Published in final edited form as:
*Mol Immunol*. 2009 August ; 46(13): 2505–2514. doi:10.1016/j.molimm.2009.05.023.

# Ragweed pollen-mediated IgE-independent release of biogenic amines from mast cells via induction of mitochondrial dysfunction

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## Abstract

Normal functions of mitochondria are required for physiological dynamics of cells, while their dysfunction contributes to development of various disorders including those of immune system. Here we demonstrate that exposure of mast cells to ragweed pollen extract increases production of $H_2O_2$ via mitochondrial respiratory complex III. These mitochondrial ROS (mtROS) enhance secretion of histamine and serotonin from mast cells, but not enzymes such as β-hexosaminidase, independently from FcεRI-generated stimuli. The release of biogenic amines is associated with inhibition of secretory granules' $H^+$-ATPase activity, activation of PKC-δ and microtubule-dependent motility, and it is independent from intracellular free $Ca^{2+}$ levels. To asses differences from IgE-mediated mast cell degranulation we show that mtROS decrease antigen-triggered β-hexosaminidase release, while they are synergistic with antigen-induced IL-4 production in sensitized cells. Taken together, these data indicate that mitochondrial dysfunction can act independently from adaptive immunity, as well as augments Th2-type responses. Pharmacological maintenance of physiological mitochondrial function could have clinical benefits in prevention and treatment of allergic diseases.

### Keywords

Mast cells; Mitochondria; Degranulation; Reactive oxygen species



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## 1. Introduction

Mast cells are one of the most important effectors involved in elicitation of allergic inflammation and immune responses to many pathogens including parasites (Metcalfe et al., 1997). Antigenic activation of mast cells via the high-affinity receptor for IgE (FcεRI) mediates exocytosis of cytoplasmic granules containing preformed mediators, secretion of lipid-derived factors, and *de novo* synthesis of cytokines, chemokines and growth factors (Burgoyne and Morgan, 2003; Logan et al., 2003; Metcalfe et al., 1997; Rivera and Gilfillan, 2006). In addition to FcεRI-mediated signals, exposure to a variety of stimuli can lead to the release of mast cell mediators (Frossi et al., 2004). Pathogen-associated molecules may activate mast cells and basophils via receptors selectively expressed on their surfaces (Kojima et al., 2007). Eosinophil-derived major basic protein, compound 48/80 or substance P also induces degranulation of mast cells (Munitz et al., 2003). Several lines of evidence indicate that oxidative stress is also a stimulus for mast cell activation (Frossi et al., 2003; Ohmori et al., 1979; Swindle et al., 2002). During allergic and other inflammatory reactions mast cells are exposed to an oxidative microenvironment because ROS are produced by various cell types in the peripheral tissues as a consequence of their effector function (Nagata, 2005). We have previously reported that pollen grains, sub-pollen particles, and pollen extracts contain intrinsic NAD(P)H oxidases, which generate ROS [superoxide anions ($O_2^{\cdot -}$)] (Bacsi et al., 2006a; Boldogh et al., 2005). These radicals induce oxidative stress in cultured cells, as well as in airway and conjunctival epithelium within minutes of exposure (Bacsi et al., 2005; Boldogh et al., 2005).

There is a close correlation between the exclusively maternal inheritance of mitochondria and the fact that maternal history of atopy and asthma is one of the substantial risk factors for the development of asthma in children (Litonjua et al., 1998). A mitochondrial haplogroup has been shown to be associated with elevated total serum IgE levels in asthmatic patients (Raby et al., 2007). Oxidative stress and mitochondrial metabolism are involved in antigen-induced release of mast cell mediators, including IL-4, which is essential for naive T cell polarization toward Th2 phenotype (Frossi et al., 2003; Inoue et al., 2008). Studies with metabolic inhibitors have demonstrated a close link between mitochondrial energy production and mast cell degranulation (Johansen, 1987). Furthermore, release of $Ca^{2+}$ from mitochondria is involved in antigen-induced mast cell degranulation (Suzuki et al., 2006).

Here we report for the first time that treatment with short ragweed (*Ambrosia artemisiifolia*) pollen extract (RWE) induces elevated mitochondrial ROS production in non-sensitized RBL-2H3 cells, a model of mucosal mast cells (Park and Beaven, 2009; Seldin et al., 1985). We show that increased production of ROS from mitochondrial respiratory complex III, but not intrinsic pollen NAD(P)H oxidase-generated ROS directly, enhances secretion of histamine and serotonin from non-sensitized mast cells. Mitochondrial ROS trigger the release of biogenic amines, but not enzymes such as β-hexosaminidase, via inducing PKC δ- and microtubule-dependent motility of secretory granules and inhibiting activity of vacuolar $H^+$-ATPase independently from intracellular $Ca^{2+}$ levels. We demonstrate that mtROS are also able to enhance FcεRI-mediated IL-4 production of mast cells. These findings may shed light on a new role for mitochondrial dysfunction in the regulation of mast cell activation.

## 2. Materials and methods

### 2.1. Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

### 2.2. Cell cultures

The RBL-2H3 cells were obtained from the American Type Cell Collection and cultured at 37°C in a humidified atmosphere with 5% $CO_2$ in Minimum Essential Medium containing Earle's salts and L-glutamine (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml).

### 2.3. Analysis of inflammatory mediator release

Cells were treated with RWE (Greer Laboratories, Lenoir, NC) at the indicated concentrations for 1 h at 37°C. To induce mitochondrial ROS generation, cells were incubated with antimycin A, an inhibitor of complex III. Mitochondrial ROS-induced inflammatory mediator release was determined in the presence or absence of $Ca^{2+}$ or antigenic stimuli. For antigenic stimulation, cells were first sensitized with mouse IgE anti-DNP (0.1 μg/ml; Sigma, clone SPE-7) for 1 h at 37°C and then treated with antigen (DNP-BSA; 50 ng/ml). Collected supernatants were centrifuged at 400 × *g* in 4°C to remove any remaining cells. Histamine secretion was determined according to Shore's method (Alfonso et al., 2000). The fluorescence was measured in an Flx800 microplate fluorescence reader at 360/460 nm.

The release of radioactively-labeled serotonin ([$^3$H]-serotonin) was measured as previously described (Isersky et al., 1978). Briefly, 1.5 × $10^4$ cells per well (96-well plates) in 100 μl of culture medium were incubated with 1 μCi/ml of [$^3$H]-serotonin for 18 h at 37°C and 5% $CO_2$. Labeled cells were washed with pre-warmed (37°C) assay buffer, HBSS containing 0.1% BSA. Cells were then further incubated for 30 min at 37°C. Radioactivity in the supernatant fluids was determined by scintillation spectroscopy (Beckman Coulter, Fullerton, CA). Results are expressed as a fraction of analyzed mediator concentration in the supernatant with respect to its total content in a corresponding number of non-treated cells.

Release of β-hexosaminidase was assayed fluorimetrically with 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (MUNAG) (Demo et al., 1999). Briefly, equal volumes (25 μl) of supernatant and substrate solution (2 mM MUNAG in 0.2 M citric buffer, pH 4.5) were added to wells of a 96-well plate, and the enzymatic reaction was developed for 30 min at 37°C and terminated with 100 μl of 1 M $Na_2CO_3$ solution (pH 10). The fluorescence of released 4-methylumbelliferone was measured in an Flx800 microplate fluorescence reader at 360/460 nm. Results were further confirmed by measuring the β-hexosaminidase enzymatic activity spectrophotometrically using 4-nitrophenyl-N-acetyl-β-D-glucosamine as substrate (Suzuki et al., 2006).

