Oxidant stress inhibits the store-dependent Ca^{2+} -influx pathway of vascular endothelial cells

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Oxidant stress induced by t-butyl hydroperoxide (t-BuOOH) inhibits bradykinin-stimulated Ca^{2+} signalling in vascular endothelial cells. The effect of t-BuOOH on intracellular Ca^{2+} pools was determined by addition of Ca^{2+} -releasing agents to fura-2-loaded cells suspended in Ca^{2+} -free/EGTA buffer. In control cells, sequential additions of bradykinin and ionomycin produced similar increases in cytosolic free $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$). By contrast, incubation with t-BuOOH progressively decreased the response of $[\text{Ca}^{2+}]_i$ to bradykinin and increased that to ionomycin, suggesting that the total (ionomycin-releasable) Ca^{2+} pool remains replete during oxidant stress. The effect of t-BuOOH on the InsP_3 -sensitive Ca^{2+} pool was measured by the increase in $[\text{Ca}^{2+}]_i$ or efflux of $^{45}\text{Ca}^{2+}$ stimulated by 2,5-di-t-butylhydroquinone (BHQ). Incubation with t-BuOOH did not inhibit BHQ-stimulated increases in $[\text{Ca}^{2+}]_i$ or $^{45}\text{Ca}^{2+}$ efflux, suggesting

that the InsP_3 -sensitive Ca^{2+} pool remains replete and releasable. Activity of the Ca^{2+} -influx pathway stimulated by release of internal Ca^{2+} stores was determined via re-addition of Ca^{2+} to BHQ-stimulated cells suspended in Ca^{2+} -free/EGTA buffer and via BHQ-stimulated $^{45}\text{Ca}^{2+}$ uptake. Incubation of cells with t-BuOOH for 1 h significantly inhibited the influx pathway. At later time points, t-BuOOH increased basal $[\text{Ca}^{2+}]_i$ and potentiated the response of $[\text{Ca}^{2+}]_i$ to BHQ. Similar results were demonstrated with thapsigargin. Together, these findings suggest that (1) the inhibitory effect of t-BuOOH on bradykinin-stimulated release of Ca^{2+} from internal stores is not related to depletion of these stores, and (2) inhibition of the store-dependent Ca^{2+} -influx pathway occurs by a direct effect of the influx pathway or by inhibition of the mechanism which links the internal Ca^{2+} store to plasmalemmal Ca^{2+} influx.

INTRODUCTION

Ca^{2+} -dependent signalling mechanisms play a key role in the regulation of vascular-endothelial-cell paracrine function. As a result of endothelial-cell receptor stimulation, activation of Ca^{2+} influx and release of Ca^{2+} from internal stores each contribute to increased cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), leading to release of vasoactive secretory products such as NO [1–3]. Many aspects of Ca^{2+} signalling in non-excitable cells, such as the mechanism(s) responsible for activation of Ca^{2+} entry, have yet to be fully characterized. Regulation of Ca^{2+} influx has been linked to the state of repletion of intracellular Ca^{2+} stores [4–6]. In this regard, numerous studies have employed the microsomal Ca^{2+} -ATPase inhibitors 2,5-di-t-butylhydroquinone (BHQ) or thapsigargin to deplete internal stores of releasable Ca^{2+} and stimulate Ca^{2+} influx in the absence of activation of the inositol polyphosphate signalling pathway [4,7].

Under normal conditions, mammalian cells exist in a redox environment which is homeostatically stable and which reflects a balance between the activities of oxidant and reductant chemical species. Oxidant stress may be operationally defined as that condition in which one or more oxidant moieties elicits a measurable biological response. The oxidant t-butyl hydroperoxide (t-BuOOH) is metabolized predominantly via the action of glutathione peroxidase, an enzyme that links the reduction of t-BuOOH to the oxidation of GSH. In this respect, t-BuOOH has proved to be useful in several experimental models, because the glutathione system forms the most important antioxidant defence mechanism possessed by many cell types, including vascular endothelial cells [8].

Various forms of oxidant stress alter vascular tone in an endothelium-dependent manner, and previous work in cultured

vascular endothelial cells has indicated that oxidative stress significantly alters Ca^{2+} signalling and homeostasis in this cell type in a reproducible time-dependent manner [9–11]. Initially, t-BuOOH inhibits bradykinin-stimulated Ca^{2+} influx without effect on basal $[\text{Ca}^{2+}]_i$ [10], whereas after longer incubations t-BuOOH inhibits receptor-activated release of Ca^{2+} from internal stores, and increases basal $[\text{Ca}^{2+}]_i$ [10,11].

Oxidant stress may inhibit release of Ca^{2+} from internal stores by inhibition of one or more of the steps responsible for activation of $\text{Ins}(1,4,5)\text{P}_3$ receptors at the endoplasmic reticulum. In this regard, t-BuOOH has recently been shown to decrease agonist-stimulated production of InsP_3 in vascular endothelial cells [12]. It is not known, however, whether this effect alone accounts for the inhibitory action of t-BuOOH. For example, oxidant stress may also deplete internal stores of releasable Ca^{2+} , resulting in diminished release of Ca^{2+} upon stimulation by agonists. Such an effect may be mediated by increased leak of Ca^{2+} from internal stores under basal conditions, as observed in rat hepatocyte microsomes [13], and/or inhibition of endoplasmic-reticular Ca^{2+} -ATPase, as has been shown for the plasmalemmal Ca^{2+} pump [14,15]. To investigate the possibility that incubation of vascular endothelial cells with t-BuOOH results in depletion of internal Ca^{2+} stores, two series of experiments were performed. First, we hypothesized that, compared with controls, release of Ca^{2+} by ionomycin remains unchanged in t-BuOOH-treated cells. Thus, unaltered or increased response to ionomycin in the presence of decreased response to bradykinin would support the hypothesis that internal Ca^{2+} stores remain intact, despite treatment with t-BuOOH. Second, we hypothesized that if decreased receptor-activated Ca^{2+} release is due solely to inhibition of inositol polyphosphate signalling, then the agonist-sensitive Ca^{2+} pool would remain accessible to agents which cause Ca^{2+} release

Abbreviations used: BAECs, bovine aortic endothelial cells; BHQ, 2,5-di-t-butylhydroquinone; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; DME, Dulbecco's modified Eagle's medium; ECS, extracellular solution; t-BuOOH, t-butyl hydroperoxide.

