Disruption of Fas Receptor Signaling by Nitric Oxide in Eosinophils

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Summary

It has been suggested that Fas ligand–Fas receptor interactions are involved in the regulation of eosinophil apoptosis and that dysfunctions in this system could contribute to the accumulation of these cells in allergic and asthmatic diseases. Here, we demonstrate that nitric oxide (NO) specifically prevents Fas receptor–mediated apoptosis in freshly isolated human eosinophils. In contrast, rapid acceleration of eosinophil apoptosis by activation of the Fas receptor occurs in the presence of eosinophil hematopoietins. Analysis of the intracellular mechanisms revealed that NO disrupts Fas receptor–mediated signaling events at the level of, or proximal to, Jun kinase (JNK), but distal to sphingomyelinase (SMase) activation and ceramide generation. In addition, activation of SMase occurs downstream of an interleukin 1 converting enzyme–like (ICE-like) protease(s) that is not blocked by NO. However, NO prevents activation of a protease that targets lamin B1. These findings suggest a role for an additional NO-sensitive apoptotic signaling pathway that amplifies the proteolytic cascade initialized by activation of the Fas receptor. Therefore, NO concentrations within allergic inflammatory sites may be important in determining whether an eosinophil survives or undergoes apoptosis upon Fas ligand stimulation.

Inhibition of eosinophil apoptosis has been proposed as a key mechanism for the development of blood and tissue eosinophilia in diseases such as bronchial asthma and other allergic disorders (1, 2). The delay of eosinophil death might be due, at least in part, to overproduction of T cell–derived cytokines (2–9). Besides cytokines, eosinophil apoptosis also seems to be regulated by at least one member of the TNF/nerve growth factor (NGF) receptor superfamily, namely the Fas receptor (CD95/APO-1) (10–13). Cross-linking of the Fas receptor is associated with the induction of apoptosis in eosinophils from normal individuals. In contrast, blood and tissue eosinophils derived from eosinophilic donors often do not undergo cell death after Fas receptor cross-linking, although Fas protein is normally expressed in these cells, suggesting that receptor activity is regulated as previously observed in other systems (13). Fas receptor susceptibility does not seem to be regulated by cytokines that promote eosinophil survival (10, 11).

Patients with bronchial asthma and allergic rhinitis show an increased level of nitric oxide (NO)1 in exhaled air (14, 15). NO originates from the biotransformation of l-arginine to l-citrulline by an enzyme called NO synthase (NOS; 16). There is evidence that the inducible isoform of NOS (iNOS) is expressed in the bronchial mucosa of patients with bronchial asthma, but not of normal control individuals (17), suggesting that increased levels of NO may result from increased NO production by iNOS. Recently, it has been demonstrated that eosinophils themselves are a source of NO production in eosinophilic inflammation (18).

The pathophysiological consequences of increased NO production in allergic diseases are not yet known. However, it is clear that NO has effects on immune responses. For example, NO inhibits both the proliferation of Th1s and their production of IL-2 and IFN-γ, as demonstrated in several infectious disease models (19, 20). In contrast, Th2s are not affected by NO (21). These data suggest that increased amounts of NO may contribute to a preferential Th2 response in allergic diseases of the respiratory tract (22).

Abbreviations used in this paper: DAG, 1,2-diacyl-sn-glycerol; db, dibutyryl; DISC, death-inducing signaling complex; ECL, enhanced chemiluminescence; IBMX, 3-isobutyl-1-methylxanthine; ICE, IL-1 converting enzyme; iNOS, inducible isoform of NOS; JNK, Jun kinase; LY 83583, 6-anilinoquinoline-5,8-quinone; MAP, mitogen-activated protein; mRNA, messenger RNA; NMMA, N6-monomethyl-l-arginine; NO, nitric oxide; NOS, NO synthase; PS, phosphatidylserine; SNAP, S-nitroso-N-acetylpenicillamine.
Consequently, we were interested in whether NO may also play a role in later events of Th2 responses such as inhibition of eosinophil apoptosis (1, 2). A possible involvement of NO in defective Fas ligand–Fas receptor interactions was concluded from studies performed in mice where NO protected liver cells from TNF-induced apoptosis (23). Since at least one signaling cascade in the induction of cell death is common to both TNF and Fas receptors (24), we hypothesized that NO, at least in some cellular systems, may also counterregulate Fas receptor–mediated apoptosis. In this study, we demonstrate that NO mediates a functional defect in the Fas receptor signal transduction cascade in human eosinophils.

