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Mitochondrial Uncoupling Protein 2 Inhibits Mast Cell Activation and Reduces Histamine Content

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Mast cells are critical mediators of allergic diseases, as well as innate and acquired immunity (1). Mast cells are found in areas exposed to the external environment and are activated by diverse stimuli, including IgE receptor (FceRI) cross-linking and neuropeptides such as substance P (SP) that act on G protein-coupled receptors. Mast cell granules store a number of preformed mediators, including histamine and proteases that are released by degranulation; activated mast cells also de novo synthesize numerous proinflammatory eicosanoids and cytokines, many of which can be released selectively (2). These mediators cause immediate hypersensitivity reactions, characterized by vasodilation and increased vascular permeability, as well as late phase allergic inflammation (3). Despite the increasing importance of mast cells in regulating immune processes (4), little is still known about how mast cell activation is regulated.

Uncoupling proteins (UCPs) reside on the inner mitochondrial membrane where they alter the mitochondrial membrane potential and reactive oxygen species (ROS) production (5). UCP2 in particular has been implicated in control of mitochondrial ROS production. Both mitochondrial and cytosolic ROS are produced by mast cell activation (6). Inhibition of ROS production reduces degranulation, indicating that ROS may play a role in regulating exocytosis (7, 8). UCP2 activity inhibits exocytosis of insulin granules from pancreatic β cells (9) and dopamine-containing vesicles from rat PC12 cells (10). UCP2 also dampens the inflammatory response of macrophages (11–13). We, therefore, hypothesized that UCP2 could inhibit mast cell activation and decrease mast cell-mediated inflammatory responses.

In this article, we show that UCP2 is expressed in both murine and human mast cells. Ucp2−/− bone marrow-derived mast cells (BMMCs) have greater release of proinflammatory molecules after both allergic and nonallergic triggers, in addition to having increased histamine content. Ucp2−/− mice also develop significantly greater skin vascular permeability in response to SP and Ag after passive sensitization. Our results indicate that UCP2 could inhibit mast cell activation and decrease mast cell-mediated inflammatory responses.

Materials and Methods

Animals, isolation, and culture of mast cells

Ucp2−/− and Ucp2+/+ littermates were generated as previously described (11). Bone marrow cells were isolated from the femurs and tibias of age (8–12 wk) and sex-matched mice. These cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, nonessential amino acids, sodium pyruvate, 10 ng/ml murine stem cell factor (SCF), and 10 ng/ml murine IL-3 (both from PeproTech). Cells were cultured from 4 to 8 wk of culture. Purity was tested by staining granules with toluidine blue (1%, pH 2). Fetal skin mast cells (FSCMs) were isolated and cultured as previously described (10). Cell suspensions were prepared from excised trunk skin of day 16 fetal mice by limited trypsinization. Cells were cultured for 3 wk in

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2 Abbreviations used in this paper: SP, substance P; BMMCs, bone marrow mast cell; C4H8/80, compound 4H80; HSA, human serum albumin; EB, Evans blue dye; FSCMC, fetal skin mast cell; HDC, histidine decarboxylase; SOD, superoxide dismutase; ROS, reactive oxygen species; UCP, uncoupling protein; SCF, stem cell factor; TBAP, tetraakis(4-benzoic acid) porphyrin.

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medium described above and purified by Percoll density gradient centrifugation before use in experiments. LAD2 cells (supplied by Dr. A. S. Kirshenbaum, National Institutes of Health, Bethesda, MD) derived from a human mast cell leukemia (14) were cultured in StemPro-34 medium (Invitrogen) supplemented with 100 U/ml penicillin/streptomycin and 100 ng/ml recombinant human SCF, supplied by Amgen.

Skin histamine content

Age- and sex-matched mice were sacrificed and an 8-mm diameter piece of dorsal skin was immediately removed and weighed. The skin was homogenized in 10 μl of 2% perchloric acid and boiled for 10 min. After centrifugation at 10,000 × g for 10 min, the supernatant was removed and neutralized with an equal volume of 2 M NaOH. Histamine levels were determined by commercial ELISA (Beckman Coulter) according to the manufacturer’s instructions.

