L-NAME differentially alters ventilatory behavior in Sprague-Dawley and Brown Norway rats

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Our laboratory has previously described two strains of rats, Sprague-Dawley (SD) and Brown Norway (BN), with markedly different ventilatory responses to steady-state hypoxia and hypercapnia (23). Differences are present in the ventilatory response to rapid reoxygenation after brief hypoxic exposure, i.e., Dejours phenomenon (25). SD exhibit a tendency for short-term potentiation, whereas, in the BN, a tendency for post-hypoxic frequency declines (PHFD). These observations are consistent with the concept that inherited factors determine such differences. Indeed, for steady-state responses, an intercross of SD and BN results in second-generation progeny with a wider range of ventilatory chemosensitivity than is present in either pa-
rental strain (24). This information indicates that, in the rat, naturally occurring NOS polymorphisms may shape such differences; however, direct evidence of this is lacking. A physiological approach to this issue is to determine the effects of a NOS inhibitor in the two strains (11). Administration of a broad NOS inhibitor to unanesthetized SD and BN would address the overall influence of NOS on differences in the ventilatory responses that inheritance currently provides in these two rat strains.

We hypothesized that NOS blockade would produce different effects on ventilatory behavior in these two rat strains in the unanesthetized state. If the null hypothesis is rejected by evidence showing differences, then one could reason that the genetic background of the animals plays a role in the pharmacological effect.

**METHODS**

Adult male SD (SD/Harlan; n = 8) and BN (BN-SSN/Harlan; n = 8) between the ages of 12 and 16 wk were obtained from Harlan (Indianapolis, IN), housed in our animal facility, and fed rat chow and water ad libitum. The animals were tested 8–10 wk after arrival. The study protocol was approved by the Louis Stokes Veterans Affairs Medical Center Institutional Animal Care and Use Committee and was in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Ventilation and metabolism were assessed by whole body plethysmography via the open-circuit method, as previously described (23). The chamber consisted of a 14-cm-diameter Plexiglas cylinder of 8.4-liter volume, with air intake and output ports to allow for different gas mixtures to be flushed through the chamber at a rate of 30 l/min and a low continuous flow of the gas to be drawn through the chamber during the testing period at a rate of 600 ml/min, which our laboratory has previously found to be sufficient in studies of adult rats to keep CO₂ levels below 0.03% and to maintain a constant chamber temperature and humidity at or near ambient room temperature and humidity, yet prevent excessive chamber noise (23). Flow rates lower than ~550 ml/min over time produce CO₂ buildup that is accompanied by a fall in oxygen consumption; higher flow rates increase chamber noise and possibly heat loss through evaporation, as well as prevent the accuracy of measurement of metabolic parameters. A small opening in the top of the chamber permitted sampling of chamber air for assessment of oxygen and carbon dioxide concentration within the chamber. Ventilatory parameters included minute ventilation (Ve), Vt, f, and metabolic parameters: oxygen consumption and carbon dioxide production. Two setups allowed two animals to be tested at a time.

**Protocol.** Testing was done between 10:00 AM and 1:00 PM to limit circadian effects. Animals were brought to the laboratory at 9 AM and allowed 45 min to acclimatize to the testing chamber. Baseline resting ventilation and carbon dioxide and oxygen concentrations were continuously recorded during this acclimatization period. At the end of the acclimatization period, animals were injected intraperitoneally with 2 ml of vehicle (saline), and five measurements were taken over a 15-min period to provide resting values for ventilation and metabolism. Animals were then exposed to a 5-min presentation of the test gases in the following order: 10% O₂/3% CO₂/balance N₂ followed rapidly by reoxygenation with 100% O₂. During the challenges, ventilatory parameters were continuously recorded. Representative values of f, Vt, and Ve were obtained at baseline on room air and at the following time points: end of minute 5 of hypoxia and, after the switch to hyperoxia, at the end of the minute 1. Animals were returned to room air and observed until values for ventilation returned to within 5% of baseline values. Approximately 20 min later, animals were exposed to 7% CO₂/93% O₂ (hypercapnia), and similar measurements were made after minute 5 of exposure. This was followed by a 30-min recovery period. Afterward, 100 mg/kg of N°-nitro-L-arginine methyl ester (L-NAME) dissolved in 2 ml of vehicle were injected intraperitoneally in each animal. Metabolic values were again measured, and the protocol using test gases was repeated in an identical manner (baseline followed by 5 min of hypoxia, hyperoxia, and, after a 20-min period, hypercapnia).