Materials and Methods
Reagents and Antibodies
All cell cultures were performed using complete culture medium, which was RPMI 1640 supplemented with 10% fetal calf serum (both Life Technologies, Basel, Switzerland). SNAP (S-nitroso-N-acetylpenicillamine), dibutyryl-cGMP, dibutyryl-cAMP, C2-ceramide, C2-ceramide, C2-dihydroceramide, DAG (1,2-diacyl-sn-glycerol), IBMX (3-isobutyl-1-methylxanthine), LY 83583 (6-anilinoquinoline-5,8-quinone), and GST-fusion protein of c-Jun (GST-c-Jun, 1-79) were from Biomol (Plymouth Meeting, PA). Azide and hydroxyamine hydrochloride were obtained from Fluka (Buchs, Switzerland). The IL-1 converting enzyme (ICE) inhibitor II (N-acetyl-Tyr-Val-Ala-Asp-chloromethylketone; Ac-YVAD-cmk) was obtained from Bachem (Bubendorf, Switzerland). Nω-monomethyl-L-arginine (NωMA) was from Alexis Corp. (Läufelfingen, Switzerland). The concentrations used for all these reagents are indicated in the text and figure legends. Anti-CD16 mAb microbeads were from Miltenyi Biotec (Bergisch-Gladbach, Germany). Unconjugated (clone CH-11, IgM) and FITC-conjugated (clone UB2, IgG1) anti-CD16 mAb were from Immunotech (Marseille, France). For functional assays, anti-Fas mAb (IgM) was used at 1 

Eosinophil Purification
Eosinophils were purified as previously described (7–9, 13, 25, 26). The resulting cell populations contained 99% eosinophils as controlled by staining with Diff-Quik (Baxter, Düdingen, Switzerland) and light microscopy.

Determination of NO Production in Eosinophilic Inflammation (Nasal Polyposis)
NO was measured in the nasal cavity of 20 patients with nasal polyps and 20 normal control individuals. Both groups were age and sex matched. Analysis was performed with a chemiluminescence analyzer (Eco Physics, Dünten, Switzerland), which measures emitted light caused by the reaction NO + O$_2$ = NO$_2$ + O$_2$. One nasal olive was closed using an occlusive nasal olive from a rhinomanometer (H. Omo hoy, Hamburg, Germany), and connected to a syringe that had access to the nasal vestibulum. The contralateral nostril was open. Subjects were asked to keep the mouth closed. Thus, breathing was carried out through the open nostril only. This allowed analysis of the local NO concentration with free flow of air from one nostril to the other via the nasopharynx. Using the syringe, 20 ml of nasal air was aspirated over a time period of 15 s. The aspirated air was injected into the analyzer. NO concentrations were recorded by an on-line microcomputer and given in parts per billion. The analyzer was calibrated before each experiment using certified NO mixtures. NO concentrations in the room were <5 parts per billion.

Determination of C Cell Death
Eosinophils (109/ml) were cultured in the presence or absence of anti-Fas mAb or ceramides in the indicated conditions and for the indicated times. Cell death of eosinophils was assessed by uptake of anti-Fas mAb or ceramides in the indicated conditions and for the indicated times. Cell death of eosinophils was assessed by uptake of anti-Fas mAb or ceramides in the indicated conditions and for the indicated times. Cell death of eosinophils was assessed by uptake of anti-Fas mAb or ceramides in the indicated conditions and for the indicated times.
Determination of Acidic Sphingomyelinase Activity

Eosinophils were cultured in the presence or absence of C2-Ceramide (C2) in the indicated conditions for 60 min. Acidic sphingomyelinase activity in eosinophils was determined with an in vitro assay as previously described (29).

