Nitric oxide protects mast cells from activation-induced cell death: the role of the phosphatidylinositol-3 kinase-Akt-endothelial nitric oxide synthase pathway

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Abstract: NO is known to suppress mast cell activation, but the role of NO in mast cell survival is unclear. Ligation of the high-affinity receptor for IgE (FcεRI) resulted in NO production in mast cells within minutes. This NO production was largely dependent on NO synthase (NOS) activity and extracellular Ca^{2+}. The NO production required an aggregation of FcεRI and was accompanied by increased phosphorylation of endothelial NOS (eNOS) at Ser1177 and Akt at Ser473. The phosphorylation of eNOS and Akt and the production of NO were abolished by the PI-3K inhibitor wortmannin. Although thapsigargin (TG) induced NO production as well, this response occurred with a considerable lag time (>10 min) and was independent of FcεRI aggregation and PI-3K and NOS activity. Mast cells underwent apoptosis in response to TG but not upon FcεRI ligation. However, when the NOS-dependent NO production was blocked, FcεRI ligation caused sizable apoptosis, substantial mitochondrial cytochrome c release, caspase-3/7 activation, and collapse of the mitochondrial membrane potential, all of which were inhibited by the caspase-3 inhibitor z-Asp-Glu-Val-Asp-fluoromethylketone. The data suggest that the NO produced by the PI-3K-Akt-eNOS pathway is involved in protecting mast cells from cell death. J. Leukoc. Biol. 83: 000–000; 2008.

Key Words: antigen · eNOS · apoptosis · cell survival

INTRODUCTION

Mast cells play a critical role in allergic and inflammatory reactions as well as innate and acquired immunity [1]. Crosslinking of the IgE-bound, high-affinity IgE receptor (FcεRI) on the cell surface by antigen initiates a cascade of intracellular signaling events that leads to degranulation, inflammatory mediator release, and cytokine production, contributing to allergic and inflammatory reactions. FcεRI is a tetramer of α-, β-, and γ-chain homodimers (αβγε), of which the α-chain binds IgE, and the β- and γ-chains mediate intracellular signaling through the receptor. FcεRI is a member of the multichain immunorecognition receptors, including TCR and BCR, and the signal transduction pathways present in mast cells have many similarities to those of T and B cells. Like TCR and BCR, FcεRI lacks intrinsic enzyme activity, but the β- and γ-chains contain the immunoreceptor tyrosine-based activation motif (ITAM), which is critical for cell activation through these receptors [2–4]. Activation of these receptors results in the phosphorylation of the tyrosine residues in the ITAM. It is believed that the aggregation of FcεRI causes tyrosine phosphorylation of the β-chain ITAM by the activated Lyn and subsequent phosphorylation of Syk and the γ-chain ITAM, which in turn leads to downstream signals, including the elevation in cytosolic Ca^{2+} and the activation of protein kinase C (PKC) and MAPK family members [5].

NO is a potent radical with diverse roles in biological systems. It is a mediator of vasodilatation, platelet aggregation, and neuronal transmission, and depending on cell type and concentration, NO regulates function, death, and survival of various cell types including many of those involved in immunity and inflammation [6, 7]. NO is synthesized from l-arginine and molecular oxygen catalyzed by the NO synthase (NOS). There are three members of the NOS family enzyme: neuronal NOS (nNOS or NOS-1), endothelial NOS (eNOS or NOS-3), and inducible NOS (iNOS or NOS-2). nNOS and eNOS are characterized by constitutive expression, regulation via increased intracellular Ca^{2+} levels, and low NO production, and iNOS is expressed only upon cell activation by a variety of inflammatory mediators and causes relatively slow, sustained, and high levels of NO production independently of Ca^{2+} [8]. NO is shown to suppress mast cell activation and subsequent features of inflammation. NO suppresses antigen-induced degranulation, leukotriene secretion, and cytokine expression and release in mast cells [9–11]. Nonetheless, little is known about the mechanisms by which NO is produced in mast cells and how NO elicits the biological effects. Although NO is known to act as antiapoptotic and proapoptotic signals depending on cell

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types and extracellular stimuli used [12], the role of NO in mast cell survival is unclear.

In the present study, we studied NO production in response to antigen and elucidated the potential role of NO in mast cell survival. Our results show that mast cells produce NO via the PI-3K-Akt-eNOS pathway and that this NO plays an important role in protecting mast cells against activation-induced cell death via a mitochondrial death pathway.

