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Research paper

Didox (3,4-dihydroxybenzohydroxamic acid) suppresses IgE-mediated mast cell activation through attenuation of NFκB and AP-1 transcription

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ABSTRACT

Mast cell activation via the high-affinity IgE receptor (FcεRI) elicits production of inflammatory mediators central to allergic disease. As a synthetic antioxidant and a potent ribonucleotide reductase (RNR) inhibitor, Didox (3,4-dihyroxybenzohydroxamic acid) has been tested in clinical trials for cancer and is an attractive therapeutic for inflammatory disease. We found that Didox treatment of mouse bone marrow-derived mast cells (BMMC) reduced IgE-stimulated degranulation and cytokine production, including IL-6, IL-13, TNF and MIP-1a (CCL3). These effects were consistent using BMMC of different genetic backgrounds and peritoneal mast cells. While the RNR inhibitor hydroxyurea had little or no effect on IgE-mediated function, high concentrations of the antioxidant N-acetylcysteine mimicked Didox-mediated suppression. Furthermore, Didox increased expression of the antioxidant genes superoxide dismutase and catalase, and suppressed DCFH-DA fluorescence, indicating reduced reactive oxygen species production. Didox effects were not due to changes in FcεRI expression or cell viability, suggesting it inhibits signaling required for inflammatory cytokine production. In support of this, we found that Didox reduced FcεRI-mediated AP-1 and NFκB transcriptional activity. Finally, Didox suppressed mast cell-dependent, IgE-mediated passive systemic anaphylaxis *in vivo*. These data demonstrate the potential use for Didox as a means of antagonizing mast cell responses in allergic disease.

1. Introduction

Mast cells are most recognized for their role in allergic disease. Antigen-induced cross-linkage of surface-bound IgE activates the high-affinity IgE receptor, FcεRI, and leads to the two distinct phases of mast cell activation [1–6]. The first phase, degranulation, occurs immediately and is noted by release of preformed inflammatory mediators such as histamine and proteases. The second phase occurs several hours later, and is marked by *de novo* production and secretion of arachidonic acid metabolites and many cytokines, including IL-4, IL-6, IL-13, MIP-1a, MCP-1, TNF, GM-CSF and VEGF. While these mediators are necessary for inflammation associated with acute allergic responses, recent evidence suggests that chronic mast cell activation also contributes to harmful inflammatory diseases such as rheumatoid arthritis, multiple sclerosis and heart disease [7–10]. As such, mast cells have become an important target for therapeutic intervention in these maladies.

The synthetic antioxidant and potent ribonucleotide reductase (RNR) inhibitor Didox (3,4-dihyroxybenzohydroxamic acid) has become an attractive therapeutic for treatment of inflammatory diseases [11–13]. Originally developed as an antineoplastic and antiproliferative agent to improve upon the activities of hydroxyurea, Didox possesses both iron chelating and free-radical scavenging function. Didox exhibits greater RNR inhibition than hydroxyurea, with minimal *in vivo* toxicity [11,14–16]. In addition to its anti-neoplastic activity, more recent studies have shown suppressive effects on immune cell activation. Inayat and colleagues found that Didox suppresses T cell proliferation and cytokine production following anti-CD3 activation that models organ rejection or graft-versus-host disease [17]. Didox treatment of LPS-stimulated RAW264.7 macrophage cells *in vitro* reduced the expression of inflammatory genes without causing cytotoxicity [18]. Furthermore, we recently published that Didox suppresses IL-33-mediated mast cell activation *in vitro* [19]. These observations prompted us to study Didox

Abbreviations: BMMC, bone marrow-derived mast cell; XL, cross-linking; IL, interleukin; RNR, ribonucleotide reductase; HU, hydroxyurea; NAC, N-acetylcysteine

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effects on IgE-mediated mast cell activation. Here we report that Didox antagonizes IgE-induced degranulation, cytokine production, transcription factor function, and passive systemic anaphylaxis. These data support further study of this drug's potential for understanding and treating allergic disease.

2. Material and methods

2.1. Reagents

3,4-Dihydroxybenzohydroxamic acid (Didox) was synthesized by Molecules for Health, Inc. (Richmond, VA). Lyophilized Didox was resuspended in DEPC-treated water at concentrations of 100 mM, briefly sonicated, and filter sterilized (0.45 mm syringe filter, Cell Treat). Didox was added to cultures at a final concentration of 100 μM unless otherwise indicated. Recombinant mouse IL-3 and SCF were purchased from Biolegend (San Diego, CA). DNP-specific purified mouse IgE was purchased from BD Pharmingen (San Jose, CA). Dinitophenyl-coupled human serum albumin (DNP-HSA), propidium iodide, N-acetylcysteine (NAC), and hydroxyurea (HU) were purchased from (Sigma, St Louis, MO).

2.2. Mice

Most experiments utilized C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME) and used at a minimum of 10 weeks old with approval from the Virginia Commonwealth University Institutional Animal Care and Use Committee. To ensure the reproducibility of findings in another genetic background, 129/SvJ mice were also used in Fig. 3.

2.3. Mast cell culture

Most experiments were conducted using mouse bone marrow-derived mast cells (BMMC), which were derived by culture for at least 21 days in complete RPMI supplemented as previously described, with ≥90% FceRI$^+$ and cKit$^+$ mast cells present by day 21 [19–21]. Cultures were used for up to 8 weeks of age and age-matched in experiments. Additionally, mast cells from mouse peritoneal lavage were expanded in complete RPMI 1640 medium containing 10% FBS and IL-3 + SCF (10 ng/mL) for 7–10 days to yield ~85% FceRI$^+$ and cKit$^+$ mast cells. These cells matured *in vivo* with *ex vivo* expansion and were used to support BMMC data to ensure results were not an effect of *in vitro* differentiation.