Detection of JNK Activation

JNK activation was determined with an in vitro assay as previously described (31). In brief, purified eosinophils were cultured in the presence or absence of anti-Fas mAb in the indicated conditions for the indicated times. Cells were washed twice with 1 ml homogenization buffer (10 mM Hepes, 1 mM EGTA, pH 7.4, and 10 mM PM SF), and then resuspended in 100 μl of this buffer. Cells were lysed by sonication. 500 μl of fluorescamine solution (0.3 mg/ml in acetonitrile) were added to a quartz cuvette (Hellma, Basel, Switzerland) containing 1 ml of a 1:100 dilution of cell lysates in homogenization buffer. Fluorescence was measured at excitation and emission wavelengths of 390 nm and 480 nm, respectively, using a Hitachi F-2000 spectrophotometer. Fluorescence was quantified by measuring the change in fluorescence intensity (cps) of the standard using a fluorescamine assay kit (Pierce, Rockford, IL).

Detection of Total Intracellular Proteolytic Activity

Protein fragmentation occurring during Fas receptor-mediated apoptosis in eosinophils was first determined by the fluorescamine assay as previously described (31). In brief, purified eosinophils were cultured in the presence or absence of anti-Fas mAb in the indicated conditions and for the indicated times. Cells (10^6) were washed twice with 1 ml homogenization buffer (10 mM Hepes, 1 mM EGTA, pH 7.4, and 10 mM PM SF), and then resuspended in 100 μl of this buffer. Cells were lysed by sonication. 500 μl of fluorescamine solution (0.3 mg/ml in acetonitrile) were added to a quartz cuvette (Hellma, Basel, Switzerland) containing 1 ml of a 1:100 dilution of cell lysates in homogenization buffer. Fluorescence was measured at excitation and emission wavelengths of 390 nm and 480 nm, respectively, using a FluorMax spectrophotometer (Spx Industries Inc., Edison, NJ). Intracellular proteolytic activity was determined as fluorescence intensity (cps)/μg protein cell lystate added to the cuvette. Protein concentrations in the cell lysates were measured by a Bradford protein assay (BioRad Labs., München, Germany).

Zymogram Gels

To further confirm the data obtained using the fluorescamine assay, we used Zymogram gels (Novex, San Diego, CA). These 0.1% gelatin-containing and standardized 10% SDS-polyacrylamide gels can be used to detect a wide variety of proteases that can use gelatin as a substrate (32). After cell culture, eosinophils (5 x 10^6) were washed with cold PBS, resuspended in 50 μl of sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 0.0025% bromophenol blue, and 1% glycerol), and lysed by sonication. Electrophoresis was conducted under standard conditions according to the manufacturer’s instructions. Gels were incubated in renaturing buffer (0.25% Triton X-100) for 30 min, and then briefly washed with developing buffer (5 mM Tris-HCl, pH 7.6, 20 mM NaCl, 0.5 mM CaCl2, and 0.002% Brj 35). The gels were stained in 0.5% Coomassie Blue R 250, and partially destained in destaining solution (10% CH3COOH, and 40% CH3OH) to make digested gelatin spots visible.

Immunoblotting

We measured expression and/or degradation of lamin B2 in eosinophils that had been cultured in the presence or absence of anti-Fas mAb in the indicated conditions and for the indicated times. Eosinophils (10^6) were washed, resuspended in Lämmli buffer, and lysed by sonication. Equal sample volumes of whole cell lysates were applied to an 8% SDS polyacrylamide gel, and separated proteins were electrotransferred onto a nitrocellulose filter (Hyclone-enhanced chemiluminescence [ECL]; Amersham Intl., Buckinghamshire, UK). The blots were blocked at room temperature for 1 h in blocking solution (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, and 5% BSA)). Filters were incubated in 1% BSA blocking solution containing 0.5 μg/ml anti-lamin B2 mAb at room temperature for 1-2 h, followed by incubation with horseradish peroxidase (HRP)-conjugated antimurine IgG1 mAb (1:2,000; Amersham Intl.). Blots were developed by an ECL technique (ECL kit; Amersham Intl.) according to the manufacturer’s instructions.

