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Biochimica et Biophysica Acta 1450 (1999) 406–413

BIOCHIMICA ET BIOPHYSICA ACTA

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Nitric oxide can function as either a killer molecule or an antiapoptotic effector in cardiomyocytes

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Received 5 August 1998; received in revised form 17 March 1999; accepted 13 April 1999

Abstract

Caspase enzymes are a family of cysteine proteases that play a central role in apoptosis. Recently, it has been demonstrated that caspases can be *S*-nitrosylated and inhibited by nitric oxide (NO). The present report shows that in chick embryo heart cells (CEHC), NO donor molecules such as *S*-nitroso-*N*-acetylpenicillamine (SNAP), *S*-nitrosoglutathione, spermine-NO or sodium nitroprusside inhibit caspase activity in both basal and staurosporine-treated cells. However, the inhibitory effect of NO donors on caspase activity is accompanied by a parallel cytotoxic effect, that precludes NO to exert its antiapoptotic capability. *N*-Acetylcysteine (NAC) at a concentration of 10 mM blocks depletion of cellular glutathione and cell death in SNAP-treated CEHC, but it poorly affects the ability of SNAP to inhibit caspase activity. Consequently, in the presence of NAC, SNAP attenuates not only caspase activity but also cell death of staurosporine-treated CEHC. These data show that changes in the redox environment may inhibit NO-mediated toxicity, without affecting the antiapoptotic capability of NO, mediated by inhibition of caspase enzymes. NO may thus be transformed from a killer molecule into an antiapoptotic agent.

Keywords: Apoptosis; Caspase; *N*-Acetylcysteine; Nitric oxide

1. Introduction

Nitric oxide (NO) participates in diverse biological processes including the modulation of cell death in which it is implicated both as a mediator of cell injury and as a cytoprotective agent [1–4].

Three processes control the fate of NO and consequently its effect in biological systems: formation, diffusion and reactivity. NO is formed by the action of constitutive calcium-dependent NO synthase (NOS), principally expressed in neurons and endothelial cells, or by inducible NOS, whose activity is induced in several cell types after exposure to inflammatory stimuli [5]. NO rapidly diffuses and, owing to its high reactivity, may have a number of cellular targets. NO can react with several biomolecules, typically sulfhydryl-containing molecules or redox metal-containing proteins and interactions with these redox-sensitive sites can mediate several signaling or cytotoxic NO actions [2,5–7].

S-Nitrosylation of proteins is an important biological action of NO that can mediate several of its

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0167-4889/99/$ – see front matter
PII: S0167-4889(99)00045-2

effects [5–7]. Recently it has been shown that NO can *S*-nitrosylate and inactivate caspase-3 [8] as well as other members of the caspase family [9]. Caspases are conserved aspartate-specific proteases that play a central role in apoptosis [10]. These enzymes are synthesized as inactive proenzymes that are activated by cleavage to specific aspartate residues to release smaller subunits [10]. Caspases are cysteine proteases and, consequently, potential targets of *S*-nitrosylation by NO. This reaction has been demonstrated for caspase-3, where NO causes specific nitrosylation of Cys-163, a functionally essential amino acid [8]. As a consequence, NO inhibits the activity of caspase-3 and other caspases [8,9,11–16] which, depending on the cell model, could result in protection against apoptosis [8,13,15] or in the shift of apoptotic toward necrotic cell death [11]. Since caspases play a pivotal role in apoptosis, NO potentially has an anti-apoptotic capability. However, in several cell types NO elicits toxic effects, which preclude it exerting a cytoprotective action against inducers of apoptosis. Furthermore, NO itself may induce apoptosis [1–4], suggesting that cell sensitivity and NO concentration are crucial to determine the effect of NO on cell survival.

This report shows that the ability of NO donor molecules to inhibit caspase activity in chick embryo heart cells (CEHC) in vivo is parallel to their cytotoxic effects. In the presence of relatively low concentrations of *N*-acetylcysteine (NAC), that protects cells against NO-induced injury, the ability of NO to inhibit caspase activity is partially retained, thus transforming NO from a killer molecule into an apoptosis-attenuating agent.