MATERIALS AND METHODS

Materials

Thapsigargin (TG) and anti-2,4-dinitrophenol (anti-DNP) IgE mAb (clone SPE-7) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-2,4,6-trinitrophenyl (TNP) IgE mAb (clone IgE-3) was obtained from BD PharMingen Japan (Tokyo). TNP-BSA conjugate (25 molecules of TNP coupled to one molecule of BSA) was purchased from Cosmo Bio (Tokyo, Japan). DNP-BSA conjugate (33 molecules of DNP coupled to one molecule of BSA) and z-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk) were obtained from Callbiochem (San Diego, CA, USA). Recombinant IL-3 (IL-3) was purchased from PeproTech (Rocky Hill, NJ, USA). Wortmannin, PP1, and 2,4-diamino-6R,8R,6-O-(1-erythro-1’,2’-dihydroxypropyl)-5,6,7,8-tetrahydropteridine (H_{2}aminohydropterin), a structural analog of 6R,6,7,8-tetrahydropterin-1-, which is the natural cofactor of all NOS isoenzymes, were obtained from BioMol (Plymouth Meeting, PA, USA). Diamonodihydroflavene-diacetate (DAF-2-DA) was obtained from Daichi Pure Chemicals (Tokyo, Japan). Rabbit polyclonal antibody against phosphorylated eNOS (Ser1177) and phosphorylated Akt (Ser473) and Akt were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The hybridoma-producing, anti-rat β-chain mAb (clone JRK) was kindly provided by Dr. J. Rivera [National Institutes of Health (NIH), Bethesda, MD, USA], and the antibody was prepared in our laboratory.

RBL-2H3 cells

The RBL-2H3 cells obtained from the National Institute of Health Sciences [Japanese Collection of Research Biosources (JCRB), Cell No. JCRB0023] were grown in DMEM (Sigma Chemical Co.), supplemented with 10% FBS (JRH Bioscience, Lenexa, KS, USA) in a 5% CO_{2}-containing atmosphere.

Cells were harvested by incubating them in HBSS (pH 7.4) containing 1 mM EDTA and 0.25% trypsin for 5 min at 37°C. For IgE sensitization, cells suspended in complete DMEM were plated on a 100-mm culture dish (10^{6}/ml) or in a 24-well plate (10^{5}/well). Stained cells were considered to be living cells. These stained cells were visualized using an ECL Plus kit (GE Healthcare, Little Chalfont, UK), according to the recommendations of the manufacturer.

Bone marrow-derived mast cells (BMMCs)

All animal experiments were performed according to Nihon University guidelines. BMMCs were prepared from femurs of 4- to 8-week-old C3HeBFeJ mice, as described previously [13]. The cells were cultured in the RPMI-1640 medium (Sigma Chemical Co.) supplemented with 10% FBS, 100 U/ml penicillin, and streptomycin (Invitrogen Corp., Carlsbad, CA, USA), 5 × 10^{-5} M 2-ME (Wako Pure Chemicals, Osaka, Japan), 100 μg/ml sodium pyruvate (Invitrogen), 1% MEM nonessential amino acid solution (Invitrogen), and 5 ng/ml IL-3 (PeproTech) in a 5% CO_{2}-containing atmosphere at 37°C. After 4–6 weeks of culture, the cells were stained with a cell-surface expression of FcεRI, and BMMCs were used for experiments after 4–8 weeks of culture (>95% mast cells).

Measurement of intracellular NO production

The production of intracellular NO was measured by flow cytometry using DAF-2-DA, a cell-permeable, NO-sensitive fluorescent dye. For cytometrical analysis, IgE-sensitized cells (1×10^{6}/ml), suspended in HBSS, were incubated with 5 μM DAF-2-DA for 15 min at 37°C in a final volume of 450 μl. Then, 50 μl stimulated (10^{6}) was added and incubated at 37°C for the time indicated before harvest. The cells were then washed, resuspended in HBSS on ice, and analyzed in a FACSCalibur (Becton Dickinson, San Jose, CA, USA; excitation and emission at 488 and 575 nm, respectively). In inhibition experiments, the agents tested were added to cells 15 min before stimulation.