Temperature measurements were made by using an implantable chip and transponder during the acclimatization period and at the end of each testing session. In a separate protocol, eight animals (4 SD, 4 BN) were monitored for temperature every 30 min before and after L-NAME. Continuous monitoring during chemosensory challenge was not possible due to the technical limitations of the telemetry signal. To assess the effects of the protocol on changes in ventilation over time, 3 SD underwent the testing protocol using sham injections of vehicle (saline) instead of L-NAME.

**Data analysis.** Values for ventilatory behavior were obtained by using computer scoring of breaths (BGPLLOT). Sniffing and sighs were not included in the calculations of Vt and f. A mean value of 10 breaths at each time point was entered for each animal. Values are reported as the mean and standard deviation. Vt measurements were not corrected for the temperature of the animal. Chamber temperature did not systematically vary according to the protocol or strain. At the end of testing, the animal was removed from the chamber, and body weights and body length were obtained. HVR and hypercapnic ventilatory response (HCVR) was defined as percent change in f, Vt, and Ve from air to the end of minute 5 of gas challenge. Deploys phenomenon was defined as change in f, Vt, and Ve from baseline to 50–60 s after the switch to breathing 100% O₂.

Models used to test for statistical significance (SPSS, version 10, Chicago, IL) consisted of ANOVA and Levine tests with t-test models (paired within each strain and unpaired between strains) for comparisons of ventilation and its components, metabolism, and derivative values (e.g., Ve and carbon dioxide production). A P value of <0.05 was considered significant.

**RESULTS**

Mean values for age (24 ± 4 wk in BN vs. 23 ± 3 wk in SD; P > 0.05) were similar in the two strains. Chamber temperature during the studies of BN was 24.4 ± 1.0°C and, during the SD studies, was 24.9 ± 1.0°C (P > 0.05); corresponding barometric pressures were 754 ± 9 and 754 ± 8 Torr, respectively (P > 0.05).

Mean values for temperature before and after vehicle were similar in strains and temperature (37.8 ± 0.4°C in BN and 38.0 ± 0.3°C in SD; P > 0.05). Baseline values for resting ventilation and ventilation after vehicle for the two strains are shown in Table 1. Values for oxygen consumption and carbon dioxide production were significantly different between strains (Table 1). SD rats were significantly heavier (482 ± 50 g for SD vs. 300 ± 41 g for BN; P < 0.0001), but the values of carbon dioxide production corrected for body
weight were not significantly different between the two strains. Although the values for f, VT, and VE were significantly higher in SD than in BN, there were no significant strain differences in VE corrected for body weight or in VE corrected for carbon dioxide production. In response to 100% O₂, a significant depression in f [PHFD (5)] and VE (Dejours phenomenon) was present in BN but not consistently observed in SD (Table 2, Fig. 1). There were differences in hypoxic and hypercapnic responses between the two strains for f, VT, and VE after administration of vehicle, with SD showing more brisk hypoxic and hypercapnic ventilatory drive, consistent with previous studies (data not shown) (25).

After administration of L-NAME, there occurred a fall in temperature of 1.7 ± 0.2°C in BN (P = 0.011) and a 0.7 ± 0.2°C fall in SD (P < 0.05); the difference between strains was 1.1 ± 0.09°C (P < 0.01). As shown in Table 1, a significant reduction in oxygen consumption occurred in both strains, and carbon dioxide production decreased significantly only in BN (Fig. 2, Table 1). After L-NAME, there was an increase in baseline f and VE that was significantly greater in SD compared with BN (Fig. 2, Table 1). Changes in values for VE corrected for carbon dioxide production showed no differences between the two strains (Fig. 1). In the posthypoxic period, a significant decline in f and VE, i.e., Dejours phenomenon, was now observed in SD as well as in BN (Table 2, Fig. 1).

With vehicle and after L-NAME, animals were given ~20 min to recover before hypercapnic testing; values for ventilation were not significantly different from baseline values obtained before hypoxic and hyperoxic challenges. There were no significant differences between strains in values (f, VT, and VE) for HVRs and HCVRs expressed as percent difference between vehicle and L-NAME (Fig. 3).

In a protocol to control for the time effect of the second injection, three SD underwent testing after vehicle and then with a second vehicle injection rather than L-NAME, and there were no significant differences in body temperature, metabolism, or ventilation after the second sham injection.

