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The nitric oxide donors, azide and hydroxylamine, inhibit the programmed cell death of cytokine-deprived human eosinophils

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Abstract Azide and hydroxylamine release nitric oxide (NO) enzymatically in biological conditions. We observed that both compounds were able to inhibit in vitro the programmed cell death of human eosinophils from peripheral blood. This protective effect could be mimicked by permeable cGMP analogs and by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. Moreover, the soluble guanylate cyclase inhibitor LY-83583 inhibited in a dose–response manner the effects of the NO donors. Consequently, via the increase of eosinophil survival, NO could contribute to the amplification of inflammatory and allergic processes. This effect appears to be mediated, at least in part, by the soluble guanylate pathway.

Key words: Eosinophil; Programmed cell death; Apoptosis; Nitric oxide

1. Introduction

Eosinophils are thought to play an important role in many allergic diseases such as allergic rhinitis, bronchial asthma or atopic dermatitis [1]. Under such circumstances, eosinophils infiltrate the tissues and release mediators (eosinophil cationic proteins, paf-acether and leukotriene C4) which are responsible for tissue damages and inflammation [2]. Therefore, the knowledge of the factors which regulate the survival of eosinophils in tissues is important for the understanding of allergic diseases. Some cytokines and mediators (e.g. IL-3, IL-5 or GM-CSF), possibly present in inflamed tissues, have been shown to increase eosinophil half-life in vitro by inhibiting eosinophil apoptosis [3]. In this article, we propose that nitric oxide (NO) – a free radical derived from the oxidative metabolism of arginine – is able to up-regulate eosinophil survival.

Although there is a growing interest for NO in immunology and in inflammation [4], the possible effects of NO on the physiology of the eosinophils remain to be studied. Among the cells involved in inflammation, the synthesis of NO by murine macrophages has been clearly established [5]. However, NO synthesis by neutrophils and by human macrophages is questioned [6–8]. In human, NO has been shown to down-modulate T cell proliferation [9], to activate peripheral blood mononuclear cells [10], to inhibit neutrophil degranulation [11], but to increase the release of TNF-α by neutrophils [12].

Azide and hydroxylamine generate NO in a well-documented enzymatic reaction catalyzed by endogenous catalase [13,14]. In this paper we report the effects of these two compounds on the survival of human eosinophils in vitro. We propose that NO is able to increase the eosinophil viability and that activation of the soluble guanylate cyclase pathway could up-regulate the eosinophil survival.

2. Materials and methods

2.1. Reagents

Azide (Merck, Darmstadt, Germany), hydroxylamine hydrochloride, 3-isobutyl-1-methylxanthine, dibutyryl-cGMP, 8-bromo-cGMP (Sigma, Saint-Louis, MO, USA), dibutyryl-cAMP, LY-83583 (Biomol, Plymouth Meeting, PA, USA) were obtained as mentioned.

2.2. Human blood eosinophil preparation

Human blood eosinophils from healthy donors were isolated using Percoll gradients as described [15]. Venous blood was obtained from the local Center for Blood Transfusion. The granulocyte population was recovered by dextran sedimentation and centrifugation through Percoll ($d = 1.077$). After washing, the residual erythrocytes were eliminated by hypotonic lysis. The cell suspension was resuspended in RPMI medium at a concentration of 50×10^6 cells/ml. Two ml of this cell suspension were layered on a Percoll gradient (1.080, 1.085, 1.090, 1.095, 1.105) and centrifuged at $600 \times g$ for 30 min. The cells were harvested from the bottom through a pinhole and collected in 500 μl fractions. Cytocentrifuge smears of the different fractions were prepared, fixed in methanol and stained by Wright's stain (kit RAL 555, Prolabo, Paris, France). The eosinophil percentage was assessed for each fraction and fractions with >95% eosinophils were pooled. The only contaminating cells were neutrophils. The ability of freshly isoblated eosinophils to exclude Trypan blue was >98%.

2.3. Cell culture

Freshly isolated eosinophils ($2–4 \times 10^5$ cells/ml) were suspended in RPMI 1640 (Gibco BRL) supplemented with 0.1 mM non-essential amino-acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM L-glutamine and 10% (v/v) fetal calf serum. Aliquots of 180 μl were placed into 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA, USA) containing 20 μl of compound to be tested at defined concentrations or diluting medium alone. The plates were maintained at 37°C in a 5% CO_2 atmosphere for 48 h.

2.4. Viability and cytology of apoptotic eosinophils

The viability of the eosinophils was assessed by Trypan blue exclusion. The percentages of viable cells were calculated over the number of viable cells present at the beginning of the culture (i.e. around 100% of viable cells).

