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Nitric Oxide Donor SNAP Induces Apoptosis in Smooth Muscle Cells through cGMP-Independent Mechanism

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Recent evidence suggests that nitric oxide (NO) may function as a second messenger in the intracellular signal transduction pathways. We explored the possibility that NO was involved in the signal for triggering apoptosis in smooth muscle cells (SMCs). Chemical NO donors induced SMCs apoptosis in a concentration- and time-dependent manner. The membrane-permeable cGMP analogue, dibutyryl-cGMP, did not induce SMCs apoptosis, and the highly selective inhibitor of cGMP-dependent protein kinase, KT5823, was unable to inhibit the induction of NO-induced SMCs apoptosis. Inhibitor of ADP-ribosyltransferase slightly attenuated the induction of SMCs apoptosis by S-nitroso-N-acetyl penicillamine (SNAP). The inhibitor of Na^+-H^+ antiporter, amiloride, completely inhibited the induction of SMCs apoptosis by SNAP. These results demonstrate for the first time that NO can induce apoptosis in SMCs, suggesting that NO acts as a mediator in the development of atherosclerosis lesion via alterations in the number of SMCs. In addition, the results suggest that NO exert these effects through a pathway that does not involve guanylate cyclase and cGMP-dependent protein kinase.

It is now well established that apoptosis is a vital aspect of normal development, and dysregulated apoptosis may have an important role in the pathogenesis and progression of a variety of diseases including cancer, acquired immune deficiency syndrome, heart disease and neurological disorders.

A characteristic feature of atherosclerosis blood vessel disease is excessive proliferation of SMCs, macrophages and T lymphocytes. Simultaneously, apoptosis of SMCs has recently been demonstrated to occur in the advanced atherosclerotic lesions(1). But the mechanism of apoptosis is still not well understood.

NO is now known to act as an intracellular mediator in several cells(2)(3). It is released by cells containing the enzyme nitric oxide synthase following an increase in the intracellular calcium level, whereas cytokine-activated SMCs(4), macrophages, or endothelial cells have an inducible form of the enzyme that can ultimately generate a much higher level of NO than that produced by constitutive enzyme in endothelial cells. It then diffuses to affect neighboring cells. Many, but not all, of the actions of NO in the target cells involve the activation of guanylate cyclase, which subsequently increases intracellular cGMP levels and stimulation of cGMP-dependent protein kinase. Recent work has shown that NO inhibits the proliferation of subcultured aortic smooth muscle cells(5), raising the possibility that NO may act in the inhibition of proliferation via apoptosis. In this study, we showed that NO can induce apoptosis in SMCs in a concentration- and time-dependent manner, and we have provided evidence that the activation of guanylate cyclase and cGMP-dependent protein kinase do not play a major role in this effect.

MATERIALS AND METHODS

Materials. S-nitroso-N-acetyl penicillamine(SNAP) and 3-Morpholinosydnonimine Hydrochloride (SIN-1) were obtained from BIOMOL Research Laboratories inc; Dibutyl-cGMP, nicotinamide and phylloquinone from Sigma, KT5823 and 5-(N-Methyl-N-isobutyl) amiloride from BIOMOL Research Laboratories inc, and all cell culture materials from Life Technologies.

Cell culture. Aortic SMCs were obtained from thoracic aorta of the Japanese white rabbit by the method described(6).

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The cells (1×10^5) were seeded into 35-mm diameter dishes and maintained in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were used between the third and fifth passage. After Subconfluence, the medium was exchanged for 2 ml of serum-free DMEM. The cells were used for experiments after 24 hr.

Analysis of DNA fragmentation in agarose gels. SMCs (1×10^5) were lysed at 37°C for 60 min in a buffer containing 0.5% sodium N-lauroyl-sarcosinate, 50 mM Tris buffer (PH7.8) and 10 mM EDTA. The lysate was then incubated in RNase A (100 μg/ml) for 0.5 hr at 50°C. After proteinase (100μg/ml) treatment for 1 hr at 50°C, DNA was extracted with an equal volume of phenol/chloroform, 1;1. and precipitated with 1/10 th vol 7 M ammonium aceteate and 2.5 vol ice-cold ethanol at room temperature for 1hr. DNA samples were loaded onto 2% agarose gels and run at 100V≈3hr(7).

