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Adaptation to Oxidative Stress: Quinone-Mediated Protection of Signaling in Rat Lung Epithelial L2 Cells

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ABSTRACT. Cells can respond to a sublethal oxidative stress by up-regulating their intracellular glutathione (GSH) pool. Such increased GSH concentration is likely to be protective against further oxidative challenge, and, in fact, pre-exposure to low levels of oxidants confers increased cellular resistance to subsequent greater oxidative stress. Previously, we have shown that pretreatment of rat lung epithelial L2 cells with sublethal concentrations of *tert*-butylhydroquinone (TBHQ) increases intracellular GSH concentration in a concentration- and time-dependent manner. This increase resulted from up-regulation of both γ-glutamyltranspeptidase (GGT) and γ-glutamylcysteine synthetase (GCS). Therefore, we investigated whether such increased GSH concentration protected these cells against a subtle loss in function caused by a subsequent challenge with sublethal concentrations of *tert*-butyl hydroperoxide (tBOOH) (≤200 μM), mimicking a physiological oxidative stress. Activation of L2 cell purinoreceptors with 100 μM ADP caused an elevation of intracellular Ca^{2+}. This response was suppressed by a brief pre-exposure to tBOOH. The inhibition, however, was alleviated dramatically by a 16-hr pretreatment with 50 μM TBHQ. The same TBHQ pretreatment also protected the cells from ATP-depletion induced by tBOOH. L-Buthionine *S,R*-sulfoximine (BSO), an irreversible inhibitor of GCS, prevented the increase in intracellular GSH and also completely removed the protection by TBHQ in maintaining the ATP level. Thus, pre-exposure to a sublethal level of TBHQ results in protection of cell functions from hydroperoxide toxicity. This protection appears to depend on alteration of the intracellular GSH pool, the modulation of which constitutes an adaptive response to oxidative stress. BIOCHEM PHARMACOL 53;7:987–993, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. glutathione; quinone; hydroperoxide; calcium; ATP; adaptation

GSH† is the most abundant intracellular nonprotein thiol, found ubiquitously in cells at levels as high as 1–10 mM. GSH has been strongly implicated as playing a pivotal role in cellular defense against oxidative stress and xenobiotics. Accordingly, the relative level of GSH has been shown to dictate the susceptibility of a given cell to oxidative injury [1]. Therefore, maintaining the GSH pool may be important in cellular resistance to the toxicity of various oxidants, whose effects can range from rapid cell mortality to subtle disruption of signal transduction cascades.

Attempts to increase intracellular GSH concentration have led to a paradoxical finding, which is that cells respond to low levels of oxidants by elevation in GSH. Notably, various redox cycling quinones are capable of inducing GSH up-regulation: BHA, menadione (2-methyl-1,4-naphthoquinone), DMNQ, and, recently, TBHQ have all been demonstrated to increase intracellular GSH concentration [2–6].

TBHQ is a monofunctional Phase II enzyme inducer that is one of the major metabolites of BHA, a synthetic antioxidant [7–9]. Although used as a food additive antioxidant, TBHQ can be oxidized to semiquinone and then to TBQ either enzymatically or by auto-oxidation, producing reactive oxygen species via redox cycling [10–12]. We have shown that the effect of TBHQ on GSH content is accompanied by increased activities and mRNA levels of both GGT (EC 2.3.2.2; γ-glutamyltransferase) and GCS (EC 6.3.2.2; glutamate–cysteine ligase) [13]. GGT is a membrane-bound enzyme that breaks down extracellular GSH to provide substrates for intracellular *de novo* synthesis of GSH. GCS catalyzes the first and rate-limiting reaction in this *de novo* synthesis pathway. GCS was found to be transcriptionally induced by TBHQ treatment, while increased

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† *Abbreviations:* BHA, butylated hydroxyanisole; BSO, L-buthionine *S,R*-sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; GCS, γ-glutamylcysteine synthetase; GGT, γ-glutamyltranspeptidase; GSH, glutathione; 4-HC, 4-hydroperoxycyclophosphamide; IP_3, inositol(1,4,5)triphosphate; $[Ca^{2+}]_i$, intracellular calcium; KRPH, Krebs–Ringer phosphate buffer; OPT, *ortho*-phthaldialdehyde; tBOOH, *tert*-butyl hydroperoxide; TBHQ, *tert*-butylhydroquinone; and TBQ, 2-*tert*-butyl(1,4)paraquinone.

