

## Full Paper

**Prior Antagonism of Endothelin-1A Receptors Alleviates Circulatory Shock and Cerebral Ischemia During Rat Heatstroke**Chia-Chyuan Liu<sup>1</sup>, Zhih-Cherng Chen<sup>2</sup>, Bor-Chih Cheng<sup>3</sup>, and Mao-Tsun Lin<sup>4,\*</sup><sup>1</sup>Institute of Physiology, National Yang-Ming University Medical School, Taipei, Taiwan 112<sup>2</sup>Division of Cardiology, Department of Internal Medicine, Chi-Mei Medical Center, Tainan, Taiwan 710<sup>3</sup>Division of Cardiac Surgery, Department of Surgery, Chi-Mei Medical Center, Tainan, Taiwan 710<sup>4</sup>Department of Medical Research, Chi-Mei Medical Center, Tainan, Taiwan 710

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**Abstract.** In this study, we investigated the acute hemodynamic effects of an infusion of the endothelin-1 (ET-1)-A-selective receptor antagonists BQ-610 and BQ-123 in heatstroke rats with circulatory shock and cerebral ischemia. Heatstroke was induced by putting the anesthetized adult Sprague-Dawley rats into an ambient temperature of 42°C. The moment in which the mean arterial pressure dropped irreversibly from the peak for an extent of 25 mmHg was taken as the onset of heatstroke. The interval between initiation of heat exposure and heatstroke onset was found to be about 80 min for rats treated with vehicle solution. When the animals were exposed to 42°C for 80 min, hyperthermia, arterial hypotension, decrement of cardiac output (due to decreased stroke volume and decreased total peripheral resistance), increment of plasma ET-1 and tumor necrosis factor- $\alpha$ , and increment of cerebral ischemia and injury markers were manifested. Prior antagonism of ET-1 A receptors with BQ-610 (0.5 mg/kg, i.v.) or BQ-123 (1 mg/kg, i.v.), but not ET-1B receptors with BQ-788 (0.5 mg/kg, i.v.), 60 min before the initiation of heat exposure, appreciably alleviated hyperthermia, arterial hypotension, decreased cardiac output, increment of tumor necrosis factor- $\alpha$ , and increment of cerebral ischemia (e.g., glutamate and lactate/pyruvate ratio) and injury (e.g., glycerol) markers exhibited during heatstroke. The data indicates that ET-1A receptor antagonism may maintain appropriate levels of mean arterial pressure and cerebral circulation during heatstroke by reducing production of tumor necrosis factor- $\alpha$ .

**Keywords:** blood pressure, cardiac output, endothelin, heatstroke, receptor

**Introduction**

The clinical diagnosis of heatstroke was strongly suggested when hyperthermia was accompanied by arterial hypotension, cerebral edema, intracranial hypertension, and cerebral ischemia and injury (1–3). The heatstroke-induced central nervous system dysfunction induces delirium, convulsion, or coma (4).

Endothelin (ET)-1 exerts multiple biologic action via its type A (ET<sub>A</sub>) and B (ET<sub>B</sub>) receptors (5). ET-1 can play an important pathogenic role in arterial hypertension and congestive heart failure (6). Evidence has accumulated to indicate that endogenous ET-1 levels are

elevated in both the plasma and cerebrospinal fluids of patients with cerebral ischemia, stroke, or brain trauma (7–10). Endothelial cell activation/injury also occurs in heatstroke victims (11–13). Thus, it is likely that ET-1 mechanisms are involved in the pathogenesis of heatstroke. The development of ET<sub>A</sub> and ET<sub>B</sub> selective receptor antagonists (5), which can be administered intravenously in rats with heatstroke, has made it possible to elucidate this issue.

Therefore, in the present study, we investigated the effects of heat stress on colonic temperature (T<sub>co</sub>), mean arterial pressure (MAP), stroke volume (SV), total peripheral resistance (TPR), cardiac output (CO), heart rate (HR), cerebral blood flow (CBF), cerebral neuronal damage, and extent of plasma levels of ET-1 and tumor

\*Corresponding author. FAX: +886-6-2517850  
E-mail: mtlin@ym.edu.tw

necrosis factor- $\alpha$  (TNF- $\alpha$ ) in rats with or without prior administration of ET<sub>A</sub>- or ET<sub>B</sub>-selective receptor antagonist. The lactate/pyruvate ratio is a well-known marker of cellular ischemia, whereas glycerol is a marker of how severely cells are affected by ongoing pathology (14–17). Excessive accumulation of glutamate has been shown in ischemic brain tissue (18, 19). We have also assessed the preventive effect of ET<sub>A</sub>- and ET<sub>B</sub>-selective receptor antagonists on the extracellular levels of lactate/pyruvate ratio, glycerol, and glutamate in brain associated with heatstroke (2, 3).

## Materials and Methods

### *Experimental animals*

Adult male Sprague-Dawley rats weighing  $325 \pm 50$  g were obtained from the Animal Resource Center of the Chi-Mei Medical Center (Tainan, Taiwan). The animals were housed individually at an ambient temperature (Ta) of  $24 \pm 1^\circ\text{C}$  with a 12-h light-dark cycle. Pelleted rat chow and tap water were administered ad libitum. All experiments were approved by the Animal Research Committee of the Chi-Mei Medical Center on compliance with NIH regulations. Adequate anesthesia was maintained to abolish the corneal reflex and pain reflexes induced by tail pinch throughout the course of all experiments (about 8 h) following a single dose of urethane (1.4 g/kg body wt, i.p.).

### *Animal surgery and physiological parameter monitoring*

The right femoral artery and vein of rats were cannulated with a polyethylene tube (PE50), under urethane anesthesia, for blood pressure monitoring in artery and blood sampling (for ET-1 or TNF- $\alpha$  assay) and drug administration in vein, respectively. The animals were positioned in a stereotaxic apparatus (model 1460; David Kopf Instruments, Tujunga, CA, USA) to allow insertion of a probe for measurement of CBF. Physiological monitoring included Tco, MAP, HR, and CBF values in the corpus striatum. Tco was monitored continuously by a thermocouple.