Table 1. Effect of vehicle and L-NAME on resting ventilation in SD and BN

<table>
<thead>
<tr>
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<th>BN</th>
<th>SD</th>
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<tbody>
<tr>
<td>f-al after vehicle, breaths/min</td>
<td>92.1 ± 8.8</td>
<td>104.8 ± 13.2*</td>
</tr>
<tr>
<td>f-al after L-NAME, breaths/min</td>
<td>134.4 ± 19.8†</td>
<td>184 ± 28.2†</td>
</tr>
<tr>
<td>Vt after vehicle, ml</td>
<td>0.83 ± 0.08</td>
<td>1.25 ± 0.13*</td>
</tr>
<tr>
<td>Vt after L-NAME, ml</td>
<td>0.66 ± 0.09†</td>
<td>0.99 ± 0.09†</td>
</tr>
<tr>
<td>Ve after vehicle, ml/min</td>
<td>76.3 ± 5.64</td>
<td>128.6 ± 17.4*</td>
</tr>
<tr>
<td>Ve after L-NAME, ml/min</td>
<td>88.8 ± 16.5†</td>
<td>182 ± 29.7†</td>
</tr>
<tr>
<td>O₂ consumption after vehicle, ml/min</td>
<td>0.93 ± 0.10</td>
<td>1.35 ± 0.12*</td>
</tr>
<tr>
<td>O₂ consumption after L-NAME, ml/min</td>
<td>0.69 ± 0.04†</td>
<td>1.09 ± 0.28†</td>
</tr>
<tr>
<td>CO₂ production after vehicle, ml/min</td>
<td>0.49 ± 0.07</td>
<td>0.78 ± 0.07*</td>
</tr>
<tr>
<td>CO₂ production after L-NAME, ml/min</td>
<td>0.38 ± 0.06†</td>
<td>0.72 ± 0.05*</td>
</tr>
</tbody>
</table>

Values are means ± SD. f, respiratory frequency; Vt, tidal volume; Ve, minute ventilation; al, baseline on room air; l-NAME, N⁵-nitro-L-arginine methyl ester; SD, Sprague-Dawley rats; BN, Brown Norway rats. *Significant difference between strains (P < 0.05). †Significant difference between vehicle and L-NAME (P < 0.05).

Table 2. Effect of vehicle and L-NAME on Dejours phenomenon in SD and BN

<table>
<thead>
<tr>
<th></th>
<th>BN</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>f-al after vehicle, breaths/min</td>
<td>81.9 ± 9.4*</td>
<td>99 ± 11.5</td>
</tr>
<tr>
<td>f-al after L-NAME, breaths/min</td>
<td>95.3 ± 15.3*</td>
<td>119.4 ± 26.3*</td>
</tr>
<tr>
<td>Vt-al after vehicle, ml</td>
<td>0.78 ± 0.12</td>
<td>1.22 ± 0.11</td>
</tr>
<tr>
<td>Vt-al after L-NAME, ml</td>
<td>0.69 ± 0.08</td>
<td>0.96 ± 0.09</td>
</tr>
<tr>
<td>Ve-al after vehicle, ml</td>
<td>64.0 ± 11.2*</td>
<td>121 ± 18.4</td>
</tr>
<tr>
<td>Ve-al after L-NAME, ml/min</td>
<td>66.3 ± 11.9*</td>
<td>114.3 ± 23.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. After hypoxia at the end of minute 1. *Significant difference between ol and baseline (P < 0.05).
DISCUSSION

NOS blockade with L-NAME alters both metabolism and ventilation in these rat strains. With L-NAME, there occurred some fall in body temperature, which was greater in magnitude in BN than in SD. Nevertheless, ventilation while breathing room air was increased in both strains, primarily because of an increase in f; this effect was more pronounced in SD. The Dejours phenomenon, not consistently seen in SD after vehicle, was present after NOS blockade, whereas the phenomenon was still present in BN.

Effects on metabolism. The literature involving inhibition of endogenous generation of NO have yielded mixed results. Shen et al. (19) studied conscious dogs and showed that administration of a specific NOS inhibitor, N-nitro-L-arginine, increased whole body oxygen consumption. However, Crystal and Zhou (6) administered L-NAME to anesthetized dogs and found no effect on oxygen consumption. Kline et al. (14) found that hypoxia induced a decrease in oxygen consumption that was greater in a NOS-3 knockout mutant mouse compared with a wild-type mouse. Barros and Branco (2) showed that administration of a nonspecific NOS blocker, NG-nitro-L-arginine, to awake unrestrained Wistar rats produced a significant drop in oxygen consumption and body temperature. Our results indicate a drop in temperature and a reduction in the production of carbon dioxide that differs quantitatively between SD and BN. In aggregate, the studies in unrestrained rodents lend support for a role of NOS in maintenance of whole body metabolism.

