Nitric oxide induces and inhibits apoptosis through different pathways

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Abstract Physiological levels of nitric oxide (NO) regulate vascular tone and protect the microvasculature from injury whereas excessive NO may be harmful. The present study explored the effects of NO on human endothelial cell apoptosis. We found that the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) inhibited TNFα-induced endothelial apoptosis and that this was mediated partly through the cGMP pathway. In contrast, high SNAP concentration induced endothelial apoptosis via cGMP-independent pathways and the cGMP pathway protected against NO-induced apoptosis. These findings demonstrate that low NO concentrations contribute to human endothelial cell survival, whereas higher NO concentrations are pathological and promote destruction of endothelial cells.

Key words: Nitric oxide; Tumor necrosis factor α; Apoptosis; Endothelial cell; cGMP; Atherosclerosis

1. Introduction

The endothelium provides an anti-thrombotic and anti-inflammatory barrier for the normal blood-vascular wall interface. Intact endothelium also acts as a sensor and transducer of signals that regulate physiological functions of vascular wall. Dysfunction of endothelial cells has been shown to promote atherogenesis. However, mechanisms maintaining the integrity of the endothelium are unclear. Endothelial apoptosis is a physiological process which contributes to vessel homeostasis by eliminating damaged cells from the vessel wall; if increased it may lead to disturbed endothelial function and this may promote atherogenesis. It has been demonstrated that endothelial cell damage plays an important role in the progression of glomerular sclerosis [1–3] and in acute tubular necrosis occurring in the generalised Shwartzman reaction [4]. Endothelial cell damage also occurs during hypertension-induced microvascular rarefaction [5] and septic shock [6]. In addition, regions of atherosclerotic lesions are characterised by an enhanced endothelial cell 'turnover' [7], which could be due to increased endothelial cell apoptosis. These findings suggest a possible link between endothelial cell apoptosis and atherosclerosis. Although a causative role for apoptosis in atherogenesis remains hypothetical, it has been shown that apoptotic endothelial cells up-regulate the expression of intercellular adhesion molecules on normal endothelial cells resulting in hyperadhesiveness [8].

Nitric oxide (NO) is a free radical synthesised from L-arginine by NO synthases (NOS). Three isoforms of NOS (neuronal (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS)) are expressed in various tissues and cells. NO plays a vital role in diverse biological responses that include the regulation of vascular tone, neurotransmission, anti-viral defense and immune responses. Recent studies have also demonstrated that NO is an important regulator of apoptosis. Many lines of evidences suggest that NO may be involved in the pathogenesis and associated tissue destruction of immune disease. Enhanced NO production is observed in inflammatory arthritis (inflammatory synovium and cartilage) and the onset of arthritis in rodent models can be successfully blocked by a NOS inhibitor [9,10]. The presence of activated macrophages expressing high levels of iNOS and peroxynitrite formation have been demonstrated in brain tissue from patients with multiple sclerosis [11]. Moreover, increased nNOS expression has been detected in the cerebral cortex and spinal cord during the development of experimental allergic encephalomyelitis in Lewis rats [12]. On the other hand, an anti-apoptotic role of NO in cultured cells has been demonstrated. NO induces resistance to TNFα-induced hepatotoxicity [13], inhibits Fas-induced apoptosis in B Lymphocytes [14] and modulates CD95-induced apoptosis in T Lymphocytes [15].

NO has important functional effects in the cardiovascular system. It regulates vascular tone, protects endothelium from vascular injury, inhibits platelet aggregation and modulates adhesion of inflammatory cells [16,17]. Thus it has anti-inflammatory and anti-atherogenesis effects. However, excessive NO production induced by cytokines is also implicated in the tissue injury of inflammatory disease. Both the cytoprotective and cytotoxic actions of NO are relevant to the pathogenesis of vascular disease and the anti-inflammatory/anti-atherogenesis and pro-inflammatory/pro-atherogenesis actions of NO may be mediated by its effects on endothelial cell apoptosis. The present study was designed to explore the effect of NO on human endothelial cell apoptosis.

2. Materials and methods

2.1. Cell extraction and culture

Human venous endothelial cells (HUVEC) were extracted from umbilical veins. The cells were cultured in DMEM with 20% foetal calf serum (15 mM HEPES, 2.8 mg/ml sodium bicarbonate, 2 mM L-glutamine, 5 U/ml heparin, 20 μg/ml endothelial cell growth factor (ECGF), 100 μg/ml penicillin) and apoptosis of the cells assessed under different conditions.