2. Materials and methods

2.1. Materials and cells

SNAP and other NO donor molecules, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC) and other biochemicals were products of Sigma. Media, cell culture supplements and fetal calf serum were purchased from Gibco.

CEHC cultures were prepared from the hearts of 10-day-old chick embryos by a trypsin disaggregation procedure [17]. To minimize non-myocyte cell contamination, the dissociated cells were preplated for 2 h at 37°C, after which unattached cells (cardiomyocytes) were resuspended in Dulbecco minimal essential medium supplemented with 10% fetal calf serum and 1% penicillin and streptomycin. 2×10^6 cells were seeded in 35 mm dishes and grown to confluence. Confluent cultures, maintained for 20 h in a serum-free medium, were then treated as described. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Cell death was evaluated by trypan blue exclusion and quantified by counting 100 cells per dish with the percent survival being defined as the percentage of cells that excluded the dye.

2.2. Determination of caspase activity

The activity of caspase enzymes was measured by the cleavage of the fluorogenic peptide substrate Ac-DEVD-AMC which represents a substrate for caspase-3 and other members of the caspase family [18]. At the end of the experiment, the cells were collected, washed in phosphate-buffered saline and suspended in 0.4 ml of lysis buffer consisting of 20 mM HEPES, pH 7, 5 mM dithiothreitol (DTT), 2 mM EDTA, 0.1% CHAPS, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each aprotinin, pepstatin and leupeptin. The cells were then subjected to two cycles of freeze-thawing. The lysates were centrifuged for 10 min at $28\,000\times g$ and the supernatant was used as enzyme source. Ten microliters of this extract (containing about 10 μg of protein) were combined with 20 μl of assay buffer containing 100 mM HEPES, pH 7, 5 mM dithiothreitol, 0.1% CHAPS, 10% sucrose and 0.15 mM Ac-DEVD-AMC and incubated for 15 min at 37°C. The reaction was stopped in ice by adding 0.1 ml of 2% sodium acetate in 0.2 M acetic acid. The samples were diluted with 2.5 ml of water and the specific cleavage of Ac-DEVD-AMC was monitored by aminomethylcoumarin release using 370 nm excitation and 455 nm emission wavelength. One unit is defined as the amount of enzyme activity cleaving 1.0 nmol of substrate per minute in the standard conditions described. This activity will be simply referred to as caspase activity. In order to evaluate the direct effect of NO donors on the function of caspases, an extract containing high caspase activity was

prepared from cells incubated 6 h with 1 μM staurosporine. Caspase activity in this extract was assayed in the presence of the indicated concentration of NO donors.

2.3. Other assays

To measure the intracellular level of reduced glutathione (GSH), the cells from five dishes were collected, pooled and lysed in 0.6 N perchloric acid. GSH in the acid extract was determined by capillary electrophoresis, as previously described [19]. Protein concentration was measured by Coomassie staining.

3. Results

The effect of NO on cell death widely differs depending on the cell type [1–4]. In preliminary experiments we observed that NO donors were toxic to CEHC without causing the typical morphological changes of apoptosis. A study of the dose-effect of SNAP on caspase activity and cell survival was then carried out. Caspase activity was assayed following a 5 h incubation, whereas cell viability was measured after 24 h (Fig. 1A). The treatment of CEHC with SNAP caused a dose-dependent decrease of caspase activity that accompanied cell death. In these experiments caspase activity was not stimulated, and the basal activity measured in cell extracts is evidently due to background spontaneous apoptosis in cell cultures.

Recently, it was demonstrated that NO directly inhibits caspase-3 [8]. In agreement with these observations, SNAP, as well as the other NO donor molecules *S*-nitrosoglutathione (GSNO) and spermine-NO (SpmNO), dose-dependently inhibited caspase activity in extracts obtained from staurosporine-treated CEHC (Fig. 1B). This in vitro inhibitory effect of NO donors is probably underestimated, because, in order to optimize caspase activity, DTT was present in the assay mixture and DTT is known to counteract the inhibitory effect of NO on caspases [9]. Fig. 1C shows that several NO donor molecules that release NO as nitrosothiols (SNAP, GSNO) or with different mechanisms (SpmNO, SNP), inhibited the basal caspase activity in whole CEHC.