Cytocentrifuge slides were stained as described above in cell preparation section and were examined under a light microscope ($\times 400$) using oil immersion. The proportion of eosinophils exhibiting morphologic features of apoptotis was assessed as previously described [16]. Briefly, after 24 h of incubation, apoptotic eosinophils showed nuclear pycnosis and cytoplasmic condensation. In some eosinophils, the limit between nucleus and cytoplasm vanished. At 48 h, these effects were more marked, affecting increasing numbers of eosinophils. Moreover, the cell size decrease was particularly marked and frequently no nucleus was visible. The apoptotic eosinophil appeared then as a 'granule bag'. At least 200 cells were examined for each slide.

2.5. Electronic volume analysis

An aliquot of the cells was diluted in phosphate-buffered saline and the volume distribution of the eosinophils was determined by electronic analysis using a Coulter Counter (model ZM) coupled to a Channelyzer

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(Coulter Electronics, Hialeah, FL, USA) [17]. The following settings were used: gain 1, attenuation 2, aperture current 1,000 μA, aperture 140 μm, lower threshold 3.5, upper threshold 100. The instrument was calibrated with latex microspheres (Coulter) to allow calculation of absolute volumes.

As previously described, eosinophils underwent a strong volume decrease (approximately 60%) during apoptosis [16]. Therefore, small apoptotic eosinophils and normal volume eosinophils appeared as well-separated populations with mean volumes of 150 and 400 μm^3. The limit between shrunken and normal volume populations was fixed at 300 μm^3 [16].

2.6. DNA fragmentation

The DNA fragmentation of eosinophils was evidenced as described [18]. Briefly, purified eosinophils ($1–2 \times 10^6$ cells) were washed in phosphate-buffered saline at 4°C and resuspended in lysis buffer (NaCl 100 mM, Tris 10 mM, EDTA 1 mM, sodium *N*-lauroyl-sarcosine 1%, pH 8) containing proteinase K (0.2 mg/ml) (Sigma). The suspensions were incubated overnight at 37°C and the DNA was twice extracted with phenol and 24 : 1 chloroform/isoamyl alcohol (1 : 1, v/v). Sodium acetate was then added (0.3 M final) and the DNA was precipitated in 80% final ethanol. The DNA precipitates were recovered by centrifugation, air dried and resuspended in 20 μl of Tris-EDTA buffer. Two μl of loading buffer was added to each sample. Electrophoresis was performed at 6 V/cm of gel in Tris-EDTA-acetate buffer. After electrophoresis, DNA was visualized by soaking the gel in distilled water containing 1 μg/ml ethidium bromide.

2.7. Statistical analysis

Statistical analysis was performed by using the Student's *t*-test for paired variates. Differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Effect of azide and hydroxylamine on the survival in culture of human eosinophils

In the absence of added cytokine, eosinophils in culture undergo a program of cell death via an apoptotic process. The cell death and the apoptosis were evidenced and quantified by four parameters: Trypan blue exclusion, morphological examination using light microscopy, cell volume measurement and DNA fragmentation. In our culture conditions, in control wells, 24.5 ± 2.9% of the eosinophils present at the beginning of the culture still excluded Trypan blue after 48 h of culture (Fig. 1A). Conversely, 81.6 ± 2.0% of the eosinophils present in the culture were apoptotic, as judged by cytological examination (Fig. 1B and Fig. 2B) and 67.0 ± 3.4% were shrunken as measured by electronic cell sizing (Fig. 1C and Fig. 2A). The hallmark of apoptosis, namely the DNA fragmentation with a ladder pattern, was present (Fig. 3).

In the presence of azide (1–300 μM) or hydroxylamine (0.01–1 mM), the survival of eosinophils was increased. This effect was dose-dependent with an optimal effect at 100 μM and 300 μM, for azide and hydroxylamine respectively. Indeed, after 48 h of culture, 61.9 ± 6.6% ($P < 0.001$) of the eosinophils present at the beginning of the culture were viable in the presence of 100 μM azide and 64.8 ± 8.2% ($P < 0.01$) in the presence of 300 μM hydroxylamine vs. 24.5 ± 2.9% in the presence of the diluting medium alone (Fig. 1A). This was paralleled by a decrease of the percentages of apoptotic and shrunken eosinophils present in the culture (Fig. 1B and Fig. 1C). Moreover, the DNA fragmentation was markedly reduced in the presence of azide or hydroxylamine at optimal concentration (Fig. 3).

