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Cytosolic calcium increase in coronary endothelial cells after H_2O_2 exposure and the inhibitory effect of U78517F

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1 Cytosolic calcium concentrations ($[Ca^{2+}]_i$ were determined with fura-2 on both resting (unstimulated) and A23187-stimulated coronary endothelial cells following injury by hydrogen peroxide (H_2O_2).

2 Treatment of cells with H_2O_2 (10^{-4} M) caused an increase in the resting $[Ca^{2+}]_i$, which reached a maximum of five fold after 3 h.

3 The increase in resting $[Ca^{2+}]_i$ was significantly attenuated by treatment with U78517F, a potent inhibitor of lipid peroxidation, at a concentration of 10^{-6} M or greater. Catalase (50 u ml^{-1}) also markedly inhibited the H_2O_2-induced rise in $[Ca^{2+}]_i$. Pretreatment with verapamil (10^{-5} M), nifedipine (10^{-6} M) or diltiazem (10^{-5} M) had no effect on the increase in $[Ca^{2+}]_i$ following addition of H_2O_2.

4 A23187 produced a transient increase in $[Ca^{2+}]_i$ followed by a sustained plateau. The initial peak and plateau phase responses to A23187 were augmented by H_2O_2. This augmentation of $[Ca^{2+}]_i$ was suppressed by U78517F or catalase but not by Ca-entry blockers.

5 Thus, it is likely that lipid peroxidation plays a critical role in the sustained increase in $[Ca^{2+}]_i$ that occurs following treatment with H_2O_2 and that this continues in the presence of agonists which stimulate the endothelium. Voltage-gated Ca^{2+} channels do not seem to be involved in the genesis of cellular damage associated with sustained large increases in $[Ca^{2+}]_i$.

Keywords: Intracellular calcium concentration; endothelial cell; cell injury; lipid peroxidation; hydrogen peroxide; U78517F

Introduction

There is a growing body of evidence which suggests that endothelial dysfunction occurs in a number of cardiovascular diseases, including hypertension (Lüscher *et al.*, 1986), diabetes (Mayhan, 1989), atherosclerosis (Förstermann *et al.*, 1988) and ischaemia/reperfusion (Mehta *et al.*, 1989) and thus results in impaired endothelium-dependent vasodilatation. Since the endothelium is often exposed to a reactive oxygen burst, it is likely to be the first site of damage in the vascular wall. Xanthine oxidase (XO)-derived oxidation is a critical pathway in microvascular and parenchymal tissue injury (Granger, 1988). Following production of superoxide anion, H_2O_2 generated extracellularly can readily diffuse into the endothelial cell, resulting in degradation of ATP (Spragg *et al.*, 1985) and increased substrate availability for XO. Highly reactive oxidants (Kvietys *et al.*, 1989) such as hydroxyl radical are formed through interaction of H_2O_2 and intracellular iron. Prime targets for free radical reactions are the unsaturated bonds in membrane lipids. Consequent lipid peroxidation results in a loss of membrane fluidity, receptor alignment, and potentially cellular lysis (Machlin & Bendich, 1987), associated with uncontrolled, sustained rises in cytosolic Ca^{2+} concentration (Nicotera *et al.*, 1988).

Release of endothelium-derived relaxing factor (EDRF) and prostaglandin I_2 (PGI_2) following agonist-stimulation in endothelial cells requires the presence of extracellular Ca^{2+} (Adams *et al.*, 1989). When the endothelium is stimulated by an agonist such as bradykinin, the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) increases with an initial peak due to inositol-1, 4,5-triphosphate (IP_3)-mediated Ca^{2+} release from intracellular stores, followed by a sustained plateau that is dependent on the presence of extracellular Ca^{2+}. However, it is not clear how $[Ca^{2+}]_i$ changes in response to an agonist under oxidative stress and subsequent cell injury.

