Hydrogen peroxide activates agonist-sensitive Ca^{2+} -flux pathways in canine venous endothelial cells

Thanh N. DOAN, Deborah L. GENTRY, Addison A. TAYLOR and Stephen J. ELLIOTT*

Departments of Pediatrics, Molecular Physiology & Biophysics, Pharmacology and Medicine, Baylor College of Medicine, Houston, TX 77030, U.S.A.

The effect of the biological oxidant H_2O_2 on purinergic-receptor-stimulated Ca^{2+} signalling was determined in canine venous endothelial cells. H_2O_2 increased cytosolic free $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$), the rate of rise of which was dose-dependently related to H_2O_2 concentration. The response of $[\text{Ca}^{2+}]_i$ to H_2O_2 resulted in part from release of Ca^{2+} from internal stores. The H_2O_2 -sensitive intracellular Ca^{2+} pool was characterized in cells suspended in Ca^{2+} -free/EGTA buffer and stimulated in sequence with H_2O_2 and ionomycin or ATP. Under this condition, the rank order of apparent compartment size sensitive to each compound was ionomycin > H_2O_2 > ATP. Stimulation of cells with H_2O_2 eliminated any response of $[\text{Ca}^{2+}]_i$ to subsequent addition of ATP. To test more directly whether H_2O_2 accesses the inositol trisphosphate-sensitive Ca^{2+} store, cells were pretreated with

thapsigargin, a selective inhibitor of that store's Ca^{2+} pump. Release of Ca^{2+} from internal Ca^{2+} stores by H_2O_2 declined as the interval after thapsigargin addition increased, a finding that supports the contention that H_2O_2 accesses the inositol trisphosphate-sensitive Ca^{2+} store. H_2O_2 -stimulated Ca^{2+} influx across the cell membrane was sensitive to Ni^{2+} , La^{3+} , and 1- $\{\beta$ -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole HCl (SKF-96365), a selective inhibitor of the agonist-stimulated Ca^{2+} -influx pathway. Ca^{2+} entry triggered by H_2O_2 appears to occur via the agonist-sensitive Ca^{2+} influx pathway. Together, these results suggest that H_2O_2 , which is normally secreted by activated neutrophils and monocytes, may act as an intercellular messenger and stimulate Ca^{2+} signalling in target endothelial cells.

INTRODUCTION

By virtue of its location, the vascular endothelium may be exposed to various blood-borne agents that shift the redox balance within endothelial cells to a more oxidized state. Changes in redox status may result in altered endothelial-cell function and may ultimately alter cell viability. We have previously used the membrane-permeant compound *t*-butyl hydroperoxide (*t*-buOOH) as a model oxidant in order to examine the effect of oxidative stress on endothelial-cell function. Because *t*-buOOH is metabolized predominantly via the glutathione redox cycle, the major antioxidant defence system of endothelial cells, this oxidant has proven to be a useful model of oxidant stress in this cell type. Incubation of arterial endothelial cells with *t*-buOOH inhibits bradykinin-stimulated Ca^{2+} signalling in a time-dependent manner [1]. Initially, *t*-buOOH inhibits the agonist-stimulated Ca^{2+} -influx pathway, whereas at subsequent times the oxidant inhibits receptor-activated release of Ca^{2+} from internal stores and decreases extrusion of Ca^{2+} from the cytosolic compartment. Inhibition of Ca^{2+} release from internal stores during agonist stimulation is related to inhibition of $\text{Ins}(1,4,5)\text{P}_3$ production rather than depletion of internal Ca^{2+} stores [2,3].

Additional oxidants have also been demonstrated to alter endothelial-cell function. H_2O_2 is an endogenous oxidant that may be derived from the superoxide anion produced by NADPH oxidase of activated neutrophils and monocytes [4,5]. H_2O_2 is generally regarded as a harmful agent, because its reactivity can result in cell injury in many different cell types. In addition, H_2O_2 may induce injury by formation of hypochlorous acid or the hydroxyl radical ($\text{OH}^\bullet$). For these reasons, the effects of H_2O_2 on endothelial-cell function and viability have been extensively studied using reagent H_2O_2 , activated neutrophils, or H_2O_2

generated enzymically by the actions of xanthine oxidase on hypoxanthine or glucose oxidase on glucose. H_2O_2 (0.5–10 mM) stimulates release of the cytosolic marker enzyme, lactate dehydrogenase [6], and increases efflux of ^{51}Cr [7,8] from pulmonary artery endothelial cells. Exposure of vascular endothelial cells to H_2O_2 has also been associated with decreases in cellular ATP [6,8,9], scission of DNA strands [6,10], and inhibition of protein synthesis [11]. In addition, this oxidant substantially inhibits production of InsP_3 in human umbilical-vein endothelial cells stimulated with thrombin or histamine [12]. Under certain circumstances, however, H_2O_2 apparently activates several endothelial-cell functions. H_2O_2 stimulates adherence of neutrophils to isolated canine carotid arteries, veins and cultured endothelial cells by a process that promotes production of platelet-activating factor [13] and expression of adhesive ligands on the cell surface membrane [4,14,15]. In addition, H_2O_2 initiates hydrolysis of endothelial-cell inositol phospholipids [16], stimulates phospholipase A_2 activity [17], and induces release of prostaglandin I_2 [18].

The aim of the present study was to investigate the effect of H_2O_2 on endothelial-cell Ca^{2+} signalling, and explore the possibility that this oxidant is an intercellular messenger. The results of these experiments demonstrate that H_2O_2 activates Ca^{2+} signalling in cultured venous endothelial cells. In this regard, H_2O_2 stimulates release of Ca^{2+} from intracellular stores, accessing a Ca^{2+} compartment that includes the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} store, and stimulates the entry of Ca^{2+} from the extracellular space via the agonist-sensitive Ca^{2+} -influx pathway. These results suggest that, rather than acting simply as a toxin deleterious to vascular endothelial cells, H_2O_2 may serve an important agonist function to stimulate Ca^{2+} signalling in this cell type.

