

Rapid publication

Regulation of eosinophil apoptosis by nitric oxide: Role of c-Jun-N-terminal kinase and signal transducer and activator of transcription 5

Xianzhi Zhang, MSc,^a Eeva Moilanen, MD, PhD,^{a,b} Aleksi Lahti, MD,^a
Mari Hämäläinen, MSc,^a Mark A. Giembycz, PhD,^c Peter J. Barnes, MD, DSc, FRCP,^c
Mark A. Lindsay, PhD,^d and Hannu Kankaanranta, MD, PhD^{a,e} Tampere, Finland, and
Macclesfield and London, United Kingdom

Background: Reduced eosinophil apoptosis is considered to be a key mechanism for eosinophilia in allergic diseases such as asthma, rhinitis, and eczema.

Objective: The aim of our study was to investigate the possible modulatory effect of nitric oxide (NO) in human eosinophils.

Methods: Apoptosis in isolated eosinophils was assessed by relative DNA fragmentation assay, annexin-V binding, and morphologic analysis. The activation of c-Jun-N-terminal kinase (JNK) and signal transducer and activator of transcription 5 (STAT5) was assessed by immunoblot analysis.

Results: The NO donor S-nitroso-N-acetylpenicillamine (SNAP) reversed the survival-prolonging effect of IL-5 by inducing apoptosis. This effect was blocked by the NO scavenger (2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide.potassium salt), indicating that reversal of IL-5-mediated eosinophil survival was due to NO. The effect of NO on IL-5-afforded cell survival was not mediated by cyclic guanosine 3': 5'-monophosphate (cGMP), because neither an inhibitor of guanylyl cyclase nor inhibitors of phosphodiesterases had any effect on SNAP-induced eosinophil apoptosis in IL-5-treated cells. SNAP induced a time-dependent increase in the activity of JNK, and an inhibitor peptide specific for

JNK, L-JNK11, completely reversed SNAP-induced apoptosis in IL-5-treated eosinophils. In contrast, SNAP did not inhibit IL-5-induced STAT5 activation. Inhibition of the activity of caspases by Z-Asp-CH₂-DCB reversed the effect of SNAP, suggesting that NO promotes apoptosis in IL-5-treated human eosinophils in a caspase-dependent manner. However, this effect of NO was not mediated by means of activation of caspases 3, 8, or 9.

Conclusions: Our results suggest that exogenous NO reverses IL-5-mediated eosinophil survival by inducing apoptosis, and this is mediated by means of activation of JNK in a cGMP-independent manner. (J Allergy Clin Immunol 2003;112:93-101.)

Key words: Eosinophils, apoptosis, nitric oxide, c-Jun-N-terminal kinase, cGMP, STAT5, caspases

Eosinophils are thought to play a critical role in allergic diseases, such as allergic rhinitis, asthma, and atopic dermatitis.¹ In patients with asthma, activation of eosinophils in the airways is thought to cause epithelial tissue injury, contraction of airway smooth muscle, and increased bronchial responsiveness.² Apoptosis or programmed cell death might be an important feature in the resolution of asthmatic inflammation.³ Apoptosis is characterized by specific biochemical and morphologic changes, including cell shrinkage, surface blebbing, DNA fragmentation, and loss of nucleoli,⁴ so that the apoptotic cell is phagocytosed intact without release of its contents. In vitro, eosinophil apoptosis is inhibited by cytokines, such as IL-3, IL-5, and GM-CSF.^{1,3,4} Eosinophil apoptosis is upregulated by Fas (CD95/APO-1), a 45-kDa transmembrane protein belonging to the TNF receptor family.⁵ We and others have previously shown that eosinophil apoptosis is delayed in patients with asthma or inhalant allergy.^{6,7} The number of eosinophils in the asthmatic lung is elevated and is inversely correlated with the number of apoptotic eosinophils.⁸

Nitric oxide (NO) is enzymatically synthesized from the amino acid L-arginine by enzymes called NO synthases (NOS), which are classified into the constitutive neu-

From ^aThe Immunopharmacological Research Group, Medical School, University of Tampere; ^bthe Department of Clinical Chemistry, Tampere University Hospital; ^cthe Department of Thoracic Medicine, Imperial College School of Medicine at the National Heart and Lung Institute, London; ^dEST-Biology, AstraZeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield; and ^eDepartment of Respiratory Medicine, Tampere University Hospital.

Supported by Jalmari and Rauha Ahokas Foundation (Finland), Tampere Tuberculosis Foundation (Finland), the Finnish Anti-tuberculosis Association Foundation (Finland), the Academy of Finland, and the Medical Research Fund of Tampere University Hospital (Finland).

Received for publication October 30, 2002; revised April 3, 2003; accepted for publication April 8, 2003.

Reprint requests: Hannu Kankaanranta, MD, PhD, Medical School, FIN-33014, University of Tampere, Tampere, Finland.

© 2003 Mosby, Inc. All rights reserved.

0091-6749/2003 \$30.00 + 0

doi:10.1067/mai.2003.1587

Abbreviations used

Carboxy-PTIO: 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide.potassium salt)
 cGMP: Guanosine 3': 5'-monophosphate
 GEA 3175: 1,2,3,4-oxatriazolium,-3-(3-chloro-2-methylphenyl)-5-[[4-(4-methylphenyl)sulfonyl] amino]-, hydroxide inner salt
 IBMX: Isobutylmethylxanthine
 iNOS: Inducible NO synthase
 JNK: c-Jun-N-terminal kinase
 NO: Nitric oxide
 ODQ: 1H-[1,2,4] oxadiazolo [4,3,- α]quinoxalin-1-one
 PDE: Phosphodiesterase
 SNAP: S-nitroso-N-acetylpenicillamine
 STAT5: Signal transducer and activator of transcription 5

ronal (nNOS) and endothelial (eNOS) enzymes and the inducible enzyme (iNOS).⁹ There is little or no expression of iNOS under normal circumstances, but its synthesis is rapidly stimulated by a wide range of inflammatory cytokines and bacterial products.⁹ NO has many physiologic and pathophysiologic functions, such as regulation of vascular tone, platelet aggregation, and the immune response.⁹ The biologic effects of NO can be divided into cyclic guanosine 3': 5'-monophosphate (cGMP)-mediated and cGMP-independent mechanism. Although NO can affect the cellular functions through posttranslation modifications of proteins directly (nitrosylation and nitration) and indirectly (methylation and ribosylation), the main physiologic signaling pathway of NO is considered to be the activation of guanylyl cyclase, formation of cGMP, and concomitant protein phosphorylation by means of the activation of protein kinase G.^{10,11} Increased evidence suggests that NO is involved in the pathogenesis of inflammation and associated tissue destruction.¹² The role of NO in bronchial asthma is still incompletely understood. Patients with bronchial asthma and allergic rhinitis show increased levels of NO in exhaled air.¹³ Increased concentration of products of NO are also found in induced sputum in patients with asthma.¹⁴ Moreover, iNOS is expressed in the bronchial mucosa of patients with asthma but not in normal control individuals.¹⁵

Because exhaled NO and sputum eosinophils are raised in subjects with asthma, a relationship between them is plausible. This idea is supported by the finding that production of NO and expression of iNOS have been reported in the human eosinophil.¹⁶ NO disrupts Fas-receptor signaling in human eosinophils, suggesting that it might affect eosinophil survival.¹⁷ The aim of this study was to investigate the effect of NO on apoptosis in IL-5-treated or cytokine-deprived human eosinophils and to explain the molecular mechanism of action of NO on these cells.