The exact mechanisms involved in the regulation of intracellular calcium levels are not yet elucidated. The present study was carried out to delineate the relationship between $[Ca^{2+}]_i$ in resting and A23187-stimulated endothelial cells following treatment with the oxidant, H_2O_2. To avoid excess radical formation which is likely to cause untreatable severe cell damage accompanied with massive Ca^{2+} influx into the cell, relatively low concentrations of H_2O_2 were used. The present study clearly demonstrates that U78517F (2-[[4-(2, 6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]methyl]-3, 4-dihydro-2, 5, 7, 8-tetramethyl-2H-1-benzopyran-6-ol, dihydrochloride), an inhibitor of lipid peroxidation (Hall *et al.*, 1990), protects cultured coronary endothelial cells from reactive oxygen-induced increases in $[Ca^{2+}]_i$.

Methods

Endothelial cells in culture

Porcine hearts were obtained at a local slaughter house, and kept under sterile conditions for the short period (approximately 30 min) of transportation. The coronary arterial segments of left circumflex, right circumflex and left descending arteries were cleaned of surrounding adipose and connective tissue, and cut open longitudinally. The endothelial cells were harvested by scraping the luminal surface of the coronary artery with a razor blade gently under aseptic conditions. The collected cells were suspended in Dulbecco's modified Eagle's medium (DME) supplemented with 10% foetal bovine serum (FBS), 100 u ml^{-1} penicillin G and 100 $\mu g\ ml^{-1}$ streptomycin, seeded onto gelatin-coated culture dishes (60 mm of diameter), and cultured under a humidified atmosphere containing 5% CO_2 at 37°C. After the first medium change, the concentration of penicillin G and streptomycin was reduced to 10 u ml^{-1} and 10 $\mu g\ ml^{-1}$, respectively. The cells used in the study had undergone less than

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eight passages. Cells which reached confluence were dispersed in the medium containing 0.05% trypsin and 0.53 mM EDTA for 3 to 5 min at 37°C. Thereafter, cells were centrifuged at 800 r.p.m. in HEPES-buffered solution (HBS) of the following composition (mM): NaCl 150, KCl 5.6, $CaCl_2$ 2.0, $MgCl_2$ 1.0 and HEPES 10.0 at pH of 7.4 for 6 min and resuspended with fresh HBS.

Intracellular calcium concentration

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined with the fluorescent calcium indicator fura-2 (Grynkiewicz *et al.*, 1985). The cells were incubated in 10 ml of HBS containing 3×10^{-6} M fura-2 AM and 1 mg ml^{-1} bovine serum albumin (BSA) at 25°C. Detergent was not used for loading of fura-2 to avoid damage to the cell membrane. Sixty minutes later, the cells were centrifuged, and rinsed with HBS containing 0.5 mg ml^{-1} BSA. The concentration of the cells was adjusted to 3 to 7×10^5 ml^{-1} and cells were stored at 2°C on ice. Before the measurement of fluorescence, aliquots of 0.5 ml were placed in a cuvette, and centrifuged to eliminate the leaked fura-2 in HBS. Cells were put into fresh HBS at the same volume, and kept in suspension in the cuvette at 22°C with a magnetic stirrer before measurement of $[Ca^{2+}]_i$.

Fluorescence was measured with a fluorometer of dual wavelength excitation (CAF-100, Japan Spectroscopic, Tokyo, Japan) to detect changes in Ca^{2+}-sensitive fura-2 fluorescence. Dye-stained specimens were alternately excited at wavelengths of 340 (F_{340}) and 380 nm (F_{380}) at a frequency of 48 Hz, and the ratio (R) of F_{340} to F_{380} at an emission wavelength of 500 nm was estimated. $[Ca^{2+}]_i$ was assessed according to the following equation:

