Selective Inhibition of Inducible Nitric Oxide Synthase Attenuates Renal Ischemia and Damage in Experimental Heatstroke

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Abstract. The aim of the present study was to determine whether the possible occurrence of renal ischemia and damage during heatstroke can be suppressed by prior administration of L-N6-(1-iminoethyl) lysine (L-NIL), a selective inducible nitric oxide synthase (iNOS) inhibitor. Urethane-anesthetized rats were exposed to heat stress (43°C) to induce heatstroke. Control rats were exposed to 24°C. Mean arterial pressure and renal blood flow after the onset of heatstroke both were significantly lower in vehicle-treated heatstroke rats than in normothermic controls. However, both the body temperature and renal damage scores were greater in vehicle-treated heatstroke rats compared with normothermic controls. Plasma nitric oxide (NO), creatinine, and blood urea nitrogen (BUN), as well as the renal immunoreactivity of iNOS and peroxynitrite all were significantly higher in vehicle-treated heatstroke rats compared with their normothermic controls. Pretreatment with L-NIL (3 mg/kg, administered intravenously and immediately at the onset of heat stress) significantly attenuated heatstroke-induced hyperthermia, arterial hypotension, renal ischemia and damage, increased renal levels of immunoreactivity of iNOS and peroxynitrite, and increased plasma levels of NO, creatinine, and BUN. Accordingly, pretreatment with L-NIL significantly improved survival during heatstroke. The results suggest that selective inhibition of iNOS-dependent NO and peroxynitrite formation protects against renal ischemia and damage during heatstroke by reducing hyperthermia and arterial hypotension.

Keywords: renal ischemia, hyperthermia, nitric oxide, peroxynitrite, heatstroke

Introduction

Described clinically as either classical (nonexertional) or exertional in nature, heatstroke is strongly suggested when hyperthermia occurs in a hot and humid environment or severe exercise and may result in significant neurological abnormalities and multiple organ failures with ischemic insults in the heart, liver, kidney, and brain (1). Furthermore, acute renal failure (ARF) associated with damage to both renal tubules and their blood supply system is a common complication of heatstroke (2).

Nitric oxide (NO) has been shown to play an important role in various physiological processes in the kidney including salt and fluid reabsorption (3), renal hemodynamics (4), rennin secretion, and tubuloglomerular feedback (5). Recently, several investigators have suggested that the activity of inducible nitric oxide synthase (iNOS) is an important contributor to the pathophysiology of ARF (6).

In rats, heat stress leads to increased metabolic demand and reduced splanchnic blood flow, which in turn induce intestinal and hepatocellular hypoxia, and the hypoxia results in generation of high reactive nitrogen species that accelerate mucosal injury (7, 8). Intestinal mucosal permeability to endotoxin increase in hyperthermia rats (9). This alteration allows leakage of endotoxins and increases production of inflammatory

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cytokines that induce release of NO and endothelins (10, 11). Both pyrogenic cytokines can interfere with normal thermoregulation, thereby precipitating arterial hypotension, hyperthermia, and heatstroke (1). Recent reports have also shown that aminoguanide, an iNOS inhibitor, slows the rate of NO production, preserves the splanchnic (8) and cerebral blood flow (10), and improves heat tolerance in rats. However, relative little evidence is available about the effects of iNOS inhibition on the renal ischemia and damage during heatstroke.

Therefore, in order to validate the matter, the present experiments were performed to assess the effect of heat stress on renal blood flow (RBF) values, renal damage scores, and the extent of iNOS in rat kidney with or without prior administration of L-N6-(1-iminoethyl)lysine (L-NIL), a relative selective iNOS inhibitor (12, 13). In addition, the preventive effects of L-NIL on the plasma levels of NO, blood urea nitrogen (BUN), and creatinine during heatstroke were assessed.