3.2. Guanylate cyclase pathway and eosinophil apoptosis

Guanylate cyclase is one of the most important target for

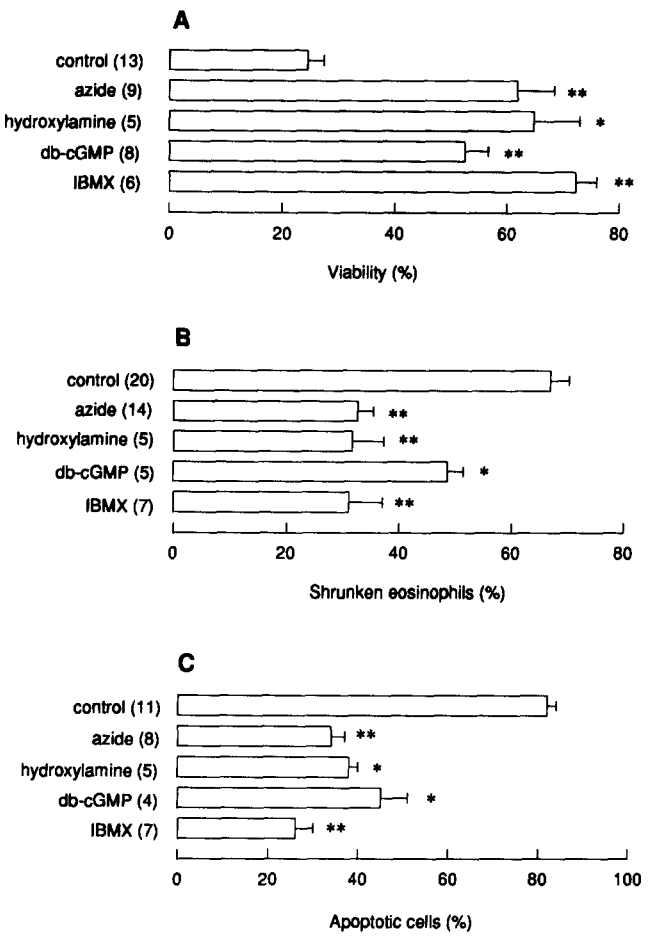

Fig. 1. Effect of azide, hydroxylamine, db-cGMP and IBMX on eosinophil viability and apoptosis. purified human eosinophils from peripheral blood were incubated ($2–4 \times 10^5$ cells/ml) in the presence of azide (100 μM), hydroxylamine (300 μM), db-cGMP (1 mM), IBMX (1 mM) or diluting buffer alone as control. After 48 h, the percentages of viable eosinophils remaining in the culture (A), the percentages of shrunken (B) and apoptotic (C) eosinophils present in the culture were assessed as described in methods. In this figure and the followings, the results are given as means ± S.E.M. of independent experiments with different donors. The numbers of experiments are indicated between parenthesis. $*P < 0.01$, $**P < 0.001$ as compared to control values.

NO. We thus tempted to mimic the effect of NO donors by adding permeable analogs of cGMP. Dibutyryl-cGMP (db-cGMP; 1 mM) significantly increased the number of eosinophils excluding Trypan blue and decreased the percentages of apoptotic and shrunken eosinophils (Fig. 1). The same results were obtained with 8-bromo-cGMP (1 mM) (data not shown).

IBMX (3-isobutyl-1-methylxanthine) is a phosphodiesterase inhibitor and thus it increases the concentration of the cyclic nucleotides in the cell by decreasing their degradation. As shown in Fig. 1, IBMX inhibited eosinophil shrinkage, eosinophil apoptosis and increased eosinophil viability. However, IBMX is a non-specific inhibitor of phosphodiesterases and the observed effect could be due to an increase of cAMP. Indeed, in the same experimental conditions, dibutyryl-cAMP increased apoptosis (data not shown). This strongly suggested that the protective effect of IBMX was linked to an increase of cGMP levels.

LY-83583 is an inhibitor of NO-mediated soluble guanylate

cyclase activation [19]. LY-83583 (1–30 μM) inhibited, in a dose–response manner, the effect of azide 100 μM (Fig. 4). The IC_{50} for inhibition of the azide effect was approximately 1–3 μM. This agrees with the IC_{50} of LY-83583 (2 μM) previously reported for guanylate cyclase inhibition [19]. Inhibition by LY-83583 of the hydroxylamine effect on eosinophil survival was similarly observed (data not shown).

