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Protein Tyrosine Nitration of Aldolase in Mast Cells: A Plausible Pathway in Nitric Oxide-Mediated Regulation of Mast Cell Function

Yokananth Sekar,* Tae Chul Moon,* Carolyn M. Slupsky,† and A. Dean Befus*,†

NO is a short-lived free radical that plays a critical role in the regulation of cellular signaling. Mast cell (MC)-derived NO and exogenous NO regulate MC activities, including the inhibition of MC degranulation. At a molecular level, NO acts to modify protein structure and function through several mechanisms, including protein tyrosine nitration. To begin to elucidate the molecular mechanisms underlying the effects of NO in MCs, we investigated protein tyrosine nitration in human MC lines HMC-1 and LAD2 treated with the NO donor S-nitrosogluthathione. Using two-dimensional gel Western blot analysis with an anti-nitrotyrosine Ab, together with mass spectrometry, we identified aldolase A, an enzyme of the glycolytic pathway, as a target for tyrosine nitration in MCs. The nitration of aldolase A was associated with a reduction in the maximum velocity of aldolase in HMC-1 and LAD2. Nuclear magnetic resonance analysis showed that despite these changes in the activity of a critical enzyme in glycolysis, there was no significant change in total cellular ATP content, although the AMP/ATP ratio was altered. Elevated levels of lactate and pyruvate suggested that S-nitrosogluthathione treatment enhanced glycolysis. Reduced aldolase activity was associated with increased intracellular levels of its substrate, fructose 1,6-bisphosphate. Interestingly, fructose 1,6-bisphosphate inhibited IgE-mediated MC degranulation in LAD2 cells. Thus, for the first time we report evidence of protein tyrosine nitration in human MC lines and identify aldolase A as a prominent target. This posttranslational nitration of aldolase A may be an important pathway that regulates MC phenotype and function.

The mechanisms underlying the effects of NO on MCs are poorly understood.

At a molecular level, NO acts to modify protein structure and function through several mechanisms, including nitration of tyrosine and nitrosylation of cysteine residues and heme and thiols groups (16, 17). Tyrosine nitration is a marker of reactive nitrogen species and has been used as a biomarker in inflamed tissues. Protein tyrosine nitration can modify cellular signaling pathways, and nitration may be a pathogenic component in disease (18, 19). Protein conformational changes due to tyrosine nitration can generate novel antigenic epitopes, alter enzymatic activity, modulate metabolic pathways, and inhibit tyrosine phosphorylation by kinases (20). The targets for protein tyrosine nitration are diverse, and multiple functions can be affected by nitration of selected tyrosine residues on a protein (21, 22).

There are no reports of protein tyrosine nitration in MCs, although Ag/Ab-mediated activation of lung MCs from guinea pig induced peroxynitrite production, an important mediator of tyrosine nitration and, in turn, modulated the release of inflammatory mediators from MCs (23). Given the vast number of potential protein targets for this posttranslational modification by NO, we investigated protein tyrosine nitration in MCs using a proteomic approach. We identified that aldolase A, a critical enzyme in glycolysis, is one target for tyrosine nitration in human MC lines HMC-1 and LAD2. Nitration of aldolase inhibits its enzymatic activity and is associated with elevated levels of its substrate, fructose 1,6 bisphosphate (FBP), which can inhibit MC activation. Thus, the posttranslational tyrosine nitration of aldolase may be one of the mechanisms involved in NO-mediated regulation of MC function.

Materials and Methods

**MC culture**

HMC-1, an immature human MC line derived from a patient with MC leukemia (a gift from J.H. Butterfield, Rochester, MN) was cultured in Iscove’s medium with 5% heat-inactivated FBS, 2 mM l-glutamine, and 40 U/ml...
penicillin/streptomycin (all from Invitrogen, Burlington, Ontario, Canada). The cells were harvested at ~75% confluency (48 h) and were used if the passage number was <20. The seeding concentration between each passage was 1 × 10^5 cells/ml. LAD2, the growth factor-dependent human MC line (generously provided by Dr. A.S. Kirshenbaum and D.D. Metcalf, National Institutes of Health, Bethesda, MD), was cultured in Stem-Pro-34 medium (Invitrogen) supplemented with 100 ng/ml recombinant human stem cell factor (PeproTech, Rocky Hill, NJ). LAD2 were cultured by replenishment of the medium every 7 days and maintained at a cell concentration <5 × 10^5 cells/ml. LAD2 cells are used as an alternative to primary human MCs because they express IgE receptors and can be activated by IgE cross-linking (24).

Human cord blood-derived MCs (CBMCs) were cultured as described previously (25, 26). Briefly, placentae were obtained within 45 min of delivery in adherence with ethics approval from the University of Alberta and Capital Health Region and with informed patient consent at Royal Alexandra Hospital, Edmonton, Alberta, Canada. EDTA-treated umbilical cord blood was diluted with the same volume of 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and layered over Histopaque-1077 (Sigma-Aldrich, Oakville, Ontario, Canada) at room temperature within 4 h of collection. The CBMC progenitor fraction was obtained by centrifugation at 1000 × g for 20 min at room temperature. The cells were washed twice with PBS and grown in tissue-culture flasks in AIM-V medium (Invitrogen) with 100 ng/ml recombinant human stem cell factor. Nonadherent cells were transferred to fresh culture flasks and grown for ≥8 wk. CD117 and IgE expression were confirmed by flow cytometry. All cells were maintained at 37°C in a humidified incubator at 5% CO2.