Whether this is an effect mediated through the central nervous system or through a direct effect on cellular metabolism was not identified in this study. It is reasonable to suspect that it would be a central mechanism, given the literature on the central role of NO in determining the set point of body temperature and mitigating the fall in temperature with hypoxia (9, 21).

Effects on resting ventilation. Compared with vehicle, L-NAME increased resting ventilation in both strains primarily because of an increase in f and was much greater in SD. Gozal et al. (10) also showed that there was a marked increase in \( V_E \) response to L-NAME in rats, primarily because of an increase in f, and this was accompanied by a decrease in \( V_T \). Barros and Branco (2) describe a similar increase in \( V_E \), but, in contrast to Gozal et al. (10), this was primarily due to an increase in \( V_T \). The increase in f and \( V_E \) was more in SD than in BN, suggesting that there may be a more tonic inhibitory effect of NOS under resting conditions in SD. In the study by Gozal et al. (10), an increase in \( V_E \) did not occur with a selective nNOS blocker. In the present study, there were no differences between strains in the increase in \( V_E \) corrected for carbon dioxide production. This would suggest that the greater increase in \( V_E \) in SD is not due to differential effects of NOS blockade on the pulmonary circulation and/or dead space fraction in the two strains. Taken together, these data support the hypothesis that there is a strong tonic NOS-mediated modulation of resting ventilation in the rat. The strain differences may be attributable to qualitative and quantitative differences in this modulation.

One potential site for this would be the carotid body, a mechanism for which we have no direct evidence. It is intriguing that although metabolism appeared to decrease, resting ventilation increased when one might expect the opposite. The respiratory quotient was no different among strains after either vehicle or L-NAME, so the strain differences in ventilatory behavior may well be independent of the load of carbon dioxide. The effect of NOS inhibition may be due to differences in L-NAME action at a local cellular level in the carotid body or in the transduction processes for respiratory rhythmogenesis. NO has been shown to induce inhibition of various mitochondrial enzymes in the respiratory chain (3). Addressing this issue was beyond the scope of the present study.

Effects on ventilatory behavior with hypoxia-reoxygenation. We have previously reported that BN exhibits posthypoxic frequency decline and SD shows posthypoxic potentiation (25). In this report, measurements were made after administration of the vehicle used for L-NAME and again confirm BN response. There was, however, no statistical increase in posthypoxic f from baseline f in the SD, perhaps indicating that the intraperitoneal vehicle injection had an effect. For this reason, a time control study of two consecutive vehicle injections was performed in SD; in this sub-study, the second injection had no effect. Therefore, we conclude that L-NAME was the active intervention in uncovering posthypoxic f decline in the SD.

The absence of a significant fall in \( V_E \) in response to hyperoxia is generally attributed to resting carotid body activity (7), but more recently Coles and Dick (5) have assigned the frequency component of the response to specific pontine region. Our pharmacological study would not distinguish mechanisms but suggests that the phenomenon may be related to the degree of tonic NOS suppression at baseline. For instance, NOS activity at the carotid body level in the SD would prevent
any further depression of ventilation in the posthyperoxic period, whereas blockade of NOS activity not only increases baseline ventilatory values but also uncovers a depression in \( V_E \) in the response to reoxygenation.

Another process to consider is PHFD, an event in large part controlled by pontine cell groups in the A5 region. (5). A role of NOS in modulating these neurons has not been identified. Given other evidence showing a role for central NOS activity in metabolic and temperature responses to hypoxia (8, 22), the working hypothesis is that NOS may be involved in shaping this response at the level of the ventrolateral pons. Strain differences in rats may reflect differences in the distribution and/or activity of nNOS-containing neurons at this location.

In addition to the pontine A5 region, NO acts as a neurotransmitter in other regions of the brain, such as the cortex and medulla, either directly or indirectly through cardiovascular effects. The metabolic effect of L-NAME would be something to consider if only one strain had been examined; however, the observations between SD and BN suggest that the posthypoxic frequency decline occurs independently of body temperature.

NO also acts at the level of the carotid body. A role of the peripheral chemoreceptor in the Dejours phenomenon, and the posthypoxic decline in ventilation in particular, is supported by studies in both animals (13, 14).