METHODS**Eosinophil purification**

Blood (50-100 mL) was obtained from normal individuals. Eosinophils were isolated to >99% purity under sterile conditions as previously reported.^{6,18} The cells were resuspended at 10⁶ cells/mL and cultured in RPMI 1640 or L-arginine, nitrate, and nitrite-free Dulbecco modified Eagle medium supplemented with 100 μ mol/L L-arginine (for nitrite production and NOS inhibitor cultures), 10% fetal calf serum, and antibiotics.

Determination of eosinophil apoptosis

Unless otherwise stated, eosinophil apoptosis was determined by propidium iodide staining of DNA fragmentation and flow cytometry as previously described.^{6,18,19} The cells showing decreased relative DNA content were considered to be apoptotic. Annexin-V binding and morphologic analysis were performed as previously reported.¹⁹ Oligonucleosomal DNA fragmentation in eosinophils was analyzed by agarose gel DNA electrophoresis as previously described.^{18,20}

Nitrite determination, RNA extraction, and RT-PCR

Nitrite was measured as previously described by adding 100 μ L of Griess reagent to 100- μ L samples of medium.²¹ RNA was extracted as previously described.²¹ First-strand cDNA was synthesized using sequence-specific downstream primer for human iNOS or human β -actin, which was used as reference. The Mg²⁺ concentration in the reaction mixture was 1.0 mmol/L. Primers for amplification of β -actin were 5'-TGACTGACTACCTCATGAAGATCCTCACCG-3' (sense) and 5'-CCACGTCACACTTCATGATGGAGTTG-3' (antisense), resulting in a 304-bp product. Primers for amplification of iNOS were 5'-ATGCCAGATGGCAGCATCAGA-3' (sense) and 5'-CACTTCCTCCAGGATGTTGTA-3' (antisense), resulting in a 372-bp product.²² Products were analyzed on 1.5% agarose gel containing ethidium bromide and visualized in UV light.

Immunoblot analysis

Eosinophils were suspended at 10⁶ cells/mL and cultured at 37°C. At the indicated time points, cells were centrifuged at 12,000g for 10 minutes. The cell pellet was lysed by boiling for 5 minutes in 30 μ L of Laemmli buffer ($\times 6$), centrifuged at 12,000g, and debris was carefully removed. Samples were then stored at -20°C until immunoblot analysis. For immunoblot analysis, each protein sample was loaded on 8% to 12% SDS-PAGE gel and electrophoresed for 2 hours at 100 V. The separated proteins were transferred to nitrocellulose membrane (Hybond ECL) with semi-dry blotter and blocked using 5% nonfat dry milk in TRIS-buffered saline containing Tween 20 (0.1%). Proteins were labeled using specific antibody and subsequently detected using horseradish peroxidase-linked anti-rabbit or anti-mouse IgG and imaged on film using ECL Western blotting detection systems. After phospho-signal transducer and activator of transcription 5 (STAT5) was detected, the membrane was striped at 50°C for 30 minutes and washed by TRIS-buffered saline containing Tween 20 (0.1%). To confirm equal protein loading, immunoblots for total STAT5 proteins were carried out using antibody against STAT5 and detected as previously.

Materials

Reagents were obtained as follows: 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide. potassium salt (Carboxy-PTIO), L-arginine, L-c-Jun N-terminal kinase peptide inhibitor 1, L-stereoisomer (L-JNKI1), and L-TAT control peptide (Alexis Corp, L  ufelfingen, Switzerland); Z-Asp-CH₂-DCB, AC-