$$[Ca^{2+}]i = K_d \cdot B \cdot (R - R_{min})/(R_{max} - R) \quad [1]$$

The dissociation constant (K_d) for Ca^{2+}-fura-2 complex at 20°C was assumed to be 135 nM (Grynkiewicz *et al.*, 1985). B is the ratio of F_{380} in Ca^{2+}-free solution with 20 mM EGTA to that in Ca^{2+} containing solution with 0.2% Triton X-100. Cells were treated with 0.2% Triton X-100 in HBS and 20 mM EGTA in 60 mM Tris solution (pH 8.3), to obtain maximum (R_{max}) and minimum (R_{min}) fluorescence ratios, respectively; slight fluorescence (2% or less of baseline fluorescence) of Triton X-100 and EGTA solution was calibrated for net fluorescence of fura-2. $MnCl_2$ at 20 mM was added to the cell suspension in Ca^{2+}-free solution to obtain the autofluorescence generated spontaneously from cells. Autofluorescence was subtracted to obtain values of $[Ca^{2+}]_i$ of the cell according to equation [1]. The slight fluorescence of 10^{-6} M A23187 did not influence measurement of $[Ca^{2+}]_i$.

After the cells in the cuvette were equilibrated for 10 to 15 min, the resting fluorescence of the cells was measured. Cells were then exposed to hydrogen peroxide (H_2O_2) at 10^{-4} M, and each cuvette incubated for 0.5, 1, 2 and 3 h at 22°C. Before the measurement of fluorescence was taken in the presence and absence of H_2O_2, cells were centrifuged, washed and resuspended. The fluorescence of the cell suspension for the calcium transient response to A23187 (10^{-6} M) was measured after an equilibration period of 5 min. Cells were treated with U78517F, Ca^{2+} antagonists and catalase 5 min before the addition of H_2O_2 to the cell suspension. Saline was used as a control.

Since fura-2 loaded in cells may leak out due to injury with consequent influence on net assessment of $[Ca^{2+}]_i$, care was taken to minimize leak from the cell just before measurement of $[Ca^{2+}]_i$ as follows: (1) centrifugation of the cell suspension and subsequent replacement with fresh HBS, and (2) maintenance at a low temperature of 22°C to reduce any leak of fura-2 (Schilling *et al.*, 1988).

Release of lactate dehydrogenase

Confluent cells plated on 24-well were incubated in DME medium containing 10^{-4} M H_2O_2 for 3 h. Lactate dehydrogenase (LDH) released into the external medium from cells was assessed by the first order rate of NADH oxidation at 340 nm using pyruvate and NADH (Kornberg, 1955).

Materials and statistical analysis

The following were used: Dulbecco's modified Eagles medium (Sigma Chemical, St. Louis, MO, U.S.A.), foetal bovine serum (FBS, Hyclone Lab., Logan, Utah, U.S.A.), penicillin G sodium salt (Sigma), streptomycin sulphate (Sigma), trypsin-EDTA (Gibco, Grand Island, NY, U.S.A.), ethylenediamine tetraacetic acid sodium salt (Na_2EDTA, Sigma), N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (HEPES, Sigma), fura-2 acetoxymethyl ester (Dojin, Kumamoto, Japan), bovine serum albumin (Sigma), ethyleneglycol *bis*-(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, Sigma), tris (hydroxymethyl) aminomethane (Tris, Sigma), $MnCl_2$ (Wako, Osaka, Japan), calcium ionophore A23187 (Sigma), catalase (Sigma), hydrogen peroxide (H_2O_2, Wako), diltiazem hydrochloride (Wako), verapamil hydrochloride (Wako), nifedipine (Sigma), catalase (Sigma), ionomycin (Carbiochemical Co., Belmont, CA, U.S.A.) and U78517F (2-[[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]methyl]-3, 4-dihydro -2, 5, 7, 8- tetramethyl-2H-1- benzopyran-6-ol, dihydrochloride) (Upjohn, Kalamazoo, MI, U.S.A.).

Statistical analyses were made according to the unpaired Student's *t* test. A *P* value less than 0.05 was regarded as significant.

Results

Treatment of coronary endothelial cells with H_2O_2 at a concentration of 10^{-4} M resulted in gradual increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). After a 3 h treatment, $[Ca^{2+}]_i$ was increased five fold over the control levels (Figure 1).

There was a release of LDH into external medium following treatment of cells with 10^{-4} M H_2O_2 for 3 h; when expressed as a percentage increase over total LDH content (0.199 ± 0.013 u/well) in cells, it was 11% (0.022 ± 0.02 u/well) for control and 19% (0.038 ± 0.013 u/well) for H_2O_2-treatment.