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GGT message appeared to result from message stabilization. Both enzymes were then increased due to increased translation.

The resulting increase in GSH concentration is likely to be protective against further oxidative insults. Moreover, such an active regulation occurring at the gene level is reminiscent of some inherent adaptation mechanism that the cells use to mount their own defense against environmental stress. Low H_2O_2 "priming" doses have been shown to increase cellular resistance to H_2O_2 [14]. Preconditioning by brief anoxia and reoxygenation cycles protects myocytes from the subsequent sustained ischemic injury [15]. Similarly, pre-exposure to a sublethal level of TBHQ has been demonstrated to render rat lung epithelial L2 cells resistant to a previously toxic level of the quinone itself [6]. These studies have focused on cell injury. In the present study, the focus was on whether such increase in GSH could protect cells against the more subtle effects of oxidative stress in altering signal transduction.

Recently, intracellular thiol levels have been shown to modulate oxidant-induced changes in Ca^{2+} signaling; an exogenous supply of dithiothreitol, cysteine ethyl ester and cysteine methyl ester reversed a hypochlorous acid-mediated rise in intracellular Ca^{2+} in rat ventricular myocytes [16]. Also, elevation of cellular thiol content with GSH monoethyl ester protected cardiac myocytes from 4-HC-induced changes in intracellular Ca^{2+} concentration and ATP, while GSH depletion with BSO made the cells more susceptible to the toxicity of 4-HC [17]. Furthermore, GSH depletion with either BSO or CDNB potentiated the inhibition of agonist-stimulated Ca^{2+} entry and Ca^{2+} release from intracellular stores by tBOOH [18].

Therefore, we hypothesized that yet another means of elevating the GSH level, which is by pretreatment with a sublethal concentration of TBHQ, would also protect Ca^{2+} signaling in L2 cells from subsequent challenge with physiologically relevant levels of hydroperoxide. If such protection occurred, then this model would present us with a novel pharmacological method to protect cells against oxidative stress. Using both intracellular Ca^{2+} signaling and ATP concentration as sensitive and subtle measures of cellular toxicity, we demonstrated that TBHQ pretreatment indeed protects L2 cells against subsequent oxidative injury.

MATERIALS AND METHODS

Chemicals

TBHQ was purchased from the Aldrich Chemical Co. (Milwaukee, WI). OPT, the somatic cell ATP assay kit, and probenecid were obtained from the Sigma Chemical Co. (St. Louis, MO). BSO was from Chemalog (South Plainfield, NJ) and Indo-1AM, from Molecular Probes, Inc. (Eugene, OR).

Cell Culture and Pretreatment

The rat lung epithelial L2 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in Ham's F12 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in the presence of 5% CO_2 at 37°. Culture medium was changed every 2–3 days. TBHQ and BSO were dissolved in DMSO and culture medium, respectively. All experimental and control incubations contained a 0.1% final concentration of DMSO. L2 cells, after grown to confluence, were treated with different agents for the times indicated in Results.

Intracellular Ca^{2+} Measurements

L2 cells were grown as a monolayer on 1 × 2 cm glass coverslips (Hitachi, San Jose, CA). Cells were seeded directly onto the coverslips at 1–2 × 10^4 cells/coverslip. After the cells adhered to the glass surface, culture medium was added and the cells were grown to confluence. Then cells were pretreated for 16 hr with TBHQ in DMSO or DMSO alone, as described above. On the day of the experiment, the medium was changed to KRPH (pH 7.4; containing 1.0 mM $MgSO_4$, 1.3 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, 125 mM NaCl, 5 mM KCl, and 10 mM sodium phosphate), and the cells were equilibrated for 2–3 hr in a 37° CO_2-free incubator. Then the cells were washed gently on a shaker at room temperature for half an hour and subsequently loaded with 10 μM indo-1AM (100 μL/coverslip) for 30 min at 37° while protected from light. Excess indo was quickly washed off. Then the attached cells were bathed in KRPH containing 11 μM probenecid at 37° until used.