2.4. IgE-mediated activation

Mast cells were sensitized overnight with 0.5 mg/mL anti-DNP mouse IgE (k isotype). Next, cells were washed and resusupended at 1×10^6 cells/mL with IL-3 and SCF (10 ng/mL). Crosslinking (XL) was induced by the addition of DNP-HSA (50 ng/mL) for 18 h. Didox or vehicle control (DEPC water) was added for 6 h prior to IgE activation unless otherwise stated.

2.5. ELISA

Following Didox treatment and IgE activation for 18 h, cytokine levels were measured in the cell culture supernatant via ELISA. Murine ELISA kits were purchased from BioLegend (San Diego, CA) for IL-6, TNF, and MCP-1 (CCL2) and Peprotech (Rocky Hill, NJ) for IL-13 and MIP-1a (CCL3). ELISAs were performed using duplicate samples according to the manufacturers' protocols.

2.6. mRNA analysis

BMMC were activated by IgE XL for 4 h for IL-6 analysis or 24 h for SOD1 and catalase analysis. Cells were harvested and total RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY). Nucleic acid was quantified and assessed for purity using the Thermo Scientific NanoDrop™ 1000 UV–vis Spectrophotometer (ThermoScientific, Waltham, MA). For mRNA detection, cDNA was synthesized using qScriptTM cDNA Synthesis from Quanta Biosciences (Gaithersburg, MD). BioRad CFX96 Touch Real-Time PCR Detection System (Hercules, VA) was used to amplify message using PerfeCTa SYBR Green SuperMix (Quantabio, Gaithersburg, MD). The following primers were purchased from Eurofins MWG Operon (Huntsville, AL): IL-6 (forward: 5′-TCCAGTTGCCTTCTTGGGAC-3′; reverse, 5′-TCCAG TTGCCTTCTTGGGAC-3′), GAPDH (forward: 5′-GATGACATCAAGAA GGTGGTG-3′, reverse: 5′-GCTGTAGCCAAATTCGTTGTC-3′), SOD1 (forward: 5′-CGGATGAAGAGAGGCATGTT-3′, reverse: 5′-CACCT TTGCCCAAGTCATCT-3′), catalase (forward: 5′AAGACAATGTCACT CAGGTGCGGA3′, reverse: 5′-GGCAATGTTCTCACAGAGGCGTTT-3′), and β-actin (forward: 5′-GATGACGATATCGCTGCGC-3′), reverse: 5′-CTCGTCACCCACATAGGAGTC-3′). Amplification conditions consisted of a heat-activation step at 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 60 °C for 1 min. All melting curve analysis was performed between 50 °C and 95 °C. Results were normalized to the housekeeping gene GAPDH or actin and the DEPC treated water control using the Livak Method.

2.7. Flow cytometry

For all experiments, flow cytometry was run on the FACsCelesta (BD Biosciences). For cell cycle analysis, propidium iodide DNA staining was used. Cells were fixed at 4 °C for at least 4 h in the dark in Fixation Buffer (52.5% ethanol, 12.5% fetal calf serum, 35% 1X PBS). Following fixation, cells were washed and stained in a solution of PBS, RNase A (100 mg/ml), EDTA, and propidium iodide (200 mg/mL) for 2–3 h in the dark before flow cytometric analysis. For assessing viability, propidium iodide was added to cells at a final concentration of 10 mg/mL immediately before flow cytometric analysis. For degranulation, APC-anti-CD107a (2 mg/ml, clone 1D4B, Biolegend), PE-anti-mouse CD63 (2 mg/mL, clone NVG-2, Biolegend), their respective isotype controls (APC-anti-IgG2 a (clone RTK2758, Biolegend) and PE rat IgG2 b (BD Pharmagen)), and anti-CD16/32 (10 mg/ml, clone 2.4G2, BD Pharmingen) were added to the cell culture media ~30 min prior to IgE activation. Following 10 min activation, cells were washed and analyzed by flow cytometry. For analysis of oxidative stress, cells were treated with Didox for 6 h, then resuspended in Hank's buffered saline solution (HBSS) + 2′,7′ Dichlorofluorescein Diacetate (DCFH-DA, 5 μM, Millipore) ± Didox for 30 min at 37 °C. Cells were then activated with DNP (50 ng/mL) for 2 h, washed, and analyzed in the FITC channel by flow cytometry. For surface expression of FcεRI, anti-CD16/32 (clone 2.4G2) and APC-anti-FcεRI (2 mg/ml, clone MAR1, Biolegend) were added to pelleted cells, incubated for 30 min at 4 °C, washed in FACS buffer (PBS, 3% FBS, 0.1% sodium azide), and analyzed by flow cytometry.

2.8. Luciferase reporter Assay

BMMC (3×10^6) were co-transfected with pGL4.74 [hR*luc*/TK] encoding the luciferase gene from *Renilla reniformis* under the HSV-TK promoter, and either pGL4.32[luc2 p/NFκB RE/Hygro] or pGL4.44[luc2 p/AP1 RE/Hygro] vectors encoding luciferase gene from *Photinus pyralis* (Firefly) under AP-1 and NFκB response elements, respectively. The co-transfection protocol utilized a 1:5 ratio of hR*luc*:NFκB/AP1. The protocol was as previously described [22] using the Amaxa Nucleofector (Lonza, Allendale, NJ). All experiments were performed 48 h after transfection. Luciferase expression within cell lysates was measured with the Dual-Luciferase Reporter Assay System and the GloMax 20/20 Luminometer (Promega, Madison, WI).