A23187 at 10^{-6} M produced a transient increase in $[Ca^{2+}]_i$ within 3 min, followed by the sustained plateau phase (Figures 2 and 3). Both peak and sustained responses to A23187

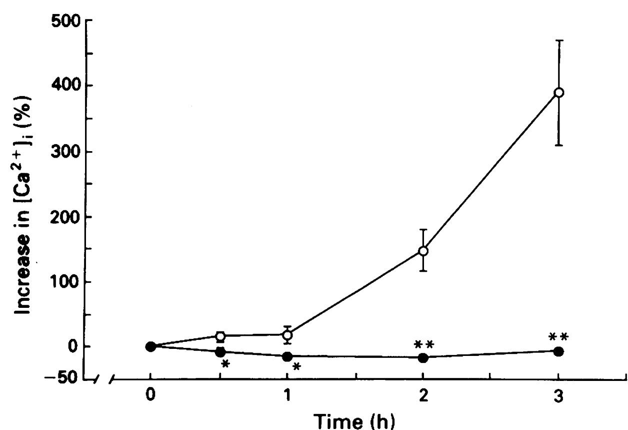

Figure 1 The time-dependent increase in resting $[Ca^{2+}]_i$ following addition of 10^{-4} M H_2O_2 to cell suspensions in the absence (○) and presence of U78517F at 10^{-6} M (●). Endothelial cells were exposed to 10^{-4} M H_2O_2 at zero time on the axis. The increase in the $[Ca^{2+}]_i$ is expressed as a percentage increase over the original $[Ca^{2+}]_i$ determined before treatment with 10^{-4} M H_2O_2, which ranged from 28.3 to 32.6 nM. Each point with vertical bars shows the mean ± s.e.mean ($n = 5$). *Significantly different from cells not exposed to U78517F; $*P < 0.05$; $**P < 0.01$.

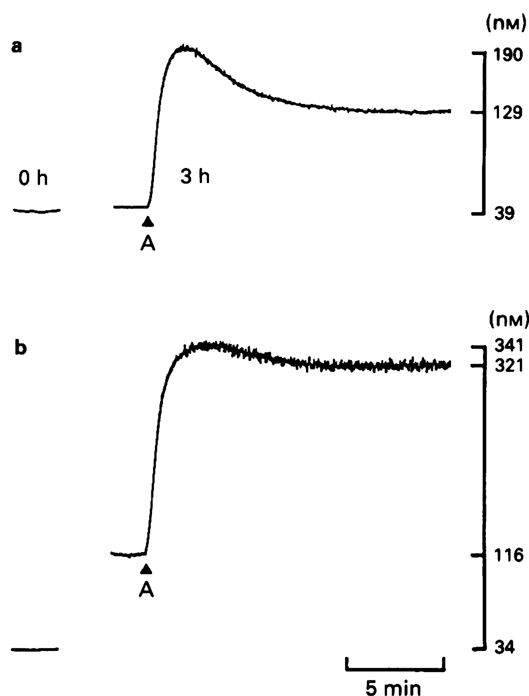

Figure 2 Typical tracing for calcium transient responses to A23187 (A, 10^{-6} M) of endothelial cells. After 3 h of treatment with (b) or without (a) 10^{-4} M H_2O_2, A23187 was added to the medium.