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free $[\text{Ca}^{2+}]$; CVECs, canine venous endothelial cells; DME, Dulbecco's modified Eagle's medium; ECGF, endothelial-cell growth factor; SKF-96365, 1- $\{\beta$ -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole HCl; *t*-buOOH, *t*-butyl hydroperoxide; ECS, extracellular solution.

* To whom all correspondence and reprint requests should be addressed.

MATERIALS AND METHODS

Reagents and solutions

H₂O₂, ATP and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ionomycin was purchased from Calbiochem (San Diego, CA, U.S.A.), and fura-2/AM from Molecular Probes (Eugene, OR, U.S.A.). Dulbecco's modified Eagle's medium (DME) was obtained from GIBCO (Grand Island, NY, U.S.A.), and endothelial-cell growth factor (ECGF) from Collaborative Research Inc. (Lexington, MA, U.S.A.). 1- β -[3-(4-Methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole HCl (SKF-96365) was kindly provided by SmithKline Beecham Pharmaceuticals (Welwyn, U.K.).

Extracellular solution (ECS) contained: 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM D-glucose and 15 mM Hepes, pH adjusted to 7.40 at 37 °C with NaOH. Ca²⁺-free/EGTA buffer contained 0.3 mM EGTA and the same salts as ECS but without added CaCl₂. All chemicals were of reagent grade.

Culture of endothelial cells

Canine jugular-venous endothelial cells (CVECs) were harvested by a modification of the technique described by Ford et al. [19]. Freshly isolated vessels were everted on glass rods and placed in a phosphate buffer solution containing type III collagenase (50 units/ml; Worthington Biochemical Corp.) for 10 min. Detached cells were centrifuged and resuspended in DME supplemented with 10% (v/v) fetal-bovine serum (Hyclone, Logan, UT, U.S.A.), 100 μ g/ml streptomycin, 100 μ g/ml penicillin, 100 μ g/ml neomycin, 2 mM glutamine, ECGF (50 μ g/ml) and heparin (50 μ g/ml). After plating, cells were grown to confluency in monolayer conformation, and endothelial identity was confirmed by uptake of di-iodoacetyl-low-density lipoprotein observed by using epifluorescence microscopy [20]. In addition, monolayers demonstrated the cobblestone appearance typical of cultured vascular endothelial cells observed under phase-contrast light microscopy. All experiments were performed with confluent cells at passages 2–5.

Measurement of cytosolic free Ca²⁺ concentration ([Ca²⁺]_i)

[Ca²⁺]_i was measured by using the fluorescent indicator fura-2, as previously described [1]. Briefly, attached cells were dispersed by brief incubation with 0.05% trypsin/0.53 mM NaEDTA (GIBCO), the action of which was terminated by addition of an equal volume of supplemented DME. CVECs were centrifuged, washed, and incubated in ECS (2.3 ml) containing 20 μ M fura-2/AM for 30 min at 37 °C. After dilution of cells into a volume of 25 ml, an additional incubation for 30 min was carried out before washing. The suspension was divided into control and treated samples, and maintained at 37 °C throughout each experiment. Samples (2 ml) were subsequently removed and washed twice immediately before fluorescence measurement with an SLM 8000 spectrophotofluorimeter (SLM Instruments, Urbana, IL, U.S.A.). The excitation wavelength alternated between 340 and 380 nm every 0.5 s during recording of fluorescence emission at 510 nm. All measurements were corrected for autofluorescence by using cells treated as above except for absence of fura-2. [Ca²⁺]_i was calculated by using the equation of Grynkiewicz et al. [21]. Calibration of the fluorescent signal was determined on individual samples by sequential addition of Triton X-100 (0.1%) and EGTA (5 mM). None of the com-

pounds used altered the calibration values of fura-2 or significantly altered autofluorescence. Values for [Ca²⁺]_i are reported as means $\pm$ S.E.M.

To determine whether fura-2 became sequestered within cytoplasmic organelles, fura-2-loaded cells were monitored at 510 nm as excitation alternated between the Ca²⁺-sensitive wavelength of 340 nm and the Ca²⁺-insensitive wavelength of 360 nm. Addition of Mn²⁺ (1 mM MnCl₂) had no effect on emission fluorescence generated by excitation at 360 nm, but caused a rapid 15% decrease in emission fluorescence at excitation wavelength 340 nm. Subsequent addition of digitonin (25 μ M), an agent that selectively permeabilizes the plasmalemma without significant effect on organellar membranes, dramatically decreased fluorescence at both excitation wavelengths. Addition of Triton X-100 (final concn. 0.1%) caused no further decrease in fluorescence, demonstrating that all measurable fura-2 had been released by the action of digitonin.

RESULTS

Stimulation of Ca²⁺ signalling by ATP

In the presence of extracellular Ca²⁺, addition of ATP (100 μ M) stimulated [Ca²⁺]_i to increase from a basal level of 229 ± 21 nM to a peak value of 351 ± 45 nM ($n = 11$). Activation of endothelial-cell purinergic receptors increases [Ca²⁺]_i via release of Ca²⁺ from endoplasmic reticulum and influx of extracellular Ca²⁺ across the plasma membrane. To determine the contribution of release of Ca²⁺ from internal stores to the overall response of CVECs to ATP, addition of nucleotide was performed in the absence of extracellular Ca²⁺. With cells suspended in Ca²⁺-free/EGTA buffer, basal [Ca²⁺]_i was 191 ± 21 nM ($n = 7$), and increased to 231 ± 30 nM upon application of ATP. Subsequent addition of Ca²⁺ (2 mM CaCl₂) to the extracellular buffer rapidly increased [Ca²⁺]_i, a response not observed in absence of ATP (results not shown). Together, these results demonstrate that stimulation of CVECs with ATP releases Ca²⁺ from internal stores and stimulates Ca²⁺ entry across the cell membrane.