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independently of inositol polyphosphate signalling. To this end, the response of $[Ca^{2+}]_i$ to application of the microsomal Ca^{2+} -pump inhibitors BHQ or thapsigargin was determined under control and oxidant-treated conditions. In addition, the effect of t-BuOOH on BHQ-stimulated $^{45}Ca^{2+}$ efflux, a measure of the internal Ca^{2+} -store release pathway, was determined. The results of these experiments provide evidence that inhibition of receptor-activated release of Ca^{2+} from internal stores is not related to depletion of releasable Ca^{2+} .

Evidence from previous investigations which employed fura-2 to measure $[Ca^{2+}]_i$ [10] or $^{45}Ca^{2+}$ to measure directly the activity of several Ca^{2+} -flux pathways [11] indicates that incubation of vascular endothelial cells with t-BuOOH inhibits agonist-stimulated Ca^{2+} influx. The mechanism by which this action of t-BuOOH is produced is largely unknown. In this respect, one possible mechanism is decreased production or increased metabolism of a key second messenger, such as a hydrolysis product of phosphatidylinositol 4,5-bisphosphate. Alternatively, t-BuOOH may inhibit Ca^{2+} influx via a direct effect on the influx pathway. To investigate the latter possibility, we developed a strategy in which the effect of t-BuOOH on BHQ- or thapsigargin-stimulated influx of Ca^{2+} was determined. Inhibition by t-BuOOH of the store-dependent Ca^{2+} -influx pathway, similar to that observed with agonist-stimulated Ca^{2+} influx, would suggest that the oxidant directly inhibits the plasmalemmal influx pathway. In addition, the effect of oxidant stress on BHQ- or thapsigargin-stimulated $^{45}Ca^{2+}$ uptake, employed as a more direct measure of the activity of the Ca^{2+} -influx pathway, was determined in cell monolayers incubated in absence or presence of t-BuOOH.

The results of these studies indicate that, although t-BuOOH progressively inhibits bradykinin-stimulated release of Ca^{2+} from internal stores in a time-dependent manner, the response to ionomycin, and the response to the Ca^{2+} -pump inhibitors BHQ or thapsigargin, remain intact. Indeed, after more prolonged incubation of cells with t-BuOOH, basal $[Ca^{2+}]_i$ is elevated and the rise in $[Ca^{2+}]_i$ elicited by BHQ or thapsigargin is increased, suggesting that at these later times t-BuOOH enlarges the BHQ- and thapsigargin-sensitive Ca^{2+} pools. Inhibition of BHQ- and thapsigargin-stimulated Ca^{2+} influx, despite preservation of release of Ca^{2+} from internal stores induced by these agents, suggests that the oxidant either directly inhibits the Ca^{2+} -influx pathway or inhibits the mechanism which links regulation of plasmalemmal Ca^{2+} entry to the repletion status of the internal Ca^{2+} store.

MATERIALS AND METHODS

Reagents and solutions

Thapsigargin and t-BuOOH [70% (w/v) aq. solution] were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). BHQ ([88-58-4]) was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Bradykinin and ionomycin were obtained from Calbiochem (San Diego, CA, U.S.A.), and fura-2/AM was from Molecular Probes (Eugene, OR, U.S.A.). All chemicals were of reagent grade. Extracellular solution (ECS) contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM D-glucose and 15 mM Hepes, pH adjusted to 7.40 at 37 °C with NaOH. Ca^{2+} -free/EGTA buffer contained 0.3 mM EGTA and the same salts as ECS without added $CaCl_2$.

Culture of endothelial cells

Bovine aortic endothelial cells (BAECs) were cultured as previously described [10] in Dulbecco's modified Eagle's medium

(DME; GIBCO, Grand Island, NY, U.S.A.) supplemented with 10% fetal-bovine serum (Hyclone, Logan, UT, U.S.A.), 100 μ g/ml streptomycin, 100 μ g/ml penicillin, 100 μ g/ml neomycin and 2 mM glutamine (complete-DME). Endothelial-cell identity was confirmed by uptake of di-iodoacetyl-low-density-lipoprotein examined by epifluorescence microscopy [16]. Monolayers demonstrated the cobblestone appearance typical of vascular endothelial cells when observed under light microscopy by phase contrast. Experiments were performed on cells at passage 10–15, at 1–2 days post-confluency.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured with the fluorescent indicator fura-2, as previously described [10]. Briefly, cells grown to confluency in 100-mm dishes 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 complete-DME. Cells were centrifuged, washed, and resuspended in ECS containing 20 μ M fura-2/AM for 30 min at 37 °C. After dilution into a volume of 40 ml, cells were incubated for a further 30 min 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.). Excitation wavelength alternated between 340 and 380 nm every 0.5 s during recording of emission fluorescence at 510 nm. All measurements were corrected for autofluorescence by using cells treated as above except for the absence of fura-2. Calibration of the fluorescent signal was determined on individual samples by sequential addition of Triton X-100 (0.1%) and EGTA (5 mM), and $[Ca^{2+}]_i$ was calculated by the equation of Grynkiewicz et al. [17]. None of the compounds used altered the calibration values of fura-2, or significantly altered autofluorescence.