### 2.4. Isolation of mitochondria

Mitochondria were isolated and purified as we had previously described (Bacsi et al., 2006b). Briefly, cell pellets were incubated in a hypotonic buffer A (220 mM mannitol, 70 mM sucrose, 2 mM MOPS and 1 mM EGTA, pH 7.4) containing protease inhibitor cocktail (catalog No. P8340, Sigma). Cell suspensions kept in ice bath were sonicated with a Branson sonifier by 4 pulses of 20 % power and cell homogenates were centrifuged for 10 min at 4700 × *g*. Mitochondria were sedimented from supernatants by centrifugation at 7168 × *g*. Pellets were resuspended in buffer B (220 mM mannitol, 70 mM sucrose, 2 mM MOPS, pH 7.4) and centrifuged at 9072 × *g*. Crude mitochondrial solutions were layered on discontinuous sucrose gradients (1.5, 1.0 and 0.5 M sucrose in 10 mM MOPS and 1 mM EDTA, pH 7.4) and ultracentrifuged for 1.5 h at 82705 × *g* (SW28 rotor, Beckman Coulter, CA). All centrifugation procedures were carried out at 4°C. The band containing mitochondria was removed and washed in 10 times volume of buffer B. Mitochondrial pellets were resuspended in buffer B containing protease inhibitor cocktail (Sigma).

### 2.5. Assessment of ROS

To measure the release of $H_2O_2$ from mitochondria, we used Amplex® Red (Molecular Probes) assay as we previously described (Bacsi et al., 2006b). Briefly, mitochondria (100 μg/ml) were suspended in 50 μl (per well) reaction buffer and incubated with 0.25 U/ml Amplex® Red and 1 U/ml of HRP at 25°C for 30 min. The changes in fluorescence intensity were measured using a microplate reader (SpectraMass M2, Molecular Devices, Sunnyvale, CA) at 530/590 nm.

Changes in intracellular ROS levels were determined by 2',7'-dichlorodihydro-fluorescein diacetate ($H_2$DCF-DA; Molecular Probes, Invitrogen, Carslbad, CA) as we previously described (Bacsi et al., 2006b; Boldogh et al., 2005). Changes in DCF fluorescence intensity were determined using an Flx800 microplate fluorescence reader (BioTek Instruments, Winooski, VT) at 485 nm excitation and 528 nm emission.

### 2.6. Assessment of V-ATPase activity

Secretory granule fraction from RBL-2H3 cells was collected as it has previously been described (Lindstedt and Kovanen, 2006). ATP hydrolysis of vacuolar membranes was assayed by using an ATP determination kit (Molecular Probes) (Ha and Snyder, 1999). Secretory granules in ATPase buffer (Lu et al., 2002) were preincubated for 15 min at room temperature in the presence and absence of bafilomycin A. The reaction was initiated by addition of ATP at a final concentration of 2 mM and luminescence was measured using an Flx800 reader by adding 90 μl of luciferase reagent to 10 μl of sample. ATP values were calculated from the standard curve based on a series of ATP concentrations.

### 2.7. Annexin V binding assay

Fusion of secretory granules with the cytoplasmic membrane was determined as previously described (Demo et al., 1999). Briefly, stimulated cells ($7.5 \times 10^5$/dish) were washed with HBSS and stained with 25 μl of Annexin V-Phycoerythrin (PE) in the manufacturer's 1 × binding buffer (Annexin V-PE Apoptosis Detection Kit I, Becton Dickinson, San Jose, CA) for 15 min. Fluorescence was analyzed directly by a FACSCanto™ Flow Cytometer (BD Becton Dickinson). A minimum of 15,000 cells per sample was acquired.

### 2.8. Assessment of pH in acidic cellular compartments

Cells adhered to cloverslips were loaded with 5 μg/ml acridine orange for 10 min at 37°C and treated with AA (10 μM) or bafilomycin A (100 nM), a specific inhibitor of vacuolar ATPases. Acridine orange fluorescence was visualized by a NIKON Eclipse TE 200 UV microscope (Lewisville, TX). Images were taken with a Photometrix CoolSNAP Fx digital camera using Metamorph software (version 6.09; Universal Imaging, Downingtown, PA).

In parallel experiments, changes in acridine orange-mediated fluorescence intensity at 10 min upon treatment were assessed in an FLx800 micro plate reader (Bio-Tek Instruments, Winooski, VT) at 485 nm excitation and 528 nm emission.

### 2.9. Measurement of extracellular IL-4 levels

Levels of IL-4 were measured in supernatant fluids of plated cells 5 h after stimulation using ELISA CytoSet™ from BioSource International (Camarillo, CA) specific for rat IL-4 according to manufacturer's instructions.

### 2.10. Statistical analysis

Data were analyzed by analysis of variance with *post hoc* tests: Bonferroni's (samples with equal variances) and Dunnett's T3 (samples with unequal variances) with SPSS 14.0 software. Differences were considered to be statistically significant at $P < 0.05$.

## 3. Results

### 3.1. RWE induces the release of biogenic amines in an IgE-independent manner

Previous studies have demonstrated that exposure to a variety of non-antigenic stimuli leads to the activation of mast cells (Frossi et al., 2004; Stassen et al., 2002; Yoshimaru et al., 2006). Here we investigated whether RWE induces degranulation of RBL-2H3 cells in the absence of sensitization with IgE antibodies. Treatment of the cells with RWE induced the release of histamine in a dose-dependent manner (Fig 1A). Administration of RWE (100 μg/ml) to the cells also increased the secretion of serotonin (Fig. 1B); however, it did not change the level of the β-hexosaminidase (data not shown). Elimination of NAD(P)H oxidase activity from RWE by heat-treatment (10 min at 72°C) (Boldogh et al., 2005), or pre-treatment of the cells with the antioxidant N-acetyl-$_L$-cysteine (10 mM), abrogated the effect of RWE on the biogenic amine release (Fig. 1B). Unexpectedly, inhibitors of mitochondrial complex I (10 μM rotenone, an inhibitor of NADH-decylubiquinone reductase activity) or complex II (3 mM 3-nitropropionic acid, a succinate dehydrogenase inhibitor) significantly ($p<0.05$) decreased the release of serotonin from RWE-treated cells (Fig. 1B). Combined use of rotenone and 3-nitropropionic acid lowered the serotonin secretion to the basal level (Fig. 1B). These inhibitors block ROS generation only from mitochondria, suggesting that mtROS [but not pollen NAD(P)H oxidase-generated ROS directly] are implicated in the biogenic amine release.

### 3.2. Mitochondrial ROS induce histamine and serotonin release

Oxidative stress has been shown to induce mitochondrial dysfunction leading to elevated mitochondrial $O_2^{\cdot -}$ production (Xia et al., 2007), which is rapidly converted to $H_2O_2$ by enzymatic or non-enzymatic pathways (Loschen et al., 1974). To test whether exposure of the cells to RWE leads to mitochondrial dysfunction, mitochondria were isolated from RWE-treated and mock-treated cells 60 min after exposure and changes in their $H_2O_2$ production were determined. As shown in Fig. 2, mitochondria from RWE-treated cells released significantly ($p<0.05$) higher amounts of $H_2O_2$ than those from mock-treated cells, suggesting that exposure to RWE enhances mtROS generation in the RBL-2H3 cells and mtROS may be involved in the release of biogenic amines.

