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FcεRI- and Fcγ Receptor-Mediated Production of Reactive Oxygen Species by Mast Cells Is Lipoxygenase- and Cyclooxygenase-Dependent and NADPH Oxidase-Independent

Emily J. Swindle,* John W. Coleman,* Frank R. DeLeo,† and Dean D. Metcalfe*†

We investigated the enzymes responsible for FcεRI-dependent production of reactive oxygen species (ROS) and the influence of ROS on mast cell secretory responses. 5-Lipoxygenase (5-LO) was the primary enzyme involved in ROS production by human mast cells (huMC) and mouse bone marrow-derived mast cells (mBMMC) following FcεRI aggregation because incubation with 5-LO inhibitors (AA861, nordihydroguaiaretic acid, zileuton) but not a flavoenzyme inhibitor (diphenyleneiodonium) completely abrogated Ag-induced dichlorodihydrofluorescein (DCF) fluorescence. Furthermore, 5-LO-deficient mBMMC had greatly reduced FcεRI-dependent DCF fluorescence compared with wild type mBMMC or those lacking a functional NADPH oxidase (i.e., gp91phox- or p47phox-deficient cells). A minor role for cyclooxygenase (COX)-1 in FcεRI-dependent ROS production was demonstrated by inhibition of Ag-mediated DCF fluorescence by a COX-1 inhibitor (FR122047) and reduced DCF fluorescence in COX-1-deficient mBMMC. Complete abrogation of FcεRI-dependent ROS production in mast cells had no effect on degranulation or cytokine secretion. In response to the NADPH oxidase-stimulating agents including PMA, mBMMC and huMC produced negligible ROS. IgG-coated latex bead-induced ROS production in human polymorphonuclear leukocytes occurred by the NADPH oxidase pathway. Thus mBMMC and huMC generate ROS by 5-LO and COX-1 in response to FcεRI aggregation; huMC generate ROS upon exposure to IgG-coated latex beads by 5-LO and COX; and ROS appear to have no significant role in FcεRI-dependent degranulation and cytokine production. The Journal of Immunology, 2007, 179: 7089–7071.

Abbreviations used in this paper: ROS, reactive oxygen species; AUC, area under the curve; COX, cyclooxygenase; mBMMC, mouse bone marrow-derived mast cell; PMN, polymorphonuclear leukocyte; LT, leukotriene; DCF, dichlorodihydrofluorescein; HSA, human serum albumin; DPI, diphenyleneiodonium; huMC, human mast cell; 5-LO, 5-lipoxygenase; DHE, dihydroethidium; DHR, dihydrorhodamine; IgG-LB, IgG-coated latex bead; PHGP, phospholipid hydroperoxide glutathione peroxidase; WT, wild type.

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generated appreciable levels of ROS compared with PMN via this pathway. As our results will show mouse bone marrow–derived mast cells (mBMMC) and human mast cells (huMC) generate ROS in response to FcεRI aggregation enzymatically through 5-lipooxygenase (5-LO) and cyclooxygenase (COX)-1 but not NAPDH oxidase. Inhibition of ROS production by 5-LO and COX-1 inhibitors had negligible effects on degranulation and cytokine secretion. Following stimulation with IgG-coated latex beads (IgG-LB), ROS production in huMC was dependent on 5-LO and COX-1; this was not the case in PMN that used primarily NAPDH oxidase in this response.

Materials and Methods

Animals and materials

Wild-type (WT) C57Bl/6 mice (&lt; 6 mo-old, 20 g) and 5-LO-deficient mice were obtained from The Jackson Laboratory. Aged-matched WT, CD34+ cell–grown from CD34+ PBMC obtained following informed consent from volunteers by standard methods (24) using a protocol approved by the NIAID review committee. Following three washes in PBS the resulting IgG-LB were resuspended in double-distilled H2O containing 0.0035% 2-ME, and 30 ng/ml mouse IL-3 as previously described (19). mBMMC were

Cell isolation and mast cell culture

The mBMMC collected were cultured from femoral marrow cells in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 &amp;#34;g/ml streptomycin, 25 mM HEPES, 1 mM sodium pyruvate, nonenzy-

Cell activation

HuMC or mBMMC optimally sensitized with mouse IgE anti-DNP or biotinylated human IgE, respectively (both 100 ng/ml, overnight) were washed to remove excess IgE and resuspended in either mBMMC or huMC medium at the required cell density for a specific assay. After stimulation of mBMMC with DNPp-HSA (30 ng/ml) or huMC with streptavidin (30 ng/ml) cell-free supernatants were removed after 20 min for LTC4 and PGD2 analysis, after 4 h for mouse TNF-α and IL-6 analysis or after 10 h for human GM-CSF and IL-8 determination.

Cell degranulation assay

IgE-sensitized mast cells were seeded at 1 × 10⁴ (huMC) or 5 × 10⁴ (mBMMC) per well in 80 μl of HEPES plus 0.04% BSA buffer (HEPES buffer) in a 96-well cell culture plate. Enzyme inhibitors or ROS scavengers (10 μl) were added for 10 min before addition of DNP-HSA (mBMMC) or streptavidin (huMC), and degranulation was measured as the percentage of release of β-hexosaminidase as described (20).

