Carmustine Augments the Effects of tert-Butyl Hydroperoxide on Calcium Signaling in Cultured Pulmonary Artery Endothelial Cells*

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The effects of oxidant stress and inhibition of glutathione reductase on the bradykinin-stimulated changes in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) of calf pulmonary artery endothelial cells were determined using the intracellular fluorescent probe, fura-2. Changes in [Ca\(^{2+}\)] \(_{i}\) upon stimulation with bradykinin were measured after incubation of cells with the chemical oxidant tert-butyl hydroperoxide (0.4 \(\mu\)M) for various times. After 60 min, bradykinin-stimulated Ca\(^{2+}\) influx was significantly decreased. With more prolonged incubations with the peroxide, bradykinin had little effect on cytosolic calcium concentration. Preincubation of cells with the glutathione reductase inhibitor, carmustine, led to elevated basal [Ca\(^{2+}\)], yet the cells remained responsive to bradykinin. However, incubation of carmustine-treated cells with tert-butyl hydroperoxide for 30 min dramatically reduced both bradykinin-stimulated release of Ca\(^{2+}\) from internal stores and influx of Ca\(^{2+}\) from the extracellular space. These results suggest that inhibition of glutathione reductase alters cytosolic Ca\(^{2+}\) homeostasis and enhances the effects of oxidative stress on signal transduction in vascular endothelial cells.

In response to a number of agonist agents, vascular endothelial cells secrete vasoactive substances such as prostacyclin and endothelial-derived relaxation and constriction factors (Vanhoutte et al., 1986). These paracrine substances alter vascular tone via their effects on the underlying smooth muscle cells of the blood vessel wall. Results of several studies suggest that alteration in endothelial cell function plays an important role in mediation of oxidant-induced lung injury. Oxidant stress has been shown to alter reactivity of the vasculature (Gartner et al., 1983; Burke and Wolin, 1987), and, recently, Collesky and Evans (1988) found that endothelium-dependent relaxations are substantially diminished in rat pulmonary arterial vessels following hyperoxic injury to the lung, suggesting that oxidant-induced changes in endothelial cell signal transduction could lead to a decreased release of endothelium-derived relaxation factors.

Cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]) is an important second messenger associated with the signal transduction process of vascular endothelial cells. In a previous study, we examined the effect of oxidant stress on agonist-induced changes in [Ca\(^{2+}\)] of fura-2-loaded bovine aortic endothelial cells (BAECs) (Elliott et al., 1989). After brief incubations (30 min) of the BAECs with the chemical oxidant tert-butyl hydroperoxide (t-bu-OOH), bradykinin-stimulated Ca\(^{2+}\) influx was substantially inhibited, while basal (resting) [Ca\(^{2+}\)], and bradykinin-stimulated release of Ca\(^{2+}\) from internal stores were unchanged from control. With longer incubations in t-bu-OOH (60–180 min), basal [Ca\(^{2+}\)], increased, and the cells became completely unresponsive to bradykinin. These results clearly indicate that signal transduction of systemic arterial endothelial cells can be altered by oxidant stress, and that one of the early events is a diminished cellular response to vasoactive agents.

The major antioxidant mechanism of vascular endothelial cells is provided by the tripeptide, glutathione (GSH) (Meister, 1983; Suttrop et al., 1986). Peroxides such as t-bu-OOH are metabolized via the action of glutathione peroxidase (glutathione: hydrogen-peroxide oxidoreductase, EC 1.11.1.9) which couples the oxidation of GSH to the reduction of t-bu-OOH (Bellomo et al., 1982).