Treatment of MCs with NO donor

We chose an NO donor S-nitrosoglutathione (SNOG) (Calbiochem, San Diego, CA), whose decomposition rate is ~5% per hour in water at room temperature, and the half-life is ~80 h at 37°C (27). SNOG is a well-known NO donor that favors a cGMP-independent mechanism of action of NO in different systems (28), and 500 μM SNOG has been widely used in different studies on MCs and inhibits MC function (6, 29–31). Moreover, SNOG has been used at up to 2 mM for 4 h to induce nitration in different systems (28), and 500 μM SNOG over 4 h was designed to be consistent with physiological release of NO under some in vivo conditions. For the sham control, cells were treated with equivalent volumes of the vehicle (dH2O) in the culture media (v/v ratio of 1/40 of vehicle/media). The viability of the cells was documented before and after treatment with SNOG using trypan blue dye exclusion; the viability in all experiments was >95%.

Two-dimensional gel electrophoresis

MCs were centrifuged at 300 × g for 5 min at 4°C and washed three times with ice-cold PBS. Cell pellets were stored at −80°C until further use. The cell pellet was lysed by vortexing at high speed for 2 min in two-dimensional electrophoresis (2-DE) cell lysis buffer (9 M urea, 4% CHAPS, 50 mM DTT, 0.5% immobilized pH gradient [IPG] 3-10 ampholytes [Bio-Rad, Mississauga, Ontario, Canada], 10 μl Protease Arrest kit from Genotech [St. Louis, MO]) and incubated on ice for 60 min for protein denaturation and solubilization. Cell lysates were collected and stored at −80°C in 100-μl aliquots after centrifuging the homogenate at 17,530 × g for 20 min at 4°C. Protein concentration was determined using the Bradford reducing agent (Bio-Rad). Western blot analysis.

The specificity of anti-nitrotyrosine Ab was demonstrated by treating the PVDF membranes after transferring the proteins with 100 mM sodium dithionite (Sigma-Aldrich) in 50 mM sodium borate buffer (pH 9) for 2 h at room temperature. Dithionite treatment reduces the nitroso group into an amino group, thereby preventing the Ab from binding with the nitrated epitope. After dithionite treatment, membranes were washed three times with distilled water (5 min each wash), followed by blocking with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE). Secondary Abs were goat anti-rabbit 100 (1:10,000 dilution) and goat anti-mouse 680 (1:10,000; both from LI-COR Biosciences). Blots were visualized with an Odyssey imager (LI-COR Biosciences) by scanning simultaneously at 700 and 800 nm. Odyssey software was used for molecular mass determination and quantitation of Western blots.

Aldolase enrichment

An immunofluorescent column was constructed by coupling cyanogen bromide-activated Sepharose 4B with polyclonal goat anti-aldolase Ab using standard protocols (33). HMC-1 and LAD2 cell lysates were passed through this column and washed extensively with wash buffer. The bound protein fractions were eluted using glycine (2.5 mM) pH 11.2. The eluted fractions containing enriched MC aldolase were neutralized with 2 M Tris buffer (pH 11.2), and the fractions were concentrated using Centricron centrifugal filter 10K devices (Millipore). The concentrated fractions were separated in a 10% SDS-PAGE gel and transferred to PVDF membrane for Western blot analysis.

Mass spectrometry

In-gel tryptic digestion, peptide extraction, and mass spectrometry (MS) analysis were performed using standard protocols at the Mass Spectrometry Facility, Department of Chemistry, University of Alberta. Briefly, proteins in the gel were treated with 5 mM DTT and carboxymethylated with 10 mM iodoacetamide, followed by tryptic digestion overnight with 0.06 μg/μl modified bovine trypsin (Promega, Madison, WI) at 35°C. Five microliters of the resultant peptide digests was loaded onto a nanoAcquity UPLC system with peptide trap (180 μm × 20 mm, Symmetry C18 nanoAcquity column,) and a nano analytical column (75 μm × 100 mm, Atlantis dC18 nanoAcquity column, both from Waters, Milford, MA). Desalting on the peptide trap was achieved by flushing it with 1% acetonitrile, 0.1% formic acid at a flow rate of 10 μl/min for 3 min. Peptides were separated with a gradient of 2–95% solvent B (acetonitrile, 0.1% formic acid) over 35 min at a flow rate of 300 nl/min. The column was connected to a q-TOF premier (Waters) for electrospray ionization-tandem mass spectrometry (MS/MS) analysis. Obtained MS/MS data were analyzed through proteomic software PEAKS (Bioinformatics Solutions, Waterloo, Ontario, Canada). Database searches were done with the following settings: carboxymethylated as fixed modification, and oxidation as the variable modification. Peptide identifications were further confirmed by examining the scores and manual inspection of the original MS/MS spectra. Good spectra with significant numbers of matched high-intensity peaks were considered important for the identification.