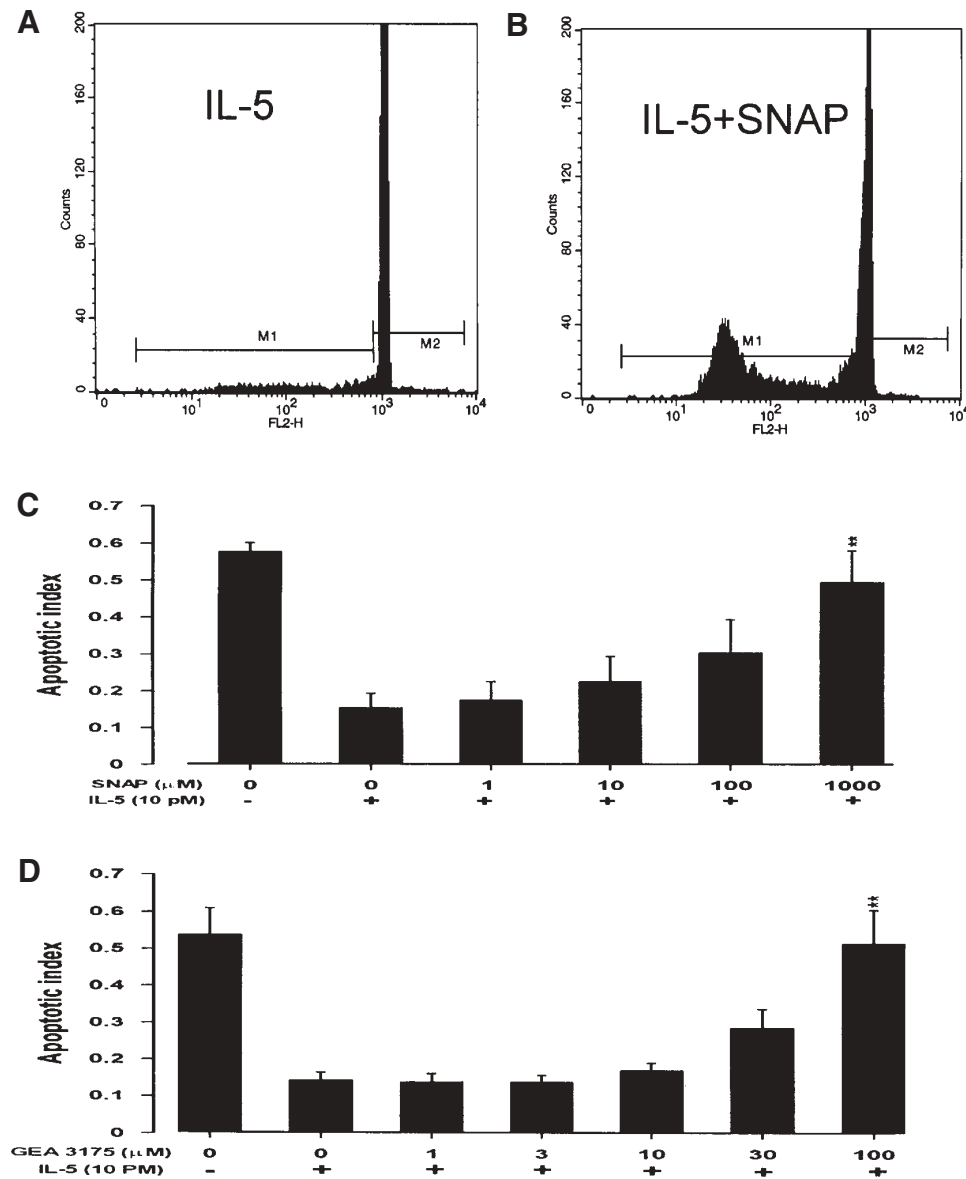


FIG 1. Assessment of percentages of apoptotic (hypodiploid DNA content) and nonapoptotic (diploid DNA) cells in IL-5 (10 pmol/L)-treated eosinophils in the absence (**A**) and presence (**B**) of S-nitroso-*N*-acetylpenicillamine (SNAP; 1000 μ mol/L). The effect of nitric oxide (NO) donors SNAP (**C**, 1-1000 μ mol/L) and GEA3175 (**D**, 1-100 μ mol/L) on apoptosis in IL-5 (10 pmol/L)-treated eosinophils. ** $P < .01$ compared with the respective control in the absence of NO donors and in the presence of IL-5. Each data point represents the mean $\pm$ SEM of 5 (**C**) or 7 (**D**) independent determinations using eosinophils from different donors.

DMQD-CHO, AC-IETD-CHO, AC-LEHD-CHO (Peptide Institute, Inc, Osaka, Japan); ODQ (Tocris, Bristol, UK); IBMX (isobutylmethoxyxanthine; EGA-Chemie, Steinheim, Germany); 8-Br-cGMP, 8-Br-cAMP, dibutyl-cGMP, dibutyl-cAMP, zaprinast (Sigma Chemical Co, Poole, Dorset, UK); polyclonal Bax and monoclonal caspase 3, polyclonal STAT5b, monoclonal pJNK antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif); caspase 8 mAb and Bcl-2 mAb (Oncogene Research Products, Boston, Mass); caspase 9 mAb (NeoMakers, Fremont, Calif); phospho-STAT5 mAb (New England Biolabs, Hitchin, UK); horseradish peroxidase-linked anti-rabbit or anti-mouse IgG (Amersham Life Science, Amersham, UK). Unless otherwise stated, the reagents were obtained as previously

described.^{6,18,19,21-23} The incubation time is 40 hours unless otherwise stated. Carboxy-PTIO, L-JNK1, L-TAT, ODQ, IBMX, zaprinast, and caspase inhibitors were added a moment before S-nitroso-*N*-acetylpenicillamine (SNAP).

Statistics

Results are expressed as means $\pm$ SEMs. Apoptosis is expressed as apoptotic index. Apoptotic index = number of measured apoptotic cells/number of total measured cells. Statistical significance was calculated by analysis of variance for repeated measures supported by the Student-Newman-Keuls test. Differences are considered significant if $P < .05$.

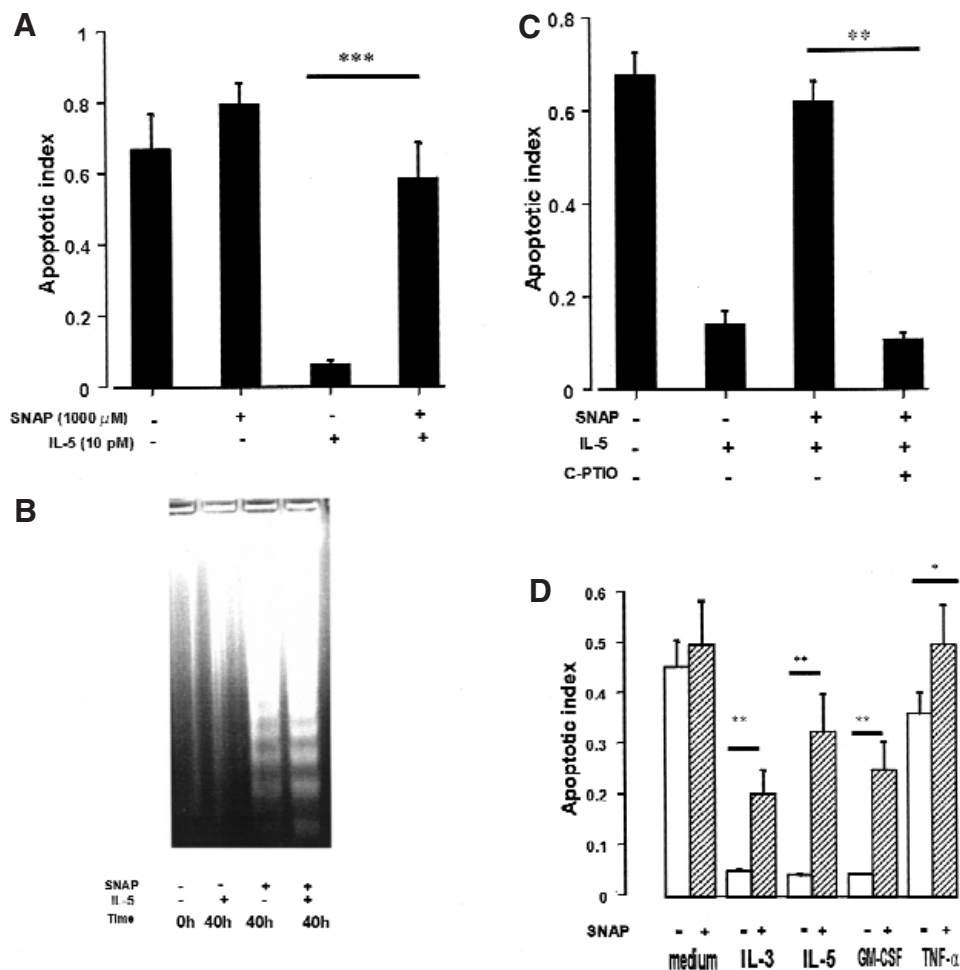


FIG 2. A, The effect of S-nitroso-N-acetylpenicillamine (SNAP) (1000 μ mol/L) on apoptosis in IL-5 (10 pmol/L)-treated cells as analyzed by morphologic criteria. B, The effect of SNAP (1000 μ mol/L) on oligonucleosomal DNA fragmentation in eosinophils in the presence of IL-5 (10 pmol/L). A representative of 2 similar experiments is shown. C, 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (C-PTIO) (50 mmol/L) abolished the apoptosis-inducing effect of SNAP (1000 μ mol/L) in IL-5 (10 pmol/L)-treated eosinophils. D, The effect of SNAP on apoptosis in the presence of IL-3 (100 pmol/L), IL-5 (10 pmol/L), GM-CSF (70 pmol/L), and TNF- α (600 pmol/L) as assessed by annexin-V binding during 24-hour incubation. ** P < .01; *** P < .001 compared with the respective solvent control. Each data point represents the mean $\pm$ SEM of 6 independent determinations using eosinophils from different donors.

