Effects of nitric oxide on the eosinophil survival in vitro. A role for nitrosyl-heme

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Abstract In a previous paper we showed that the nitric oxide (NO) donors azide and hydroxylamine inhibited eosinophil apoptosis. Azide and hydroxylamine generate a nitrosyl-heme complex – due to endogenous catalase activity – which activates soluble guanylate cyclase. In contrast, in the present paper, we show that NO donors (SNAP, SIN-1, *S*-nitroso-L-cysteine, NOC-18) which spontaneously release NO in physiological solutions did not support the survival of eosinophils and induced apoptosis or necrosis. However, the addition of hematin (the ferric form of heme) together with low doses of NO (SNAP 10 μM) promoted eosinophil survival. In conclusion, we propose that NO and heme (e.g. from heme-containing enzymes such as peroxidase or catalase), both released in inflammation sites, could form nitrosyl-heme and thus promote eosinophilic inflammation.

Key words: Apoptosis; Eosinophil; Nitric oxide

1. Introduction

Eosinophils accumulate at the sites of allergic inflammation and further enhance inflammation through the release of lipid mediators, cationic proteins and cytokines [1]. Although the eosinophils are short-lived cells, they can survive in tissues for several days. Some cytokines – such as granulocyte/macrophage colony stimulating factor (GM-CSF), interleukin (IL)-3 or IL-5 – which are released at the sites of inflammation promote eosinophil survival in vitro [2]. In their absence, eosinophils spontaneously undergo a program of cell death with the biochemical and morphological features of apoptosis [3,4].

In a previous paper, we pointed out the role of soluble guanylate cyclase (sGC) and its product cGMP in the inhibition of the spontaneous apoptosis of cytokine-deprived eosinophils [3]. sGC is a heme-containing enzyme and its activation can be achieved by the binding of nitric oxide (NO) to heme. Activation of sGC can also be obtained by compounds such as azide or hydroxylamine. The sGC activation by these compounds requires an enzymatic step which is catalyzed by the heme-containing enzyme catalase in the presence of H_2O_2 [4,5]. A nitrosyl-heme complex is formed which competes with the free heme of sGC and thus activates the formation of cGMP [6].

In the present paper we studied the effect of pharmacological compounds which spontaneously release NO in the external medium without enzyme requirement. We show that – in contrast with enzymatically formed NO – free NO induces eosinophil death by apoptosis or necrosis. Finally, we propose that NO is able to promote eosinophil survival when presented to the sGC in the nitrosyl-heme form.

2. Materials and methods

2.1. Reagents

SIN-1 (3-morpholino-sydnonimine) and SIN-1C (synthesized by Cassela AG, Frankfurt, Germany, graciously provided by Dr. Winicki, Hoechst, France), *S*-nitroso-*N*-acetyl-penicillamine (SNAP: Biomol, Tebu, Paris, France), recombinant human IL-5 and GM-CSF (Genzyme, Cambridge, MA, USA), sodium azide (Merck, Darmstadt, Germany), hydroxylamine hydrochloride, hematin (Sigma, St. Louis, MO, USA), were obtained as mentioned. *S*-Nitrosocysteine was prepared as described [7]. Briefly, 100 mM of L-cysteine (Gibco BRL, Gaithersburg, MD, USA) and 100 mM of $NaNO_2$ were added in 0.5 N HCl for 30 min at room temperature. The solution was neutralized with NaOH just before use and diluted in RPMI medium. The synthesis of *S*-nitroso-L-cysteine was confirmed by the method of Saville [8]. The NO donors were assayed for release of free NO in culture conditions (RPMI medium, see above) using an NO meter (Iso-NO, World Precision Instrument, Aston, Stevenage, Hertfordshire, UK). As expected, SNAP and SIN-1 released NO for hours, whereas *S*-nitroso-L-cysteine released NO in a few minutes. NOC-18 (ICN Pharmaceuticals France, Orsay, France), which releases NO for periods of >18 h, was also used.