Immunoblotting

Cells were washed once with PBS and then lysed in buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na3VO4, and 50 mM NaF. Equal amounts of protein were electrophoresed on 10% SDS-polyacrylamide gels (Invitrogen) and then transferred to a 0.45-μm polyvinylidene difluoride membrane (Invitrogen). After blocking with 5% milk, membranes were probed overnight at 4°C with either goat anti-UCP2 (1/400; Santa Cruz Biotechnology) or rabbit anti-histidine decarboxylase (HDC; 1/300; Abcam). For detection, the membranes were incubated with the appropriate secondary Ab conjugated to HRP (1/5000; Santa Cruz Biotechnology) or 400 g/ml aprotinin, 10 μg/ml aprotinin, 10 μg/ml aprotinin, 10 μg/ml aprotinin, and values were normalized to the tissue weight. Intravascular EB and IL-6 and PGD2 assays

For IL-6 release, BMMCs (5 × 10^6 cells/500 μl) were distributed in 48-well microtiter assay plates and stimulated in complete culture medium with the indicated concentrations of stimulus for 24 h. IL-6 content was determined in cell-free supernatants with commercial ELISA kits (R&D Systems) according to the manufacturer’s instructions. IL-6 and PGD2 (PGD2-MOX kit; Cayman Chemical) were performed according to the manufacturer’s instructions.

ROS production

Cells were loaded with 5 μM dihydroethidium (Sigma-Aldrich) for 30 min at 37°C. The cells were washed twice, resuspended in Tyrode’s buffer, and stimulated as described. After 5 min of stimulation, cells were placed on ice until being analyzed using a FACSCalibur flow cytometer.

Lentiviral production and infection

Lentiviral vectors were generated as previously described (16). UCP2-encoding or pWPI vector with expression vectors encoding VSV-G gag-pol and env proteins were transiently transfected into 293T cells using Lipofectamine 2000 (Invitrogen). Supernatants were collected at 24 and 48 h, filtered through 0.45-μm filters, and centrifuged at 50,000 × g for 2 h at 4°C to pellet the virus. Viral pellets were resuspended in 300 μl of culture medium and frozen at −80°C until use. Cells were infected by spinoculation: 5 × 10^5 cells were resuspended in 200 μl of fresh medium and 50 μl of viral suspension with 8 μg/ml polybrene (Sigma-Aldrich) in 12-well plates and spun at 1500 × g for 90 min at 30°C. Alternatively, 1 ml of medium was added and the next day the cells were resuspended in fresh medium. Cells were used for experiments 5 days after infection.

Evans blue (EB) extravasation

EB extravasation was performed as previously described (17). Sex- and age-matched mice were administered 1% EB in sterile normal saline (0.1 ml) via tail vein injection. They were then anesthetized by an i.p. injection (0.1 ml) of ketamine (100 mg/kg)/xylazine (10 mg/kg), after which the dorsal subcapsular skin was shaved and either 50 pmol of SP or 10 μg of histamine (both from Sigma-Aldrich) were injected intradermally in a total volume of 50 μl in the subcapsular region with a tuberculin syringe. After 20 min, mice were sacrificed and an 8-mm diameter circular piece of skin at each injection site was removed and weighed. Alternatively, we used passive cutaneous anaphylaxis: normal saline or anti-DNP IgE (20 μg/50 μl; Sigma-Aldrich) was administered intradermally at two skin sites each. Two days later, DNP-HSA (200 μg) along with 1% EB in sterile normal saline (100 μl) was injected in the tail vein. The mice were sacrificed 30 min later and skin injection sites were cut, weighed, and placed in Eppendorf tubes. The mice were then handled the same as described above. EB was extracted in 1 ml of 10% triethylamine in methanol and the OD was measured at 620 nm using a PerkinElmer Luminescence Spectrophotometer. The EB concentration was calculated using a standard curve and values were normalized to the tissue weight. Intravascular EB and nonspecific effects of injection were accounted for by subtracting the EB extracted after injection of PBS.

