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Hypertonic Saline Inhibits Arachidonic Acid Priming of the Human Neutrophil Oxidase

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Abstract

Background—Arachidonic acid (AA, and its leukotriene derivatives e.g.: LTB₄) 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 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 minutes at 37°C. The PMNs were then primed for 10 minutes with AA [5 μM] or 5 minutes with LTB₄ [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 LTB₄ [1 μM] significantly primed the PMA activated respiratory burst ($p < 0.05$, ANOVA, Newman-Keuls, $n = 4$). HTS inhibited both AA and LTB₄ 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.

Keywords

Multiple organ failure (MOF); G-protein coupled receptors (GPCR); hypertonic saline (HTS); clathrin-mediated endocytosis (CME); arachidonic acid (AA); leukotriene B₄ (LTB₄); neutrophil (PMN); superoxide anion; acute lung injury (ALI)

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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 B₄ (LTB₄) 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].

LTB₄, 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 LTB₄ and AA.

Materials and Methods

All reagents, unless otherwise specified, were purchased from Sigma Chemical Company (St. Louis, MO). Solutions were made from sterile water for injection, United States Pharmacopeia (USP), from Baxter Healthcare Corp. (Deerfield, IL). All buffers were made from the following stock USP solutions: 10% CaCl₂, 23.4% NaCl, 50% MgSO₄ (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). LTB₄ and AA were purchased from Cayman Chemical (Ann Arbor, MI). The calcium binding fluorometric dye Indo-1 AM was purchased from Invitrogen Corporation (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 cell) was done in either isotonic buffer at a Na⁺ concentration of 135mmol/L (control), or HTS at a Na⁺ concentration of 180mmol/L, for 5 minutes at 37°C. PMNs were then primed for 10 minutes with AA [5 μ M] or 5 minutes with LTB₄ [100 nM, 1 μ M, 10 μ M] 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 resuspended 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 $\pm$ 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 vs. 0.97 ± 0.07 nmol O_2^- / 3.75×10^5 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 vs 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 when compared to its isotonic counterpart. HTS did not significantly inhibit buffer-treated controls when compared to 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, vs 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 $72\% \pm 22\%$, $58\% \pm 13\%$, and $78\% \pm 28\%$ in the 100 nM, 1 μM , and 10 μM LTB_4 primed-human neutrophils, respectively.

AA and LTB_4 stimulation increases intracellular neutrophil calcium concentration

Intracellular calcium ion concentration, as seen in figures 3 and 4, shows a sudden spike as a result of AA or LTB_4 addition. This calcium flux was a qualitative test used to ensure the activation of GPCR by its corresponding ligand and subsequent heterotrimeric G protein signaling that is crucial for the release of Ca^{2+} from the calciosomes. Therefore, as a result of LTB_4 addition to PMNs, an increase in intracellular Ca^{2+} signifies ligand activation of its BLT receptors and subsequent release of G proteins to increase intracellular Ca^{2+} [7]. Whether in isotonic or HTS conditions, the receptor-mediated increases in cytosolic Ca^{2+} is unaffected, and that HTS inhibition of the superoxide anion assay is likely downstream of the release of G proteins. Increase in intracellular calcium following AA stimulation suggests activation of a GPCR receptor and subsequent release of G proteins to increase intracellular Ca^{2+} .