2.2. Human blood eosinophil preparation

Venous blood from healthy subjects was sedimented in the presence of dextran. The cell-rich supernatant was layered onto a cushion of Ficoll (density 1.077). The cell pellet containing eosinophils and neutrophils was harvested after hypotonic lysis of red cells. An immunomagnetic cell separation system (MACS system; Miltenyi Biotec) was used to purify the eosinophils by negative separation with anti-CD16 magnetic microbeads. After passage through the column for magnetic separation, the purity of the cell suspension (above 95%) was assessed after specific staining of the eosinophils.

2.3. Cultures of eosinophils

Freshly isolated eosinophils ($2–4\times10^5$ cells/ml) were suspended in RPMI 1640 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). 20 μl of IL-5, nitric oxide donors (SNAP, SIN-1, *S*-nitroso-L-cysteine, NOC-18) or hematin at defined concentrations or diluting medium alone were added. The plates were maintained at 37°C in a 5% CO_2 atmosphere for 48 h.

2.4. Eosinophil viability and apoptosis

2.4.1. Trypan blue exclusion. The viability of 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).

2.4.2. Apoptosis of eosinophils

2.4.2.1. Eosinophil cytology. Cytocentrifuge slides were stained as described above in Section 2.2 and were examined under a light microscope (×400) using oil immersion. The proportion of intact eosinophils was assessed as previously described [9]. Typically, apoptotic

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Abbreviations: sGC, soluble guanylate cyclase; SNAP, *S*-nitroso-*N*-acetyl-penicillamine

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eosinophils show nuclear pycnosis and cytoplasmic condensation. At a further state, the limit between the nucleus and the cytoplasm vanishes and the nucleus structures disappear. Moreover, the cell size decrease is particularly marked. The eosinophils appear then as 'granule bags'. At least 200 cells were examined for each slide.

2.4.2.2. Acridine orange method. In some experiments, the acridine orange method was used as an alternative method to show apoptosis. An aliquot of cell suspension was added to an equal volume of acridine orange (Sigma) at 10 μg/ml in phosphate-buffered saline and observed in fluorescence microscopy. Apoptotic eosinophils appeared with a bright condensed pycnotic nucleus and at a further step nucleus structures disappeared. At least 200 cells were examined for each experimental point.

2.4.2.3. In situ DNA fragmentation. The presence of fragmented DNA was evidenced in situ using the TUNEL method. This method was used according to the manufacturer's instructions (Apoptosis Detection System, Promega). Briefly, the eosinophils of each well were cytocentrifuged and fixed in a formaldehyde solution at 4% (25 min; 4°C). After washing, the preparations were permeabilized in a 2% Triton X-100 solution (5 min; 20°C). The cells were then incubated (1 h; 37°C) in the presence of a solution containing fluorescein-12-dUTP and terminal deoxynucleotidyl transferase. The samples were examined under a fluorescence microscope using a fluorescein filter set.

2.5. Statistical analysis

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

3. Results

3.1. NO released in the external medium in large amounts inhibits apoptosis but does not support the survival of cytokine-deprived human eosinophils

Freshly isolated human eosinophils were incubated in the presence of three different NO donors: SIN-1 and SNAP, which release NO in a sustained manner, and *S*-nitroso-L-cysteine which releases NO in a short time. Monitoring the apoptosis of the eosinophils with the acridine orange method indicated that apoptosis at 48 h was strongly inhibited by SNAP and SIN-1. The appearance of the nuclear events of apoptosis was strongly decreased in the presence of SIN-1 (1–5 mM) and SNAP (1–5 mM) in a dose-responsive manner (Fig. 1). Thus, in the presence of 5 mM SNAP, $12 \pm 3\%$ ($P < 0.01$; $n = 5$) of the eosinophils present in the 48-h culture were apoptotic and $25 \pm 4\%$ ($P < 0.001$; $n = 5$) in the presence of 5 mM SIN-1 vs. $89 \pm 3\%$ in the presence of the diluting medium alone (Fig. 1). The brief NO release from *S*-nitroso-L-cysteine was enough to inhibit apoptosis (Fig. 1). However, in sharp contrast with apoptosis inhibition, SNAP and SIN-1 did not increase the eosinophil survival as expected. Indeed, after 48 h, only $34 \pm 4\%$ ($P < 0.05$; $n = 11$) of the eosinophils present at the beginning of the culture excluded trypan blue in the presence of 3 mM SIN-1 vs. $21 \pm 2\%$ in the presence of the diluting medium alone (Fig. 1). In the presence of SNAP, the eosinophil viability was not significantly increased. As negative controls, SIN-1C, the inactive metabolite of SIN-1, $NaNO_2$, and degraded SNAP were without effect on both eosinophil survival and apoptosis (data not shown).