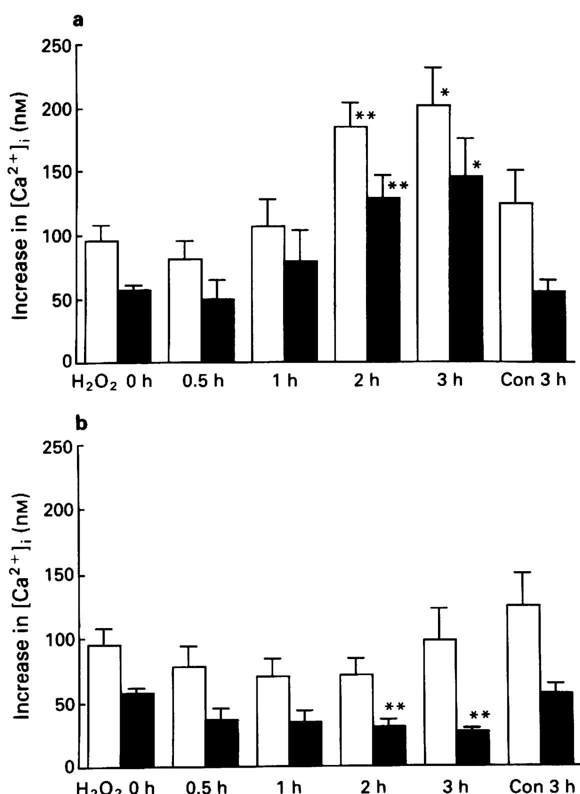

Figure 3 Calcium responses to A23187 at 10^{-6} M after exposure of the endothelial cells to 10^{-4} M H_2O_2 in the absence (a) and presence of U78517F at 10^{-6} M (b). Open and shaded columns indicate increase in $[Ca^{2+}]_i$ of peak and plateau phases in response to A23187 after the exposure to H_2O_2, respectively. A23187-stimulated increase in $[Ca^{2+}]_i$ was assessed before (0 h) and 0.5 h, 1 h, 2 h and 3 h after the exposure to H_2O_2. For the control, cells were left in HBS without exposure to H_2O_2 for 3 h (Con 3h). Each column with vertical bars shows the mean ± s.e.mean ($n = 5$). *Significantly different from H_2O_2 at 0 h; *$P<0.05$; **$P<0.01$.

for $[Ca^{2+}]_i$ were augmented in endothelial cells treated with 10^{-4} M H_2O_2; significantly augmented responses were seen at 2 h and 3 h. U78517F at 10^{-6} M inhibited the H_2O_2-induced potentiation of the rise in $[Ca^{2+}]_i$ in response to A23187 (Figure 1), while there was no significant alteration of the $[Ca^{2+}]_i$ rise in the absence of H_2O_2 (data not shown).

The H_2O_2-induced increase in resting $[Ca^{2+}]_i$ was attenuated by U78517F dose-dependently; the maximum inhibitory effect was obtained at 10^{-6} M (Figure 4). In the absence of H_2O_2, cells which were treated with U78517F at 10^{-6} M for 3 h did not show any change in the resting $[Ca^{2+}]_i$ (data not shown). The augmented responses to A23187 in H_2O_2-treated cells was inhibited by U78517F in a concentration-dependent fashion (Figure 5). U78517F at 10^{-6} M had no effect on A23187-stimulated responses in the absence of H_2O_2 (Table 1).

Treatment with 10^{-6} M nifedipine, 10^{-5} M diltiazem or 10^{-5} M verapamil, failed to attenuate the increase in $[Ca^{2+}]_i$ resulting from exposure of the cells to either H_2O_2 or H_2O_2 plus A23187 (data not shown). None of the Ca^{2+}-entry blockers altered either the resting $[Ca^{2+}]_i$ in the absence of H_2O_2 or the A23187-stimulated $[Ca^{2+}]_i$ in the absence and presence of H_2O_2 (Table 1).

Catalase at 50 u ml^{-1} attenuated both the increase in resting $[Ca^{2+}]_i$ (Figure 4) and augmented responsiveness to A23187 (Figure 5) following treatment of cells with H_2O_2 for 3 h.

Discussion

Disruption of calcium homeostasis, leading to a sustained and excess increase in cytosolic Ca^{2+} levels, is associated with cytotoxicity of hepatocytes (Nicotera *et al.*, 1988). The present study clearly demonstrated slowly increasing $[Ca^{2+}]_i$ in endothelial cells in response to H_2O_2. The concentration of H_2O_2 employed in the present study (10^{-4} M) was low relative to that associated with injury-induced release of lactate dehydrogenase; a cytotoxic effect has previously been reported at 10^{-3} M after 60 min (Sacks *et al.*, 1978; Weiss *et al.*, 1981; Harlan *et al.*, 1984). After a latent period of 1 h, H_2O_2 gradually increased resting $[Ca^{2+}]_i$ in coronary endothelial cells by as much as five times over the pretreatment level, suggesting sustained accumulation of cytosolic calcium. It is