Measurement of $^{45}Ca^{2+}$ efflux

To determine the effect of oxidant stress on BHQ-stimulated efflux of $^{45}Ca^{2+}$, radioisotope was added to confluent monolayers of BAECs cultured in 35-mm dishes containing 2 ml of complete-DME. After equilibration of the cells with the radioisotope for 15–18 h, the medium was aspirated from the dish, and the monolayer washed with ECS. Cells were washed three times, and after the final aspiration 3 ml of ECS was added to the dish. Cells were allowed to equilibrate for 5 min, at which time duplicate 100 μ l samples were withdrawn (zero time) and BHQ (10 μ M) was added. At each subsequent time point, duplicate samples were withdrawn and placed in scintillation vials. Radioactivity in each sample was determined by standard liquid-scintillation technique. Upon removal of the final sample, the remaining solution was washed once, and NaOH (0.5%) added for determination of radioactivity remaining associated with the monolayer. Values were calculated as percentage of radioisotope remaining associated with the monolayer relative to the value at the end of the 5 min equilibration period. Basal efflux rate constants were determined for the slopes of the line relating the logarithm of the percentage of radioisotope remaining associated with the cells versus time. Rate constants for stimulated efflux were determined from the initial slope of the efflux curve by using the calculated value at the time of BHQ addition and the values measured at 0.5 and 1.0 min after addition of BHQ. The efflux rate constants are reported with the respective correlation coefficients obtained from linear regression analysis.

Measurement of $^{45}\text{Ca}^{2+}$ uptake

Uptake of radiolabelled Ca^{2+} was determined at 37°C in control cells and cells preincubated with t-BuOOH for 1 h. After incubation (5 min) in the absence or presence of BHQ ($10\ \mu\text{M}$) or thapsigargin ($200\ \text{nM}$), uptake was initiated by addition of $^{45}\text{CaCl}_2$ ($1.8\ \mu\text{Ci/ml}$). Uptake of $^{45}\text{Ca}^{2+}$ was terminated by rapid aspiration of medium and washing of the monolayer three times with ice-cold ECS containing LaCl_3 ($0.2\ \text{mM}$). Monolayers were digested with 0.5% NaOH, and radioactivity associated with the cells was determined by standard liquid-scintillation technique using a Beckman LS3801 scintillation counter. Stimulated uptake was determined from the difference in uptake between that in the absence and that in the presence of releasing compound. Monolayer integrity of a random sampling of culture dishes was performed during each experiment, by phase-contrast light microscopy.

Incubation of cells with t-BuOOH

Fura-2-loaded cells were incubated in ECS at 37°C containing t-BuOOH ($0.4\ \text{mM}$) for various durations. Cells were washed twice at the completion of the incubation period and immediately transferred to a quartz cuvette for measurement of fluorescence. Control cells were subjected to identical incubations except for absence of oxidant. For radioisotope-flux experiments, cells grown to confluency in 35-mm culture dishes were preincubated in the absence or presence of t-BuOOH ($0.4\ \text{mM}$) for 1 h before determination of uptake or efflux as described above.

RESULTS

Effect of t-BuOOH on Ca^{2+} release by bradykinin and ionomycin

Cells were preincubated in the absence or presence of t-BuOOH and resuspended in Ca^{2+} -free/EGTA buffer before stimulation with bradykinin ($50\ \text{nM}$) followed by ionomycin ($1\ \mu\text{M}$). Although incubation of cells with t-BuOOH for 1 h increased basal $[\text{Ca}^{2+}]_i$, the response to bradykinin and ionomycin was similar to that observed under control conditions (Figure 1a). In cells incubated with the oxidant for 2 h, the bradykinin-stimulated increase in $[\text{Ca}^{2+}]_i$ was clearly diminished compared with the control (Figure 1b). In contrast, the response to ionomycin was slightly greater in t-BuOOH-treated cells compared with that observed under control conditions. After 3 h, the increase in $[\text{Ca}^{2+}]_i$ due to addition of ionomycin is larger than that seen under control conditions (Figure 1c), and the response of oxidant-treated cells to ionomycin is clearly greater than the increase produced by bradykinin.

Effect of t-BuOOH on internal Ca^{2+} store

To investigate the effect of t-BuOOH-induced oxidant stress on (a) the size of the ionomycin-releasable internal Ca^{2+} store and (b) ionomycin-stimulated Ca^{2+} influx, cells were incubated in the absence or presence of t-BuOOH, before suspension in Ca^{2+} -free EGTA buffer and sequential addition of ionomycin and Ca^{2+} . At all time points (0.5–3 h), the ionomycin-stimulated increase in $[\text{Ca}^{2+}]_i$ in oxidant-treated cells was nearly identical with that seen in control cells (Figure 2), a finding which suggests that incubation of cells with t-BuOOH does not deplete the ionomycin-releasable Ca^{2+} pool. Under control conditions, re-addition of Ca^{2+} to the extracellular buffer produced a large increase in $[\text{Ca}^{2+}]_i$, reflecting influx of Ca^{2+} across the plasmalemma. In contrast, re-addition

of Ca^{2+} after ionomycin to cells incubated with t-BuOOH for 0.5–1 h resulted in an attenuated rise in $[\text{Ca}^{2+}]_i$ compared with controls (Figures 2a and 2b). After more prolonged incubation (2–3 h) with the oxidant, re-addition of Ca^{2+} to ionomycin-treated cells resulted in large increases in $[\text{Ca}^{2+}]_i$, which then continued to increase progressively and rapidly with time (Figures 2c and 2d).