In situ labeling of nuclear DNA fragments. SMCs were stained by the TdT-mediated dUTP nick end-labeling(TUNEL) method(7) and imaged with Nikon optics. Apoptotic cells (TUNEL-positive cells) are expressed as a percent of total SMCs(TUNEL-positive cells+TUNEL-negative cells)

RESULTS AND DISCUSSION

1. NO-Induced Apoptosis in SMCs

Two commonly used NO donors, SNAP and SIN-1, were used to induce apoptotic cell death in SMCs. Apoptotic DNA strand breaks induced by in vitro incubation of SMCs with the chemical NO-donor were visualized by in situ nick translation.

The percentage of apoptotic cells increased with increasing NO donor concentrations (Fig. 1.). The time course of NO-induced strand breaks in cultured SMCs in shown in Figure 2. After 8hr of incubation, 75% of NO-treated SMC, nuclei were positive, whereas only 5% of untreated cells were positive. Fig. 1 and 2 show that NO caused concentration- and time-dependent induced apoptotic cells. Hemoglobin(20μM), which acts as a NO scavenger, blocked the ability of SNAP to induce apoptosis(Fig.4, 5). This result indicated that NO alone is sufficient to induce apoptosis.

2. Inhibition of NO-Induced Apoptosis by Na^+-H^+ Antiport Inhibitor

Searching for the involvement of cGMP in NO-induced apoptosis, the selective cGMP-dependent protein kinase inhibitor, KT5823(20μM), applied alone to SMCs did not affect basal levels of apoptic cells(data not shown) or the increase observed following exposure to SNAP(100μM)(Fig. 5). Furthermore, the cGMP analogue, dibutyryl-cGMP(100μM), did not induce apoptosis, compared with NO-induced apoptosis. Two inhibitors of ADP-ribosyltransferase, nicotinamide(10mM) and phylloquinone(100μM) appear to slightly reduce apoptotic cells, but this is not a significant difference when compared to the controls(Fig3,4,5). We then investigated the effect of Na^+-H^+ antiport inhibitor, amiloride, because NO is reported to stimulate the ability of Na^+-K^+ ATP ase activity secondary to increases in Na^+-H^+ exchange(9). Amiloride (0.3mM), at the

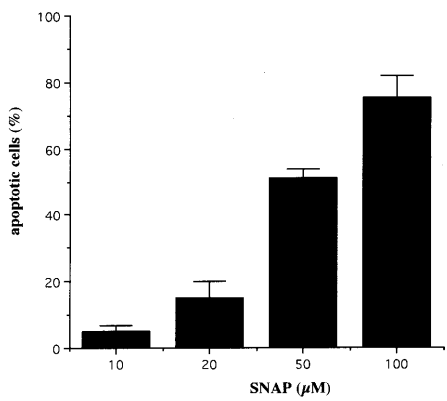

FIG. 1. Concentration-dependent induction of apoptosis in SMCs by NO donor. Serum-deprived SMCs were treated with different concentrations of SNAP as indicated for 8hr. Apoptotic cells are expressed as a percentage compared to the total number of SMCs and represents the average and range of two experiments.

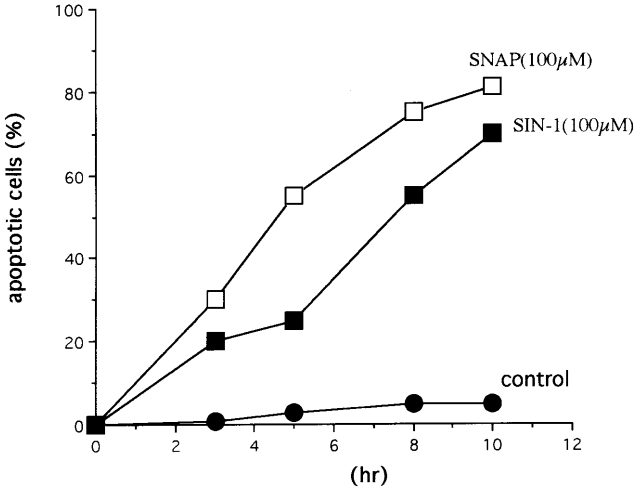

FIG. 2. Time-dependent induction of apoptosis in SMCs by NO donor Serum-deprived SMCs were treated with NO donor, SNAP and SIN-1, for the indicated time. Apoptotic cells(TUNEL-positive adherent cells) are expressed as a percentage of the total number of SMCs.

highest non-toxic concentration, inhibited completely NO-induced apoptosis(Fig 3,4,5). This result indicated that NO-induced apoptosis does not depend on NO-induced cGMP level elevation.