Results

Increased NO Production in Nasal Polyp Tissues

We have previously reported that eosinophils in nasal polyp tissues often have a decreased susceptibility to undergo apoptosis after Fas receptor activation (13). Recently published work suggests that NO may protect liver cells from TNF receptor-mediated apoptosis (23). To verify a potential role of NO for Fas receptor resistance in eosinophils, we measured NO concentrations in the nasal cavity in 20 healthy subjects and 20 patients with nasal polyps. As shown in Fig. 1, NO concentrations were increased by fourfold in patients with nasal polyps, suggesting increased production of NO in this model of eosinophilic inflammation.

Increased NO production is often achieved by activation of the iNOS gene (22). Consequently, we investigated the pattern of iNOS gene expression by reverse transcription PCR in nasal polyp tissues. The results are shown in Fig. 1. A, NO concentrations in nasal polyps compared to normal nasal controls were expressed detectable iNOS messenger RNA (mRNA), as indicated by the presence of a PCR product with the expected size of 257 bp. NO mRNA specificity of this PCR product was demonstrated by hybridization to an iNOS-specific probe. β-actin-specific amplification products were of similar intensity between all samples, suggesting equality of the RNA populations. Thus, it is likely that the expression of iNOS in nasal polyp tissues is increased in eosinophilic inflammation.

Figure 1. Role of NO in eosinophilic inflammation. (A) Increased NO concentrations in the nasal cavity of patients with nasal polyps compared to normal control individuals. Measurements were performed with a chemiluminescence analyzer as described in Materials and Methods. Values are means ± SEM. This figure includes results from 20 different individuals in each group (*, P < 0.001). (B) iNOS mRNA is expressed in nasal polyp tissues. In contrast, under the same experimental conditions, control nasal tissues expressed little or no iNOS mRNA. RNA was isolated, and first-strand synthesis was performed. Human iNOS cDNA was amplified by PCR using specific iNOS primers. The PCR products were electrophoresed on a 1% agarose gel, transferred onto a nitrocellulose filter, and hybridized with a random-primed fluorescein-12-dUTP-labeled iNOS probe. As a control, human β-actin cDNA was amplified and PCR products were stained with ethidium bromide in an agarose gel. Data from four different patients with nasal polyps and two control individuals are presented.
Moreover, addition of 1 mM NMMA (NOS inhibitor) or a factor that counterregulates Fas receptor–mediated death. This suggests that activated U937 cells produce eosinophil numbers averaged 63 ± 2% after 24 h of in vitro culture. Anti-Fas mAb treatment accelerated eosinophil death by twofold (31 ± 3%). Azide, in the range of 0.02 to 1.2 mM, hydroxylamine, in the range of 0.3 to 1.2 mM, and SNAP, in the range of 0.001 to 1 mM, significantly inhibited Fas receptor–mediated eosinophil death. At optimal concentrations, the rescue effect was 95% for azide (1.2 mM), 89% for hydroxylamine (1.2 mM), and 100% for SNAP (1 mM) (Fig. 3 B). Similar to the coculture system, LY 83583 abrogated the protective effect of 1 mM SNAP on anti-Fas mAb-induced eosinophil death. Moreover, the specific eosinophil survival factor IL-5 (3, 4) had only little rescue effects (17%) in this system (Fig. 3 B). In addition, similar to IL-5, IFN-γ, IL-3, and GM-CSF were unable to significantly protect eosinophils from Fas receptor–mediated death (not presented). Furthermore, and in contrast to the strong rescue effect of NO on Fas receptor–activated cells, it is important to note that all NO donors only slightly inhibited spontaneous eosinophil death (16% for azide, 18% for hydroxylamine, and 5% for SNAP).