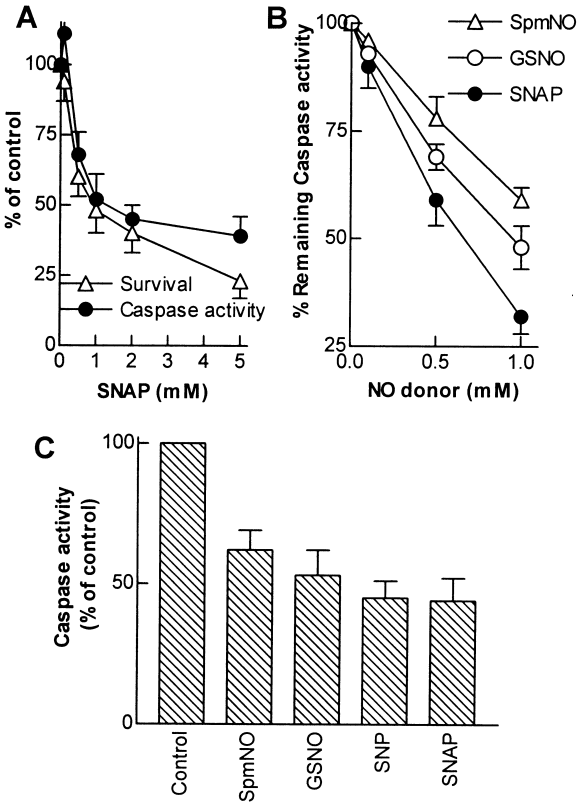

Fig. 1. Nitric oxide inhibits caspase activity of CEHC. (A) CEHC were treated with the indicated doses of SNAP. Caspase activity was measured following a 5 h incubation, while cell survival was determined after 24 h. The data are expressed as % of the control values measured in untreated cells. The control values were 0.16 ± 0.03 units/mg of protein ($n = 4$) for caspase activity and $91 \pm 3\%$ ($n = 4$) for survival. The data are means ± S.E. of four determinations. (B) Extracts obtained from staurosporine-treated CEHC were assayed in the presence of the indicated concentrations of NO donor molecule. Caspase activity in the absence of any NO donor was 2.35 units/mg of protein. The graph reports the mean and the range of results obtained in two separate experiments. (C) Caspase activity was measured in CEHC incubated for 5 h in the presence of 1 mM of SNAP, GSNO or SpmNO or 0.1 mM SNP. Data are means ± S.E. of four determinations.

Subsequently, the effect of NO donors on caspase activity was studied in intact cells undergoing apoptosis. Staurosporine is known to cause apoptosis in virtually all cell types. The treatment of CEHC with 1 μM staurosporine caused an early and marked activation of caspase activity, that increased more than tenfold within 6 h (Fig. 2A). Caspase activity from extracts of staurosporine-treated CEHC was lowered by about 40, 65 or 68% when the cells were cultured

in the presence of 1 mM GSNO, SNAP or SNP, respectively. A greater inhibition was obtained when CEHC were treated with 300 μM DEVD-CHO, a caspase inhibitor (Fig. 2B). DEVD-CHO also inhibited the death of staurosporine-treated CEHC, indicating that caspase activation was sufficient to mediate cell death in this model (Fig. 2C). On the other hand, while they inhibited caspase activity, NO donors did not increase cell survival after staurosporine treatment but, on the contrary, they increased cell death. As expected, DEVD-CHO did not give any protection against NO-induced cytotoxicity (data not shown).