Effect of BHQ in control endothelial cells

Under control conditions, addition of BHQ ($10\ \mu\text{M}$) to cells suspended in Ca^{2+} -free/EGTA buffer resulted in a step-like increase in $[\text{Ca}^{2+}]_i$, reflecting net release of Ca^{2+} from internal stores (Figure 3a). Subsequent addition of Ca^{2+} ($2\ \text{mM}\ \text{CaCl}_2$) to the extracellular buffer produced a large biphasic increase in $[\text{Ca}^{2+}]_i$, a response which was not observed in cells not treated with BHQ (results not shown). Stimulation of cells with BHQ in the presence of extracellular Ca^{2+} resulted in a sustained increase in $[\text{Ca}^{2+}]_i$, which rapidly decreased toward basal levels upon addition of La^{3+} ($20\ \mu\text{M}$) (Figure 3b). When La^{3+} was added before application of BHQ, the amplitude of the $[\text{Ca}^{2+}]_i$ response to BHQ was unchanged, but the increase in $[\text{Ca}^{2+}]_i$ was transient

Figure 1 Effect of t-BuOOH on relative responses of BAECs to bradykinin and ionomycin

$[\text{Ca}^{2+}]_i$ was measured in fura-2-loaded cells incubated in the absence (trace a in each panel) or presence (trace b) of t-BuOOH ($0.4\ \text{mM}$) for (a) 1, (b) 2 or (c) 3 h. At the completion of the incubation period, cells were washed twice and resuspended in Ca^{2+} -free/EGTA buffer. Bradykinin (BK; final concn. $50\ \text{nM}$) was added at 1 min, followed by ionomycin (Iono; $1\ \mu\text{M}$) 2 min later. In this and subsequent Figures, all incubations and experiments were carried out at 37°C . The experiment shown is representative of eight independent experiments.

Figure 2 Effect of t-BuOOH on ionomycin-stimulated Ca^{2+} influx

Fura-2-loaded cells were suspended in ECS and incubated in the absence or presence of t-BuOOH (0.4 mM) for (a) 0.5, (b) 1.0, (c) 2.0 or (d) 3.0 h. At the completion of the incubation period, cells were washed twice and resuspended in Ca^{2+} -free/EGTA buffer. Fluorescence was monitored within 30 s of resuspension. Shown are representative traces obtained in control cells (traces labelled a) and t-BuOOH-treated cells (traces labelled b). Ionomycin (final concn. 1 μM) was added at 2 min, followed by Ca^{2+} (2 mM CaCl_2) 2 min later. The experiment shown is representative of four independent experiments.

in nature. These results demonstrate that BHQ stimulates Ca^{2+} influx from the extracellular space in this cell type.

Effect of t-BuOOH on BHQ-stimulated change in $[\text{Ca}^{2+}]_i$

The absence of an inhibitory effect of t-BuOOH on ionomycin-stimulated release of Ca^{2+} from internal stores suggests that the size of the ionomycin-sensitive Ca^{2+} compartment remains unchanged during incubation of BAECs with this oxidant. To investigate more specifically whether t-BuOOH alters the size of the agonist-sensitive internal Ca^{2+} pool, cells were incubated in the absence or presence of t-BuOOH before suspension in Ca^{2+} -

Figure 3 Stimulation of Ca^{2+} influx by BHQ

(a) $[\text{Ca}^{2+}]_i$ was measured in fura-2-loaded BAECs suspended in Ca^{2+} -free/EGTA buffer. Addition of BHQ (10 μM) was made at 2 min, followed by addition of Ca^{2+} (2 mM CaCl_2) 2 min later. The experiment shown is representative of three independent experiments. (b) Cells loaded with fura-2 were suspended in Ca^{2+} -containing ECS. Two traces are superimposed to show the effect of La^{3+} on BHQ-induced changes in $[\text{Ca}^{2+}]_i$. In the lower trace, La^{3+} (LaCl_3 ; 20 μM) was added at 1 min, followed by addition of BHQ (10 μM) at 2 min. In the upper trace, BHQ was added at 2 min, followed by addition of La^{3+} at 6 min.

free/EGTA buffer and addition of BHQ. The increase in $[\text{Ca}^{2+}]_i$ upon application of BHQ was employed as a measure of the state of repletion of the BHQ-sensitive Ca^{2+} pool, whereas the change in $[\text{Ca}^{2+}]_i$ observed with subsequent re-addition of Ca^{2+} to the extracellular buffer was used as a measure of activity of the store-dependent Ca^{2+} -influx pathway. Representative traces of control and t-BuOOH-treated cells after each period of incubation (0.5–3 h) are shown in Figure 4.

In control cells incubated for 0.5 h (i.e. in the absence of t-BuOOH), basal $[\text{Ca}^{2+}]_i$ was 44 ± 3 nM ($n = 5$). Addition of BHQ (10 μM) increased $[\text{Ca}^{2+}]_i$ to 65 ± 2 nM, and subsequent re-addition of Ca^{2+} to the extracellular buffer (2 min after BHQ) increased $[\text{Ca}^{2+}]_i$ to 246 ± 21 nM. Incubation of BAECs with t-BuOOH (0.5 h) failed to alter either basal $[\text{Ca}^{2+}]_i$ (42 ± 1 nM; $n = 5$) or BHQ-stimulated $[\text{Ca}^{2+}]_i$ (65 ± 2 nM). Similarly, both basal and BHQ-stimulated $[\text{Ca}^{2+}]_i$ were unaffected by incubation with t-BuOOH for 1 h.