Materials and Methods

Experimental animals

Adult Sprague-Dawley rats weighing between 300–350 g were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals were housed 4 in a group at an ambient temperature (Ta) of 22 ± 1°C, with a 12-h light-dark cycle, with the lights being switched on at 0600 h. Dry rat chow and tap water were available ad libitum. All protocols were approved by the Animal Ethics Committee of the National Yang-Ming University School of Medicine. Adequate anesthesia was maintained to abolish the corneal reflex and pain reflexes induced by tail pinching throughout all experiments (approx 8 h), by a single dose of urethane (1.4 g/kg body weight, i.p.). At the end of the experiment, control rats and any rats that had survived heatstroke were killed with an overdose of urethane.

Animals were randomly assigned to one of 3 groups. One group of rats was treated with normal saline (NS, 1 ml/kg, i.v.) before initiation of heat stress and used as vehicle-treated heatstroke controls. For heatstroke induction, animals were exposed to a Ta of 43°C (with relative humidity of 60% in a temperature-controlled chamber). When mean arterial pressure (MAP) began to decrease from their peak levels; this moment was considered as the onset of heatstroke (4, 5). Immediately after the onset of heatstroke, heat stress was terminated and the Ta was restored to 24°C. Our pilot results showed that the latency for the onset of heatstroke (i.e. the internal between the start of heat exposure and the onset of heatstroke) were 70 ± 2 min for vehicle-treated rats (n = 8). The second group of animals was treated with L-NIL (1–3 mg/kg, i.v.) at the start of heat exposure. For comparison with the vehicle-treated heatstroke group, this group of animals were exposed to 43°C for exactly 70 min and then allowed to recover at 24°C. The survival times [i.e., interval between the termination of heat exposure (70 min after initiation of heat exposure) and animal death] were recorded for the heatstroke groups. The third group of NS-treated rats was kept at room temperature (24°C) and used as normothermia controls. Different groups of animals were used for the different sets of experiments: i) measurements of core temperature (Tco), mean blood pressure (MBP), and heart rate (HR); ii) measurements of plasma creatinine, BUN, and RBF in renal cortex; and iii) determination of nitrite/nitrate (NOx) concentration in plasma and immunoreactivity of iNOS and 3-nitrotyrosine in renal tissue.

Surgery and physiological parameters monitoring

The femoral artery and vein were cannulated with polyethylene tubing (PE 50), under urethane anesthesia, for blood pressure monitoring and drug administration. The blood samples were collected (200 µl each) for analysis of BUN, and creatinine content at three time points: 0, 70, or 80 min after the initiation of heat exposure. Local RBF in the kidney was monitored with a laser-Doppler flowmeter (T206; Transonics, Ithaca, NY, USA), a surface Laser Doppler probe placed directly over the renal cortex. The Tco was monitored continuously by a thermocouple inserted into the rectum and connected to a thermometer (HR 1300; Yokogawa, Tokyo). Pulsatile arterial blood pressure, mean arterial pressure, and HR were monitored continuously with a pressure transducer (Statham PA23AC; Gould, Valley View, OH, USA) and a chart recorder (2107, Gould).

The data are expressed as a percentage of the control value. During the experiments, blood samples were collected and centrifuged. Then both the serum urea nitrogen and creatinine concentration were determined by a Kodak Ektachem dry chemistry (Eastman Kodak, Rochester, NY, USA) analyzer.

Renal histology and morphological evaluation

At the end of the experiments, animals were sacrificed by an overdose of urethane, and kidneys were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Serial (5 µm) sections were stained with hematoxylin and eosin for microscopic evaluation. The extent of renal damage was scored on a scale of 0–3 according to the semiquantitative manner (14): 0: normal structure; 1: areas of tubular epithelial cell swelling, brush-border...
loss, vacuolar degeneration and necrosis with up to 1/3 of tubular profile showing nuclear loss; 2: as for score 1, but greater than 1/3 and less than 2/3 of tubular profile showing nuclear loss; and 3: greater than 2/3 of tubular profile showing nuclear loss.

