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Stimulation of Nitric Oxide Synthase during Oxidative Endothelial Cell Injury

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ABSTRACT. The purpose of this study was to determine changes in nitric oxide synthase (NOS) activity during the process of lethal oxidative cell injury following H_2O_2 treatment of endothelial cells. NOS activity was determined by measuring the conversion of [^{3}H]arginine ([^{3}H]Arg) to [^{3}H]citrulline ([^{3}H]Cit). Cell death was assessed by measuring the release of intracellular lactate dehydrogenase (LDH). Moreover, cell death and changes in cytosolic free Ca^{2+} (Ca_i^{2+}) were measured simultaneously using a confocal laser scanning system, and propidium iodide and fluo-3 as fluorescent indicators, respectively. Treatment with H_2O_2 (125–1000 μM) concentration dependently increased L-Cit formation from L-Arg, and a peak was obtained at 90 min after the addition of 500 or 1000 μM H_2O_2. The H_2O_2-induced increase in L-Cit formation was blocked completely by N^G-nitro-L-arginine (L-NNA) or N^G-methyl-L-arginine (L-NMA), both inhibitors of NOS. LDH release from endothelial cells was evoked from 120 min after the addition of H_2O_2 (125–1000 μM) in a concentration-dependent manner. Moreover, H_2O_2 increased Ca_i^{2+} before cell death, and addition of Ca^{2+} chelator inhibited both the increase in L-Cit formation and LDH release by H_2O_2. The H_2O_2-induced LDH release was reduced by L-NNA, but not by L-NMA. These results suggest that H_2O_2 treatment of endothelial cells increases Ca_i^{2+} before cell death, and stimulates NOS activity. The activation of NOS may be involved in oxidative endothelial cell death. BIOCHEM PHARMACOL 55;1:77–83, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. nitric oxide synthase; hydrogen peroxide; cell injury; intracellular Ca^{2+}; vascular endothelial cells; bovine aorta

Vascular endothelium generates NO[‖] via a Ca^{2+}/calmodulin-dependent constitutive enzyme that catalyzes the conversion of L-Arg to L-Cit [1–4]. NO is important in the regulation of vascular tone and immune surveillance. Moreover, NO has been shown to react with superoxide and H_2O_2 to produce highly cytotoxic species such as peroxynitrite and singlet oxygen [5–7]. In fact, there have been many reports of the toxic effects of NO in ischemia–reperfusion [8, 9]. On the other hand, NO has been shown to attenuate ischemia–reperfusion injury [10–12] and ROS-induced cytotoxicity [13]. Thus, the role of NO under pathological conditions is complex.

Interestingly, NOS has been shown to release ROS at a low concentration of L-Arg or tetrahydrobiopterin [14, 15]. We previously found that L-NNA analogues, inhibitors of NOS, attenuate H_2O_2-induced endothelial cell injury, but not L-NMA, also an inhibitor of NOS, although both inhibitors block NOS activity [16]. Moreover, we reported that L-NAME, one of the L-NNA analogues, does not affect H_2O_2-induced cell death in the rat fetal lung fibroblast cell line (RFL-6), which lacks NOS [17]. L-NAME has been shown to block the generation of ROS from NOS, whereas L-NMA has no effect on this reaction [15, 18]. Therefore, we speculated that L-NNA analogues may inhibit ROS production from NOS during H_2O_2 treatment, and attenuate H_2O_2-induced endothelial cell injury [16]. Not only NO but also NOS activity may be very important in the development of tissue injury. However, changes in NOS activity in the process of endothelial cell death induced by oxidative stress are not known. In this study, we determined the changes in NOS activity during lethal endothelial cell injury induced by H_2O_2 treatment.

MATERIALS AND METHODS

Materials

H_2O_2 (30% solution) was purchased from Wako Chemicals (Japan). Fluo-3 AM was from Dojindo (Japan). PI, L-NNA,

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‖ *Abbreviations*: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetraacetic acid; Ca_i^{2+}, intracellular Ca^{2+}; cNOS, constitutive nitric oxide synthase; fluo-3 AM, fluo-3 acetoxymethyl ester; iNOS, inducible nitric oxide synthase; L-Arg, L-arginine; L-Cit, L-citrulline; LDH, lactate dehydrogenase; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMA, N^G-methyl-L-arginine; L-NNA, N^G-nitro-L-arginine; NOS, nitric oxide synthase; NO, nitric oxide; PI, propidium iodide; and ROS, reactive oxygen species.