Analyses of eNOS and Akt phosphorylation

The phosphorylation status of eNOS and Akt was determined by immunoblot analysis using antiphospho-eNOS (Ser1177), antiphospho-Akt (Ser473), and anti-Akt antibody. After treatment with the indicated agents, cells were washed with PBS, lysed with SDS sample buffer, subjected to SDS-PAGE using a 10% separation gel under reducing conditions, and then transferred to polyvinylidene difluoride (PVDF) membranes, which were incubated with 3% Block AceTM (Snow Brand Milk Products, Tokyo, Japan) in PBS at 4°C for 1 h at room temperature. The PVDF membranes were incubated with each primary antibody (1:1000 dilution) in Tris-buffered saline containing 0.1% Tween-20 overnight at 4°C and then incubated with HRP-conjugated, species-specific, anti-rabbit Ig (GE Healthcare, Little Chalfont, UK) for 1 h at room temperature. After extensive washing of the membrane, the immunoreactive proteins were visualized using an ECL Plus kit (GE Healthcare), according to the recommendations of the manufacturer.

Evaluation of cell death

Apoptotic cell death and overall cell death were evaluated by double staining with FITC-conjugated Annexin V and propidium iodide (PI). Briefly, after the cells, adhered in a 24-well plate (4×10^{5}/well), were treated with the inhibitor tested in 10% FBS/DMEM and then stimulated with antigen or TG for 18–22 h, phosphatidylserine (PS) translocated from the inner to the outer leaflet of the plasma membrane was detected by Annexin V staining using a commercially available kit (Annexin V-FITC apoptosis detection kit I, BD PharMingen, San Diego, CA, USA), according to the manufacturer’s instructions. By double-staining cells with Annexin V-FITC and PI, the subsets of cells that were Annexin V-positive, PI-negative (apoptotic) and Annexin V-positive and PI-positive (necrotic and/or advanced apoptotic cells) were determined. Double-negative cells were considered to be living cells. These stained cells were evaluated in a FACScaliber and analyzed using CellQuest software (Becton Dickinson).

Cytochrome c release assay

Cells adhered in a six-well plate (3×10^{5}/well) were washed and treated with inhibitor in 10% FBS/DMEM and stimulated with antigen or TG for 18 h. To assess cytochrome c content in the mitochondria, the cells were fractionated into mitochondrial and cytosolic fractions by differential centrifugation using a mitochondrial isolation kit (Fierce, Rockford, IL, USA), and the mitochondrial fraction was lysed with 1% Triton X-100. In regards to RBL-2H3 cells, we collected the cultured medium and cytosolic fraction together as an extra-mitochondrial fraction. Cytochrome c content in each fraction was determined using a commercially available, solid-phase sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s protocol.

Measurement of caspase-3/7 activation and mitochondrial membrane potential

Cells adhered in a six-well plate (3×10^{5}/well) were treated with the inhibitor tested in 10% FBS/DMEM and then stimulated with antigen for 18 h. Caspase-3/7 activation and changes in mitochondrial membrane potential were determined using the dual-sensor MitoCasp™ (Cell Technology Inc., Mountain View, CA, USA), according to the manufacturer’s protocol. Stained cells were evaluated in a FACScaliber and analyzed using CellQuest software.

Statistical analysis

The Student’s t-test was performed to determine statistical significance among the experimental groups; P < 0.05 was considered significant.
RESULTS