Intracellular ROS detection by microfluorimetry

Intracellular ROS were measured in a 96-well plate assay using the fluorescent probe DCB (the intracellular product of DCF diacetate that fluoresces in the presence of ROS) that includes H2O2, hydroperoxides, and NO (25). Mast cells or PMN (1 × 10⁶/ml) were incubated with DCF diacetate (20 μM) in cell culture medium for 15–20 min at 4°C with rotation. Cells were then washed in HEPES buffer (10 ml) and seeded at 1–2 × 10⁵ cells per well in a black opaque 96-well microplate in the presence or absence of enzyme inhibitors or ROS scavengers (10 μl). For FcεRI aggregation experiments, DCF fluorescence was monitored at 37°C in 10-s intervals for 5 min before and 10 min after the addition of DNP-HSA (mBMMC) or streptavidin (huMC). For IgG-LB experiments, huMC or PMN were kept at 4°C and added to the microplate at 2 × 10⁵ cells per well on ice containing enzyme inhibitors and stimulants. The microplate was then synchronized by centrifugation (170 × g, 5 min at 4°C) before monitoring of DCF fluorescence at 30-s intervals for 60 min at 37°C. In all experiments, DCF fluorescence was monitored using a GENios fluorescent plate reader (ReTiSoft) at 37°C with an excitation wavelength of 492 nm and emission wavelength of 535 nm. Fluorescence was expressed as relative fluorescent units. The kinetic data were collected using an XF fluoroscan macro within Microsoft Excel. For confocal microscopy, cells were visualized and photographed using an Axioskop microscope (Zeiss) at a lens objective of ×63.

Intracellular ROS detection by flow cytometry

To determine whether O2•− was produced following FcεRI aggregation on mast cells, we used DHE, which is an O2•−–specific intracellular probe, and when used in combination with flow cytometry is a sensitive method for cell–specific O2•− production. For a specific assay for ROS production by flow cytometry, we used the nonspecific ROS probe DHE and dichlorodihydrofluorescein (DHR123). Briefly, mast cells were incubated with DCF diacetate (2 μM), DHR123 diacetate (2 μM), or DHE (2 μM) for 15 min at 37°C. Following stimulation of the cells with either DNP-HSA (mBMMC) or streptavidin (huMC) over various time points (0–30 min), samples were cooled on ice until analyzed by flow cytometry. Cell fluorescence was analyzed (10,000 events) on a gated forward light scatter and side light scatter area previously determined as mast cell–specific using a FACSCaliber flow cytometer (BD Biosciences) and associated CellQuest software (AppleMAC).

LTC4 and PGD2 measurements

IgE-sensitized mast cells were seeded at 1 × 10⁴ (huMC) or 5 × 10⁴ (mBMMC) cells per well in 80 μl of HEPES buffer in a 96-well cell culture plate. Enzyme inhibitors or ROS scavengers (10 μl) were added for 20 min before the addition of 10 μl of DNP-HSA (mBMMC) or streptavidin (huMC). Following 20 min of stimulation, cell-free supernatants were removed and analyzed for LTC4 and PGD2 by competitive enzyme immunoassay (Cayman Chemicals), according to the manufacturer’s instructions. Ag-stimulated mBMMC supernatants were diluted 1/1000 before analysis of LTC4 release, and mBMMC cell number was increased to 2 × 10⁵ per well before analysis of PGD2 release in order for samples to be read within the range of the respective standard curve.
Cytokine measurements

IgE-sensitized huMC and mBMMC were seeded at 2 × 10^6 cells per well in 160 µl of huMC or mBMMC medium (medium contained 0.04% FCS for mBMMC cultures) in a 96-well cell culture plate. Enzyme inhibitors or ROS scavengers (20 µl) were added for 20 min before addition of 20 µl of streptavidin (100 ng/ml) for huMC and of DNP-HSA (100 ng/ml) for mBMMC (medium contained 20% FCS for mBMMC cultures). Cell-free supernatants were removed 4 h poststimulation for analysis of mouse TNF-α and IL-6 by ELISA (BioSource International) and 24 h poststimulation for human IL-8 and GM-CSF by ELISA (R&D Systems). ELISA was done according to the manufacturer’s instructions.

Data presentation and statistical analysis

Results shown are for real-time kinetic readings and as grouped data from a minimum of three separate experiments performed in duplicate. Area under the curve (AUC) data for total ROS production was determined after baseline subtraction by Prism (GraphPad Software). Differences between individual concentration response curves for inhibitors or Ag was tested for statistical significance using ANOVA. If statistical significance was found by ANOVA (p < 0.05), differences between groups within concentration response curves or between responses of WT and 5-LO- or COX-1-deficient mBMMC were determined by Student’s t test. Significant difference at a value of p < 0.05 was displayed at +, p < 0.01, or ++, p < 0.001 when comparing responses to Ag alone or control unstimulated cells or was displayed as *, p < 0.05, **, p < 0.01, or ***, p < 0.001 when comparing WT to 5-LO- and COX-1-deficient responses.