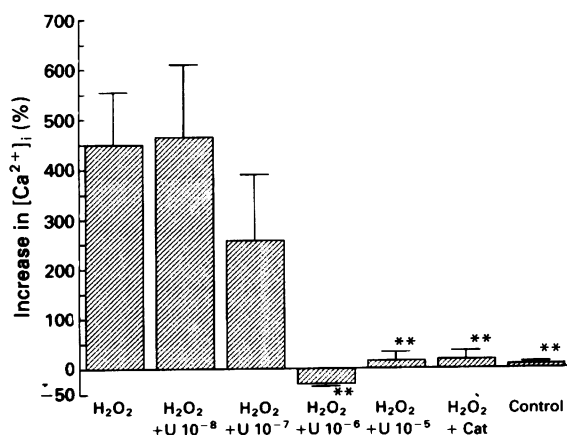

Figure 4 The inhibitory effect of U78517F or catalase against H_2O_2-induced increases in resting $[Ca^{2+}]_i$. The increases in $[Ca^{2+}]_i$ are expressed as a percentage increase over the original $[Ca^{2+}]_i$, which ranged from 32.8 to 39.0 nM. Endothelial cells were exposed to H_2O_2 for 3 h in the absence and presence of U78517F at 10^{-8} M (H_2O_2 + U 10^{-8}), 10^{-7} M (H_2O_2 + U 10^{-7}), 10^{-6} M (H_2O_2 + U 10^{-6}), 10^{-5} M (H_2O_2 + U 10^{-5}) or 50 unit ml^{-1} catalase (H_2O_2 + Cat). For the controls, cells were placed in HBS without H_2O_2 for 3 h (Control). Each column with vertical bars shows the mean ± s.e.mean ($n = 5$ to 7). **Significantly different from H_2O_2 alone; $P<0.01$.

Table 1 Effects of inhibitors on calcium responses to A23187 at 10^{-6} M in the absence of H_2O_2

Treatment	Concentration (M)	Increase in $[Ca^{2+}]_i$ (nM) Peak phase[1]	Plateau phase[2]
Control		229.2 ± 14.5	53.9 ± 11.9
U78517F	10^{-6}	219.3 ± 51.4	51.4 ± 8.6
Diltiazem	10^{-5}	204.8 ± 15.7	62.9 ± 8.7
Verapamil	10^{-6}	194.7 ± 9.4	56.5 ± 8.9
Nifedipine	10^{-6}	237.6 ± 0.6	54.5 ± 9.1

Increases in $[Ca^{2+}]_i$ in responses to A23187 are expressed as both peak[1] and plateau[2] phases. There was no significant difference between control and drug-treated values ($n = 3$, $P > 0.05$).