Measurements of NOx level in plasma
Blood samples were taken at 0, 70, or 80 min after initiation of heat exposure for determination of NO levels. The samples were centrifuged for 5 min at approximately 10,000 x g, and then the serum was taken for analyses. NOx levels were determined by the Griess reaction, which relies on a colorimetric reaction between nitrite, sulphanilamide, and N-(1-naphthyl) ethylenediamine dihydrochloride to produce a pink Azo product. Prior to the Griess reaction, all nitrate was converted to nitrite using bacterial enzyme nitrate reductase. Concentrations were determined by comparison to a standard solution of sodium nitrite.

Immunohistochemical staining
Paraffin sections of the kidney were deparaffinized, and the endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide for 15 min at room temperature. The sections were washed in 0.01 M phosphate-buffered saline, pH 7.2 (PBS), containing 10% normal goat serum and incubated overnight at 4°C with rabbit anti-iNOS antibody (1:100 dilution) or rabbit anti-nitrotyrosine antibody (1:100 dilution). Horseradish-conjugated goat anti-rabbit Ig antibody was used at 1:200 after washing. Immunocomplexes were detected with a solution of 3,3-diaminobenzidine (0.2 mg/ml) and hydrogen peroxide in 0.05 M Tris-HCl buffer. Sections were counterstained with methyl green. In the negative control sections, rabbit antiserum against ovalbumin (1:500) was used as an irrelevant antibody. Semiquantitation of the expression of iNOS and 3-NT in kidney tissue from rats was performed according to the methods of Furusu et al. (15). Briefly, the following scoring scale from 0 to 3 was used: 0: no specific staining, 0.5: possibly positive, 1+: weakly positive, 2+: moderately positive, and 3+: strongly positive. Scoring was generally influenced by the extent rather than the intensity of staining.

Statistical analyses
All values, except those in Table 2, are expressed as means ± S.E.M. and were analyzed by two-way analysis of variance (ANOVA). Duncan’s multiple range test was used for post-hoc multiple comparison among means. For the Table 2 data, the Wilcoxon signed rank test was used. The Wilcoxon tests convert the scores or values of a variable to ranks, require calculation of sum of the ranks, and provide critical values for the sum necessary to test the null hypothesis at a given significant level. The data were treated by ‘median’ followed by first and third quartile. All results were considered statistically significant at P<0.05.

Results
Effects of heat exposure on survival time
Table 1 summarizes the effects of heat exposure (Ta = 43°C for 70 min) on survival time in NS- and drug-treated rats. The survival time values obtained from eight rats pretreated with i.v. administration of NS solution at the start of heat exposure were found to be 96 – 104 min. Treatment with L-NIL (1 – 3 mg/kg, i.v.) dose-dependently increased the survival time values (145 – 202 min) during heatstroke.

Effects of heat exposure on physiological and biochemical parameters
Both Figs. 1 and 2 show the effects of heat stress (Ta = 43°C) on MBP, HR, Tco, RBF, and plasma levels of NOx, BUN, and creatinine in rats treated with NS

Table 1. Effects of heat exposure (HE: 43°C for 70 min) on survival time in normal saline (NS)-treated heatstroke rats and L-NIL-treated heatstroke rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. NS (ml/kg, i.v.)-treated rats kept at 24°C</td>
<td>480 ± 2 (8)</td>
</tr>
<tr>
<td>2. NS (ml/kg, i.v.)-treated rats kept at 43°C</td>
<td>100 ± 4 (8)*</td>
</tr>
<tr>
<td>3. L-NIL (1 mg/kg, i.v.)-treated rats kept at 43°C</td>
<td>97 ± 5 (8)*</td>
</tr>
<tr>
<td>4. L-NIL (2 mg/kg, i.v.)-treated rats kept at 43°C</td>
<td>145 ± 10 (8)*†</td>
</tr>
<tr>
<td>5. L-NIL (3 mg/kg, i.v.)-treated rats kept at 43°C</td>
<td>202 ± 37 (8)*†</td>
</tr>
</tbody>
</table>