In contrast, more prolonged incubation (2 h) with t-BuOOH significantly increased basal $[\text{Ca}^{2+}]_i$ to 88 ± 5 nM ($P < 0.05$ compared with control). In addition, the response of oxidant-treated cells to BHQ under this condition was slightly increased; BHQ increased $[\text{Ca}^{2+}]_i$ by 17 ± 1 nM in control cells and 22 ± 3 nM in BAECs treated with t-BuOOH ($P > 0.05$). After incubation of cells with oxidant for 3 h, basal $[\text{Ca}^{2+}]_i$ was further increased to 124 ± 15 nM, compared with 69 ± 5 nM ($P < 0.001$) observed in control BAECs incubated for the same duration. In addition, the BHQ-stimulated increase in $[\text{Ca}^{2+}]_i$ (24 ± 2 nM) was significantly greater than that of controls (13 ± 2 nM; $P = 0.01$). Thus, incubation of BAECs with t-BuOOH for 2 h or longer

Figure 4 Effect of t-BuOOH on BHQ-stimulated changes in $[\text{Ca}^{2+}]_i$, determined in Ca^{2+} -free/EGTA buffer

$[\text{Ca}^{2+}]_i$ was measured in fura-2-loaded cells incubated in absence (trace *a* in each panel) or presence (trace *b* in each panel) of t-BuOOH (0.4 mM) for (a) 0.5, (b) 1.0, (c) 2.0 or (d) 3.0 h. At the completion of the incubation period, cells were washed twice and resuspended in Ca^{2+} -free/EGTA buffer, and fluorescence was monitored within 30 s of resuspension. BHQ (final concn. 10 μM) was added at 2 min, followed by Ca^{2+} (2 mM CaCl_2) at 4 min. Traces shown are representative of five experiments.

results in elevation of basal $[\text{Ca}^{2+}]_i$, and a larger increase in $[\text{Ca}^{2+}]_i$ upon stimulation with BHQ.

Effect of t-BuOOH on BHQ-stimulated Ca^{2+} Influx

BHQ-stimulated influx of Ca^{2+} was determined via addition of Ca^{2+} to BHQ-stimulated cells suspended in Ca^{2+} -free/EGTA buffer. As with the responses to application of BHQ, increases in $[\text{Ca}^{2+}]_i$ upon addition of Ca^{2+} are shown in Figure 4 for each period of incubation. Although incubation of BAECs with t-BuOOH for 0.5 h did not alter basal or BHQ-stimulated $[\text{Ca}^{2+}]_i$, peak $[\text{Ca}^{2+}]_i$ stimulated by addition of Ca^{2+} was significantly decreased to 171 ± 15 nM, compared with control (246 ± 21 nM; $P < 0.001$). Similarly, neither basal nor BHQ-stimulated $[\text{Ca}^{2+}]_i$ was affected by incubation of cells with t-BuOOH for 1 h, yet the increase in $[\text{Ca}^{2+}]_i$ upon re-addition of Ca^{2+} was further diminished to 160 ± 7 nM ($P < 0.001$ compared with control).

In contrast with inhibition of BHQ-stimulated Ca^{2+} influx observed in BAECs treated with t-BuOOH for 1 h or less, incubation of cells with the oxidant for 2 h resulted in a large increase in $[\text{Ca}^{2+}]_i$ upon addition of Ca^{2+} to the extracellular buffer. This effect was even more pronounced after an incubation period of 3 h, when re-addition of Ca^{2+} to t-BuOOH-treated cells dramatically increased $[\text{Ca}^{2+}]_i$ to 340 ± 75 nM, after which $[\text{Ca}^{2+}]_i$ progressively increased with time.

To determine the effect of t-BuOOH on Ca^{2+} influx in unstimulated cells, experiments were performed in which Ca^{2+} was added to the Ca^{2+} -free/EGTA buffer before addition of BHQ. Representative traces for each incubation period are shown in Figure 5. Addition of Ca^{2+} to BAECs incubated for 0.5 h in the presence of t-BuOOH increased $[\text{Ca}^{2+}]_i$ from a baseline of 40 ± 2 nM to a new level of 108 ± 9 nM ($n = 3$). By

contrast, addition of Ca^{2+} to control cells increased $[\text{Ca}^{2+}]_i$ to 153 ± 11 nM. After incubation of BAECs with t-BuOOH for 2 or 3 h, basal $[\text{Ca}^{2+}]_i$ was elevated and addition of Ca^{2+} markedly increased $[\text{Ca}^{2+}]_i$ to 258 ± 77 nM and 406 ± 136 nM, respectively. At these later time points, subsequent addition of BHQ resulted in a marked elevation of $[\text{Ca}^{2+}]_i$, and $[\text{Ca}^{2+}]_i$ failed to decrease toward baseline with time.

Effect of t-BuOOH on response to thapsigargin

To evaluate whether the effects of t-BuOOH on BHQ-stimulated changes in $[\text{Ca}^{2+}]_i$ reflect an interaction between BHQ and the oxidant, experiments were performed in which endoplasmic-reticular Ca^{2+} -ATPase was inhibited by using the structurally unrelated compound thapsigargin. Cells were incubated in the absence or presence of t-BuOOH for various times before suspension in Ca^{2+} -free/EGTA buffer and addition of thapsigargin and Ca^{2+} . Incubation of BAECs with t-BuOOH for 0.5–1 h resulted in inhibition of thapsigargin-stimulated Ca^{2+} influx. After longer treatment periods with t-BuOOH, basal $[\text{Ca}^{2+}]_i$ increased, and the responses of $[\text{Ca}^{2+}]_i$ to thapsigargin and Ca^{2+} significantly increased. Mean values of $[\text{Ca}^{2+}]_i$ at key points during measurement of thapsigargin-stimulated changes in $[\text{Ca}^{2+}]_i$ are plotted in Figure 6.