These deleterious effects on survival were confirmed by microscopic observations which indicated that these NO donors profoundly modified cell morphology.

(1) Prolongation of the culture of the eosinophils of the SIN-1- or SNAP-treated eosinophils at NO concentrations which blocked the apoptosis process finally led to cell lysis

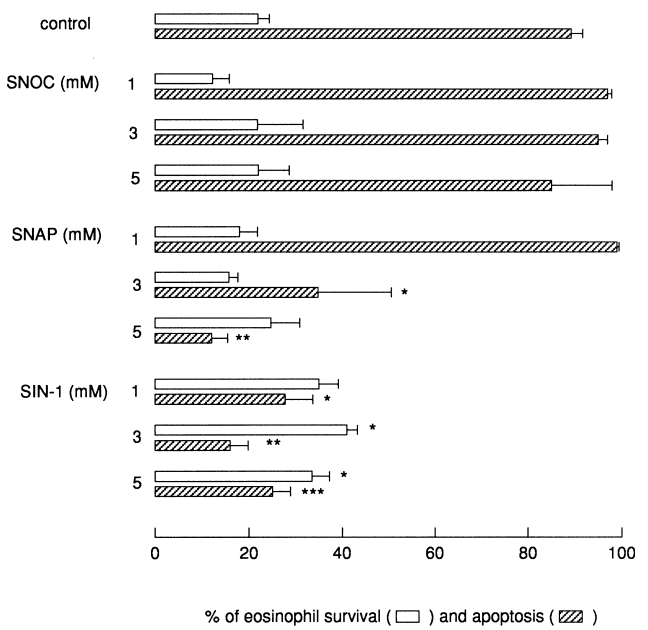

Fig. 1. Effect of NO donors on apoptosis and survival of cytokine-deprived eosinophils. Eosinophils were incubated in the presence of *S*-nitroso-L-cysteine (SNOC), SNAP, SIN-1 at defined concentrations, or diluting buffer alone as control. After 48 h, the percentages of viable eosinophils and the percentages of apoptotic cells were assessed ($n = 3$–11 independent experiments). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to control values.