RT-PCR

Total RNA was extracted with an RNAasy Plus mini kit (Qiagen, Mississauga, Ontario, Canada), according to the manufacturer’s instructions, quantified by measuring OD at 260 nm, and assessed by applying to 1% formaldehyde-agarose gels. cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Five micrograms of total RNA from each sample was used as a template for the reverse transcription reaction. The RNA/primer mixture (5 μg total RNA, 0.5 μg oligonucleotide, 10 pmol of primers, and 0.5 μM 2′-deoxynucleoside 5′-triphosphate mixture in RNase-free water) was incubated for 5 min at 65°C and then on ice for 1 min.
The reaction mixture (40 U RNase inhibitor in 20 mM Tris-HCl [pH 8.4], 50 mM KCl, 5 mM MgCl2, 10 mM DTT) was added and incubated at 42 °C for 2 min. Then, 50 U SuperScript II reverse transcriptase was added, and the reaction was continued for 50 min at 42 °C, 15 min at 70 °C, and chilled to 4 °C on ice. Before proceeding to PCR, 2 U RNase H was added and incubated for 20 min at 37 °C. Twenty microliters cDNA was diluted to 100 μl for use in PCR amplification. Ten microliters cDNA was used for PCR with recombinant Taq DNA Polymerase (Invitrogen). Human kidney cDNA was a kind gift from Dr. Philip F. Halloran, Division of Nephrology, University of Alberta. PCR was carried out with iCycler (Bio-Rad). Isomeric-specific primers were designed based on the published human aldolase sequences: aldolase A sense 5’-AGCCAATGCTCTCACAATA-3’, antisense 5’-ACGA- CACCACACACCCGTTG-3’, human aldolase B sense 5’-GATCTGGTGGT- GAAATCGTTG-3’, antisense 5’-CCGTTTAAAGCCCTGGT-3’, human aldolase C sense 5’-GATCTGGTGGTGGAAATCGTTG-3’, antisense 5’-GATCACC-TACCCCCGTTG-3’, antisense 5’-AGGGCATACCCCTGCTGAT-3’. The conditions for PCR amplification were denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension for 72 °C for 1 min, 25 cycles. The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining and confirmed by automated sequencing using a CEQ2000XL DNA sequenator (Beckman Coulter, Mississauga, Ontario, Canada) at the Biochemistry Department, University of Alberta.

**Aldolase enzyme assay**

HMC-1 and LAD2 cells (2 10⁶ cells) were treated with 50 μl water (sham) or 500 μM SNOG in 2 ml media for 4 h, and the cell pellets in 1 ml Tris buffer (pH 7.4) containing protease inhibitors were rapidly frozen in liquid N₂. The frozen cell lysate was subjected to ultrasound treatment for 5 min in an ultrasonic bath (FS 30H, Fisher Scientific, Ottawa, Ontario, Canada). Three cycles of freeze–thawing were performed, followed by centrifugation at 17,530 x g for 15 min at 4 °C. The supernatant of cell lysate was stored on ice. The hydrazine assay (34, 35) was used to measure total aldolase activity in 100 μl cell lysate using FBP as substrate and hydrazine sulfate as detection reagent for the 3-phosphoglyceraldehyde produced by the reaction. One enzyme unit is defined as a change of absorbance of 1.00/min at 25 °C (pH 7.5) and 240 nm wavelength. To calculate the maximum velocity (Vmax) and the Michaelis–Menten constant (Km), the enzymatic activity was measured using a range of substrate (FBP) concentrations (3.125–800 μM). Km and Vmax values from aldolase activity, in terms of change in OD values (ΔA₂₄₀/2 × 10⁶ cells/min), were calculated using the Michaelis–Menten equation in a nonlinear fitting solver function in Microsoft Excel, Microsoft, Redmond, WA).

**FBP measurement**

HMC-1 and LAD2 cells (1 10⁶) were sham treated or treated with 500 μM SNOG for 4 h. The cells were washed with PBS, and the cell pellet was deproteinized using 100 μl 5% trichloroacetic acid for 15 min on ice and then neutralized with 12 μl 2 M Tris (pH 11.2). The cell extracts were centrifuged at 17,530 x g for 20 min, and the supernatants containing the metabolite pools were analyzed for their FBP content. The standard protocol for measuring FBP (34) was used with modifications. A 0.5 ml sample was incubated with aldolase (rabbit muscle aldolase, Sigma-Aldrich), the enzyme that cleaves FBP into dihydroxyacetone phosphate (DAP) and glyceraldehyde 3-phosphate. DAP and glyceraldehyde 3-phosphate are interconverted by the enzyme triose-phosphate isomerase. Glyceraldehyde-3-phosphate dehydrogenase catalyzes the reduction of DAP by NADH. The kinetic measurement of depletion of NADH at 340 nm was measured for 5 min in a 48-well plate (Corning, Lowell, MA) containing 170 μl standard medium (400 μM D-glucose) in triplicate. ATPFlite 1step Luminescence ATP detection assay kit was used to quantify ATP in the viable cells by luminescence measured using an I450 Micro ß Scintillation and Luminescence detector (both from PerkinElmer, Woodbridge, Ontario, Canada).

**Statistical analysis**

Data were analyzed with the unpaired Student t test and the Tukey–Kramer multiple comparisons test (one-way ANOVA). A p value <0.05 was considered significant.

**Results**

**Constitutive nitration among different MCs**

To investigate whether there was constitutive nitration among MCs, we performed Western blot with normally cultured, unstimulated HMC-1 and LAD2 cell lysates using anti-nitrotyrosine Ab. The anti-nitrotyrosine Ab detected many nitrated proteins (Fig. 1A), whereas dithionite treatment diminished the immunoreactivity, thereby confirming the existence of constitutive protein tyrosine nitration in MCs.