RESULTS

Effects of NO on IL-5-mediated eosinophil survival

When eosinophils were cultured in cytokine-deprived conditions for 40 hours, the apoptotic index was 0.43 ± 0.06 ($n = 6$). IL-5 increased cell survival in a concentration-dependent manner during 40-hour incubation by delaying apoptosis. The maximal antiapoptotic effect of IL-5 was obtained at 10 pmol/L concentration (apoptotic index 0.15 ± 0.05 , $n = 6$). NO-donor SNAP (1-1000 μ mol/L) reversed IL-5-mediated cell survival in a concentration-dependent manner by inducing apoptosis (Fig 1, A-C). Structurally unrelated NO-donor 1,2,3,4-oxatriazolum, -3-(3-chloro-2-methylphenyl)-5-[(4-methylphenyl) sulfonyl] amino]-, hydroxide inner salt;

GEA 3175; GEA Ltd., Copenhagen, Denmark; 1-100 μ mol/L)²³ also reversed the IL-5-afforded eosinophil survival (Fig 1, D). When morphologic criteria for apoptosis were used, SNAP increased the apoptotic index in IL-5-treated cells (Fig 2, A). Furthermore, SNAP induced fragmentation of DNA in IL-5-treated cells (Fig 2, B). To see whether the SNAP-induced reversal of IL-5-afforded eosinophil survival was due to the release of NO from SNAP, the effect of a NO-scavenging compound carboxy-PTIO²⁴ was tested. Carboxy-PTIO abolished the apoptosis-inducing effect of SNAP in IL-5-treated eosinophils (Fig 2, C), confirming that the effect is mediated by NO and not the penicillamine moiety of SNAP.

SNAP also reversed the effects of IL-3, IL-5, GM-CSF, and TNF- α on eosinophil survival (Fig 2, D) as analyzed by the annexin-V binding assay. SNAP (1 mmol/L)

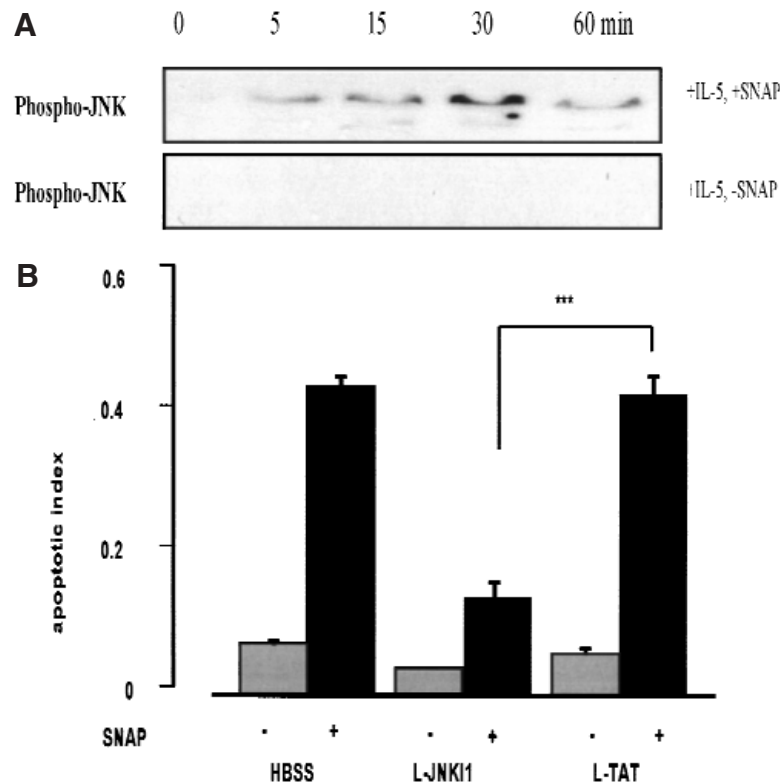


FIG 3. **A**, Effect of S-nitroso-*N*-acetylpenicillamine (SNAP) on phosphorylation of c-jun-N-terminal kinase (JNK). JNK activation was measured using an antibody recognizing the phosphorylated JNK. **A**, representative of 3 similar experiments is shown. **B**, The effect of JNK inhibitor, L-JNKI1 (10 μ mol/L), and the negative control peptide L-TAT (10 μ mol/L) on SNAP (1000 μ mol/L)-induced apoptosis in IL-5-treated eosinophils. Each data point represents the mean $\pm$ SEM of 4 independent determinations using eosinophils from different donors. *** P < .001 compared with the respective SNAP-treated sample in the presence of L-TAT.

also increased constitutive eosinophil apoptosis (apoptotic index 0.40 ± 0.03 and 0.56 ± 0.03 in the absence and presence of SNAP, respectively, $n = 5$, $P < .05$). GEA 3175 (100 μ mol/L) did not enhance constitutive eosinophil apoptosis (apoptotic index 0.54 ± 0.07 and 0.51 ± 0.09 in the absence and presence of GEA 3175, respectively, $n = 7$).

The effect of NO on human eosinophil apoptosis is independent of cGMP

To test whether the induction of apoptosis by NO in IL-5-treated eosinophils was mediated by cGMP, several pharmacologic tools were used. The cell permeant cyclic nucleotide analogs, dibutyl-cGMP and 8-Br-cGMP (both at 10-1000 μ mol/L) failed to reverse IL-5 (10 pmol/L)-induced eosinophil survival (data not shown, $n = 4$). Neither an inhibitor of guanylyl cyclase ODQ (10 μ mol/L), nor an inhibitor of phosphodiesterase (PDE)5 zaprinast, nor a nonselective PDE inhibitor IBMX affected the effect of SNAP (33-1000 μ mol/L) in the presence of IL-5 (10 pmol/L) (data not shown, $n = 4$). Furthermore, stable and nonhydrolyzable analogs of cAMP, dibutyl-cAMP, and 8-Br-cAMP (both at 10-1000 μ mol/L) failed to reverse IL-5-induced eosinophil survival ($n = 4$, data not shown).