Response of [Ca²⁺]_i to H₂O₂

Application of H₂O₂ to CVECs suspended in Ca²⁺-containing ECS increased [Ca²⁺]_i in a dose-dependent manner. The rate of

Figure 1 Dose-response for H₂O₂-stimulated increase in [Ca²⁺]_i

CVECs were loaded with fura-2 and suspended in ECS containing 2 mM CaCl₂. Increase in [Ca²⁺]_i above basal is plotted versus time after addition of 1 (●), 5 (▲) or 10 (■) mM H₂O₂ (corresponding to 1, 5, or 10 μ mol/10⁶ cells per ml). In this and subsequent Figures, all experiments used the highest concentration of H₂O₂ and were carried out at 37 °C. Values represent means $\pm$ S.E.M. ($n = 3-8$).

Figure 2 Effect of H₂O₂ on [Ca²⁺]_i determined in Ca²⁺-free/EGTA buffer

Fura-2-loaded CVECs were suspended in Ca²⁺-free/EGTA buffer and stimulated with H₂O₂ at the time indicated by the arrow. Re-addition of Ca²⁺ (2 mM CaCl₂) to the extracellular buffer was carried out 6 min after application of H₂O₂.

Figure 3 Effect of ATP and H₂O₂ on [Ca²⁺]_i in CVECs

CVECs were loaded with fura-2 and suspended in Ca²⁺-containing ECS immediately before measurement of [Ca²⁺]_i. (a) Addition of ATP (100 μM) was performed at 2 min as indicated by the first arrow, followed by addition of H₂O₂ 4 min later. (b) Reversal of addition order, with H₂O₂ added at 2 min, and ATP added at approx. 9 min. No response to ATP was observed when added after H₂O₂. Traces shown are representative of 7 (a) and 4 (b) independent experiments.

rise of [Ca²⁺]_i increased from 1.15 ± 0.10 nM/min in cells treated with 1 mM H₂O₂ to 7.05 ± 0.01 nM/min in cells treated with 10 mM H₂O₂ (Figure 1). For all subsequent experiments, an H₂O₂ concentration equal to 10 μmol/10⁶ cells per ml (10 mM) was employed.

In the presence of extracellular Ca²⁺, H₂O₂ increased [Ca²⁺]_i from a basal value of 303 ± 25 nM to a peak level of 699 ± 54 nM ($n = 12$), after which [Ca²⁺]_i gradually declined toward baseline. The response of CVECs to H₂O₂ may result from release of Ca²⁺ from internal stores and/or influx of Ca²⁺ across the plasma-membrane. To address the first possibility, cells were suspended in Ca²⁺-free/EGTA buffer immediately before addition of H₂O₂. Under this condition, application of H₂O₂ rapidly increased [Ca²⁺]_i from 260 ± 25 nM ($n = 8$) to a peak of 390 ± 14 nM, before declining to 273 ± 20 nM 2 min later (Figure 2), a response consistent with H₂O₂-induced release of Ca²⁺ from internal

Figure 4 Ionomycin- and H₂O₂-sensitive intracellular Ca²⁺ stores

(a) Fura-2-loaded CVECs were suspended in Ca²⁺-free/EGTA buffer and stimulated with ionomycin (Iono; 1 μM) followed by H₂O₂. (b) Reversal of addition order is shown, with H₂O₂ added at 2 min, and ionomycin added 4 min later. In this and subsequent Figures, traces are representative of at least 3 independent experiments.

stores. Subsequent addition of Ca²⁺ (6 min after addition of H₂O₂) produced a large increase in [Ca²⁺]_i to 680 ± 32 nM, a value similar to that obtained when H₂O₂ was added to CVECs suspended in Ca²⁺-containing buffer. The response of [Ca²⁺]_i to addition of Ca²⁺ provides evidence that H₂O₂ activates Ca²⁺ entry across the cell membrane.

Activation of ATP-sensitive Ca²⁺ flux pathways by H₂O₂

To investigate whether H₂O₂ increases [Ca²⁺]_i via activation of ATP-sensitive Ca²⁺-flux pathways, CVECs were stimulated in sequence with ATP and H₂O₂. In the presence of extracellular Ca²⁺, H₂O₂, added 4 min after ATP, increased [Ca²⁺]_i from 284 ± 21 nM to 548 ± 52 nM ($n = 7$) (Figure 3a). By contrast, addition of ATP (after H₂O₂) failed to alter [Ca²⁺]_i (Figure 3b).

In order to identify the intracellular Ca²⁺ pool to which H₂O₂ has access, stimulation of cells was performed in the absence of extracellular Ca²⁺, a strategy that temporally isolates internal store release from transplasmalemmal influx. When cells were suspended in Ca²⁺-free/EGTA buffer and stimulated with ionomycin, [Ca²⁺]_i increased from 242 ± 52 nM to 501 ± 108 nM ($n = 5$). Subsequent application of H₂O₂ (added 2–6 min after ionomycin) failed to elicit any detectable response (Figure 4a). Conversely, when cells were stimulated first with H₂O₂, subsequent addition of ionomycin produced an increase in [Ca²⁺]_i similar to that observed with H₂O₂ (Figure 4b). To examine more specifically whether H₂O₂ releases Ca²⁺ from the ATP-sensitive Ca²⁺ store, cells were suspended in Ca²⁺-free/EGTA buffer before sequential additions of ATP and H₂O₂. When added after ATP, H₂O₂ increased [Ca²⁺]_i from 157 ± 15 nM to 238 ± 22 nM (Figure 5). Reversal of addition order eliminated the response of [Ca²⁺]_i to ATP (result not shown).