**References**

1. The abbreviations used are: [Ca\(^{2+}\)], cytosolic free calcium concentration; BAECs, bovine aortic endothelial cells; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CPAs, calf pulmonary artery endothelial cells; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, HEPES-buffered saline; t-bu-OOH, tert-butyl hydroperoxide.
Oxidant Stress and Ca\textsuperscript{2+} Signaling in Endothelial Cells

Effect of t-bu-OOH on Ca\textsuperscript{2+} Signaling in BCNU-treated CPAs—To determine the effect of inhibition of glutathione reductase on agonist-stimulated changes in [Ca\textsuperscript{2+}]. CPAs were examined after treatment with the glutathione reductase inhibitor, BCNU. A representative control trace for these experiments is shown in Fig. 5A. Under control conditions, bradykinin increased [Ca\textsuperscript{2+}] from a basal value of 103 ± 5.7 nM to a peak value of 367 ± 22 nM (Fig. 6). CPAs preincubated with BCNU (75 μM) for 20 min were subsequently incubated for 30 min in HBS either with or without t-bu-OOH (0.4 mM). Basal [Ca\textsuperscript{2+}], in BCNU-treated CPAs without oxidant was increased to 129 ± 9.8 nM (p < 0.01 compared to control cells), with no effect on the responsiveness of the cells to bradykinin (Figs. 5B and 6). In comparison, basal [Ca\textsuperscript{2+}], of BCNU-treated cells incubated with t-bu-OOH for 30 min increased to 148 ± 7.0 nM (p < 0.0001 compared to control) and addition of bradykinin resulted in a peak response of 244 ± 6.8 nM (Figs. 5C and 6), a value significantly lower than that obtained in either control CPAs (p < 0.0001) or CPAs treated with BCNU alone (p < 0.0001). Note that these changes in Ca\textsuperscript{2+} signaling were observed after incubation with t-bu-OOH for only 30 min, a duration that produced little effect in cells not pretreated with BCNU (Figs. 1 and 2).

DISCUSSION

Regulation of vasoreactivity depends upon the functional integrity of the vascular endothelium. This aspect of endothelial cell function, which appears to be altered under conditions of oxidative stress, is governed, at least in part, by the concentration of cytosolic free Ca\textsuperscript{2+} within the endothelial cell. The aim of the present study was to examine the effect of oxidative stress on the changes in [Ca\textsuperscript{2+}], that occur in pulmonary arterial endothelial cells when stimulated by the...
To address these two possibilities, the response of CPAs to bradykinin stimulation was determined in Ca"-free/EGTA buffer. Under these conditions, neither basal \([\text{Ca}^2+]\), nor the flux and/or an inhibition of \([\text{Ca}^2+]\) release from internal stores, was affected during the first 60 min of incubation with t-bu-bradykinin-stimulated release of \([\text{Ca}^2+]\) from internal stores. Thus, a change in basal \([\text{Ca}^2+]\) was observed after 30 min of incubation with the oxidant. t-bu-OOH is an inhibition of bradykinin-stimulated \([\text{Ca}^2+]\) influx and efflux. Thus, it appears that during the early stages of oxidant stress, the mechanism responsible for the agonist-stimulated influx of \([\text{Ca}^2+]\) is inhibited, while many of the other cellular pathways associated with \([\text{Ca}^2+]\) signaling (e.g. release of \([\text{Ca}^2+]\) from internal stores) appear to remain unchanged.

With more prolonged incubation in t-bu-OOH (120-180 min), basal \([\text{Ca}^2+]\), significantly increases over control levels and the bradykinin-stimulated change in \([\text{Ca}^2+]\), progressively decreases when measured in both HBS and in Ca"-free/EGTA buffer. These results suggest that release of \([\text{Ca}^2+]\) from internal stores is diminished either by inhibition of the pathways leading to release of \([\text{Ca}^2+]\), by depletion of the internal stores of \([\text{Ca}^2+]\), or perhaps by the rise in \([\text{Ca}^2+]\), that occurs during this incubation time period. The present studies cannot distinguish between these possibilities. However, the elevated basal \([\text{Ca}^2+]\), at longer incubation times was observed even when measured in the absence of extracellular \([\text{Ca}^2+]\) suggesting that the mechanism responsible for \([\text{Ca}^2+]\) efflux from the cell (presumably plasmalemma \([\text{Ca}^2+]\)-ATPase) is inhibited by prolonged oxidant stress.