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and L-NMA were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-[2,3,4,5-^{3}H]Arginine monohydrochloride (2.29 TBq/mmol) was from Amersham. All other reagents were of the highest grade commercially available.

Endothelial Cell Culture

Endothelial cells were obtained from fresh bovine thoracic aortae and cultured in monolayers as described [16]. Cells were finally grown on Cytodex 3 microcarrier beads (Pharmacia, Uppsala, Sweden) to measure L-Arg metabolism and LDH release. Ca_i^{2+} and PI staining (cell death) were measured in endothelial cells grown in 35-mm culture dishes. Cells at 5–8 passages were used for the experiments.

Endothelial cells were characterized as previously described [16].

L-Arg Metabolism

Confluent cells on microcarrier beads were washed five times with Krebs solution (pH 7.4) containing 118.5 mM NaCl, 4.74 mM KCl, 2.5 mM $CaCl_2 \cdot 2H_2O$, 1.18 mM $MgSO_4 \cdot 7H_2O$, 1.18 mM KH_2PO_4, 2.5 mM $NaHCO_3$, 11 mM glucose, and 10 mM HEPES. The washed cells (50 μL of microcarrier beads, approximately 1×10^6 cells) were treated with H_2O_2 for various periods (0–4 hr) at 37° in 1 mL of Krebs solution. After treatment with H_2O_2, 700 μL of incubation medium was removed, and 37 KBq of [^{3}H]Arg was added followed by incubation for 9 min. The reaction was terminated with 10 μL of perchloric acid (final concentration of 2%), and after 30 min the reaction mixture was sonicated (5 sec) and centrifuged for 10 min at 1500 × g. The supernatant was subjected to high-performance liquid chromatography using an ODS-80Ts (4.6 mm i.d. × 150 mm; Tosoh Co., Japan) with a mobile phase of 25 mM sodium acetate (pH 4.35) containing 15 mM sodium hexanesulfonate. [^{3}H]Cit and [^{3}H]Arg were eluted at *ca.* 4 min and *ca.* 18 min after the injection of the supernatant, respectively. The radioactivity in the [^{3}H]Cit fraction was determined with a liquid scintillation spectrometer [19].

Simultaneous Measurement of Ca_i^{2+} and Cell Death

We measured Ca_i^{2+} and cell death using fluo-3 and PI, respectively. Confluent cells in 35-mm culture dishes were washed three times with Krebs solution. The washed cells were incubated with 5 μM fluo-3/AM in 2 mL of Krebs solution for 1 hr at room temperature. The cells were washed three times with Krebs solution, and 1 μM PI in 2 mL of Krebs solution was added to the cells. The fluorescent images of the endothelial cells were recorded with a confocal laser-scanning microscope (BioRad, MRC-500) using an argon-krypton ion laser (488 nm, for excitation). Emission wavelengths of 522 and 570 nm long path (LP) were used to measure fluo-3 and PI fluorescence, respectively. Fluorescence intensity of fluo-3 was increased by the H_2O_2 treatment and then decreased quickly. Therefore, changes in Ca_i^{2+} were expressed as percentages of the maximum fluorescent intensity in individual cells. PI staining was expressed as the percentage of total cells.

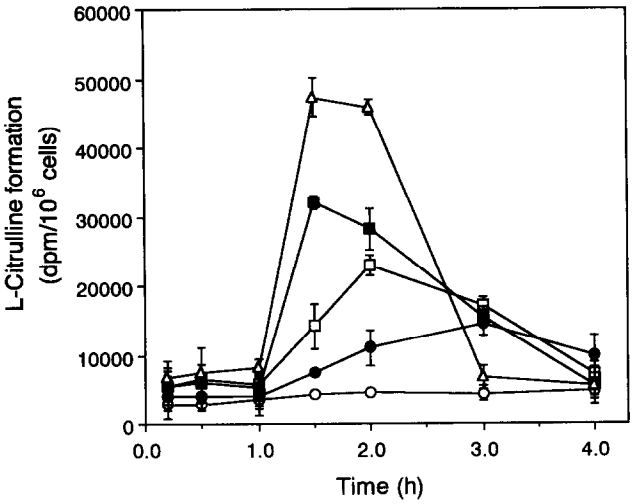

FIG. 1. Effects of H_2O_2 on L-Cit formation from L-Arg in cultured endothelial cells. Cells were incubated for the indicated periods at 37° in the absence or presence of various concentrations of H_2O_2 (control, ○; 125, ●; 250, □; 500, ■; and 1000 μM, △), and then L-Arg metabolism was measured. Results are the means ± SEM of 6 assays.