Pollen grains and their extracts contain several compounds, which can alter the function of human cells (Bagarozzi et al., 1998; Gunawan et al., 2008; Traidl-Hoffmann et al., 2002). To evaluate if increases in mtROS levels are the cause of the release of biogenic amines (histamine and serotonin), cells were treated with 10 μM of antimycin A (AA, an inhibitor of cytochrome *b* reoxidation), which generates mtROS by blocking electron transport at complex III resulting in mitochondrial dysfunction (Bacsi et al., 2006b; Panduri et al., 2004). Elevated mtROS levels induced a significant ($p<0.05$) increase in the extracellular histamine (from 4.9%±0.2 to 23.0%±0.2) and serotonin (from 3.4%±0.9 to 23.4%±4.3) levels (Fig. 3A and B). Mitochondrial ROS did not further enhance antigen-induced histamine or serotonin release from sensitized cells (Fig. 3A and B). Since cell loading with radio-labeled serotonin was shown to change the properties of secretory granules (Pihel et al., 1998), we investigated the effect of mtROS on histamine release in detail. Time course experiments showed that a statistically significant ($p<0.05$) increase in histamine secretion correlated well with the kinetics of increases in intracellular ROS levels (Fig. 3C and D).

### 3.3. Inhibition of mtROS generation abolishes the release of histamine

Addition of AA increased the release of $H_2O_2$ from isolated mitochondria respiring from pyruvate+malate or succinate (Fig. 4A). Administration of rotenone prevented AA-induced $H_2O_2$ production in the presence of complex I substrates (pyruvate/malate); however, it did not affect AA-induced ROS generation in mitochondria respiring on succinate, a complex II substrate (Fig. 4A). Stigmatellin, which inhibits complex I at 10 μM and the $Q_o$ site of complex

III at 0.06 μM concentration (Degli Esposti et al., 1993), abolished mitochondrial $H_2O_2$ generation mediated by AA (Fig. 4A). As expected, stigmatellin (10 μM) inhibited AA-induced ROS production and subsequent histamine release (Fig. 4B).

To provide additional evidence that the histamine release from RBL-2H3 cells is due to mtROS, antioxidants with distinct mechanisms of action were used. Treatment of cells with N-acetyl-$_L$-cysteine, a glutathione precursor, significantly decreased spontaneous and mtROS-induced release of histamine (Fig. 5A). Phenyl N-*tert*- butylnitrone (1 mM), a direct scavenger of superoxide anion and hydroxyl radicals (Kotake, 1999), completely abolished mtROS-triggered release of histamine and decreased its basal secretion (Fig. 5B). In parallel experiments, we confirmed that these inhibitors decreased intracellular ROS levels to or below basal levels (data not shown). These data together suggest that inhibition of mtROS generation or overall decrease in intracellular ROS levels was sufficient to block histamine release.

### 3.4. Mitochondrial ROS-induced histamine release does not require complete exocytosis

In antigen-exposed, sensitized cells histamine release is mediated via a series of events including migration, fusion, and release of granule/vesicle contents into the cell exterior (Nishida et al., 2005). Since β-hexosaminidase was not released from cells with mitochondrial dysfunction (Fig. 6A), it suggests that migration of secretory granules and exocytosis may not required for histamine liberation. When cells were treated with colchicine, an inhibitor of granule motility (Smith et al., 2003) (100 μM, optimal concentration was determined in preliminary studies), mtROS-induced release of histamine was decreased (from 24.7%±0.6 to 15.9%±0.7). These data suggest that granule migration to the proximity of cytoplasmic membrane is required for histamine release (Fig. 6B).

PKC δ regulates movement of endosomes, lysosomes and secretory vesicles (Chen et al., 2004; Llado et al., 2004; Ma et al., 2008). Because mtROS-induced histamine release required vesicles migration (Fig. 6B), we investigated a potential involvement of PKC. Treatment of cells with chelerythrine chloride (5 μM), a specific, but not isoform-selective inhibitor of PKC, decreased mtROS-induced histamine release indicating the participation of PKC in this process (Fig. 6C). Furthermore, PMA (100 nM), a PKC activator (Wrede et al., 2003), significantly increased histamine release, but did not influence the β-hexosaminidase secretion (Fig. 6D). In control, ionomycin induced release of both histamine and β-hexosaminidase (Fig. 6D). Together these data indicate that PKC-dependent migration of secretory granules is a requirement for mtROS-induced histamine release and they are in line with oxidative stress-mediated activation of PKC δ via tyrosine phosphorylation (Konishi et al., 1997).

To sort out whether PKC-dependent histamine liberation requires free cytoplasmic $Ca^{2+}$ ($[Ca^{2+}]_i$), we investigated effects of $Ca^{2+}$ chelators. Administration of EGTA (1 mM, data not shown) or BAPTA [1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester); 10 μM for 30 min], chelators of extracellular $Ca^{2+}$ and $[Ca^{2+}]_i$, respectively resulted in insignificant changes in histamine release (Fig. 7A). These results indicate that $[Ca^{2+}]_i$ is not an absolute requirement for mtROS-mediated release of biogenic amines in RBL-2H3 cells, and also support the involvement of PKC δ, a $Ca^{2+}$-independent isoform of PKC family (Pernas-Sueiras et al., 2006), in these processes.

Appearance of phosphatidylserine at the outer side of the cytoplasmic membrane is a hallmark of exocytosis (Demo et al., 1999). To test the requirement of exocytosis for histamine release by mtROS, we analyzed the appearance of phosphatidylserine by Annexin V assay. In cells with mitochondrial dysfunction, there was only insignificant change in Annexin V binding, suggesting the lack of complete exocytosis (Fig. 7B). These results are consistent with non-degranulative secretion of biogenic amines as reported previously (Uvnas, 1991). In control, intense binding of Annexin V to the cell surface after addition of antigen to IgE-sensitized cells

indicated exocytosis of granules (Fig. 8A). These results imply that mtROS-generated signals induce granule migration, but are not sufficient for release of the granules' total content.

Since mtROS did not induce β-hexosaminidase release from RBL-2H3 cells, we investigated whether mtROS can alter the β-hexosaminidase release from antigen-treated sensitized cells. Unexpectedly, mtROS inhibited β-hexosaminidase release from antigen-treated sensitized cells; thus, its level in the supernatant was decreased by 50% (Fig. 6A). Although this phenomenon was also observed in RWE-treated RBL-2H3 cells and warrants further investigations, these results suggest that mitochondrial dysfunction (mtROS) inhibits events required for fusion of vesicles with cytoplasmic membrane, which could be a possible explanation for the lack of β-hexosaminidase release.

### 3.5. Vacuolar $H^+$-ATPase is inhibited by mtROS

Release of histamine and serotonin from secretory granules thought to be regulated by changes in intravesicular pH (Williams and Webb, 2000). Intraluminal pH of granules is maintained by vacuolar $H^+$-ATPase (V-ATPase) activities (Schumacher, 2006). Therefore, we assessed V-ATPase activity by determining ATP consumption of isolated secretory granules. Secretory granules isolated from cells with mitochondrial dysfunction, utilized significantly lower amounts of externally added ATP, compared to those from untreated control cells, so we found higher levels of unutilized ATP in the reaction mixture (Fig. 8A). Treatment of cells with bafilomycin A (100 nM), a specific inhibitor of V-ATPases (Camacho et al., 2008), resulted in similar decrease in ATP utilization (Fig. 8A). Suppression of ATP utilization by mtROS is consistent with $H_2O_2$-mediated inhibition of V-ATPases (Wang and Floor, 1998). In control, treatment of cells with bafilomycin A increased histamine release from the RBL-2H3 cells, confirming that a decrease in V-ATPase activity results in secretion of biogenic amines (Fig. 8B). In support, V-ATPase-dependent acidification of secretory granules was shown by microscopic imaging (Fig. 8C) and fluorimetrically (Fig. 8D) using acridine orange, a dye that accumulates in secretory granules and other cellular acidic compartments (Williams and Webb, 2000). In acridine orange-loaded, untreated cells, secretory granules emitted red fluorescence (Fig. 8C), while green fluorescence was observed in cells with mitochondrial dysfunction or treated with bafilomycin A, indicating an increase in intraluminal pH (Fig. 8C and D). Together, these data indicate that inhibition of V-ATPase activity, consequently an increase in pH of secretory vesicles is a possible cause of mtROS-induced release of biogenic amines from mast cells.

