PREMARIN CAN ACT VIA ESTROGEN RECEPTORS TO RESCUE MICE FROM HEATSTROKE-INDUCED LETHALITY

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ABSTRACT—The present study was conducted to assess whether Premarin, a water-soluble estrogen sulfate, can act via estrogen receptors (ERs) to rescue mice from heat-induced lethality. Unanesthetized, unrestrained mice were exposed to ambient temperature of 42.4°C to induce heatstroke (HS). Another group of mice was exposed to room temperature (24°C) and used as normothermic controls. They were given isotonic sodium chloride solution, Premarin (0.1–1.0 mg/kg of body weight, i.p.), or Premarin (1 mg/kg of body weight, i.p.) plus the nonselective ER antagonist ICI 182, 780 (0.25 mg/kg of body weight, i.p.) 1 h after the termination of heat stress. Their physiologic and biochemical parameters were continuously monitored. Mice that survived on day 4 of heat treatment were considered survivors. When the vehicle-treated mice underwent heat, the fraction survival and core temperature at +4 h of body heating were found to be 0 of 12 and 34.4°C ± 3°C, respectively. Administration of Premarin (1 mg/kg) 1 h after the cessation of heat stress rescued the mice from heat-induced death (fraction survival, 12/12) and reduced the hypothermia (core temperature, 37.3°C). The beneficial effects of Premarin in ameliorating lethality and hypothermia can be abolished by simultaneous administration of ICI 182, 780. Both IL-10 (an anti-inflammatory cytokine) and estradiol in the serum were increased significantly in heat-stressed mice administered Premarin compared with vehicle-treated HS group. Heat-induced apoptosis, as indicated by terminal deoxynucleotidyl-transferase-mediated dUDP-biotin nick end-labeling staining, in the spleen, liver, and kidney were significantly reduced by Premarin. The increased levels of cellular ischemia (e.g., glutamate, lactate-to-pyruvate ratio, and nitrite) and damage (e.g., glyceral) markers and iNOS expression in the hypothalamus during HS were decreased significantly by Premarin therapy. The levels of proinflammatory cytokines (e.g., IL-1β and TNF-α) and renal and hepatic dysfunction markers in plasma that are up-regulated in heat stressed mice were significantly lower in Premarin-administered mice. The data indicate that Premarin may act via ERs to rescue mice from HS-induced lethality.

KEYWORDS—Heatstroke, Premarin, estrogen, liver, kidney, thermoregulation

INTRODUCTION

Heatstroke (HS) is characterized by hyperpyrexia and multiorgan dysfunction (1–3). The full spectrum of the signs and symptoms occurring during HS in humans can be mimicked by the rodent HS model (2). Multiorgan dysfunction ensuing from severe HS contains systemic inflammation, hypotension, hepatic, renal and thermoregulatory dysfunction, and hypercoagulable state.

Premarin, a water-soluble estrogen sulfate, is widely used as estrogen replacement therapy in clinical practice (4). Recently, we have successfully demonstrated that Premarin therapy may improve survival during HS by ameliorating inflammatory responses and cardiovascular dysfunction in an anesthetized rat model (5). However, it is not known whether Premarin can act via estrogen receptors (ERs) to rescue mice from HS-induced lethality.

To deal with the question, the effects of systemic administration of Premarin and/or the nonselective ER antagonist ICI 182, 780 (6) on the HS-induced hypothermia and lethality were assessed in unanesthetized, unrestrained mice (7–9). In addition, the temporal profiles of cellular ischemia and damage markers in the hypothalamus, plasma levels of cytokines, apoptosis of spleen, liver, and kidney, and hepatic and renal dysfunction during HS were assessed in mice with or without Premarin therapy.

MATERIALS AND METHODS

Mice

All the experiments were carried out in accordance with the ethical guidelines laid down by the committee for the purpose of control and supervision of experiments on animals, Chi Mei Medical Center (Tainan, Taiwan). The studies also conform to the Guiding Principles of American Physiologic Society. Institute of Cancer Research inbred female mice were given food and water ad libitum and acclimatized to room temperature at 24°C, relative humidity of 50% ± 8%, and a 12-h dark/light cycle for 1 week before the start of the experiment at least.