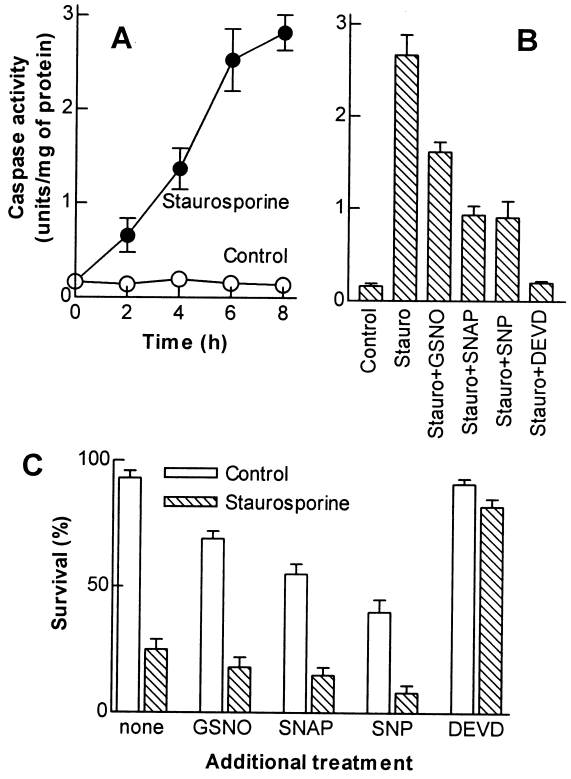

Fig. 2. NO donors inhibit caspase activity but not cell death of staurosporine-treated CEHC. (A) CEHC were incubated for the indicated times without any treatment (control) or in the presence of 1 μM staurosporine, then caspase activity was measured. (B) CEHC were incubated for 5 h without any treatment (control) or with staurosporine alone (stauro) or with staurosporine together with 1 mM GSNO or 1 mM SNAP or 0.1 mM SNP or 0.3 mM DEVD-CHO (DEVD). (C) Untreated (control) and staurosporine-treated CEHC were incubated without any further treatment or in the presence of 1 mM GSNO or 1 mM SNAP or 0.1 mM SNP or 0.3 mM DEVD-CHO (DEVD). Cell survival was measured after 24 h of incubation. All the panels report the means ± S.E. of results obtained in multiple determinations (three to six).

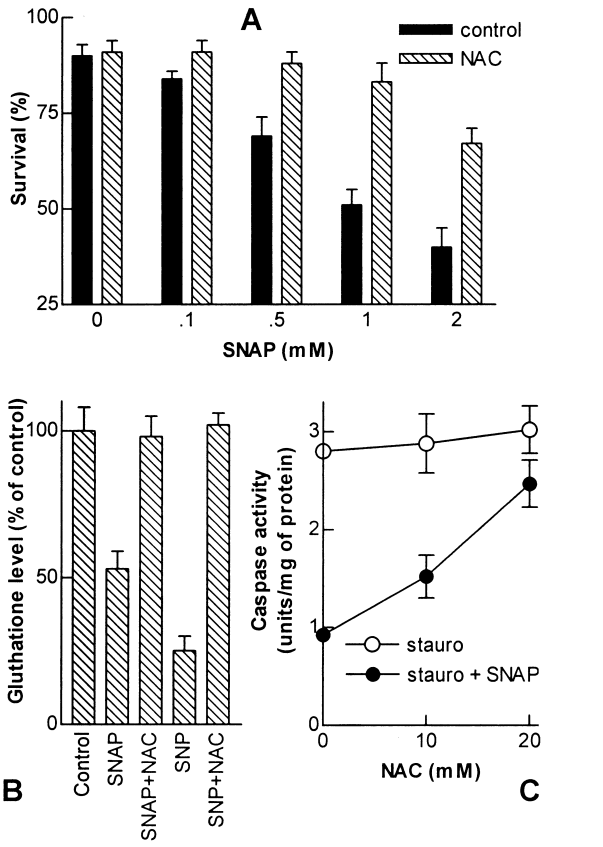

Fig. 3. Effect of NAC on cell death and inhibition of caspase activity caused by SNAP. (A) CEHC were incubated for 24 h with the indicated concentrations of SNAP without any further treatment or in the presence of 10 mM NAC. (B) The cells were incubated for 5 h in the presence of the indicated treatments (concentrations were: 1 mM SNAP, 0.1 mM SNP, 10 mM NAC), then collected for the determination of GSH content. (C) CEHC were treated with 1 μM staurosporine alone (stauro) or together with 1 mM SNAP. The cells were incubated for 5 h in the presence of the indicated concentrations of NAC, and then collected for caspase assay. Results reported in all the panels are means ± S.E. of four determinations.