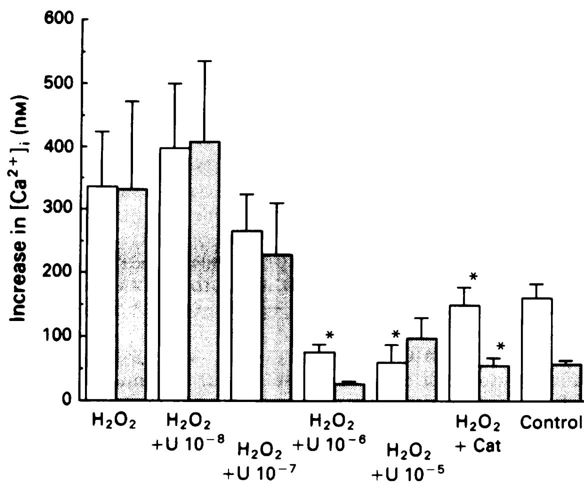

Figure 5 Inhibitory effect of U78517F or catalase on calcium responses to A23187 in the presence of H_2O_2. Open and shaded columns indicate $[Ca^{2+}]_i$ of peak and plateau phases in response to A23187 (10^{-6} M) after exposure to H_2O_2, respectively. Endothelial cells were exposed to 10^{-4} M H_2O_2 for 3 h in the absence (H_2O_2) and presence of U78517F at 10^{-8} M (H_2O_2 + U 10^{-8}), 10^{-7} M (H_2O_2 + U 10^{-7}), 10^{-6} M (H_2O_2 + U 10^{-6}), 10^{-5} M (H_2O_2 + U 10^{-5}) and 50 unit ml^{-1} catalase (H_2O_2 + Cat). For the controls, cells were placed in HBS without H_2O_2 for 3 h. Each column with vertical bars shows the mean ± s.e.mean (n = 5 to 7). *Significantly different from H_2O_2 alone; $P < 0.05$.

postulated that the sustained increase in $[Ca^{2+}]_i$ of cells arises from disturbed membrane Ca^{2+} permeability, depressed extrusion of Ca^{2+} from the cell secondary to an energy deficit, and/or altered release/sequestration of Ca^{2+} within the cell. The cytosolic Ca^{2+} concentration was assessed, assuming that fura-2 is located exclusively in the cell cytoplasm and that the fluorescence reflects cytosolic free Ca^{2+} (Grynkiewicz *et al.*, 1985; Morgan-Boyd *et al.*, 1987; Schilling *et al.*, 1988). However, microscopic examination of bovine aortic endothelial cells loaded with fura-2 reveals fluorescence associated with discrete intracellular structures rather than the homogeneous distribution expected for a cytosolic stain (Steinberg *et al.*, 1987). The precise location of the signal from each of the fluorescent Ca^{2+} indicator dyes after exposure of cells to H_2O_2 remains to be determined. With respect to lactate dehydrogenase activity, it was seen even in cells that had not been injured by reactive oxygen species. However, this does not suggest that cells are exposed to an injurious environment irrespective of H_2O_2-treatment, since there is baseline level of lactate dehydrogenase activity observed in cultured endothelial cells (Acosta & Li, 1979; Brigham *et al.*, 1987).

There is a great deal of evidence that release of EDRF in response to endothelium-dependent vasodilators depends on the presence of extracellular Ca^{2+} (Peach *et al.*, 1987; Luckhoff *et al.*, 1988), while release of PGI_2 is less dependent (Luckhoff *et al.*, 1988). Therefore, facilitated transport of Ca^{2+} across the membrane seems a prerequisite for triggering endothelial activation. It is conceivable that a sustained rise of $[Ca^{2+}]_i$ in the resting stage is induced by the reactive oxygen through an altered transport mechanism for Ca^{2+} across the membrane. A critical role of sustained increases in $[Ca^{2+}]_i$ in cell injury is supported by a study showing that deletion of extracellular Ca^{2+} inhibits cytotoxicity induced by lymphokine-activated killer cells (Kotasek *et al.*, 1988). Our findings in endothelial cells are consistent with those obtained in hepatocytes where oxidative stress by tert-butyl hydroperoxide at toxic concentrations increases $[Ca^{2+}]_i$ (Nicotera *et al.*, 1988). Voltage-gated Ca^{2+} channels are not involved in excitation-secretion coupling in endothelial cells (Spedding *et al.*, 1986; Jayakody *et al.*, 1987), but could possibly be involved in cell injury. We found, however, that Ca^{2+}-entry blockers of three different types, verapamil, nifedipine and diltiazem, at concentrations high enough to be effective (Hayashi & Toda, 1977; Godfraind & Miller, 1983), failed to suppress the rise of $[Ca^{2+}]_i$ following addition of H_2O_2 to endothelial cells. Our findings are consistent with those in non-injured cells where verapamil at 10^{-5} M does not alter resting $[Ca^{2+}]_i$ (Peach *et al.*, 1987; Morgan-Boyd *et al.*, 1987). Thus, the voltage-gated Ca^{2+} channel is unlikely to be activated by oxygen radicals. As suggested in hepatocytes, inhibition of the Ca^{2+}-ATPase that extrudes cytosolic Ca^{2+} may also be involved in the intracellular calcium accumulation through oxidation of the enzyme (Nicotera *et al.*, 1985).