Effects on steady-state chemosensitivity. With acute hypoxia NOS activity in the carotid body is probably inhibited, decreasing NO concentrations and increasing afferent peripheral chemoreceptor activity (4, 18). Responsiveness, measured as the difference between resting ventilation and minute 5 of a chemochallenge, was similarly affected in all components of ventilation in SD and BN (Fig. 3). The reciprocal effects on \( f \) and \( V_T \) was such that \( V_E \) was unaffected.

In the study by Teppema et al. (26), NOS blockade with \( N^G \)-nitro-L-arginine depressed HCVR in anesthetized cats. However, hypercapnic responses were found not to be affected by NOS blockade in the Wistar rat (1), and Kline et al. (13) found that the HCVR was comparable between unanesthetized mutant mice deficient in nNOS or eNOS compared with WT. Our data in SD and BN indicate that NOS may not have a major role to play in the magnitude of the ventilatory response to hyperoxic hypercapnia.

Limitations. We only administered a single dose of L-NAME. The weight of the animals in each strain was significantly different; however, the dose by weight that produced a significant change in the Dejours phenomenon would be significantly lower than that used for BN, in which the phenomenon was still present and unchanged. It is also possible that different doses may alter ventilatory responses and do so differently among these and other strains.

Only L-NAME, a general NOS blocker, was utilized, and we could did not determine the mechanisms, isoforms, or location of the effects of NO suppression. L-NAME has known effects on heart rate and blood pressure, which also might contribute directly or indirectly to strain differences. The effects of NOS blockade in the Wistar rat are to reduce the fall in body temperature (average of \(-0.73^\circ C\)) with hypercapnic challenge without affecting HCVRs. We could not detect significant differences among strains in regard to oxygen consumption or temperature at rest. There was a fall in carbon dioxide production (less in SD) so that it is possible that other systemic differences could account for the differences in the strains in regard to the Dejours effect.

Animals were without food and water for the duration of \(-80\) min while the testing protocol was being followed. This might result in changes in metabolism, as the order was always a study after vehicle followed by one after L-NAME. However, in control experiments using vehicle followed by vehicle in place of L-NAME and following an identical protocol, metabolism increased by a small albeit insignificant degree. Therefore, we do not believe the effect is the result of the order of testing or of brief periods of fasting. The advantages of this study design were an avoidance of day-to-day effects and the fact that each animal acted as its own control.

Although we restricted the time of testing to the midday hours and after a time of acclimatization to the laboratory setting, there was no attempt to objectively record state. Observational determinations of the body position and relative quiescence of the animal suggest that during and after challenges there occurred increased movements and alertness, and during resting breathing measures were made during observed quiet wakefulness. However, the time of testing was during the inactive period of the day for these animals, and changes in vigilance might have affected results.

There are limitations to the barometric measurement of ventilation, despite its attractiveness in regard to making measurements in unrestrained animals. \( V_T \) estimation is critically dependent on chamber attractiveness in regard to chamber size, and the temperature of both the chamber and the animal. Absolute accuracy of \( V_T \) is always a problem, even with the use of calibration volumes, as there exist differences in the pressure wave that is detected as \( V_T \) created by spontaneous breathing or by a given calibrating syringe at a given calibrating frequency. In the best-case scenario, differences of up to 10% may occur between \( V_T \) measured by plethysmograph and directly from an anesthetized animal; correlation coefficients remain high enough (>0.75) to show correlation even under the more extreme conditions. In the present study, unanesthetized animals were tested under environmentally similar circumstances (chamber size, material, temperature, humidity, etc. being equal). The fact that the temperature of the animal was similar after vehicle indicates that the relative \( V_T \) differences reflect biological changes induced by L-NAME. The quantitative error introduced by a falling body temperature and by the substantial temperature difference (1°C) after L-NAME means that the estimates of relative \( V_T \) are altered. We chose not to computationally adjust for
these temperature changes. Such an approach would have to consider a correction for humidity and, therefore, introduces other uncertainties. The interpretation of the data can be made by using frequency alone, which is an excellent signal and is not altered as a signal by temperature or humidity.

In conclusion, there occur strain differences between SD and BN both in metabolism and ventilation at rest both after vehicle and after L-NAME. Administration of a broad NOS inhibitor increases resting ventilation in SD to a far greater extent than in BN. The Dejours phenomenon, observed consistently in BN, is also seen in SD after L-NAME administration. Both HVR and HCVR are similarly affected in both strains. These findings suggest that SD has a higher level of NO function at baseline compared with BN. NOS polymorphisms, or other variations in the genetic mechanisms influencing the NO systems, may play a significant role in metabolic behavior, baseline ventilation, and ventilatory responses to reoxygenation.

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