Results

ROS production in mast cells following FceRI aggregation is NADPH oxidase-independent

We initially visualized ROS production following FceRI aggregation of DCF-loaded mBMMC using confocal microscopy. As shown in Fig. 1A, FceRI-dependent ROS production was observed as soon as 60 s poststimulation. To determine the enzymes responsible for ROS generation in mast cells, we initially examined the effect of DPI, an inhibitor of flavoenzymes including NADPH oxidase and NO synthase, on the DCF signal generated following FceRI aggregation. IgE-sensitized, DCF-loaded mBMMC were incubated with increasing concentrations of DPI, and DCF fluorescence was monitored for 5 min before (baseline reading) and 10 min after FceRI aggregation induced by the addition of Ag. FceRI-dependent ROS production rose steadily over 5 min after cell stimulation and reached a plateau after 10 min (Fig. 1B). Incubation of mBMMC with DPI caused a concentration-dependent inhibition in FceRI-dependent ROS production (p < 0.05 by ANOVA), which was significant at 10 µM (data not shown, n = 5 experiments, p < 0.01 by Student’s t test). These data suggest a NADPH oxidase enzyme could be partially responsible for FceRI-dependent...
FIGURE 2. 5-LO as the primary source of Ag induced intracellular ROS production in mast cells. Experiment was conducted as in Fig. 1 except mBMMC (A and B) and huMC (C and D) were incubated with the 5-LO inhibitors AA861 at 100 nM (thick dark gray line), 1000 nM (thick light gray line), or 10,000 nM (thin light gray line) (A and E) or nordihydroguaiaretic acid (NDGA) at 100 nM (thick dark gray line), 1000 nM (thick light gray line), 10,000 nM (thin light gray line) (B and F) or zileuton at 2 μM (thick dark gray line), 10 μM (thick light gray line), 20 μM (thin light gray line) (C and G), or the 5-LO-activating protein inhibitor MK886 at 10 nM (thick dark gray line), 100 nM (thick light gray line), 1000 nM (thin light gray line) (D and H) followed by addition of DNP-HSA (mBMMC) or streptavidin (huMC) (thick black line) or HEPES plus 0.04% BSA as control (thick black line). DCF fluorescence was monitored for a total of 15 min at 10-s intervals throughout the entire experiment. ROS production was also monitored in WT (I and K) and 5-LO-deficient (J and K) mBMMC following stimulation with increasing concentrations of...
ROS production in mast cells. To further examine this possibility, DCF fluorescence was monitored in mBMMC that were deficient in the major NADPH oxidase subunits gp91^phox^- or p47^phox^-deficient mBMMC, indicating that the effect of this metabolic inhibitor on mast cells at these concentrations is not related to inhibition of NADPH oxidase (data not shown). In similar experiments, FcεRI-mediated ROS production was completely abrogated by the antioxidants dihydrocaffeic acid (Fig. 1, D and E) or Trolox (Fig. 1, F and G) in both mBMMC (Fig. 1, D and F) and huMC (Fig. 1, E and G) further supporting the hypothesis that ROS are produced as a consequence of Ag stimulation in mast cells. These data taken together are consistent with the conclusion that ROS production by mast cells following FcεRI aggregation is NADPH oxidase-independent.

5-LO is the major enzyme responsible for FcεRI-dependent generation of ROS in mast cells, a minor role for COX-1

Because ROS production following FcεRI aggregation was NADPH oxidase-independent, we turned our attention to other ROS-producing enzymes present in mast cells, namely those involved in arachidonic acid metabolism. In both mBMMC and huMC, the 5-LO inhibitors AA861 (Fig. 2, A and E, respectively), nordihydroguaiaretic acid (Fig. 2, B and F, respectively), and zileuton (Fig. 2, C and G, respectively) reduced FcεRI-mediated DCF fluorescence in a concentration-dependent manner (p < 0.05 by ANOVA). This reduction by AA861 and nordihydroguaiaretic acid (1000 and 10,000 nM) and zileuton (20 μM) was statistically significant when total ROS production was calculated from AUC (data not shown, n = 3–6 donors per group, p < 0.05 by Student’s t test). The 5-LO-activating protein inhibitor MK886 was also used to investigate the requirement of 5-LO-activating protein in ROS production by mast cells. In mBMMC, MK886 caused a partial reduction in Ag-mediated ROS production (Fig. 2D), whereas it was ineffective in huMC (Fig. 2H). Therefore ROS generation following FcεRI aggregation was primarily lipoygenase-dependent.

To confirm these data regarding the effect of 5-LO inhibitors on FcεRI-mediated ROS production, we obtained mBMMC from 5-LO-deficient and WT mice and compared the DCF signal in response to increasing concentrations of Ag. The concentration-dependent increase in DCF fluorescence typically associated with WT mBMMC following FcεRI aggregation (Fig. 3J) was reduced (p < 0.05 by ANOVA) in COX-1-deficient mBMMC (Fig. 3K). These reduced ROS levels in COX-1-deficient mBMMC were significant when total ROS production was compared (p < 0.05 by Student’s t test) between the same Ag concentrations in WT mBMMC (Fig. 3L). However, the DCF response in COX-1-deficient mBMMC to Ag was never completely abrogated to baseline levels, suggesting the residual ROS was 5-LO-specific. These data unequivocally show that FcεRI aggregation leads to ROS production in mast cells, which is primarily 5-LO-dependent and partially COX-1-dependent.