Effect of Oxidant Stress on BCNU-treated Cells—Glutathione reductase assumes a key role in the antioxidant defense mechanism of most cells, including pulmonary artery endothelial cells. As an integral part of the glutathione system, the enzyme catalyzes the reduction of oxidized glutathione disulfide at the expense of NAD(P)H. BCNU selectively inhibits the activity of glutathione reductase (Frischer and Ahmad, 1977; Rabson and Reed, 1978; Shinohara and Tanaka, 1979; Kehrer, 1983; Reed, 1985) and may enhance the effects of oxidative stresses under various experimental conditions (Miccadi et al., 1988; Paradisathathu et al., 1985; Riley, 1984; Smith, 1987; Tsokos-Kuhn, 1988). In the present study, we investigated whether inhibition of glutathione reductase alters the autacoid-stimulated changes in \([\text{Ca}^2+]\). The results of these experiments clearly indicate that treatment of CPAs with BCNU changes basal \([\text{Ca}^2+]\), to an elevated and stable level. This finding suggests that BCNU alters the cellular mechanism associated with the steady state between \([\text{Ca}^2+]\), influx and efflux. Since BCNU alone did not alter the bradykinin-stimulated release of \([\text{Ca}^2+]\) from internal stores, the signaling mechanisms responsible for the rise in \([\text{Ca}^2+]\), following activation of bradykinin receptors appear to be intact. The increase in basal \([\text{Ca}^2+]\), value was obtained even when measured in Ca"-free/EGTA buffer. Thus, the most likely explanation for the elevated resting \([\text{Ca}^2+]\), is a partial inhibition of \([\text{Ca}^2+]\) flux out of the cells.

Subsequent incubation of BCNU-treated cells with t-bu-OOH for 30 min caused an increase in basal \([\text{Ca}^2+]\), to a value significantly higher than that observed in cells treated with BCNU alone. A similar result was obtained when \([\text{Ca}^2+]\), measurements were performed with the CPAs incubated in Ca"-free/EGTA buffer. Likewise, incubation of BCNU-treated cells with t-bu-OOH for 30 min produced a dramatic reduction in both bradykinin-stimulated \([\text{Ca}^2+]\) release from internal stores and influx of \([\text{Ca}^2+]\) from the extracellular space. The profile obtained after incubation of BCNU-treated cells with t-bu-OOH for 30 min was essentially identical with the profile observed in untreated cells incubated with t-bu-OOH for 120 min. Thus, oxidant stress appears to have an enhanced effect on \([\text{Ca}^2+]\) signaling in pulmonary artery endothelial cells when glutathione reductase is inhibited by BCNU.

In conclusion, the effect of oxidant stress produced by t-bu-OOH on \([\text{Ca}^2+]\) signaling of cultured pulmonary artery endothelial cells is shown to be time-dependent. After short incubations with the oxidant, the changes in \([\text{Ca}^2+]\), produced by bradykinin, an agent known to cause vasorelaxation and increased vascular permeability, are substantially altered. These changes in \([\text{Ca}^2+]\) signaling occur without a change in...
basal $[Ca^{2+}]$. Following more prolonged incubation with the peroxide, $[Ca^{2+}]$ increases and the cells become completely unresponsive to bradykinin. Pretreatment of the cells with BCNU dramatically reduces the time necessary for the effects of t-bu-OOH to become evident, suggesting that glutathione reductase plays an important role in the physiologic effect of oxidative stress on $Ca^{2+}$ signaling in pulmonary artery endothelial cells. In addition, these findings suggest that, within the lung, one of the early effects of oxidative injury on vascular permeability and tone may be mediated by an inhibition of agonist-induced changes in $[Ca^{2+}]$.