2.2. Cell viability assay

Cell viability was determined using CellTiter 96AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI). HUVEC grown in a 96-well plate were incubated with and without apoptotic

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Abbreviations: NO, nitric oxide; TNFα, tumour necrosis factor alpha; SNAP, *S*-nitroso-*N*-acetylpenicillamine; NOS, nitric oxide synthase; HUVEC, human venous endothelial cells; ECGF, endothelial cell growth factor; ODQ, guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; LDH, lactate dehydrogenase

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stimuli, followed by incubation with [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS*] for 4 h at 37°C. The amount of converted formadan by viable cells was determined by measuring absorbance at 490 nm using a 96-well plate reader photometer.

2.3. Cytotoxic assay (lactate dehydrogenase activity, LDH assay)

Cytotoxicity of endothelial cells was assessed using the CytoTox 96Cytotoxicity Assay System (Promega). The HUVEC were incubated with and without apoptotic stimuli. Cells were lysed with lysis solution (1% Triton X-100, PBS) at 37°C for 45 min followed by centrifugation at 250×*g* for 4 min. The supernatant was incubated with assay buffer and substrate mix (diaphorase, lactate and NAD^+ mix) at room temperature for 30 min. The lactate dehydrogenase activity in the supernatant was determined by measuring the absorbance at 490 nm using a 96-well plate reader photometer.

2.4. Apoptosis assay (DNA fragmentation detection)

DNA fragmentation was detected using the KLENOW FrgEL DNA fragmentation detection kit (Calbiochem, San Diego, CA). Briefly, apoptotic endothelial cells (floating and adherent) were harvested, fixed with 4% formaldehyde and applied on to glass slides. Fixed cells were permeabilised with 0.04% Triton in TBS and endogenous peroxidase was inactivated by 3% H_2O_2 in methanol. Apoptosis was detected by labeling the 3′-OH ends of fragmented DNA with biotin-dNTP using klenow at 37°C for 1 h. The slides were then incubated with streptavidin horseradish peroxidase conjugate, followed by incubation with 3,3′-diaminobenzidine and H_2O_2. Apoptotic cells were identified by their dark brown nuclei seen under a light microscope. The degree of apoptosis was determined by the percentage of apoptotic cells over total cells.

3. Results

3.1. NO inhibits TNFα-induced apoptosis

To assess whether NO has an anti-apoptotic effect in endothelial cells, TNFα was used to induce apoptosis as it has been shown to trigger apoptosis in many cell lines including endothelial cells. TNFα induced apoptosis in the HUVEC in a dose-dependent fashion (data not shown). To determine whether NO could prevent this TNFα-induced apoptosis, the HUVEC were treated with TNFα together with the NO donor SNAP. SNAP substantially delayed the onset and re-

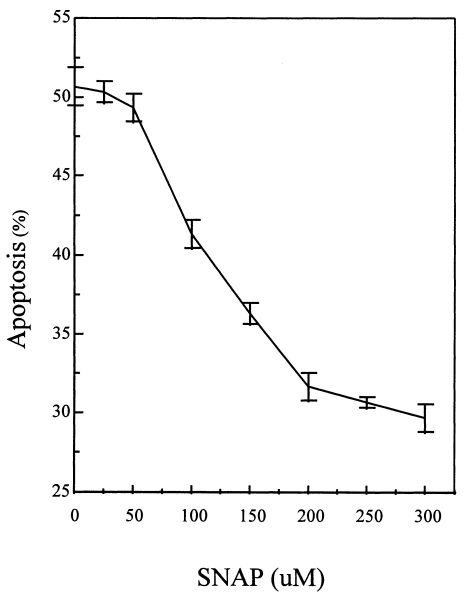

Fig. 1. Dose response of SNAP inhibition of TNFα-induced apoptosis. HUVEC were treated with 25 ng/ml of TNFα in the absence or presence of increasing concentrations of SNAP for 24 h and the apoptosis was determined as described in Section 2.