Ca^{2+} measurements were made, using a F-2000 Fluorescence Spectrophotometer (Hitachi), thermostatted at 37°. Cells on coverslips were individually inserted into a coverslip holder and submerged into probenecid-free KRPH. Cells were thus incubated inside the fluorimeter for 3 min and then with various tBOOH concentrations for an additional 10 min. Next, intracellular Ca^{2+} was monitored continuously for 20 min with a 0.1-sec response time. Cells were illuminated through a 10-nm slit with excitation at 380 nm and emission at 400 and 450 nm. The 33% light cut-off filter and an interference filter were in place at all times. $[Ca^{2+}]_i = K_d (R - R_{min})/(R_{max} - R) \cdot S_{f2}/S_{b2}$, where R stands for the ratio of fluorescence at 400 nm to fluorescence at 450 nm [19]. S_{f2}/S_{b2} represents the ratio of fluorescence of the Ca^{2+}-free form of indo to the Ca^{2+}-bound form at the wavelength used to monitor the Ca^{2+}-free indo. K_d was experimentally determined for our system, using a calcium calibration buffer kit supplied by Molecular Probes, Inc. For each run, 100 μM ADP was added at 60 sec. R_{max} was determined by adding 80 μM digitonin, and R_{min}, with 4 mM EGTA.

The baseline, which represented unstimulated cells scanned over time, was plotted along with the rest of the calcium measurements for direct comparison. Apparently, the upslope in calcium curves resulted largely from indo leakage since the addition of probenecid, an inhibitor of

dye exclusion, could reduce much of this upslope. However, the fluorescence of probenecid interfered significantly with that of indo, precluding its use in our system.

Intracellular GSH Measurements

The cells were washed twice with ice-cold phosphate-buffered saline. Protein was precipitated with 5% trichloroacetic acid. Upon centrifugation, GSH content in the supernatant was determined by OPT fluorescence [20]. This assay was suitable for routine GSH measurements and was determined to be comparable to the HPLC method in its specificity and sensitivity for GSH. Protein assays were performed using the bicinchoninic acid protein assay system, supplied by Pierce (Rockford, IL) [21].

ATP Assay

ATP concentration was determined with the Somatic Cells ATP Assay Kit. L2 cells were grown in 6-well plates (Costar, Cambridge, MA) and were gently washed twice with KRPH. Then the cells were incubated for various time intervals with 0–200 μM tBOOH in KRPH at 37°. At the end of the incubation, ATP was released with 0.5 mL of somatic cell ATP releasing solution. ATP concentration was measured by the firefly luciferin-luciferase reaction, with a Perkin–Elmer LS-5 Fluorescence Spectrophotometer in the phosphorescence mode. The ATP concentration thus obtained was normalized by protein measurements and then reported as the percentage of control ATP level measured at the respective time points.

Statistics

Data are expressed as mean ± SEM and were evaluated by one-way ANOVA. Statistical significance was determined by the post-hoc Tukey A test in which $P < 0.05$ was considered significant.

RESULTS

TBHQ-Mediated Protection of L2 Cells from the Inhibition of ADP-Mediated Ca^{2+} Signaling by tBOOH

We used a protocol to increase GSH with TBHQ, which we had established previously [6]. Incubation with TBHQ (0–100 μM) resulted in significant elevation in the intracellular GSH level in rat lung epithelial L2 cells in a concentration- and time-dependent manner. Maximal elevation in GSH was achieved after 16 hr of treatment and 50 μM TBHQ, when there was an ~2.5-fold increase in GSH concentration compared with the basal level. Even at 48 hr post-treatment, the GSH level was still about two times above the basal concentration; however, all subsequent experiments were conducted after 16 hr of incubation with or without 50 μM TBHQ.