Using a DNA fragmentation assay and flow cytometric analysis of surface PS expression, we demonstrated that the anti-Fas mAb triggered apoptotic cell death. This apoptotic cell death was inhibited by NO. We found 35 ± 2% DNA fragmentation in nontreated purified eosinophils after 16 h of in vitro culture (Fig. 4 A a). Anti-Fas mAb treatment of eosinophils increased the levels of DNA fragmentation to 64 ± 4% DNA (Fig. 4 A b). In this system, SNAP inhibited Fas receptor–mediated apoptosis in a dose-dependent manner (Fig. 4 A, c, and d and right). Similar results were obtained using azide and hydroxylamine as NO donors (not presented). Moreover, anti-Fas mAb–induced externalization of PS, an early marker of apoptosis (28), was inhibited in the presence of 1 mM SNAP, further suggesting that NO inhibits typical apoptotic processes induced by Fas receptor activation in human eosinophils (Fig. 4 B).

NO Inhibits Fas Receptor–induced Apoptosis of Eosinophils. To more directly determine a possible role of NO for the reduction of eosinophil sensitivity to Fas receptor–mediated apoptosis, we cocultured stimulated monocytic-like U937 cells with anti-Fas receptor–activated freshly purified eosinophils. U937 cells were stimulated with LPS and IFN-γ to induce iNOS expression and NO production. As shown in Fig. 2, eosinophils undergo normal Fas receptor–mediated apoptosis under conditions where U937 cells were not stimulated. In contrast, LPS– and IFN-γ–stimulated U937 cells prevented eosinophil apoptosis in this coculture system. This suggests that activated U937 cells produce a factor that counterregulates Fas receptor–mediated death. Moreover, addition of 1 mM NMMA (NOS inhibitor) or 10 μM LY 83583 (sGC inhibitor; NO signals via sGC; reference 27) abolished the effect of stimulated U937 cells on Fas receptor–mediated eosinophil apoptosis (Fig. 2). In addition, since activated U937 cells may also produce eosinophil survival factors such as GM-CSF, we also used a neutralizing anti-human GM-CSF mAb in this system, and observed no effect on Fas receptor–mediated eosinophil apoptosis (not presented). These data suggest that NO, but not GM-CSF, is involved in U937-mediated Fas receptor resistance of eosinophils.

To complement these studies and to establish a functional approach without the need of a coculture system, we generated NO in pure eosinophil cultures either by endogenous catalase from azide or hydroxylamine or by hydrolysis from SNAP (33, 34). As shown in Fig. 3 A, cell viability of purified eosinophils from individuals with normal or slightly increased eosinophil numbers averaged 63 ± 2% after 24 h of in vitro culture. Anti-Fas mAb treatment accelerated eosinophil death by twofold (31 ± 3%). Azide, in the range of 0.02 to 1.2 mM, hydroxylamine, in the range of 0.3 to 1.2 mM, and SNAP, in the range of 0.001 to 1 mM, significantly inhibited Fas receptor–mediated eosinophil death. At optimal concentrations, the rescue effect was 95% for azide (1.2 mM), 89% for hydroxylamine (1.2 mM), and 100% for SNAP (1 mM) (Fig. 3 B). Similar to the coculture system, LY 83583 abrogated the protective effect of 1 mM SNAP on anti-Fas mAb-induced eosinophil death. Moreover, the specific eosinophil survival factor IL-5 (3, 4) had only little rescue effects (17%) in this system (Fig. 3 B). In addition, similar to IL-5, IFN-γ, IL-3, and GM-CSF were unable to significantly protect eosinophils from Fas receptor–mediated death (not presented). Furthermore, and in contrast to the strong rescue effect of NO on Fas receptor–activated cells, it is important to note that all NO donors only slightly inhibited spontaneous eosinophil death (16% for azide, 18% for hydroxylamine, and 5% for SNAP).