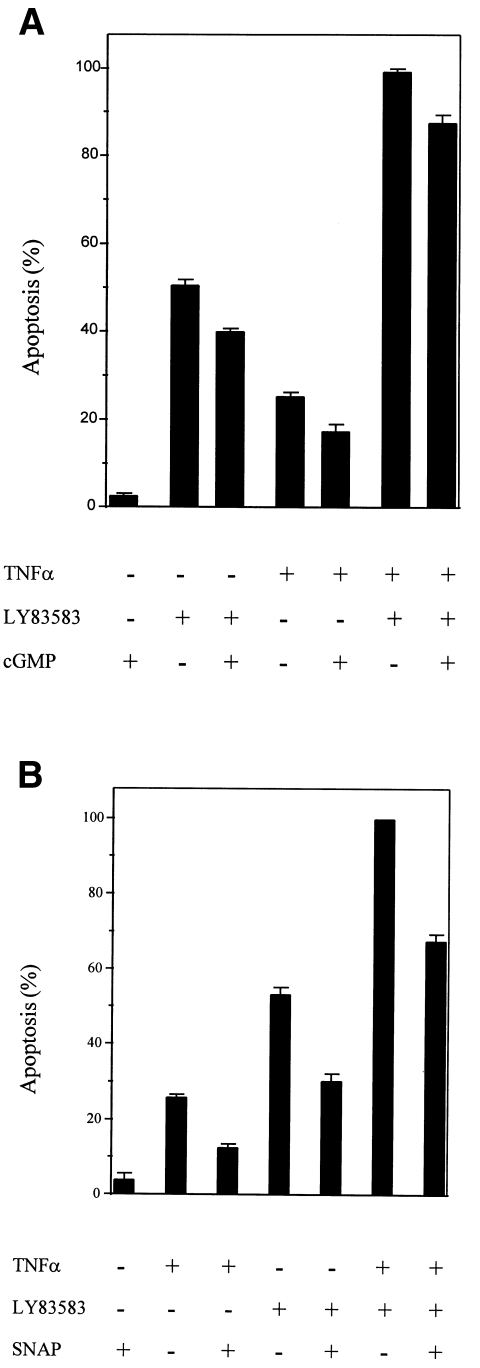

Fig. 2. cGMP pathway dependence of NO anti-apoptotic effect. A: Involvement of cGMP pathway in anti-apoptotic effect in endothelial cells. HUVEC were treated with 25 ng/ml TNFα, 10 μM LY83583, or 25 ng/ml TNFα+10 μM LY83583 in the presence or absence of 500 μM 8-bromo-cGMP. Apoptosis was determined by detection of DNA fragmentation and cell viability. B: cGMP pathway dependence of NO anti-apoptotic effect. HUVEC were treated with 25 ng/ml TNFα, 10 μM LY83583, or 25 ng/ml TNFα+10 μM LY83583 in the presence or absence of 200 μM SNAP. Apoptosis was determined by detection of DNA fragmentation and cell viability.

