific NOS inhibitor No-nitro-L-arginine (L-NNA, 20 mg/kg).
Following this experiment, rabbits were conditioned to the environment of the experiment by placing them in the box for 2-5 hours a day for at least 5 days. Some rabbits required longer conditioning periods of up to 25 days. Rabbits were considered conditioned and unstressed when HR was less than 170 bpm. At this point the experiment was repeated, and baroreflex curves were again generated before and 107-144 min after the administration of L-NNA.

**Plasma corticosterone measurements.** To determine if blockade of nitric oxide returns plasma corticosterone levels to normal during stress, in five additional rabbits, blood samples were collected for plasma corticosterone measurements. Samples (3 ml) were drawn from the arterial catheter while the animals were resting quietly before and 90-120 minutes after L-NNA on the first day they were placed in the experimental box. Samples were also collected after they had been conditioned to the box. Again, conditioning lasted 1-4 weeks, until resting HR was less than 170 bpm. Blood was replaced with an equal volume of sterile isotonic saline. Samples were placed into chilled heparinized tubes and centrifuged at 4°C. Plasma was stored at -20°C until assayed. Corticosterone levels were determined by the Endocrine Services Laboratory of the Oregon National Primate Research Center by radioimmunoassay (RIA). Fifty µl of rabbit serum was first diluted in 950 µl of redistilled ethanol. The samples were centrifuged to pellet precipitated proteins, and 50 and 100 µl of ethanol aliquots were dried in 13 x 100 mm assay tubes using room air. RIA of these reconstituted samples utilized a corticosterone antisera as described previously (21). Solvent blanks were routinely less than 5 pg/tube and the intra- and inter-assay coefficients of variation did not exceed 9%.

**Data Analysis.** Data for the baroreflex curves were collected at 200 Hz and processed using a Biopac (Santa Barbara, CA) MP100 data acquisition and analysis system. Raw data were
grouped into 1-s bins from which mean values were obtained. Since more than one curve was generated using the vena caval occluder, curves that were free from movement artifact and exhibited the best sigmoidal fit were selected for further analysis (see Figure 2 for representative curve). Often the basal MAPs and HRs before the pressor (phenylephrine) part of the reflex curve were slightly different from the depressor (occluder) part. When this occurred, to avoid erroneous measurements of baroreflex gain, half of the pressure difference was added to all pressure values in the segment with the lower basal pressure and half the difference was subtracted from the pressures in the segment with the higher pressure, so that the two segments were aligned, as previously described (15). The same was done for HR.

The sigmoidal baroreflex relationships between MAP and HR generated in each experiment were fitted and compared using the Boltzmann sigmoidal equation (HR=A+B/1+e(C-MAP)/D, where A equals the minimum HR, B equals the HR range, C equals the MAP at the midpoint between the minimum and maximum HR, and D is the slope coefficient). Maximum gain was calculated by dividing the HR range by four times the slope coefficient. Because of the exponential nature of baroreflex gain and the high variability of plasma corticosterone levels in stressed animals, the log of these parameters was used for statistical analysis.

Basal MAPs, HRs, curve fitting parameters, and plasma corticosterone levels were compared between groups using ANOVA for repeated measures and the post-hoc Bonferroni test. All data are reported as mean ± standard error mean. In the figures, the sigmoidal curves were derived by averaging the curve parameters from all animals and are shown along with basal points ± standard error mean.
RESULTS

Effects of stress. Animals exposed to the novel environment exhibited a higher basal MAP (73 ± 4 mmHg, p<0.05), HR (185 ± 6 bpm, p<0.05) and plasma corticosterone levels (15.5 ± 5.0 ng/ml, p<0.05) compared to after conditioning (65 ± 2 mmHg, 159 ± 3 bpm, 5.4 ± 0.7 ng/ml). Baroreflex gain (Table 1, Figure 3A) was suppressed in the rabbits when they were first exposed to the experimental environment, compared to after conditioning. Stress also increased the midpoint of the baroreflex curve (BP50) and the minimum heart rate (Table 1, Figure 3A).

Effects of NOS blockade on the baroreflex control of HR and corticosterone levels. The effect of L-NNA on baroreflex control of HR was examined in the same rabbits before and after conditioning to the experimental environment. L-NNA increased basal MAP (p<0.05) and decreased basal HR (p<0.05) in animals in both the stressed (MAP to 84 ± 2 mmHg; HR to 118 ± 3 bpm) and unstressed states (MAP to 80 ± 3 mmHg; HR to 116 ± 3 bpm). The increase in MAP was less and the decrease in HR was greater when the animals were stressed (P<0.05), so that after L-NNA, neither basal MAP nor HR were different between the stressed and unstressed states (Figure 3B).