Statistical analysis

Data are expressed as the mean ± SD. Groups were compared with a Student’s t test using the statistical software program SigmaStat. A value of p < 0.05 was considered significant.

Results

Mast cells express UCP2

We used RT-PCR and immunoblotting to assess expression of UCP2 in murine BMMCs. The Ucp2 transcript could be detected by RT-PCR in BMMCs (Fig. 1A). Immunoblotting showed that UCP2 protein was expressed in BMMCs as well as in human leukemic LAD2 mast cells (Fig. 1B). No UCP2 was detected in lysate from Ucp2−/− BMMCs, showing the specificity of the Ab (Fig. 1B).

Normal development of Ucp2−/− mast cells

BMMCs were obtained by culturing nonadherent bone marrow progenitor cells for 4 wk in SCF- and IL-3-supplemented medium. Bone marrow from both wild-type and Ucp2−/− mice generated...
highly pure BMMC cultures shown to be morphologically normal with numerous distinguishable granules upon staining with May-Grünwald-Giemsa (Fig. 1C). Expression of the mast cell markers c-kit and FcεRI was assessed by flow cytometry, which showed similar levels of these molecules in wild-type and Ucp2−/− BMMCs. Original magnification, ×100. D, Flow cytometry of the surface expression of c-kit and FcεRI in wild-type and Ucp2−/− BMMCs. Dotted lines are BMMCs incubated with isotype control Ab. Data are representative of three experiments with similar results. Pos, Positive.

Histamine secretion from Ucp2−/− BMMCs

Following overnight sensitization with DNP-specific IgE (100 ng/ml), BMMCs were stimulated for 20 min with DNP-HSA (10 ng/ml). Ucp2−/− BMMCs released significantly more histamine (1.4-fold) after challenge with 10 ng/ml DNP-HSA (Fig. 2A). Levels of basal release were similar in wild-type and Ucp2−/− BMMCs (Fig. 2A). We then compared the effect of three different nonimmune triggers also inducing more calcium influx. All of these nonimmune triggers also induced more histamine release from Ucp2−/− mast cells (Fig. 2B), indicating that UCP2 may regulate signaling steps downstream of cytosolic calcium ion influx.

Histamine secretion from UCP-overexpressing human LAD2 mast cells

We next examined the effect of UCP2 on degranulation of human mast cells by transducing LAD2 cells with a lentivirus encoding human UCP2 and GFP or a lentivirus expressing GFP alone as a control (16). The transduction efficiency was ~50% as assessed by flow cytometric analysis of GFP expression; overexpression of UCP2 was confirmed by immunoblot (Fig. 3A). IgE-sensitized UCP2-overexpressing LAD2 cells challenged with anti-IgE (10 ng/ml) had 38% less histamine release compared with control cells (Fig. 3B). Levels of basal histamine release were similar in UCP2-overexpressing and control cells (Fig. 3B). UCP2-overexpressing LAD2 cells stimulated with SP (1 μM) had 23% less histamine release compared with control cells (Fig. 3C). LAD2 cells transduced with the control virus showed similar histamine release to untransduced controls (data not shown). Together, these results suggest that UCP2 down-regulates degranulation in both mouse and human cells regardless of the stimulus used.

UCP2 regulates mast cell histamine content

We next addressed the possibility that UCP2 may affect mast cell histamine content before stimulation. Histamine was extracted from the untreated skin of wild-type and Ucp2−/− mice and measured by ELISA. Histamine levels were 241 ± 70 mmol/g for wild-type vs 352 ± 90 mmol/g for Ucp2−/− mice (Fig. 4A), a 60% increase over wild-type mice.