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NO Causes Reduced Fas Receptor Sensitivity via sGC in Eosinophils. sGC is generally accepted as being the main molecular target of NO leading to the production of cGMP. The subsequent regulation of cGMP–dependent protein kinases, nucleotide phosphodiesterases, and ion channels mediates most of the physiological NO functions (35). Therefore, we mimicked intracellular cGMP increases after sGC activation by adding cell membrane-permeable dibutyryl (db)-cGMP to the in vitro cultures. Similarly to SNAP, azide, or hydroxylamine, db-cGMP reduced anti-Fas mAb–induced death in purified eosinophils in a dose-dependent manner (Fig. 5 A). The inhibition of cell death was 100% at...
we excluded the possibility of Fas receptor downregulation in eosinophils. In preliminary experiments, we demonstrated enhancement of intracellular cGMP and cAMP levels. Previously published work suggested that acidic SMase-generated ceramide is involved in the Fas receptor-mediated activation of SMase (Fig. 6A). In fact, the db-cAMP rescue effect was observed at concentrations between 1 and 50 mM, even more potent than db-cGMP.

cGMP and cAMP are both targets of regulatory phosphodiesterases that cleave these molecules to 5'-GMP and 5'-AMP, respectively. To confirm the roles for cGMP and cAMP in the reduction of eosinophil sensitivity to Fas receptor-mediated apoptosis, we next inhibited phosphodiesterases by using IBMX to increase intracellular cAMP. Similarly to db-cGMP, db-cAMP was an effective blocker of Fas receptor-mediated eosinophil death (Fig. 5A). In fact, the db-cAMP rescue effect was observed at concentrations between 1 and 50 mM, even more potent than db-cGMP.

We next investigated whether SMase-generated ceramide could induce apoptosis in eosinophils as it has been reported in other systems (29, 37, 38). Indeed, 10-50 µM C2- or C6-ceramide induced, in a dose-dependent manner, Fas receptor-mediated apoptosis (not presented). Rather, this implied that the reduced sensitivity of NO-treated eosinophils to Fas receptor-mediated apoptosis reflected a functional defect to transduce signals via this receptor. Previously published work suggested that acidic SMase-generated ceramide is involved in the Fas receptor signaling pathway leading to apoptosis (29, 37, 38). Therefore, the effect of anti-Fas mAb treatment on SMase activity was studied in the absence and presence of optimal concentrations of SNAP. Enzyme activity was determined by degradation of radioactively labeled sphingomyelin (SM) in an in vitro assay. As shown in Fig. 6A, anti-Fas mAb treatment was followed by induction of SMase activity in both SNAP-untreated and -treated eosinophils, indicating that NO may act distally from SM breakdown. In contrast, the ICE inhibitor II Ac-YVAD-cmk abrogated Fas receptor-mediated activation of SMase (Fig. 6A), suggesting that SM breakdown is distal to early ICE protease activation in the Fas receptor signaling pathway.

We next investigated whether SMase-generated ceramide could induce apoptosis in eosinophils as it has been reported in other systems (29, 37, 38). Indeed, 10-50 µM C2- or C6-ceramide induced, in a dose-dependent manner, eosinophil death (Fig. 6B). In contrast, the biologically inactive structural analogue of C2-ceramide, C2-dihydroceramide, had no effect on cell death in the same concentration range. Higher ceramide concentrations were found to damage the cell membrane. To determine whether NO can counterregulate ceramide-induced apoptosis, untreated and C2-ceramide-treated eosinophils were incubated in the absence and presence of optimal concentrations of SNAP, db-cGMP, and db-cAMP. SNAP, db-cGMP, and db-cAMP inhibited C2-ceramide-induced cell death (Fig. 6C) and DNA fragmentation (not presented). These data suggest that NO, as well as its second messengers, counterregulate Fas receptor signaling distal to ceramide generation.
NO prevents Fas receptor–mediated apoptosis in freshly purified eosinophils. (A) SNAP inhibited, in a dose-dependent manner, Fas receptor–mediated DNA fragmentation. Eosinophils were cultured for 16 h. DNA fragmentation (black) was determined by DNA staining with propidium iodide and flow cytometric analysis. (Left) a, untreated; b, anti-Fas mAb; c, anti-Fas mAb plus 0.5 mM SNAP; d, anti-Fas mAb plus 1 mM SNAP. (Right) Values are means ± SEM of three independent experiments. (B) Inhibition of phosphodiesterase activity by IBMX enhanced low-dose NO effects. 0.5 mM, but not 0.1 mM, SNAP inhibited Fas receptor-mediated eosinophil death. However, 0.1 mM SNAP could completely block death when IBMX was added. Experiments and analysis were performed as described in A. Values are means ± SEM of three independent experiments.