### *Experimental groups*

Animals were assigned randomly to one of following 3 major groups. One group of rats, treated with vehicle solution per ml per kilogram of body weight 60 min before testing, were exposed to Ta of  $24^\circ\text{C}$  and their physiological parameters were continuously recorded for up to 450 min (at the end of the experiments). This group of animals was used as normothermic controls. The second group of rats, treated with an i.v. dose of per ml per kilogram of body weight of vehicle solution 60 min before the start of heat exposure (Ta of  $42^\circ\text{C}$

for 80 min in a temperature-controlled chamber with relative humidity of 60%) were used as vehicle-treated heatstroke controls. The moment in which MAP dropped to a value of 25 mmHg from the peak level was taken as the onset of heatstroke. After the onset of heatstroke, the animals were allowed to recover at room temperature. As shown in Fig. 1, the latency for the onset of heatstroke (interval between the start of heat exposure and the onset of heatstroke) was found to be  $80 \pm 4$  min for the vehicle-treated heatstroke group. Then, both physiological parameters and survival time (interval between the initiation of heat exposure and animal death) were observed for up to 450 min (or the end of experiments). The third group of rats, treated with an i.v. dose of 0.5 mg/ml per kg body wt of BQ-610 (an ET<sub>A</sub>-receptor antagonist; Phoenix Pharmaceuticals, Inc., Mountain View, CA, USA), BQ-123 (an ET<sub>A</sub>-receptor antagonist, Phoenix Pharmaceuticals), or BQ-788 (an ET<sub>B</sub>-receptor antagonist, Phoenix Pharmaceuticals) 60 min before the initiation of heat stress, were exposed to heat stress (Ta  $42^\circ\text{C}$  for 80 min) to induce heatstroke. Again, after 80-min heat exposure, the animals were allowed to recover at room temperature ( $24^\circ\text{C}$ ). The 1 mg of BQ-788 was dissolved in 0.01% ammonium hydroxide in 2 ml normal saline, while 5 mg of BQ-610 or 10 mg of BQ-123 was dissolved in 400  $\mu\text{l}$  acetonitrile containing 0.1% trifluoroacetic acid and then diluted to 10 ml with normal saline.

### *CBF monitoring*

Local CBF in the striatum (SBF) was monitored with a Laserflo BPM2 laser Doppler flowmeter (Vasamedics, St. Paul, NM, USA). A 24-gauge stainless steel needle probe (diameter, 0.58 mm; length, 40 mm) was inserted into the right corpus striatum using the coordinates of A, interaural 9.7 mm; L, 2.0 mm from midline; and H, 4.5 mm from the top of the skull (20).

### *Assay for serum ET-1*

Blood samples were taken at 0, 80, and 95 min after heat exposure, for determination of ET-1 levels. Blood samples were allowed to clot for 2 h at room temperature or overnight at  $2 - 8^\circ\text{C}$  before centrifuging for 20 min at approximately  $2000 \times g$ . Serum was quickly removed from these plasma samples and assayed for ET-1 immediately. The ET-1 in 3 ml plasma was extracted with a Sep-Pak C18 cartridge in accord with the procedures described by Xuan et al. (21) and then assayed with a radioimmunoassay kit purchased from Peninsula Laboratories (Belmont, CA, USA).

### *Assay for serum TNF- $\alpha$*

Blood samples were taken at 95 min after heat expo-

sure for determination of TNF- $\alpha$  levels. Blood samples were allowed to clot for 2 h at room temperature or overnight at 2–8°C before centrifuging for 20 min at approximately 2000  $\times$  g. Serum was quickly removed from these plasma samples and assayed for TNF- $\alpha$  immediately. The DuoSet Enzyme-linked Immunosorbent Assay (ELISA) Development System rat TNF- $\alpha$  kit (R & D Systems, Minneapolis, MN, USA) was used for measuring the levels of active rat TNF- $\alpha$  present in serum. This assay employs the quantitative colorimetric sandwich ELISA technique. The assay was based on the competition between rat TNF- $\alpha$  in the test sample and enzyme-linked recombinant rat TNF- $\alpha$  for binding sites on a specific TNF- $\alpha$  primary antibody. An affinity purified antibody specific for rat TNF- $\alpha$  had been pre-coated onto a microplate. Standards, controls, and samples are pipetted into the wells and any rat TNF- $\alpha$  present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody for rat TNF- $\alpha$  is added to the wells. Following a wash to remove any unbound antibody-enzyme reagents, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. After the addition of stop solution, plates were read in an ELISA plate reader at 450 nm. Recombinant rat TNF- $\alpha$  represented the standards for calibration and the detection limit of all assays was 2 pg/ml.

#### *Measurement of cardiac output*

In separate experiments, the animal's trachea was intubated, and the animal was artificially respired at 50 breaths/min, with a tidal volume of 20 ml and inspiration-to-expiration ratio at 1:2. A 3S Transonic flow probe (Transonic System, Taconic, NY, USA) was implanted around the ascending aorta as described by Smith (22). Briefly, the chest was opened at the third intercostal space to expose the heart. A small section (1-cm-long) of the ascending aorta was freed from connective tissue. The flow probe was then implanted around the root of the ascending aorta. The chest incision was closed, and a negative intrathoracic pressure was restored. The cardiac output was monitored continuously by the Transonic flow probe. The values of TPR were obtained by dividing MAP by CO. The values of SV were obtained by dividing CO by HR.

#### *Measurement of extracellular ischemia and damage markers in brain*

A microdialysis probe (4 mm in length, CMA/12; Carnegie Medicine, Stockholm, Sweden) was stereotaxically implanted into the left corpus striatum. An equilibrium period of 120 min without sampling was

allowed after probe implantation. The microdialysis was perfused at 2.0  $\mu$ l/min, and the dialysates were sampled in microvials. The dialysates were collected every 10 min in a CMA/140 fraction collector. Aliquots of dialysates (5  $\mu$ l) were injected onto a CMA 600 microdialysis analyzer for measurement of lactate, glycerol, pyruvate, and glutamate as described previously (2, 3).

