Objective: We investigated the effect of estrogen therapy on inflammatory responses, cardiovascular functions, and survival in a rat model of heatstroke.

Design: Controlled, prospective study.

Setting: Hospital medical research laboratory.

Subjects: Sprague-Dawley rats (280–312 g of body weight, males and females).

Interventions: Four major groups of anesthetized rats were designated for experiments: a) vehicle-treated male rats; b) vehicle- or premarin-treated estrus female rats; c) vehicle- or premarin-treated ovariectomized rats; and d) vehicle- or premarin-treated leuprolide-treated rats. All animals were exposed to heat stress (ambient temperature 43°C for 70 mins) and then allowed to recover at room temperature (24°C). Their survival time (interval between the onset of heatstroke and animal death) and physiologic and biochemical variables were monitored. Vehicle (normal saline 1 mL/kg of body weight, intravenously) or premarin (1 mg/mL/kg of body weight, intravenously) was administered 70 mins after initiation of heat stress. Ovariectomy or leuprolide (100 μg/kg/day, subcutaneously) injection was conducted 4 wks before the start of heat stress experiments. Another group of rats were exposed to 24°C and used as normothermic controls.

Measurements and Main Results: Compared with the estrus female rats, the ovariectomized rats, the leuprolide-treated rats, and male rats all had lower levels of plasma estradiol and lower survival time values. However, after an intravenous dose of premarin, both the plasma estradiol and survival time values were significantly increased. Compared with the nonthermocold controls, the vehicle-treated male and ovariectomized rats all displayed higher levels of serum tumor necrosis factor-α, which could be suppressed by premarin therapy. In contrast, the serum levels of IL-10 in these groups were significantly elevated by premarin during heatstroke. Furthermore, the heatstroke-induced hyperthermia, arterial hypotension, intracranial hypertension, and cerebral hypoperfusion, hypoxia, and ischemia were significantly attenuated by premarin therapy in ovariectomized rats.

Conclusions: We successfully demonstrated that estrogen replacement may improve survival during heatstroke by ameliorating inflammatory responses and cardiovascular dysfunction. (Crit Care Med 2006; 34:1113–1118)

Key Words: heatstroke; rat; premarin; brain ischemia; hypotension; cytokine

Evidence obtained from studies in humans and animals indicates that the inflammatory and hemostatic responses of the host to heat stress contribute to multiple organ injury in those who suffer heatstroke (1, 2). High levels of pro- and anti-inflammatory cytokines in the peripheral bloodstream as well as dopamine correlate with the severity of circulatory shock, cerebral ischemia and hypoxia, and neuronal damage occurring during heatstroke (3–8).

Another line of evidence has suggested that estradiol influences the severity of injury associated with cerebrovascular stroke. For example, ovariectomy was shown to eliminate the endogenous protective effect observed in female rats following cerebral ischemia (9–11). Additionally, plasma estradiol levels have been shown to relate inversely to ischemic stroke damage in female rats (12). Furthermore, estrogen replacement in adult ovariectomized (OVX) rats significantly restored neuroprotection to a level similar to that observed in intact animals (9–11). Estradiol was also shown to regulate pro- and anti-inflammatory cytokine release after trauma-hemorrhage (13, 14) or autoimmune encephalomyelitis (15). However, it is not known whether estrogen replacement has an effect on inflammatory responses, cardiovascular functions, and survival in a rat model of heatstroke.

In the present study we measured the cerebral cardiovascular responses of normal adult male and female rats with or without estrogen replacement during heat stress. In addition, we determined whether estrogen replacement with premarin (USP) 1 mg/kg intravenously (16), immediately after the onset of heatstroke, confers cardiovascular protection in surgically or chemically OVX rats by altering production of tumor necrosis factor (TNF)-α, a proinflammatory cytokine (17), and interleukin (IL)-10, an anti-inflammatory cytokine (18).

**METHODS**

Animals. Adult female and male Sprague-Dawley rats (weight, 287 ± 16 g) were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The stage of the female reproductive cycle was determined by regular examination of vaginal smears by the same examiner. Proestrus was defined as 24°C and used as normothermic controls.
in approximately equal numbers were present on the vaginal smears. Estrus was characterized by large, squamous-type epithelial cells without nuclei. In female rats, experiments were performed only after at least one complete estrus cycle had been documented. All protocols were approved by the Animal Ethics Committee of the Chi-Mei Medical Center (Tainan, Taiwan) in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act.