Caspase activation has a pivotal role in the execution phase of apoptosis [10] and one can speculate that by blocking NO toxicity, its potential antiapoptotic capabilities, mediated by caspase inhibition, may also be exerted in cells sensitive to NO-induced toxicity. In order to test this hypothesis, we searched for treatments able to protect CEHC against NO-induced injury and found that NAC inhibited CEHC death caused by NO donors. Incubation of

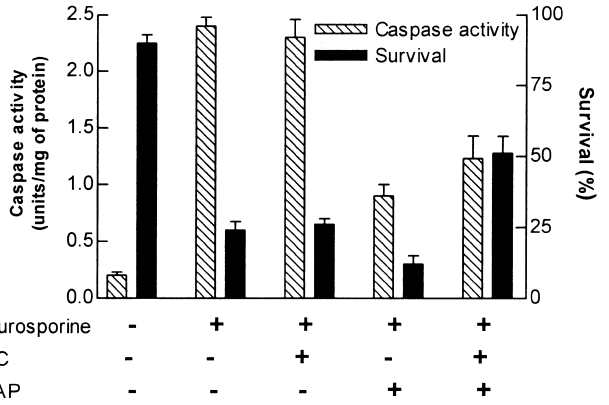

Fig. 4. In the presence of 10 mM NAC, the NO donor SNAP attenuates staurosporine-induced apoptosis of CEHC. CEHC were treated as indicated, then caspase activity was measured after 5 h and cell survival after 24 h of incubation. Results are means ± S.E. of four determinations.

CEHC in the presence of 10 mM NAC did not affect the survival of these cells, but restored the viability of SNAP-treated cells (Fig. 3A). The cytoprotective action of NAC, however, decreased at high concentrations of the NO donor. Interestingly, NAC also blocked the depletion of cellular GSH triggered by NO donors (Fig. 3B), suggesting that GSH depletion could be involved in the toxicity of NO in CEHC, as observed in different cell types [2,20,21].

Although SNAP toxicity was reduced in the presence of NAC, the NO donor molecule continued to inhibit caspase activity. In fact, at the concentration of 10 mM, NAC only slightly attenuated the inhibition of caspase activity exerted by 1 mM SNAP in whole cells treated with staurosporine (Fig. 3C). At higher NAC concentration, however, the inhibitory effect of SNAP on caspase activity was lost.

In staurosporine-treated CEHC, NAC alone did not significantly affect survival or caspase activity, but when 2 mM SNAP was present at the same time, survival increased from less than 20% to about 50% (51 ± 6%, $n = 6$). In the meantime, caspase activity was reduced by more than 50% (Fig. 4). If NAC was omitted from the medium, SNAP continued to reduce the caspase activity induced by staurosporine, while cell death was not inhibited, but actually enhanced, according to separate experiments depicted in Fig. 2C. Similar results were obtained when SNP was used as NO donor (not shown).

4. Discussion

Growing evidence has shown that NO may have either cytotoxic or cytoprotective effects [1–4]. Several mechanisms may participate to NO-induced toxicity [2,22,23]: NO may react with molecular oxygen or with the superoxide radical, generating potent cell damaging agents [24–26]. NO also inhibits DNA synthesis and repair, antioxidant enzymes and mitochondrial respiration [22,23,27] and depletes the cellular GSH [2,20,21]. Furthermore, induction of p53 [28] and of mitochondrial permeability transition [29] are mechanisms that can mediate NO-induced apoptosis in sensitive cells. On the other hand, antioxidant capability [30], induction of stress [31] or antiapoptotic [32] proteins and increased cGMP content [15,33] are the main mechanisms that have been implicated in the cytoprotective effect of NO.