#### *Neuronal damage score*

At the end of each experiment, the brain was removed, fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Serial (10  $\mu$ m) sections through the cortex and striatum were stained with hemotoxylin and eosin for microscopic evaluation. The extent of cortical and striatal neuronal damage was scored on a scale of 0–3, modified from the grading system of Pulsinelli et al. (23), in which 0 is normal, 1 means that approximately 30% of the neurons are damaged, 2 means that approximately 60% of that neurons are damaged, and 3 means that 100% of that neurons are damaged. Each hemisphere was evaluated independently without the examiner knowing the experimental conditions. When examined for neuronal damage in gray matter, only areas other than those invaded by probes were assessed. Our previous results (24, 25) have shown that the striatal, hypothalamic, and cortical neurons are susceptible to cerebral ischemia following heatstroke. However, in the present study, only the striatal and cortical regions were chosen for histological examination of neuronal damage.

#### *Statistical analyses*

Data are presented as the mean  $\pm$  S.E.M. Repeated-measures analysis of variance was used for factorial experiments, whereas Duncan's multiple-range test was used for post hoc multiple comparisons among means. For scoring neuronal damage, Wilcoxon signed rank test was used when only two groups were compared. The Wilcoxon tests convert the scores or values of a variable to ranks, require calculation of a 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 given by "median" and first and third quartile. A *P* value less than 0.05 was considered as statistical significance.

## **Results**

#### *Effects of heat exposure on survival time during heatstroke*

Table 1 summarizes the effects of heat exposure (42°C Ta for 80 min) on survival time in rat heatstroke. It can be seen from the table that survival time was found to be 102  $\pm$  5 min (*n* = 8) for rats treated with vehicle

**Table 1.** Effects of heat exposure (HE: Ta = 42°C for 80 min) on survival time in vehicle-treated or drug-treated rats

Treatments	Survival time (min) <sup>a</sup>
1. Vehicle (ml/kg, i.v., 60 min before HE)-treated rats at 24°C	>450
2. Vehicle (ml/kg, i.v., 60 min before HE)-treated rats at 42°C	21 ± 6
3. BQ-610 (0.1 mg/ml per kg, i.v., 60 min before HE)-treated rats at 42°C	24 ± 4
4. BQ-610 (0.5 mg/ml per kg, i.v., 60 min before HE)-treated rats at 42°C	169 ± 15*
5. BQ-788 (0.5 mg/ml per kg, i.v., 60 min before HE)-treated rats at 42°C	20 ± 5
6. BQ-610 (0.5 mg/ml per kg, i.v., 0 min before HE)-treated rats at 42°C	25 ± 8
7. BQ-123 (1 mg/ml per kg, i.v., 60 min before HE)-treated rats at 42°C	197 ± 25*

<sup>a</sup>All drug-treated groups, exposed to 42°C, had heat exposure withdrawn at 80 min. Except vehicle-treated group, data are means ± S.E.M. for eight rats per group. \* $P < 0.05$ , compared with the vehicle-treated rats at 42°C. One-way ANOVA followed by Duncan's test.

solution 60 min before the initiation of heat exposure. Pretreatment with an i.v. dose of 0.5 mg/ml per kg of BQ-610 or 1 mg/kg of BQ-123, but not BQ-788, 60 min before the initiation of heat exposure significantly increased the survival time. However, pretreatment with a lesser dose (e.g., 0.1 mg/ml per kg) of BQ-610 60 min before heat stress had an insignificant effect on survival time. Furthermore, intravenous administration of BQ-610 at a dose of 0.5 mg/kg "0" min before the start of heat stress did not affect the survival time.

#### *Effects of heat exposure on Tco, MAP, CO, SV, and TPR*

As shown in Fig. 1, 95 min after initiation of heat exposure in the vehicle-treated group, all the MAP, CO, SV, and TPR were significantly decreased as compared with those of normothermic controls. In contrast, the values of Tco were significantly higher than those of the normothermic controls. Treatment with an i.v. dose of BQ-610 (0.5 mg/kg) 60 min before the initiation of heat exposure significantly attenuated the heat stress-induced arterial hypotension, decreased CO, decreased SV, decreased TPR, and increased hyperthermia.

#### *Effects of heat exposure on MAP, SBF, and cerebral glutamate, glycerol and lactate/pyruvate ratio*

As shown in both Figs. 2 and 3, 95 min after the initiation of heatstroke in the vehicle-treated group, all the values of MAP and SBF were significantly decreased as compared with those of normothermic controls. On the other hand, the values of extracellular concentrations of glutamate, glycerol, and lactate/pyruvate ratio in the corpus striatum were significantly greater than those of the normothermic controls. Treatment with an i.v. dose of BQ-610 (0.5 mg/kg) (Fig. 2) or BQ-123 (1 mg/kg) (Fig. 3) 60 min before the initiation of heat stress significantly reduced the heat stress-induced arterial hypotension, cerebral ischemia, and increased levels of glutamate, glycerol, and lactate

/pyruvate ratio in the striatum.

#### *Effects of heat exposure on neuronal damage score*

Table 2 summarizes the effects of heat exposure (42°C for 80 min) on neuronal damage in the striatum and cortex of rats pretreated with vehicle solution, BQ-610 (0.5 mg/kg, i.v.), or BQ-123 (1 mg/kg, i.v.). Again, it was found that the scores for striatal or cortical neuronal damage in heatstroke rats receiving vehicle solution [median (Q1, Q3), 2 (2, 2)] were significantly greater ( $P < 0.05$ ) than those of the normothermic controls [median (Q1, Q3), 0 (0, 0.75)]. However, the striatal or cortical neuronal damage score in heatstroke rats receiving BQ-610 (0.5 mg/kg, i.v.) or BQ-123 (1 mg/kg, i.v.) 60 min before the initiation of heat exposure [median (Q1, Q3), 0 (0, 1) or 0 (0.25, 1)] or [median (Q1, Q3), 0 (0, 1) or 0 (0.25, 1)] were significantly lower ( $P < 0.05$ ) than those of the heatstroke rats that received vehicle as the controls. Figure 4 shows that heatstroke-induced cell body shrinkage, pyknosis of the nucleus, and loss of Nissl substance in the cortex or striatum were attenuated by pretreatment with BQ-610.