FcεRI-dependent superoxide production by mast cells

DCF reacts with several oxidant species including H_2O_2 and hydroperoxides but not O_2^-•. Thus to determine whether O_2^-• were produced following FcεRI aggregation on mast cells we used DHE, which is a O_2^-•-specific intracellular probe, in combination with flow cytometry to provide a sensitive method for cell-specific O_2^-• production. We used this probe in addition to the nonspecific ROS probes DCF and DHR123, which detect hydroperoxides H_2O_2 and NO (not produced by mast cells). As shown in Fig. 4A following FcεRI aggregation on huMC and mBMMC (WT), a statistically significant increase in DHE fluorescence was observed (p < 0.05 by Student’s t test). This FcεRI-dependent increase in DHE fluorescence in mBMMC was absent in 5-LO-deficient mBMMC (p < 0.05 by Student’s t test) and partially reduced in COX-1-deficient mBMMC (p < 0.01 by Student’s t test), suggesting O_2^-• was derived from 5-LO and COX-1. Following FcεRI aggregation of DCF and DHR123 loaded cells, an increase in DCF and DHR123 fluorescence was observed in huMC and WT mBMMC (Fig. 4B), which is consistent with data obtained by microfluorimetry (Figs. 2 and 3). Furthermore, both the DCF and DHR123 signals were greatly reduced in the 5-LO-deficient...
mBMMC and partially reduced in the COX-1-deficient mBMMC compared with the WT mBMMC (Fig. 4B). These data are consistent with the conclusion that ROS produced by mast cells following FceRI aggregation, in addition to hydroperoxides and H$_2$O$_2$, include O$_2^-$ generated through 5-LO and COX-1.

Complete abrogation of ROS in WT and 5-LO-deficient mBMMC does not alter FceRI-dependent degranulation and cytokine secretion

Once the enzymes responsible for ROS production had been identified and the resulting ROS production could be fully inhibited, the next step was to determine the effect of ROS inhibition on mast cell secretory responses following FceRI aggregation because previously published data suggested a positive role in some responses (21). Initially, mBMMC were incubated with increasing concentrations of the COX-1 inhibitor FR122047 before Ag stimulation and levels of H$_2$O$_2$-hexosaminidase, LTs, PGs, and cytokines released into cell-free supernatants were determined. For WT mBMMC, a concentration-dependent partial reduction in ROS ($p < 0.05$ by ANOVA) was observed following incubation with FR122047 before FceRI aggregation (Fig. 5A, open histogram), but this had little effect on $\beta$-hexosaminidase, LTC$_4$, TNF-$\alpha$, or IL-6 release

FIGURE 3. Contribution of 5-LO and COX-1 in the production of ROS induced by Ag-stimulated mBMMC and huMC. Experiments were conducted as in Fig. 1 for measurement of ROS. mBMMC (A and B, D–F, and J–L) or huMC (C, and G–I) were incubated with increasing concentrations of FR122047 at 3 nM (thick black line), 30 nM (thick light gray line), 300 nM (thin light gray line) (A–C) or FR122047 alone at 300 nM (thick light gray line) (D–I), zileuton alone at 10 $\mu$M (thick dark gray line) (D and G), AA861 alone at 100 nM (E and H) or 1000 nM (F and I) (thick dark gray line) or FR122047 (300 nM) in combination with zileuton (10 $\mu$M) or AA861 (100–1000 nM) (thin light gray line) for 5 min before stimulation with DNP-HSA or streptavidin, respectively (thick black line), or HEPES plus 0.04% BSA as control (thin black line) for a further 10 min. DCF fluorescence was monitored for a total of 15 min at 10-s intervals throughout the entire experiment. Results shown are representative of three independent experiments performed in duplicate for WT mBMMC, 5-LO-deficient mBMMC, and huMC (A–I). ROS production was also monitored in WT and COX-1-deficient mBMMC following stimulation with increasing concentrations of DNP-HSA and shown as representative kinetic curves of six independent experiments performed in duplicate (J and K) or averaged AUC data calculated from individual kinetic curves and shown as mean ± SE ($n = 6$ experiments in duplicate) (L). Statistical significance for inhibitor (A–I) or Ag (J–L) concentration response curves was determined by ANOVA from calculated AUC data. If statistical significance was achieved following ANOVA ($p < 0.05$), differences between individual groups within inhibitor curves (WT or 5-LO-deficient mBMMC and huMC) or between WT and COX-1-deficient mBMMC Ag responses was determined by Student’s $t$ test. †, $p < 0.05$ and ‡, $p < 0.05$ for comparison with Ag between WT and COX-1-deficient mBMMC.
FR122047 caused a complete inhibition (p < 0.001 by ANOVA) of Ag-dependent PGD₂ release (Fig. 5 D). In 5-LO-deficient mBMMC incubated with FR122047, complete abrogation of Ag-mediated ROS production was observed (p < 0.001 by ANOVA) at the highest concentration tested (300 nM; p < 0.01 by Student’s t test) (Fig. 5 A, gray histogram). However, no effect on -hexosaminidase, TNF- or IL-6 release was observed (Fig. 5, B, E, and F, respectively). As expected, LTC₄ could not be detected in 5-LO-deficient mBMMC (Fig. 5 C), whereas PGD₂ levels were enhanced compared with WT mBMMC; furthermore, FR122047 incubation caused complete blockade of PGD₂ levels (Fig. 5 D). When directly comparing WT and 5-LO-deficient mBMMC secretory responses, complete abrogation of ROS production in 5-LO-deficient mBMMC caused no change in LTC₄, TNF-α, or IL-6 release, whereas differences in LTC₄ and PGD₂ levels were observed even though cells had comparable levels of FcεRI bound IgE (data not shown). Taken together, these data suggest that even though ROS are generated following FcεRI aggregation in mBMMC, they are not required for degranulation or cytokine secretion.