**Figure 5.** Fas receptor–mediated death is prevented by NO-induced second messengers in freshly purified eosinophils. (A) cGMP and cAMP inhibited, in a dose-dependent manner, Fas receptor–mediated death. In contrast, DAG had no effect in this system (not presented). The dashed line represents cell viability of untreated eosinophils. Eosinophils were cultured for 24 h. Cell viability was assessed by uptake of 1 μM ethidium bromide and flow cytometric analysis. Values are means ± SEM of three independent experiments. (B) Inhibition of phosphodiesterase activity by IBMX enhanced low-dose NO effects. 0.5 mM, but not 0.1 mM, SNAP inhibited Fas receptor-mediated eosinophil death. However, 0.1 mM SNAP could completely block death when IBMX was added. Experiments and analysis were performed as described in A. Values are means ± SEM of three independent experiments.
SNAP by Western blotting. As shown in Fig. 7C, complete cleavage of lamin B1 occurred within 4 h after anti-Fas mAb treatment. In contrast, optimal concentrations of SNAP completely inhibited Fas receptor-mediated cleavage of lamin B1, whereas a suboptimal SNAP concentration was only partially protective. In addition, a second protein with a molecular mass of ~70 kD reacted with the antibody, probably representing an early degradation product of lamin B1. These data suggest that NO blocks the proteolytic action of proteases on specific target proteins that are important for the apoptotic process, such as lamin B1.

Discussion

Allergic diseases of the respiratory tract are associated with a marked eosinophilic inflammation (46) and enhanced cytokine production (47). Some of these cytokines inhibit eosinophil apoptosis and may, therefore, contribute to tissue eosinophil accumulation (1, 2). In addition to these effects, there is evidence to suggest upregulation of iNOS in eosinophilic inflammation airways (17). Consequently, measurements of NO in exhaled air have demonstrated elevated levels in asthma and seasonal allergic rhinitis patients (14, 15, 22). In agreement with these studies, we measured increased NO levels within the nasal cavity of patients with nasal polyps. We also provided evidence for increased iNOS mRNA expression in polyp tissues. Together with the previously published work (14, 15, 22), these data suggest that...
increased NO production may represent a general feature of eosinophilic inflammation.

Fas receptors are broadly expressed on many different cell types. Activation of the Fas receptors leads to induction of apoptosis in many, but not all systems (48). For example, hematopoietic and non-hematopoietic tumor cells resistant to Fas receptor-mediated apoptosis have been found, although they express the receptor on the cell surface (49, 50). Therefore, the mechanisms of Fas receptor resistance have generated great interest. The results presented in this study suggest a novel NO-mediated mechanism causing non-functional Fas receptors by disruption of the death signaling pathway in human eosinophils.

Extensive analysis of the signaling pathways associated with Fas receptor-mediated apoptosis has revealed that oligomerization of the receptor induces conformational changes of the intracellular domains leading to binding of cytoplasmic proteins and formation of the death-inducing signaling complex (DISC; 51). One of the DISC proteins is an ICE-like protease (24, 52). Thus, perhaps there is a direct physical connection between initialization of the death signal at the cell membrane and the death machinery (48). However, other cell signaling events involving tyrosine phosphorylation (53), SMase-ceramide (29, 37, 38), and Ras/MAP kinase pathways (39, 40) have also been reported to be involved in cell death. The contribution of all of these pathways to the induction of apoptosis as well as possible interactions between different pathways have not yet been clearly elucidated.

