Peroxynitrite Induces Apoptosis in Rat Aortic Smooth Muscle Cells: Possible Relation to Vascular Diseases

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An emerging body of evidence is accumulating to suggest that *in vivo* formation of free radicals in the vasculature, such as peroxynitrite ($ONOO^-$), and programmed cell death (i.e., apoptosis) play important roles in vascular diseases such as atherosclerosis, hypertension, and restenosis. The present study was designed to determine whether primary rat aortic smooth muscle cells (SMCs) undergo apoptosis following treatment with $ONOO^-$. Direct exposure of primary rat aortic SMCs to $ONOO^-$ induced apoptosis in a concentration-dependent manner, as confirmed by means of quantitative fluorescence staining and TUNEL assays. $ONOO^-$-induced apoptosis in rat aortic SMCs appears to involve activation of Ca^{2+}-dependent endonucleases. Although the precise mechanisms by which peroxynitrite induces apoptosis in rat aortic SMCs need to be further investigated, the present, preliminary findings could be used to suggest that $ONOO^-$ formation in the vasculature may play roles in the processes of vascular diseases, such as atherosclerosis, hypertension, and restenosis, *via* adverse actions on blood vessels. Exp Biol Med 229:264–269, 2004

Key words: peroxynitrite; apoptosis; vascular smooth muscle cells; rat aorta

An emerging body of evidence is emerging to suggest that reactive oxygen species (ROS) and reactive nitrogen species (RNS) of diverse types play roles in vascular diseases such as atherosclerosis, hypertension, and restenosis (1, 2). Peroxynitrite ($ONOO^-$), the product of a reaction between nitric oxide and superoxide, is a potent and versatile oxidant implicated in a number of pathophysiological processes (3, 4). The activity of $ONOO^-$ is related to its accessibility. In *in vitro* studies, exposure of cells to $ONOO^-$ evokes responses that depend on the environment and cell types (5–7). It has been demonstrated that $ONOO^-$ can diffuse freely across phospholipid membrane bilayers to react with a wide variety of molecular targets, including lipids, proteins, and DNA, leading to cell death *via* necrosis or apoptosis (8, 9).

Apoptosis is termed a physiological type of cell death that is involved in a number of critical events occurring during normal development and differentiation and plays key roles in a wide variety of diseases, including vascular-related diseases of diverse types (for review, see Ref. 10). Vascular diseases are characterized by alterations of blood vessel structure determined mainly by VSMC growth, which is now viewed as the result of the opposing effects of cell proliferation and apoptosis (8). Apoptosis is a prominent feature of the vascular remodeling process that occurs in atherosclerosis, hypertension, and restenosis (1, 2, 11).

A few years ago, Salgo *et al.* first reported that $ONOO^-$ can induce apoptosis in thymocytes (12). Following this report, a series of publications on $ONOO^-$-induced apoptosis in different cell types *in vitro* have appeared (13–15), but the effects of RNS, including $ONOO^-$ on SMCs, have been studied only sparingly (16) and not in primary vascular smooth muscle cells in culture. The present study was designed to determine whether primary rat aortic SMCs undergo apoptosis following treatment with $ONOO^-$ and whether the process can occur in a concentration-dependent manner and its relationship with Ca^{2+} mobilization.

Materials and Methods

Primary Cell Culture and $ONOO^-$ Treatment. The experiments were performed on single vascular smooth muscle cells of aorta, which were obtained from

This work was supported in part by NIH Grant AA-08674.

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Received July 3, 2003.
Accepted October 30, 2003.

1535-3702/04/2293-0001$15.00

male Wistar rats (250–350 g) after pentobarbital sodium anesthesia and sacrifice. The procedures employed to isolate and culture rat aortic SMCs have been reported (17). Briefly, the SMCs were cultured in Dullbecco's modified Eagle's medium, mixed 1:1 with Ham's nutrient mixture F-12 at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2 (17). The culture medium contained 10% fetal calf serum, 100 U/ml penicillin, and 100 μg streptomycin. More than 97% of the primary cultured cells were SMCs as confirmed by immunostaining with a monoclonal α-smooth muscle actin antibody (17). Cells were regularly subcultured with 0.05% trypsin and seeded onto 12-mm circular coverslips. Cells at the second to sixth passages, at 80% confluence, were used for our experiments. Twenty-four hours before $ONOO^-$ treatment, the medium was replaced with 1% serum medium. Peroxynitrite was diluted in phosphate-buffered saline (PBS; pH 8.3) and was directly added to the culture medium at final concentrations ranging from 10 to 200 μM. Control samples were treated with PBS (pH 8.3) only. The effect of decomposed peroxynitrite was also tested after the solution of peroxynitrite was kept in PBS (pH 7.2) at 37°C for 30 mins (18).