### 3.6. Mitochondrial ROS do not induce de novo synthesis of enhance IgE-mediated IL-4 production

Antigenic stimuli result in release of preformed low and high molecular weight inflammatory mediators, as well as *de novo* synthesis of cytokines in mast cells (Rivera and Gilfillan, 2006). Next, we investigated whether mtROS affect the production of IL- 4, which is characterized as one of the key cytokines of allergic responses (Corry and Kheradmand, 2002). As shown in Figure 9, mtROS alone did not enhance synthesis of IL-4 in RBL-2H3 cells; however, they further increased IL-4 production of antigen-treated sensitized cells. When RWE was administered to the antigen-treated, sensitized cells, it also significantly enhanced the level of IL-4 in the culture supernatant (Fig. 9). These data suggest that mtROS are synergistic with FcεRI-induced cell activation signals for IL-4 production and release. These data are in line with previous findings showing that environmental oxidative stress- and FcεRI-mediated signals are additive, enhancing IL-4 production from mast cells (Frossi et al., 2007).

## 4. Discussion

Mitochondria are involved in regulation of various cellular functions as they store/release/generate signaling mediators including $Ca^{2+}$ and ROS. These mediators are required in differentiation and function of various immune cells participating in innate and adaptive responses (Del Prete et al., 2008; Hunt et al., 1991). In this study, we show that increased production of mtROS, induced by treatment with RWE, triggers secretion of biogenic amines, i.e. histamine and serotonin, but not β-hexosaminidase from RBL-2H3 cells, independently from FcεRI-mediated signals. This phenomenon occurs in the absence of complete exocytosis of secretory granules and without changes in intracellular $Ca^{2+}$ levels, but it is exclusively associated with activation of protein kinase C-δ and inhibition of vacuolar $H^+$-ATPase. We also show that mtROS significantly increase FcεRI-mediated IL-4 production. These findings provide evidence for a relationship between mitochondrial dysfunction and antigen dependent and independent activation of mast cells. Thus, pharmacological protection from mitochondrial dysfunction may have clinical significance in prevention and treatment of allergic diseases.

In our studies, we utilized extract from ragweed pollen, which known to cause severe allergic symptoms (Hunt et al., 2002). Ragweed pollen-induced clinical symptoms are highly dependent on ROS production by pollen grains' intrinsic NADPH oxidases, which are activated after hydration on mucosal membranes of airways or conjunctiva (Bacsi et al., 2005; Boldogh et al., 2005), raising the possibility that these ROS are implicated in mast cell degranulation. However, our results showing that mitochondrial respiratory chain inhibitors (rotenone and 3NPA), which have no effect on pollen grains' NADPH oxidases, prevented RWE-induced release of allergic mediators were unexpected. These results strongly suggest that ROS generated by pollen grains' NADPH oxidase have no direct implication in degranulation processes, but induce damage to mitochondrial macromolecules (Boldogh et al., unpublished data), thereby dysfunction and elevated release of ROS from mitochondria. Indeed, mitochondria isolated from RWE-treated cells released nearly 3-fold higher amount of $H_2O_2$ (the major product of $O_2^{\cdot -}$ dismutation (Turrens, 2003)) compared to control mitochondria. Using site-specific inhibitors we identified the complex III-$Q_i$ as site of ROS generation in the respiratory chain. Indeed, previous studies have been identified complex III as one of the main sites of $O_2^{\cdot -}$ production (Raha and Robinson, 2000; St-Pierre et al., 2002).

Antimycin A, by binding to cytochrome *b*, inhibits the electron flow from semiquinone to ubiquinone, consequently increasing the steady state concentration of semiquinone, and resulting in electron escape from complex III $Q_i$ site (Turrens, 2003). Stigmatellin does not allow entry of electrons into complex III at the $Q_o$ site and thus it abolishes the AA (and RWE)-induced ROS production. Because complex III-$Q_i$ is the site of ROS generation in RWE treated cells and exposure to RWE may trigger a myriad of cellular effects, we utilized AA, a complex III inhibitor, to study effects of mtROS on release of the content of secretory vesicles.

Our data demonstrated the release of biogenic amines by mtROS (AA or RWE exposure). Inhibitors of mitochondrial $H_2O_2$ release (and antioxidants NAC or PBN) significantly decreased histamine/serotonin release, confirming that both AA- and RWE-induced mast cell activations are specific to mtROS. These results are in line with previous findings showing that $H_2O_2$ induce histamine release from mast cells (Menon et al., 1989; Ohmori et al., 1979). Interestingly, there was no β-hexosaminidase release detectable by mtROS (after AA or RWE exposure) from RBL-2H3 cells. However, these results are not surprising because oxidative stress did not trigger release of β-hexosaminidase from either RBL-2H3 cells (Bachelet et al., 2002) or murine bone marrow-derived mast cells (Mortaz et al., 2008). Similarly, organophosphate compounds induce histamine but not β-hexosaminidase release from RBL-1 cells after 1 h of exposure (Xiong and Rodgers, 1997). Worm (e.g. Trichinella) antigens induce the release of histamine but not β-hexosaminidase from sensitized rat mast

cells (Yepez-Mulia et al., 2009). Although our data suggest that mtROS inhibit complete exocytosis required for β-hexosaminidase release, an alternative explanation for the lack of its secretion could be the existence of various compartments storing different allergic mediators (Puri and Roche, 2008; Rajotte et al., 2003; Smith et al., 2003).

Upon activation of RBL-2H3 cells, secretory granules migrate to cytoplasmic membrane, the sites of exocytosis, and this event is microtubule-dependent and can be inhibited by colchicine (Smith et al., 2003). Release of histamine/serotonin by mtROS required colchicine-sensitive movement of secretory granules, suggesting microtubule-dependent release of these mediators. During typical degranulation process, migration of secretory granules is followed by exocytotic events resulting in release of vesicles content into extracellular milieu. However, in mtROS activated RBL-2H3 cells full exocytosis may not occur, as β-hexosaminidase was not released. It is possible that granules may fuse with the plasma membrane transiently (Hide et al., 1993; Obermuller et al., 2005; Williams and Webb, 2000) or the size of the fusion pores between vesicle and plasma membrane (Alvarez de Toledo et al., 1993; Fernandez-Chacon and Alvarez de Toledo, 1995) does not allow release of β-hexosaminidase.