Mast cells produce NO within minutes upon FceRI ligation

First, we examined whether mast cells produced NO upon FceRI ligation. RBL-2H3 cells that had been sensitized with anti-TNP IgE were stimulated with TNP-BSA, and then NO production was measured. We initially attempted to detect the production of NO by measuring NO\(_3^-\)/NO\(_2^-\) but obtained no reliable data as a result of their low (frequently undetectable) accumulation during a short time (within minutes). Next, we sought to detect the production of NO by fluorometry using DAF-2-DA, a NO-sensitive dye. DAF-2-DA is rapidly taken up by cells and hydrolyzed into a nonfluorescent DAF-2 by cellular esterase activity, which is oxidized to its fluorescent derivative by NO [14]. Thus, DAF-2-DA has been widely used as a selective probe for intracellular NO in various cell types [15–18]. FceRI ligation induced a small but significant increase in DAF-2 fluorescence. The effect was observed in an antigen concentration-dependent manner with a minimal effective concentration of 0.3 ng/ml (Fig. 1A). This NO production was observed within minutes and increased gradually with time (Fig. 1B). Addition of antigen to the cells that had not been treated with IgE resulted in no increase in DAF-2 fluorescence for up to 30 min (Fig. 1B), indicating that an aggregation of FceRI is essential for the NO production. Basically, the same results were obtained by sensitization with anti-DNP IgE and stimulation with DNP-BSA. In this case, the presence of an excess amount of the monovalent hapten DNP-lysine, which blocks the aggregation of FceRI [19], completely abolished the increase in DAF-2 fluorescence (Fig. 1C). Collectively, the data indicate that the increase in DAF-2 fluorescence is a result of the increased intracellular NO level induced by an aggregation of FceRI but is not merely a result of auto-oxidation of the dye. To exclude the possibility that such NO production is an aberrant special case with tumor mast cells such as RBL-2H3, we performed similar experiments using the primary mast cells BMMCs. Furthermore, FceRI ligation induced substantial NO production in these cells within minutes (Fig. 1D). This effect was seen clearly at relatively high concentrations of antigen (\(\geq 3\) ng/ml).

NO production is largely dependent on NOS activity and Ca\(^{2+}\)

In most experiments, there were no significant differences in the NO production properties of RBL-2H3 cells and BMMCs; therefore, we performed a further analysis using RBL-2H3 cells as a result of their ease of use. Unlike nNOS and eNOS, iNOS is not expressed in resting cells but is expressed following cell activation. There is a lag phase of several hours between cell activation and NO production, reflecting the time taken for mRNA and protein synthesis [20, 21]. Thus, the rapid NO production following FceRI ligation suggested that this response may be mediated by eNOS isozymes rather than iNOS. To demonstrate that NO production is dependent on NOS activity, we tested the effect of H\(_4\)-aminobiopterin, a structural analog of H\(_4\)-biopterin in which the keto group at C4 of H\(_4\)-biopterin is replaced by an amino group [22, 23]. H\(_4\)-aminobiopterin competes with H\(_4\)-biopterin, the natural cofactor of all NOS [24]. As a result, when applied to purified enzymes, the activity of all three NOS isoenzymes was inhibited by H\(_4\)-aminobiopterin. In addition, H\(_4\)-aminobiopterin has...
However, H4-aminobiopterin alone had no effect on basal NO production partially but not completely (75% maximum inhibition) even when used at 100 μM, indicating that the response may occur dependently or independently of NOS activity. It is noteworthy that H4-aminobiopterin blocked NO production (Fig. 2A), indicating the involvement of NOS activity. It is noteworthy that H4-aminobiopterin blocked NO production partially but not completely (75% maximum inhibition) even when used at 100 μM, indicating that the response may occur dependently or independently of NOS activity. However, H4-aminobiopterin alone had no effect on basal NO production in the cells (Fig. 2A). It is well-established that activation of nNOS and eNOS is strictly dependent on intracellular Ca2+ levels, and iNOS functions independently of the levels [6, 7]. In an attempt to demonstrate the role of eNOS, we studied the Ca2+ dependence of NO production. RBL-2H3 cells placed in Ca2+-containing medium (in the presence of 1 mM CaCl2) or in nominally Ca2+-free medium (in the presence of 1 mM EGTA) were compared for their ability to produce NO upon FcεRI ligation. NO production was profoundly but not completely reduced when extracellular Ca2+ was removed (Fig. 2B). Notably, the effect was clearer at an earlier time-point than at a later time-point; at 10 min after FcεRI ligation, the effect was strong (70% maximum reduction), and at 30 min, the effect was only modest (40% maximum reduction; Fig. 2B). A similar residual response (~30%) was seen even when the cells were treated with EGTA up to 10 mM.

FcεRI ligation induces eNOS phosphorylation at Ser1177 via a PI-3K-Akt pathway

An earlier work demonstrated that small amounts of eNOS mRNA could be detected basally, whereas neither mRNA of iNOS nor nNOS was detected in unstimulated rat peritoneal mast cells [26]. Thus, eNOS is the most likely candidate for the NOS isoform responsible for the rapid NO production. eNOS activity is basically regulated by intracellular Ca2+ levels but is also modulated by the phosphorylation of the enzyme [27–29]. Phosphorylation at Ser1177 (corresponding to Ser1179 of human eNOS) is considered to be an important mechanism to increase eNOS activity [27, 28]. We therefore assessed the levels of eNOS phosphorylation by Western blotting analysis with antibody to the phosphorylated form of eNOS (phospho-eNOS). As shown in Figure 3A, within minutes after FcεRI ligation, the level of eNOS phosphorylation increased significantly compared with the 0 time-point and increased gradually with time (2.6-fold at 60 min). H4-aminobiopterin at a concentration of as little as 10 μM completely blocked the eNOS phosphorylation (Fig. 3B), but the drug inhibited the production of NO only modestly (~40% inhibition), suggesting that another pathway may also be involved.