Cell Viability Assays. Cell viability was assessed and quantified by using trypan blue exclusion. Briefly, the SMCs in complete medium were plated into wells of a six-well cluster plate overnight. After treatment with different doses of peroxynitrite for 24 hrs, the cells were harvested by trypsin digestion. The cells were centrifuged at 1200 *g* for 10 mins at 4°C, examined by adding an equivalent volume of a 0.4% trypan blue solution to an aliquot of the re-suspended cells, and incubated for 5 min. The stained and unstained cells were counted by means of a hemacytometer. The mean values obtained represent data of triplicates from each separate experiment.

Morphological and Quantitative Analysis of Apoptotic Cells. Quantitative morphologic evaluation of apoptosis was performed by staining cells with propidium iodide (PI; Ref. 19). Briefly, primary cells cultured on coverslips were fixed with 4% paraformaldehyde on ice for 15 mins and rinsed with PBS for 5 mins, and 0.2 ml of an RNAse A stock solution (1 mg/ml) were added at 37°C for 30 mins, washed with PBS for 5 mins, and stained with PI (5 μg/ml) DNA-binding dye for 20 mins. After decolorization with distilled water, the cells were examined with a confocal ultraviolet-fluorescence microscope and an ultraviolet-laser microscope. Cells with typical features of nuclear fragmentation and/or marked condensation of chromatin with reduced nuclear size were interpreted as apoptotic cells (20). The number of apoptotic cells and total cells were counted in six randomly selected high-power fields under a fluorescence microscope. The percentage of apoptotic cells was calculated as the number of apoptotic cells/number of total cells × 100% (19).

TUNEL Assay. DNA fragmentation *in situ* was detected by means of terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick-end labeling (TUNEL) assays, according to the kit instructions (Roche, Germany). Briefly, cells were fixed with 4% paraformaldehyde in PBS for 1 hr at 25°C, coverslips were rinsed with PBS for 5 mins and incubated in permeabilization solution (1% Triton X-100 in 0.1% sodium citrate) for 2 mins on ice, and the TUNEL reaction mixture was added for 1 hr at 37°C in a humidified atmosphere in the dark. Negative controls were set up by adding labeling solutions without terminal transferase instead of the TUNEL reaction mixture. After rinsing three times with PBS, samples were analyzed in a drop of PBS under fluorescence using an excitation wavelength in the range of 450–500 nm, and detection was in the range of 515–565 nm.