LDH Release

Lethal cell injury was estimated by determining LDH release as previously described [16]. Confluent endothelial cells on microcarrier beads were washed five times with Krebs solution. Cells (50 μL of microcarrier beads, approximately 1×10^6 cells) were then treated with H_2O_2 at 37° in 1 mL of Krebs solution. LDH activity was determined in cell supernatants and cell fractions of endothelial cells solubilized in 2% Triton X-100. The percentage of the total LDH activity (supernatant fraction + cell fraction) released into the supernatant fraction was then calculated.

Statistical Analysis

Data are presented as means ± SEM of N observations. The statistical significance of observed differences was determined by analysis of variance followed by Bonferroni's method. Differences among means were considered significant when $P < 0.05$.

RESULTS

Effects of H_2O_2 Treatment on NOS Activity

NOS activity was determined by measuring the conversion of [^{3}H]Arg to [^{3}H]Cit. Figure 1 shows the effects of various periods of pretreatment with H_2O_2 (125–1000 μM) on changes in L-Cit formation from L-Arg. The formation of L-Cit in untreated cells did not change during the experimental period. On the other hand, pretreatment with H_2O_2

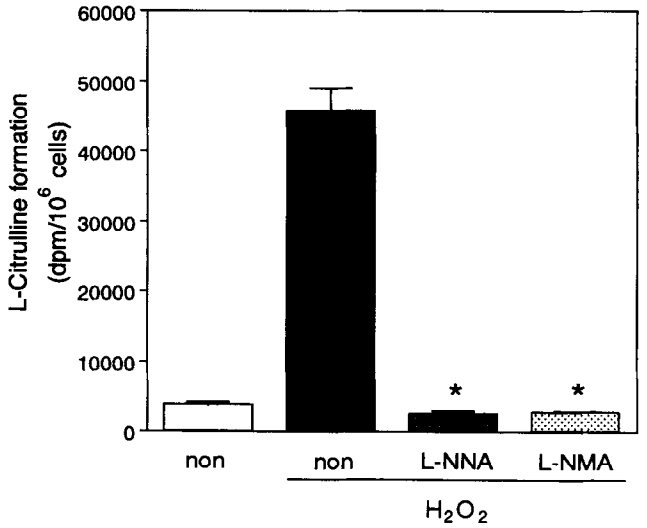

FIG. 2. Effects of NOS inhibitors on the increase in L-Cit formation from L-Arg during H_2O_2 treatment. Cells were incubated with H_2O_2 (1000 μM) for 90 min at 37°, and then arginine metabolism was measured. L-NNA (100 μM) or L-NMA (100 μM) was added to the cell suspensions 10 min before measurement of L-Arg metabolism. Results are the means ± SEM of 6 assays. Key: (*) $P < 0.05$ vs H_2O_2 alone.

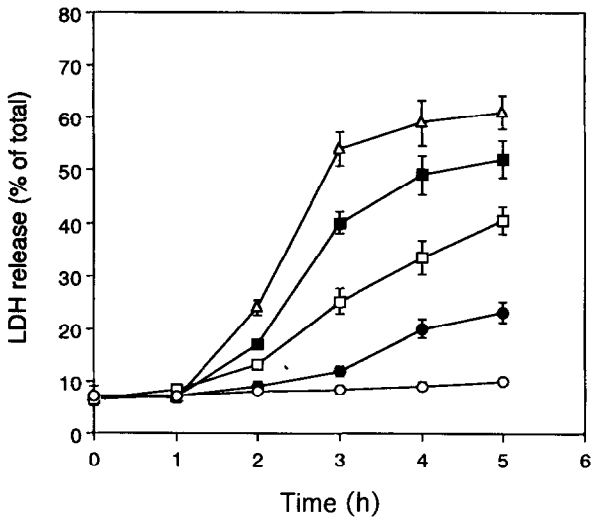

FIG. 3. Time-course of LDH release from endothelial cells exposed to H_2O_2. Cells were incubated with various concentrations of H_2O_2 (control, ○; 125, ●; 250, □; 500, ■; and 1000 μM, △) at 37°. At the indicated times after addition of H_2O_2, the percentage of the total LDH released into medium was determined. Results are the means ± SEM of 6 assays.