To gain insight into signaling pathways involved in the NO production, we examined the effects of divergent pharmacological inhibitors of selective signaling pathways on the response. As shown in Figure 3C, treatment with the Src family kinase inhibitor PP1 or the PI-3K inhibitor wortmannin for 15 min before FcεRI ligation inhibited antigen-induced NO production. In particular, wortmannin almost completely abolished the response. These results show that the NO production requires PI-3K activity and is dependent on Src family kinase activity. We also elucidated the role of Src family kinase activity and PI-3K activity in eNOS phosphorylation. PP1 and wortmannin blocked antigen-induced eNOS phosphorylation at concentrations that effectively inhibited the NO production (Fig. 3D). Although eNOS-Ser1179 can be phosphorylated by several protein kinases including Akt [29, 30], adenosine monophosphate-activated protein kinase [31], PKA, and PKG [32], the serine/threonine protein kinase Akt/PKB is one of the best-characterized targets of PI-3K lipid products [33]. As the above data indicate that eNOS is phosphorylated at Ser1177 for activation, we next examined whether FcεRI ligation induced Akt phosphorylation at Ser473, the residue linked to eNOS activation. As shown in Figure 3E, FcεRI ligation could increase Akt phosphorylation, which was blocked completely by PP1 and wortmannin. Even in nonstimulated (medium-treated) cells, substantial Akt phosphorylation could be seen, and this phosphorylation was abolished completely by PP1 and wortmannin. This suggests that FcεRI ligation stimulates the Akt phosphorylation pathway, which may be constitutively acti-
activated regardless of the presence or absence of this stimulus. Collectively, the data show that FcεRI ligation induces NO production via the PI-3K-Akt-eNOS pathway.

TG induces NO production with a considerable lag time, which is independent of FcεRI aggregation, PI-3K, and NOS activity To determine whether NO production is a general phenomenon associated with mast cell activation or is specific to FcεRI ligation, we examined the ability of the SERCA pump-blocker TG to induce NO production, as TG is capable of activating mast cells by bypassing the FcεRI-dependent signaling pathway [34]. TG at concentrations of ≥1 μM significantly increased intracellular NO levels in RBL-2H3 cells. However, the effect was slow, and no apparent NO production was observed for up to at least 10 min (Fig. 4A). Similarly, TG induced NO production in BMMCs with a lag time of >20 min (Fig. 4B). The TG-induced NO production was not affected by treatment with DNP-Lys but was reduced significantly when the cells were placed in nominally Ca2+-free medium (Fig. 4, C and D). We next elucidated the role of Src family kinase activity and PI-3K activity in TG-induced NO production. Cells were treated with inhibitors tested for 15 min before TG addition, incubated for 30 min, and then NO production was measured. As preliminary experiments revealed that in this longer incubation, the PI-3K inhibitor wortmannin at concentrations of 30 nM abolished even ambient NO production, we used a lower concentration (10 nM) of wortmannin in this longer incubation protocol. PP1 inhibited the TG-induced NO production profoundly (70% maximal inhibition), but wortmannin, up to 10 nM, had no effect (Fig. 5A). Under these conditions, antigen-induced NO production was almost completely abolished by wortmannin (Fig. 5B). Lastly, to determine whether TG-induced NO production is dependent on NOS activity, we examined the effect of H4-aminobiopterin on the response. The results showed that unlike FcεRI ligation, this
response was completely resistant to H₄-aminobiopterin up to 10 μM (Fig. 5C). It was found that TG-induced NO production was largely insensitive to H₄-aminobiopterin up to 100 μM (data not shown). These data show that TG may induce NO production independently of FcεRI aggregation, PI-3K, and NOS activity.