Effect of t-BuOOH on BHQ-stimulated $^{45}\text{Ca}^{2+}$ efflux

As described above, incubation of BAECs with t-BuOOH for 0.5 or 1 h resulted in inhibition of BHQ- and thapsigargin-stimulated Ca^{2+} influx, in the absence of change in the response of $[\text{Ca}^{2+}]_i$ to BHQ or thapsigargin. To investigate further the effect of t-

Figure 5 Effect of *t*-BuOOH on addition of Ca^{2+} and BHQ

$[\text{Ca}^{2+}]_i$ was measured in fura-2-loaded cells incubated in the absence (trace *a* in each panel) or presence (trace *b* in each panel) of *t*-BuOOH (0.4 mM) for (a) 0.5, (b) 1.0, (c) 2.0 or (d) 3.0 h. At the completion of the incubation period, cells were washed twice and fluorescence was monitored within 30 s of resuspension. Ca^{2+} (2 mM CaCl_2) was added at 2 min, followed by BHQ (final concn. 10 μM) at 6 min. Traces shown are representative of three experiments. Note change in ordinate scale in panels (c) and (d).

Figure 6 Effect of *t*-BuOOH on thapsigargin-stimulated changes in $[\text{Ca}^{2+}]_i$ determined in Ca^{2+} -free/EGTA buffer

Fura-2-loaded cells were incubated in the absence (○) or presence (●) of *t*-BuOOH (0.4 mM) for (a) 0.5, (b) 1.0, (c) 2.0 or (d) 3.0 h. At the completion of the incubation period, cells were washed twice and resuspended in Ca^{2+} -free/EGTA buffer. Thapsigargin (TG; final concn. 200 nM) was added at 2 min, followed by Ca^{2+} (2 mM CaCl_2) at 4 min. Shown are mean $\pm$ S.E.M. values of $[\text{Ca}^{2+}]_i$ at key points during measurement. Note change in ordinate scale in panel (d).

BuOOH at these early times on BHQ-stimulated release of Ca^{2+} from internal stores, we measured BHQ-stimulated $^{45}\text{Ca}^{2+}$ efflux in radioisotope-equilibrated cells in control and oxidant-treated cells (Figure 7). Under control conditions, the basal efflux rate constant for $^{45}\text{Ca}^{2+}$ was $(5.76 \pm 0.22) \times 10^{-2} \text{ min}^{-1}$ ($r = 0.96$)

compared with $(5.76 \pm 0.60) \times 10^{-2} \text{ min}^{-1}$ ($r = 0.99$) in cells incubated with *t*-BuOOH. Upon application of BHQ, efflux increased to $(10.5 \pm 0.7) \times 10^{-2} \text{ min}^{-1}$ ($r = 0.96$) and $(10.9 \pm 0.4) \times 10^{-2} \text{ min}^{-1}$ ($r = 0.94$) ($P > 0.05$) in control and *t*-BuOOH-treated cells respectively.

Figure 7 Effect of t-BuOOH on BHQ-stimulated $^{45}\text{Ca}^{2+}$ efflux

Efflux of $^{45}\text{Ca}^{2+}$ was determined in confluent monolayers of BAECs grown in 35-mm dishes under control conditions (a) or after incubation with t-BuOOH (0.4 mM) for 1 h (b). Experiments were performed in the absence ($\circ$, $\triangle$) or presence ($\bullet$, $\blacktriangle$) of BHQ (final concn. $10 \mu\text{M}$) added at 1 min (arrow). Data represent means $\pm$ S.E.M. ($n = 4$); where not shown, S.E.M. was smaller than the symbol size employed.

Figure 8 Effect of t-BuOOH on BHQ- and thapsigargin-stimulated $^{45}\text{Ca}^{2+}$ uptake

Uptake of $^{45}\text{Ca}^{2+}$ was determined in confluent monolayers of BAECs grown in 35-mm culture dishes and preincubated in the absence ($\circ$) or presence ($\bullet$) of t-BuOOH for 1 h. (a) Addition of BHQ (final concn. $10 \mu\text{M}$) or vehicle was carried out 5 min before addition of $^{45}\text{Ca}^{2+}$ (final concn. $1.8 \mu\text{Ci}/\text{ml}$). Uptake was terminated at the times indicated by rapid aspiration of reaction medium and washing with ice-cold ECS containing 0.2 mM LaCl_3 . Radioactivity associated with cells was determined on NaOH digests of each monolayer. Plotted values represent BHQ-stimulated $^{45}\text{Ca}^{2+}$ uptake, determined from the difference in uptake measured in the absence and presence of BHQ. Data represent means $\pm$ S.E.M. of four experiments, each performed in triplicate. (b) As for (a), except that thapsigargin (final concn. 200 nM) was employed rather than BHQ. Data represent means $\pm$ S.E.M. ($n = 5$).

Effect of t-BuOOH on BHQ- and thapsigargin-stimulated $^{45}\text{Ca}^{2+}$ uptake

To test more directly the effect of oxidant stress on store-dependent Ca^{2+} influx, uptake of $^{45}\text{Ca}^{2+}$ was measured in cells preincubated in the absence or presence of t-BuOOH. BHQ- or thapsigargin-stimulated uptake was determined from the difference in flux measured in the absence and presence of the respective releasing agent. Incubation of cells with t-BuOOH decreased BHQ-stimulated Ca^{2+} uptake at all time points; at 2 min after addition of BHQ, for example, Ca^{2+} uptake was $150 \pm 5 \text{ pmol}/10^6$ cells in control cells, compared with $60 \pm 10 \text{ pmol}/10^6$ cells ($P < 0.01$) in oxidant-treated cells (Figure 8a). The effect of t-BuOOH on thapsigargin-stimulated Ca^{2+} uptake was more pronounced (Figure 8b), resulting in little response to application of the pump inhibitor at all time points.