Intracellular Ca^{2+} Measurement. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was measured in isolated primary SMCs in the presence or absence of peroxynitrite, using the Ca^{2+}-sensitive membrane-permeant fluorescent dye fura 2-AM (acetoxymethylester of 1-2-[5-carboxyoxazol-2-yl]-6-aminobenzofuran-5-oxy-2-[2′-amino-5′methylphenoxy]ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (22), according to previously established methods (17, 23). Monolayers of the aortic SMCs, grown on the coverslips, were loaded with 2.0 μM fura 2-AM and 0.12% pluronic acid F-127 (60 mins, 37°C). The monolayers were washed two to three times with PBS and 20 mM HEPES (pH 7.4) and incubated with this buffer at room temperature until ready to use. The monolayers were inserted in a leakproof coverslip holder. Buffer was added to the monolayer on the coverslip. The coverslip holder was mounted onto the stage of a temperature-controlled Nikon TMS inverted microscope with a long working distance Nikon Fluor objective (n.a. 0.5), attached to a 300-W xenon light source and the CCD camera for image acquisition. Buffer (control) and peroxynitrite were added to the monolayers. The primary cultured aortic SMC monolayers, preloaded with fura 2-AM, were excited alternatively, at 340 and 380 nm, and the emission intensity was recorded at 510 nm using a silicon-intensified target camera (23). Background autofluorescence for both excitation wavelengths was acquired from blanks for each experiment and subtracted from each pair of images separately before ratioing. Fluorescence ratios (R) were obtained by dividing the 340-nm image by the 380-nm image. No image misalignments occurred when those two ratiometric images were superimposed. The resulting images were then used to calculate $[Ca^{2+}]_i$ in smooth muscle cells using external standards containing 2.54 mM Ca^{2+} and 0 mM Ca^{2+} plus 10 mM EGTA for maximum (R_{max}) and minimum (R_{min}) fluorescence ratios of the 340-nm and 380-nm images. $[Ca^{2+}]_i$ was calculated according to the following equation (21): $[Ca^{2+}]_i = K_d \times B \times (R - R_{min})/(R_{max} - R)$. A K_d of 224 nM was used for the fura-2/Ca^{2+} complex (17). B is the ratio of fluorescence intensity of fura-2 to Ca^{2+}:fura-2 complex excited at 380 nm.

Materials. Peroxynirite was purchased from CALBIOCHEM (San Diego, CA; catalog no. 516620) and kept deep frozen at −70°C before use, as the product is heat and

light sensitive. Activity decreases approximately 2% per day at −20°C. Propidium iodide was purchased from Sigma Chemical (St. Louis, MO). Fura-2 was purchased from Molecular Probes (Eugene, OR). All other organic and inorganic chemicals were obtained from Fisher Scientific (Fair Lawn, NJ) and were of the highest purity.

Statistics. Where appropriate, results are expressed as means ± SD and were examined for statistical significance by means of Student's *t* tests and ANOVA. Values of $P < 0.05$ were considered to be statistically significant.

Results

Cytotoxicity of $ONOO^-$. As shown in Figure 1, treatment with $ONOO^-$ resulted in a concentration-dependent reduction of survival of rat aortic SMCs as determined by trypan blue exclusion. The greater the concentration of $ONOO^-$, the less the number of surviving cells. Threshold effects took place between 10 and 50 μM peroxynitrite. Use of decomposed $ONOO^-$ (see *Materials and Methods*) failed to exert any effects on cell viability or any of other parameters tested below ($n = 6$).

Morphological Changes Induced by $ONOO^-$. In order to find out whether cell death induced by $ONOO^-$ was due to necrosis or apoptosis, we evaluated the morphological changes induced in $ONOO^-$-treated cells using nuclear chromatin staining with fluorescence dyes. Figure 2 (top panel) shows the typical morphology of apoptotic nuclei stained with propidium iodide (PI). The chromatin is condensed and aggregated along the nuclear membrane as indicated by bright fluorescence. The predominant characteristics in the nuclei of $ONOO^-$-treated cells were the fragmented states of their nuclei (Fig. 2, top panel B). As shown in Figure 3, the percentage of apoptotic cells increased in a concentration-dependent manner as the concentration of $ONOO^-$ increased. This indicates that $ONOO^-$ can elicit apoptosis in rat aortic SMCs, which is reflected by decreased cell viability. The threshold concentration for induction of apoptosis is ~10 μM.

DNA Fragmentation by TUNEL Assay. Cells that simultaneously exhibit condensed nuclei and positive labeling by the TUNEL assay are regarded as apoptotic cells (24). In addition, the TUNEL assay allows the detection of apoptotic cells before the DNA is extensively cleaved. In the present study, cells treated with $ONOO^-$, as illustrated by a strong green fluorescence in the nuclei, are considered positive cell labeling by the TUNEL assay (24). In contrast, only a few positive cells were detected in untreated cells (Fig. 2 ,bottom panel B).