4. Discussion

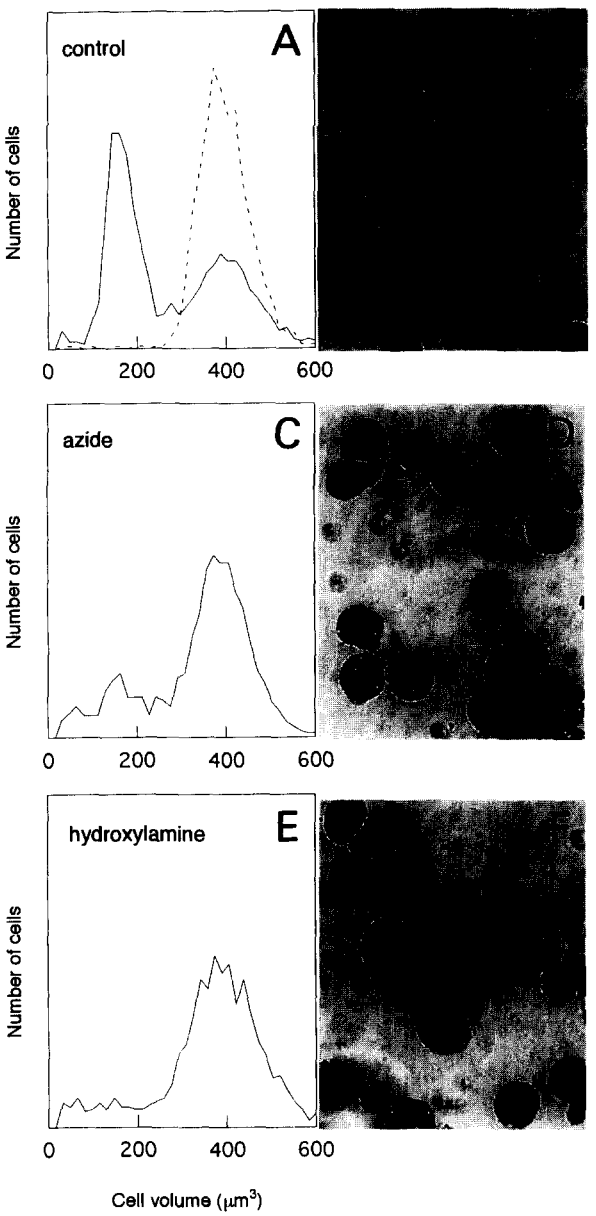

Fig. 2. Inhibition of the morphological features of apoptosis in eosinophils treated by NO donors. The morphological changes associated with apoptosis are characteristics in eosinophils (A): at 48h, most of the eosinophils appeared as 'granule bags' without visible nucleus structures. In some cells, a pycnotic nucleus was still visible. Moreover, the cell volume was strongly reduced. This latter characteristic was precisely measured by electronic cell sizing. The population in dashed line is the volume distribution of the eosinophils at the beginning of the culture (B). In the presence of azide (100 μM) or hydroxylamine (300 μM), the percentages of eosinophils exhibiting the features of apoptosis (C,E) and the percentages of shrunken eosinophils (D,F) were strongly reduced.

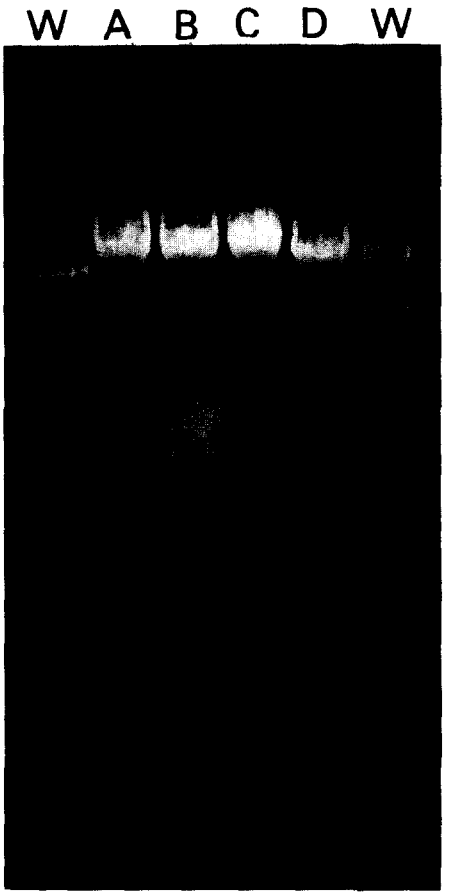

Fig. 3. Electrophoresis of DNA from eosinophils treated by azide and hydroxylamine. Agarose gel electrophoresis of DNA extracted from 2×10^6 eosinophils was performed at the initiation of the culture (lane A) or after a 48 h-culture in the presence of the diluting buffer alone (lane B), azide 100 μM (lane C), hydroxylamine 300 μM (lane D). Lanes W show the migration of λ/*Hin*dIII digested fragments.