DISCUSSION

Previous studies have demonstrated that oxidant stress inhibits agonist-stimulated release of Ca^{2+} from internal stores [10,11] of vascular endothelial cells. To investigate whether this effect is related to oxidant-induced depletion of internal Ca^{2+} stores, we determined the effect of t-BuOOH on the release of Ca^{2+} from internal Ca^{2+} pools induced by several pharmacological agents which act in the absence of activation of the inositol polyphosphate signalling system. In addition, we sought to characterize further the mechanism by which oxidant stress inhibits bradykinin-stimulated Ca^{2+} influx [10,11] in this cell type.

Effect of oxidant stress on internal Ca^{2+} stores

The technique by which agonist is added to cells suspended in Ca^{2+} -free/EGTA buffer affords temporal dissociation of release of Ca^{2+} from $\text{Ins}(1,4,5)\text{P}_3$ -sensitive internal Ca^{2+} pools from influx of Ca^{2+} [18]. Under this condition, the bradykinin-stimulated increase in $[\text{Ca}^{2+}]_i$ solely reflects release of Ca^{2+} and occurs in the absence of Ca^{2+} entry across the cell membrane. This protocol, in addition to $^{45}\text{Ca}^{2+}$ -radioisotope flux studies, has been previously utilized to show that t-BuOOH inhibits agonist-stimulated release of Ca^{2+} in a progressive time-dependent manner [10,11]. The underlying mechanism, however, by which oxidant stress inhibits receptor-activated release of Ca^{2+} from internal stores has not been fully elucidated. Inhibition of the inositol polyphosphate signalling pathway appears to be involved, because t-BuOOH decreases agonist-stimulated production of InsP_3 in endothelial cells in a time-dependent manner [12]. Depletion of internal Ca^{2+} stores by t-BuOOH may contribute to decreased receptor-activated release of Ca^{2+} , and to test this hypothesis, three separate strategies were employed. First, in fura-2-loaded cells suspended in Ca^{2+} -free/EGTA buffer, t-BuOOH progressively attenuated the bradykinin-stimulated increase in $[\text{Ca}^{2+}]_i$, yet the response of $[\text{Ca}^{2+}]_i$ to subsequent addition of ionomycin appeared to increase as the incubation time with t-BuOOH increased. This effect of t-BuOOH might be explained by inhibition of the mechanism responsible for agonist-stimulated release of Ca^{2+} from internal stores without alteration in basal Ca^{2+} release or activity of the internal store Ca^{2+} pump. Under this circumstance, incubation of cells with t-BuOOH may not significantly decrease the size of the ionomycin-releasable Ca^{2+} pool.

Second, to evaluate more directly the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool, a protocol was employed using the Ca^{2+} -ATPase inhibitors BHQ or thapsigargin. These agents irreversibly inhibit Ca^{2+} -pump units by binding to the pump protein in its E_2 state

[19], and shifting the E_2 - E_1 equilibrium toward the E_2 state, resulting in decreased pump affinity for Ca^{2+} [20]. Application of either of these agents induces net release of Ca^{2+} from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} compartment in the absence of activation of the inositol polyphosphate signalling pathway [21–24]. Furthermore, when added to cells suspended in Ca^{2+} -free/EGTA buffer, BHQ or thapsigargin increases $[\text{Ca}^{2+}]_i$ independently of Ca^{2+} influx from the extracellular space, enabling isolation of Ca^{2+} release from internal stores. Application of either agent to cells incubated with t-BuOOH initially produced an increase in $[\text{Ca}^{2+}]_i$ that was equal to that seen in control cells, providing further evidence that the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool is not depleted by oxidant stress, at least under the conditions employed. After more prolonged incubation of BAECs with t-BuOOH, the response to BHQ or thapsigargin was greater than that seen in controls. This observation may be related to the increased basal $[\text{Ca}^{2+}]_i$ observed in t-BuOOH-treated cells, since elevation of basal $[\text{Ca}^{2+}]_i$ would be expected to increase the driving force for Ca^{2+} -pumping units at the endoplasmic reticulum. Increased compartment size of the intracellular Ca^{2+} pool as a result of increased basal $[\text{Ca}^{2+}]_i$ would suggest that endoplasmic-reticular Ca^{2+} -ATPase activity increases in response to incubation of BAECs with the oxidant.

Third, the effect of t-BuOOH on BHQ-stimulated $^{45}\text{Ca}^{2+}$ efflux was determined in cells maintained in monolayer formation and equilibrated with radioisotope. Release of radioisotope from internal stores is followed by its extrusion to the extracellular compartment, where its appearance is measured [25]. In calf pulmonary-artery endothelial cells, t-BuOOH inhibits bradykinin-stimulated $^{45}\text{Ca}^{2+}$ efflux [11], an effect that appears to be linked to inhibition of the inositol polyphosphate signalling pathway [12]. However, the present results indicate that incubation with t-BuOOH for 1 h does not alter $^{45}\text{Ca}^{2+}$ efflux under basal or BHQ-stimulated conditions, providing further evidence that the oxidant does not alter the size of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool.