As activation of PKC δ is required for oxidant-induced regulation of microtubule cytoskeleton (Banan et al., 2002), granule movement and exocytosis (Ma et al., 2008), we showed that chelerythrine chloride, a PKC inhibitor decreased mtROS-induced histamine release, while depletion of intracellular $Ca^{2+}$ had no effect. Chelerythrine chloride is a specific but not a PKC isoform-selective inhibitor; however, only PKC δ and PKC θ from the novel subfamily have been identified as regulators of antigen-induced mast cell degranulation (Cho et al., 2004; Liu et al., 2001; Parravicini et al., 2002). Presently there are no data demonstrating any effects of oxidative stress on PKC θ activity; therefore, PKC δ could be the key kinase in regulation of microtubule-dependent granule motility. In support, PKC δ is a $Ca^{2+}$-independent isoform and it can be activated by PMA (Pernas-Sueiras et al., 2006) as well as by ROS (Inoue et al., 2008), suggesting that mtROS regulate activity of PKC-δ in microtubule-dependent migration of secretory granules for release of biogenic amines from non-sensitized mast cells.

Biogenic amines are low molecular weight, positively charged mediators retained by low intravacuolar pH (Williams and Webb, 2000), and an increase in pH may be sufficient for their liberation as suggested by bafilomycin A-mediated release of histamine. Bafilomycin A is specific inhibitor of vacuolar $H^+$-ATPase (V-ATPase) (Camacho et al., 2008). V-ATPases function exclusively as ATP-dependent proton pumps, and the proton-motive force generated by them (in organelles' membranes including secretory vesicles) maintains pH gradient toward the cytosol (Nelson et al., 2000) and it is utilized as a driving force for transport processes (Beyenbach and Wieczorek, 2006; Saroussi and Nelson, 2008). Changes in pH gradient due to altered activity of V-ATPases in the vesicles' membrane thought to be responsible for the accumulation and release of amines (Camacho et al., 2008). Correspondingly, a decrease in the V-ATPases' function causes granule alkalization leading to release of serotonin from cells (Williams and Webb, 2000). Alkalization of secretory vesicles shown by acridine orange fluorescence suggests inhibition of the V-ATPase in mtROS producing cells. The fact that secretory vesicles isolated from these cells utilized lower amount of ATP than those from control cells strongly suggests the inhibition of V-ATPase activity by mtROS. These observations are in line with previous data showing inhibition of the V-ATPase by $H_2O_2$ due to oxidation of reactive cysteine sulfhydryl group in the ATP binding site (Wang and Floor, 1998).

Mast cell activation leads to increased synthesis of various cytokines (Metcalfe et al., 1997) and oxidative stress exacerbates their production (Frossi et al., 2007). Thus, mtROS-mediated augmentation of IL-4 production from antigen-activated cells is not particularly surprising. $H_2O_2$ is a well-characterized signaling molecule, which influence mast cell behavior (Guerin-

Marchand et al., 2001; Matsui et al., 2000). Narrow concentration range of externally added $H_2O_2$ (5-50 nM) alone was sufficient to trigger IL-4 release from RBL-2H3 cells (Frossi et al., 2003), while in our hand mtROS ($H_2O_2$) did not do so. This phenomenon could be explained simply by concentration or by the site of $H_2O_2$ action. Although, it is highly speculative, but it is possible that externally added $H_2O_2$ could trigger gene transcription via membrane receptor (s) and signals amplified by second messengers, while mtROS act directly on redox-sensitive kinases, phophatases, thereby signal intensity could be lower. Together these results raise the possibility that mitochondrial dysfunction is directly implicated in early events of allergic responses via inducing release of biogenic amines and it is indirectly involved in Th2 differentiation, B cell activation, and IgE production by its synergistic effect on IL-4 production.

In conclusion, we show that mitochondrial dysfunction triggers release of histamine/serotonin from non-sensitized mast cells by inducing PKC δ- and microtubule-dependent motility of secretory granules and inhibiting activity of vacuolar $H^+$-ATPase independently from intracellular $Ca^{2+}$ levels. The synergistic effect of mtROS on antigen-induced IL-4 production warrants further investigation and suggests that mitochondrial dysfunction may also have a role in the development of adaptive immune responses. These findings are particularly important because mitochondrial dysfunction was induced by extract of ragweed pollen, one of the most potent inducers of severe allergic symptoms. Thus, therapeutic strategies that decrease mitochondrial dysfunction will offer benefits in intervention and prevention of allergic symptoms.

## Acknowledgements

This work was supported by NIAID, P01 AI062885-01 (I.B., S.S., T.H), NIH HL071163 (S.S., I.B), NIEHS Center Grant, EOS 006677 and the Hungarian Scientific Research Fund (73347).

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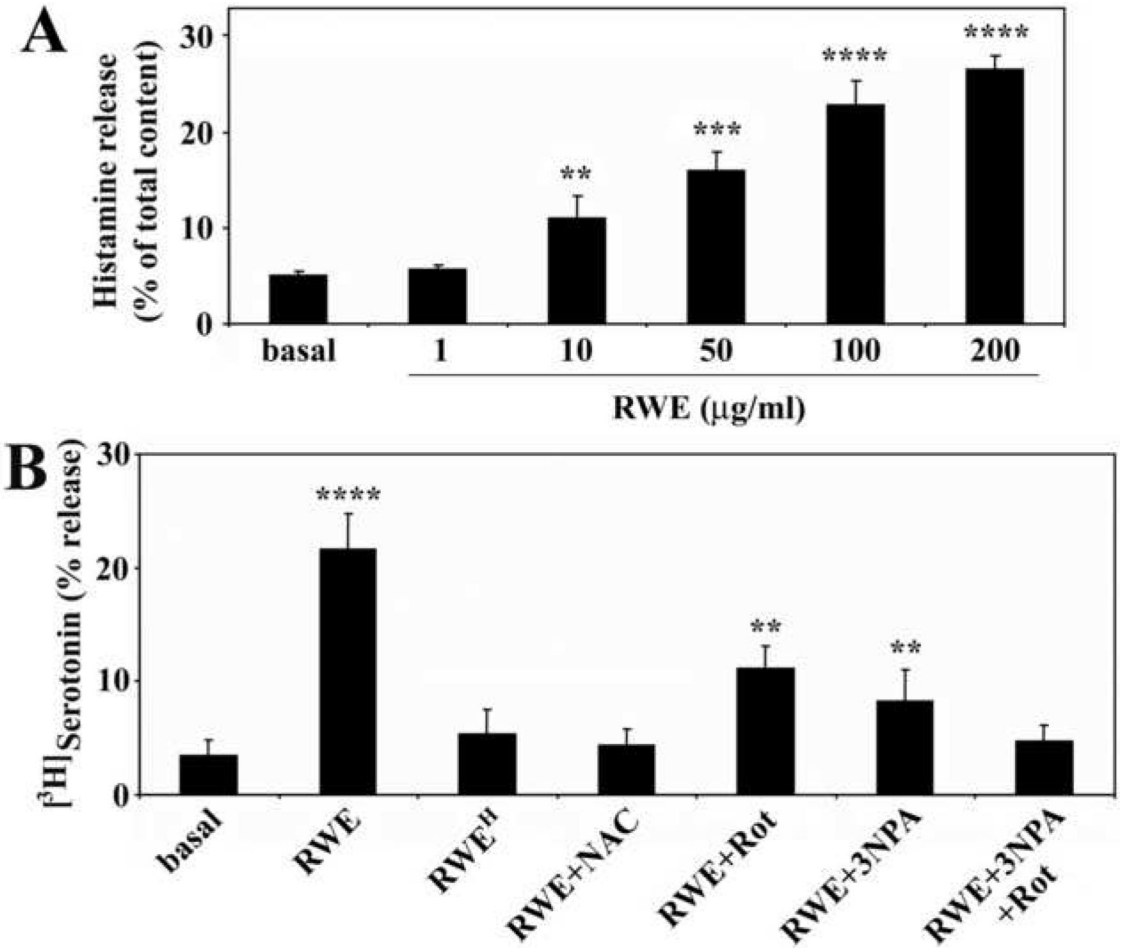


**Figure 1.**

Ragweed pollen extract (RWE) induces release of biogenic amines from RBL-2H3 cells in the absence of IgE antibodies. Exposure to RWE triggers release of histamine in a dose-dependent manner (A). Heat-treatment of RWE ($RWE^{H}$), administration of antioxidant (N-acetyl-$_{L}$-cysteine; NAC) as well as mitochondrial complex I and complex II inhibitors together (rotenone; Rot, 3-nitropropionic acid; 3-NPA) abrogates the effect of RWE on serotonin release (B). Data are presented as means ±SEM of 5-6 measurements. ***P*< 0.01, ****P*< 0.001, *****P*< 0.0001.