In support of the hypothesis, blockade of NOS in stressed animals increased baroreflex gain (Table, Figure 4B); however, there was no effect of NOS blockade on baroreflex gain in unstressed animals (Table, Figure 4A). In animals in both the unstressed and stressed states NOS blockade decreased the minimum HR and increased the BP50 (Table, Figure 4). After L-NNA, baroreflex curves were superimposed, and baroreflex parameters in the stressed and unstressed states were not different (Table, Figure 3B).
Contrary to our hypothesis, blockade of NOS did not decrease corticosterone levels in stressed animals (14.0 ± 3.9 ng/ml). Moreover, L-NNA administration in unstressed rabbits increased plasma corticosterone levels (to 14.3 ± 2.1 ng/ml, p<0.05).

**DISCUSSION**

The purpose of this study was to investigate the role of nitric oxide in the decrease in baroreflex gain during psychological stress. The novel findings are 1) acute psychological stress decreases gain in rabbits, 2) this decrease is completely reversed by blockade of NOS, and 3) blockade of NOS increases corticosterone levels in unstressed but not stressed rabbits. These data support the hypothesis that stress decreases baroreflex gain through actions of nitric oxide, but that these actions are independent of changes in corticosterone release.

Previous studies have shown that exposure to a novel environment induces stress in rats as demonstrated by an increased MAP and HR (59), as well as increased plasma corticosterone levels (36). Rabbits exhibit similar responses, including increases in MAP, HR and plasma corticosterone levels on the first day they were placed in the experimental box as compared to when they had been conditioned. In addition, we observed that this stress was associated with depressed baroreflex gain.

Several previous studies have investigated the effects of acute stress on baroreflex gain in humans (1; 48; 55; 56), rats (24; 43) and rabbits (49). While many of the human studies demonstrated a decrease in baroreflex gain with stress, studies in rats and rabbits usually reported no change or an increase in baroreflex gain. The discrepancy between the present study and the previous study of Schadt et al. (49), who found no effect of stress on baroreflex gain in rabbits, may be due to differences in the type of stress employed. The earlier study examined the
effects of airjet and shaker stress, which may evoke different emotional responses than that of exposure to a novel environment. Sawada et al. (48) found that various types of stress differentially altered baroreflex gain in humans. Exposure of their subjects to intermittent pink noise or the cold pressor test caused a decrease in gain, whereas isometric handgrip and mental arithmetic did not affect gain.

Previous studies used male rabbits (49) and rats (24), whereas we used female rabbits; therefore, the action of stress on the baroreflex in females may differ from males. We have observed that female rabbits tend to exhibit higher baroreflex gain (6; 8; 9) compared to male rabbits (7), and Steptoe et al. (82) have demonstrated a similar phenomenon in humans. Nevertheless, these authors further reported that men and women responded with similar reductions in baroreflex gain during stress (82). Thus, gender differences do not appear to underlie the discrepancies between the present study and previous animal work.

One final difference between the current study and previous studies in rabbits and rats was the method used to generate baroreflex curves. Schadt et al. (49) and Hatton et al. (24) quantified HR responses to rapid changes in blood pressure produced by bolus injections of phenylephrine and nitroprusside. Rapid pressure changes may not allow enough time for the sympathetic nervous system to completely respond so that primarily vagal-mediated alterations in HR are observed (14). In the current study, occluder inflations and phenylephrine infusions were purposely performed slowly to allow complete sympathetic activation and withdrawal.

The major purpose of the present study was to determine the mechanism by which stress decreases baroreflex gain. Previous studies have shown that NOS activity can decrease baroreflex gain (4; 31; 33; 38; 41), but this study is the first to demonstrate that nitric oxide underlies impaired baroreflex function during stress. In fact, after NOS blockade, baroreflex
curves of animals in the stressed and unstressed state were superimposed, suggesting that stress-induced increases in NOS activity may be entirely responsible for the decrease in baroreflex gain. One complicating factor, however, is that the control and L-NNA experiments were conducted sequentially in the stressed rabbits. As a result, post-L-NNA curves were constructed after the rabbits had been in the experimental box for approximately three hours. Thus, a possibility that should be considered is that the rabbits acclimated to the novel environment and, therefore, were no longer stressed during the second curve generation. However, we have observed that several exposures to the experimental box are required before a rabbit exhibits HR below 170 bpm. In the present study, for example, HR was reexamined after 5-10 days of conditioning to the box. Of ten animals, only four demonstrated reduced HR within this time frame. The other six rabbits required between 11 and 25 days (average = 18 ± 2 days) of exposure before their HRs dropped into the non-stressed range below 170 bpm. Therefore, it is highly unlikely that baroreflex gain increased because of rapid acclimatization to the new environment, rather than to blockade of NOS. Thus, we conclude that nitric oxide contributes to impaired baroreflex function induced by stress.