In addition to the rise in resting $[Ca^{2+}]_i$, H_2O_2 enhanced the calcium responses of cultured cells to the calcium ionophore A23187 (Reed & Lardy, 1972). Potentiation of the calcium responses was seen for both phases, i.e. the initial peak and sustained plateau of $[Ca^{2+}]_i$, suggesting an increase in inositol-1,4,5-trisphosphate-mediated Ca^{2+} release from intracellular stores and greater membrane permeability to Ca^{2+}. A23187 stimulates not only Ca^{2+} influx but also Ca^{2+} release from the internal stores (Itoh *et al.*, 1985). Therefore, it seems reasonable to suggest that the increase in $[Ca^{2+}]_i$ continues in the presence of agonists which stimulate the endothelium during the course of cell injury.

The increases in $[Ca^{2+}]_i$ of resting and A23187-stimulated cells following treatment with H_2O_2 was attenuated by U78517F at a very low concentration compared with other inhibitors of reactive oxygen species (10^{-6} M U78517F vs. 5×10^{-3} M deferoxamine, an iron chelator: Kvietys *et al.*, 1989). H_2O_2 which is implicated as a primary effector of endothelial cell lysis by neutrophils (Sacks *et al.*, 1978; Weiss *et al.*, 1981; Harlan *et al.*, 1984) is relatively innocuous, but can readily pass through cell membranes and interact with intracellular iron to form highly reactive oxidants such as hydroxyl radical (Grisham & Granger, 1988). The radicals can attack many biological molecules, including membrane lipids, which undergo metal iron-dependent peroxidation (Braughler *et al.*, 1988; Gutteridge & Halliwell, 1990), and as a consequence may allow more Ca^{2+} to enter the cell. Although the rise in cytosolic Ca^{2+} after oxidative stress is not a universal factor in cell damage (Starke *et al.*, 1986; Herman *et al.*, 1990), there is evidence that cell death can be mediated by a sustained elevation of $[Ca^{2+}]_i$ (Chien *et al.*, 1978; Nicotera *et al.*, 1986). Thus, measuring resting $[Ca^{2+}]_i$ may be a useful means of assessing cytotoxicity. Since U78517F at 10^{-6} M had no effect in non-injured cells on resting or A23187-stimulated levels of $[Ca^{2+}]_i$, it is unlikely to inhibit Ca^{2+} mobilization. U78517F has a ring portion, i.e. a chromanol moiety of α-tocopherol, in which the hydroxyl group at the 6 position of the methylchroman ring structure serves as a reducing agent of lipid peroxyl radicals (Hall *et al.*, 1990; 1991). In a preliminary study (unpublished data, Maeda *et al.*), U78517F markedly suppressed formation of malonyldialdehyde, which is presumed to be closely related to progress of lipid peroxidation, in cultured coronary artery

endothelial cells exposed to a reaction mixture of xanthine (5×10^{-4} M) and xanthine oxidase (0.02 u ml^{-1}); $83 \pm 6\%$ ($n = 3$) inhibition by 10^{-6} M U78517 was obtained. Hall and colleagues have also demonstrated that U78517F at 10^{-6} M inhibits iron catalyzed lipid peroxidation in rat brain homogenates by approximately 90% (Hall *et al.*, 1991). Thus, U78517F is likely to exert an inhibitory action on lipid peroxidation and consequently suppress the sustained increase in $[Ca^{2+}]_i$ of endothelial cells exposed to cytotoxic reactive oxygen species.

In summary, H_2O_2 at a low concentration gradually increased $[Ca^{2+}]_i$ of coronary endothelial cells in culture. In the course of injury, the increase in $[Ca^{2+}]_i$ continues in the presence of agonists which stimulate the endothelium. Voltage-gated Ca^{2+} channels do not play a role in the genesis of cellular damage associated with increased $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ increases simultaneously with cell injury presumably as a consequence of lipid peroxidation which is inhibited by U78517F.

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