The finding that NO can directly inhibit caspases [8,9,11–16] has revealed another major mechanism that can be involved in the effects of NO on cell death. The members of the caspase protease family represent the executioners of the cell death program, and their activation may be considered the 'point of no return' in apoptosis [10]. The effect of NO on caspases is likely to be a primary event mediating its inhibition of apoptosis in appropriate cell models. Inhibition of caspases may also be important in determining the shape of cell death [11]. In fact, it seems reasonable to think that NO may induce cell death accompanied by the typical features of apoptosis, as happens in some cell models [1–4], only when the activation of caspases prevails over the inhibitory effects of NO.

In CEHC, relatively high concentrations of NO donor molecules are cytotoxic. However, their toxicity is inhibited by NAC, in agreement with other reports indicating a protective effect of thiols against NO-induced injury [34–36]. Cell death is accompanied by a drastic reduction in GSH level, as observed in other cell models [20,21], that is fully prevented by NAC, suggesting that GSH depletion may be responsible for NO toxicity. The cytoprotective action of NAC could be mediated by its ability to preserve cellular levels of GSH, that, in turn, could inhibit the toxic effects of NO [27]. However, it cannot be ruled out that GSH depletion is a consequence of cell death and that NAC promotes cell survival by mech-

anisms independent of GSH [37]. Further work is required to answer this question.

NO donors do not cause CEHC apoptosis, since their toxic effect is not accompanied with activation of caspases, whose activity is actually decreased. NO also inhibits caspase activity stimulated by staurosporine, a universal inducer of apoptosis. This effect of NO, however, does not result in protection against staurosporine-induced apoptosis, as observed in the presence of the caspase inhibitor DEVD-CHO, probably because NO itself is toxic. Actually, SNAP and other NO donors increase the death of staurosporine-treated CEHC. However, in the presence of 10 mM NAC, that protects CEHC from NO toxicity, SNAP yet inhibits caspase activity, demonstrating that this inhibition is not a simple unspecific consequence of cytotoxicity, but an independent event that can be separated from cell death. As a consequence, in the presence of 10 mM NAC the antiapoptotic power of NO may be exerted, and SNAP attenuates cell death induced by staurosporine.

The chemistry of NO is extraordinarily complex, and its fate in the cell is determined by interaction with several kind of compounds, especially oxidants or thiols [7,38–43]. The inhibition of caspase activity by SNAP in the presence of NAC in vivo is quite unexpected, because thiol compounds should competitively counteract the *S*-nitrosylation of caspase enzymes as described in in vitro experiments [9,12–14]. Actually, in the presence of high concentrations of NAC (>10 mM) the inhibitory effect of SNAP against caspase activity is lost, but at NAC concentration of 10 mM this effect is at least partially retained. To explain this fact, it should be considered that thiol-bearing compounds such as NAC act as buffering agents for NO and not as scavengers, because *S*-nitrosothiols are not stable compounds and decompose, again yielding NO [2,38]. Consequently, the presence of NAC, both directly and by preserving GSH level, could stabilize NO in a nitrosothiol form and render it available for a prolonged time. On the contrary, in the absence of NAC, NO released from donor molecules is more abundant, but its life is probably much shorter. Thus, *S*-nitrosothiols may be regarded as NO-storage compounds, and this NO-buffering effect could also be responsible for the inhibition of NO toxicity, avoiding an excessive NO accumulation.

The complex redox chemistry [6,7,38–43] of NO can modulate several cell processes including cell death [34]. The reported data suggest that changes in the redox environment allow for NO interconversion from a cytotoxic into an antiapoptotic specie, mediated by the effects on caspase activity. Differences in the redox status could be responsible for the divergent effects of NO on cell death in different cell types, highlighting another example of the double edged role of NO in cell biology.

Acknowledgements

This research was supported by the Italian Ministry of University and Scientific Research (fondi MURST 40% and 60%) and Consiglio Nazionale delle Ricerche (CNR).

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