To support our conclusion that ROS have a minimal participation in mast cell secretory responses, we next incubated WT mBMMC or huMC with 5-LO and COX-1 inhibitors alone or in combination to assess the effect of varied levels of ROS on mast cell secretory responses including β-hexosaminidase, LTs, PGs, and cytokines. As shown in Fig. 6, WT mBMMC incubated with the 5-LO inhibitors zileuton, AA861 or the COX-1 inhibitor FR122047 (Fig. 6 A) caused partial reduction in ROS production, whereas a combination of 5-LO and COX-1 inhibition completely abrogated ROS levels (Fig. 6 A). However, when ROS production was absent no effect on -hexosaminidase release (Fig. 6 B) and TNF- or IL-6 secretion was observed (Fig. 6, E and F, respectively) but LTC₄ and PGD₂ secretion was fully inhibited (Fig. 6, C and D, respectively). Note that the mean TNF-α production reported in Fig. 6 E is lower than TNF-α mean presented in Fig. 5 E, but within the range of TNF-α data averaged for Fig. 5 E. We attribute this variation to reported differences in measurements of TNF-α levels possibly due to differences in mast cell cultures, which has also been noted by others (26 – 28). These data were similar for huMC (n = 3 donors) (data not shown). These results further demonstrate that ROS generated as a consequence of FcεRI aggregation do not participate in mast cell secretory responses.

Selective phagocytic stimuli induce ROS production in mast cells

Because 5-LO and COX-1 were responsible for ROS generation following FcεRI aggregation, we next assessed whether mast cells exclusively used these enzymes in response to stimuli known to
induce ROS in other cell types. Phagocytic cells such as PMN primarily use NADPH oxidase to generate ROS and this enzyme can be activated by soluble and/or particulate stimuli including PMA, fMLP, zymosan, and IgG-LB. Therefore, we tested the ability of mast cells to generate ROS in response to these stimuli and assessed whether the resultant ROS were derived from the 5-LO and COX-1 pathway.

mBMMC or huMC were incubated with the ROS-sensitive probe DCF before stimulation with PMA (1000 nM), fMLP (1 μM), zymosan (1/20), IgG-LB (1/20), or ionomycin (300 nM) as a positive control. ROS generation was then monitored for 60 min. As shown in Fig. 7, mBMMC generated low levels of ROS in response to zymosan and IgG-LB, whereas PMA and fMLP were ineffective (Fig. 7A). The lack of responsiveness to fMLP was not surprising because mast cells do not express the fMLP receptor (29, 30). However, the response to zymosan and IgG-LB was variable and overall negligible ROS were detected. In contrast, huMC stimulated with IgG-LB but not zymosan, fMLP or PMA generated low levels of ROS (Fig. 7B) and this phenomenon was reproducible with cells from several donors (data not shown). The levels of ROS generated by huMC were less than observed in PMN, which generated high levels in response to PMA or IgG-LB and low levels in response to fMLP and ionomycin (Fig. 7C). However, the rate of ionomycin-induced ROS production in PMN was reduced compared with huMC (Fig. 7, B and C).

Because it is well documented that NADPH oxidase activity can be enhanced following exposure of PMN to various cytokines and bacterial products including IFN-γ, GM-CSF, TNF-α, and LPS, we next investigated whether ROS generation could be induced in mBMMC by the same stimuli following pre-exposure to IFN-γ, GM-CSF, and LPS for 1–24 h. However, no ROS production was observed with stimulation alone or following pre-exposure to these cytokines or LPS (n = 2) (data not shown). When huMC were pre-exposed to IFN-γ or LPS for 1–24 h no increase in ROS production was observed following stimulation with PMA, fMLP, or zymosan (n = 2) (data not shown). However, when huMC were preincubated with IFN-γ (24 h) before IgG-LB stimulation, an increase in ROS production was observed (Fig. 8A). When AUC data were calculated over replicate donors, a significant increase in ROS production was observed in the presence (p < 0.01 by Student’s t test) or absence (p < 0.05 by Student’s t test) of IFN-γ following IgG-LB stimulation and the enhancing effect of IFN-γ was also significant (p < 0.01 by Student’s t test) (Fig. 8B).