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REFERENCES


SUPPORTING MATERIAL

Supplementary Information

Materials and Methods

Materials

Fura-2/AM was obtained from Molecular Probes (Eugene, OR) and bradykinin was obtained from Calbiochem Corp. (San Diego, CA). Triton X-100, test-N,N'-bis(carboxymethyl) ethylene diamine tetraacetic acid (EDTA) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3-bis-[2-(methoxy carbonyl)ethyl]-1-(2-methyl-4-nitro-6-sulfophenyl) tetrazolium chloride (MTS) was obtained from Boehringer-Mannheim (Indianapolis, IN). Di-4-aleph-keto-lysinoprotein was obtained from Biomedical Technologies Inc. (Stratton, MA). All other chemicals were of the highest quality available.

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Endothelial Cell Culture

Endothelial cells from umbilical cords and umbilical arteries were obtained from Human Growth Strategies (Orange, CA). Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 15% fetal bovine serum (HyClone, Logan, UT), 100 μg/ml streptomycin, and 100 μg/ml amphotericin. Cultures showed contact-inhibition and typical cobblestone appearance under phase contrast microscopy. Confirmation of endothelial cell identity was obtained by incorporation of the dye Hoechst 3342 (Hoechst, Toronto, ON) and by positive staining with anti-endothelial cell antibodies.

Measurement of Intracellular Free $Ca^{2+}$ Concentration

$[Ca^{2+}]$ was measured using the fluorescent $Ca^{2+}$ indicator, fura-2 as previously described (Elliott et al., 1988). Briefly, cells were incubated in the presence of fura-2/AM for 30 min at 37°C. Following incubation, cells were washed and resuspended in fresh medium containing 2% fetal bovine serum. The fluorescence of the cells was determined using a Perkin Elmer spectrophotofluorimeter (SPEX Instruments, Urbana, IL). Emission wavelengths were alternated every 0.5 sec between 340 and 365 nm. The excitation wavelength was 380 nm. Measurements were corrected for autocorrelation using windows cells. Calibration of the fura-2 concentration was performed using a standard curve obtained from a $Ca^{2+}$ standard solution.

Effect of Bradykinin on $Ca^{2+}$ Signaling in CAFs

The response of CAFs to stimulation with bradykinin was assessed by the addition of 20 nM of bradykinin to the perfusate. The response was determined by the increase in $Ca^{2+}$ concentration, as measured by the fluorescent $Ca^{2+}$ indicator, fura-2.

RESULTS

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After more prolonged incubation, basal [Ca^{2+}] was steadily increased to 144 ± 7.0 vs. 144 ± 7.1 in cells treated with t-bu-OH for 120 and 180 min, respectively. Additionally, the fold-increase in the [Ca^{2+}] (peak [Ca^{2+}] (maximal) / basal [Ca^{2+}]) produced by bradykinin progressively decreased with incubation time. Thus, in comparison to a 1.5-fold change observed in control cells, bradykinin stimulated a 1.0-fold increase in [Ca^{2+}] in cells treated with t-bu-OH for 120 min, and only a 1.3-fold increase in cells treated with t-bu-OH for 180 min. These results clearly indicate that the cells become progressively unresponsive to bradykinin stimulation when subjected to oxidant stress.

**Effect of t-bu-OH on Ca^{2+} in Ca^{2+}-free/EgTA buffer.**

Experiments were performed using Ca^{2+}-free/EgTA buffer in order to separate the two components of the biphasic response. Under these conditions, the initial increase in [Ca^{2+}] after stimulation with bradykinin reflects the release of Ca^{2+} from internal stores. The subsequent diminution of [Ca^{2+}] to the base level is indicative of Ca^{2+} influx across the plasma membrane. In control cells, the increase in [Ca^{2+}] measured in Ca^{2+}-free/EgTA buffer was 23 ± 2.4 nM. Subsequent addition of Ca^{2+} to the extracellular buffer 2 min after stimulation with bradykinin resulted in an increase in [Ca^{2+}] from 23 ± 2.4 nM to 85.3 ± 3.6 nM, respectively. Thus, when released in Ca^{2+}-free/EgTA buffer, Ca^{2+} cannot change the responsiveness of the cells to bradykinin.