In this article, we show that azide and hydroxylamine are able to increase the viability of cytokine-deprived eosinophils in culture as evidenced by Trypan blue exclusion, and to inhibit the eosinophil apoptosis as evidenced by the classical parameters of apoptosis (characteristic morphological modification, DNA fragmentation). Azide and hydroxylamine are NO donors in biological conditions. Indeed, azide or hydroxylamine, in the presence of endogenous catalase and H_2O_2, give a NO-heme complex which is able to activate guanylate cyclase [13]. Although – due to low numbers of eosinophils in healthy human donors – we were unable to measure directly the cytosolic cGMP, our experiments with LY-83583 (Fig. 4) strongly suggested that guanylate cyclase was involved in the present model. Indeed, LY-83583 has been reported to be a specific inhibitor of NO-mediated soluble guanylate cyclase activation and we calculated an IC_{50} compatible with inhibition of guanylate cyclase [19]. Furthermore, both permeable analogs of cGMP and IBMX mimicked the effect of NO. Although we cannot rule out other targets for NO, the activation of guanylate cyclase appears to be an important mechanism for eosinophil survival.

Both cytotoxic and cytoprotective properties have been reported for NO. Thus, NO has been shown to be responsible for the apoptosis observed during the LPS-induced macrophage

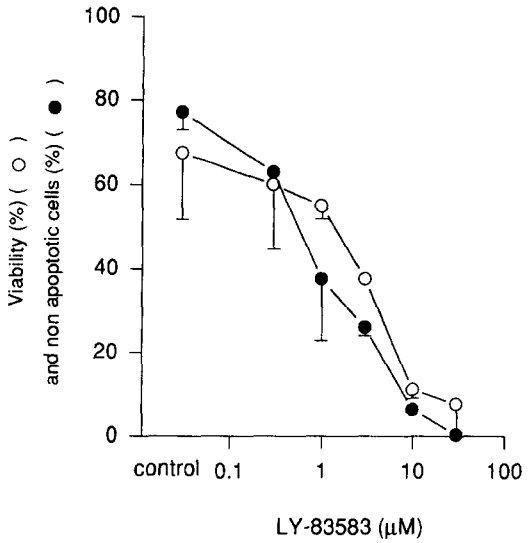

Fig. 4. Effect of the soluble guanylate cyclase inhibitor LY-83583 on eosinophil survival and apoptosis. Purified human eosinophils were incubated (2×10^5 cells/ml) in the presence of azide 100 μM with defined concentrations of LY-83583. After 48 h, the percentages of remaining viable cells and the percentages of non-apoptotic eosinophils were assessed ($n = 3$).

activation [20, 21] and for the apoptosis of murine mastocytoma cells [22]. In neuronal cells, exogenous NO released from NO donors, was shown, according to the environmental redox potential, to have either protective or cytotoxic properties. Cytotoxic properties were thought to be due to the formation of peroxynitrites whereas cytoprotective properties appeared to be due to the *S*-nitrosylation of a membrane receptor [23]. In other systems, NO has been shown to protect from the cellular damages induced by the reactive oxygen species [24]. Since some compounds which react with the reactive species of oxygen have a protective effect [25], one could speculate that, in our experiments, NO also forms intermediate products with reactive species of oxygen and thus indirectly protects eosinophils from deleterious effects of reactive oxygen species by diverting them from their usual targets. However, the simultaneous addition of superoxide dismutase and catalase to the eosinophil cultures did not inhibit apoptosis (unpublished data). Thus, this indirect mechanism does not seem to be at work in our system.

In contrast with azide and hydroxylamine which release NO enzymatically, other NO donors spontaneously release NO in physiological conditions (e.g. *S*-nitroso-*N*-acetyl-penicillamine, *S*-nitroso-cysteine, SIN-1). We have also used such compounds, but the cGMP-dependent protective effect of NO on eosinophils appeared to be counteract by cytotoxic effects (data not shown). These NO donors, however, release NO in a relatively short time as compared to the culture length. Thus, a tempting hypothesis could be that eosinophil protection is obtained at best by low NO doses released continuously (for example, when NO is released enzymatically in the presence of azide and hydroxylamine). In contrast, a burst of high amounts of NO from compounds such as SIN-1 should lead to eosinophil injury. Experiments aimed to measure the kinetics of NO release from these two types of NO donors have been undertaken in the eosinophil model to address this question.

In conclusion, our results suggest an important role for both the soluble guanylate cyclase pathway and its natural agonist NO in inflammation and allergy by increasing eosinophil survival.

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