Murine model of HS

Institute of Cancer Research female or male mice 8 to 10 weeks old were exposed to heat stress treatment (42°C, relative humidity, 50%–55%; 1 h) in an environment-controlled chamber. The time at which mice were removed from the environmental chamber was called 0 h. The heat-stressed mice were returned to the normal room temperature (24°C) after the end of the heat exposure. Mice that survived on day 4 of heat treatment were considered survivors, and the data were used for analysis of the results. Core temperatures were measured every 5 min with a copper constantan thermocouple inserted into the rectum and connected to a thermometer (HR1300; Yokogawa, Tokyo, Japan). After the 1-h heating period, animals were properly fed and hydrated. Heatstroke resembles sepsis in many aspects (1–3). Like many sepsis studies, we use death as an end point in conscious mice in this study.
**Experimental groups**

Two major groups of animals were designated for the experiment. In the normothermic control (NC) groups, the animals were treated with a dose of vehicle (1 mL 0.9% NaCl solution/kg body weight, i.p.) or Premarin (USP; 1 mg in 1 mL/kg body weight, i.p.; Wyeth Laboratories, Rouse Point, NY). Their core temperatures were found to be 37.1°C to 37.7°C at a room temperature of 24°C throughout the entire experiments. In the HS groups, the animals were treated with a dose of 10% NaCl solution (1 mL/kg body weight, i.p.), Premarin (0.1 – 1 mg in 1 mL/kg body weight, i.p.), and/or the nonselective ER antagonist ICI 182, 780 (Tocris Cookson, Inc., Ellisville, Mo; 0.25 mg in 1 mL/kg body weight, i.p.) 1 h after termination of heat exposure.

As depicted in Figure 1, percent survival values of vehicle-treated HS mice for 0, 2, 4, and 96 h post-whole-body heating (WBH) are 100%, 100%, 100%, and 0%, respectively (n = 12 for each group). The core temperature values of vehicle-treated HS mice for 0, 2, 4, and 96 h post WBH are 41.6°C ± 0.4°C, 37.2°C ± 0.5°C, 34.4°C ± 0.3°C, and 26.2°C ± 0.4°C, respectively (n = 8 for each group).

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**Plasma E2 concentrations**

Plasma E2 concentrations were determined using the double-antibody radioimmunobassent assay (R&D Systems, Minneapolis, Minn) according to the manufacturer’s instruction. The concentration of TNF-α, IL-1β, or IL-10 in the serum samples was calculated from the standard curve multiplied by the dilution factor and was expressed as picograms per milliliter.

**Biochemical assay**

The plasma levels of activated partial thromboplastin time, prothrombin time, and d-dimer were measured by automated coagulation instruments.

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**RESULTS**

**Table 1. Plasma levels of E2 for different groups of mice**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Plasma E2, pg/mL</th>
</tr>
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<tbody>
<tr>
<td>NCs</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>Saline-treated HS mice</td>
<td>76 ± 5</td>
</tr>
<tr>
<td>Premarin (0.1 mg/kg)-treated HS mice</td>
<td>112 ± 11*</td>
</tr>
<tr>
<td>Premarin (0.5 mg/kg)-treated HS mice</td>
<td>346 ± 23*</td>
</tr>
<tr>
<td>Premarin (1 mg/kg)-treated HS mice</td>
<td>673 ± 38*</td>
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</table>

*P < 0.01 in comparison with groups 1 or 2. Vehicle or Premarin was or at the termination of heat exposure, administered 1 h post-WBH (42.4°C), and the blood sampling was obtained for E2 assay 4 h post-WBH. Data are mean ± SE of eight mice per group.