The Effects of $ONOO^-$ on $[Ca^{2+}]_i$ in Isolated Smooth Muscle Cells. The quantitative effects of peroxynitrite on $[Ca^{2+}]_i$ in SMCs isolated from rat aorta were determined by using the direct technique of Ca^{2+} visualization in single cells as revealed by the digital imaging microscope using fura 2-AM (17). The control basal level of $[Ca^{2+}]_i$ was 88.5 ± 4.09 nM. As shown in Figure 4, $ONOO^-$ elicited a concentration-dependent increase in $[Ca^{2+}]_i$ within 30 secs, the threshold being ~10 μM; 100 μM peroxynitrite resulted in an almost a 2.5-fold rise in $[Ca^{2+}]_i$.

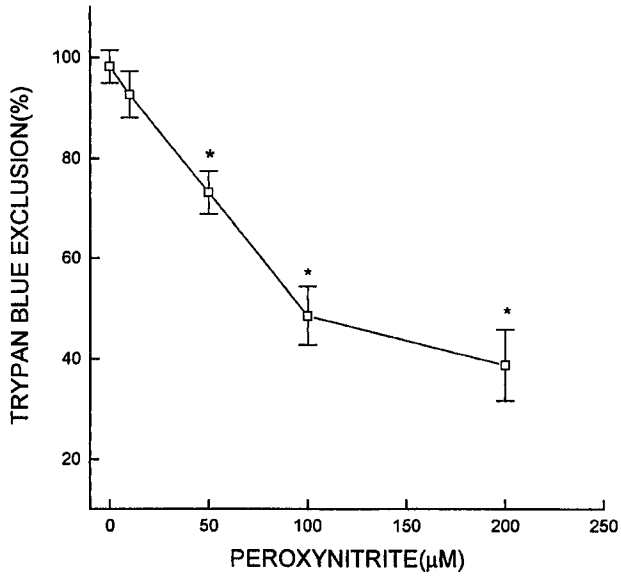

Figure 1. Concentration-dependent manner of reduction of survival of rat aortic SMCs induced by peroxynitrite, as measured by trypan blue exclusion (see *Methods*).

Discussion

Previous studies have shown that apoptosis in certain vascular SMCs (e.g., human coronary arteries) appears to have a profound effect on many vascular or vascular-related diseases (25, 26). In normal conditions, the net balance between cell proliferation and apoptosis determines the extent of vascular SMC growth (10, 25). Considerable evidence suggests that the pathogenesis of vascular diseases involves a perturbation of the balance between cell proliferation and cell death (27). Many mediators, including ROS and RNS, can trigger apoptosis in different cell types (15, 25). In the present study, we clearly demonstrate that $ONOO^-$ can trigger apoptosis in rat aortic primary SMCs based on the following two criteria. First, rat aortic primary SMCs treated with $ONOO^-$ show condensation of nuclear chromatin and apoptotic bodies using PI staining. These morphological changes are characteristic of apoptosis (20). Second, the TUNEL assay is a relatively specific tool to assist in detection of apoptotic cells (28). In addition, the TUNEL assay allows the detection of apoptotic cells before the DNA is extensively cleaved (11). In the present study, TUNEL-positive cells appear in cells treated with $ONOO^-$ but almost nil in untreated cells. Furthermore, $ONOO^-$-induced apoptosis in rat aortic SMCs occurs in a concentration-dependent manner (Fig. 3). Although others have demonstrated that $ONOO^-$ can result in accumulation of histones (a probable indicator of DNA fragmentation) in rat aortic SMCs (18), our study is the first to quantify morphological changes characteristic of apoptosis and allow

