Free Radical Biology and Medicine 93 (2016) 84–93

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Therapeutic treatment with ascorbate rescues mice from heat stroke-induced death by attenuating systemic inflammatory response and hypothalamic neuronal damage

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ARTICLE INFO

Article history:
Received 5 July 2015
Received in revised form
28 November 2015
Accepted 14 December 2015
Available online 15 December 2015

Keywords:
Ascorbate
Heat stroke
Systemic inflammatory response

ABSTRACT

The impact of ascorbate on oxidative stress-related diseases is moderate because of its limited oral bioavailability and rapid clearance. However, recent evidence of the clinical benefit of parenteral vitamin C administration has emerged, especially in critical care. Heatstroke is defined as a form of excessive hyperthermia associated with a systemic inflammatory response that results in multiple organ dysfunctions in which central nervous system disorders such as delirium, convulsions, and coma are predominant. The thermoregulatory, immune, coagulation and tissue injury responses of heatstroke closely resemble those observed during sepsis and are likely mediated by similar cellular mechanisms. This study was performed by using the characteristic high lethality rate and sepsis-mimic systemic inflammatory response of a murine model of heat stroke to test our hypothesis that supra-physiological doses of ascorbate may have therapeutic use in critical care. We demonstrated that parenteral administration of ascorbate abrogated the lethality and thermoregulatory dysfunction in murine model of heat stroke by attenuating heat stroke-induced accelerated systemic inflammatory, coagulation responses and the resultant multiple organ injury, especially in hypothalamus. Overall, our findings support the hypothesis and notion that supra-physiological doses of ascorbate may have therapeutic use in critical care.

1. Introduction

Vitamin C (ascorbic acid) dissociates at physiological pH to form ascorbate. It is well known that ascorbate acts physiologically as an antioxidant and co-factor in the biosynthesis of many neurotransmitter sand neuropeptides [1]. The impact of ascorbate on oxidative stress-related diseases is moderate because of its limited oral bioavailability and rapid clearance. However, recent evidence of the clinical benefit of parenteral vitamin C administration has emerged, especially in critical care [2]. Supra-physiological doses of ascorbate by parenteral administration were recently shown to exert inflammation inhibitory and organ-protective effects in several critical conditions, such as sepsis, cardiac arrest and burn injury [3]. In animal models of sepsis, intravenous ascorbate increases survival and protects several microvascular functions, microvascular permeability barrier, and arteriolar responsiveness to vasoconstrictors and vasodilators [4,5]. The effects of parenteral ascorbate on microvascular function are both rapid and persistent. It is suggested that ascorbate quickly accumulates in microvascular endothelial cells, which scavenges reactive oxygen species (ROS) and modulates redox-sensitive signaling pathways to diminish septic induction of NADPH oxidase and inducible nitric oxide synthase [6].

Heatstroke (HS) is defined as a form of excessive hyperthermia associated with a systemic inflammatory response that results in multiple organ dysfunctions in which central nervous system disorders such as delirium, convulsions, and coma are predominant [7]. HS-induced deaths are increasing with global warming and with a world-wide increase in the frequency and intensity of heat waves [8]. The thermoregulatory, immune,

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.12.017
0891-5849/

coagulation and tissue injury responses that ensue during long-term progression of heatstroke closely resemble those observed during sepsis and are likely mediated by similar cellular mechanisms [9]. In addition, it is suggested that ischemic and oxidative damage to the hypothalamus may be responsible for HS [10]. Hence, we focused on measuring HS-induced neuronal cell damage in the hypothalamus for its pivotal role in thermoregulation and control of autonomic and endocrine activity [11].

In this study, by using the characteristic high lethality rate and sepsis-mimic systemic inflammatory response of a murine model of HS, we tested our hypothesis that supra-physiological doses of ascorbate may have therapeutic use in critical conditions, HS as an exemplar. We measured the lethality rate, systemic inflammatory (serum levels of TNF-α and IL-10), coagulation (tissue factor) and multiple tissue injury (iNOS, cell apoptosis) responses in lung, kidney and hypothalamus (the thermoregulatory center) tissues in HS mice, and we evaluated whether ascorbate administration can attenuate these parameters.