**Constitutive nitration and NO donor-induced nitration in HMC-1**

In addition to constitutive protein tyrosine nitration in MC, we tested whether specific proteins would undergo nitration upon cell treatment with an NO donor. We treated HMC-1 cells with different doses and at different time periods (50, 250, and 500 μM for 0.5, 2, and 4 h for each dose) with SNOG, a slow-releasing NO donor. 2-DE Western blots with anti-nitrotyrosine Ab were used to detect changes in the pattern of nitrated proteins in SNOG-treated HMC-1 cells. Treatment with 500 μM SNOG for 4 h induced a significant change in the nitration pattern (compare panels in Fig. 1B). Isotope controls using rabbit IgG did not identify any immunoreactivity (data not shown). SNOG-induced nitration was reproducibly evident on a protein at ~40 kDa and pl 8.3 (arrowhead in right panel, Fig. 1B). In additional
studies, 500 μM SNOG was used for 4 h. The SNOG-induced nitrated spot at pI 8.3, ~40 kDa was matched with the corresponding spot in a silver-stained gel run in parallel, and the spot was removed under sterile conditions and submitted for spectroscopy (MS) identification of the protein.

MS analysis of aldolase

MS analysis of the SNOG-induced nitrated spot (~40 kDa, pI 8.3) revealed that peptides matched human aldolase A. To ensure that the spot submitted to MS analysis and the protein selectively nitrated upon SNOG treatment were the same, we reprobed the membranes with anti-aldolase Ab and confirmed that the spot at pI 8.3, ~40 kDa was aldolase (arrowhead in right panel, Fig. 1C). We also documented multiple immunoreactive spots for aldolase near molecular mass ~40 kDa but at different pI, ranging from 7.0 to 9.0, in sham- and SNOG-treated HMC-1 cells (Fig. 1C). Aldolase at pI 9.0 matched the nitrated spot at pI 9.0 (compare and see arrows in left panel, Fig. 1B, 1C), indicating constitutive nitration of aldolase. SNOG-induced nitration was observed for aldolase A at pI 8.3 (arrowhead in right panel, Fig. 1B, 1C). Similar
results were obtained when LAD2 cells were treated with SNOG. The region around 40 kDa in the 2-DE gel was highlighted to demonstrate that SNOG induces selective nitration of aldolase at pI 8.3 in LAD2 cells, as well as in HMC-1 cells (Fig. 1D). More- ever, in addition to aldolase, other spots were nitrated following SNOG treatment in LAD2 cells (data not shown). To further con- firm constitutive nitration of aldolase, we enriched aldolase from HMC-1 or LAD2 cells using an aldolase affinity column and performed Western blot with anti-nitrotyrosine Ab (Fig. 1E). Dithionite treatment significantly reduced the nitro tyrosine reac- tivity of aldolase fractions, thereby confirming constitutive nitra- tion of aldolase in these two MC lines. Similar results were observed when we preincubated nitrotyrosine Ab with free 3- nitrotyrosine (data not shown).

Identification of aldolase isoforms in MCs

Because there were multiple pI forms of aldolase in MCs, we defined the different isoforms of aldolase in MCs. To investigate the mRNA expression of the isoforms of aldolase in MCs, we used RT- PCR with isofrom-specific primers for aldolase A, B, and C with cDNA of HMC-1 cells, LAD2 cells, and CBMCs. cDNA from non-tumor areas of kidney from a patient who underwent nephrectomy was used as a positive control for all three isoforms of aldolase. We identified mRNA for two isoforms, aldolase A and C, in HMC-1 cells, LAD2 cells, and CBMCs, whereas mRNA for all three isoforms was found in human kidney (Fig. 2). The products were sequenced and confirmed. Similarly, the affinity-purified fractions of aldolase from HMC-1 cells were analyzed using MS. We found peptides for aldolase A (Supplemental Table I) and aldolase C (Supplemental Table II).

SNOG-induced changes in the enzymatic activity of aldolase

To determine whether nitration of aldolase affects the activity of the enzyme, we measured the \( K_m \) and \( V_{\text{max}} \) of aldolase from HMC-1 and LAD2 cell homogenates before and after treatment with SNOG. SNOG induced a reduction in the \( K_m \) and the \( V_{\text{max}} \) of aldolase in HMC-1 cells (Fig. 3A, 3B) and a reduction in the \( V_{\text{max}} \) of aldolase in LAD2 cells (Fig. 3D). The raw data representing the aldolase activity in terms of change in OD values (\( \Delta A_{236} \times 10^8 \) cells/min) between sham- and SNOG-treated HMC-1 and LAD2 cells are shown in Supplemental Fig. 1. Because inhibition of the activity of an enzyme can result in accumulation of the substrate, we measured levels of FBP, the substrate for aldolase A, before and after treatment with SNOG. SNOG treatment significantly increased the intracellular FBP levels by 61% in HMC-1 cells and by 61% in LAD2 cells (Fig. 4).