Discussion

Multiple Organ Failure (MOF) remains the leading cause of in-hospital mortality following trauma/hemorrhagic shock (T/HS) and lipid mediators in the post hemorrhagic shock lymph (PHSML) have been implicated in its development [19]. The conduit for these pro-inflammatory agents are the mesenteric lymphatics [20] [21]. PHSML contains significant amounts of AA and lymph flow increases three-fold following T/HS and has been linked to the pathogenesis of ALI following gut ischemia reperfusion [22]. AA, which is in the PHSML, primes PMNs, the effector cell in MOF [23]. Thus, research has focused on decreasing the pro-inflammatory potential of PMNs following T/HS, particularly by HTS. With data showing that isotonic saline may actually cause increased neutrophil adhesion molecules and activation [24], coupled with *in vitro* evidence that HTS favorably dampens PMN cytotoxicity [5], there is ongoing research on the use of HTS as a potential resuscitation fluid of choice following T/HS. Our data presented here shows that HTS inhibits LTB₄ and AA priming of the PMN oxidase *in vitro*, from 58–100%. Since HTS is a CME antagonist, these data support the hypothesis that both AA and LTB₄ induce CME upon ligation of their respective receptors, followed by priming of the neutrophil oxidase. Our previous work with PAF showed that HTS also inhibited the neutrophil. Identical to previous data using PAF, HTS had no effect on the LTB₄ and AA mediated increases in cytosolic Ca²⁺ [17]. These data indicate that the inhibition of AA and LTB₄ priming did not affect ligand:receptor avidity and this increase in cytosolic Ca²⁺ is likely the result of the release of a G_q subunit which, in turn, release intracellular Ca²⁺ from the calciosomes [18]. Of note, HTS-incubated PMNs had a higher calcium concentration in either LTB₄ or AA primed neutrophils. The exact reason for this occurrence is not entirely understood, however we believe it is because a defect in proper clathrin formation allows for a longer stimulated receptor, and therefore more dissociation of G-proteins followed by subsequent increase in calcium, before the receptor is completely desensitized. Whether this plays a role in increasing the cytotoxicity of neutrophils after they're activated remains to be proven. As a qualitative test, however, it confirmed our belief that the receptor:ligand interaction was not inhibited by presence of HTS. The full mechanistic detail of neutrophil metabolism of exogenous AA is currently unknown [12], although, AA has been reported to bind to the 5-oxo-ETE receptor [25], a known GPCR linked to increases in cytosolic Ca²⁺ upon ligand occupancy [26]. AA has also been shown to activate the arachidonate-regulated calcium (ARC) channel, a calcium-selective cation channel [27], although to date neutrophils have no known ARC channels. Increases in intracellular calcium is necessary for activation of 5-Lipoxygenase, the enzyme responsible for the synthesis of leukotrienes from AA, further propagating a pro-inflammatory state [28, 29]. Since the lymph flow is increased threefold in PHSML, thereby increasing the amount of AA delivered into the circulation, AA may represent an important mediator in the development of ALI post T/HS.

Our previous work has shown that HTS inhibits superoxide production of neutrophils primed by PAF, another lipid mediator of acute inflammation, via inhibition of CME [30, 31]. The data presented here further supports the immunomodulatory role of HTS. In concert with HTS administration regarding timing [32], neutrophils were preincubated with either HTS or buffer for 5 minutes, which was the optimal time for HTS to have an effect on PMNs. Regardless of the percentage of HTS used, *in vitro* concentration of Na⁺ was 180 mmol/L for the HTS group, which was the achievable Na⁺ concentration in swine hemorrhagic/shock models.

In summary, we have shown that HTS inhibits LTB₄ and AA priming of the PMN oxidase *in vitro*, and this inhibition suggests that AA primes human PMNs via activation of a GPCR. Future work is needed to elucidate the mechanisms of AA-priming in PMNs via known GPCRs, including: BLT1, BLT2, and 5-oxo-ETE receptors. Discovery of these signal

transduction pathways may allow for pharmacological intervention to attenuate or preclude the proinflammatory activation of PMNs by these lipids and ultimately decrease the incidence and/or the morbidity and mortality of MOF in injured patients.

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Figure 1.

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

Figure 2.

HTS inhibits neutrophil priming by LTB₄. PMNs were incubated for 5 minutes with either isotonic or HTS buffer, primed with buffer (control) or 100 nM, 1 μM, and 10 μM LTB₄ for 5 minutes, activated with 200 ng/ml PMA for 5 minutes, and the superoxide anion release measured. This figure is the average superoxide release from of n=4. `\*' and `π' denotes (p<0.05 ANOVA, Newman-Keuls) significant priming versus buffer-treated control and significant superoxide attenuation of HTS group versus its isotonic counterpart, respectively.

Figure 3.

HTS does not inhibit AA receptor binding. Changes in cytosolic calcium concentration were measured in indo-1 AM-loaded PMNs (1×10^6 cells/ml) in a dual-wavelength spectrofluorimeter in real time. PMNs were pretreated with isotonic or HTS buffer for 5 mins at 37°C and subsequently stimulated with AA (5 μM) at 30 seconds. Both isotonic or HTS group showed a spike a calcium burst, suggesting succesful activation of a GPCR via ligand-binding followed by release of G proteins to increase intracellular Ca^{2+} . This figure represents the average increase in intracellular Ca^{2+} of two separate experiments.

Figure 4.

HTS does not inhibit LTB_4 receptor binding. Changes in cytosolic calcium concentration were measured in iIndo-1 AM-loaded PMNs (1×10^6 cells/ml) in a dual-wavelength spectrofluorimeter in real time. PMNs were pretreated with isotonic or HTS buffer for 5 mins at 37°C and subsequently stimulated with LTB_4 ($1 \mu\text{M}$) at 30 seconds. Both isotonic or HTS group showed a spike a calcium burst, indicating succesful activation of the GPCRs BLT1 via ligand-binding followed by release of G proteins to increase intracellular Ca^{2+} . This figure represents the average increase in intracellular Ca^{2+} of two separate experiments.