**Mast cells undergo apoptosis in response to TG but not upon FcεRI ligation**

TG has been shown to display strong proapoptotic activity in various cell types including mast cells [35], but it is known that mast cells stimulated through FcεRI do not always undergo cell death. We initially confirmed this in our cell systems. RBL-2H3 cells were incubated for 18–22 h after FcεRI ligation, and PS exposure and PI permeability were analyzed in a flow cytometer. As shown in Figure 6, A and B, in most studies, there were no significant changes in PS exposure and PI permeability even when cells were stimulated with antigen (30 ng/ml TNP-BSA or 62.5 ng/ml DNP-BSA), an optimal dose for mast cell activation, as measured with DAF-2-DA-loaded RBL-2H3 cells (5×10⁶/450 μl) incubated with 1 μM TG for 30 min, with or without 30 μM DNP-Lys (C), and then DAF-2 fluorescence was measured. DAF-2-DA-loaded RBL-2H3 cells (5×10⁶/450 μl) incubated with 1 μM TG for 30 min, and then DAF-2 fluorescence was measured. The data represent the mean ± SEM of three to four independent experiments.

**The role of NO in protecting mast cells from mitochondrial cell death**

The data presented so far indicated that FcεRI ligation but not TG treatment can induce rapid NO production and that the latter but not the former can evoke considerable cell death in mast cells. These observations led us to elucidate the potential role of NO in protecting mast cells from cell death. For this purpose, we used two different agents, DNP-Lys and H₄-aminobiopterin, because as mentioned above, they could inhibit the antigen-induced NO production strongly with a minimal effect on the TG-induced NO production. As shown in Figure 7A, 30 μM DNP-Lys enhanced antigen-induced apoptosis remarkably, although the hapten on its own had minimal cytotoxicity and a minimal effect on TG-induced apoptosis (Fig. 7A). Treatment with H₄-aminobiopterin also significantly enhanced antigen-induced apoptosis in RBL-2H3 cells and BMMCs (Fig. 7, B and C). However, H₄-aminobiopterin alone had no effect on mast cell survival (Fig. 7B).

The intrinsic or mitochondrial apoptotic pathway is a major death pathway, where mitochondria act as central gateway controllers through the release of a number of apoptogenic factors including cytochrome c into the cytoplasm. To elucidate the role of mitochondria in mast cell death, we examined whether FcεRI ligation/TG could evoke mitochondrial release of cytochrome c. After treatment with the tested agents, the cells were fractionated into mitochondrial and cytosolic fractions, and cytochrome c content in each fraction was determined by ELISA. In preliminary experiments, we found that in BMMCs the cytochrome c released from the mitochondria was predominantly detected in the cytosolic fraction, but in RBL-2H3 cells, a considerable amount of cytochrome c was released into the cultured medium. We therefore considered the cytochrome c detected in the cytosolic and extracellular fractions as the cytochrome c released from the mitochondria. In RBL-2H3 cells and BMMCs, TG induced cytochrome c release considerably, and antigen alone had a minimal effect (Fig. 8, A and B). However, when antigen and H₄-aminobiopterin were used together, sizable cytochrome c release was observed. Moreover, treatment with the caspase-3 inhibitor z-DEVD-fmk completely blocked the cytochrome c release (Fig. 8C). In contrast, H₄-aminobiopterin or z-DEVD-fmk had a minimal effect on TG-induced apoptosis (data not shown).
Cytochrome c released to the cytosol forms a multimeric complex with apoptotic protease factor-1 and procaspase-9, leading to the activation of caspase-9 and downstream caspases. Caspase-3 is a major effector caspase that plays a critical role in the apoptotic cascade. Moreover, decrease of mitochondrial membrane potential is one important characteristic during apoptotic cell death. Therefore, we next examined whether antigen plus H4-aminobiopterin affects caspase-3/7 activation and changes in mitochondrial membrane potential. As shown in Figure 9, antigen plus H4-aminobiopterin evoked substantial caspase-3/7 activation and the collapse of mitochondrial membrane potential.