(125–1000 μM) concentration dependently increased L-Cit formation from L-Arg, and a peak was observed at 90 min after addition of 500 or 1000 μM H_2O_2 (Fig. 1). The peak was shifted to 120 and 180 min dependent on decreases in H_2O_2 concentration to 250 and 125 μM, respectively (Fig. 1). Longer exposure to H_2O_2 significantly inhibited L-Cit formation.

To determine whether H_2O_2-stimulated L-Cit formation is mediated by NOS, we studied the effects of L-NNA and L-NMA, both specific inhibitors of NOS (Fig. 2). H_2O_2-stimulated L-Cit formation was inhibited completely by 100 μM L-NNA or 100 μM L-NMA.

Effects of H_2O_2 on LDH Release

To compare the time-courses of L-Cit formation and cell death induced by H_2O_2, LDH release induced by H_2O_2 (125–1000 μM) was measured under the same L-Arg metabolism conditions. LDH release occurred from 120 min after the addition of H_2O_2 in a concentration-dependent manner (Fig. 3).

Changes in Ca_i^{2+} and Lethal Cell Injury Induced by H_2O_2

Endothelial NOS activity is dependent on Ca^{2+} [1–4]. Disruption of Ca_i^{2+} homeostasis has been shown to play an important role in oxidative stress-induced injury [20, 21]. Therefore, we measured the effects of H_2O_2 (1000 μM) on Ca_i^{2+} (fluo-3 intensity) and cell death (PI staining), simultaneously (Fig. 4). Ca_i^{2+} increased from approximately 40 min after the addition of H_2O_2. Maximum Ca_i^{2+} was observed between 80 and 90 min after the addition of H_2O_2, following which the fluorescence intensity decreased quickly. This rapid decrease in fluo-3 fluorescence may have been caused by leakage of fluo-3 accompanied by cell injury. On the other hand, cell death (PI staining) occurred from 80 min after the addition of H_2O_2.

Effects of BAPTA on H_2O_2-Induced L-Arg Metabolism and Lethal Cell Injury

To determine whether influx of Ca^{2+} into cells evoked by H_2O_2 treatment activates NOS, we studied the effects of BAPTA, a Ca^{2+} chelator, on L-Cit formation. Addition of 5 mM BAPTA completely inhibited the stimulation of L-Cit formation by H_2O_2 (Fig. 5).

Moreover, the addition of 5 mM BAPTA strongly inhibited the H_2O_2 (1000 μM)-induced LDH release at 3 hr after the addition of H_2O_2 (Fig. 6).

Effects of NO Synthase Inhibitors on H_2O_2-Induced Cell Death

L-NNA (1000 μM) or L-NMA (1000 μM) was added to the incubation medium, and H_2O_2 (500 μM)-induced cell injury was assessed. L-NNA, but not L-NMA reduced the H_2O_2-induced LDH release (Fig. 7). In the absence of H_2O_2, both L-NNA and L-NMA had no effect on LDH release (data not shown).

DISCUSSION

We have shown previously that a high concentration (3–10 mM) of H_2O_2 stimulates NO synthesis immediately [19].