#### *Effects of heat exposure on plasma levels of ET-1 and TNF- $\alpha$*

Figure 5 summarizes the values of plasma ET-1 in rats ( $n = 8$ ) at 0 min before initiation of heat exposure in rats ( $n = 8$ ) at 80 min after initiation of heat exposure and in rats ( $n = 8$ ) at 95 min after initiation of heat exposure. The values of ET-1 in the plasma of rats at 80 or 95 min after initiation of heat exposure were significantly increased as compared with those at 0 min before initiation of heat exposure.

Figure 6 summarizes the values of plasma TNF- $\alpha$  in rats ( $n = 8$ ) kept at 24°C for 95 min, in vehicle-pretreated rats ( $n = 8$ ) kept at 42°C for 80 min plus 15 min room temperature exposure, and in BQ-610-pretreated rats ( $n = 8$ ) kept at 42°C for 80 min plus 15 min room

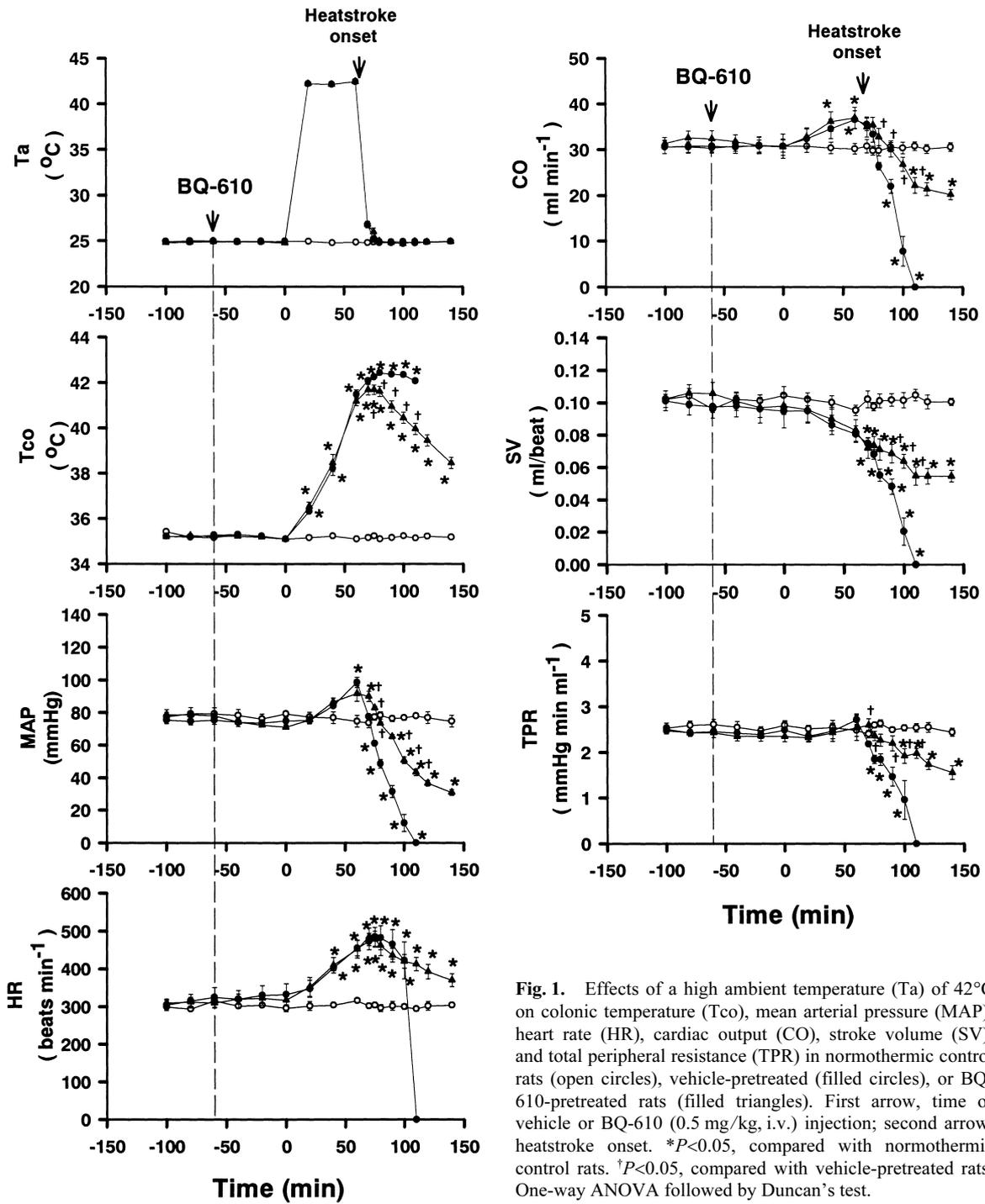
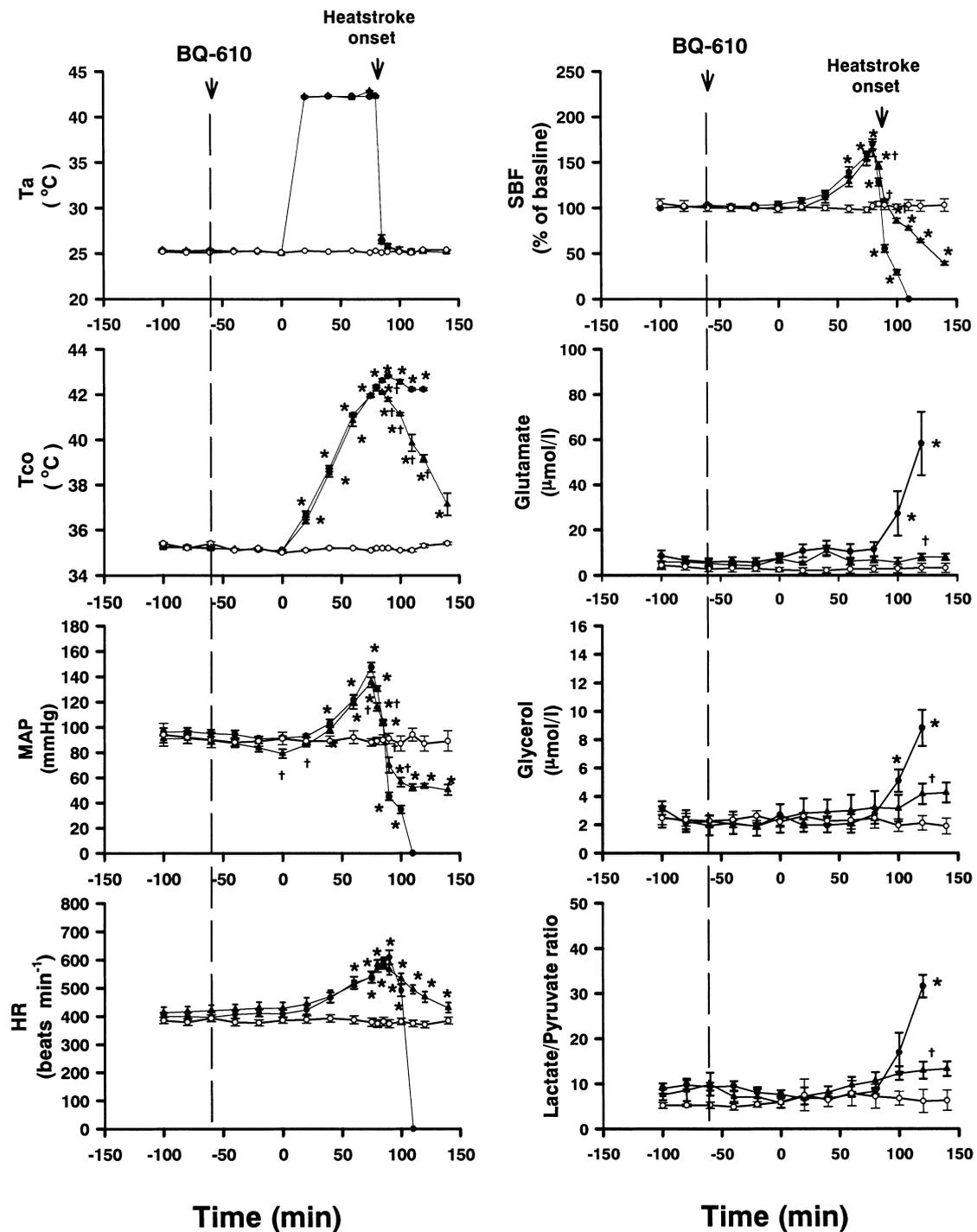