SNOG-induced changes in glycolytic metabolites

Because aldolase is a crucial enzyme in the glycolytic pathway, we studied whether inhibition of aldolase A activity might alter ATP levels in MCs. We used \(^{[1]}\text{H}\) NMR to begin to define components of the metabolome (set of <1500-Da metabolites) (39, 40) of HMC-1 cells treated with sham or SNOG. Surprisingly, in contrast to the expectation from the inhibition of aldolase activity, ATP levels in cell extracts were similar in SNOG- and sham-treated MCs (Fig. 5A), although the AMP/ATP ratio was increased follow- ing SNOG treatment (Fig. 5B). Interestingly, pyruvate and lactate, measures of cellular glycolytic activity, were significantly elevated in culture media of SNOG-treated MCs (Fig. 5C, 5D). Similar results with HMC-1 and LAD2 cells were obtained using a different assay system (ATPlite 1-step ATP kit; Supplemental Fig. 2).

FBP inhibits the degranulation of LAD2 cells

To simulate the effects of increased intracellular FBP in MCs and to study the effects of FBP on MC function, we used an in vitro IgE-dependent degranulation assay of MCs. Because exogenously ap- plied FBP was shown to enter intracellular compartments poorly (41), we used 5 mM exogenous FBP in our model, as other inves- tigators used previously (42). Indeed, when we tested intracellular levels of FBP following treatment with exogenous FBP (100 nmol to 100 \( \mu \)mol), only a few nanomoles of FBP were available in the intracellular compartment of LAD2 cells (Supplemental Fig 3).

IgE/anti-IgE stimulation induced release of 21.8 ± 2.1% of the total cellular \( \beta \)-hex from LAD2 cells, whereas pretreatment with SNOG or FBP significantly inhibited this degranulation of LAD2 by 59 or 48%, respectively (Fig. 6). However, F1P and F6P, as negative controls for FBP, did not inhibit MC degranulation.

Discussion

Endogenous and exogenous NO have been widely reported to modulate several MC functions (43). One common pathway in- volved is that NO activates soluble guanylate cyclase and increases intracellular cGMP, which, in turn, regulates numerous physiolog- ical events in the cell (44). However, many studies indicated that non-cGMP-mediated pathways are also important in the effects of NO on MC function (6, 12, 31, 45). Earlier, we suggested that S- nitrosylation of calpain was responsible for the NO-mediated in- hibition of MC adhesion (31). In the current study, we provide the first evidence that protein tyrosine nitration plays a role in NO- mediated inhibitory effects on MC function.

Using Western blot with an anti-nitrotyrosine Ab, we identified constitutive protein tyrosine nitration in MCs, thereby providing evidence that this posttranslational modification of proteins occurs in a variety of MCs (Fig. 1). Our observation is supported by evi- dence that constitutive nitration occurs in other leukocytes, such as macrophage cell lines (46), eosinophils (47, 48), and neutro- phils (49). It is interesting that a recent study claims that there are no nitrotyrosine+ proteins in MCs on lung sections of cystic fibro- sis patients (48). Unfortunately, isotype controls were not reported in that study, and there was no information about optimization of sensitivity of the immunohistochemistry to detect nitrotyrosine reactivity in MCs or the numbers of MCs assessed on the tissues.

In addition to the constitutive nitration of multiple MC proteins, we identified SNOG-induced changes in protein nitration (Fig. 1B). It is interesting that aldolase at pI 9.0 was nitrated constitutively, whereas NO-mediated nitration of aldolase A was associated with an 8.3 pI form (Fig. 1B, 1C). The presence of multiple pI forms (\( \sim 7.0–9.3 \)) of aldolase in MCs is consistent with earlier findings in rat diaphragm and mouse sperm cells (50, 51). Aldolase can also be phosphorylated (52) and regulated by different posttransla- tional modifications.

Interestingly, aldolase A can be found in the cytoplasm (53), mi- tochondria (54), and the heterochromatin region of nucleus (55), and in vivo nitration can be seen in many intracellular compartments.
FIGURE 3. SNOG-induced reduction in the total aldolase enzymatic activity of MCs. HMC-1 and LAD2 cells (2 × 10^6) were treated with 50 µl of water or 500 µM SNOG for 4 h, and the total aldolase activity in the cell lysates was measured. To calculate the V_{max} and the K_m, the 1.5125–800 µM (serial 2-fold dilution) of substrate was used. SNOG treatment reduced the K_m and V_{max} of aldolase in HMC-1 cells (A, B), but it only reduced the V_{max} in LAD2 cells (C, D). Results are from seven (HMC-1) and five (LAD2) independent experiments (mean ± SE). *p < 0.05.

FIGURE 4. SNOG-induced elevation of FBP in MCs. HMC-1 cells (A) or LAD2 cells (B) (1 × 10^6) were treated with 250 µl of water or 500 µM SNOG in 10 ml media for 4 h, and the total cell pellet were deproteinized using 5% TCA followed by neutralization by 2 M Tris. The FBP concentration was estimated in the metabolite fractions. Results are from five (A) or eight (B) independent experiments (mean ± SE). *p < 0.05.