DISCUSSION

Although NO is known to suppress activation of in vivo-derived rodent mast cells (mouse and rat peritoneal mast cells), there is still considerable debate as to whether mast cells themselves or contaminating cells such as macrophages are the source of NO. The demonstration of NO production by cultured mast cells can validate the cellular capacity of mast cells to produce NO. In this respect, it is noteworthy that in two different human mast cell lines, HMC-1 and LAD-2, A23187 or IgE-mediated FcεRI cross-linking induces cytoplasmic NO formation [36]. However, one cannot exclude the possibility that this is an aberrant special case with tumor mast cells. The data presented here clearly demonstrate that not only a tumor mast cell line (RBL-2H3) but also primary cultured mast cells (BMMCs) can produce NO upon FcεRI cross-linking. This strongly supports the view that mast cells are indeed a NO producer in response to physiological stimuli.

It is necessary to understand the detailed mechanism of NO production including the source and the signal transduction pathway. As antigen-induced NO production can be seen within minutes and is largely dependent on the presence of extracellular Ca^{2+}, constitutively expressed NOS isoforms (eNOS, nNOS) are more likely to be involved than iNOS. An earlier work has revealed that small amounts of eNOS mRNA can be detected basally, whereas neither mRNA of iNOS nor nNOS is detected in unstimulated rat peritoneal mast cells [36]. Thus, eNOS appears the most likely source of the short-term NO production in mast cells. Consistent with this view, we here demonstrated that in mast cells, antigen induces phosphorylation of eNOS at Ser1177 (equivalent to human eNOS-Ser1179), the well-characterized event essential for maximal activation of the enzyme [27, 28]. Earlier works have demonstrated that phosphorylation at Ser1179 of human eNOS by Akt increases enzyme activity [29, 30]. Consistent with these reports, we here demonstrated that FcεRI stimulation can induce eNOS phosphorylation at Ser1177 and Akt phosphorylation at Ser473, the residue linked to eNOS activation. Moreover, our data indicate that PI-3K activity is necessary for antigen-induced NO production and eNOS phosphorylation. Collectively, eNOS activation through the PI-3K/Akt pathway is the most likely mechanism of the rapid NO production. Our finding is quite similar to that made in T cells by Ibiza et al. [37], who have shown that within a few minutes of antigen binding, T cells produce NO via eNOS and that this process requires increased intracellular Ca^{2+} and PI-3K activity. Thus, in mast cells and T cells, NO may be at least in part produced in a similar, eNOS-dependent mechanism.

It is noteworthy that TG can induce substantial NO production despite its inability to evoke surface signaling events including FcεRI aggregation. This indicates that FcεRI-mediated signaling events such as receptor-component phosphorylation are not necessary for NO production on its own. However, the pharmacological properties of NO produced by TG

Fig. 5. TG induces NO production independently of PI-3K and NOS activity. (A, B) DAF-2-DA-loaded RBL-2H3 cells (5x10^6/450 μl) were incubated with 30 μM PP1 or 10 nM wortmannin for 15 min before stimulation with 1 μM TG (A) or 30 ng/ml TNP-BSA (B) for 30 min, and then DAF-2 fluorescence was measured. (C) DAF-2-DA-loaded RBL-2H3 cells (5x10^6/450 μl) were incubated for 15 min, with or without H2-aminobiopterin at the indicated concentrations, and stimulated with 30 ng/ml TNP-BSA, and then DAF-2 fluorescence was measured. The data are expressed as a percentage of the control stimulated with TG or TNP-BSA alone and represent the mean ± SEM of three to four independent experiments. (A and B) Statistical significance versus untreated control was determined using an unpaired Student’s t-test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. N.S., Not significant.
were clearly distinct from that of NO produced upon FcεRI ligation. In particular, TG-induced NO production appears to be independent of PI-3K and NOS activity. The enzymatic, NOS-dependent pathway is generally accepted as a source of NO. However, generation of NO that is independent of NOS has been demonstrated in several cell types, which is likely a result of direct reduction of nitrite to NO under acidic and highly reducing conditions, including ischemia [38, 39]. Thus, it is conceivable that in mast cells, NO is produced by at least two distinct mechanisms: One mechanism is the PI-3K-Akt-eNOS pathway mediated by FcεRI aggregation, and the other is a NOS-independent pathway, although further studies may be necessary to prove this view.

