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Flutamide, an androgen receptor antagonist, improves heatstroke outcomes in mice

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ABSTRACT

Flutamide has been used as an adjunct for decreasing the mortality from subsequent sepsis. Heatstroke resembles septic shock in many aspects. We hypothesized that heat-induced multiple organ dysfunction syndromes and lethality could be reduced by flutamide therapy. In heatstroke groups, mice were exposed to whole body heating (41.2°C, for 1 h) in a controlled-environment chamber. The heat-stressed mice were returned to normal room temperature (24 °C) after whole body heating. Mice still alive on day 4 of WBH treatment were considered survivors. Physiological and biochemical parameters were monitored for 2.5 h post-WBH. Heatstroke mice were subcutaneously treated with flutamide (12.5–50 mg/kg body weight in 0.05 ml) or vehicle solution (0.05 ml/kg body weight) once daily for 3 consecutive days post-WBH. We evaluated the effect of flutamide in heatstroke mice and showed that flutamide significantly (i) attenuated hypothermia, (ii) reduced the number of apoptotic cells in the hypothalamus, the spleen, the liver, and the kidney, (iii) attenuated the plasma index of toxic oxidizing radicals (e.g., nitric oxide metabolites and hydroxyl radicals), (iv) diminished the plasma index of the organ injury index (e.g., lactate dehydrogenase), (v) attenuated plasma systemic inflammation response molecules (e.g., tumor necrosis factor-α and interleukin-6), (vi) reduced the index of infiltration of polymorphonuclear neutrophils in the lung (e.g., myeloperoxidase activity), and (vii) allowed three times the fractional survival compared with vehicle. Thus, flutamide appears to be a novel agent for the treatment of mice with heatstroke or patients in the early stage of heatstroke.

1. Introduction

Heatstroke is defined as a form of excessive hyperthermia associated with an activated inflammation that results in multiple organ dysfunction or injury in which central nervous system disorders such as delirium, convulsions, and coma are predominant (Bouchama and Knochel, 2002). Most of heatstroke responses can be reproduced by exposing rodents under general anesthesia to a high ambient temperature (Chang et al., 2006; Lin et al., 2011; Shen et al., 2008).

Multiple organ dysfunction syndrome in septic shock (Srinivasan et al., 2010; Wu et al., 2008) or heatstroke (Chang et al., 2006; Lin et al., 2011) includes the production and release of proinflammatory cytokines, including tumor necrosis factor- (TNF)-α, the toxic oxidizing radicals, including nitric oxide metabolites (NO_x^-) and 2,3-dihydroxybenzoic acid (2,3-DHBA), and the organ injury indicator lactate dehydrogenase (LDH) in rats. Testosterone depletion by castration has recently been reported (Lin et al., 2010) to protect mice from heat-induced multiple organ damage and lethality. Flutamide, a non-steroidal androgen receptor antagonist, has been used as an adjunct after trauma (e.g., hemorrhage) for restoring immune and cardiovascular function and for decreasing the mortality from subsequent sepsis (Ba et al., 2001; Raju and Chaudry, 2008). This raises the possibility that heat-induced multiple organs dysfunction syndrome and lethality can be attenuated by flutamide.

The aim of this study, therefore, was to determine whether chemical castration with the androgen receptor blocker flutamide, like surgical castration, attenuates heat-induced multiple organs dysfunction syndrome and lethality. To test this hypothesis, after heatstroke therapy in mice, we assessed the effects of flutamide on heat-induced thermoregulatory deficits (in particular, hypothalamic apoptosis and hypothermia), multiple organ apoptosis, increased serum levels of toxic oxidizing radicals and LDH, increased serum levels of proinflammatory cytokines, increased lung levels of an indicator of the accumulation of polymorphonuclear cells, (e.g., myeloperoxidase), and lethality.

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2. Materials and methods

2.1. 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). Institute of Cancer Research inbred male mice, 8 week old, were housed individually and 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.

2.2. Murine model of heatstroke and experimental groups

Mice were subcutaneously (s.c.) injected with 0.05 ml/g body weight of the nontoxic vehicle 1,2-propanediol or flutamide (12.5–50 mg/kg) (Sigma Chemical Co., St. Louis, MO, USA) solution to be tested in 0.90% saline, at the concentration mentioned, and were directly exposed to whole body heating (41.2 °C for 1 h at 50–55% relative humidity, using a temperature-controlled environmental chamber) from 24 °C. Rectal temperature was measured using a copper-constantan thermocouple probe inserted into the rectum and connected to a thermometer (HR1300; Yokogawa, Tokyo, Japan). After the 1-h heating period, the mice were returned to their home cages and given food and water *ad libitum*. Survival of the mice was monitored initially every 8 h for the first 24 h and then each day for another 4 days. Mice that survived the first 4 days lived for more than 30 days. Mice alive on day 4 post-whole body heating (WBH) treatment were considered survivors and their data were used for analysis of the results. Mice kept in the environmental chamber for 1 h at a temperature of 24 °C and relative humidity of 50% were the sham group.