Bilateral O VX. All adult female rats were anesthetized with pentobarbital (40 mg/kg) and subjected to operation. An incision was made two thirds of the way down on each side of the back, and the ovaries were seized along with the periovarian fat and delivered through the incision. The juncture between the fallopian tube and uterine horn was severed with scissors, freeing the ovaries. Incisions were then sutured, and the animals recovered on a heating pad.

Chemical Castration. Forty-two-day-old female Sprague-Dawley rats were administrated leuprolide (100 µg/kg/day, subcutaneous injection) beginning 4 wks before heat stress (19).

Surgery and Physiologic Variable Monitoring. On the day of thermal experiments, the animals were anesthetized with urethane (1.4 g/kg, intraperitoneally) to abolish the corneal reflex and pain reflexes. The right femoral artery and uterine horn were cannulated with polyethylene tubing (PE 50), under urethane anesthesia, for blood pressure monitoring and drug administration. Core temperature was monitored continuously by a thermocouple, whereas both mean arterial pressure (MAP) and heart rate were continuously monitored with a pressure transducer.

Induction of Heatstroke. The core temperature of the anesthetized animals was maintained at about 36°C with an infrared light lamp except during the heat stress experiments. Heatstroke was induced by putting the animals in a folded heating pad of 43°C controlled by circulating hot water. The instant at which the MAP dropped irreversibly from the control value to approximately 20 mm Hg was taken as the onset of heatstroke (6, 8). After the onset of heatstroke, the heating pad was removed and the animals were allowed to recover at room temperature (24°C). Our pilot results showed that the latency for onset of heatstroke was 70 ± 2 min (n = 8). Therefore, in the following heatstroke groups of rats, all animals were exposed to 43°C for exactly 70 mins and then were allowed to recover at room temperature (24°C).

Experimental Groups. Four major groups of animals were designated for experiments: a) vehicle- or premarin-treated male rats; b) vehicle- or premarin-treated estrus female rats; c) vehicle- or premarin-treated OVX rats; and d) vehicle- or premarin-treated leuprolide-treated rats. In vehicle- or premarin-treated rats, normal saline (1 mL/kg, intravenously) or premarin (1 mg/kg, intravenously), respectively, was administered 70 mins after the initiation of heat stress (or immediately after the onset of heatstroke). Different groups of animals were used for different sets of experiments: a) determination of plasma levels of estradiol; b) measurements of survival time (interval between onset of heatstroke and animal death); c) measurements of MAP, intracranial pressure, cerebral perfusion pressure (CPP) (CPP = MAP – ICP), cerebral blood flow, brain PO2, and brain temperature; d) measurements of core temperature, MAP, heart rate, and striatal levels of glutamate, glyceral, and lactate/pyruvate; and e) measurements of serum levels of both TNF-α and IL-10.

Measurements of Cerebral Blood Flow, Brain Oxygen, and Brain Temperature. A 100-µm diameter thermocouple and two 230-µm fibers were attached to the oxygen probe. This combined probe measures oxygen, temperature, and microvascular blood flow. Under urethane anesthesia, the animal was placed in a stereotaxic apparatus, and the combined probe was implanted into the striatum using the atlas and coordinates of Paxinos and Watson (20). The detailed procedures for measurement of brain temperature, PO2, and temperature were described previously (20).

Measurement of Extracellular Glutamate, Glyceral, and Lactate/Pyruvate Ratio in the Striatum. Animals were anesthetized with urethane administered intraperitonely. The animal’s head was mounted in a stereotaxic apparatus (Davis Kopf Instruments). The microdialysis probe was stereotaxically implanted into the striatum. The detailed procedures for measurements of cellular ischemic and damage markers were described previously (21).

Measurement of Serum TNF-α and IL-10. Blood samples were taken at 0, 70, or 90 mins after the start of heat exposure for determination of TNF-α and IL-10 levels. For measurement of serum cytokines, 5 mL of blood was withdrawn from the femoral vein of each rat. The amounts of the cytokines including TNF-α and IL-10 in serum were determined by using double-antibody sandwich enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) according to the manufacturer’s instruction. Optical densities were read on a plate reader set at 450 nm for TNF-α and IL-10. The concentration of TNF-α or IL-10 in the serum samples was calculated from the standard curve multiplied by the dilution factor and was expressed as pg/mL.