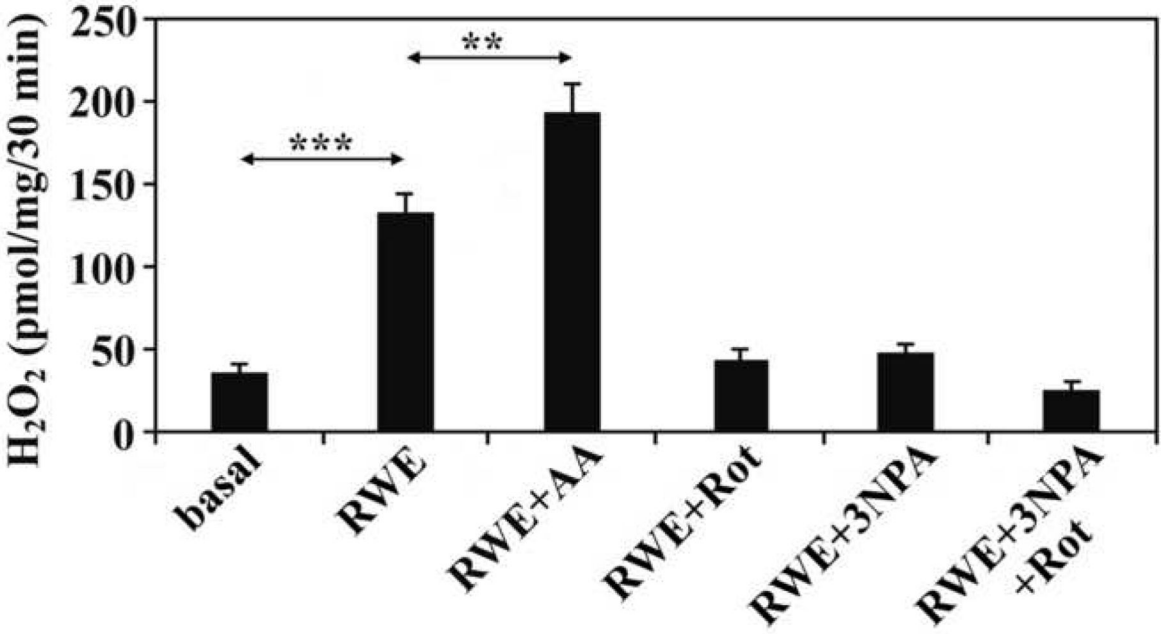


**Figure 2.**

Mitochondria isolated from RWE-treated cells produce higher amount of $H_2O_2$ than those from mock-treated cells. Co-administration of complex I or complex II inhibitors (Rot as well as 3-NPA) with RWE decreases the mtROS generation; however, co-administration of a complex III inhibitor (antimycin A; AA) enhances RWE-induced mtROS. Data are presented as means ±SEM of 3 measurements. $^{**}P < 0.01$, $^{***}P < 0.001$.

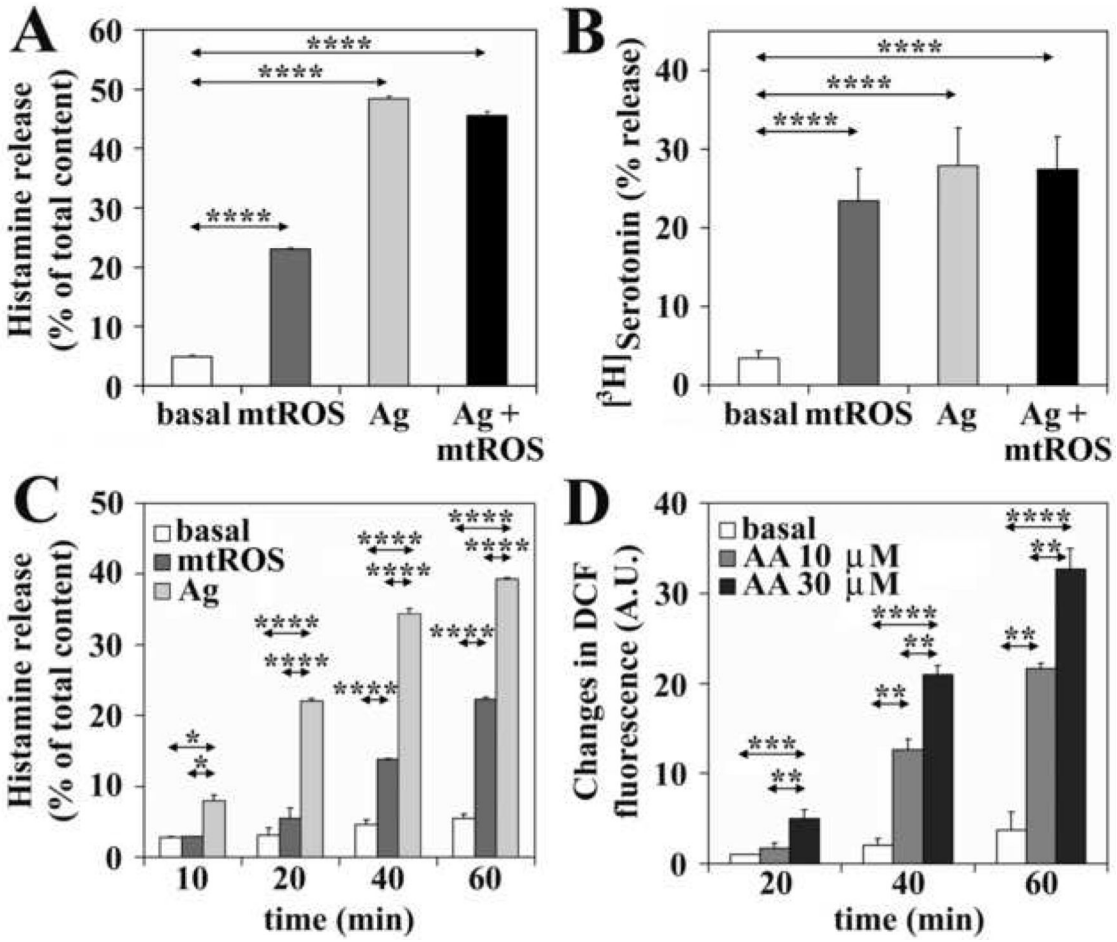


**Figure 3.**

Mitochondrial dysfunction induces biogenic amine release. Treatment of RBL-2H3 cells with antimycin A (AA) induces histamine (A) and serotonin (B) release. Mitochondrial ROS-induced histamine release is delayed compared to that triggered by the antigen (Ag) in sensitized cells (C). Kinetics of increase in intracellular ROS levels after induction of mitochondrial dysfunction by AA treatment (D). Data are presented as means ± SEM of 7-10 measurements. **P*< 0.05, ***P*< 0.01, ****P*< 0.001, *****P*< 0.0001.