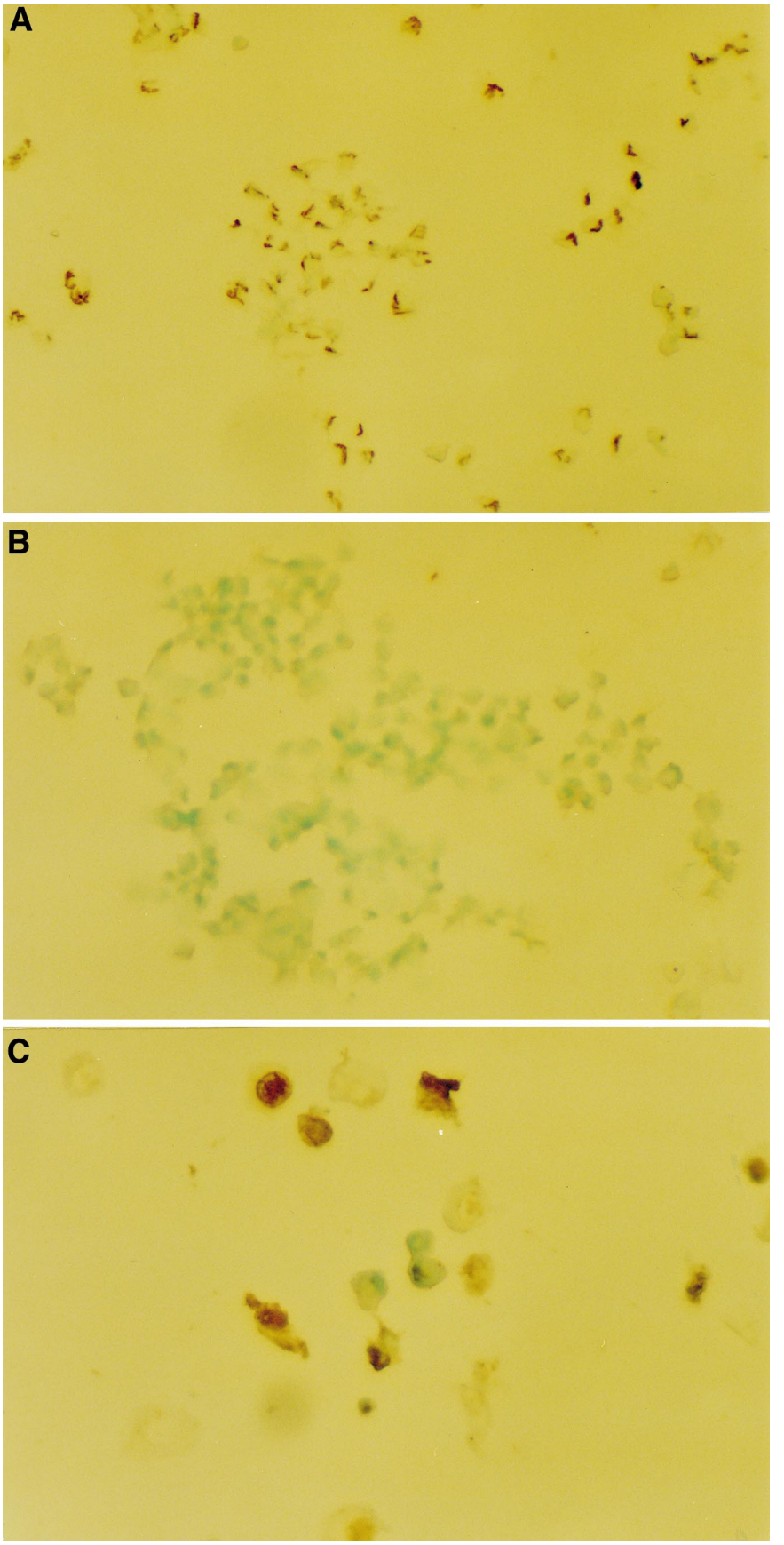

Fig. 3. Pro-apoptotic effect of NO in endothelial cells. HUVEC were treated (A) with 750 μM SNAP or (B) without SNAP for 48 h and apoptotic cells were identified by their dark brown nuclei (C) seen under a light microscope as described in Section 2.

duced the severity of TNFα-induced apoptosis. Within the low concentration range (25–300 μM), SNAP inhibited TNFα-induced apoptosis in a dose-dependent fashion with maximum effects (60%) at a concentration of 250 μM (Fig. 1). The anti-apoptotic effects of SNAP was correlated with inhibition of TNFα-induced decrease of cell viability and increase of DNA fragmentation. However, higher concentrations of SNAP (>500 μM) increased the TNFα-induced apoptosis. The concentrations of SNAP used (25–300 μM) did not affect cell viability in the absence of TNFα (data not shown). These results suggest that low concentrations of exogenous NO protect endothelial cells from TNFα-induced apoptosis.

3.2. NO anti-apoptotic effect is partially mediated through cGMP pathway

The above observation indicates that NO is a potent inhibitor of apoptosis in endothelial cells. We next investigated the mechanisms mediating the NO anti-apoptotic effect in these cells. The secondary messenger cGMP signaling pathway is a major mediator of the physiological effects of NO. NO has been shown to prevent apoptosis via either cGMP-dependent or cGMP-independent mechanisms in other cell lines [15,18]. To determine whether NO inhibits TNFα-induced endothelial cell apoptosis through a cGMP pathway, we first investigated the involvement of the cGMP pathway in anti-apoptotic activity. The cGMP donor, 8-bromo-cGMP, inhibited TNFα-induced apoptosis (Fig. 2A), but itself did not induce apoptosis. The guanylyl cyclase inhibitor LY83583 (10 μM) not only induced apoptosis by itself, but also enhanced TNFα-induced apoptosis. The apoptosis induced by LY83583, or TNFα+LY 83583 could be inhibited by the addition of 8-bromo-cGMP. Similar results were also obtained with another guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). These findings indicate that cGMP pathway is involved in the anti-apoptosis activity in endothelial cells. However, 8-bromo-cGMP did not completely abolished the apoptosis induced by these TNFα or LY83583 indicating that mechanisms other than that of the cGMP pathway may be involved in the suppression of apoptosis in endothelial cells.