Although FcεRI ligation and TG are powerful activators of mast cells, there is an agreement in the literature that their ability to induce mast cell death is quite different. TG constantly induces apoptosis in various cell types, including mast cells [35, 40–42], and FcεRI ligation induces mast cell death only under limited conditions [43]. Rather, FcεRI ligation is known to transduce a positive signal for mast cell survival. It has been demonstrated that FcεRI activation promotes mast cell survival by up-regulating proapoptotic Bcl-2 family member A1 [44], Bim, and Bcl-Xl [45] in BMMCs and the human A1 homologue Bfl-1 in human mast cells [46]. However, the mechanisms by which mast cells escape activation-induced cell death are not fully understood. It has been reported that low levels of NO act as an antiapoptotic factor [47, 48]. As our data indicate that FcεRI ligation specifically stimulated NO production via the PI-3K-Akt-eNOS pathway, and the PI-3K-Akt signaling pathway has been shown to be an important mechanism of cell survival [33, 49], we were interested in examining the role of this NO production in mast cell survival.

No significant cell death or mitochondrial cytochrome c was observed following FcεRI ligation. However, when FcεRI-mediated NO production was blocked by DNP-Lys or H4-aminobiopterin, FcεRI ligation induced sizable apoptosis, mitochondrial cytochrome c, caspase-3/7 activation, and a decrease of mitochondrial membrane potential. The release of mitochondrial proteins such as cytochrome c results in activation of the caspase-dependent cell death pathway. Consistent with the role of the mitochondrial pathway, the caspase-3 inhibitor z-DEVD-fmk completely blocked the antigen plus H4-aminobiopterin-induced apoptosis. Moreover, we found that z-DEVD-fmk blocked the antigen plus H4-aminobiopterin-induced cytochrome c release and caspase-3/7 activation. It is noteworthy that z-DEVD-fmk blocked cytochrome c release, as this suggests the existence of a feedback activation of a mitochondrial apoptotic pathway. In fact, it has been reported that caspase-3-dependent cleavage of Bcl-2 promotes cytochrome c release [50]. Moreover, caspase-3-mediated feedback activa-

Fig. 6. Mast cells undergo apoptosis upon exposure to TG but not FcεRI ligation. RBL-2H3 cells (A–C) or BMMCs (D) adhered in a 24-well plate (5×10^4/200 µl) were stimulated with 30 ng/ml TNP-BSA (A, D), 62.5 ng/ml DNP-BSA (B), or TG at the indicated concentrations (C) for 18 h at 37°C, and these cells were harvested. Cells were stained with FITC-conjugated Annexin V and PI and analyzed by flow cytometry. Double-negative cells are considered to be living cells. The data are representative of three to five independent experiments.
Fig. 7. Blockade of NOS-dependent NO production facilitates FcεRI-mediated apoptosis. RBL-2H3 cells (A, B) or BMMCs (C) in a 24-well plate (5×10⁴/200 μl) were stimulated with 62.5 ng/ml DNP-BSA (A), 30 ng/ml TNP-BSA (B, C), or 1 μM TG (A) in the absence or presence of 30 μM DNP-Lys (A), 100 μM H₁-aminobiopterin (B, C), or 10 μM z-DEVD-fmk (B) for 18 h at 37°C, and these cells were harvested. The cells were stained with FITC-conjugated Annexin V and PI and analyzed by flow cytometry. Double-negative cells are considered to be living cells. The data are representative of three to five independent experiments.
tion of apical caspases (caspase-8 and -9) and cytochrome c release has been shown in doxorubicin- and TNF-α-induced apoptosis [51]. In addition, it is suggested that NO suppresses a key step in the positive-feedback amplification of apoptotic signaling by preventing Bcl-2 cleavage and cytochrome c release [52].

Although TG can induce NO production, this NO may play a minor role in protecting mast cells from cell death, as TG...
alone is a powerful, proapoptotic agent. We observed that FceRI-mediated NO production or z-DEVD-fmk failed to block TG-induced apoptosis, although caspase-3/7 activation was observed. This is consistent with the view that the endoplasmic reticulum stress rather than mitochondrial dysintegrity is important in TG-induced apoptosis and suggests that FceRI-mediated NO production is selectively involved in regulating the mitochondrial death pathway. Thus, our data suggest that NO produced by the PI-3K-Akt-eNOS pathway protects mast cells from activation-induced cell death.

In conclusion, the current study demonstrates that FcεRI ligation induces the production of intracellular NO by the PI-3K-Akt-eNOS pathway and that this NO plays an important role in maintaining mitochondrial integrity, thereby protecting cells from mitochondrial cell death. To our knowledge, this is the first study demonstrating that endogenous NO acts as a prosurvival factor in mast cells.

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