Effect of oxidant stress on Ca^{2+} influx

To investigate the mechanism by which t-BuOOH inhibits the agonist-stimulated Ca^{2+} influx pathway, we employed BHQ to stimulate Ca^{2+} influx in the absence of receptor activation [7,26]. By using the manoeuvre in which BHQ and Ca^{2+} are added in sequence to cells suspended in Ca^{2+} -free/EGTA buffer, incubation of cells with t-BuOOH for 0.5–1 h was clearly seen to inhibit BHQ-stimulated influx of Ca^{2+} . Because the effect of t-BuOOH on BHQ-stimulated Ca^{2+} influx might be explained by an interaction between the oxidant and BHQ, experiments were performed with the unrelated compound, thapsigargin. The effects of t-BuOOH on thapsigargin-stimulated Ca^{2+} influx are similar to those seen with BHQ. In addition, t-BuOOH inhibited both BHQ-stimulated and thapsigargin-stimulated $^{45}\text{Ca}^{2+}$ uptake to a similar degree. When Ca^{2+} was added to unstimulated cells (i.e. before any other addition), the increase in $[\text{Ca}^{2+}]_i$ was attenuated in cells treated with t-BuOOH for 0.5–1 h compared with control cells, a result that may reflect partial depletion of internal stores and activation of store-dependent Ca^{2+} influx upon suspension of BAECs in Ca^{2+} -free/EGTA buffer. Together, these findings suggest that t-BuOOH either has a direct effect on the store-dependent Ca^{2+} -influx pathway independent of the agent employed to deplete internal Ca^{2+} stores, or inhibits the mechanism which links the internal store to plasmalemmal Ca^{2+} influx.

Rather than via a direct effect of t-BuOOH, inhibition of store-dependent Ca^{2+} influx might conceivably be explained by

decreased BHQ- or thapsigargin-stimulated release of Ca^{2+} from internal stores. In this regard, t-BuOOH may directly interact with the Ca^{2+} -ATPase-binding site(s) for BHQ and/or thapsigargin, or allosterically alter the enzyme protein. This possibility appears unlikely, because (a) the increase in $[\text{Ca}^{2+}]_i$ produced by application of BHQ or thapsigargin to t-BuOOH-treated cells suspended in Ca^{2+} -free/EGTA buffer is not attenuated compared with that seen in control cells, and (b) t-BuOOH does not alter BHQ-stimulated $^{45}\text{Ca}^{2+}$ efflux.

There are several lines of evidence that, after more prolonged incubation of cells with t-BuOOH (2–3 h), the mechanisms responsible for cellular Ca^{2+} homeostasis and extrusion of Ca^{2+} from the cytosol are compromised. First, at these later times, basal $[\text{Ca}^{2+}]_i$ is elevated, even when determined in the absence of extracellular Ca^{2+} . The increase in $[\text{Ca}^{2+}]_i$ observed under these conditions may in part reflect influx of Ca^{2+} through a non-selective cation channel recently found to be activated by t-BuOOH in vascular endothelial cells [27] or may occur through the putative leak channel. Second, addition of Ca^{2+} to unstimulated or BHQ- or thapsigargin-stimulated cells suspended in Ca^{2+} -free/EGTA buffer results in sustained elevation of $[\text{Ca}^{2+}]_i$, which fails to return toward baseline, suggesting that cell membrane Ca^{2+} -ATPase is significantly inhibited after treatment of cells for these durations. A similar response is obtained if cells are stimulated with bradykinin rather than with BHQ or thapsigargin [10]. Third, inhibition of plasmalemmal Ca^{2+} -ATPase may contribute to decreased bradykinin-stimulated $^{45}\text{Ca}^{2+}$ efflux after incubation of cells with t-BuOOH for 2 h or longer [11].

Treatment of cells with t-BuOOH for more prolonged periods also increases Ca^{2+} influx under basal (unstimulated) conditions, suggesting that at these time points the oxidant increases Ca^{2+} permeability. This conclusion is supported by the large increase in $[\text{Ca}^{2+}]_i$ observed when extracellular Ca^{2+} is added to cells incubated with t-BuOOH for 2–3 h and suspended in Ca^{2+} -free/EGTA buffer. Further, in radioisotope studies, t-BuOOH has been shown to increase uptake of $^{45}\text{Ca}^{2+}$ under basal conditions [11].

Although intracellular Ca^{2+} homeostasis appears compromised after incubation of endothelial cells with t-BuOOH for 3 h, there is considerable evidence that the cells remain viable at this time point. First, the fura-2 technique determines $[\text{Ca}^{2+}]_i$ in only those cells that retain the fluorescent probe; that is, $[\text{Ca}^{2+}]_i$ is not determined in cells that have lost cell membrane integrity and sustained efflux of probe. Second, the large Ca^{2+} gradient across the cell membrane is maintained, and third, the size of the intracellular ATP pool is unaltered by incubation of cells with t-BuOOH for at least 3 h [28]. Further, in response to increased cellular Na^+ content at this time point, Na^+ - K^+ -ATPase activity increases appropriately [28]. Thus the changes in Ca^{2+} homeostasis that occur in vascular endothelial cells after incubation with t-BuOOH for 3 h appear to represent relatively early events in the cell injury process.

In summary, although t-BuOOH inhibits bradykinin-stimulated release of Ca^{2+} from internal stores in vascular endothelial cells, the oxidant fails to inhibit release of Ca^{2+} triggered by addition of ionomycin, BHQ or thapsigargin. These results suggest that decreased receptor-activated release of Ca^{2+} from internal storage pools is related to inhibition of one or more of the steps responsible for activation of Ca^{2+} release, rather than depletion of internal stores. In addition, t-BuOOH inhibits the store-dependent Ca^{2+} -influx pathway in the absence of altered basal $[\text{Ca}^{2+}]_i$ or altered response to BHQ or thapsigargin. This finding suggests that oxidant stress mediated by t-BuOOH either directly inhibits the store-dependent Ca^{2+} -influx pathway or

uncouples the link between the internal Ca^{2+} store and plasmalemmal influx.

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