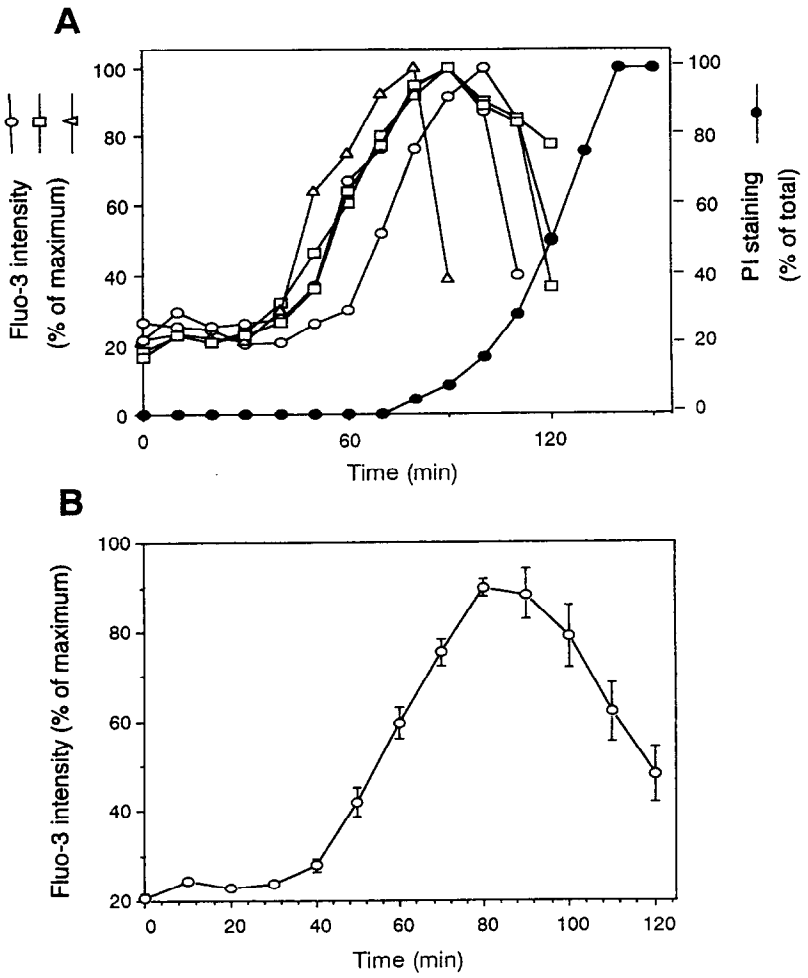

FIG. 4. Simultaneous measurement of changes in Ca_i^{2+} and cell death (PI staining) induced by H_2O_2 treatment. H_2O_2 (1000 μM) was added to cells, and then changes in Ca_i^{2+} in individual cells (○, □, △) and PI staining (●) were measured at 10-min intervals (panel A). A summary of the changes in Ca_i^{2+} is shown in panel B. Results are the means ± SEM of 23 cells.

However, the effects of long-term treatment with H_2O_2 on NOS activity are not known. In the present study, we found that H_2O_2 treatment of cultured endothelial cells increased Ca_i^{2+} and stimulated NOS activity before cell death.

Previous investigations have indicated that increases in Ca_i^{2+} are involved in oxidative stress-induced lethal endothelial cell injury [20, 21]. Reactive oxygen species, including H_2O_2, cause an increase in Ca_i^{2+} due to the influx of Ca^{2+} from the extracellular medium [20–22]. In the present study, we measured H_2O_2-induced changes in Ca_i^{2+} and lethal cell injury simultaneously. The results clearly showed that H_2O_2 treatment of endothelial cells caused an increase in Ca_i^{2+} before cell death. Moreover, the increase in Ca_i^{2+} induced by H_2O_2 may be involved in lethal endothelial cell injury, since exclusion of extracellular Ca^{2+} by the Ca^{2+} chelator BAPTA strongly inhibited H_2O_2-induced lethal endothelial cell injury.

H_2O_2 treatment of endothelial cells stimulated the formation of L-Cit from L-Arg. In vascular endothelial cells, L-Cit is formed from L-Arg either by metabolism to L-Cit via L-ornithine formation by arginase or direct conversion by NOS. Arginase has been suggested to be either absent or inactivated in endothelial cells, since neither urea nor L-ornithine formation is observed in these cells [23]. We have also shown that NOS activity can be determined by measuring L-Cit formation from L-Arg [24] in endothelial cells. In the present study, the H_2O_2-induced increase in formation of L-Cit from L-Arg was strongly inhibited by NOS inhibitors. These findings show that H_2O_2 treatment of endothelial cells stimulates the conversion of L-Arg to L-Cit via NOS. Moreover, removal of extracellular Ca^{2+} by BAPTA inhibited the increase in NOS activity during H_2O_2 treatment. Thus, NOS may be stimulated by an increase of Ca_i^{2+} due to the influx of Ca^{2+} from the extracellular medium. Longer periods of exposure to H_2O_2 significantly inhibited L-Cit formation and induced lethal cell injury. This inhibition of NOS activity may be dependent on cell injury. Murphy *et al.* [25] reported that thiol depletion-induced loss of NO production roughly parallels the appearance of cell damage.