Fig. 1. Effects of a high ambient temperature ( $T_a$ ) of 42°C on colonic temperature ( $T_{co}$ ), mean arterial pressure (MAP), heart rate (HR), cardiac output (CO), stroke volume (SV), and total peripheral resistance (TPR) in normothermic control rats (open circles), vehicle-pretreated (filled circles), or BQ-610-pretreated rats (filled triangles). First arrow, time of vehicle or BQ-610 (0.5 mg/kg, i.v.) injection; second arrow, heatstroke onset. \* $P < 0.05$ , compared with normothermic control rats. † $P < 0.05$ , compared with vehicle-pretreated rats. One-way ANOVA followed by Duncan's test.

temperature exposure. It was found that the values of plasma TNF- $\alpha$  was significantly increased in vehicle-pretreated rats kept at 42°C as compared with rats kept at 24°C. In addition, the value of plasma TNF- $\alpha$  in vehicle-treated rats kept at 42°C was significantly higher than those of BQ-610-pretreated rats kept at 42°C.

**Discussion**

As demonstrated by previous results that endothelial cell activation/injury and marked increases in plasma levels of ET occurred in heatstroke patients (13, 26), the present results also showed that the plasma levels of ET-1 were remarkably elevated during the rat heatstroke. During heatstroke, the excessive accumulation



**Fig. 2.** Effects of a high ambient temperature ( $T_a$ ) of  $42^\circ\text{C}$  on colonic temperature ( $T_{co}$ ), mean arterial pressure (MAP), heart rate (HR), striatal blood flow (SBF), and the extracellular concentrations of glutamate, glycerol, and lactate/pyruvate ratio of the corpus striatum in normothermic control rats (open circles), vehicle-pretreated (filled circles), or BQ-610-pretreated rats (filled triangles). First arrow, time of vehicle or BQ-610 (0.5 mg/kg, i.v.) injection; second arrow, heatstroke onset. \* $P < 0.05$ , compared with normothermic control rats. † $P < 0.05$ , compared with vehicle-pretreated rats. One-way ANOVA followed by Duncan's test.

of ET-1 was accompanied by arterial hypotension, cerebral ischemia, and neuronal damage. The present results further demonstrated that prior antagonism of

ET<sub>A</sub>, but not ET<sub>B</sub>, receptors significantly attenuated the heatstroke-induced arterial hypotension, cerebral ischemia, and neuronal damage and prolonged the