Activation of JNK mediates the induction of apoptosis by NO in IL-5-treated eosinophils

When IL-5-treated eosinophils were incubated at 37°C in the presence of SNAP (1000 μ mol/L), a time-dependent increase in the activity of JNK was detected using Western blotting with an anti-pJNK antibody that recognizes the dual phosphorylated (ie, activated) JNK (Fig 3, A). To evaluate the functional role of JNK activation in NO-induced apoptosis in IL-5-treated cells, a novel cell-permeant inhibitor peptide specific for JNK, L-JNKI1,²⁵ was used. L-JNKI1 alone did not affect apoptosis in IL-5-treated eosinophils (Fig 3, B). However, L-JNKI1 (10 μ mol/L), but not the negative control peptide L-TAT, almost completely reversed SNAP (1000 μ mol/L)-induced apoptosis in IL-5-treated eosinophils (Fig 3, B). To see whether IL-5 downregulates JNK activation and whether SNAP simply restores basal JNK activation independent of IL-5, JNK phosphorylation was measured in eosinophils cultured for 30 minutes in the presence or absence of IL-5 and SNAP. The immunoblots showed some basal activity of JNK, which was not affected by IL-5 (10 pmol/L). In contrast, SNAP (1 mmol/L) significantly increased the phosphorylation of JNK in the presence of IL-5 compared with the respective control in the presence of IL-5 ($n = 7$, data not shown).

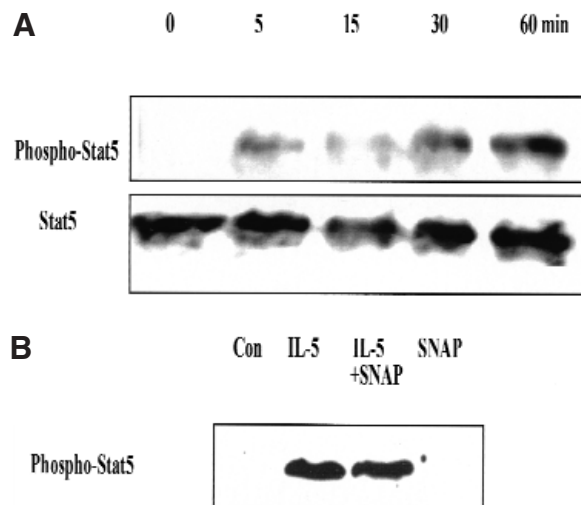


FIG 4. A, Time course of phosphorylation of signal transducer and activator of transcription 5 (STAT5) by IL-5 (10 pmol/L). STAT5 activation was detected using an antibody directed toward the phosphorylated STAT5 (upper lane). The lower lane shows the total STAT5 content in the same sample. **B,** The effect of S-nitroso-N-acetylpenicillamine (SNAP) on IL-5-induced phosphorylation of STAT5. Eosinophils were incubated with medium (Con), IL-5 (10 pmol/L), IL-5 (10 pmol/L), and SNAP (1000 μ mol/L) for 60 minutes. Representatives of 3 experiments with similar results are shown.

Early IL-5 signaling pathways in eosinophils are not affected by NO

Recently in rat eosinophils, IL-5 has been shown to promote the phosphorylation of STAT5 by means of a JAK2-dependent mechanism.²⁶ When the eosinophils were incubated in the presence of 10 pmol/L IL-5, STAT5 was phosphorylated in a time-dependent manner, and the maximal activation was obtained at 30 to 60 minutes (Fig 4, A). When the eosinophils were incubated in the presence of SNAP and IL-5 for 60 minutes, the phosphorylation of STAT5 was not decreased, however (Fig 4, B). This suggests that NO does not affect STAT5 phosphorylation or earlier IL-5 signaling steps in eosinophils.

Bcl-2 expression in IL-5-treated eosinophils: no effect by NO

The role of Bcl-2 family proteins in eosinophil apoptosis or its prevention is not clear, and the reported data are conflicting.²⁷⁻²⁹ We did not detect any expression of Bcl-2 ($n = 2$). In contrast, the proapoptotic species Bax was strongly expressed as previously reported.²⁷ We could not see any change in the expression of Bax between the samples of freshly isolated cells and cells aged for 24 hours in the absence or presence of IL-5 with or without SNAP ($n = 2$, data not shown).

Effects of NO on activation of caspase cascades in IL-5-treated eosinophils

A pan-caspase inhibitor, Z-Asp-CH₂-DCB (100-200 μ mol/L) significantly reversed SNAP (1000 μ mol/L)-induced apoptosis in IL-5-treated eosinophils during 40

hours of incubation (Fig 5, A). Caspase 3 seemed to be activated in the dying eosinophils cultured for 24 hours, because the active 17-kDa subunit was detectable. IL-5 prevented the cleavage of procaspase 3. SNAP increased caspase 3 cleavage in the presence of IL-5 (Fig 5, B), thus suggesting a role for caspase 3 in the actions of NO. However, SNAP reversed IL-5-mediated survival completely, and the activation of caspase 3 in SNAP-treated cells was clearly less than in cells aged for 24 hours in the absence of IL-5. In addition, the specific caspase 3 inhibitor (AC-DMQD-CHO, 2-200 μ mol/L) was not able to reverse the effect of SNAP in IL-5-treated eosinophils (data not shown, $n = 4$). Caspases 8 and 9 have been proposed to be involved in the extrinsic and intrinsic death pathways, respectively. Some cleavage of procaspase 8 was detected both in IL-5-treated and IL-5-untreated cells, but this was not increased by SNAP ($n = 2$, data not shown). We were not able to detect any activation of caspase 9 in eosinophils cultured for 24 hours in the presence or absence of IL-5 or SNAP ($n = 3$; data not shown). Furthermore, when the functional role of caspases 8 and 9 was evaluated by use of specific inhibitors (AC-IETD-CHO and AC-LEHD-CHO, 2-200 μ mol/L, respectively), they did not have any effect on apoptosis induced by SNAP in IL-5-treated cells (data not shown, $n = 4$).

Endogenous NO is not a major regulator of constitutive eosinophil apoptosis

Eosinophils contain the enzyme NOS and produce NO.¹⁶ To see whether endogenous NO production modulates eosinophil apoptosis, we studied the effects of NOS inhibitors, L-NMMA (10-1000 μ mol/L), L-NIO (10-1000 μ mol/L), and the selective iNOS inhibitor 1400W (1-1000 μ mol/L) and that of a substrate for NOS, L-arginine (1000 μ mol/L). Neither the NOS inhibitors nor L-arginine affected spontaneous eosinophil apoptosis during 18 to 40 hours of incubation ($n = 3-4$, data not shown). Consistently, spontaneous cell death did not induce any release of NO measured as NO₂⁻ ($n = 2$, data not shown). Similarly, IL-5 did not induce any production of NO ($n = 2$, data not shown). Consistently, no iNOS mRNA could be detected by RT-PCR in eosinophils cultured for 0, 6, or 20 hours in the absence or presence of 10 pmol/L of IL-5 ($n = 2$, data not shown).

