Journal of Surgical Research **174,** 24–28 (2012)
doi:10.1016/j.jss.2011.06.022

ASSOCIATION FOR ACADEMIC SURGERY

Hypertonic Saline Inhibits Arachidonic Acid Priming of the Human Neutrophil Oxidase[1]

Luis Lee, M.D.,* Marguerite R. Kelher, M.S.,*,§ Ernest E. Moore, M.D.,*,† Anirban Banerjee, Ph.D.,* and Christopher C. Silliman, M.D., Ph.D.*,‡,§,[2]

*Department of Surgery, University of Colorado Denver, Aurora, Colorado; †Department of Surgery, Denver Health Medical Center, Denver, Colorado; ‡Department of Pediatrics, University of Colorado Denver, Aurora, Colorado; and §Research Department, Bonfils Blood Center, Denver, Colorado

Originally submitted January 8, 2011; accepted for publication June 10, 2011

Background. **Arachidonic acid (AA, and its leukotriene derivatives, e.g., LTB_4) is an inflammatory mediator in post-shock mesenteric lymph that appears to act as an agonist on G-protein coupled receptors (GPCRs). These mediators prime neutrophils (PMNs) for an increased production of superoxide, implicated in the development of acute lung injury (ALI). Hypertonic saline (HTS) has also been shown to have immunomodulatory effects such as attenuation of PMN priming by precluding appropriate clathrin-mediated endocytosis of activated GPCRs, thereby potentially attenuating ALI. We hypothesize that HTS inhibits priming of the PMN oxidase by these lipid mediators.**

Methods. **After PMNs were isolated from healthy donors, incubation was done in either isotonic buffer (control) or HTS (180 mmol/L) for 5 min at 37°C. The PMNs were then primed for 10 min with AA [5 μM] or 5 min with LTB_4 [1 μM] and the oxidase was activated with 200 ng/mL of phorbol 12-myristate 13-acetate (PMA), a non-GPCR activator, and superoxide anion generation was measured *via* reduction of cytochrome *c*.**

Results. **Both AA [5 μM] and LTB_4 [1 μM] significantly primed the PMA activated respiratory burst ($P < 0.05$, ANOVA, Newman-Keuls, $n = 4$). HTS inhibited both AA and LTB_4 priming of the respiratory burst.**

Conclusions. **These data indicate that HTS reduces the cytotoxicity of PMNs stimulated by these lipid mediators *in vitro* and further support the immunomodulatory effects of HTS.**

Key Words: **multiple organ failure (MOF); G-protein coupled receptors (GPCR); hypertonic saline (HTS); clathrin-mediated endocytosis (CME); arachidonic acid (AA); leukotriene B_4 (LTB_4); neutrophil (PMN); superoxide anion; acute lung injury (ALI).**

INTRODUCTION

Arachidonic acid (AA) is a 20-carbon, ω-6 polyunsaturated fatty acid that is a precursor to leukotrienes and other important mediators of inflammation [1]. Both leukotriene B_4 (LTB_4) and AA prime human neutrophils (PMNs) for a subsequent increased superoxide production [2]. Hypertonic saline (HTS) is a known inhibitor of clathrin-mediated endocytosis (CME) [3], a process crucial in the internalization of G-protein coupled receptors (GPCRs), which are seven transmembrane proteins that form the largest family of signal transducing membrane receptors [4]. Moreover, HTS inhibits priming of PMNs for numerous priming agents, such as platelet activating factor (PAF) that induce pro-inflammatory changes in PMNs through activation of CME [5, 6].

LTB_4, an effective PMN chemoattractant and priming agent, signals through B leukotriene receptor 1 (BLT1), a GPCR [7]. Although a number of reports have postulated that AA primes PMNs through a GPCR, the data is inconclusive [8–12]. We hypothesize that HTS, a CME antagonist, inhibits the human PMN priming activity of LTB_4 and AA.

MATERIALS AND METHODS

All reagents, unless otherwise specified, were purchased from Sigma Chemical Company (St. Louis, MO). Solutions were made from sterile

[1] Presented at the Academic Surgical Congress, February 3, 2011.

[2] To whom correspondence and reprint requests should be addressed at Research Department, Bonfils Blood Center, 717 Yosemite St., Denver, CO 80230. E-mail: Christopher.Silliman@ucdenver.edu.

0022-4804/$36.00

water for injection, [United States Pharmacopeia (USP); Baxter Healthcare Corp., Deerfield, IL]. All buffers were made from the following stock USP solutions: 10% $CaCl_2$, 23.4% NaCl, 50% $MgSO_4$ (American Reagent Laboratories, Inc., Shirley, NY), sodium phosphates (278 mg/mL monobasic and 142 mg/mL dibasic), and 50% dextrose (Abbott Laboratories, North Chicago, IL). Furthermore, all solutions were sterile-filtered with Nalgene MF75 series disposable sterilization filter units purchased from Fisher Scientific Corp. (Pittsburgh, PA). Ficoll-Paque was purchased from GE Healthcare Biosciences (Piscataway, NJ). LTB_4 and AA were purchased from Cayman Chemical (Ann Arbor, MI). The calcium binding fluorometric dye Indo-1 AM was purchased from Invitrogen Corp. (Grand Island, NY).

