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Arginine metabolic pathways determine its therapeutic benefit in experimental heatstroke: Role of Th_1/Th_2 cytokine balance

Saurabh Chatterjee [a], Sudha Premachandran [a], Raghavendra S. Bagewadikar [a], Sayanti Bhattacharya [b], Subrata Chattopadhyay [c], T.B. Poduval [a,*]

[a] *Immunology and Hyperthermia Section, Radiation Biology and Health Sciences Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085, India*
[b] *Dr. B.C. Roy Post Graduate Institute of Basic Medical Sciences, 244, A.J.C. Bose Road, Kolkata 700 020, India*
[c] *Bio-Organic Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India*

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Abstract

We have demonstrated that therapeutic administration of L-arginine (L-arg) (120 mg/kg) at +2 h of whole body hyperthermia (WBH) could rescue the mice from heatstroke-induced death. Studies were undertaken to elucidate the role of L-arg in the immunomodulation of the heat-stressed mice. Administration of L-arginine (L-arg), (120 mg/kg, i.p.), at +2 h of WBH, rescued the mice from heat-induced death and reduced the hypothermia. At +4 and +24 h of WBH, levels of IL-1β, IFN-γ, nitrite, TNF-α, IL-4, TGF-β1, inducible form of nitric oxide synthase (iNOS), and corticosterone significantly increased compared to the sham group. The elevated levels of Th_1 cytokines, namely TNF-α, IL-1β, IFN-γ, nitrite, and iNOS, decreased significantly both at +4 and +24 h of WBH, following L-arg administration. However, L-arg administration did not reduce the increased levels of Th_2 cytokines, namely IL-4 and TGF-β1, in WBH mice at +4 h of WBH. L-arg administration significantly increased the levels of Th_2 cytokines at +24 h of WBH, compared to the saline-treated WBH mice. L-arg administration significantly increased both the splenic and hepatic arginase activity at +4 and +24 h of WBH compared to the saline-treated WBH mice. L-NAME treatment at +2 h of WBH and anti-TGF-β antibody treatment at 0 h of WBH significantly increased the mortality compared to the saline-treated WBH mice. Altered liver histopathology was attenuated following the administration of L-arg at +2 h of WBH. These results suggest that therapeutic administration of L-arg at appropriate concentration and time attenuates the acute inflammatory response, leading to the rescue of mice from heatstroke.

Keywords: Arginase; Nitrite; Corticosterone; Nitric oxide synthase

The consequences of thermal trauma like heat wave are disastrous. The recent heat waves in 2003 that killed more than 35,000 people in Europe and more than 1600 people in India [1] are the worst weather-related natural disasters in the human memory. Heatstroke is associated with multiple organ failure, which includes cerebrovascular disorder [2]. The ensuing multi-organ injury results from the complex interplay among the cytotoxic effects of heat and the inflammatory responses of the host, which result in the generation of highly reactive oxygen and nitrogen species that lead to cell injury. The reaction of the nitrergic system to ischemic/hypoxia is always associated with an increase in nitric oxide ($NO^{\cdot}$) production leading to increased nitrosative stress [3].

Our earlier results demonstrated that heatstroke induced by WBH initiated an acute inflammatory response associated with increased production of inflammatory cytokines and upregulation of iNOS. Subsequently, therapeutic treatment with L-arg rescued the mice from lethal heatstroke [1]. Earlier studies indicated the requirement of constitutive NOS for successful adaptation to acute heat stress, and iNOS expression lead to a decreased heat tolerance [1,4,5].

* Corresponding author. Fax: +91 022 25505151.
E-mail address: tbpodu@magnum.barc.ernet.in (T.B. Poduval).

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Since Th_2 cytokines and arginase are known to be involved in the regulation of acute inflammation and tissue repair [6–9], we hypothesized that the upregulation of Th_2 cytokines and arginase as well as the downregulation of iNOS, by the administration of L-arg, is central to the rescue of the mice from heatstroke-induced death.