Biological effects of NO that are cGMP independent have recently been a subject of considerable interest. For example, SNP has been reported to inhibit glucose-induced insulin release by activating the ATP-sensitive K^+ channel(10). This is presumably a cGMP-independent effect, since cGMP itself potentiates insulin release without affecting ionic movement. In addition, NO-generating compounds have been shown to inhibit mitogenesis, proliferation of cells(11) and

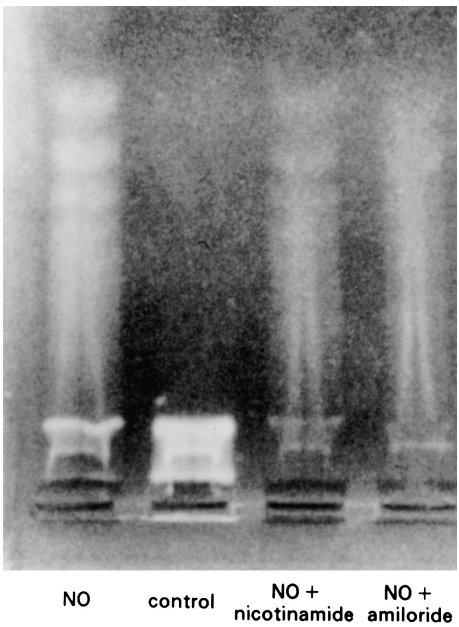

FIG. 3. Effect of amiloride and nicotinamide on DNA fragmentation in SMCs. DNA, isolated from SNAP-treated SMCs with or without amiloride or nicotinamide for 8hr, was subjected to electrophoresis in 2% agarose and visualized with ethidium bromide. lanes 1–4 are SNAP(100μM), control, SNAP+amiloride (0.3mM), SNAP+nicotinamide (10mM).