Ceramide, generated by activated SMase, triggers apoptosis in response to Fas receptor activation (29, 37, 38) and many other death stimuli (54). Thus, ceramide production appears to be a pleiotropic activator of apoptosis. Therefore, we first analyzed activation of SMase after Fas receptor activation in the presence and absence of NO or second messengers of NO. This strategy allowed us to determine whether the possible disruption of the Fas receptor signaling pathway by NO occurs proximal or distal to SMase activation. We observed a Fas receptor-mediated activation of SMase in both NO-untreated and -treated eosinophils, suggesting that NO may block the death signal distal to SMase. The possibility to inhibit Fas receptor-initiated signaling events downstream of SMase activation has been previously demonstrated in cells overexpressing Abl tyrosine kinases (55). In contrast to the effects observed with NO donors, activation of SMase was abrogated when the tetrapeptide YVAD was used to block activation of the ICE protease. In agreement with a previously published work (56), these data demonstrate that activation of ICE proteases is required for SMase activation. Moreover, these data indicate that NO did not disrupt DISC formation in Fas receptor-activated eosinophils.

There has been some progress in the identification of ceramide targets. The first reported target for its activity was a serine/threonine protein kinase, termed ceramide-activated protein kinase, that was only recently identified (57). The target of this kinase appears to be the protooncogene Raf (57). Moreover, Fas receptor activation and ceramide induction activation of another, alternative MAP kinase pathway resulting in JNK stimulation. JNK activation has been shown to be critical for induction of apoptosis in many systems (30, 39). To examine the possibility of a functional JNK inactivation by NO, we monitored kinase activity after anti-Fas mAb or ceramide treatment of eosinophils in the presence and absence of NO or second messengers of NO. The results suggest that JNK activation is also necessary for Fas receptor-mediated apoptosis in eosinophils. Moreover, since second messengers of NO prevent c-Jun phosphorylation, NO may act at the level of, or proximal to, JNK activation to prevent death.

It is now clear that proteases play a key role in apoptosis (43, 48). This study provides evidence that NO prevents Fas receptor-mediated proteolysis. However, the observation that it is possible to block activation of SMase by using an inhibitor of ICE suggests that there is some protease activation even in the presence of NO. It has recently been demonstrated that activation of a protease can lead to further activation of other proteases within the apoptotic pathway (58), generating a protease cascade (43, 48). We hypothesize at this point that the generation of ceramide and subsequent JNK activation may represent a signaling event responsible for amplification of the proteolytic cascade. Therefore, disruption of ceramide-induced signals prevents further proteolysis. This idea is further supported by very recent reports demonstrating that the central ICE-like protease CPP32 (Yama/Apopain) is not only a target of ICE (58) but, at least in a later phase of the apoptotic death process, also of MAP kinase and JNK signaling pathways (59). Thus, it is possible that, even in the presence of NO, activation of eosinophils via the Fas receptor leads to an immediate but limited activation of proteases able to degrade only a limited number of substrates. These can then be replaced without any damage to the cell. In contrast, in the absence of NO, ceramide-mediated amplification of the proteolytic cascade takes place and the apoptotic process initialized via the Fas receptor proceeds, causing irreversible damage to the cell.

In summary, the data reported here indicate that NO, a secretory product released in increased amounts within chronic eosinophilic inflammatory responses such as bronchial asthma and other chronic-allergic disorders, disrupts Fas receptor-mediated apoptosis in eosinophils. Therefore, we have identified a mechanism of Fas receptor resistance that might contribute to the eosinophilia associated with these diseases. In this context, it is tempting to speculate that glucocorticoids, which are known to suppress NO concentration in asthmatic patients (15), decrease eosinophil numbers, besides other possible mechanisms, by sensitization of the Fas receptor. Moreover, we have localized the disruption of the Fas signaling pathway by NO at the level of, or proximal to, JNK, but distal to SMase activation. We also provide evidence that the ceramide-induced apoptotic response may serve as an amplification step within the proteolytic cascade of the apoptotic process that, on the other hand, can be counterregulated by additional signals.
References


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