**Extracellular levels of glutamate, lactate-to-pyruvate ratio, glyceral, and NO in hypothalamus**

The brain (hypothalamus) samples were prepared according to previous reports (10, 11). The NO concentrations in the dialysates were measured with the Eicom ENO-20 NOx analysis system (Eicom, Kobe, Japan) (12). In the Eicom ENO-20 NOx analysis system, after the NO2− and NO3− in the sample have been separated by the column, the NO2− reacts in the acidic solution with the primary amine amine to produce an azo compound. After this, the addition of aromatic amines to the azo compound results in a coupling that produces a diazo compound, and the absorbance rate of the red color in this compound is then measured. The dialsates were injected onto a CMA 600 microdialysis analyzer (Carnegie Medicine, Stockholm, Sweden) for measurement of lactate, glyceral, pyruvate, and glutamate.

**Immunohistochemical staining of INOS in hypothalamus**

Mice were killed with overdose of an anesthetic and were transcardially perfused with heparinized 0.05-M phosphate-buffered saline (PBS), followed by ice-cold 15% sucrose in PBS. The brains were rapidly removed and frozen in liquid nitrogen. Coronal brain sections (5-μm thick) were cut in a cryostat and were thaw-mounted on gelatin-coated slides. Sections were incubated with commercially available rabbit anti-NOS antiserum (1:50) diluted in 0.2% Triton X-100 1% azide (Sigma, St. Louis, Mo)/PBS at 4°C overnight then rinsed with PBS for 30 min and incubated in biotinylated goat antirabbit immunoglobulin G (1:500) for 1 to 2 h. The INOS-positive cells were counted in each section through the hypothalamus.

**Terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end-labeling assay**

Terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining was performed on paraffin-embedded sections of the spleen, liver, and kidney. Color was developed using 3,3′-diaminobenzidine tetrachloride. Sections were treated with xylene and ethanol to remove paraffin and for dehydration. They were then washed with PBS and incubated in 3% H2O2 solution for 20 min. The sections were treated with 5 μg/mL proteinase k for 2 min at room temperature and rewarshed with PBS (0.1 M, pH 7.4). The sections were treated with a TUNEL reaction mixture (Roehl, Mannheim, Germany) at 37°C for 1 h, and then the sections were washed with distilled water. The TUNEL-positive cells were counted in each section.

**Statistical analysis**

Statistical significance of survival was assessed using a chi-square method and followed by Fisher exact probability test. Core temperatures, levels of E2, cytokines, NO, glutamate, glyceral, lactate-to-pyruvate ratio, and values of immunoassayed sections were analyzed using the Kruskal-Wallis H test, followed by several post hoc comparisons with Dunn method. Significance was set at P < 0.05.
Premarin attenuates heat-induced lethality and hypothermia

As summarized in both Tables 2 and 3, heat-induced lethality and hypothermia at 4 h post-WBH were attenuated by Premarin in a dose-dependent manner over a dose range of 0.1 to 1 mg/kg. It was further found that the beneficial effects exerted by Premarin in WBH-induced lethality and hypothermia were significantly attenuated by simultaneous administration of the broad-spectrum ER antagonist ICI 182, 780. We chose 0.5 mg/kg of body weight of Premarin for studying its therapeutic effects in the following experiments.

Premarin reduces heat-induced increased levels of glutamate, glycerol, lactate-to-pyruvate ratio, and nitrite in hypothalamus

Figure 2 depicts the effects of heat exposure (42.4°C for 1 h) on cellular levels of glutamate, glycerol, lactate-to-pyruvate ratio, and nitrite in the hypothalamus in NCs, vehicle-treated HS mice, and Premarin-treated HS mice. In vehicle-treated HS groups (HS + saline), the cellular levels of ischemia markers (e.g., glutamate, lactate-to-pyruvate ratio, and nitrite) in the hypothalamus were all significantly higher at 2 to 4 h after the termination of heat exposure than they were for NCs. However, the cellular levels of damage marker (e.g., glycerol) started to increase at 4 h post-WBH exposure in vehicle-treated HS mice. Resuscitation with Premarin (0.5 mg/kg, i.p.) 1 h post-WBH significantly attenuated the heat-induced increased levels of glutamate, glycerol, lactate-to-pyruvate ratio, and nitrite in the hypothalamus. The basal levels of biochemical parameters in NCs treated with Premarin (0.5 mg/kg, i.p.) were indistinguishable from those of NCs that received no treatment (data not shown).