(56), including nuclear histones (57). Tyrosine nitration of a protein can be mediated by multiple mechanisms, including ONOO^− (22). In MCs, ONOO^− can be generated by the reaction of NO and MC-produced O2^−, which could, in turn, nitrate aldolase. The half-life of ONOO^− is only a few seconds; thus, ONOO^− reactivity may act in the local microenvironment on a limited spectrum of proteins. In our experiment, we decided to use a slow-releasing NO donor SNOG (decomposition rate is ~5% per hour in water at room temperature, and the half-life is ~80 h at 37˚C), which would likely combine with physiologically relevant, MC-derived O2^− and, thus, generate a more physiological nitration event. In addition to ONOO^−, there are other mechanisms of nitration (58); however, the precise mechanisms underlying the nitration of aldolase are beyond the scope of the current study. Another consideration is that NO might facilitate selective nitration of aldolase A in different intracellular compartments of HMC-1 cells and modulate compartment-specific functions of aldolase (see below).

There are three isoforms of mammalian aldolase: A, B, and C (59). Aldolase A and C are preferentially involved in the glycolytic cycle and predominantly expressed in muscle and brain, respectively, whereas aldolase B is reported to be preferentially involved in gluconeogenesis and expressed in liver (59). However, the expression of these isoforms is not restricted to specific tissues, (e.g., all three isoforms are expressed in kidney) (60, 61). Tyrosine nitration of aldolase A, B, and C was reported in a wide range of tissues and under different inflammatory conditions (50, 62–65). To define specific tyrosine residues that are nitrated in aldolase A or in aldolase C, MS identification of nitration-specific sites requires large amounts of protein; we are working toward enriching the nitrated aldolase fractions with specific techniques (66).

Aldolase A is the predominant isoform expressed in lymphocytes (67). Aldolase C expression was reported in platelets, but it was not detectable in erythrocytes and lymphocytes (68). It is interesting that MCs express aldolase C, the isoform predominantly expressed in neuronal tissues. The potential significance of aldolase C expression in MCs is multiple. For example, if there is inactivation of one isoform of the enzyme, the other isoform, in this case aldolase C, might act as a backup enzyme in the glycolytic cycle. Interestingly, aldolase C was reported to be involved in a stress-response pathway for lung epithelial cell function during hypoxia (69). Moreover, subsets of cerebellar Purkinje cells expressing aldolase C were resistant to excitotoxic insult (70), and aldolase C small interfering RNA transfection resulted in the death of these subsets of Purkinje cells. Because MCs play a major role in TGF-β1–mediated excitotoxicity (71), expression of aldolase C may be of functional significance in MC homeostasis.

It is intriguing that aldolase A has been identified as a target of tyrosine nitration in several other cells and tissues, including diaphragm (50), human skin fibroblasts (35), quadriceps muscle of patients with severe chronic obstructive pulmonary disease (72), and in lung tissue in a model of asthma (65). However, this is the first report in human MCs. The discovery of aldolase as one of the targets for nitration in MCs might be due to the abundance of this protein, the extent of nitration of tyrosines in aldolase, and the sensitivity of the methods used. Interestingly, other proteins were...
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FIGURE 5. SNOG-induced glycolytic intermediates change in HMC-1 cells. HMC-1 cells (1 × 10⁶) were treated with sham (2.5 ml water in 100 ml media) or with 500 μM SNOG for 4 h, and glycolytic metabolites were analyzed using NMR. SNOG did not increase the ATP level (A), but it significantly increased the AMP/ATP ratio (B) and the pyruvate (C) and lactate (D) levels (enhanced glycolysis). Error bars represent SE from three independent experiments. *p < 0.05.

nitrated (e.g., pl 7.0, molecular mass 25 kDa) upon SNOG treatment in LAD2 cells (data not shown). We are currently working toward identifying these targets using MS.

An earlier in vitro study using rabbit muscle aldolase treated with ONOO− demonstrated four tyrosine residues (Y222, Y243, Y342, and Y363) that were targets for tyrosine nitration (35). Although, we do not know which tyrosine residues in MC aldolase A are nitrated following SNOG treatment, NO treatment reduced total activity of MC aldolase (Fig. 3), as previously shown using rabbit muscle aldolase (35). Because it is difficult to separate the two isoforms of aldolase A and C from the whole-cell lysate, we studied total aldolase activity, as reported earlier in different tissues and under different conditions (55, 73). The tyrosine at the carboxy terminal end of the aldolase A (i.e., tyrosine 363) was critical for the enzymatic activity; nitration of this residue significantly reduced the Vₘₐₓ of the enzyme activity (35). Interestingly, our observation that NO induced a reduction in the Kₘ and Vₘₐₓ of HMC-1 aldolase is consistent with earlier studies in which substitution of tyrosine 363 with serine in human aldolase A also reduced enzyme Kₘ and Vₘₐₓ (74). SNOG reduced the Kₘ of HMC-1 aldolase, whereas there was no change in the Kₘ of LAD2 cells (Fig. 3). Moreover, there was a 10-fold difference between the Kₘ values of HMC-1 and LAD2 cells (Fig. 3). Thus, there may be cell-type–specific differences in the effects of nitration (i.e., LAD2 is a mature MC line, whereas HMC-1 is an immature cell line). Moreover, the expression and intracellular distribution of aldolase isoforms A and C may be different between these two cell lines, and the tyrosine nitration sites may differ as well.