Considering the above, we used dexamethasone, which is an inhibitor of iNOS, and L-ω-nitro arginine methyl ester (L-NAME) at 3 mg/kg, which is a preferential inhibitor of eNOS, to demonstrate the importance of arginine pathways in modifying the heatstroke. Since cytokine profile has a bearing on the balance of inflammatory and repair mechanisms, the regulation of constitutive NOS, iNOS, and arginase (EC 3.5.3.1) was investigated at various times after heatstroke. The levels of Th_1 and Th_2 cytokines in the serum of mice subjected to heatstroke and its modification by the administration of L-arg was also investigated. Potential peripheral markers such as nitrite, Th_1 cytokines, Th_2 cytokines, corticosterone, iNOS, and arginase in various tissues are important prognostic indicators of experimental heatstroke [1]. The levels of these markers in heat-stressed mice and its modulation by the administration of L-arg was investigated to explain the therapeutic benefit of L-arg administered at appropriate time and concentration in experimental heatstroke.

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 (CPCSEA), Government of India. Swiss/Bh inbred male mice were given food and acidified water ad libitum and acclimatized to room temperature at 23.1 °C, relative humidity (RH) of 50 ± 10% and a 12 h dark/light cycle for 1 week before the start of the experiment.

Murine model of heatstroke

Swiss male mice, 8–10 weeks old, were exposed to a LD_{50} dose of WBH treatment (41.2 °C, RH-50–55%, 1 h) in an environment-controlled chamber, as reported earlier [1]. All mice were administered 0.5 ml of normal physiological saline just before exposure. The time at which mice were removed from the environmental chamber was called 0 h. The heat-stressed mice were returned to the normal surroundings after the end of the exposure. Mice that survived on day 4 of WBH treatment were considered survivors and the data were used for analysis of the results. Rectal temperatures were measured by a thermocouple probe (Bailey Instruments, Saddlebrook, NJ, USA) as described elsewhere [1].

Drug administration

L-arg/D-arg (Sigma Chemical Co., St. Louis, USA), dexamethasone as sodium phosphate salt (Wockhardt Laboratories, India), L-ω-nitro arginine methyl ester (L-NAME) (Calbiochem, USA), and anti-pan TGF-β polyclonal antibody raised in rabbit (A-TGF-β) (Sigma Chemicals, USA) were administered intraperitoneally. Sham group was exposed to 23 °C, RH of 55%, for 1 h and received normal physiological saline in place of L-arg.

Preparation of serum samples

Mice were sacrificed at 2, 4, and 24 h of WBH and blood was collected through retro-orbital puncture. Serum samples were stored at −80 °C until further use.

Serum cytokine levels

Serum levels of TNF-α, IL-1 β, IFN-γ, and IL-4 were estimated by commercially available ELISA kits (BD-Opt EIA kits, BD Pharmingen, USA) using manufacturer's protocols. The method of TGF-β1 estimation [10] in sera involved only the active form of the cytokine and acidification of the samples was carried out to include the latent TGF-β1 content. Serum samples were acidified with 1 N HCl and incubated at room temperature for 15 min, neutralized with 1 N NaOH and used for the sandwich ELISA procedure. Briefly, 96-well high binding ELISA plates (Nunc, Germany) were coated with purified rat anti-mouse TGF-β1 monoclonal antibody. Recombinant human TGF-β1 (from CHO cells) was used as a standard. Biotinylated rat anti-human TGF-β 1 monoclonal antibody was used as the detection antibody. Avidin–horseradish peroxidase was used as an enzyme conjugate. 3,3′,5,5′-Tetramethyl benzidine was used as a substrate. The reaction was stopped with 1 N H_2SO_4 and absorbance was read at 450 nm in an ELISA Plate reader (Biotek Instruments Inc., USA).

Serum nitrite levels

Serum nitrite levels were estimated according to the method described earlier [1]. Briefly, 100 µl of diluted serum samples (1:2) was incubated with 100 µl of Griess reagent (1% sulfanilamide, 0.1% NEDDH, and 2.5% phosphoric acid in glass distilled water). The absorbance of the formed color was read at 550 nm on a ELISA plate reader (Biotek Inc., USA).

Serum corticosterone levels

Serum corticosterone levels in mice, subjected to heatstroke, were estimated by commercially available colorimetric enzyme immunoassay kit (Correlate-EIA, Assay Designs Inc., USA) using manufacturer's protocol. Briefly, diluted serum samples (1:4) were treated with the steroid displacement reagent provided in the kit to get the actual corticosterone concentration in the sample. The assay utilized donkey anti-sheep IgG as the coated antibody and sheep polyclonal antibody to corticosterone. Alkaline phosphatase conjugated with corticosterone and its substrate