The above findings provide evidence that H₂O₂ releases Ca²⁺ from internal stores, and suggest that the H₂O₂-sensitive Ca²⁺ store includes the ATP-sensitive pool and is a subset of the

Figure 5 ATP- and H_2O_2 -stimulated release of Ca^{2+} from intracellular Ca^{2+} stores

Fura-2-loaded CVECs were suspended in Ca^{2+} free/EGTA buffer and stimulated in sequence with ATP and H_2O_2 .

Figure 7 Stimulation of Ca^{2+} influx by H_2O_2

CVECS were suspended in 2 ml of Ca^{2+} -containing ECS and stimulated with H_2O_2 . During the plateau phase of elevated $[\text{Ca}^{2+}]_i$, addition of 20 μl of EGTA (buffered with NaHepes to pH 8.5) caused $[\text{Ca}^{2+}]_i$ to decrease rapidly to baseline, whereas vehicle (upper trace) had no effect. Final concentration of EGTA in the cell suspension was 2 mM. Subsequent re-addition of Ca^{2+} (2 mM CaCl_2) restored $[\text{Ca}^{2+}]_i$ to the plateau level.

Figure 6 H_2O_2 releases Ca^{2+} from the thapsigargin-sensitive internal Ca^{2+} store

Fura-2-loaded CVECs were suspended in Ca^{2+} -containing ECS and stimulated with thapsigargin (TG; 200 nM) at 2 min. Subsequent additions of H_2O_2 were performed after various time intervals. In (a), the interrupted trace shows a typical recording in the absence of H_2O_2 addition. The response to H_2O_2 decreases with time after addition of TG. Note that in each trace, after addition of H_2O_2 , $[\text{Ca}^{2+}]_i$ continues declining toward a level below that immediately before H_2O_2 .

ionomycin-sensitive pool. Alternatively, the effect of H_2O_2 may be explained by inhibition of the signalling pathway responsible for ATP-stimulated Ca^{2+} flux. To test more directly whether H_2O_2 stimulates release of Ca^{2+} from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive internal store, cells were pretreated with the Ca^{2+} -pump inhibitor thapsigargin before stimulation with H_2O_2 . The response of $[\text{Ca}^{2+}]_i$ to H_2O_2 , when added at various times after thapsigargin, progressively declined with time after application of thapsigargin (Figure 6), suggesting that the intracellular source of Ca^{2+} to which H_2O_2 has access shares identity with or includes the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive store. An interesting feature of the response of thapsigargin-treated cells to H_2O_2 was the continued decline in $[\text{Ca}^{2+}]_i$ during the relaxation phase of the response to H_2O_2 . Thus Ca^{2+} declined toward a level lower than that immediately before addition of oxidant. This new plateau level was midway between basal and thapsigargin-stimulated levels.

Activation of Ca^{2+} entry by H_2O_2

Addition of Ca^{2+} to H_2O_2 -treated cells suspended in Ca^{2+} -free/EGTA buffer resulted in a large increase in $[\text{Ca}^{2+}]_i$ (see above and Figure 2), reflecting influx of Ca^{2+} across the cell membrane. To characterize the Ca^{2+} -entry pathway further, cells were stimulated with H_2O_2 in the presence of extracellular Ca^{2+} . During the resultant plateau phase of elevated $[\text{Ca}^{2+}]_i$, addition of 2.5 mM EGTA caused a sharp decrease in $[\text{Ca}^{2+}]_i$ to the baseline level (Figure 7). This step could be reversed by re-addition of Ca^{2+} , a manoeuvre that promptly increased $[\text{Ca}^{2+}]_i$ back to the plateau level.

In order to identify the pathway(s) responsible for the translocation of Ca^{2+} from the extracellular space to the cytosolic compartment of CVECs, cells were stimulated with H_2O_2 in the absence or presence of the Ca^{2+} -channel blocker Ni^{2+} . As shown in Figure 8, the presence of Ni^{2+} resulted in attenuation of the plateau phase of elevated $[\text{Ca}^{2+}]_i$ after application of H_2O_2 , as would be expected under conditions of inhibited Ca^{2+} influx. Addition of Ni^{2+} during the plateau phase in cells treated with H_2O_2 in the initial absence of Ni^{2+} accelerated the return of $[\text{Ca}^{2+}]_i$ towards basal levels. These findings are consistent with H_2O_2 -stimulated influx of Ca^{2+} occurring via a Ni^{2+} -inhibitable Ca^{2+} channel or entry pathway. A similar protocol was performed with La^{3+} , which had a similar, but quantitatively smaller, effect than did Ni^{2+} (trace not shown).

Figure 8 Effect of Ni²⁺ on H₂O₂-stimulated Ca²⁺ influx

CVECs suspended in Ca²⁺-containing ECS were stimulated with H₂O₂ in the presence (lower trace) of Ni²⁺ (2 mM NiCl₂). In the upper trace, cells were stimulated with H₂O₂ in the absence of Ni²⁺, which was then added during the plateau phase of elevated [Ca²⁺]_i.

Figure 9 Effect of SKF-96365 on H₂O₂-stimulated Ca²⁺ influx

CVECs were suspended in Ca²⁺-free/EGTA buffer and stimulated with H₂O₂ at 2 min. At 8 min, addition of Ca²⁺ (2 mM CaCl₂) was made in the absence (trace a) or the presence (trace b) of SKF-96365 (50 μM).

To determine whether H₂O₂ activates Ca²⁺ entry via the agonist-stimulated Ca²⁺-influx pathway, CVECs were suspended in Ca²⁺-free/EGTA buffer, and sequential additions of H₂O₂ and Ca²⁺ were made in the absence or presence of the imidazole compound SKF-96365 (50 μM). In the absence of SKF-96365, application of H₂O₂ followed by addition of Ca²⁺ resulted in the characteristic increases in [Ca²⁺]_i described above (Figure 2). In the presence of SKF-96365, the response of [Ca²⁺]_i to H₂O₂ was unchanged compared with that observed in the absence of the imidazole (Figure 9), indicating that SKF-96365 does not alter H₂O₂-induced release of Ca²⁺ from internal stores. By contrast, the increase in [Ca²⁺]_i upon addition of Ca²⁺ was significantly attenuated in the presence of SKF-96365, providing evidence that H₂O₂ activates the agonist-stimulated Ca²⁺-influx pathway.