Given the reduction in the enzymatic activity of aldolase, we postulated that this would increase the levels of its substrate FBP. Indeed, we found that the intracellular FBP in HMC-1 cell extracts significantly increased following SNOG treatment (Fig. 4A). Similarly, a statistically significant increase in FBP levels following SNOG treatment was documented in LAD2 cells (Fig. 4B). It is interesting to note that the SNOG-induced FBP levels also differed between HMC-1 and LAD2 cells, as did the Kₘ values of these two MC lines (see above). Thus, the difference in magnitude of Kₘ values, unaltered Kₘ values for LAD2 cells compared with the reduction in Kₘ of HMC-1 cells after SNOG treatment, and the variable intracellular FBP levels strongly suggest that the outcome of nitration might be different between HMC-1 and LAD2 cells. Further detailed study is necessary to understand our observations. Interestingly, a mutation of aldolase A (Glu206Lys) was reported in a patient with an inherited metabolic myopathy and hemolysis and was associated with increased FBP levels (182.9% of control) in the patient’s RBCs (75). Moreover, NO-induced inhibition of GAPDH, the enzyme downstream of aldolase in the glycolytic cycle, and a corresponding increase in FBP levels in rat intestinal tissues were reported (76). Thus, increased FBP levels upon inactivation of aldolase A may have physiological relevance.

To assess the effects of aldolase nitration on the metabolic pathways of MCs, we analyzed metabolites using NMR. ATP levels in cell extracts were similar in sham- and SNOG-treated HMC-1 cells (Fig. 5A). It is interesting that cellular ATP levels remained similar, despite a reduction in the activity of aldolase, thereby suggesting restoration of ATP levels through homeostatic mechanisms. AMP-activated protein kinase was reported to act as a fuel gauge in mammalian cells. It can enhance cellular ATP levels by switching off energy-using pathways and switching on energy-generating pathways (77, 78). AMP-activated protein kinase activation occurs when there is an increase in AMP levels and...
a decrease in ATP levels (increased AMP/ATP ratio) (77). We predicted that this regulatory pathway also exists in MCs; therefore, we measured the AMP/ATP ratio before and after SNOG treatment. Indeed, the AMP/ATP ratio increased following SNOG treatment in HMC-1 cells (Fig. 5B), providing evidence for homeostatic regulation of ATP levels in our model. In addition to in HMC-1 cells, the ATP levels in LAD2 cells were unaltered after SNOG treatment (Supplemental Fig. 2). The lower ATP levels detected using NMR (Fig. 5A) compared with live cell-based ATP assay (Supplemental Fig. 2) might be due to the loss of ATP during the preparation of samples for NMR (see Materials and Methods).

Measurement of pyruvate, the end product of glycolysis, and lactate, the end product of anaerobic glycolysis, in the media of cultured cells provides a clear understanding of the metabolic status of the cells (79). Interestingly, pyruvate and lactate were significantly elevated in culture media of NO-treated MCs (Fig. 5C, 5D), indicating enhanced glycolysis, as previously shown in astrocytes treated with NO (80). NO downregulates mitochondrial energy production, but the cells maintain energy production by regulating glycolysis in astrocytes (81). It seems paradoxical that, although aldolase enzymatic activity is depressed (Fig. 3), glycolysis seems to be enhanced. In support of our observations, studies of skeletal muscle showed that elevated FBP is associated with increased glycolysis (81) and FBP activation of 6-phosphofructo-1-kinase, a key rate-limiting enzyme in glycolysis (81). It is interesting that some cells can respond to NO by activating glycolysis (astrocytes), whereas others do not (neurons) (81). Our results suggest that SNOG fundamentally alters MC metabolism to keep energy production at a homeostatic level.

FBP has several activities, including protection in ischemia/reperfusion injury and hypoxia (76, 82) and suppression of T cell proliferation (83). FBP has been used as a component of protective solutions in the transport of donor organs, such as liver (84). Other investigators have filed a patent on FBP as a therapeutic for asthma (85), and a recent in vivo study in rats demonstrated that FBP has an inhibitory role on MC degranulation and histamine release (86). We hypothesized that increased FBP levels, following aldolase A nitration in MCs, might inhibit some MC functions. Indeed, FBP significantly inhibited the 6-β-hex release by LAD2 cells (Fig. 6), whereas similar concentrations of F1P or F6P did not have significant effects, supporting the specific effects of FBP on MC degranulation. The effects of NO donors on human MC activation depends on their NO release kinetics rather than the amount of NO released (87). Hence, our data on the effects of SNOG pretreatment followed by MC activation in an NO-free environment supports NO-mediated posttranslational modification, in this case nitration of aldolase. Moreover, the slow NO-releasing donor SNOG has been widely reported to mediate its action through non-cGMP pathways (28, 87).

Phospholipase C (PLC) cleaves phosphatidylinositol 4,5-biphosphate to diacylglycerol and inositol 1,4,5-triphosphate (IP3), a critical intracellular messenger. The PLC signaling pathway plays a pivotal role in MC degranulation (88). Interestingly, some effects of FBP in other cells depend on PLC signaling (82). Moreover, FBP shares a binding site on aldolase A with IP3 (89) and significantly inhibits IP3 binding to aldolase A (89, 90). This binding also inhibits aldolase A enzymatic activity and influences release/repartition of aldolase A from binding to cytoskeletal elements (90). The posttranslational modification of tyrosine nitration of aldolase A may alter its relative affinity for FBP and/or IP3. Thus, it is possible that NO acts through excess FBP that modifies IP3 and PLC signaling cascades critical in MC secretion in IgE-dependent responses. Interestingly, aldolase can bind to many intracellular proteins, such as actin and ryanodine receptor (91, 92). Aldolase nitration might alter some of these interactions and, in turn, regulate MC function. Further studies with mature MCs may enhance our knowledge of these pathways.