We next sought to determine whether the enzyme responsible for the enhanced ROS generation following IgG-LB stimulation was NADPH oxidase, 5-LO, or COX-1. huMC were pretreated with IFN-γ for 24 h, preloaded with DCF and added to a plate containing increasing concentrations of the flavoenzyme inhibitor DPI (1–10 μM), 5-LO inhibitors zileuton (2–20 μM), or AA861 (0.1–10 μM), or the COX-1 inhibitor FR122047 (3–300 nM) before IgG-LB stimulation. PMN were used as a positive control in all experiments and were also pretreated with inhibitors. As shown in Fig. 9, there was no effect of DPI on IFN-γ-primed IgG-LB induced ROS production in huMC (Fig. 9A), whereas FR122047 (Fig. 9D) or zileuton (Fig. 9B) partially inhibited and AA861 completely abrogated (p < 0.05 by ANOVA) IgG-LB-induced ROS production (Fig. 9C). As expected, DPI completely abrogated IgG-LB-dependent ROS production in PMN at all concentrations tested (Fig. 9E), whereas the 5-LO and COX-1 inhibitors were without effect (Fig. 9, F–H). These data demonstrate that mast cells use 5-LO and COX-1, whereas PMN use NADPH oxidase in the generation of ROS following IgG-LB stimulation.

FIGURE 5. Effect of a COX-1 inhibitor on FceRI-dependent secretory responses in WT and 5-LO-deficient mBMMC. IgE-sensitized WT or 5-LO-deficient mBMMC were pretreated with FR122047 (3–300 nM) before stimulation with DNP-HSA (30 ng/ml). Total ROS production was determined by DCF fluorescence before calculation of AUC over 10 min (A). Cell-free supernatants were collected and quantified for net β-hexosaminidase after 30 min (B), LTC4 (C) or PGD2 (D) after 20 min by competitive enzyme immunoassay, or TNF-α (E) or IL-6 (F) after 4 h by ELISA. Note the average measurements for TNF-α data of individual experiments in WT mBMMC shown as 455 ± 251 varied from 124 to 1440 pg/ml. Results are the mean ± SE for n = 3–5 experiments in duplicate. Differences in individual concentration response curves were determined by ANOVA. Following ANOVA, differences between individual groups within single concentration response curves were determined by Student’s t test. +, p < 0.05 and ++, p < 0.01 for comparison with Ag alone; #, p < 0.05 and **, p < 0.01 for comparison between WT and 5-LO-deficient mBMMC.
Discussion

In this study we have demonstrated that FcεRI aggregation on both human and mouse mast cells leads to ROS generation derived from the 5-LO and COX enzymes. Blockade of FcεRI-dependent ROS generation had negligible effects on mast cell secretory responses including degranulation and cytokine secretion but, as expected, completely abrogated PG and LT secretion. This is consistent with the hypothesis that 5-LO and COX-1 are the enzymatic sources of ROS in mast cells. In addition, mast cells also generated ROS following IgG-LB stimulation, which was enhanced by incubation with IFN-γ. IgG-LB dependent ROS production in huMC was derived from 5-LO and COX-1 and not NADPH oxidase. This was in sharp contrast to PMN where IgG-LB mediated ROS generation was exclusively through the activity of NADPH oxidase.

Our previous results (20) and those of others (22, 31-33) have revealed an inhibitory effect of the flavoenzyme inhibitor DPI on FcεRI-dependent ROS production, suggesting the involvement of a NADPH oxidase isofrom. At concentrations of 10–30 μM, DPI partially reduced ROS production in mast cells (22, 33), which is in agreement with data presented in this study. However, complete inhibition of ROS by DPI was only observed at 100–200 μM in these other studies (22, 33). These DPI concentrations are one hundred times higher than those required to inhibit NADPH oxidase activity in PMN (1–2 μM) (Fig. 8E) and as such may reflect nonspecific effects. In support of a minimal role for NADPH oxidase in FcεRI-dependent ROS production, WT, gp91phox- and p47phox-deficient mBMMC had negligible differences in ROS production following Ag stimulation. Furthermore, residual ROS levels in 5-LO-deficient mBMMC were not affected by DPI incubation. In support of a role for 5-LO and COX-1 in FcεRI-dependent ROS production, other studies (22, 33) showed that this response was inhibited by the phospholipid hydroperoxide glutathione peroxidase (PHGP) mimetic ebselen, which is a potent inhibitor of 5-LO (34-37) and COX activity (37). In addition, overexpression of PHGP in RBL-2H3 cells reduced hydroperoxide, LTC4, and PGD2 release following Ag stimulation (38, 39), and arachidonic acid pretreatment enhanced FcεRI-dependent ROS production (40). On the basis of the inhibitory effect of DPI and the ROS scavenging activity of ebselen, previous studies speculated the enzyme responsible for ROS production in mast cells was a NADPH oxidase isofrom (22, 33). Data presented in this study are consistent with the conclusion that the enzyme primarily responsible for the production of ROS following FcεRI aggregation in mast cells is 5-LO with a partial role of COX-1.
There are several possible species of oxidant generated following FcγRI aggregation in mast cells, including most notably hydroperoxides, H₂O₂, and O₂•⁻ (45–47) during the course of PG synthesis. In the presence of superoxide dismutase, 5-LO- and COX-1-dependent O₂•⁻ production could yield H₂O₂ and therefore both hydroperoxides and H₂O₂ could contribute equally to ROS levels in mast cells. In support of this conclusion, the probe used to detect ROS, namely DCF diacetate, can detect both H₂O₂ (25) and hydroperoxides (50). Also antioxidant enzymes including PHGP can scavenge both H₂O₂ and hydroperoxides (51). The increase in DHE fluorescence by huMC and mBMMC following FcγRI aggregation is consistent with the conclusion that O₂•⁻ was produced. Additionally, analysis of intracellular H₂O₂ by the amplex red assay yielded little production of H₂O₂ by mast cells following Ag stimulation. In summary we propose that the principal oxidants generated by mast cells following FcγRI aggregation are O₂•⁻, hydroperoxides and H₂O₂. The probe used to detect intracellular oxidants in this study was DCF diacetate, which is known to react with H₂O₂, hydroperoxides and NO. There are concerns associated with this probe despite its universal use to detect ROS in that the reaction with oxidants can be accelerated in the presence of peroxidases and fluorescence can be observed in the absence of ROS production. In our study the primary enzymes responsible for FcγRI-dependent ROS production (detected by DCF fluorescence) were primarily 5-LO with a minor role for COX-1. Because the activity of these enzymes and subsequent hydroperoxide generation is dependent on peroxidases the likelihood of DCF fluorescence monitoring both reactions is a possibility. However, using enzyme preparations of 5-LO no increase in DCF fluorescence was observed by others (52) consistent with the conclusion that the enzyme itself does not induce DCF.