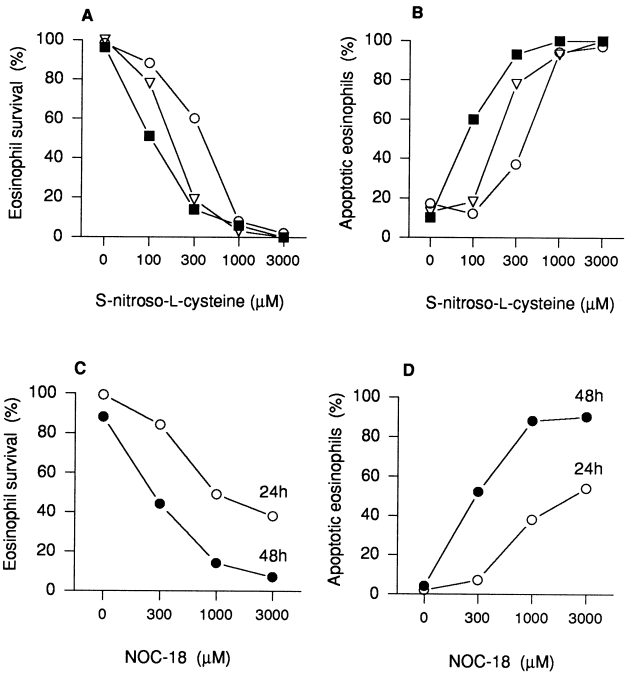

Fig. 2. Effect of brief pulses or low sustained release of NO on survival and apoptosis of IL-5-primed eosinophil. A and B: Human eosinophils were cultured in the presence of IL-5 (10 pM) and *S*-nitroso-L-cysteine at defined concentration was added at $t = 0$ (open circles), at $t = 0$ and 18 h (open triangles), at $t = 0$, 18 h, and 26 h (closed squares). The percentages of viable cells (A) and apoptotic cells (B) were assessed at 48 h (one experiment representative of three). C and D: Eosinophils were cultured in the presence of IL-5 (10 pM) and NOC-18 at defined concentrations. After 24 and 48 h of culture, the percentages of viable cells (C) and apoptotic cells (D) were assessed (one experiment representative of three).

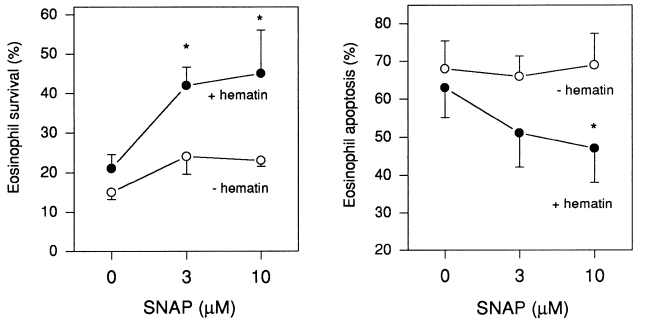

Fig. 3. Effet of low doses of SNAP in the presence of hematin on eosinophil survival and apoptosis. Eosinophils were cultured in the presence of SNAP at defined concentrations and hematin (10 μM). After 48 h of culture, the percentages of viable cells (A) and apoptotic cells (B) were assessed (n=3 independent experiments). *$P < 0.05$ as compared to control values.

(necrosis) without the apoptosis step. This was in contrast with other inhibitors of eosinophil apoptosis (e.g. IL-5 or azide) which delay apoptosis without a necrosis process (data not shown).

(2) Under phase-contrast microscopy, SIN-1- or SNAP-treated eosinophils appeared with a large, normal volume and looked like viable eosinophils. However, after cytocentrifugation on glass slides, the SIN-1- or SNAP-treated eosinophils became strongly shrunken with a condensed cytoplasm around a bilobed non-apoptotic nucleus (data not shown). Again, this was in sharp contrast with IL-5- or azide-treated eosinophils which kept a normal aspect after cytocentrifugation.

3.2. *Brief or low sustained release of external NO from pharmacological NO donors induces the apoptosis of IL-5-primed human eosinophils*

We then studied the effect of NO donors in the external medium on IL-5-primed eosinophils. *S*-Nitroso-L-cysteine, which was previously shown to be without effect on cytokine-deprived eosinophils, still induced apoptosis of IL-5-primed eosinophils. Indeed, we observed that concentrations of *S*-nitroso-L-cysteine as low as 100–300 μM induced the apoptosis of eosinophils maintained in survival by IL-5, particularly when this treatment was repeated during the culture period (Fig. 2A,B). Another NO donor, NOC-18, which releases small amounts of NO for a long time, was also used [10]. This compound also decreased the survival of eosinophils and increased their apoptosis (Fig. 2C,D). The apoptosis induced by *S*-nitroso-L-cysteine and NOC-18 was typical. Pycnotic nuclei (round, dense nuclei) due to chromatin condensation and a decreased cell diameter were observed. However, in the majority of apoptotic eosinophils, nuclear structures were lost and eosinophils appeared as 'granule bags' of small diameter. The DNA fragmentation was systematically evidenced in situ in the pycnotic nuclei (data not shown).