DISCUSSION

Previous studies have demonstrated that agonist-stimulated Ca²⁺ signalling in vascular endothelial cells is inhibited by the short-chained hydrocarbon t-buOOH [22,23]. Such effects occur in a

time-dependent manner and are characterized initially by inhibition of the agonist-sensitive Ca²⁺-influx pathway, followed by inhibition of Ins(1,4,5)P₃-activated release of Ca²⁺ from internal stores. More prolonged incubation with t-buOOH is associated with a sustained increase in resting [Ca²⁺]_i [22] and inhibition of agonist-stimulated ⁴⁵Ca²⁺ efflux [23], findings that suggest that plasmalemmal Ca²⁺-ATPase activity is decreased.

The present study set out to determine the effects on Ca²⁺ signalling when H₂O₂, a compound that occurs endogenously in biological systems, is employed as the primary oxidant. Formation of H₂O₂ occurs via reduction of molecular oxygen or dismutation of the superoxide anion, and much of its biological significance can be traced to its secretion by activated neutrophils or monocytes. Because it possesses no unpaired electrons, H₂O₂ does not strictly meet the definition required of a radical species. Nevertheless, as an oxidant, H₂O₂ is moderately reactive. In addition, H₂O₂ may accept electrons from transitional metals such as iron(II) (Fe²⁺), leading to production of the highly reactive hydroxyl radical (OH[•]) [24,25]. Alternatively, H₂O₂ may act as a co-substrate for neutrophil-derived myeloperoxidase, resulting in generation of the highly reactive compound hypochlorous acid (HOCl) [26]. The amount of H₂O₂ produced by neutrophils *in vivo* is unknown, although secretion of the oxidant by neutrophils after activation *in vitro* and subsequent adherence to endothelial cells or matrix proteins may be as much as 100 nmol/10⁶ cells [4]. Within the microenvironment created by the adhesion of an activated neutrophil to its target endothelial cell, the concentration of H₂O₂ may be quite high [5,27,28], particularly if such an area represents a limited-diffusion space. The concentration of H₂O₂ employed in the present study was based on the oxidant's dose-dependent effect on [Ca²⁺]_i, and the observation that, even at the highest dose, [Ca²⁺]_i declined toward basal levels after reaching an H₂O₂-stimulated peak. This latter finding indicates that the homeostatic mechanisms that regulate [Ca²⁺]_i remain largely intact. Additionally, the concentration of H₂O₂ used herein is similar to those used in previous studies which have investigated various effects of H₂O₂ on cell function [13,27,29–31].

The secretion of H₂O₂ by activated neutrophils raises the interesting possibility that H₂O₂ has the role of intercellular messenger rather than that of toxin. Some evidence for this already exists in both the arterial and venous vasculature. H₂O₂ pretreatment of cultured venous endothelium or isolated perfused arteries or veins increases CD18- and ICAM-1-dependent adherence of neutrophils [15]. In addition, H₂O₂ transiently increases [Ca²⁺]_i of cultured A7r5 smooth-muscle cells, an effect partially blocked by dihydropyridine compounds [32]. To investigate the possibility that H₂O₂ might serve as an agonist messenger, we determined whether H₂O₂ could stimulate Ca²⁺ signalling in vascular endothelial cells.

Addition of H₂O₂ to CVECs suspended in Ca²⁺-containing buffer resulted in an increase in [Ca²⁺]_i, the rate of rise of which was dose-dependently related to oxidant concentration. Experiments in which H₂O₂ and Ca²⁺ were added in sequence to cells suspended in Ca²⁺-free/EGTA buffer enabled temporal dissociation of release of Ca²⁺ from internal stores on the one hand from influx of Ca²⁺ on the other [33]. With this protocol, the increase in [Ca²⁺]_i stimulated by H₂O₂ results solely from release of Ca²⁺ from internal stores, and occurs in the absence of Ca²⁺ entry across the plasmalemma. Addition of Ca²⁺ to the Ca²⁺-free/EGTA buffer (i.e. after application of H₂O₂) resulted in a large increase in [Ca²⁺]_i, consistent with activation of Ca²⁺ influx from the extracellular compartment. Thus the increase in [Ca²⁺]_i observed upon addition of H₂O₂ reflects both release of internally stored Ca²⁺ and influx of extracellular Ca²⁺.

Although H_2O_2 stimulates an increase in endothelial-cell $[\text{Ca}^{2+}]_i$, the above findings do not identify the specific Ca^{2+} -flux pathways sensitive to H_2O_2 . To investigate whether H_2O_2 activates agonist-sensitive flux pathways, cells were stimulated in sequence with ATP and H_2O_2 , or vice versa. Under normal conditions, the adenine nucleotide binds to the P_{2y} subclass of purinergic receptors located on the endothelial cell surface membrane [34], and activates phosphoinositide-dependent Ca^{2+} signalling [34–36]. There is evidence that more effective delivery of ATP to the endothelial cell surface during fluid flow accounts for the effect of shear stress on endothelial cell $[\text{Ca}^{2+}]_i$ [36]. This mechanism may contribute to the somewhat elevated basal $[\text{Ca}^{2+}]_i$ of CVECs observed in the present study compared with that previously reported for arterial endothelial cells [1]. Although estimates of flow forces on cells in these and similar experiments are not available, it is possible that such forces are greater than those normally encountered by venous endothelial cells *in vivo*. In addition, preparation of CVECs before measurement of $[\text{Ca}^{2+}]_i$ may act to disperse or inactivate ecto-ATPase associated with the cells, thus potentiating the effect of ATP present in the culture medium.