2. Materials and methods

2.1. Murine model of heatstroke

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 (IACUC no: 101122426). Institute of Cancer Research (ICR) inbred male mice were given food and water ad libitum and acclimatized to room temperature at 22 ± 2 °C relative humidity of 50 ± 8%, and a 12-h dark/light cycle for 1 week before the start of the experiment at least. ICR male mice 8 to 10 weeks old were exposed to whole-body heating (WBH) (41.2 °C relative humidity 50–55%, and for 1 h) in an environment-controlled chamber [12]. The time at which mice were removed from the environmental chamber was designated as 0 h. The WBH-treated mice were returned to room temperature (24 °C) at the end of WBH. Mice that survived on day 7 of WBH were considered survivors, and the data were used for analysis of the results. Core temperatures were measured every 4 h for period of 24 h with a copper constantan thermocouple inserted into the rectum and connected to a thermometer (HR1300; Yokogawa, Tokyo, Japan). In separate experiments, 4 h after WBH, all animals were killed and their organs were removed for histology, serum enzyme linked immunoabsorbent and immunohistochemistry assays. After the 1-h heating period, animals were properly fed and hydrated. Heat stroke resembles sepsis in many aspects [7]. As in many sepsis studies, we used death as an end point in conscious mice in this study.

2.2. Experimental groups

Animals were assigned randomly to one of three groups: (1) the normothermic control (NC) mice, which were exposed to room temperature (24 °C) throughout the entire experiments. (2) heatstroke (HS) mice treated with saline (HS + saline), in which saline was injected intraperitoneally (1 mL/kg, i.p.) immediately after the end of WBH, and (3) heat stroke (HS) mice treated with ascorbate (HS + ascorbate) in which ascorbate were injected intraperitoneally (500 mg/kg, i.p.) immediately after the end of WBH. Ascorbate was dissolved in saline (1 ml/kg) with an injection volume equal to that of saline vehicle. Before the start of experiments, their core temperature was within the normal body temperature range of 37.0–37.6 °C.

2.3. Assessment of thermoregulatory function

Immediately after the termination of WBH, the animals were returned to a room temperature of 24 °C for recovery. According to the findings of Chatterjee et al. [12], WBH-treated mice became hypothermia, when they were exposed to room temperature (24 °C).

2.4. Neuronal damage score

At the end of the experiments, animals were killed by an overdose of sodium pentobarbital, and the brains were fixed in situ and left in skull in 100% neutral-buffered formalin for at least 24 h before removal from the skull. The brain was removed and embedded in paraffin blocks. Serial sections (4 μm thick) through the hypothalamus were stained with hematoxylin and eosin for microscopic examination. The extent of neuronal damage was scored on a scale of 1–3, modified from the grading system of Pulsinelli et al. [13], in which 0 is normal, 1 indicates that approximately 30% of the neurons are damaged, 2 indicates that approximately 60% of the neurons are damaged, and 3 indicates that 100% of the neurons are damaged. Each hemisphere was evaluated independently by an examiner blinded to the experimental conditions.

2.5. Serum Tumor Necrosis Factor (TNF) –α and interleukin (IL)-10 assays

Blood specimens at 4 h after termination of WBH 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 and IL-10 were determined using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. 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 picograms per milliliter.

2.6. Terminal Deoxy-nucleotidyl Transferase-mediated dUTP Nick end Labeling (TUNEL) assay

The HS mice were given an overdose of general anesthesia with urethane (1.4 g/kg body weight; i.p.) 4 h after the end of WBH treatment and then perfused and prefixed with PBS and 10% formaldehyde. The brain, lungs and kidneys were excised and post fixed in a solution containing 30% sucrose and 10% formaldehyde for at least 24 h. Paraffin samples were cryostat-sectioned (4 μm thick) and placed on slides coated with poly-L-lysine for TUNEL assay. 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 incubation, the sections were washed with PBS, and 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.