Data represent means ± S.E.M. followed by number of rats used in parentheses. All group of rats exposed to 43°C had HE withdrawn at 70 min and then were allowed to recover at 24°C. NS-treated rats kept at 24°C were terminated about 480 min after the start of HE (or at the end of the experiments). *P<0.05, compared with group 1; †P<0.05, compared with group 2 (ANOVA followed by Duncan’s test).
In iNOS Inhibition Inhibits Renal Ischemia, heatstroke was induced by exposure to high ambient temperature (43°C) for 10 min after the onset of heatstroke in rats. Normal saline (NS) or L-NIL (3 mg/kg, i.v.) was administered at the initiation of heat exposure or at time 0.

Effects of heat exposure on both iNOS and peroxynitrite (ONOO⁻) expression in renal tissues

Table 2 summarizes the effects of heat exposure on both the iNOS and ONOO⁻ immunoreactivity of the renal tissue in rats treated with vehicle solution or L-NIL.
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(3 mg/kg, i.v.) before the initiation of heat stress. In vehicle-treated heatstroke rats, killed 10 min after termination of 70-min heat exposure, both the iNOS and ONOO\(^{-}\) immunoreactivity of the renal tissues were greater than those in the normothermic controls. However, the heatstroke-induced increases of both parameters observed in the kidney were greatly attenuated by \(L\)-NIL pretreatment. A typical example for iNOS or ONOO\(^{-}\) immunoreactivity is, respectively, depicted in Figs. 3 and 4.

**Effects of heat exposure on renal pathology**

In vehicle-treated heatstroke rats, killed 10 min after termination of 70-min heat exposure, brush-border loss, intraluminal cast formation (arrow), sloughed epithelial cells with pyknotic nucleus, extrusion of cytoplasm into the lumen, and cell swelling and necrosis (arrowhead) reflected the degree of tubular cell injury (Fig. 5B). These pathological changes that occurred during heatstroke were greatly suppressed by \(L\)-NIL pretreatment (Fig. 5C). Again, it was found that the renal damage score values for vehicle-treated rats were greater than those in the normothermic controls (Table 2). However, the heatstroke-induced increase of renal damage score observed in the kidney was greatly attenuated by \(L\)-NIL treatment.

**Discussion**

As shown in the present results, after the onset of heatstroke, animals displayed renal dysfunction (as reflected by an increase in the plasma levels of iNOS-dependent NO, creatinine, and BUN) and renal ischemia and damage (as characterized by the tubular epithelial cell swelling, brush-border loss, vacuolar degeneration, necrosis, and decreased RBF in the kidney). In addition, \(L\)-NIL pretreatment significantly attenuated the heatstroke-induced hyperthermia, arterial hypotension, renal ischemia and damage, renal dysfunctions, and increased iNOS-dependent NO and peroxynitrite formation in the kidney. These results suggest that \(L\)-NIL protects against heatstroke-induced renal ischemia and damage by reducing iNOS-dependent NO-peroxynitrite formation. The present results are in part consistent with previous results (8, 10). For example, it has been shown that aminoguanidine (an iNOS inhibitor) slows the rate of NO production, preserves the splanchnic blood flow, and improves heat tolerance in rats (8). A more recent report has also shown that aminoguanidine protects against heatstroke-induced intracranial hypertension and cerebral ischemic injury by inhibition of cerebral iNOS-dependent NO production (10). The activity of iNOS and the release of lactate dehydrogenase are
believed to be involved in the integrity of tubular cell membrane (16). The formation of NO by iNOS may directly signal the mitochondrial release of cytochrome C or formation of peroxynitrite and lead to cell death, most likely necrosis (17). In fact, the excessive accumulation of ONOO⁻ resulting from superoxide anion and NO has also been indicated to be an important causative agent in the pathogenesis of cellular damage and renal dysfunction (18–20). It should be noted that in the current study, L-NIL, which inhibits the activity of iNOS, attenuates such expression of iNOS. This indicates that both over activation of iNOS and increased protein synthesis and/or release of NO occurred during heatstroke can be suppressed by L-NIL pretreatment.