Like other agonists of non-excitable cells, ATP activates Ca^{2+} -flux pathways at both the endoplasmic reticulum and plasmalemma. In the presence of extracellular Ca^{2+} , ATP increased $[\text{Ca}^{2+}]_i$, and subsequent addition of H_2O_2 produced a second peak in $[\text{Ca}^{2+}]_i$. When the addition order was reversed (i.e. ATP after H_2O_2), the nucleotide had no effect on $[\text{Ca}^{2+}]_i$. This result is consistent with H_2O_2 -induced activation of cell signalling, causing the cells to be refractory to subsequent addition of ATP. Under the experimental conditions employed, CVECs were unresponsive to ATP for at least 8 min after addition of H_2O_2 . It remains possible that H_2O_2 inhibits ATP-stimulated Ca^{2+} signalling via decreased affinity of the purinergic receptor for ATP, decreased receptor number, inhibition of G-protein activation, and/or inhibition of one or more of the subsequent steps responsible for activation of Ca^{2+} flux pathways. However, the activation of Ca^{2+} fluxes by H_2O_2 alone suggests that subsequent attenuated responsiveness of cells to ATP is linked to the preceding stimulatory action of H_2O_2 .

The response of $[\text{Ca}^{2+}]_i$ to H_2O_2 clearly involves release of Ca^{2+} from internal stores, evidenced by elevation of $[\text{Ca}^{2+}]_i$ on addition of H_2O_2 to cells suspended in Ca^{2+} -free/EGTA buffer. However, this finding alone does not identify the intracellular Ca^{2+} compartment(s) accessed by H_2O_2 . To characterize first the size of the H_2O_2 -sensitive compartment, cells were suspended in the absence of extracellular Ca^{2+} and stimulated with H_2O_2 before or after application of ionomycin. When added after ionomycin, H_2O_2 failed to alter $[\text{Ca}^{2+}]_i$, whereas H_2O_2 decreased, but did not eliminate, the response to ionomycin. Because ionomycin acts to deplete internal stores of releasable Ca^{2+} , the above finding indicates that the H_2O_2 -sensitive compartment is a subset of the ionomycin-sensitive internal Ca^{2+} pool. A similar protocol, performed with ATP and H_2O_2 , demonstrated that H_2O_2 stimulates an increase in $[\text{Ca}^{2+}]_i$, even when added after ATP. By contrast, ATP elicits no such response when added after the oxidant. Thus the rank order of internal Ca^{2+} -pool compartment sizes is ionomycin > H_2O_2 > ATP. Release of mitochondrial Ca^{2+} may contribute to the increase in $[\text{Ca}^{2+}]_i$ triggered by H_2O_2 . However, the mitochondrial compartment represents only a small fraction of intracellularly stored Ca^{2+} , and ATP-dependent uptake of Ca^{2+} by mitochondria is significantly slower than that by non-mitochondrial stores at physiological concentrations of cytosolic Ca^{2+} [37–39].

To investigate further whether H_2O_2 releases Ca^{2+} from the Ins(1,4,5) P_3 -sensitive internal Ca^{2+} store, cells were suspended in

ECS before addition of thapsigargin, a compound that irreversibly inhibits Ca^{2+} pumping units by binding to the pump protein in its E_2 state [40]. A shift in the $E_1:E_2$ equilibrium toward the E_2 state is associated with decreased pump affinity for Ca^{2+} [41]. Thapsigargin thus induces net release of Ca^{2+} from the Ins(1,4,5) P_3 -sensitive intracellular Ca^{2+} store in absence of activation of the phosphoinositide signalling pathway [42–45]. The response of $[\text{Ca}^{2+}]_i$ to addition of H_2O_2 progressively declined with increasing time interval after addition of thapsigargin, suggesting that, as the Ins(1,4,5) P_3 -sensitive pool becomes progressively depleted, the response to H_2O_2 correspondingly declines. In addition, this finding provides evidence that the lack of effect of ATP in H_2O_2 -treated CVECs is related, at least in part, to H_2O_2 -stimulated release of Ca^{2+} from the Ins(1,4,5) P_3 -sensitive Ca^{2+} store. In digitonin-permeabilized hepatocytes, pretreatment with the oxidant t-buOOH increases release of Ca^{2+} at low concentrations of Ins(1,4,5) P_3 [46], an effect that may result in release of Ca^{2+} in the absence of agonist-activated signalling. This action of t-buOOH may be related to oxidation of thiol groups on the Ins(1,4,5) P_3 -receptor/ Ca^{2+} -channel complex, because similar effects are observed in the presence of oxidized glutathione [47]. The results in the present study indicate that H_2O_2 accesses the Ins(1,4,5) P_3 -sensitive Ca^{2+} store in venous endothelial cells, although it remains to be determined if this action occurs via thiol oxidation of the Ins(1,4,5) P_3 -receptor/ Ca^{2+} -channel complex.

An unexpected finding observed in the H_2O_2 -stimulated response of thapsigargin-treated cells was seen during the declining phase of $[\text{Ca}^{2+}]_i$, when $[\text{Ca}^{2+}]_i$ continued to decline beyond the thapsigargin-stimulated level. The new plateau level was slightly higher than baseline. This phenomenon might be explained by increased plasmalemmal Ca^{2+} -ATPase activity in cells treated with both thapsigargin and H_2O_2 . Although Ca^{2+} -pump function is sensitive to oxidation of its thiol groups, it is possible that, in the presence of thapsigargin, H_2O_2 somehow increases pump function. Alternatively, H_2O_2 may have an effect on the action of thapsigargin, thus resetting the balance between Ca^{2+} flux into and out of the cytosolic compartment.