![Figure 7](image7.png)  
**FIGURE 7.** Effect of NADPH oxidase stimulants on mBMMC, huMC and PMN ROS generation. WT mBMMC (A), huMC (B), or PMN (C) were incubated with PMA (1000 nM) (thick dark gray line), IMLP (1000 nM) (dotted light gray line), zymosan (1/20) (thin black line), IgG-LB (1/20) (thick light gray line), ionomycin (300 nM) (thick black line) as a positive control or HEPES buffer as a negative control (thick black line). ROS production was then measured by DCF fluorescence for 60 min at 30-s intervals at 37°C. Results are shown as a single experiment and are representative of three separate experiments performed in duplicate.

![Figure 8](image8.png)  
**FIGURE 8.** Effect of IFN-γ on IgG-LB induced ROS generation in huMC. HuMC were incubated in the presence or absence of IFN-γ (20 ng/ml) for 24 h before stimulation with IgG-LB (1/20) (thick black line) or HEPES buffer as a negative control (thin black line). ROS production was then measured by DCF fluorescence for 60 min at 30-s intervals at 37°C. Results are shown as single kinetic curves and are representative of two experiments performed in duplicate on five different huMC donors (A) or AUC data calculated for each kinetic curve after baseline subtraction and expressed as the mean ± SE of n = 5 donors (B). Differences between individual groups were determined by Student’s t test. *p < 0.05 and ++, p < 0.01.
were reduced, supporting the hypothesis that the enzymes involved in arachidonic acid metabolism are the source of ROS in mast cells. Results presented in this study support our previous data (20) showing no effect of intracellular ROS on degranulation of mBMMC but does not support the huMC data. This maybe due to donor-to-donor variability of CD34+ blood cells. In data not shown, incubation of mBMMC and huMC with DPI before Ag stimulation had no effect on degranulation. Previous work by others (22, 31–33) showed DPI (3–33 μM) partially reduced FcεRI-dependent degranulation (22, 31–33), but complete inhibition was only achieved at higher concentrations of 100–200 μM (22, 31, 32). These higher concentrations may have nonspecific effects with global inhibition of enzymes involved in many aspects of mast cell biology. Also the sources of mast cells and culture conditions may explain apparent differences between our results and those of others regarding the role of ROS in Ag-mediated secretory responses (22, 31–33). By specifically reducing FcεRI-dependent ROS production in mast cells using a combination of 5-LO and COX-1 inhibitors or genetic deletion of 5-LO, no effect of intracellular ROS on mast cell degranulation was observed in the current study. Reduction of 5-LO activity by the PHGP mimetic ebselen (22, 33) or using specific 5-LO and COX-1 inhibitors (53) has minimal effects on mast cell degranulation. In another study using mBMMC deficient in the antioxidant enzyme thioredoxin, Ag-mediated degranulation was reduced compared with WT mBMMC (54). These data appear to contradict our current data on degranulation and may involve differences in localization of the antioxidant enzyme. However, the same study found no differences in FcεRI-dependent TNF-α or IL-6 secretion, which supports our hypothesis regarding the lack of a role for ROS in cytokine secretion. ROS derived from 5-LO are involved in physiological responses in other cell types, including TNF-α-mediated ROS generation (55) and senescence-like growth arrest (56) in fibroblasts and mitogenic signaling in keratinocytes (57). Although 5-LO- and COX-1-dependent ROS did not modulate mast cell secretory responses investigated in this study, we cannot rule out the possibility that other responses of mast cells will be altered. Generation of arachidonic acid metabolites by the action of 5-LO and other enzymes occurs at the inner nuclear membrane in mast cells (58) and this suggests novel roles of these mediators and ROS derived from 5-LO at this level. Future studies will elucidate the functional role of 5-LO-mediated ROS production in mast cells.