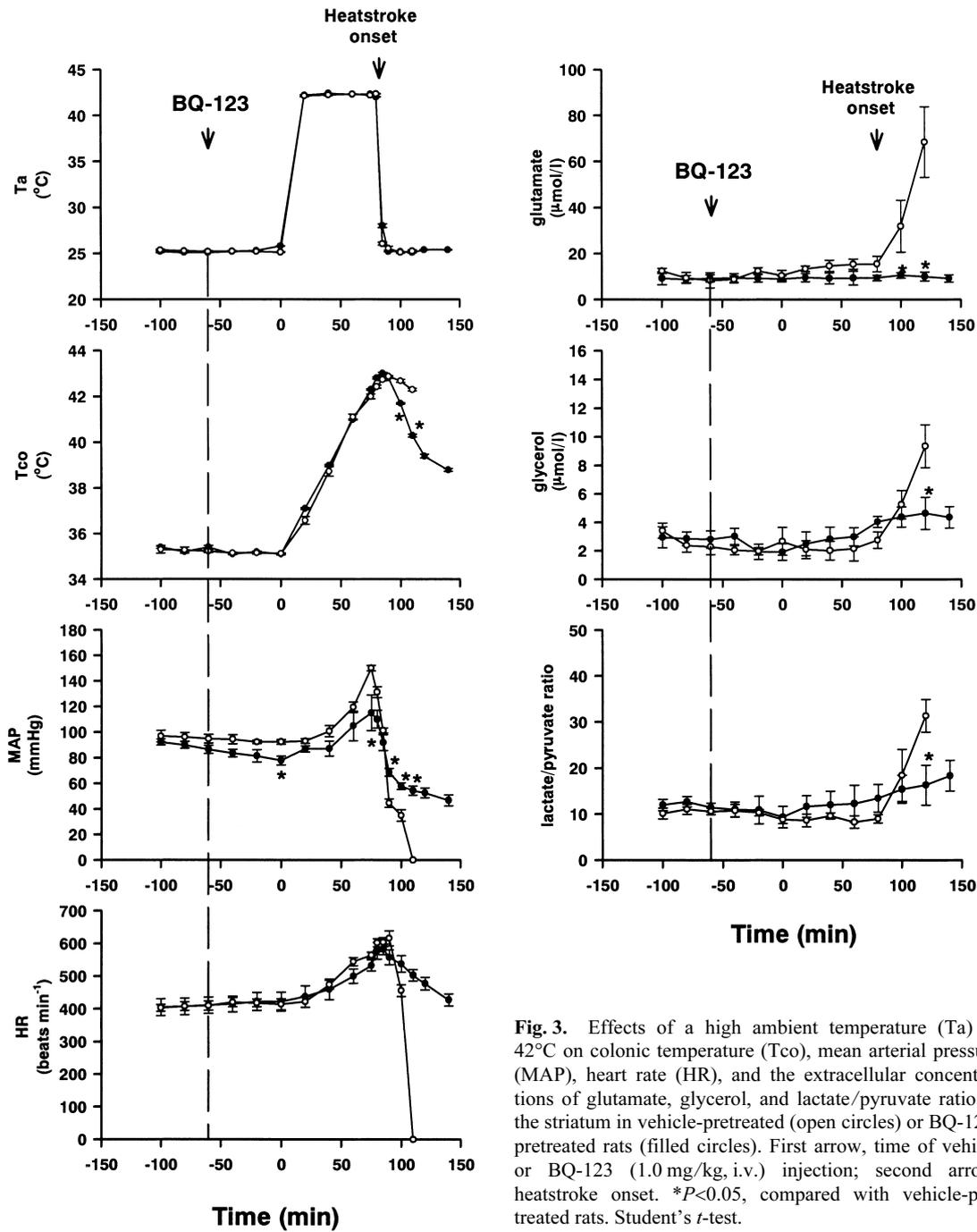


Fig. 3. Effects of a high ambient temperature ( $T_a$ ) of  $42^{\circ}\text{C}$  on colonic temperature ( $T_{co}$ ), mean arterial pressure (MAP), heart rate (HR), and the extracellular concentrations of glutamate, glycerol, and lactate/pyruvate ratio of the striatum in vehicle-pretreated (open circles) or BQ-123-pretreated rats (filled circles). First arrow, time of vehicle or BQ-123 (1.0 mg/kg, i.v.) injection; second arrow, heatstroke onset. \* $P < 0.05$ , compared with vehicle-pretreated rats. Student's  $t$ -test.

survival time (interval between the onset of heatstroke and cardiac arrest). These results implicate that high plasma levels of ET-1 may mediate the progress of pathophysiological function in rats with heatstroke, and the prior antagonism of ET<sub>A</sub> receptors may attenuate the heatstroke-induced circulatory shock, cerebral ischemia, and neuronal damage. Somewhat analogous results were obtained. For example, the amounts of ET-1 in plasma were increased in rats with cerebral