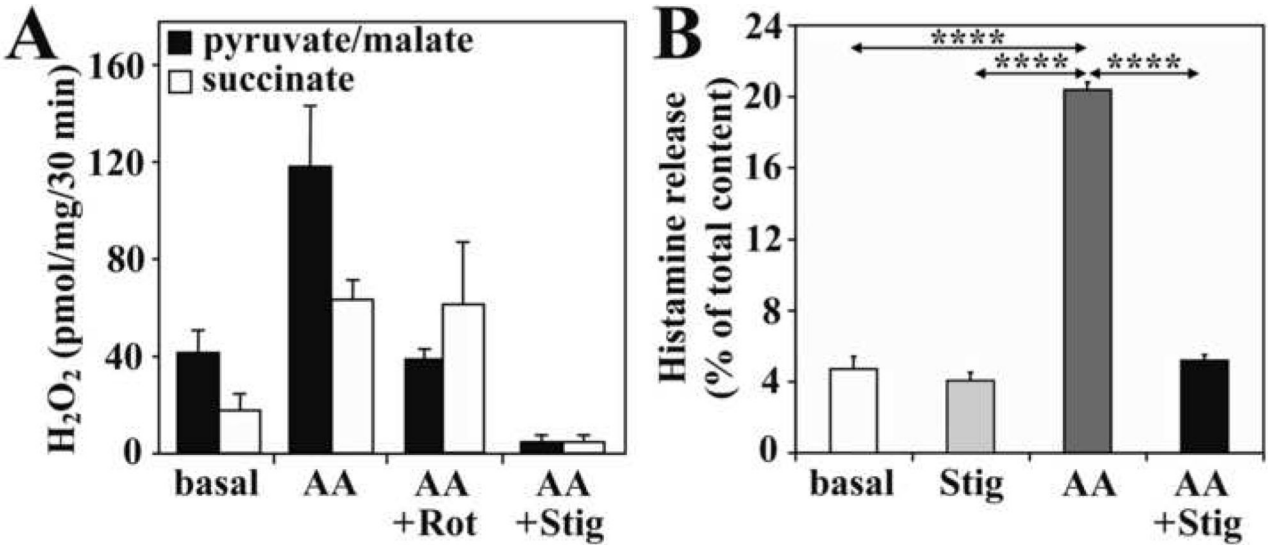


**Figure 4.**

Inhibition of mtROS generation abolishes release of histamine. Rotenone (Rot) or stigmatellin (Stig) blocks electron flow to the ubiquinone pool, consequently preventing antimycin A (AA)-induced mitochondrial $H_2O_2$ production (A). Results are representative of 3 independent experiments. Stigmatellin inhibits the AA-induced histamine release (B). Data are presented as means ±SEM of 6 measurements. ****$P$< 0.0001.

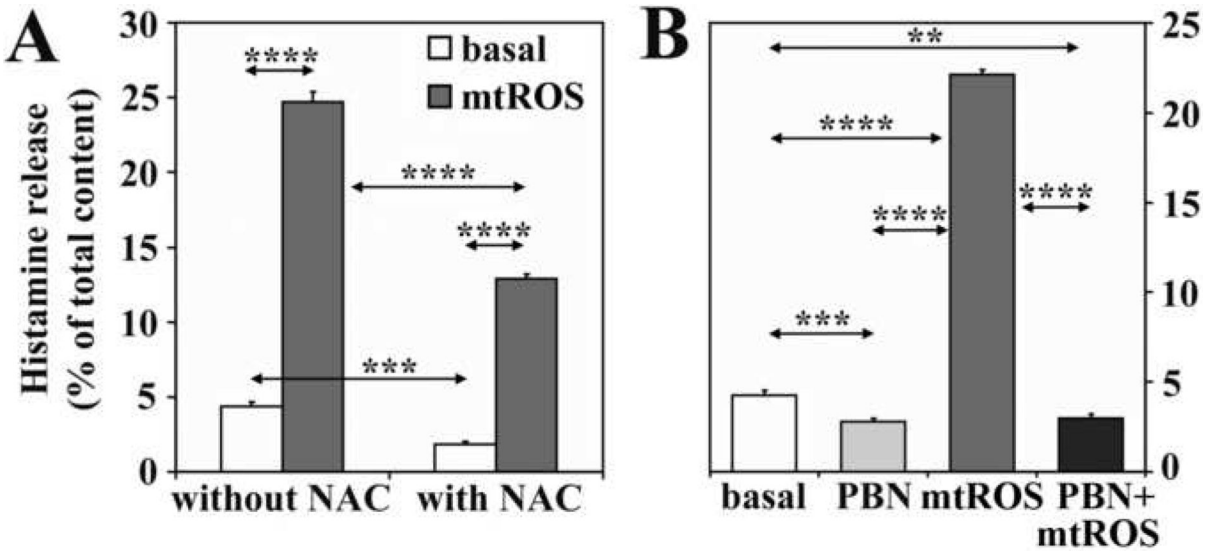


**Figure 5.**

Antioxidants decrease the mtROS-induced histamine release. N-acetyl-$_L$-cysteine (NAC)(A) or phenyl N-*tert*-butylnitrone (PBN)(B) decrease both mtROS-triggered and basal release of histamine. Mitochondrial dysfunction was induced by AA treatment (10 μM). Data are presented as means ±SEM of 6-9 measurements. ***P*< 0.01, ****P*< 0.001, *****P*< 0.0001.

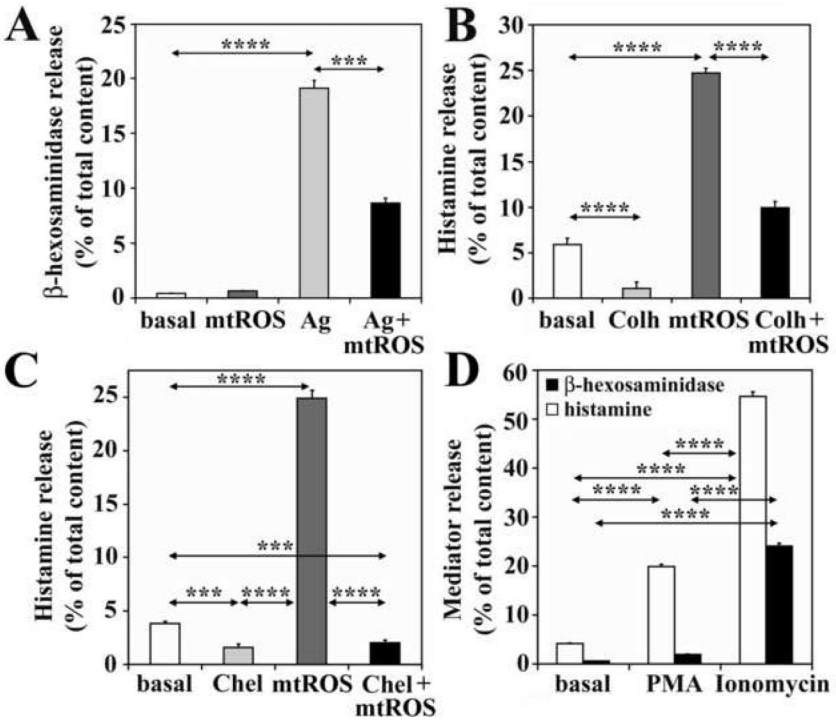


**Figure 6.**

Protein kinase C (PKC)-dependent migration of secretory granules is a requirement for mtROS-induced histamine release. Mitochondrial dysfunction does not increase β-hexosaminidase secretion from non-sensitized cells and it inhibits release of this enzyme from antigen (Ag)-treated sensitized cells (A). Colchicine (Colh), an inhibitor of the motility of secretory granules, decreases both basal and mtROS-induced release of histamine (B). Chelerythrine chloride (Chel), an inhibitor of PKC, decreases spontaneous and mtROS-induced histamine release (C). A PKC activator, 4 beta-phorbol 12-myristate 13-acetate (PMA), imitates the effects of mtROS (D). Mitochondrial dysfunction was induced by AA treatment (10 μM). ***$P$< 0.001, ****$P$< 0.0001.