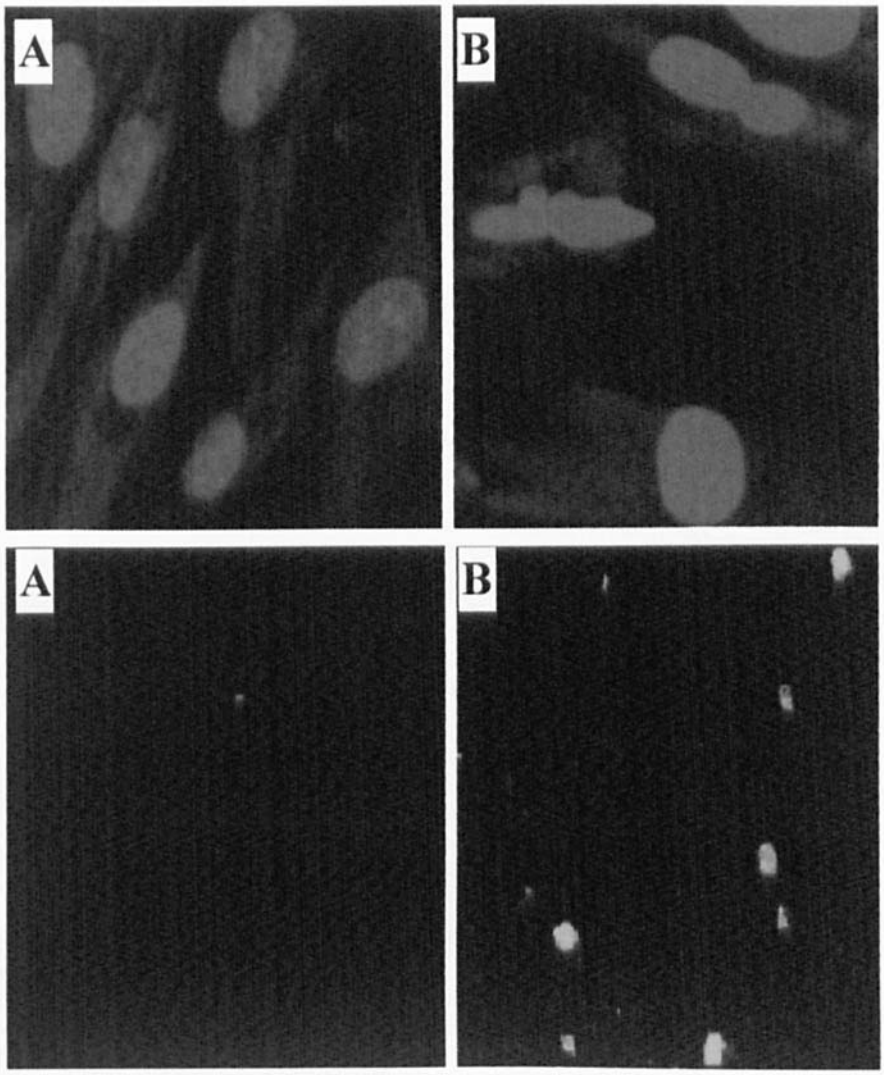

Figure 2. (Top panel) Morphological changes in rat aortic SMCs treated with peroxynitrite using PI fluorescence microscopy. (A) Controls. (B) Cells treated with peroxynitrite (50 μM) for 24 hrs. (Bottom panel) TUNEL staining in cells treated with peroxynitrite. (A) Controls. (B) Cells treated with peroxynitrite (50 μM) for 24 hrs.

detection of apoptosis before DNA is extensively cleaved in primary aortic SMCs.

There are many factors that can influence exogenous oxidants to induce apoptosis *in vitro*, especially the concentration of oxidants and cell types. $ONOO^-$-induced apoptosis is one example of such a case. In previous studies, a wide concentration range of $ONOO^-$ (10–1000 μM) has been shown to induce apoptosis in certain cells (14, 29, 30). The present research on rat aortic SMCs is clearly within the lower part of this concentration range. However, the previous studies on spinal neurons, thymocytes, and endothelial cells, like the present work, demonstrate marked differences in percentage of apoptotic cells. The explanation for the difference in reactivity might be related to different experimental conditions and different cells used. In the present study, while addition of low levels of $ONOO^-$ (e.g., 10 μM) are sufficient to trigger apoptosis, as the level of $ONOO^-$ reaches higher concentration ($\geq$200 μM), the cells clearly experience predominantly necrotic death (data not shown). This is in accordance with previous conclusions obtained from other cell types (e.g., thymocytes, cardiac myocytes); that is, low concentrations of $ONOO^-$ induce apoptosis, whereas higher concentrations of the oxidant lead to necrosis (14, 15, 24).