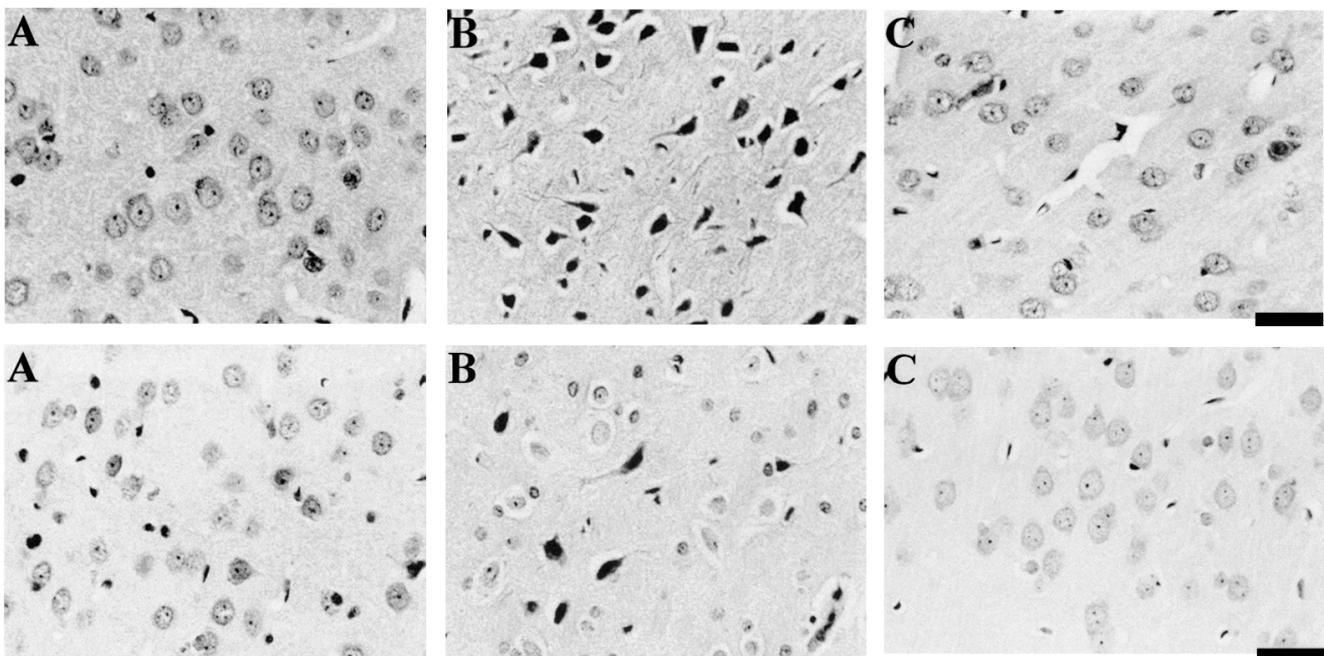
ischemia induced by middle cerebral artery occlusion (MCAO) (27, 28). The cerebral ischemic damage induced by MCAO, brain trauma, or stroke was known to be protected by ET<sub>A</sub>-receptor antagonism (29, 30).

Furthermore, the present results showed that BQ-610 administration was most efficient when initiated 60 min before the start of heat stress. However, delaying onset of BQ-610 injection (i.e., 0 min immediately after the onset of heatstroke) reduced the preventive efficiency on

**Table 2.** Effects of heat exposure (42°C for 80 min) plus 15 min room temperature (24°C) exposure on the neuronal damage score values of the cortex and corpus striatum from normal saline-treated, BQ-610-treated, or BQ-123-treated rats

Treatment	Score of neuronal damage (0–3)	
	cortex	striatum
1. Normal saline (1 ml/kg, i.v.)-treated rats at 24°C	0 (0, 0.75)	0 (0, 0.75)
2. Normal saline (1 ml/kg, i.v.)-treated rats at 42°C	2 (2, 2) <sup>a</sup>	2 (2, 2) <sup>a</sup>
3. BQ-610 (0.5 mg/kg, i.v.)-treated rats at 42°C	1 (0.25, 1) <sup>b</sup>	1 (0, 1) <sup>b</sup>
4. BQ-123 (1 mg/kg, i.v.)-treated rats at 42°C	1 (0.25, 1) <sup>b</sup>	1 (0, 1) <sup>b</sup>

Values are the median of 6 rats per group followed by Q1 and Q3 in parenthesis. For determination of neuronal damage score, animals were killed after 80 min heat exposure plus 15 min room temperature exposure. The data were evaluated by a Wilcoxon signed rank test followed by Duncan's test. <sup>a</sup> $P < 0.05$ , significance of difference from corresponding values (treatment 1, control rats). <sup>b</sup> $P < 0.05$ , significance of difference from corresponding values (treatment 2).

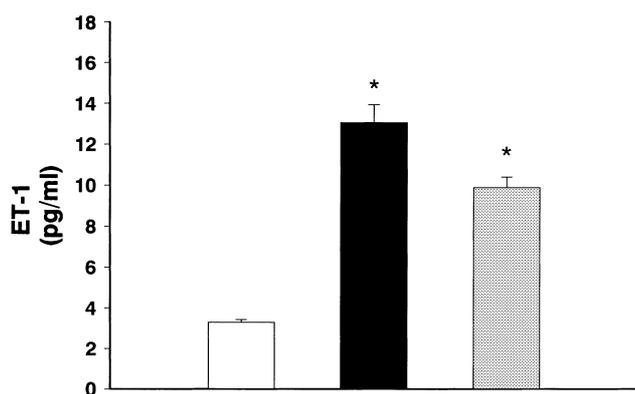


**Fig. 4.** Histological examination of neuronal damage. The photomicrographs of the corpus striatum (upper) and cortex (lower) in a normothermic control rat pretreated with vehicle solution (A), a heatstroke rat pretreated with vehicle solution (B), or a heatstroke rat pretreated with BQ-610 (0.5 mg/kg, i.v.) (C) at 60 min before the initiation of heat exposure. Ninety-five minutes after the initiation of heat exposure, both the corpus striatum and cortex of the rat pretreated with vehicle solution showed cell shrinkage and pyknosis of the nucleus (B). However, with the BQ-610 pretreatment, neuronal damage was reduced, as shown in C. Scale bar, 50  $\mu$ m.

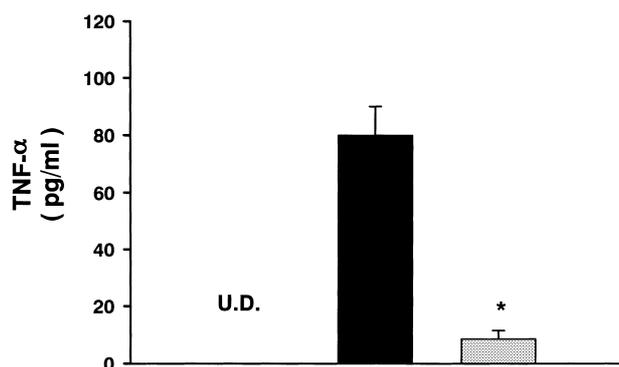
heatstroke. It should be mentioned that, in the present study, the survival time (interval between the onset of heatstroke and animal death) for the saline-treated rats was  $21 \pm 6$  min. The therapeutic window is very narrow; BQ-610 administered right after the onset of heatstroke failed to protect against heatstroke.