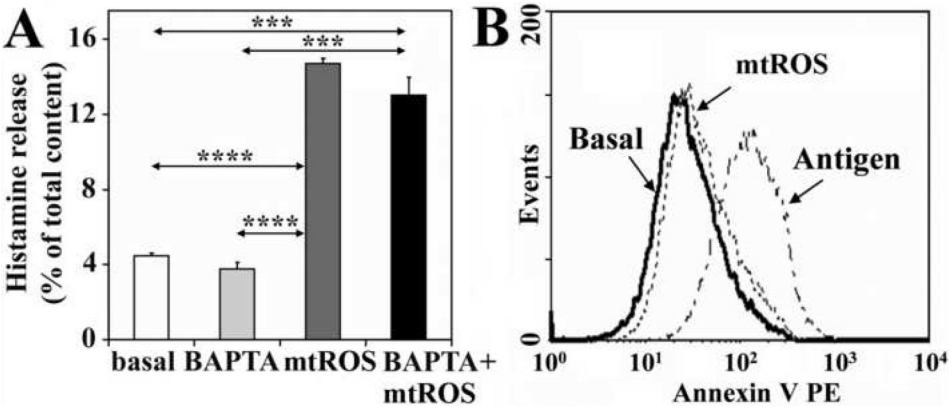


**Figure 7.**

Mitochondrial dysfunction-induced histamine release does not require intracellular free $Ca^{2+}$ and complete exocytosis. Administration of BAPTA, an intracellular $Ca^{2+}$ chelator, causes only a slight decrease in the mtROS-mediated release of histamine (A). Low levels of Annexin V binding indicate that mtROS-induced release of histamine does not require the full fusion of secretory granules with the plasma membrane (B). Antigen-induced exocytosis was used as positive control. Mitochondrial dysfunction was induced by AA treatment (10 μM). Data are presented as means ±SEM of 8-10 measurements. ***$P$< 0.001, ****$P$< 0.0001.

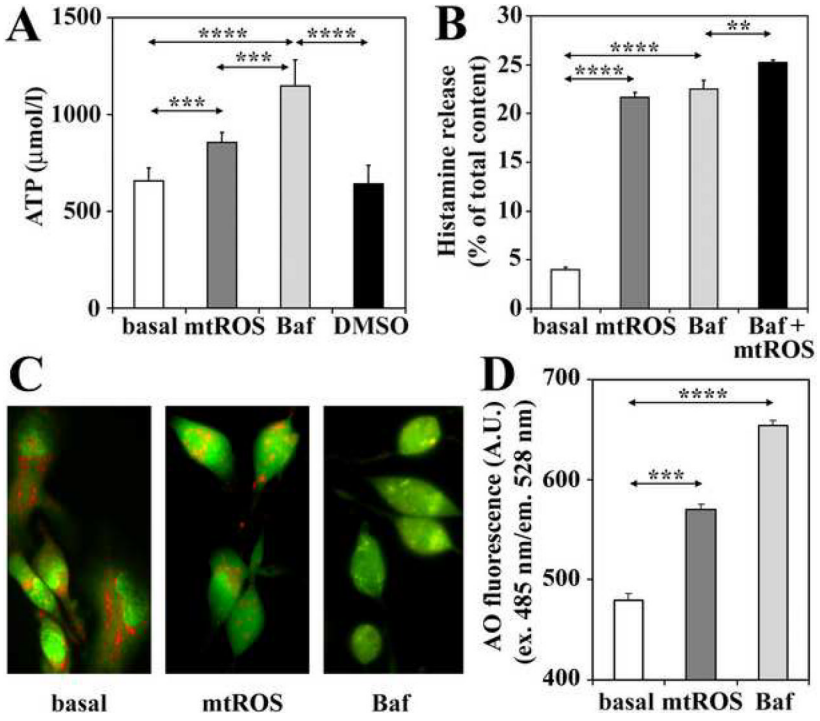


**Figure 8.**

Mitochondrial ROS induce histamine release via inhibition of vacuolar $H^+$-ATPases. Secretory granules from cells with mitochondrial dysfunction utilize significantly lower amounts of externally added ATP, compared to those from untreated control cells (A). Bafilomycin A (Baf), a specific inhibitor of vacuolar-type $H^+$-ATPases, induces histamine release from the RBL-2H3 cells (B). Cells exhibiting mitochondrial dysfunction show increases in the pH of cellular acidic compartments, which is visualized by fluorescence microscopy (C), and assessed by means of fluorimetry using acridine orange (AO) (D). In control experiments Baf was used. Mitochondrial dysfunction was induced by AA treatment (10 μM). Data are presented as means ±SEM of 6-8 measurements. ***P*< 0.01; ****P*< 0.001, *****P*< 0.0001.

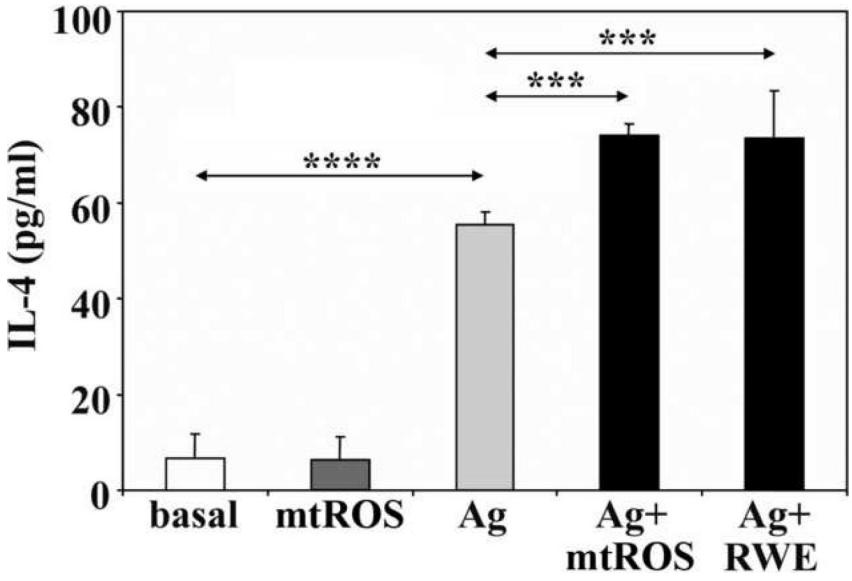


**Figure 9.**

Mitochondrial dysfunction enhances IgE-mediated IL-4 production. Mitochondrial dysfunction was induced by AA or RWE. Data are presented as means ± SEM of 5 measurements. ***$P$< 0.001, ****$P$< 0.0001.