One potential site of action of nitric oxide is the adrenal cortex. Immobilization stress increases NOS mRNA and protein levels as well as NOS enzyme activity in the adrenal cortex of rats (58). Moreover, nitric oxide contributes to increased glucocorticoid release in response to adrenocorticotropic hormone (ACTH) (39), which is increased by stress. Scheuer et al. (50; 51) report that administration of corticosterone in rats decreases baroreflex gain. In addition, blockade of cortisol production in humans before exposure to a stressful stimulus prevented the decrease in baroreflex gain normally observed (5). Therefore, we hypothesized that blockade of
NOS could improve gain in stressed animals in part by decreasing adrenal glucocorticoid production.

The current study did not support this hypothesis, however. While stress increased corticosterone levels, blockade of NOS did not reverse this response. Moreover, NOS blockade increased plasma corticosterone levels in unstressed animals. These paradoxical findings likely reflect the complex interactions between nitric oxide, ACTH and adrenal glucocorticoid production \textit{in vivo}. In mice, systemic blockade of NOS also increased plasma ACTH and corticosterone levels (19), demonstrating that nitric oxide tonically suppresses basal ACTH/glucocorticoid release. On the other hand, nitric oxide has been reported to both enhance and inhibit ACTH responses to stress [for reviews, see (45; 46)]. It has been suggested that the ultimate response is determined by the balance between opposing actions of nitric oxide, which in turn are influenced by the type and intensity of stress (46). Because NOS blockade did not alter plasma corticosterone levels in rabbits exposed to a novel environment, it appears the net effect of nitric oxide on these divergent pathways is nil.

Nitric oxide synthase inhibitors such as L-NNA can cross the blood-brain-barrier to inhibit NOS in the brain (46; 57). Therefore, L-NNA may increase baroreflex gain during stress by blocking the effects of nitric oxide in the brain. Both the PVN (12; 25; 26) and nucleus of the solitary tract (NTS) (35) are known to influence gain. Moreover, NOS-containing neurons in both brain regions are activated following stress (30). Stress increases PVN nitric oxide production and NOS mRNA levels (10; 13; 27; 28), and nitric oxide can act in the NTS (41; 53; 60) to decrease baroreflex gain. Thus, either or both of these brain regions may be involved, but further experiments are required to test this hypothesis.
In conclusion, in rabbits, exposure to a novel environment decreases baroreflex gain, which is mediated by actions of nitric oxide independent of changes in corticosterone levels.

**Perspectives**

Cardiovascular disease is the leading cause of morbidity and mortality in developed countries (34), and psychological stress is a known risk factor for cardiovascular disease (18; 47), but the mechanism for this link is not clear. Acute stress can increase sympathetic nerve activity, blood pressure, and heart rate (42) thus increasing the load on the heart and perhaps contributing to cardiovascular disease. Changes in baroreflex sensitivity may also play a role in the pathogenesis of this disease since a decrease in baroreflex sensitivity has been suggested as a predictive factor in the mortality following a myocardial infarction (3; 52). This study provides a possible mechanism for the decreased baroreflex gain during an acute psychological stress. Stress is often an unavoidable fact of life and understanding mechanisms can lead to possible therapeutic interventions to reduce the morbidity and mortality of psychological stress.
GRANTS

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REFERENCES


FIGURE LEGENDS

Figure 1. Representative tracing of changes in mean arterial pressure (MAP) and heart rate (HR) in one non-stressed, control rabbit during occluder inflation (A) and phenylephrine infusion (B).

Figure 2. Representative baroreflex curves. Curves generated in one rabbit in the unstressed (A) and stressed (B) states before and 100-120 minutes after L-NNA. Large grey points illustrate resting parameters.

Figure 3. Effect of stress on the baroreflex control of heart rate in rabbits before (A) and after (B) blockade of nitric oxide synthase (L-NNA).

Figure 4. Effect of nitric oxide synthase blockade (L-NNA) on the baroreflex control of heart rate in rabbits in the unstressed (A) and stressed (B) states.
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*p<0.05, unstressed versus stressed
#p<0.05, before versus after L-NNA
Figure 2

A

Unstressed L-NNA

Unstressed Control

Unstressed L-NNA

B

Stressed Control

Stressed L-NNA

Heart Rate (beats/min)

Mean Arterial Pressure (mmHg)
Figure 3

A

Heart Rate (beats/min)

Mean Arterial Pressure (mmHg)

- Unstressed Control
- Stressed Control

B

Heart Rate (beats/min)

Mean Arterial Pressure (mmHg)

- Unstressed L-NNA
- Stressed L-NNA
Figure 4

A

- Unstressed Control
- Unstressed L-NNA

B

- Stressed Control
- Stressed L-NNA

Mean Arterial Pressure (mmHg)

Heart Rate (beats/min)