Hall and colleagues (8) have demonstrated that hyperthermia stimulates xanthine oxidase production of reactive oxygen and nitrogen species that limit heat tolerance by promoting circulatory dysfunction. Overproduction of NO may contribute to the splanchnic vasodilation that precedes vascular collapses with heatstroke. Our previous results have further shown that in addition to overproduction of NOx in brain, decreased cardiac output (due to decreased total peripheral vascular resistance and stroke volume), intracranial hypertension, and cerebral ischemia and hypoxia, and hyperthermia occur during heatstroke in the rat (10, 11, 21). Our previous findings have also shown that accumulation of large amounts of reactive oxygen species in various organs including the kidney and the brain is associated with hyperthermia, arterial hypotension, and cerebral ischemia and injury during heatstroke (22, 23). In the present study, L-NIL may have attenuated the excessive accumulation of reactive oxygen and nitrogen species in the peripheral blood stream as well as several brain structures and resulted in attenuation of splanchnic vasodilation, arterial hypotension, ARF, intracranial hypertension, and cerebral ischemia and damage by reducing hyperthermia during heatstroke. As demonstrated in the present results, the treatment with L-NIL recovered the survival time during heatstroke. However, such a recovery rate is still small in spite of a remarkable recovery by L-NIL of the various indices in Figs. 1 and 2. We believe that such a recovery rate of survival time by L-NIL is associated with the heatstroke-induced arterial hypotension, intracranial hypertension, and cerebral ischemia and damage, rather than renal damage. It should be mentioned that the latency values for the onset of heatstroke are found to be 70 ± 2 and 68 ± 2 min for vehicle-treated and drug-treated rats, respectively. However, for determination of survival time and physiological parameters, both the vehicle-treated and the drug-treated rats were exposed to the same heat stress for the same time period (exactly 70 min) in separate experiments.

Other lines of evidence have provided evidence to indicate that an endotoxin given systemically can elicit an increase of iNOS-dependent NO production in brain and induce circulatory shock (24). In addition, exposure of animals to a hot environment induces heatstroke that is characterized by circulatory shock (22), endotoxemia (23), and reduced baroreceptor reflex response (25). Thus, it is likely that inhibition of iNOS-dependent NO and peroxynitrite production in the brain with L-NIL (present results) or aminoguanidine (10) may alleviate arterial hypotension as well as ARF exhibited during heatstroke by potentiating both the sensitivity and capacity of the baroreceptor reflex response.

Finally, it should be mentioned that the NO generated during heatstroke may react with superoxide anion to form peroxynitrite (26), which may cause injury via direct oxidant pathways and protein denature (26, 27). Thus, it appears that peroxynitrite generation is implicated in the pathophysiology of renal heat injury, ischemia/reperfusion, or hypoxia-reoxygenation injury (18–20). The present results indicate that selective inhibition of iNOS-dependent NO and peroxynitrite formation with L-NIL protects against renal ischemia and damage exhibited during heatstroke by reducing hyperthermia and arterial hypotension.

(iNOS Inhibition Inhibits Renal Ischemia)
Fig. 3. The photomicrographs of iNOS staining of the kidney of a normothermic control rat (A), a heatstroke rat pretreated with normal saline (1 ml/kg, i.v.) (B), and a heatstroke rat pretreated with L-NIL (3 mg/kg, i.v.) (C) at the start of heat exposure. Animals were killed at 80 min after initiation of heat exposure (or 10 min after the onset of heatstroke) (OM = 200×).

Fig. 4. The photomicrographs of 3-NT staining of the kidney of a normothermic control rat (A), a heatstroke rat pretreated with normal saline (ml/kg, i.v.) (B), or a heatstroke rat pretreated with L-NIL (3 mg/kg, i.v.) (C) at the start of heat exposure. Animals were killed at 80 min after the start of heat exposure (OM = 200×).
Acknowledgments

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References