There are at least two kinds of NOS, i.e. cNOS and iNOS, the former of which is Ca^{2+} dependent, while the latter is Ca^{2+} independent [26, 27]. In the present study, H_2O_2-induced stimulation of NOS was evoked by an increase in Ca_i^{2+}. Moreover, the H_2O_2-induced increase in NOS activity was not inhibited by dexamethasone (data not shown), which has been shown to inhibit an induction of iNOS [28–30]. Therefore, H_2O_2 treatment of endothe-

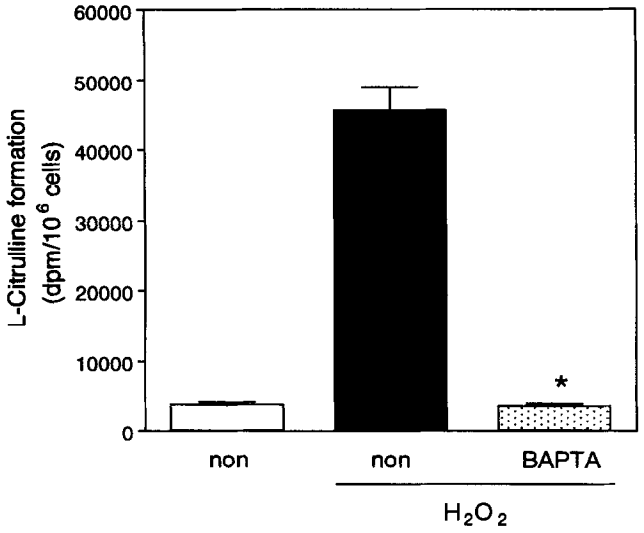

FIG. 5. Effects of BAPTA on L-Cit formation induced by H_2O_2 treatment. BAPTA (5 mM) was added to the incubation mixture 1 hr after the addition of H_2O_2 (1000 μM). L-Arginine metabolism was measured 90 min after the addition of H_2O_2. Results are the means ± SEM of 6 assays. Key: (*) $P < 0.05$ vs H_2O_2 alone.

lial cells may stimulate cNOS via an increase in Ca_i^{2+}, but not induction of iNOS.

We previously showed that L-NNA, but not L-NMA, reduces H_2O_2-induced cultured bovine endothelial cell death [16]. Under the conditions in this study, L-NNA, but not L-NMA, also reduced H_2O_2-induced cell injury. Moreover, we reported that L-NAME does not affect H_2O_2-induced cell death in the rat fetal lung fibroblast cell line (RFL-6), which lacks NOS [17]. NOS has been shown to

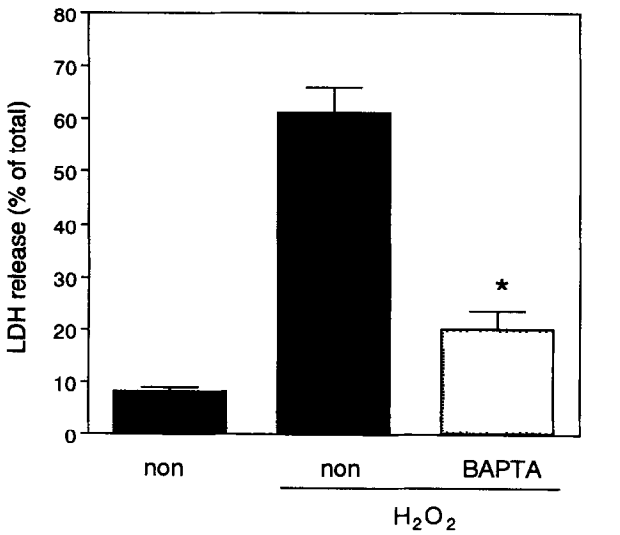

FIG. 6. Effects of BAPTA on LDH release induced by H_2O_2 treatment. BAPTA (5 mM) was added to the incubation mixture 1 hr after the addition of H_2O_2 (1000 μM). LDH release was measured 3 hr after the addition of H_2O_2. Results are the means ± SEM of 6 assays. Key: (*) $P < 0.05$ vs H_2O_2 alone.