To investigate the effect of t-bu-OH on each component of the bradykinin response, cells were incubated with the oxidant for various lengths of time before stimulation in Ca^{2+}-free/EgTA buffer and measurement of [Ca^{2+}]. (Fig. 3B-D). Again there was little effect of t-bu-OH on the Ca^{2+} signaling following 10 min of incubation with the oxidant (t-test not shown). As observed with Ca^{2+} evaluated in Ca^{2+}-containing MBS, an increase in basal [Ca^{2+}] was only observed in cells incubated with the oxidant for prolonged periods of time. Compared to control levels of basal [Ca^{2+}], treated in the absence of exogenous [Ca^{2+}], the basal [Ca^{2+}] of cells incubated with the oxidant for 120 and 180 min increased to 48.6 ± 1.9 and 85.3 ± 3.6 nM, respectively. Thus, even when released in Ca^{2+}-free/EgTA buffer, cells incubated with t-bu-OH for prolonged periods showed elevated basal [Ca^{2+}].

The bradykinin-stimulated release of Ca^{2+} from internal stores, determined by the fold-increase in [Ca^{2+}] of cells in Ca^{2+}-free/EgTA buffer, was unchanged following 10 min of incubation in t-bu-OH (data not shown). Likewise, the release of Ca^{2+} from internal stores was unchanged after 60 min of incubation with the oxidant (Fig. 4). However, with more prolonged incubations, Ca^{2+} release from internal stores progressively declined (Fig. 4). Compared to the fold-increase of 2.28 ± 0.21 observed in control cells, bradykinin stimulated a fold-increase of only 1.4 ± 0.3 and 1.0 ± 0.03 in cells incubated with t-bu-OH for 120 and 180 min, respectively.

The effects of t-bu-OH on bradykinin-stimulated Ca^{2+} influx was determined by the fold-increase in [Ca^{2+}] following the readmission of Ca^{2+} to the Ca^{2+}-free/EgTA buffer. The percent response to Ca^{2+} addition progressively decreased from 1.0 ± 0.3 fold to 88.6 ± 2.1 fold to 23.1 ± 6.5 fold at 120 and 180 min, respectively (t-test). Moreover, in Ca^{2+} buffer, the oxidant for 180 min, addition of Ca^{2+} after bradykinin produced a marked inhibitory effect (Fig. 5), that continued to increase with time (Fig. 6). These results clearly indicate that during the early stages of oxidant stress, Ca^{2+} signaling is inhibited while prolonged stress results in elevated basal [Ca^{2+}] and a pronounced alteration in Ca^{2+} homoeostasis.

**Effect of t-bu-OH and BrCN on Plasma Membrane Permeability to Trypan Blue.**

To determine the effect of t-bu-OH and BrCN on the permeability of the cell membrane, the cells were loaded with 0.5% trypan blue under the various experimental conditions. The uptake of trypan blue in Ca^{2+} buffer incubated without or with t-bu-OH was not micromolar free ions were observed, however. 14.5 ± 2.7% of cells incubated with the oxidant for 180 min demonstrated uptake of trypan blue compared to 7.1 ± 1.5% of control cells incubated for the same time period (p < 0.001). These results indicate that cell membrane permeability increases only after incubation with 1.0 mM t-bu-OH for periods greater than 120 min.

Plasmamembrane permeability was also determined after cells had been treated with BrCN or BrCN followed by incubation with t-bu-OH as described for the measurement of cytosolic Ca^{2+} (see above). The percentage of cells which stained positively for intracellular trypan blue was 0.7 ± 0.5%, 4.4 ± 0.29%, and 9.5 ± 0.24% for control, BrCN, and BrCN followed by t-bu-OH, respectively (p < 0.001). BrCN treatment produced a slight increase in plasma membrane permeability which is enhanced by subsequent incubation in t-bu-OH.
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