In separate groups, the mice were intraperitoneally (i.p.) anesthetized with urethane (1.4 g/kg of body weight) 2.5 h after the termination of heat stress. Tissue samples were then obtained for biochemical and immunological analysis. At +4 and +24 h post-WBH, serum levels of cytokines were significantly higher in the heatstroke group than in the sham group. In the present study, a time period of 2.5 h was chosen for determining biochemical and immunological parameters.

2.3. Terminal deoxy-nucleotidyl transferase-mediated dUTP Nick-end labeling (TUNEL) assays

The heatstroke mice were given an overdose of general anesthesia with urethane (1.4 g/kg body weight; i.p.) 2.5 h after the end of WBH treatment and then perfused and prefixed with PBS and 10% formaldehyde. The brain, liver, spleen, lungs, and kidneys were excised and postfixed in a solution containing 30% sucrose and 10% formaldehyde for at least 24 h. After they had been fixed, the organs were separately embedded in medium (Tissue Tek OCT; Miles Laboratories, Elkhart, IN, USA). Snap-frozen samples were cryostat-sectioned (8 μm thick) and placed on slides coated with poly-L-lysine for TUNEL assays. TUNEL staining was done using a kit (Apo Alert DNA Fragmentation Assay kit; Clontech, BD Biosciences, Palo Alto, CA, USA) using the manufacturer's instructions. In brief, tissue slides were pretreated with 20 μg/ml of proteinase K solution for 5 min and then incubated with the reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-conjugated deoxyuridine triphosphate (dUTP) for 1 h at 37 °C. After they had been incubated, the sections were washed with PBS, their nuclei were co-stained with 4,6-diamidino-2-phenylindole (DAPI) using DAPI-containing mounting medium (Vectashield R; Vector Laboratories, Burlingame, CA, USA), and subsequently analyzed using a fluorescent microscope (E800; Olympus, Tokyo, Japan) equipped with a digital camera (Coolpix 995; Olympus). Apoptosis induction efficacy was calculated as a percentage of fluorescein-positive to DAPI-stained nuclei.

2.4. Serum tumor necrosis factor (TNF)-α, interleukin-6 (IL-6), and IL-10 assays

Blood samples were drawn by heart puncture from the mice after they had been anesthetized with urethane (1.4 g/kg body weight; i.p.). The blood was centrifuged to isolate the upper layer of serum. Serum concentrations of TNF-α, IL-6, and IL-10 were determined using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. The minimum detectable doses of the assays we used for TNF-α, IL-6, and IL-10 were 1.88 pg/ml, 1.6 pg/ml, and 4.0 pg/ml, respectively. The intra- and inter-assay coefficients of variations were 3.1–3.8% and 5.8–76%, respectively, for the ELISA assay of mouse TNF-α; 3.5–6.7% and 6.2–8.8%, respectively, for IL-6; and 5.0–9.3% and 5.3–7.8% for IL-10, respectively.

2.5. Determining serum levels of the toxic oxidizing radicals

To determine NO_x^- and DHBA levels, blood samples were taken 2.5 h after the start of heat exposure. The NO_x^- concentrations in the dialysates were measured using a NO_x^- analysis system (Eicom-20; Eicom, Kyoto, Japan) (Togashi et al., 1998). The concentrations of hydroxyl radicals were measured using a modified procedure based on the hydroxylation of sodium salicylates by hydroxyl radicals, leading to production of 2,3-DHBA and 2,5-DHBA (Yang and Lin, 2002).

2.6. Quantifying LDH, the organ injury indicator

To determine LDH levels, blood samples were taken 2.5 h after the start of heat stress. The serum levels of LDH were determined by measuring the extent of organ injury using a biochemical analysis system (DRI-CHEM3030; Fujifilm, Tokyo, Japan).

2.7. Determining myeloperoxidase (MPO) activity

A spectrophotometric method (Mullane et al., 1985) was used to determine MPO activity in the lungs. A 100-μL aliquot of serum was mixed with 900 μl of 50 mmol/L phosphate buffer (pH = 6.0) containing 0.167 mg/ml of o-Dianisidine dihydrochloride and 0.0005% hydrogen peroxide. One unit of peroxidase activity was defined as equal to the amount of enzyme needed to decompose 1 μmol of hydrogen peroxide; it was calculated from the oxidation of o-Dianisidine using an absorption coefficient of 11.3/mM/cm at 460 nm.

2.8. Statistical analysis

The significance of the survival rate was assessed using the log-rank test (Mantel-Cox). Rectal temperature, levels of cytokines, nitrite, 2,3-DHBA, and TUNEL-positive cell values of immunostained sections were analyzed using Student's *t*-test. Significance was set at $P<0.05$.

3. Results

3.1. Flutamide-treated mice had a lower mortality

The mice injected with 12.5–50 mg/kg of flutamide at +1 h of WBH had significantly and dose-dependently lower mortality (Fig. 1A) than the WBH group treated with vehicle solution. WBH-treated mice had a core body temperature of >40°C at 0 h post-treatment with profound hypothermia at +4, +8, and +20 h post-WBH (Fig. 1B). Hypothermia was significantly ($P<0.05$; Fig. 1B) attenuated at +4, +8, and +20 h post-WBH in mice treated with flutamide once daily for 3 consecutive days.