In this study, we demonstrate that huMC generate ROS following stimulation with IgG-LB which was enhanced by pre-exposure to IFN-γ. The production of ROS was 5-LO- and COX-1-dependent and NADPH oxidase-independent. This was in sharp contrast to PMN, in which the primary enzyme responsible for ROS production was NADPH oxidase. In addition, huMC failed to generate appreciable levels of ROS following stimulation with other NADPH oxidase activating agents including fMLP, PMA, and zymosan. Previous studies have shown mast cells are capable of generating ROS following activation with other phagocytic stimuli including bacteria (17) and silica (59). In the case of silica 5-LO was partially responsible for the observed ROS (59). With regard to the enhancing effect of IFN-γ on IgG-LB-dependent ROS generation, previous work in our laboratory demonstrated the induction of FcγRI expression in huMC pretreated with IFN-γ and the resulting production of LTC4 and PGD2 in response to FcγRI cross-linking (60, 61). Furthermore, agents that are known to prime NADPH oxidase activity in PMN, including LPS and IFN-γ, are also known to increase 5-LO expression and activity (43). These studies support the hypothesis that IFN-γ induces the expression of FcγR and 5-LO in huMC, which results in increased

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**FIGURE 9.** Effect of enzyme inhibitors on IgG-LB induced ROS generation in huMC primed with IFN-γ and PMN. HuMC pretreated with IFN-γ (20 ng/ml) for 24 h (A–D) or PMN (E–H) were stimulated with IgG-LB (1/20) or HEPES buffer as a negative control in the presence of increasing concentrations of DPI (1–10 μM) (A and E), zileuton (2–20 μM) (B and F), AA861 (100–10,000 nM) (C and G), or FR122047 (3–300 nM) (D and H). DCF fluorescence was monitored for 60 min at 30-s intervals at 37°C. Results shown are the mean ± SE of n = 3–4 donors. Differences in individual concentration response curves were determined by ANOVA. Following ANOVA, differences between individual groups within single concentration response curves were determined by Student’s t test. +, p < 0.05 and ++, p < 0.01 for comparison with IgG-LB alone.

Arachidonic acid-mediated COX-1 activity does induce DCF fluorescence in presence of phenol, to enhance the signal, but this could be an indication of hydroperoxide and peroxidase activity (52). Incubation of BMMC with the peroxidase inhibitor sodium azide before incorporation in the DCF assay caused no reduction in ionomycin-dependent DCF fluorescence (data not shown), suggesting the DCF signal is not a result of peroxidases activity but of ROS production. These data are consistent with the conclusion that DCF fluorescence occurred independent of peroxidase activity.

Intracellular ROS production following Ag stimulation in mast cells does not influence subsequent degranulation and cytokine secretion. Incubation of mBMMC or huMC with 5-LO and COX-1 inhibitors at concentrations that completely abrogated ROS generation had no effect on degranulation and cytokine secretion following stimulation with Ag. As expected LT and PG production fluorescence. Arachidonic acid-mediated COX-1 activity does induce DCF fluorescence in presence of phenol, to enhance the signal, but this could be an indication of hydroperoxide and peroxidase activity (52). Incubation of BMMC with the peroxidase inhibitor sodium azide before incorporation in the DCF assay caused no reduction in ionomycin-dependent DCF fluorescence (data not shown), suggesting the DCF signal is not a result of peroxidases activity but of ROS production. These data are consistent with the conclusion that DCF fluorescence occurred independent of peroxidase activity.

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responsiveness to IgG-LB thereby leading to enhanced ROS production derived from 5-LO and COX-1. We have preliminary unpublished data that demonstrate phagocytosis of bacteria by mast cells also generates ROS via a 5-LO- and COX-dependent mechanism.

In PMN the primary ROS producing enzyme activated by phagocytic stimuli is NADPH oxidase (1), but there are reports of phospholipase A2 and 5-LO activation leading to subsequent LT production in these cells (62, 63). In addition, IFN-γ pre-treatment can enhance 5-LO-dependent product formation in macrophages (64, 65). This suggests both the NADPH oxidase and 5-LO pathways are activated by similar stimuli in phagocytes and there are several reports of arachidonic acid modulating NADPH oxidase dependent ROS production in PMN (66–68). Using phospholipase A2 inhibitors, a reduction in NADPH oxidase-dependent ROS production was observed with no direct effect on NADPH oxidase enzyme activity itself (67). However, a critical study using a specific phospholipase A2a inhibitor pyroliidine-1 in PMN and phospholipase A2a-deficient mouse neutrophils showed no differences ROS production induced by opsonized zymosan, JMLP, or PMA (68–70). Therefore, in professional phagocytes both NADPH oxidase and phospholipase A2 can be activated but ROS are only generated by NADPH oxidase. However, mast cells appear to exclusively derive all ROS from the 5-LO and COX-1 pathways following a phagocytic stimulus, and the role played by these ROS in host defense remains to be determined.

In summary, mBMMC and huMC generate ROS in response to FcεRI aggregation and IgG-LB stimulation through the activity of 5-LO and COX-1 enzymes but not NADPH oxidase. In terms of FcεRI aggregation inhibition of ROS had negligible effects on degranulation and cytokine secretion while having concurrent inhibition on LTC4 and PGD2 production consistent with the hypothesis that 5-LO and COX-1 were the enzymes responsible for ROS production in mast cells. The source of IgG-LB induced ROS production in huMC was in sharp contrast to that of PMN, which primarily use NADPH oxidase for ROS generation. Overall our data demonstrate that mast cells exclusively use 5-LO and COX-1 for the generation of ROS.

Disclosures
The authors have no financial conflict of interest.

References