Control L2 cells cultured as a monolayer responded to a bolus of ADP (100 μM) with an increase in intracellular Ca^{2+} concentration, from ~100 nM at resting level to ~200 nM (Fig. 1A). The amplitude of calcium response tended to vary slightly from day to day. The response started briefly after ADP addition and was sustained for at least 15 min.

Such response to ADP was suppressed concentration-dependently by a 10-min prior incubation with tBOOH (0–200 μM). Incubation with 25 μM tBOOH decreased both the amplitude and the duration of Ca^{2+} elevation, while 50–200 μM tBOOH almost completely inhibited the Ca^{2+} response and also resulted in Ca^{2+} falling below the basal level. tBOOH treatment alone did not produce such a drop in Ca^{2+} concentration below the basal level; the Ca^{2+} level dropped only after ADP stimulation (data not shown). Furthermore, although L2 cells tended to lose their intracellular Ca^{2+} rapidly in the Ca^{2+}-free medium, the cells placed in the absence of extracellular Ca^{2+} responded to ADP stimulation with a further drop in their Ca^{2+} concentration, once again to a level below the baseline (data not shown). The concentrations of tBOOH employed did not alter significantly either the basal Ca^{2+} level prior to ADP stimulation or the cell viability, as assessed by trypan blue exclusion and adherence to the coverslips.

When the cells were pretreated for 16 hr with 50 μM TBHQ, the inhibitory effects of tBOOH were alleviated

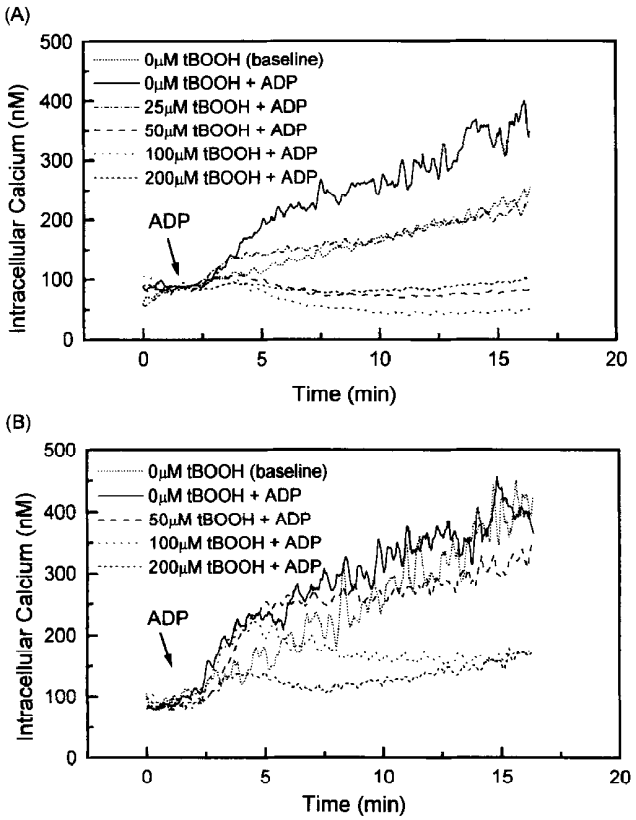

FIG. 1. Effect of TBHQ pretreatment on the inhibition of ADP-mediated Ca^{2+} signaling by tBOOH in L2 cells. L2 cells were pretreated with (A) 0.1% DMSO or (B) 50 μM TBHQ for 16 hr. After loading with indo-1AM, the cells were incubated with various concentrations of tBOOH for 10 min prior to Ca^{2+} measurements.

dramatically (Fig. 1B). A significant decrease in the ADP response did not occur until treatment with 100 μM tBOOH. Repeated experiments confirmed that neither the basal Ca^{2+} concentration nor the magnitude of the response to ADP was altered significantly by TBHQ pretreatment alone. Therefore, TBHQ pretreatment protected purinoceptor-mediated Ca^{2+} signaling in L2 cells against hydroperoxide-mediated injury.