Neutrophil Isolation

PMNs were isolated by standard techniques [13]. Heparinized whole blood was drawn from healthy human donors after obtaining informed consent employing a protocol approved by the Colorado Multiple Institutional Review Board and Human Subjects Committee at the University of Colorado School of Medicine. These donors satisfied the questions pertaining to health for all blood donors as mandated by the FDA and the industry standards for all blood banks in the United States. PMNs were isolated by dextran sedimentation, Ficoll-Paque gradient centrifugation, and hypotonic lysis as previously described [13]. Cells were resuspended to a concentration of 2.5×10^7 cells/mL in Krebs-Ringers-phosphate buffer with 2% dextrose (KRPD) (pH 7.35) and used immediately for all subsequent manipulations.

Superoxide Anion Assay

After PMNs were isolated from healthy donors, incubation of neutrophils (3.75×10^5 cells) was done in either isotonic buffer at a Na^+ concentration of 135 mmol/L (control), or HTS at a Na^+ concentration of 180 mmol/L, for 5 min at 37°C. PMNs were then primed for 10 min with AA [5 μM] or 5 min with LTB_4 [100 nM, 1 μM, 10 μM] and the oxidase was activated with 200 ng/mL of phorbol 12-myristate 13-acetate (PMA), a non-GPCR activator of the oxidase, and the maximal rate of superoxide anion generation was measured *via* reduction of cytochrome *c* at 550 nm in a Molecular Devices (Menlo Park, CA) microplate reader [13].

Cytosolic Calcium Measurements

After loading with 5 μM of the fluorometric dye Indo-1 AM (Invitrogen, Grand Island, NY), PMNs were centrifuged and resuspensed in fresh, warm KRPD to a concentration of 1×10^6 cells/mL. PMNs were then incubated in either HTS or isotonic buffer, loaded into a Perkin-Elmer LS50B spectrofluorimeter (Norwalk, CT) with constant stirring, and separately stimulated with LTB_4 and AA. Calcium concentrations were measured in real-time with excitation at 355 nm and dual emission wavelengths were monitored at 410 and 470 nm. Data was analyzed with the Grynkiewicz equation as previously described [14–16].

Statistical Analysis

Statistical differences were determined by paired or independent ANOVA followed by the Newman-Keuls test for multiple comparisons based upon the equality of variance. Statistical significance was determined by $P < 0.05$. All data are presented as the mean ± SEM.

RESULTS

HTS Inhibits Superoxide Production by AA-Primed Human Neutrophils

In the isotonic buffer, AA at 5 μM primed PMNs such that, when activated by PMA, resulted in a V_{max} of 1.29 ± 0.04 *versus* 0.97 ± 0.07 nmol $O_2^-/3.75 \times 10^5$ cells/min in the buffer-treated controls, which did not undergo AA priming. (Fig. 1; $n = 4$; $P < 0.05$ ANOVA, Newman-Keuls). In HTS-incubated neutrophils, superoxide anion production for the AA-primed PMNs was 0.72 ± 0.07 *versus* 0.79 ± 0.16 nmol O_2^- /3.75×10^5 cells/min in the buffer-treated control (Fig. 1; $n = 4$; $P < 0.05$ ANOVA, Newman-Keuls), which equated to a 100% inhibition compared with its isotonic counterpart. HTS did not significantly inhibit buffer-treated controls compared with identical PMNs under isotonic conditions.

HTS Inhibits Superoxide Production by LTB_4-Primed Human Neutrophils

Superoxide anion production for the 100 nM, 1 μM, and 10 μM LTB_4 primed-neutrophils in the isotonic group had a V_{max} of 3.56 ± 0.8, 3.9 ± 0.78, and 4.16 ± 0.84 nmol O_2^- /3.75×10^5 cells/min, respectively, *versus* 2.17 ± 0.19 nmol O_2^- /3.75×10^5 cells/min in the buffer-treated control (Fig. 2; $n = 4$; $P < 0.05$). Superoxide anion production after activation by PMA in the HTS group for the 100 nM, 1 μM, and 10 μM LTB_4 primed-human neutrophils were 2.4 ± 0.77, 2.69 ± 0.8, and 2.63 ± 1.07 nmol O_2^- /3.75×10^5 cells/min, respectively (Fig. 2; $n = 4$; $P < 0.05$). This corresponds to a V_{max} inhibition of 72% ± 22%, 58% ± 13%, and 78% ±

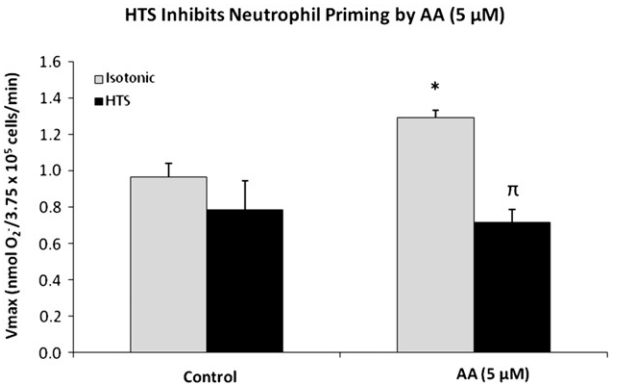

FIG. 1. HTS inhibits neutrophil priming by arachidonic acid. PMNs were incubated for 5 min with either isotonic or HTS buffer, primed with buffer (control), or AA (5 μM) for 10 min , activated with 200 ng/mL PMA for 5 min , and the superoxide anion release measured. This figure is the average superoxide release of $n = 4$. The symbols * and π denote ($P < 0.05$ ANOVA, Newman-Keuls) significant priming *versus* buffer-treated control, and significant superoxide inhibition of HTS group *versus* its isotonic counterpart, respectively.