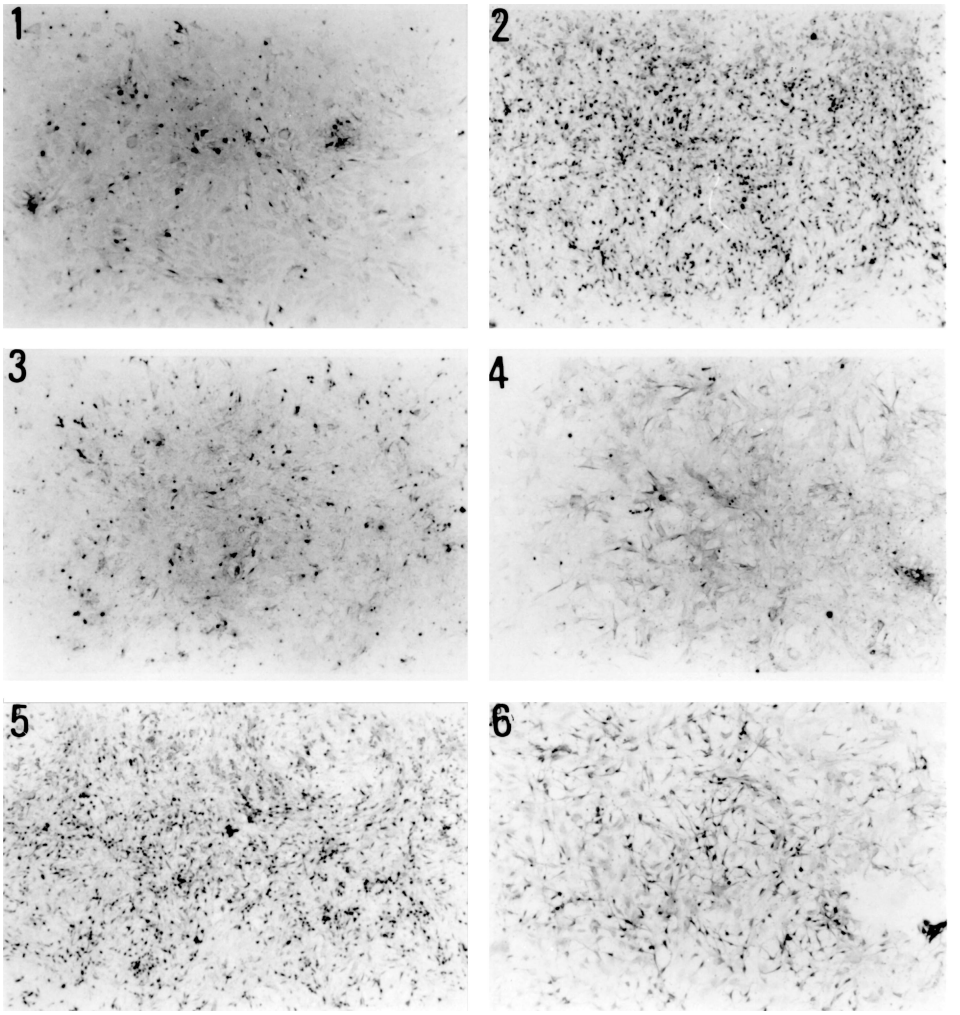

FIG. 4. Apoptosis induction in SMCs exposed to SNAP. Variously treated SMCs are shown stained by the TdT-mediated dUTP nick end-labeling (TUNEL) method and imaged with Nikon optics. (1) control, (2) SNAP, (3) cGMP only, (4) SNAP+amiloride, (5) SNAP+nicotinamide, (6) SNAP+hemoglobin.

osteoclast activating effects(12) that are not mimicked by cGMP analogues. NO has been shown to cause ADP-ribosylation of the enzyme glyceraldehyde-3-phosphate dehydrogenase(13) and of actin(14). These effects are not mimicked by the permeable cGMP analogue.

Na^+-H^+ exchanger activity plays an important role in the control of intracellular pH, maintenance of cellular volume, Na^+ reabsorption, membrane potential, and facilitation of cell proliferation in response to growth factors(15). This functional diversity is accomplished by the action of distinct isoforms of the Na^+-H^+ exchanger. To date, four members(Na^+-H^+ exchanger) of this multigene family have been identified(16)(17). Overall, they share 40–60% amino acid identity with 10–12 predicted N-terminal transmembrane-spanning regions and with the C-terminal cytoplasmic region. This latter resion exhibits the greatest divergence in amino acid sequence among the isoforms and contains one or more potential sites for phosphorylation by different serine/threonine protein kinases. Further studies are ongoing to confirm the precise molecular mechanisms involved in NO-induced apoptosis by enhancing Amiloride sensitive Na^+-H^+ exchange.

In conclusion, our study demonstrates that NO induces apoptosis in SMCs independent of the

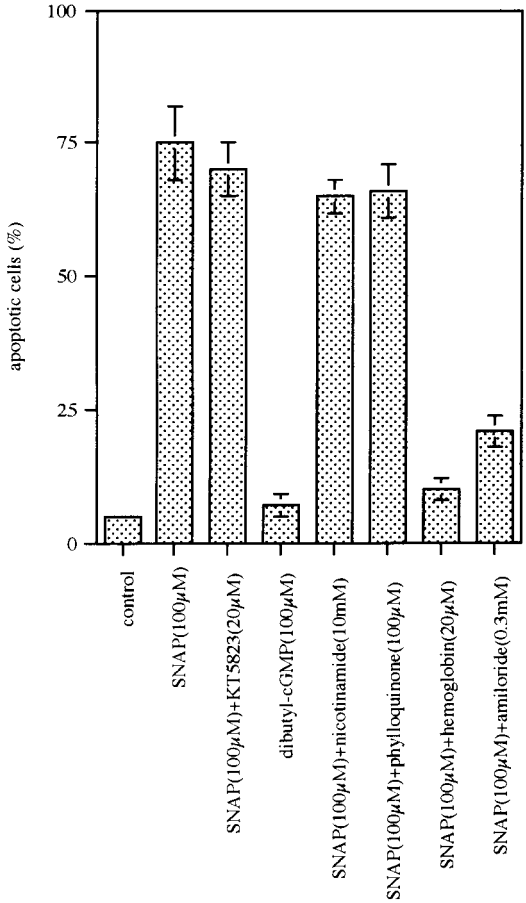

FIG. 5. Effect of various agents on NO-induced apoptosis in SMCs. Serum-deprived SMCs were treated with SNAP(100μM) and various agents for 8hr. Apoptotic cells are expressed as a percentage of the total number of SMCs and represents the average and range of two experiments.

ability to increase the intracellular cGMP concentration. The stimulation of Amiloride sensitive Na^+-H^+ exchange by NO would presumably result in apoptosis of SMCs.

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