Next, experiments were conducted to determine whether the NO anti-apoptotic effect is mediated via this cGMP pathway. SNAP not only partially prevented LY83583-induced cell death, but also decreased the LY83583 enhancement of TNFα-induced apoptosis (Fig. 2B). These results indicate that NO anti-apoptotic effect is partially mediated via the cGMP pathway. However, the finding that 8-bromo-cGMP inhibited TNFα-induced apoptosis to a lesser extent than that of SNAP indicates that mechanisms other than cGMP generation may also be involved in NO-mediated anti-apoptotic effects.

3.3. NO induces apoptosis in endothelial cells

Since NO has been reported to induce apoptosis in many cell lines, we also assessed this in endothelial cells. The HUVEC were treated with a range concentrations of SNAP for 72 h. While low concentration (25–200 μM) of SNAP did not affect cell viability, high concentrations of SNAP induced endothelial apoptosis as determined by the detection of apoptotic cells (Fig. 3). The induction was dose-dependent with a threshold concentration at 400 μM SNAP and complete induction at 1 mM SNAP (Fig. 4A). The induction of apoptosis by SNAP was a relatively slow process. It started after 48 h of SNAP treatment, and peaked after day 5, when almost all the cells lost viability (Fig. 4B). The pro-apoptotic effects of SNAP was correlated with decreased cell viability and increased DNA fragmentation. There was no increased LDH

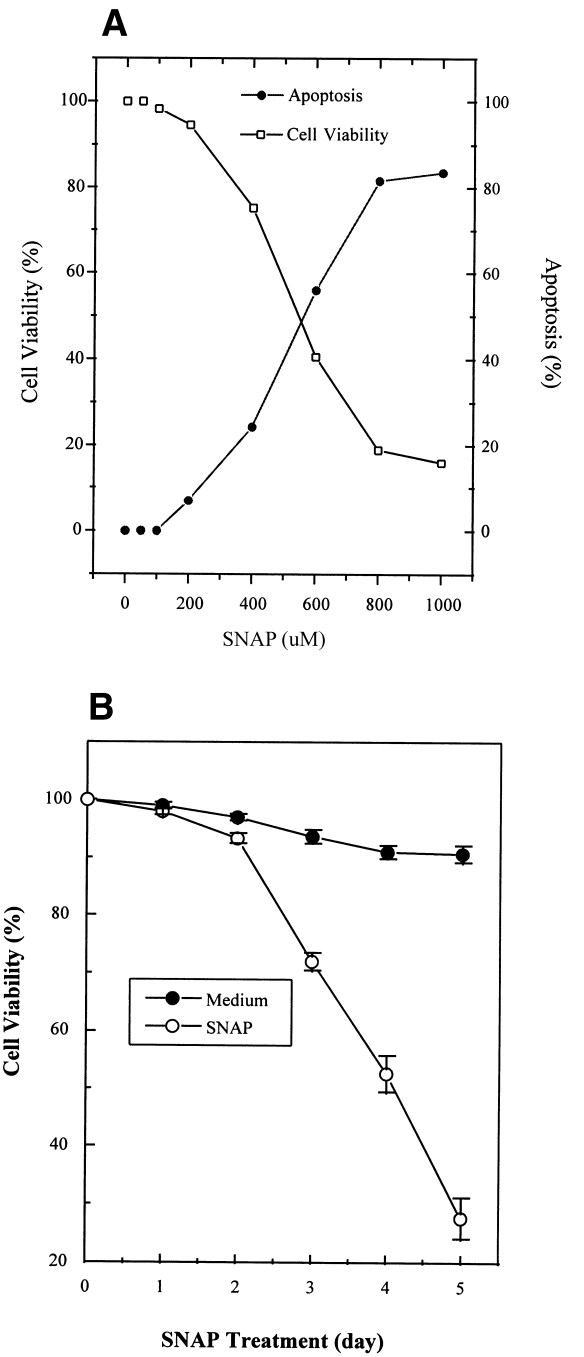

Fig. 4. Dose response and time course of NO pro-apoptotic effect in endothelial cells. A: Dose response of NO-induced apoptosis. HUVEC were treated with increasing concentrations of SNAP for 72 h and cell viability and apoptosis determined. B: Time course of NO-induced apoptosis. HUVEC were treated with 500 μM of SNAP for 1–5 days and the cell viability determined.