DISCUSSION

In this study, we found that exogenous NO reversed IL-5-mediated eosinophil survival by inducing apoptosis. The NO donors SNAP and GEA3175 increased the number of apoptotic cells in the presence of IL-5 in a concentration-dependent manner, and this effect was abolished by a NO scavenger. Our results are in agreement with the study of Beauvais and Joly,³⁰ in which it was suggested that other NO donors reduced survival and induced apoptosis of IL-5-primed eosinophils. The main physiologic signaling pathway of NO is considered to be activation of guanylyl cyclase, leading to formation of cGMP, and concomitant protein phosphorylation. We used several tools

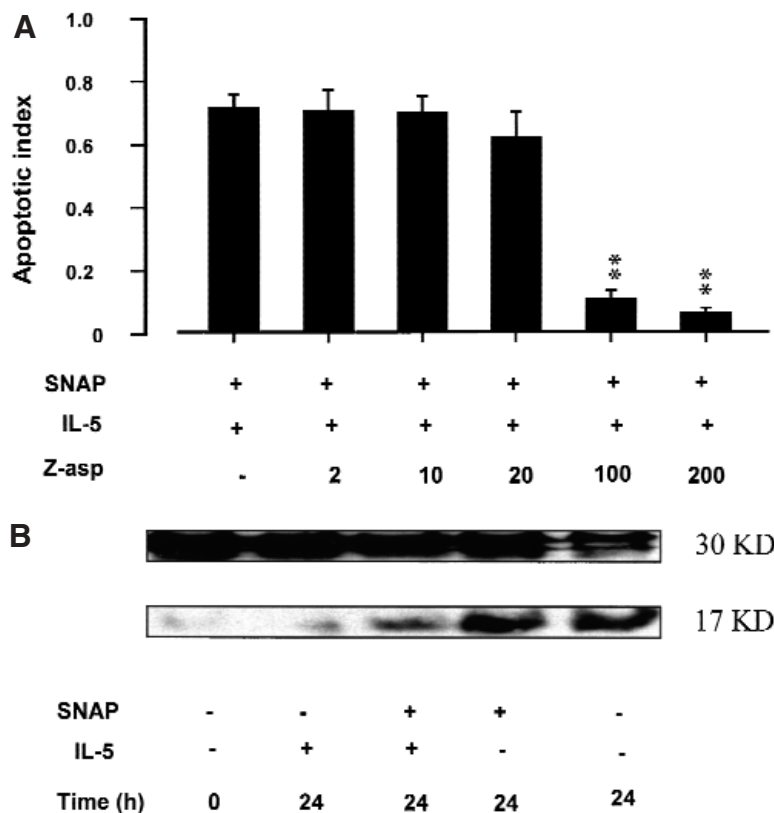


FIG 5. A, The effect of a pancaspase inhibitor Z-Asp-CH₂-DCB on eosinophil apoptosis in the presence of S-nitroso-*N*-acetylpenicillamine (SNAP) (1000 μ mol/L) and IL-5 (10 pmol/L). Each data point represents the mean $\pm$ SEM of 7 independent determinations using eosinophils from different donors. ** $P < .01$ compared with the respective control in the presence of SNAP and IL-5. **B**, The effect of SNAP on the cleavage of caspase 3. A representative experiment of 3 similar experiments is shown.

to modulate the cGMP concentrations in the eosinophils, including inhibition of guanylyl cyclase, inhibitors of PDEs, and cell permeant analogs of cGMP, but the NO-induced apoptosis or IL-5-mediated eosinophil survival was not modulated by any of them. The apoptotic effect of NO on IL-5-treated eosinophils, therefore, seems to be independent of the activation of the guanylyl cyclase. This seems to differ from anti-CD95-induced eosinophil apoptosis, because Hebestreit et al¹⁷ reported that NO was able to disrupt Fas-receptor signaling. The effect of NO on Fas-receptor signaling was reported to be due to activation of soluble guanylyl cyclase, and db-cGMP and db-cAMP induced effects similar to NO donors. Thus, the results of this study and that of Hebestreit et al¹⁷ suggest that NO can regulate human eosinophil apoptosis at 2 separate levels, namely by reversing IL-5-mediated inhibition of apoptosis and by disrupting Fas-receptor signaling and that these effects do not and do depend on the activation of guanylyl cyclase, respectively (Fig 6).

The MAPK family consists of a growing number of serine/threonine kinases that are characteristically activated by threonine and tyrosine phosphorylation. The members include ERK1, 2, 5, and 6, JNK/SAPK, and various isoforms of p38. Both p38 and JNK/SAPK are implicated in the signaling pathway of programmed cell death and

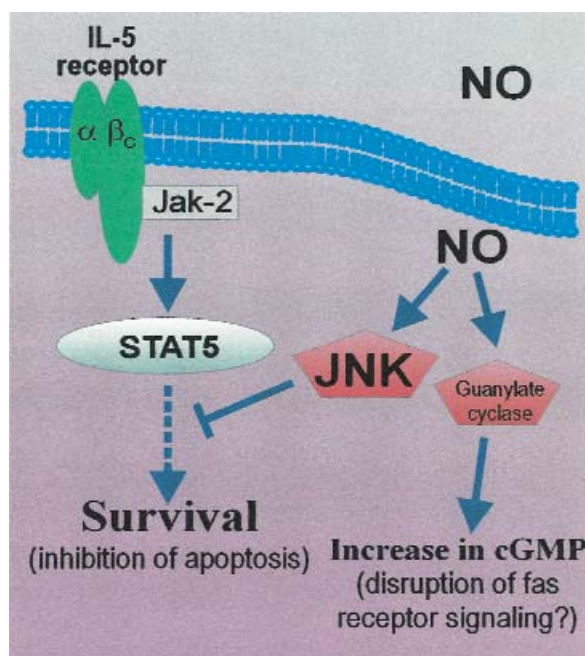


FIG 6. The mechanistic interactions involved in the regulation of eosinophil survival by IL-5 and nitric oxide.

activated in response to numerous environmental stimuli, including NO.^{11,31-33} We have previously shown that p38 MAPK activity is involved in the prolonged survival, inhibition of apoptosis, or both in human eosinophils, but it is not possible to reverse IL-5–afforded eosinophil survival by inhibiting p38 MAPK activity.¹⁸ Thus, an effect by NO on p38 activity seemed unlikely. The JNK signal transduction pathway is preferentially activated in response to environmental stress and by the engagement of several classes of cell surface receptors, including cytokine receptors, serpentine receptors, and receptor tyrosine kinases.³¹ JNK targets are mainly transcription factors, including c-Jun, ATF2, Elk1, c-myc, or p53. Thus, JNK probably acts by modifying the expression of genes that play an important role in controlling cell death or survival. We found that NO activates JNK in IL-5–treated human eosinophils. The effect of NO on apoptosis can be reversed by specifically blocking JNK activation. This confirms that the NO-induced apoptosis in human eosinophils is mediated by means of activation of JNK. Although the induction by NO of apoptosis by means of a JNK-mediated pathway seems a theoretical possibility, it is surprising that only limited data have been presented to support such a signaling mechanism in other cell types. In murine RAW 264.7 macrophages, NO induces apoptosis and activates JNK and p38 MAPK.³³ In bovine chondrocytes, NO was recently reported to mediate IL-1–dependent apoptosis in association with JNK activation.³⁴ NO-stimulated JNK activation has also been implicated in IL-1 β –activated islet beta-cell destruction in pancreatic endocrine cell lines,³⁵ in the apoptosis of H9C2 cardiac muscle cells,³⁶ rat adult cardiomyocytes,³⁷ and in serum deprivation–induced apoptosis of neuronal PC12 cells.³⁸ Thus, to our knowledge, this is the first description of JNK activation in NO-induced apoptosis in nonmalignant human cells.