3.3. *Low doses of NO in the presence of hematin increase the survival of cytokine-deprived eosinophils*

NO and heme react easily together to form a potent activator of sGC. We thus added hematin (the ferric form of heme) and SNAP at low concentrations (3–10 μM). SNAP or hematin (10 μM) alone did not significantly modify eosinophil survival and apoptosis. However, when present together the survival of eosinophils increased and their apoptosis decreased (Fig. 3). Indeed, in the presence of 10 μM SNAP and 10 μM hematin eosinophil survival after a 48-h incubation increased from 15 ± 2% to 45 ± 11% ($P < 0.05$; $n = 3$) and apoptosis decreased from 68 ± 7% to 47 ± 14% ($P < 0.05$).

4. Discussion

In a previous paper, we observed that the pharmacological activation of the sGC (by azide or hydroxylamine) and cGMP – the product of sGC – delayed eosinophil apoptosis [3]. This suggested that the sGC-cGMP pathway played an important role in eosinophil survival.

The activation of sGC by azide and hydroxylamine involves the formation of NO through an enzymatic process. Indeed, it has been shown that azide or hydroxylamine – in the presence of catalase and H_2O_2 – form a nitrosyl-heme complex which activates sGC [4,5].

In the present paper, we studied the effect of NO spontaneously released in the external medium without the need for an enzymatic step. The results show that a brief release of NO (from *S*-nitroso-L-cysteine) did not modify the survival and the spontaneous apoptosis of cytokine-deprived eosinophils. In contrast, when NO was released at high rates from SIN-1 at mM concentrations, the apoptosis was blocked but was accompanied by toxic/necrotic processes. It should be noted that SIN-1 is a donor of both NO and superoxide anion. Thus, we cannot exclude that the effects observed with SIN-1 are due – at least in part – to peroxynitrite. However, similar results were obtained with SNAP, a compound which also releases NO for hours without the simultaneous release of superoxide anion. With these high and sustained doses of NO, the cell homeostasis was most probably highly altered. Thus, the eosinophils could not undergo a programmed cell death and consequently necrosis occurred.

Recently, it has been proposed that not only azide and hydroxylamine but also SNAP – particularly at mM concentrations – inhibited the eosinophil apoptosis mediated by Fas [11]. However, trypan blue tests were not reported, thus questioning the physiological relevance of these results. Indeed, the present experiments clearly point out the necessity to carefully check that eosinophil viability is conversely increased when apoptosis is inhibited by NO donors.

When NO was released during brief pulses or at low levels for long periods (from *S*-nitroso-L-cysteine or NOC-18, respectively), it decreased the survival of IL-5-primed eosinophils through an apoptotic process. Thus, taken together the present results and our previous article [3], there is a large discrepancy between the two types of NO donors. Indeed, enzymatically formed NO – which complexes with heme – supports eosinophil survival via the activation of the soluble guanylate cyclase. In contrast, free NO released in the external medium induced the death of IL-5-primed eosinophils. The signals induced by NO which lead to eosinophil apoptosis remain unknown and will require further investigations. One can postulate that protein phosphorylations mediated by IL-5 in IL-5-primed eosinophils are possible targets for NO. Thus, inhibition of IL-5 signaling in eosinophils will lead to apoptosis.

NO has a great affinity for heme and spontaneously forms a nitrosyl-heme complex both with the ferrous (heme) or the ferric (hematin) forms of heme [12]. Moreover, both NO

and heme are present in inflammatory sites. Indeed, large amounts of heme-containing enzymes (catalase, peroxidases) are released during the degranulation of inflammatory cells (neutrophils, eosinophils, etc.) or during necrosis of tissues. Consequently, we postulated that NO which is produced during inflammation binds to heme and thus promotes eosinophil survival. This was indeed the case in vitro since the addition of low concentrations of a NO donor (SNAP 3–10 μM), which was without effect alone, significantly increased eosinophil survival in the presence of hematin (Fig. 3).

In conclusion, we show that free NO induces eosinophil death in vitro. In contrast, we propose that NO could increase eosinophil viability when it is presented to the sGC in the nitrosyl-heme form. Moreover, nitrosyl-heme could be a transport form of NO. Indeed, NO has a brief half-life and NO-heme could represent a stable form of NO. The increase of eosinophil survival by nitrosyl-heme could be a physiologically relevant mechanism at the sites of allergy and inflammation.

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