Examination of the number and distribution of mast cells in the skin of Ucp2−/− mice by May-Grünwald-Giemsa and toluidine blue stains showed no differences from wild type (Fig. 4B). The skin mast cells had a normal distribution in the superficial dermis with equal numbers in both the Ucp2−/− and wild-type skin (Fig. 4C). Given that mast cells are the only cells in the skin to produce histamine and that mast cell density is similar in wild-type and Ucp2−/− skin, it suggests that more histamine is produced per mast cell.

We also measured histamine levels in cultured BMMCs and found that wild-type BMMCs contained 0.11 ± 0.029 pg/cell, whereas Ucp2−/− BMMCs contained 0.28 ± 0.039 pg/cell, a 155% increase over wild-type cells (Fig. 4D). We next measured the histamine content in LAD2 cells after transduction with either the control or the UCP2-overexpression lentivirus. Transduction
UCP2-overexpression lentivirus. Data are the mean and individual data of six to seven mice per genotype. B, Representative photomicrographs of dorsal skin from wild-type and Ucp2<sup>−/−</sup> mice stained with toluidine blue. Metachromatic mast cells are also indicated by arrows. C, Number of mast cells per mm<sup>2</sup> in cross-section of skin. Mast cells were counted in five randomly chosen fields from three sections per mouse. Data represent the mean ± SD of five mice per genotype; *p < 0.05. D, Total histamine content of wild-type and Ucp2<sup>−/−</sup> BMMCs was measured spectrophotometrically and normalized to cell number. *p < 0.05 vs wild type (t test). Data are the mean ± SD of three experiments. E, Total histamine content of LAD2 cells 5 days after transduction with a control lentivirus or UCP2-overexpression lentivirus. *p < 0.05 vs control cells. Data are the mean ± SD (n = 3).

with the control lentivirus did not have any effect on LAD2 histamine content. LAD2 cells overexpressing UCP2 had a 27% lower histamine content 5 days after lentiviral transduction (3.63 ± 0.13 pg/cell in control cells vs 2.60 ± 0.34 pg/cell in UCP2-overexpressing cells; Fig. 4E).

We also measured expression of HDC, the enzyme responsible for histamine synthesis, to investigate whether this could account for the difference in histamine levels. Quantitative real-time PCR and immunoblot showed that Ucp2<sup>−/−</sup> BMMCs expressed greater levels of HDC mRNA (Fig. 5A) and protein (Fig. 5B). The immunoblot shows both 74- and 54-kDa bands for HDC. The full-length HDC (74 kDa) is inactive until it is cleaved to the shorter form (54 kDa). Together, these data indicate that induction of HDC may be responsible for the increased histamine content observed in Ucp2<sup>−/−</sup> mast cells.

UCP2 down-regulates superoxide production in many cell types (4, 5). It is also reported that ROS induces the HDC gene (14, 18). We therefore hypothesized that the increased histamine content in Ucp2<sup>−/−</sup> mast cells was due to increased superoxide production. We first showed enhanced ROS production in Ucp2<sup>−/−</sup> BMMCs by measuring fluorescence of cells loaded with dihydroethidium using flow cytometry. Dihydroethidium fluorescence was higher in Ucp2<sup>−/−</sup> BMMCs compared with wild-type BMMCs (Fig. 5C). We examined the effect of mitochondrial superoxide production on histamine content by treating BMMCs with the mitochondrial-targeted superoxide dismutase (SOD)-minic Mn(III) tetrakis(4-benzoic acid) porphyrin (TBAP). Culture of BMMCs for 5 days with TBAP (20–40 μM) reduced histamine content by up to 58% (Fig. 5D). Similar results were found with LAD2 cells (data not shown). Culture of LAD2 cells or BMMCs with TBAP for 5 days did not affect cell viability as shown by trypsin blue staining. These data indicate that mitochondrial superoxide increases histamine content, possibly through induction of HDC.