There are several lines of evidence that H_2O_2 stimulates influx of Ca^{2+} from the extracellular space. First, the response of $[\text{Ca}^{2+}]_i$ to addition of H_2O_2 in the presence of extracellular Ca^{2+} results in a change in $[\text{Ca}^{2+}]_i$ similar to that observed on stimulation with agonists. In this regard, the agonist-stimulated response consists of a sustained increase in $[\text{Ca}^{2+}]_i$ that reflects influx of Ca^{2+} across the surface membrane [22,33,48]. Second, addition of the Ca^{2+} -chelating agent EGTA during the H_2O_2 -stimulated sustained (plateau) phase of elevated $[\text{Ca}^{2+}]_i$ promptly returns $[\text{Ca}^{2+}]_i$ to baseline, and subsequent re-addition of Ca^{2+} rapidly restores $[\text{Ca}^{2+}]_i$ to the plateau level. Because the endothelial-cell membrane is not permeable to EGTA, this technique provides strong evidence that H_2O_2 stimulates Ca^{2+} influx. The third line of evidence that H_2O_2 activates Ca^{2+} entry comes from experiments performed in Ca^{2+} -free/EGTA buffer. Under this condition, sequential addition of H_2O_2 and Ca^{2+} affords temporal isolation of Ca^{2+} influx, observed as a dramatic increase in $[\text{Ca}^{2+}]_i$ on addition of Ca^{2+} .

Previous work has indicated that the increase in intracellular Ca^{2+} , observed on incubation of cells with H_2O_2 , is dependent on extracellular Ca^{2+} [30]. The mechanism of Ca^{2+} entry has not been extensively investigated, although in a smooth-muscle cell line the increase in $[\text{Ca}^{2+}]_i$ was inhibited by voltage-dependent Ca^{2+} -channel blockers [32]. Experiments were performed in the present study in order to characterize the Ca^{2+} -influx pathway activated by H_2O_2 . The sustained phase of elevated $[\text{Ca}^{2+}]_i$ observed in CVECs suspended in Ca^{2+} -containing buffer and

stimulated with H₂O₂ is sensitive to the bivalent cation Ni²⁺, indicating that the H₂O₂-stimulated influx pathway is Ni²⁺-inhibitable. A similar but less pronounced effect of La³⁺ was also observed. Because extracellular Ni²⁺ and La³⁺ also block the agonist-stimulated influx pathway in vascular endothelial cells, we sought to determine whether the H₂O₂-sensitive pathway shared other pharmacological characteristics with the agonist-sensitive pathway. Toward this end, the sensitivity of the H₂O₂-sensitive pathway to the imidazole compound SKF-96365 was determined. This agent has been shown previously to inhibit selectively agonist-sensitive Ca²⁺ entry in various cell types, including vascular endothelial cells [45,49]. The increase in [Ca²⁺]_i observed when Ca²⁺ is added to H₂O₂-stimulated cells is clearly inhibited by SKF-96365. The above findings do not prove that H₂O₂-activated Ca²⁺ influx occurs via the agonist-sensitive pathway, but the sharing of several pharmacological characteristics indicates that the pathways operate in a very similar manner. There is accumulating evidence that agonist-stimulated Ca²⁺ influx occurs via a channel mechanism, as suggested by the presence of Ca²⁺-conducting currents obtained via giga-seal measurements of agonist-stimulated endothelial cell clusters [50]. Thus the results of the present study may reflect a novel, H₂O₂-sensitive, Ca²⁺ channel with characteristics similar to those of the putative agonist-activated channel, or the agonist-sensitive and H₂O₂-sensitive Ca²⁺ influx pathways may share identity. If the latter is true, H₂O₂ may act by oxidizing one or more moieties within the cation-conducting protein.

In summary, the present study demonstrates that H₂O₂ stimulates both Ca²⁺ influx and release of Ca²⁺ from internal stores in venous endothelial cells. H₂O₂ accesses the agonist-sensitive intracellular Ca²⁺ pool that is normally discharged by the action of Ins(1,4,5)P₃. The H₂O₂-stimulated Ca²⁺-influx pathway shares several characteristics with the agonist-sensitive flux pathway, including sensitivity to Ni²⁺, La³⁺ and SKF-96365, suggesting that the two pathways may be identical.

This study was supported by Grant HL02595 from the National Institutes of Health and by Grant 91R-190 from the American Heart Association. S.J.E. is the recipient of a Clinical Investigator Award from the National Heart, Lung and Blood Institute.