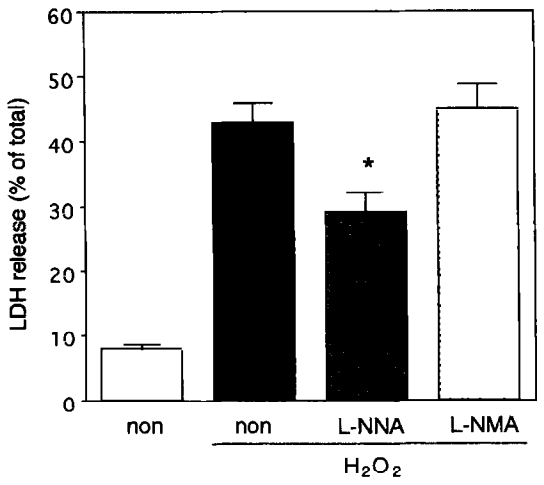

FIG. 7. Effects of L-NNA and L-NMA on H_2O_2-induced LDH release from endothelial cells. Cells were pretreated with L-NNA (1 mM) or L-NMA (1 mM) for 10 min at 37°, followed by incubation with H_2O_2 (500 μM) for 3 hr at 37°. Results are the means ± SEM of 6 assays. Key: (*) $P < 0.05$ vs H_2O_2 alone.

generate ROS from molecular oxygen in the presence of low concentrations of L-Arg or tetrahydrobiopterin [14, 15]. The tetrahydrobiopterin content of endothelial cells may be lowered by the addition of H_2O_2, since it is easily oxidized in the presence of ROS. Interestingly, L-NAME has been shown to block the ROS release from NOS, whereas L-NMA has no effect on this reaction [15, 18]. Therefore, we speculated that L-NNA analogues inhibit H_2O_2 release from NOS and consequently reduce H_2O_2-induced cell death. However, changes in NOS activity during the process of cell death were not known. In the present study, NOS was activated by increasing Ca_i^{2+} before H_2O_2-induced cell death. Interestingly, both NO production and ROS release from NOS were found to be dependent on Ca^{2+} [14]. Although it has not been determined whether NOS releases NO or ROS before cell death, activation of NOS before cell death may be involved in endothelial cell death and tissue injury. Further studies will be needed to examine NO and ROS release.

H_2O_2 concentrations of more than 125 μM were required to induce an increase in NOS activity and endothelial cell injury. Many investigators have used similar concentrations of ROS including H_2O_2 to study the mechanisms of cell injury [31–33]. Moreover, similar concentrations of H_2O_2 have been shown to initiate inositol phospholipid hydrolysis by phospholipase C in cultured Madin-Darby canine kidney cells [34] and to stimulate platelet-activating factor synthesis in cultured endothelial cells [35]. Activated neutrophils adhering to endothelial cells release large amounts of H_2O_2 [36]. Although local concentrations of H_2O_2 have not been determined, these may be high.

Cell death occurs by either apoptosis or necrosis. Necrotic death is characterized by a generalized breakdown of cellular structure followed by cell lysis. On the other hand, apoptotic death is associated with nuclear fragmentation

and endonuclease activation leading to fragmentation of nuclear DNA into oligonucleosome-length fragments. H_2O_2 has been shown to cause both apoptosis and necrosis in many types of cells [37–40]. Nosseri *et al.* [38] reported that H_2O_2 at concentrations below 2.5 mM induced apoptotic cell death, and caused necrotic cell death at concentrations in excess of 5 mM in U937 human myeloid leukemia cells. We observed no necrotic cells following H_2O_2 treatment, but fragmented nuclei, a characteristic of the apoptotic cell, were detected (data not shown). Thus, the H_2O_2-induced cell death observed in this study was most likely mediated via an apoptotic process. A rise in Ca_i^{2+} appears to serve as a common early signal for the initiation of apoptosis [41, 42], and inhibition of increases in Ca_i^{2+} has been reported to block DNA fragmentation and subsequent apoptotic cell death [43–45]. In the present study, H_2O_2-induced apoptosis, like cell death, was inhibited by the exclusion of extracellular Ca_i^{2+}. The H_2O_2-induced apoptotic cell death observed in the present study was likely to be related to increases in Ca_i^{2+}.

In summary, we showed that H_2O_2 treatment of endothelial cells increases Ca_i^{2+} before cell death and stimulates NOS activity. This stimulation of NOS activity may be involved in endothelial cell death and tissue injury induced by oxidative stress.

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