IL-5 might be the most important cytokine that primes eosinophil function in humans.³⁹ Numerous studies have indicated that one of the major functions of IL-5 is the inhibition of apoptosis in human eosinophils. Sharing a common β -chain with IL-3 and GM-CSF receptor, IL-5 activates tyrosine phosphorylation of cellular substrates.⁴⁰ In this study, we show that STAT5 is phosphorylated in IL-5–treated human eosinophils, confirming the results of Hall et al.⁴¹ Although we confirmed that IL-5 induced STAT5 phosphorylation in eosinophils, NO did not affect STAT5 phosphorylation in IL-5–treated cells, suggesting that early steps of IL-5 signaling leading to STAT5 activation are not affected by NO or JNK activation. The mechanistic interactions involved in the regulation of eosinophil survival by IL-5 and NO are summarized in Fig 6.

The Bcl-2 family has now been recognized to play a central role in the control of apoptosis in many cell types.⁴² The Bcl-2 family can be divided into 2 groups, antiapoptotic proteins (such as Bcl-2, Bcl-X_L, Mcl-1) and proapoptotic proteins (such as Bax, Bad, and Bid).⁴² The balance of these proteins seems to affect whether a cell lives or dies. Our results suggest that Bcl-2 or Bax

are not involved in the NO-induced apoptosis in IL-5–treated eosinophils.

Caspases 8 and 9 have been proposed to be involved in the extrinsic and intrinsic death pathways, respectively, leading to activation of effector caspases (caspases 3, 6, and 7).⁴³ Processing of caspase 8 and 3 proenzymes during spontaneous and Fas-stimulated eosinophil death has been reported, as well as blockade of procaspase processing by IL-5.²⁷ The effect of NO seemed to be dependent on caspase activation, because it was prevented by a broad-spectrum caspase inhibitor Z-Asp-CH₂-DCB. Also some processing of caspase 3 but not caspases 8 or 9 proenzymes by NO was detected. However, studies with specific inhibitors indicated that the effect of NO was not mediated by means of activation of caspases 8, 9, or 3. This is in line with the recent results of Daigle and Simon⁴⁴ and Zhang et al⁴⁵ that the common apoptosis-related caspases 3 and 8 are not involved in spontaneous and dexamethasone-induced apoptosis of human eosinophils.

In this study, we also found that endogenous NO was not involved in the regulation spontaneous eosinophil apoptosis. NOS inhibitors did not affect spontaneous eosinophil apoptosis. Moreover, no iNOS mRNA was detected by RT-PCR, and nitrite was not released by dying cells. These data suggested that endogenous NO production does not modulate eosinophil apoptosis in cytokine-deprived or IL-5–stimulated cells.

Taken together, we have shown that NO reverses IL-5–mediated eosinophil survival by activating JNK in a cGMP-independent manner. NO-mediated inhibition of IL-5–induced survival of eosinophils might represent a counterregulatory mechanism to limit eosinophilia in allergic diseases. The increased generation of NO in asthmatic airways might therefore counteract the effect of IL-5 in prolonging eosinophil survival.

We appreciate the skillful technical assistance of Mrs Tanja Kuusela.

REFERENCES

- Giembycz MA, Lindsay MA. Pharmacology of the eosinophil. *Pharmacol Rev* 1999;51:213-340.
- Barnes PJ. Pathophysiology of asthma. *Br J Clin Pharmacol* 1996;42:3-10.
- Simon HU. Eosinophil apoptosis in allergic diseases—an emerging new issue. *Clin Exp Allergy* 1998;28:1321-4.
- Walsh GM. Human eosinophils: their accumulation, activation and fate. *Br J Haematol* 1997;97:701-9.
- Nagata S, Golstein P. The Fas death factor. *Science* 1995;267:1449-56.
- Kankaanranta H, Lindsay MA, Giembycz MA, Zhang X, Moilanen E, Barnes PJ. Delayed eosinophil apoptosis in asthma. *J Allergy Clin Immunol* 2000;106:77-83.
- Wedi B, Raap U, Lewrick H, Kapp A. Delayed eosinophil programmed cell death in vitro: a common feature of inhalant allergy and extrinsic and intrinsic atopic dermatitis. *J Allergy Clin Immunol* 1997;100:536-43.
- Vignola AM, Chanez P, Chiappara G, Siena L, Merendino A, Reina C, et al. Evaluation of apoptosis of eosinophils, macrophages, and T lymphocytes in mucosal biopsy specimens of patients with asthma and chronic bronchitis. *J Allergy Clin Immunol* 1999;103:563-73.
- Moilanen E, Whittle B, Moncada S. Nitric oxide as a factor in inflammation. In: Gallin JJ, Snyderman R, editors. *Inflammation: basic principles and clinical correlates*. Philadelphia: Lippincott Williams & Wilkins; 1999. p. 787-800.
- Pieper AA, Verma A, Zhang J, Snyder SH. Poly (ADP-ribose) polymerase, nitric oxide and cell death. *Trends Pharmacol Sci* 1999;20:171-81.