In the present results, when the animal was exposed to heat stress, the prolongation of survival in rats with BQ-610 or BQ-123 was found to be related to main-

tenance of appropriate levels of MAP and CBF as well as reduction in cerebral neuronal damage after the onset of heatstroke. The maintenance of appropriate level of CBF may be brought about by higher cerebral perfusion pressure (CPP) resulting from lower intracranial pressure (ICP; due to reduction in cerebral edema and cerebrovascular congestion) and higher MAP during the development of heatstroke (31). BQ-610 was found to maintain an appropriate level of MAP by augmenta-



**Fig. 5.** The plasma levels of ET-1 during heatstroke. The blank, black, and gray bars, respectively, represent the values of plasma ET-1 obtained in rats ( $n=8$ ) at 0 min before the initiation of heat exposure, in rats ( $n=8$ ) at 80 min after initiation of heat exposure, and in rats ( $n=8$ ) at 95 min after initiation of heat exposure. The plasma levels of ET-1 obtained at 80 or 95 min after initiation of heat exposure are significantly increased in rats as compared with "0" time controls.  $*P<0.05$ , significantly different from the rats obtained at 0 min before the initiation of heat exposure. One-way ANOVA followed by Duncan's test.



**Fig. 6.** The serum concentrations of tumor necrosis factor-alpha (TNF- $\alpha$ ) after the initiation of heat exposure in rats pretreated with vehicle or BQ-610. The blank, black, and gray bars, respectively, represent the values of serum TNF- $\alpha$  obtained in rats ( $n=8$ ) kept at 24°C for 95 min, in vehicle-pretreated rats ( $n=8$ ) kept at 42°C for 80 min plus 15 min room temperature exposure and in BQ-610-pretreated rats ( $n=8$ ) kept at 42°C for 80 min plus 15 min room temperature.  $*P<0.05$ , significantly different from vehicle-pretreated rats. Student's  $t$ -test. U.D.: undetectable.

tion of SV as well as total peripheral vascular resistance. In fact, it has been shown that ET-1 in high concentrations was found to enhance blood-brain barrier permeability and to contribute to brain edema formation during MCAO (28). It is reasonable to assume that the heatstroke-induced intracranial hypertension may be reduced by BQ-610 or BQ-123 pretreatment.

Our present and previous (32, 33) results have shown that heatstroke induces systemic and central production

of TNF- $\alpha$  and interleukin-1 $\beta$  (IL-1 $\beta$ ) in both rats and rabbits. The plasma levels of TNF- $\alpha$  and IL-1 $\beta$  are shown to be well related to the severity of heatstroke (34, 35). Indeed, increased levels of these cytokines are associated with heatstroke-induced arterial hypotension, cerebral ischemia, and neuronal damage (32, 33; present results). Prior antagonism of ET<sub>A</sub> (present results) or IL-1 $\beta$  receptors (36, 37) protects against heatstroke-induced arterial hypotension, and cerebral ischemic damage and prolongs survival. As it is shown in the present results, prior antagonism of ET<sub>A</sub> receptors might exert its protective effects by attenuating the increased plasma level of TNF- $\alpha$  during heatstroke. According to the findings of Nakamura et al. (38), TNF- $\alpha$  might play a pathophysiological role in the progression of acute heart failure. Septic shock could be mimicked by systemic administration of TNF- $\alpha$  (39–41). Thus, it is likely that ET<sub>A</sub>-receptor antagonism might attenuate the heatstroke-induced circulatory shock by reducing TNF- $\alpha$  level in the plasma during heatstroke. In fact, the contention is supported by previous results (42). These investigators have shown that the generation of TNF- $\alpha$  by cells activated with ET-1 points to a pro-inflammatory role of ET-1 in heart failure or circulatory shock, which are associated with high ET-1 plasma levels.

Hall and colleagues (43) have concluded that hyperthermia exhibited during heatstroke stimulates xanthine oxidase production of reactive oxygen species that activate metals and limit heat tolerance by promoting circulatory and intestinal barrier dysfunction. Our recent results further showed that oxidative stress occurred after the onset of heatstroke in rats (32, 44). ET-1 may increase oxidative stress and result in vascular damage during inflammatory responses (45). In the present results, BQ-610 may antagonize ET<sub>A</sub> receptors and enhance heat tolerance by reducing oxidative stress.

During hyperthermia, vasoconstrictor tone in the viscera is lost despite high levels of sympathetic neural output and plasma catecholamines, suggesting that vascular responsiveness to adrenergic receptor stimulation is reduced. In anesthetized rats, heating to T<sub>co</sub> of 41.5°C markedly attenuated the hemodynamic responses to ET (46). In the normothermic state, the blood pressure has been lowered by administration of ET<sub>A</sub>-selective receptor antagonists (47). However, in the present results, the appropriate levels of blood pressure were maintained in hyperthermic rats treated with ET<sub>A</sub>-receptor antagonists. In the present results, ET<sub>A</sub>-receptor antagonism may improve hypotension during heatstroke by reducing hyperthermia. In fact, it has been proposed that a role for endogenous ET in mediating losses in plasma volume and albumin escape elicited by

lipopolysaccharide through ET<sub>A</sub>-receptor activation and suggested that ET<sub>A</sub>-receptor blockers may be useful agents in the therapy of septic shock (48). In the present results, ET<sub>A</sub>-receptor blockers may have improved plasma volume and albumin escape during heatstroke and resulted in maintenance of appropriate levels of blood pressure.

In summary, heatstroke caused hyperthermia, cardiac dysfunction, arterial hypotension, cerebral ischemia, neuronal damage, and an increased level of TNF- $\alpha$  and ET-1 in plasma. Systemic pretreatment with ET<sub>A</sub>-receptor antagonists attenuated the heatstroke-induced hyperthermia, cardiac dysfunction, arterial hypotension, cerebral ischemia, and neuronal damage, and this eventually resulted in prolongation of the survival time. Therefore, prior antagonism of ET<sub>A</sub>-receptor mechanisms may be a good therapeutic strategy for heatstroke syndromes.

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