Premarin attenuates heat-induced renal and hepatic dysfunction

Figure 3 depicts the BUN values and plasma levels of creatinine, SGOT, SGPT, and ALP for normothermic mice, vehicle-treated HS mice, and Premarin-treated HS mice. It can be seen from the figure that the BUN values and plasma levels of creatinine, SGOT, SGPT, and ALP for vehicle-treated HS mice were all significantly higher at 4 h post-WBH than they were for normothermic mice. Resuscitation with Premarin (0.5 mg/kg, i.p.) 1 h post-WBH significantly attenuated the heat-induced increased plasma levels of BUN, creatinine, SGOT, SGPT, and ALP. The BUN values and plasma levels of creatinine, SGOT, SGPT, and ALP measured for normothermic

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Survival, %</th>
<th>Fraction survival</th>
<th>P</th>
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<tbody>
<tr>
<td>Female NC received saline</td>
<td>100%</td>
<td>12/12</td>
<td></td>
</tr>
<tr>
<td>Female NC received Premarin (1 mg/kg)</td>
<td>100%</td>
<td>12/12</td>
<td></td>
</tr>
<tr>
<td>Female HS mice received saline</td>
<td>0%</td>
<td>0/12</td>
<td>P &lt; 0.001‡</td>
</tr>
<tr>
<td>Female HS mice received Premarin (0.1 mg/kg)</td>
<td>0%</td>
<td>0/12</td>
<td>P &lt; 0.001†</td>
</tr>
<tr>
<td>Female HS mice received Premarin (0.5 mg/kg)</td>
<td>50%</td>
<td>6/12</td>
<td>P &lt; 0.001†</td>
</tr>
<tr>
<td>Male HS mice received Premarin (1 mg/kg)</td>
<td>100%</td>
<td>12/12</td>
<td></td>
</tr>
<tr>
<td>Female HS mice received Premarin (1 mg/kg) and ICI 182, 780 (0.25 mg/kg)</td>
<td>41.6%</td>
<td>5/12</td>
<td>P = 0.001‡</td>
</tr>
<tr>
<td>Male HS mice received Premarin (1 mg/kg) and ICI 182, 780 (0.25 mg/kg)</td>
<td>50%</td>
<td>6/12</td>
<td>P = 0.001‡</td>
</tr>
</tbody>
</table>

Heat (42.4°C)-treated mice were administered different doses of Premarin or ICI 182, 780 1 h post-WBH. For each HS group, core temperature values were measured 4 h post-WBH for HS groups or the equivalent period for NCs.

*Compared with NC + saline or NC + Premarin (1 mg/kg) groups.
†Compared with HS + saline group.
‡Compared with HS + Premarin (1 mg/kg) group. When exactly 4 days post-WBH, the percent survival or fraction survival values of mice were measured.
mice treated with Premarin (0.5 mg/kg, i.p.) were indistinguishable from those of normothermic mice without treatment.

**Premarin attenuates serum levels of IL-1β and TNF-α but enhances IL-10 during HS**

Figure 4 depicts the values of serum IL-1β, TNF-α, and IL-10 in different groups of mice. The values of serum IL-1β and TNF-α of vehicle-treated HS mice obtained at 4 h post-WBH were significantly higher than those of NCs. In Premarin-treated HS mice, Premarin therapy adopted 1 h post-WBH significantly suppressed the increased levels of serum IL-1β and TNF-α obtained at 4 h post-WBH. In both normothermic and vehicle-treated HS mice, their serum levels of IL-10 were maintained at a negligible levels during the whole courses of experiments. However, 3 h after an intraperitoneal dose of Premarin (or 4 h post-WBH), the serum levels of IL-10 were greatly elevated in HS rats.