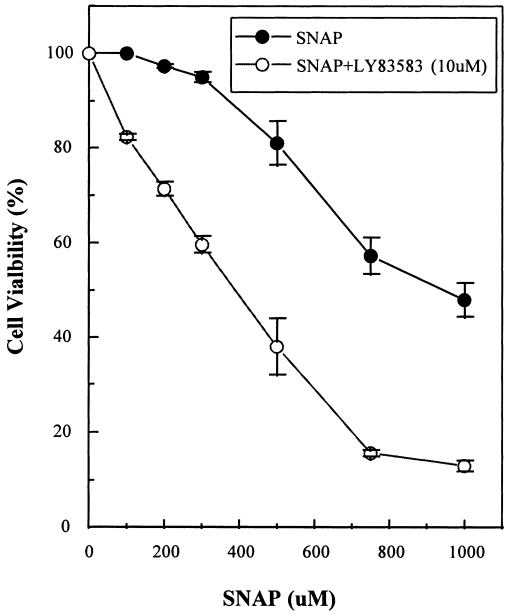

Fig. 5. Enhancement of LY83583 on SNAP-induced apoptosis. HUVEC were treated with increasing concentrations of SNAP in the presence or absence of 5 μM of LY83583 for 72 h and cell viability determined.

release detectable after SNAP incubation for 48 h which excluded the induction of cell necrosis (data not shown). However, the NO effect on endothelial cell apoptosis differed markedly depending upon the state of confluence of the cells in that apoptosis was more extensive in subconfluent cells than in confluent cells (data not shown). These results indicate that high concentrations of SNAP induces apoptosis in endothelial cells.

3.4. *NO induces apoptosis through cGMP-independent pathways*

To investigate the involvement of the cGMP pathway in this high NO concentration-induced apoptosis, the HUVEC were treated with SNAP in the absence or presence of 5 nM LY83583 for 72 h. In the absence of LY83583, SNAP began inducing apoptosis at 500 μM with maximum induction (60% of control) occurring at 1 mM (Fig. 5). However, in the presence of LY83583, SNAP induced apoptosis at 200 μM with complete induction at 1 mM. The apoptotic effect of the combined treatment of SNAP and LY83583 was stronger than a simple addition of their individual effects indicating that LY83583, by blocking the cGMP pathway, enhanced SNAP-induced apoptosis. Furthermore, 8-bromo-cGMP alone did not induce apoptosis, even at high concentration for up to 5 days (data not shown). These results indicate that a high concentration of NO induces apoptosis via cGMP-independent pathways and that the cGMP pathway has a protective effect in that it prevents high concentration NO-induced apoptosis in endothelial cells.

4. Discussion

Apoptosis is a physiological process which contributes to the control of cell population. However, excessive apoptosis of endothelial cells may be pathological and promotes atherogenesis. The results of this study indicate that, in endothelial cells, low concentrations of NO inhibit TNFα-induced apoptosis but high concentrations of NO induce apoptosis. The study also shows that the cGMP pathway is involved in the anti-apoptotic action in endothelial cells and that the NO anti-apoptotic effect is partially mediated through this pathway. However, high concentrations of NO induce endothelial cell apoptosis through cGMP-independent pathways.

Opposing effects of NO on apoptosis in other cells have been reported and the role of NO in apoptosis appears to be cell type specific. Induction of apoptosis by NO has been described in various cell lines [9,19–22], whilst other studies have demonstrated inhibitory effects [13–15,23,24]. In endothelial cells, it has been shown that shear stress inhibits TNFα- or oxidative stress-induced apoptosis and that NO is involved in the anti-apoptotic effect of shear stress [25]. Moreover, NO produced in iNOS transfected endothelial cells inhibited lipopolysaccharide-induced apoptosis and it was suggested that iNOS gene transfer may promote endothelial regeneration and accelerate vascular healing [26]. In the present study, we show that NO has both anti-apoptotic and pro-apoptotic effects in endothelial cells and that this depends on the NO concentration; low concentration of NO inhibits TNFα-induced apoptosis, high concentration of NO induces apoptosis. Thus, a constant, basal level of NO, produced under physiological conditions, may protect endothelial cells from internal and external destructive factors through anti-apoptotic effects whereas a high level of NO, released under pathological conditions (e.g. in bacterial infection and inflammatory disease), may be cytotoxic to endothelium.