11. Chung HT, Pae HO, Choi BM, Billiar TR, Kim YM. Nitric oxide as a bioregulator of apoptosis. *Biochem Biophys Res Commun* 2001;282:1075-9.
12. Coleman JW. Nitric oxide in immunity and inflammation. *Int Immunopharmacol* 2001;1:1397-406.
13. Kharitonov SA, Barnes PJ. Does exhaled nitric oxide reflect asthma control? Yes, it does! *Am J Respir Crit Care Med* 2001;164:727-8.
14. Ichinose M, Sugiura H, Yamagata S, Koarai A, Shirato K. Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. *Am J Respir Crit Care Med* 2000;162:701-6.
15. Hamid Q, Springall DR, Riveros-Moreno V, Chanez P, Howarth P, Redington A, et al. Induction of nitric oxide synthase in asthma. *Lancet* 1993;342:1510-3.
16. del Pozo V, de Arruda-Chaves E, de Andres B, Cardaba B, Lopez-Farre A, Gallardo S, et al. Eosinophils transcribe and translate messenger RNA for inducible nitric oxide synthase. *J Immunol* 1997;158:859-64.
17. Hebestreit H, Dibbert B, Balatti I, Braun D, Schapowal A, Blaser K, et al. Disruption of fas receptor signaling by nitric oxide in eosinophils. *J Exp Med* 1998;187:415-25.
18. Kankaanranta H, De Souza PM, Barnes PJ, Salmon M, Giembycz MA, Lindsay, et al. SB 203580, an inhibitor of p38 mitogen-activated protein kinase, enhances constitutive apoptosis of cytokine-deprived human eosinophils. *J Pharm Exp Ther* 1999;290:621-8.
19. Zhang X, Moilanen E, Adcock IM, Lindsay MA, Kankaanranta H. Divergent effect of mometasone on human eosinophil and neutrophil apoptosis. *Life Sci* 2002;71:1523-34.
20. Kankaanranta H, De Souza PM, Giembycz MA, Lindsay MA. Human eosinophil isolation and the measurement of apoptosis. In: Chung KF, Adcock IM, editors. *Methods in molecular medicine: asthma*. Vol 44. London: Humana Press; 2000. p. 99-110.
21. Korhonen R, Korpela R, Saxelin M, Maki M, Kankaanranta H, Moilanen E. Induction of nitric oxide synthesis by probiotic *Lactobacillus rhamnosus* GG in J774 macrophages and human T84 intestinal epithelial cells. *Inflammation* 2001;25:223-32.
22. Moilanen E, Moilanen T, Knowles R, Charles I, Kadoya Y, al-Saffar N, et al. Nitric oxide synthase is expressed in human macrophages during foreign body inflammation. *Am J Pathol* 1997;150:881-7.
23. Kankaanranta H, Rydell E, Petersson AS, Holm P, Moilanen E, Corell T, et al. Nitric oxide-donating properties of mesoionic 3-aryl substituted oxatriazole-5-imine derivatives. *Br J Pharmacol* 1996;117:401-6.
24. Maeda H, Akaike T, Yoshida M, Suga M. Multiple functions of nitric oxide in pathophysiology and microbiology: analysis by a new nitric oxide scavenger. *J Leukoc Biol* 1994;56:588-92.
25. Bonny C, Oberson A, Negri S, Sauser C, Schorderet DF. Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. *Diabetes* 2001;50:77-82.
26. Ishihara K, Satoh I, Mue S, Ohuchi K. Possible participation of a JAK2 signaling pathway in recombinant rat interleukin-5-induced prolongation of rat eosinophil survival. *Biochim Biophys Acta* 2001;1536:73-84.
27. Zangrilli J, Robertson N, Shetty A, Wu J, Hastie A, Fish JE, et al. Effect of IL-5, glucocorticoid, and Fas ligation on Bcl-2 homologue expression and caspase activation in circulating human eosinophils. *Clin Exp Immunol* 2000;120:12-21.
28. Dewson G, Walsh GM, Wardlaw AJ. Expression of Bcl-2 and its homologues in human eosinophils. Modulation by interleukin-5. *Am J Respir Cell Mol Biol* 1999;20:720-8.
29. Dibbert B, Daigle I, Braun D, Schranz C, Weber M, Blaser K, et al. Role for Bcl-xL in delayed eosinophil apoptosis mediated by granulocyte-macrophage colony-stimulating factor and interleukin-5. *Blood* 1998;92:778-83.
30. Beauvais F, Joly F. Effects of nitric oxide on the eosinophil survival in vitro. A role for nitrosyl-heme. *FEBS Lett* 1999;443:37-40.
31. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell* 2000;103:239-52.
32. Jun CD, Pae HO, Kwak HJ, Yoo JC, Choi BM, Oh CD, et al. Modulation of nitric oxide-induced apoptotic death of HL-60 cells by protein kinase C and protein kinase A through mitogen-activated protein kinases and CPP32-like protease pathways. *Cell Immunol* 1999;194:36-46.
33. Jun CD, Oh CD, Kwak HJ, Pae HO, Yoo JC, Choi BM, et al. Overexpression of protein kinase C isoforms protects RAW 264.7 macrophages from nitric oxide-induced apoptosis: involvement of c-Jun N-terminal kinase/stress-activated protein kinase, p38 kinase, and CPP-32 protease pathways. *J Immunol* 1999;162:3395-401.
34. Clancy R, Rediske J, Koehne C, Stoyanovsky D, Amin A, Attur M, et al. Activation of stress-activated protein kinase in osteoarthritic cartilage: evidence for nitric oxide dependence. *Osteoarthritis Cartilage* 2001;9:294-9.
35. Ammendrup A, Maillard A, Nielsen K, Aabenhus AN, Serup P, Dragsbaek MO, et al. The c-Jun amino-terminal kinase pathway is preferentially activated by interleukin-1 and controls apoptosis in differentiating pancreatic beta-cells. *Diabetes* 2000;49:1468-76.
36. Chae HJ, So HS, Chae SW, Park JS, Kim MS, Oh JM, et al. Sodium nitroprusside induces apoptosis of H9C2 cardiac muscle cells in a c-Jun N-terminal kinase-dependent manner. *Int Immunopharmacol* 2001;1:967-78.
37. Taimor G, Rakow A, Piper HM. Transcription activator protein 1 (AP-1) mediates NO-induced apoptosis of adult cardiomyocytes. *FASEB J* 2001;15:2518-20.
38. Chung KC, Park JH, Kim CH, Ahn YS. Tumor necrosis factor-alpha and phorbol 12-myristate 13-acetate differentially modulate cytotoxic effect of nitric oxide generated by serum deprivation in neuronal PC12 cells. *J Neurochem* 1999;72:1482-8.
39. Adachi T, Alam R. The mechanism of IL-5 signal transduction. *Am J Physiol* 1998;275:C623-C633.
40. de Groot RP, Coffey PJ, Koenderman L. Regulation of proliferation, differentiation and survival by the IL-3/IL-5/GM-CSF receptor family. *Cell Signal* 1998;10:619-28.
41. Hall DJ, Cui J, Bates ME, Stout BA, Koenderman L, Coffey PJ, et al. Transduction of a dominant-negative H-Ras into human eosinophils attenuates extracellular signal-regulated kinase activation and interleukin-5-mediated cell viability. *Blood* 2001;98:2014-21.
42. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998;281:1322-6.
43. Reed JC. Mechanisms of apoptosis. *Am J Pathol* 2000;157:1415-30.
44. Daigle I, Simon HU. Critical role for caspases 3 and 8 in neutrophil but not eosinophil apoptosis. *Int Arch Allergy Immunol* 2001;126:147-56.
45. Zhang JP, Wong CK, Lam CW. Role of caspases in dexamethasone-induced apoptosis and activation of c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase in human eosinophils. *Clin Exp Immunol* 2000;122:20-7.