**Premarin attenuates heat-induced increased numbers of iNOS-positive cells in hypothalamus**

As summarized in Table 4, the numbers of iNOS-positive cells in the hypothalamus were increased significantly at 4 h post-WBH. Photomicrographs of iNOS-positive cells in the hypothalamus of a vehicle-treated HS mouse were shown in Figure 5. The heat-induced increased numbers of iNOS-positive cells were reduced significantly by Premarin therapy (Table 4 and Fig. 5).

**Premarin attenuates heat-induced increased numbers of TUNEL-positive cells in the spleen, liver, and kidney**

As summarized in Table 5, the numbers of TUNEL-positive cells in the spleen, liver, and kidney were increased significantly at 4 h post-WBH. Photographs of TUNEL-positive cells in the spleen of a vehicle-treated HS mouse were shown in Figure 6. The heat-induced increased numbers of TUNEL-positive cells in the spleen, liver, and kidney were all reduced significantly by Premarin (Table 5).

**DISCUSSION**

It has been shown that the heat tolerance of estrus female rats is superior to that of male rats (5). On the other hand, the heat tolerance of surgically or chemically ovariectomized rats is inferior to that of estrus female rats. In addition, estrogen replacement by Premarin in male, estrus female, and surgically or chemically ovariectomized rats under general anesthesia greatly improves heat tolerance. In the present study, we further demonstrate that the heat-induced lethality in unanesthetized and unrestrained male or female mice can be completely abolished by Premarin therapy. Furthermore, we show that the broad-spectrum ER antagonist ICI 182, 780 abolishes the beneficial effects of Premarin in HS mice. These data indicate that Premarin can act via ER to rescue mice from heat-induced lethality. The contention is in part supported by several experimental studies. For example, estrogen reduces injury after global (13–15) and focal (4, 16) cerebral ischemia as well as experimental subarachnoid hemorrhage (17) in rodents. The neuroprotective effects of estrogen or Premarin during brain injury requires the ER-α because estrogen therapy has no effect in ER-α-deficient mice (18).

It is well known that glutamate and lactate-to-pyruvate ratio are cellular ischemia markers, whereas glycerol is a cellular damage marker (19). Our unanesthetized and unrestrained mice display increased production of glutamate, lactate-to-pyruvate...
ratio, and glycerol in hypothalamus. The hypothermia that occurred after HS formation in mice (present results; 7–9) may have resulted from hypothalamic ischemia and neuronal damage. The heat-induced hypothalamic ischemia and neuronal damage and hypothermia can be significantly reduced after Premarin therapy (as shown in the present results). In the current study, we further show that the protective effects of Premarin in ameliorating the heat-induced hypothermia can be significantly reduced by the ER antagonist ICI 182, 780.

DNA fragmentation assays of splenocytes are done in unanesthetized mice 2 h after heat stress by Chatterjee et al. (9). DNA ladder formation in splenocytes from mice subjected to heat stress is clearly evident. The present results further demonstrate that numbers of TUNEL-positive cells in the spleen, liver, and kidney are greatly increased in mice 4 h post-WBH. These observations demonstrate that cellular apoptosis occurs in these organs from heat-stressed mice can be ameliorated by Premarin therapy.

Like anesthetized rats (20), unanesthetized and unrestrained mice also display hepatic and renal dysfunction or failure (evidenced by increased serum urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, and ALP levels in plasma) during HS. Herein, we further demonstrate that the heat-induced hepatic and renal dysfunction can be ameliorated by Premarin therapy. The results are in part consistent with a previous investigation (21). These investigations show that male and female estrus animals have a more marked depression in cardiovascular function as compared with those of female proestrus rats. Thus, it seems that the maintenance of normal multiorgan function during various diseased states can be achieved by high levels of E2 in plasma after Premarin therapy.
In fact, estrogen may act as a systemic anti-inflammatory treatment to lower the production of, or response to, proinflammatory cytokines (22). Proinflammatory cytokine gene expression is also attenuated by this hormone. Indeed, as shown in the present findings, estrogen replacement caused by Premarin therapy significantly attenuates overproduction of both TNF-α and IL-1β levels during HS. In the rodents with HS, the serum level of IL-10 is undetectable. However, after the onset of HS, treatment with Premarin produces a significant increase of IL-10 in the bloodstream. IL-10 has important anti-inflammatory properties through suppression of many proinflammatory cytokines (23). In the present results, Premarin may improve survival during HS by increasing IL-10 but decreasing both IL-1β and TNF-α production.