Radioimmunoassay. Plasma estradiol concentrations were determined using the double-antibody radioimmunoassay kits (rat estradiol radioimmunoassay kit, Immunotech, Marseille, France). One hundred (estradiol) microliter plasma samples were assayed in duplicate. The cross-reactivity of the radioimmunoassay for estradiol was 100%. The test values were determined by interpolation from the standard curves. The lowest detectable levels of estradiol were 20 ng/mL.

Statistical Analysis. Data are presented as mean ± SEM. Statistical analysis was performed by one-way analysis of variance followed by Duncan’s multiple-range test when appropriate. We considered p < .05 to indicate a statistically significant difference.

RESULTS

Plasma Levels of Estradiol for Different Groups of Rats. Plasma levels of estradiol were found to be the highest in proestrus female animals (110 ± 9 pg/mL; group 3 in Table 1) and were significantly lower in male (37 ± 9 pg/mL) and estrus female (86 ± 8 pg/mL) rats at the start of the thermal experiments (p < .05). Compared with the vehicle-treated estrus female rats, both vehicle-treated OVX rats and vehicle-treated leuprolide-treated rats had lower levels of plasma estradiol (47 ± 4 and 50 ± 5 pg/mL, respectively). In male rats, estrus female rats, OVX rats, or leuprolide-treated rats, plasma levels of estradiol were increased to new values of 2178–2473 pg/mL of estradiol 20 mins after an intravenous dose of 1 mg/kg premarin.

Effects of Heat Stress on Survival Time Values in Different Groups of Rats. Compared with the vehicle-treated male rats, the vehicle-treated female rats had a higher survival time value. On the other hand, compared with the vehicle-treated male rats, the estrus female rats had a significantly increased survival time value. Vehicle or premarin was administered 70 mins after the initiation of heat exposure, and the blood sampling was obtained for E2 assay 20 mins after vehicle or premarin injection. Bilateral OVX or chemical castration (produced by leuprolide) was conducted 4 weeks prior to heat stress. Data are mean ± SEM of eight rats per group.
hand, compared with the vehicle-treated estrus female rats, the vehicle-treated OVX rats or leuprolide-treated female rats had lower survival time values. However, the survival time values for either the male rats, the estrus female rats, the OVX rats, or the leuprolide-treated female rats were significantly increased by treatment with premarin immediately after the onset of heatstroke. In response to an intravenous dose of premarin (1 mg/kg), the survival time values for male rats were indistinguishable from those of the estrus female rats (200 ± 51 vs. 118 ± 17; p > .05). The data are summarized in Table 2.

Effects of Heat Stress on Physiologic Variables and Striatal Levels of Cellular Ischemia and Damage Markers. As shown in Figures 1 and 2, 80–100 mins after initiation of heat stress in the vehicle-treated OVX rats, MAP, CPP, cerebral blood flow, brain PO2, and heart rate were significantly decreased compared with normothermic controls. In contrast, the values of intracranial pressure, brain temperature, core temperature, and striatal levels of glutamate, glycerol, and lactate/pyruvate ratio were significantly higher than those of the normothermic controls. Treatment with an intravenous dose of premarin 70 mins after the initiation of heat exposure significantly attenuated the heat stress-induced arterial hypotension, intracranial hypertension, decreased cerebral perfusion pressure, cerebral ischemia, brain hypoxia, bradycardia, and increased levels of glutamate, glycerol, and lactate/pyruvate ratio in the striatum. However, premarin treatment did not affect the body and brain hyperthermia that occurred during heatstroke.

Effects of Heat Exposure on Serum Levels of TNF-α and IL-10. Table 3 summarizes the values of serum TNF-α and IL-10 in different groups of rats. The values of serum TNF-α of vehicle-treated male or OVX heatstroke rats obtained at 70 or 90 mins after initiation of heat exposure were significantly higher than those at 0 mins. In both male and OVX rats, premarin therapy adopted at 70 mins significantly suppressed the increased levels of serum TNF-α obtained at 90 mins during heatstroke. In male normothermic, vehicle-treated male heatstroke, estrus female normothermic, and vehicle-treated OVX rats, serum levels of IL-10 were maintained at a negligible

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Survival Time, Mins</th>
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<tbody>
<tr>
<td>1. Vehicle-treated male rats</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>2. Vehicle-treated estrus female rats</td>
<td>53 ± 4*</td>
</tr>
<tr>
<td>3. Premarin-treated male rats</td>
<td>200 ± 51b</td>
</tr>
<tr>
<td>4. Premarin-treated estrus female rats</td>
<td>118 ± 17b</td>
</tr>
<tr>
<td>5. Vehicle-treated OVX rats</td>
<td>16 ± 1c</td>
</tr>
<tr>
<td>6. Vehicle-treated leuprolide-treated rats</td>
<td>20 ± 2c</td>
</tr>
<tr>
<td>7. Premarin-treated OVX rats</td>
<td>274 ± 26d</td>
</tr>
<tr>
<td>8. Premarin-treated, leuprolide-treated rats</td>
<td>251 ± 22d</td>
</tr>
</tbody>
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OVX, ovariectomized.