Although $ONOO^-$ can induce apoptosis in a variety of cell types and the underlying mechanism(s) of $ONOO^-$-induced apoptosis is presently under intensive investigation, it is still unclear how $ONOO^-$ triggers apoptotic pathways. Previous studies have suggested that there is an increase in cytosolic free calcium ions in hepatocytes undergoing oxidative stress (31). It has been argued that part of the calcium is conveyed to the cell nucleus, where it activates nucleases that trigger DNA strand breaks, a mechanism reminiscent of apoptosis (32). In our study, we demonstrate that $ONOO^-$ causes a sustained increase in intracellular free cytosolic calcium within 30 secs; the present results are thus in agreement with previous findings in thymocytes (33). This suggests that apoptosis induced by $ONOO^-$, at least in

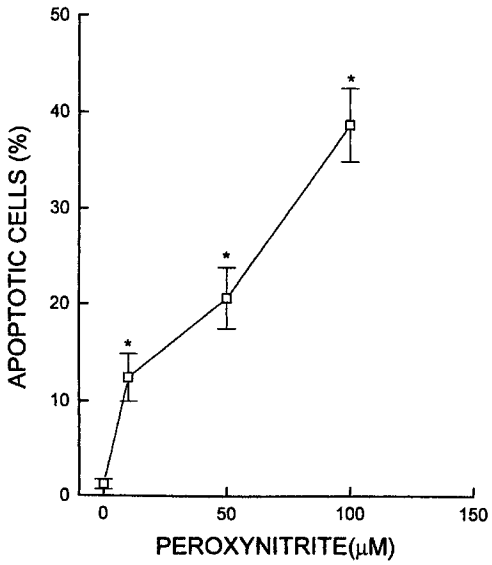

Figure 3. Percentage of apoptotic cells treated with peroxynitrite for 24 hrs. At least 400 cells were counted for each treatment under PI staining. The percentage of apoptotic cells for each treatment was the average of two independent experiments.

rat aortic primary SMCs, is mediated, at least in part, by elevation of Ca^{2+}, needed to activate the endonucleases.

In pathological vascular conditions such as atherosclerosis, hypertension, and restenosis, apoptosis has been identified as an important process in the lesions because of the conditions favoring excess formation of ROS by leukocytes, glial cells, and macrophages(12, 34). As the highly controlled apoptotic processes in vascular SMCs result in vascular remodeling following vascular injury (25), identification of both the proapoptotic and the antiapoptotic modulators may raise the possibility of being able to develop specific strategies and approaches in the treatment

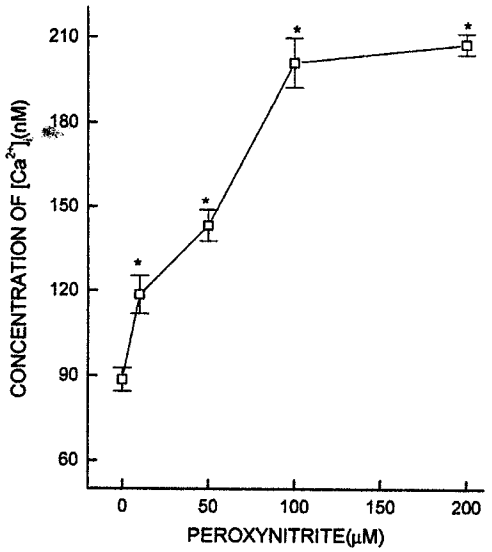

Figure 4. Peroxynitrite results in elevation in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) concentration in rat aortic SMCs. The data were obtained 30 secs after treatment with peroxynitrite. Each result indicates the mean ± SD (n = 8–10).

of vascular diseases. Therefore, our findings may have potential clinical significance in prevention and treatment of these diseases by regulation of endogenous generation of $ONOO^-$ and subsequent apoptotic processes in vascular SMCs.

In conclusion, our study demonstrates that exogenous $ONOO^-$ can trigger apoptosis in primary cultured rat aortic SMCs. This $ONOO^-$-induced apoptosis in primary rat aortic SMCs appears to involve activation of Ca^{2+}-dependent endonucleases. It is thus likely that $ONOO^-$ participates in the pathogenesis of some cardiovascular diseases, such as atherosclerosis, hypertension, and restenosis, *via* triggering apoptosis of vascular SMCs. The precise mechanism by which $ONOO^-$ induces apoptosis in rat aortic SMCs needs to be investigated further.

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