Ucp2<sup>−/−</sup> BMMCs have higher IL-6 release

Cells were sensitized overnight with anti-DNP IgE (100 ng/ml) and stimulated with DNP-HSA (10 ng/ml) for 24 h; IL-6 in the supernatant fluid was ~50% higher in the Ucp2<sup>−/−</sup> BMMCs than controls (Fig. 6).

Enhanced PG production and ERK activation in Ucp2<sup>−/−</sup> BMMCs

PGD<sub>2</sub> is a lipid produced primarily by mast cells that contributes to vasodilation, bronchoconstriction, and chemotaxis of eosinophils and lymphocytes (19). After stimulation with DNP-HSA (10 ng/ml), Ucp2<sup>−/−</sup> BMMCs produced more PGD<sub>2</sub> (5866 ± 1430 vs 3274 ± 289 pg/10<sup>6</sup> cells; Fig. 7A). A similar effect was observed...
higher in through activation of cytosolic phospholipase A2 (20) and may also allows us to measure the effects of direct mast cell activation, since increased EB extravasation was seen in the skin of wild-type mice. Furthermore, our findings cannot be explained by an augmented response to histamine in Ucp2−/− mice, because intradermal administration of histamine resulted in similar levels of plasma extravasation in both wild-type and Ucp2−/− mice. We found that activation of mast cells with both Ag and FceRI-independent stimuli (C48/80, SP, ionomycin) resulted in greater histamine release in Ucp2−/− mice, whereas overexpression of UCP2 reduced degranulation in LAD2 human mast cells. In agreement with these findings, pharmacologic uncoupling of the mitochondrial proton gradient with carbonyl cyanide p-trifluoromethoxyphenylhydrazine inhibited Ag-induced mast cell degranulation (24). UCP2 was also shown to inhibit exocytosis of insulin granules in β cells (9) and dopamine-containing vesicles in rat PC12 cells (10).

One possible explanation of our findings is the increased superoxide in the Ucp2−/− mast cells. It is known that mast cells produce ROS after stimulation with Ag and non-FceRI stimuli such as SP and C48/80 (8, 26). Inhibition of ROS production by antioxidants reduces degranulation and cytokine production (7, 27). Povidone-iodine, a superoxide scavenger, inhibited mast cell degranulation in vitro (28), and a SOD mimetic decreased mast cell response to histamine in both Ucp2−/− and wild-type mice. Furthermore, the differences seen in plasma extravasation following either SP or anti-DNP-HSA stimulation cannot be explained by an increased number of mast cells, since we showed that there were no differences in the distribution of skin mast cells in Ucp2−/− and wild-type mice (Fig. 4). These data indicate that decreased expression of UCP2 significantly increases mast cell histamine-dependent vascular permeability in vivo.

Discussion

In this study, we show that the mitochondrial protein UCP2 is expressed in both murine and human LAD2 mast cells and can regulate mast cell activation in vitro and in vivo. Expression of UCP2 negatively regulated several mast cells functions, including degranulation, histamine production, as well as IL-6 and PGD2 and release.

The increased skin plasma extravasation seen in Ucp2−/− mice can be explained by the increased release of histamine from mast cells since it occurred both after allergen challenge and stimulation with SP, a well-known trigger of mast cells (23). These findings are not due to an increased number of mast cells, because the number of mast cells in the skin of Ucp2−/− mice was similar to that of the wild-type mice. Furthermore, our findings cannot be explained by an augmented response to histamine in Ucp2−/− mice, because intradermal administration of histamine resulted in similar levels of plasma extravasation in both wild-type and Ucp2−/− mice.