*p < .05 in comparison with group 1; *p < .05 in comparison with group 1 or group 2; *p < .05 in comparison with group 2; *p < .05 in comparison with group 5 or group 6 (analysis of variance followed by Duncan’s test). In all heat stroke groups, for determination of survival time (interval between initiation of HE and animal death), the animals were exposed to HE (43°C) for exactly 70 mins and then allowed to recover at room temperature (24°C). Vehicle or premarin (1 mg/kg, intravenously) was administered 70 mins after initiation of heat exposure. Data are the mean ± SEM of eight rats per group.
level. However, 20 mins following an intravenous dose of premarin, the serum levels of IL-10 were greatly elevated in male or OVX heatstroke rats.

DISCUSSION

As shown in the present study, plasma levels of estradiol seem to be related to heat tolerance in rats. For example, the heat tolerance of estrus female rats was superior to that of male rats, whereas the heat tolerance of OVX or leuprolide-treated rats was inferior to that of estrus female rats. Furthermore, induction of high levels of plasma estradiol caused by intravenous administration of a high dose (1 mg/kg) of premarin in male, estrus female, OVX, or leuprolide-treated rats conferred protection after heatstroke occurrence, as reflected by prolonged survival time. Since the dose of premarin administered appears to be rather high, this dose of premarin functions as a pharmacologic dose.

It has been shown that the glutamate and lactate/pyruvate ratios are well-known markers of cellular ischemia, whereas glycerol is a marker of how severely cells are affected by ongoing pathology (21). Indeed, as demonstrated in the present results, cerebral ischemia and hypoxia induced by heatstroke are associated with an increased production of glycerol, lactate/pyruvate ratio, and glutamate in the brain as well as a decreased level of MAP or CPP in OVX rats. It is believed that the same nature of heatstroke reactions will be displayed by normal male or estrus female rats. In the present results, when the animal was exposed to heat stress, the prolongation of survival in the OVX rats treated with premarin was found to be related to maintenance of appropriate levels of MAP and cerebral blood flow as well as reductions in cerebral neuronal damage after the onset of heatstroke. The maintenance of an appropriate level of cerebral blood flow may be brought about by higher CPP resulting from lower intracranial pressure (due to reduction in cerebral edema and cerebrovascular congestion) and higher MAP during the development of heatstroke (17). Premarin may maintain an appropriate level of MAP by augmenting stroke volume as well as total peripheral vascular resistance (17). The present findings are in part consistent with previous investigations. For example, it has been shown that estrogen administration provides tissue protection from middle cerebral artery occlusion by increasing cerebral blood flow (22). The female proestrus rats have normalized organ functions at 24 hrs after trauma-hemorrhage, whereas male and female estrus animals have shown a marked depression in cardiovascular functions (14). The results indicate that the maintenance of cardiac functions after severe blood loss is associated with high levels of estradiol at the start of the experiment. Several investigations further demonstrate that estrogen produces a significant increase in blood flow not only to reproductive tissues (e.g., uterus, mammary gland, and vagina) but also to skin, thyroid gland, pancreas, spleen (23, 24), coronary vasculature (16), and brain (25). Estrogen may have direct vascular effects, independent of cytokines.

Our previous results (4, 5) showed that heatstroke induced overproduction of TNF-α and IL-1 in both the central...
nervous system and the peripheral bloodstream, and this was associated with hypotension, cerebral ischemia and neuronal damage, and high mortality rate. The administration of an IL-1 receptor antagonist or corticosteroids before the onset of heatstroke attenuated circulatory shock and cerebral ischemia and improved survival rate. The present findings further show that treatment with premarin protects against TNF-α overproduction, arterial hypotension, intracranial hypertension, and cerebral ischemic injury and prolongs survival during heatstroke. It is likely that premarin may attenuate circulatory shock and cerebral ischemia by reducing TNF-α levels and/or IL-1β in the serum during heatstroke.