REFERENCES

- Elliott, S. J. and Schilling, W. P. (1990) *J. Biol. Chem.* **265**, 103–107
- Elliott, S. J., Meszaros, J. G. and Schilling, W. P. (1992) *Free Radicals Biol. Med.* **13**, 635–650
- Elliott, S. J. and Doan, T. N. (1993) *Biochem. J.* **292**, 385–393
- Shappell, S. B., Toman, C., Anderson, D. C., Taylor, A. A., Entman, M. L. and Smith, C. W. (1990) *J. Immunol.* **144**, 2702–2711
- Entman, M. L., Youker, K., Shoji, T., Kukiela, G., Shappell, S. B., Taylor, A. A. and Smith, C. W. (1992) *J. Clin. Invest.* **90**, 1335–1345
- Kirkland, J. B. (1991) *Biochim. Biophys. Acta* **1092**, 319–325
- Leff, J. A., Oppegard, M. A., Terada, L. S., McCarty, E. C. and Repine, J. E. (1991) *J. Appl. Physiol.* **71**, 1903–1906
- Varani, J., Phan, S. H., Gibbs, D. F., Ryan, U. S. and Ward, P. A. (1990) *Lab. Invest.* **63**, 683–689
- Spragg, R. G., Hinshaw, D. G., Hyslop, P. A., Schraufstatter, I. U. and Cochrane, C. G. (1985) *J. Clin. Invest.* **76**, 1471–1476
- Spragg, R. G. (1991) *Am. J. Respir. Cell Mol. Biol.* **4**, 4–10
- Jornot, L., Petersen, H. and Junod, A. F. (1991) *J. Cell. Physiol.* **149**, 408–413
- Vercellotti, G. M., Severson, S. P., Duane, P. and Moldow, C. F. (1991) *J. Lab. Clin. Med.* **117**, 15–24
- Lewis, M. S., Whately, R. E., Cain, P., McIntyre, T. M., Prescott, S. M. and Zimmerman, G. A. (1988) *J. Clin. Invest.* **82**, 2045–2055
- Patel, K. D., Zimmerman, G. A., Prescott, S. M., McEver, R. P. and McIntyre, T. M. (1991) *J. Cell Biol.* **112**, 749–759
- Gasic, A. C., McGuire, G., Krater, S., Farhood, A. I., Goldstein, M. A., Smith, C. W., Entman, M. L. and Taylor, A. A. (1991) *Circulation* **84**, 2154–2166
- Shasby, D. M., Yorek, M. and Shasby, S. S. (1988) *Blood* **72**, 491–499
- Chakraborti, S. and Michael, J. R. (1991) *FEBS Lett.* **281**, 185–187
- Harlan, J. M. and Callahan, K. (1984) *J. Clin. Invest.* **74**, 442–448
- Ford, J. W., Burkel, W. E. and Kahn, R. H. (1981) *In Vitro* **17**, 44–50
- Voyta, J. C., Via, D. P., Butterfield, C. E. and Zetter, B. R. (1984) *J. Cell. Biol.* **99**, 2034–2040
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Elliott, S. J., Eskin, S. G. and Schilling, W. P. (1989) *J. Biol. Chem.* **264**, 3806–3810
- Ratti, E. and Schilling, W. P. (1991) *Am. J. Physiol.* **260**, H549–H556
- Kvietys, P. R., Inauen, W., Bacon, B. R. and Grisham, M. B. (1989) *Am. J. Physiol.* **257**, H1640–H1646
- Graf, E., Mahoney, J. R., Bryant, R. G. and Eaton, J. W. (1984) *J. Biol. Chem.* **259**, 3620–3624
- Kettle, A. J. and Winterbourn, C. C. (1991) *Biochem. Pharmacol.* **41**, 1485–1492
- Suzuki, M., Asako, H., Kubes, P., Jennings, S., Grisham, M. B. and Granger, D. N. (1991) *Microvasc. Res.* **42**, 125–138
- Grisham, M. B., Gaginella, T. S., Von Ritter, C., Tamai, H., Be, R. M. and Granger, D. N. (1990) *Inflammation* **14**, 531–542
- Hinshaw, D. B., Burger, J. M., Armstrong, B. C. and Hyslop, P. A. (1989) *J. Surg. Res.* **46**, 339–349
- Hyslop, P. A., Hinshaw, D. B., Schraufstatter, I. U., Sklar, L. A., Spragg, R. G. and Cochrane, C. G. (1986) *J. Cell. Physiol.* **129**, 356–366
- Griesmacher, A., Weigel, G., David, M., Schimke, I. and Mueller, M. M. (1992) *Thromb. Res.* **65**, 721–731
- Roveri, A., Coassin, M., Maiorino, M., Zamburlini, A., Van Amsterdam, F. T. M., Ratti, E. and Ursini, F. (1992) *Arch. Biochem. Biophys.* **297**, 265–270
- Schilling, W. P., Ritchie, A. K., Navarro, L. T. and Eskin, S. G. (1988) *Am. J. Physiol.* **255**, H219–H227
- Martin, J. W., Cusack, N. J., Carleton, J. S. and Gordon, J. L. (1985) *Eur. J. Pharmacol.* **108**, 295–299
- Piroton, S., Raspe, E., Demolle, D., Erneux, C. and Boeynaems, J. (1987) *J. Biol. Chem.* **262**, 17461–17466
- Mo, M., Eskin, S. G. and Schilling, W. P. (1991) *Am. J. Physiol.* **260**, H1698–H1707
- Somlyo, A. P., Bond, M. and Somlyo, A. V. (1985) *Nature (London)* **314**, 622–625
- Gill, D. L. and Chueh, S. H. (1985) *J. Biol. Chem.* **260**, 9289–9297
- Freay, A., Johns, A., Adams, D. J., Ryan, U. S. and Van Breeman, C. (1989) *Pflügers Arch.* **414**, 377–384
- Kijima, Y., Ogunbunmi, E. and Fleischer, S. (1991) *J. Biol. Chem.* **266**, 22912–22918
- Wictome, M., Michelangeli, F., Lee, A. G. and East, J. M. (1992) *FEBS Lett.* **304**, 109–113
- Takemura, H., Hughes, A. R., Thastrup, O. and Putney, J. W. (1989) *J. Biol. Chem.* **264**, 12266–12271
- Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2466–2470
- Lytton, J., Westlin, M. and Hanley, M. R. (1991) *J. Biol. Chem.* **266**, 17067–17071
- Schilling, W. P., Cabello, O. A. and Rajan, L. (1992) *Biochem. J.* **284**, 521–530
- Rooney, T. A., Renard, D. C., Sass, E. J. and Thomas, A. P. (1991) *J. Biol. Chem.* **266**, 12272–12282
- Renard, D. C., Seitz, M. B. and Thomas, A. P. (1992) *Biochem. J.* **284**, 507–512
- Rotrosen, D. and Gallin, J. I. (1986) *J. Cell Biol.* **103**, 2379–2387
- Merritt, J. E., Armstrong, W. P., Benham, C. D., Hallam, T. J., Jacob, R., Jaxa-Chamiec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E. and Rink, T. J. (1990) *Biochem. J.* **271**, 515–522
- Mendelowitz, D., Bacal, K. and Kunze, D. L. (1992) *Am. J. Physiol.* **262**, H942–H948