Other lines of evidence have indicated that NO plays major roles in modulating brain injury after ischemic challenges (17). iNOS is induced in reactive astrocytes and in infiltrating neutrophils after cerebral ischemia (24). Decisive evidence tends to suggest that NO from iNOS leads to neurotoxicity in the brain. The same contention can be applied to brain ischemia and damage that occurred during HS. Amino-guanidine (an iNOS inhibitor) improves survival by reducing the increased iNOS-dependent NO formation in the hypothalamus that occurred during HS in anesthetized rats (25). In unanesthetized and unrestrained mice, as shown in the present results, the increased iNOS-dependent NO production in hypothalamus during HS is also significantly suppressed by Premarin therapy. Therefore, Premarin may cause attenuation of hypothalamic ischemia and damage as well as hypothermia during HS by reducing the increased iNOS-dependent NO production in the hypothalamus.

After a heat-stress protocol, time-course changes in reactive oxygen species levels, oxidative damage markers, and glutathione–glutathione disulfide ratios are elevated in both liver (26) and brain (27) tissues. These findings suggest that an environmental challenge in rats produces exaggerated oxidative stress, which can contribute to cellular dysfunction during HS. It is likely that Premarin may maintain normal function of multiorgans by reducing the exaggerated oxidative stress during HS.

**Table 4.** Mean (± SE) number of iNOS-positive cells per hypothalamic section for different groups of mice

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>iNOS-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCs</td>
<td>0</td>
</tr>
<tr>
<td>Saline-treated HS mice</td>
<td>89 ± 6</td>
</tr>
<tr>
<td>Premarin-treated HS mice</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

*P < 0.01 in comparison to group 2. Saline or Premarin (1 mg/kg) was administered intraperitoneally 1 h post-WBH (42.4°C), and the tissue section was obtained for iNOS staining 4 h post-WBH. Data are mean ± SE of eight mice per group.

**Table 5.** Mean (± SE) number of TUNEL-positive cells per tissue section for different groups of mice

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Spleen</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCs</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Saline-treated HS mice</td>
<td>148 ± 18*</td>
<td>492 ± 98*</td>
<td>489 ± 54*</td>
</tr>
<tr>
<td>Premarin-treated HS mice</td>
<td>6 ± 2†</td>
<td>8 ± 5†</td>
<td>109 ± 37†</td>
</tr>
</tbody>
</table>

*P < 0.01 in comparison to group 1.
†P < 0.01 in comparison to group 2. Saline or Premarin (1 mg/kg) was administered intraperitoneally 1 h post-WBH, and the tissue section was obtained for TUNEL staining 4 h post-WBH. Data are mean ± SE of eight mice per group.
thermoregulatory dysfunction, which lead to death. Premarin, an estrogen sulfate, may act via ER to rescue mice from HS-induced multiorgan dysfunction and lethality.

Whether the mouse is an appropriate model for HS needs consideration. Because of their large surface-area-to-mass ratio, they have extremely different thermoregulatory responses to HS compared with rats, rabbits, and humans. However, both our (20) and others’ (8, 9) results demonstrate that mice share with rats (2) or rabbits (28, 29) similar HS reactions. Because cerebral or hypothalamic ischemia and neuronal damage occur during HS (2), the profound hypothermia observed after HS in mice (7, 8) should be observed to the same extent in rats, rabbits, or humans.

REFERENCES


FIG. 6. Apoptosis identification by TUNEL staining in the spleen of an NC, a saline-treated HS mouse (HS + saline), and a Premarin-treated HS mouse (HS + Premarin). The animals were killed at 4 h after the termination of 1-h heat exposure or the equivalent time for the NC.