Interleukin-10 has important anti-inflammatory and immunosuppressive properties through suppression of interferon-γ, TNF-α, and other proinflammatory cytokines (26). Humans with exertional heatstroke were found to have a high level of IL-10 in the serum (27). However, in the rats with nonexertional heatstroke, the serum level of IL-10 was undetectable as shown in the present study. Our present results further show that, after the onset of heatstroke in rats, treatment with premarin produces a significant increase in the serum level of IL-10 accompanied by a reduction of heatstroke reactions as mentioned in the preceding section. This implies that premarin may ameliorate cerebral ischemia and damage by increasing IL-10 but decreasing TNF-α production. In fact, both anti-inflammatory and proinflammatory reactions are normal components of the same immune response, which correlatively battle infection while preventing immune pathology (28). In the present study, premarin may have acted through this indirect neuroprotective mechanism to attenuate cerebral ischemia and damage during heatstroke.

In fact, evidence has accumulated to indicate that female hormones play a role in mediating the inflammatory response. For example, women of reproductive age are less likely than other people to develop rheumatoid arthritis (29), and they experience fewer symptoms of cardiovascular disease (30). The plasma levels of estrogen may affect outcomes after trauma and sepsis in laboratory studies (31–33). Estrogen treatments increased plasma estradiol levels, depressed burn-induced elevation in serum TNF-α levels in rats (34), restored cellular immunity in combined ethanol and burn-injured male mice via suppression of IL-6 production (35), and suppressed lung inflammatory responses in mice through an effect on vascular cell adhesion molecules and proinflammatory mediators (36). Heatstroke syndromes resemble sepsis in many of their characteristics (2). Any of the responses observed during septic shock can be mimicked by systemic administration of TNF-α (37–39). TNF-α may impair the release of nitric oxide from the vascular endothelium and lead to a reduced formation of endothelial-derived relaxing factor (40). It has also been suggested that TNF-α may induce vascular dysfunction and cause leukocytes to adhere to the vascular endothelium, where they release mediators (e.g., oxygen free radicals, cytokines, etc.) able to amplify the vascular injury. Estrogen is able to reduce enhanced macrophage and serum levels of TNF-α following splanchic ischemia-reperfusion injury (41). During heatstroke, rodents display hyperthermia, arterial hypotension, intracranial hypertension, cerebral ischemia, neural damage, and overproduction of inflammatory cytokines (including TNF-α). Hall and colleagues (42) also indicated that hyperthermia stimulates production of reactive oxygen species, which activates metals and limits heat tolerance by promoting circulatory and intestinal barrier dysfunction. In addition, overproduction of nitric oxide may contribute to the splanchic vasodilation that precedes vascular collapse with heatstroke. Thus, it appears that estradiol may improve heat tolerance by influencing vascular and cellular dysfunction (via TNF-α and/or nitric oxide production) during heatstroke.

Because many investigators have shown that monocytes, macrophages, and lymphocytes express estrogen receptors (43, 44), increases in circulating 17β-estradiol may directly alter cellular function, such as cytokine production. Knockouts of cytokines or estrogen receptors might be of interest for a future study in our heatstroke model.

REFERENCES


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Table 3. Serum levels of tumor necrosis factor (TNF)-α and interleukin (IL)-10 in different groups of rats measured at 0, 70, or 90 mins after the initiation of heat exposure in heatstroke rats

<table>
<thead>
<tr>
<th>Animal Treatment</th>
<th>Serum TNF-α, pg/mL</th>
<th>Serum IL-10, pg/mL</th>
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<tbody>
<tr>
<td>1. Male nornormeratic rats</td>
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<tr>
<td>0 mins before testing</td>
<td>1.2 ± 0.6 (8)</td>
<td>0.5 ± 0.2 (8)</td>
</tr>
<tr>
<td>70 mins after testing</td>
<td>1.0 ± 0.1 (8)</td>
<td>0.8 ± 0.5 (8)</td>
</tr>
<tr>
<td>90 mins after testing</td>
<td>1.5 ± 0.5 (8)</td>
<td>0.7 ± 0.4 (8)</td>
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For 0.05 in comparison with group 1 or group 4; a,b 0.05 in comparison with group 2 or group 5 (analysis of variance followed by Duncan’s test). In both group 1 and 4, the blood samplings were obtained at equivalent times. All heatstroke rats had heat exposure withdrawn at 70 mins and then were allowed to recover at room temperature (24°C). Data are mean ± SEM followed by numbers of animals used in parentheses. Animals used in parentheses. Vehicles or drugs were administered 70 mins after initiation of heat exposure.

OVARX, ovariectomized.


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