We found that activation of mast cells with both Ag and FceRI-independent stimuli (C48/80, SP, ionomycin) resulted in greater histamine release in Ucp2−/− cells, whereas overexpression of UCP2 reduced degranulation in LAD2 human mast cells. In agreement with these findings, pharmacologic uncoupling of the mitochondrial proton gradient with carbonyl cyanide p-trifluoromethoxyphenylhydrazine inhibited Ag-induced mast cell degranulation (24). UCP2 was also shown to inhibit exocytosis of insulin granules in β cells (9) and dopamine-containing vesicles in rat PC12 cells (10).
degranulation in vivo in a guinea pig model of allergic asthma (29). There are several other endogenous antioxidant proteins expressed in mast cells that are reported to affect mast cell activation, including thioredoxin 1 (7) and heme oxygenase 1 (30). However, UCP2 is the first protein reported to reduce mast cell degranulation that prevents the formation of ROS rather than neutralizing it.

ROS may regulate mast cell degranulation both upstream and downstream of extracellular calcium influx (6,31). Mast cell mitochondria are reported to be involved in the downstream regulation (6). This is in agreement with our results that Ucp2⁻/⁻ mast cells have enhanced degranulation even after calcium ionophore stimulation, where all cells presumably see the same intracellular calcium levels. It is possible that UCP2 affects calcium kinetics since it is involved in mitochondrial calcium uptake (32). Another possibility is that UCP2 decreases ATP levels (33), leading to increased activation of store-operated calcium channels (34).

Increased vascular permeability in the skin of Ucp2⁻/⁻ mice is likely because of increased histamine release due to both increased secretion and increased preformed histamine content. The skin of Ucp2⁻/⁻ mice had histamine levels that were 60% higher than controls. Considering the similar number of mast cells, this indicates that there is more histamine stored per cell, a finding we have confirmed in BMMCs. In conjunction with elevated histamine levels, we found that Ucp2⁻/⁻ BMMCs had a higher expression of HDC, the enzyme responsible for histamine synthesis. It has been reported that the HDC gene promoter is activated by oxidative stress in AGS gastric cancer cells (18). The higher ROS levels in Ucp2⁻/⁻ mast cells may be responsible for induction of HDC and, therefore, increased histamine synthesis. This is consistent with our finding that the SOD-mimetic TBAP decreased histamine levels in both BMMCs and LAD2 cells.

The relation of low Ucp2 expression to increased mast cell activation was not limited only to histamine since it also permitted higher IL-6 and PGD₂ production and release. Moreover, the greater PGD₂ production in Ucp2⁻/⁻ BMMCs was accompanied by greater ERK phosphorylation. Early PG synthesis in mast cells is controlled primarily by ERK (20). Oxidative stress is known to induce ERK activation in various cell types, including lymphocytes, possibly through an indirect effect on upstream kinases (22). In addition to PG, ERK may regulate degranulation, although there is conflicting evidence for this (20,21).

In addition to our results, other studies have shown that UCP2 plays an important role in other immune responses. UCP2 is also expressed in lymphocytes, dendritic cells, neutrophils, and macrophages (35). Ucp2⁻/⁻ mice develop more severe autoimmune encephalomyelitis (36) and autoimmune diabetes (37). Macrophages from Ucp2⁻/⁻ mice have a greater inflammatory response to LPS, including increased NF-κB activation and greater cytokine production (11–13). Macrophages overexpressing UCP2 produce less ROS in response to LPS (38) and have decreased transendothelial migration (39). UCP2 deficiency also confers protection to Toxoplasma gondii and Listeria monocytogenes infection in mice, due to increased inflammatory responses (11,35). Since there is evidence that ROS modulates activation of T and B lymphocytes (40,41), UCP2 may also have important regulatory functions in these cells, although this has not yet been directly explored.

In conclusion, we have identified UCP2 as a novel regulator of mast cell secretion and associated inflammation. Inhibiting UCP2 has been proposed as a method to increase insulin release from β cells in type 2 diabetes (9). However, inhibition of UCP2 may worsen allergic and inflammatory diseases, making it a less attractive target for this indication. On the other hand, induction or activation of UCP2 may be a new strategy for reducing allergic and inflammatory responses.

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Disclosures

The authors have no financial conflict of interest.

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