In conclusion, we have provided evidence that protein tyrosine nitration regulates MC function. We identified that aldolase A, a critical enzyme in the glycolytic pathway, is a target for nitration. Because of the importance of aldolase A in cellular metabolism and homeostasis, further dissection of the relevance of our observations is necessary to more fully elucidate the role of tyrosine nitration in the regulation of MC function in allergic and other diseases.

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Disclosures
The authors have no financial conflicts of interest.

References


FIGURE S1. Saturation curve for MC aldolase.

HMC-1 (A) and LAD2 (B) \(2 \times 10^6\) cells) were treated with 50 \(\mu\)l of water or 500 \(\mu\)M SNOG for 4 h and the total aldolase activity in the cell lysates were measured as change of OD values \((\Delta A_{240}/2 \times 10^5\text{cells/min})\). Value at 0 \(\mu\)M FBP were subtracted from values at each FBP concentration. Results are expressed as mean±SE from seven (HMC-1) and five independent experiments (LAD2).
FIGURE S2. Effects of SNOG on HMC-1 and LAD2 ATP levels.

HMC-1 or LAD2 were treated with or without 500 μM SNOG for 4 h and the total aldolase activity in 5x10^3 cells were measured using ATPliteTM 1step ATP kit according to the manufacturer's instructions. ATP levels in 5x10^3 cells were calculated with ATP standard curve. Results are expressed as mean±SE from three independent experiments.
FIGURE S3. LAD2 cell permeability to exogenously applied FBP.

LAD2 (1x10^7 cells) were treated with increasing concentrations (100, 1000, 10000, 100000 n mole FBP in 10 ml) for 4 h. The cell pellets were deproteinized using 5% trichloroacetic acid followed by neutralization by 2M Tris. The FBP concentration was estimated in the metabolite fractions. Results are expressed as mean±SE from four to six independent experiments.
Table S1. Peptide sequences of aldolase A identified from aldolase affinity-purified fractions of HMC-1

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Accession No. (NCBI)</th>
<th># Pept ¹</th>
<th>Coverage % ²</th>
<th>Score %</th>
<th>m.w.</th>
<th>Peptides</th>
</tr>
</thead>
</table>
| Aldolase A   | CAA30979             | 22       | 74           | 99      | 39289 | PYQYPALTPEQK  
|              |                      |          |              |         |       | KELSDIAHR   
|              |                      |          |              |         |       | GILAADESTGIAK  
|              |                      |          |              |         |       | RLQSIGTENTEENR  
|              |                      |          |              |         |       | QLLLTADDR   
|              |                      |          |              |         |       | IGGVILFHETLYQK  
|              |                      |          |              |         |       | ADDGRPFPQVIK   
|              |                      |          |              |         |       | GVVPLAGTNGETTQGLDGLSER  
|              |                      |          |              |         |       | IGEHTPSALAIMENAN  
|              |                      |          |              |         |       | YASICQQNGIVPIVEPEILPDGDHDLK  
|              |                      |          |              |         |       | AIMENTANVLR    
|              |                      |          |              |         |       | PIVEPEILPDGDHDLKR  
|              |                      |          |              |         |       | ALSDDIIYLEGTLKKPNMVTGH  
|              |                      |          |              |         |       | VLAAYVK    
|              |                      |          |              |         |       | FSHEIAMATVTALR   
|              |                      |          |              |         |       | TVPPAVTGTIF   
|              |                      |          |              |         |       | LSGGQSEEEASINLNAINK  
|              |                      |          |              |         |       | CPLLKWPWAL  
|              |                      |          |              |         |       | CPLLKWPWALTIF  
|              |                      |          |              |         |       | ALQASALK    
|              |                      |          |              |         |       | ALANSLALQGK  
|              |                      |          |              |         |       | YTPSGQAGAAASESLFVSNHAY |

¹ The number of unique peptides for which ion fragmentation was obtained is listed for each protein.
² The % of sequence coverage for each protein is listed.
Table S2. Peptide sequences of aldolase C identified from aldolase affinity-purified fractions of HMC-1

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Accession No. (NCBI)</th>
<th># Pept (^1)</th>
<th>Coverage % (^2)</th>
<th>Score %</th>
<th>m.w.</th>
<th>Peptides</th>
</tr>
</thead>
</table>
| Aldolase C   | NP_005156            | 12            | 46                | 99      | 39456| GILAADESVGSMAK  
LSQIQVENTEENR  
TPSALAILENANVLAR  
PIVEPEILPDGDHDLLKR  
YASICQQNGIVPIPEPEILPDGDHDLLKR  
RYASICQQNGIVPIPEPEILPDGDHDLLKR  
YTPEEIAMATVTALR  
ALQASALNAWR  
AEVNGLAAGK  
YEGSGEDGGIAAQSLYIANHAY  
ALSDHHYYLEGTLKPN  
GVVPLAGTDGETTTQGLGLSER |

\(^1\) The number of unique peptides for which ion fragmentation was obtained is listed for each protein.

\(^2\) The % of sequence coverage for each protein is listed.