The mechanisms mediating the NO anti-apoptotic actions in endothelial cells have not been well defined. The cGMP-dependent pathway has been demonstrated to be involved in NO-mediated inhibition of apoptosis in eosinophils [27], T lymphocytes [15] and hepatocytes [18]. However, the cGMP dependence of the NO anti-apoptotic effect seems to be cell type-specific. It has been reported that basal NOS activity in human leukocytes inhibits Fas-induced apoptosis via a cGMP-independent mechanism [14]. The involvement of cGMP in endothelial cell apoptosis has not been explored. Here we demonstrated that the cGMP-dependent pathway has anti-apoptotic activity in endothelial cells and that the NO anti-apoptotic effect is partially mediated by this pathway. Activation of caspase proteases has been shown to be associated with phosphorylation/dephosphorylation of upstream signaling [28]. Thus, cGMP generation may activate cGMP-dependent protein kinase which could in turn regulate the activities of apoptotic signal transducers (e.g. members of the caspase family or members of the Bcl-2 family) by direct or indirect modulating protein phosphorylation events.

Although it has been demonstrated that NO/cGMP action is sufficient for inhibition of the CD95 signaling cascade and protection from apoptosis in T lymphocytes [15], the failure of either NO or cGMP to completely suppress TNFα-induced apoptosis indicates that mechanisms other than NO/cGMP pathway may be responsible for the down regulation of apoptosis signaling in endothelial cells. In addition, the fact that the protective effect of SNAP is stronger than that of 8-bromo-cGMP suggests that mechanisms other than the cGMP pathway may also contribute to NO-mediated anti-apoptosis effect in endothelial cells. NO can react with oxygen radicals

thereby influence the redox balance. In hepatic cells, SNAP transiently changes the intracellular redox state by inducing glutathione oxidation and the formation of *S*-nitrosoglutathione and NO protected cells from TNF+actinomycin D-induced apoptosis through this glutathione oxidation [13]. In addition, NO may also interfere with haem proteins and enzymes containing iron-sulphur clusters, or modulate enzyme activity by *S*-nitrosylation of the proteins. Direct *S*-nitrosylation of caspases by NO may be one mechanism mediating the NO-induced anti-apoptotic effect. It has been shown that exposure of purified recombinant caspase-3 to an NO or NO^+ donor directly inhibited caspase-3-like activity through protein *S*-nitrosylation [18].

The molecular mechanisms underlying the NO pro-apoptotic effect are unclear. Our finding that cGMP did not induce apoptosis even after prolonged high concentration treatment establishes that NO induces endothelial apoptosis via cGMP-independent pathways. Also, SNAP induced stronger apoptosis in the presence of LY83583 indicating that the cGMP pathway may protect endothelial cells from NO-induced apoptosis. Mechanisms for high dose NO-induced apoptosis remain speculative. NO reacts rapidly with superoxide to form the highly toxic peroxynitrite ($ONOO^-$), and it has been demonstrated that both NO and $ONOO^-$ can damage DNA directly [29–36]. This could trigger the p53-dependent or p53-independent apoptotic cell death pathways [36,37], with subsequent activation of caspases or related proteases, and cleavage of poly(ADP-ribose) polymerase [22].

Many factors may determine whether endothelial cells undergo apoptosis when exposed to NO. These include the amounts of NO being produced, the different redox states of NO, and the local environment that may promote further production of cytotoxic moieties such as peroxynitrite. It has been shown that the interaction of NO with enzymes possessing the haem group, the iron-sulphur cluster, or thiol groups depends on its redox state [38,39] and that NO can exhibit cytoprotective or cytotoxic effects depending on the chemistry it undergoes in a given biological milieu [38–40]. The opposing effects of NO on endothelial apoptosis may also depend on the availability of anti-apoptotic signaling transducers (such as the cGMP pathway and members of Bcl-2 family) and pro-apoptotic signaling transducers (such as caspases and ICE family members). Thus, the end result may depend on the balance between a number of anti-apoptotic and pro-apoptotic factors.

In summary, the evidence reported here demonstrates both an anti-apoptotic effect of low concentration of NO and a pro-apoptotic effect of high concentration of NO in endothelial cells. The NO anti-apoptotic effect is partially mediated through the cGMP pathway, while the NO pro-apoptotic effect is cGMP-independent and the cGMP pathway has a protective effect from NO-induced endothelial apoptosis. This double-edged effect of NO may have significant implications for the initiation and progression of atherosclerosis.

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