TBHQ-Mediated Protection of L2 Cells from Decrease in ATP Concentration Caused by tBOOH Treatment

Control L2 cells exhibited decreased ATP concentration when challenged with tBOOH (0–200 μM) (Fig. 2A). The ATP level did not change significantly during a 1-hr incubation with tBOOH (0–200 μM), but it decreased to 70% of the control value after a 3-hr treatment with 100 μM tBOOH; this decrease was maintained at 4 hr. With 200 μM tBOOH, the decline was much greater, resulting in a 90% decrease by 2 hr post-treatment. Control ATP content tended to vary considerably from day to day.

When the cells were pretreated with TBHQ, the same concentrations of tBOOH failed to deplete cellular ATP content during the same time intervals (Fig. 2B). The basal ATP concentration was not changed significantly by TBHQ pretreatment alone.

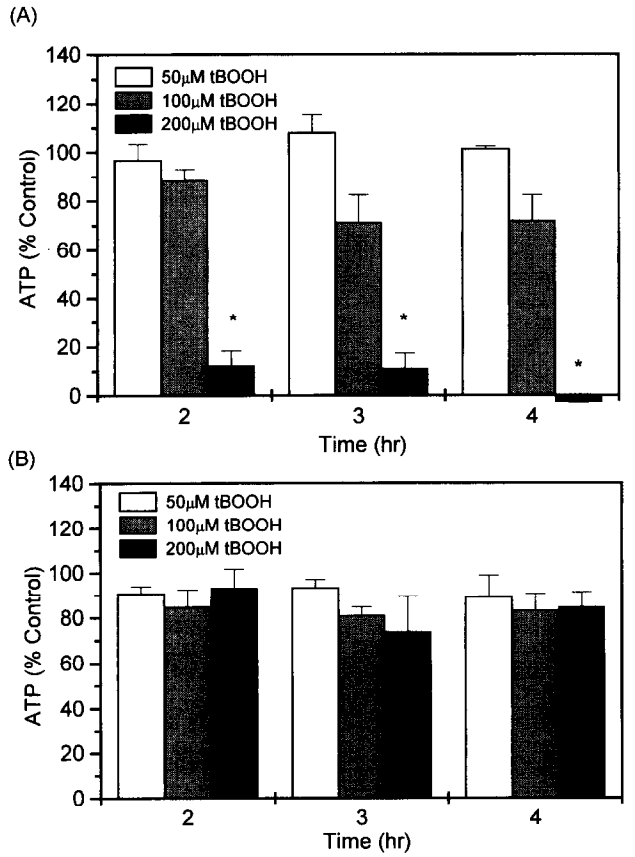

FIG. 2. Effect of TBHQ pretreatment on the decrease in ATP content by tBOOH in L2 cells. L2 cells were pretreated with (A) 0.1% DMSO or (B) 50 μM TBHQ for 16 hr before incubation with different concentrations of tBOOH for various time periods as indicated. The control ATP levels in (A) were 15.17 ± 1.06, 13.22 ± 0.58, and 12.59 ± 0.22 nmol ATP/mg protein at 2, 3, and 4 hr, respectively. ATP levels of the control cells in (B) were 15.92 ± 1.20, 13.83 ± 0.73, and 13.84 ± 1.16 nmol ATP/mg protein at 2, 3, and 4 hr, respectively. Values are means ± SEM, N = 3. Key (*) statistically significant difference ($P < 0.05$) from the control.

Role of Intracellular GSH in TBHQ-Mediated Protection of L2 Cells from ATP Depletion Caused by tBOOH Treatment

We have shown previously that the increase in GSH level by TBHQ treatment is at least partially due to an up-regulation of the *de novo* synthesis enzyme, GCS [13]. Therefore, we further tested whether inhibition of GCS function by BSO abrogated the protection afforded by TBHQ against ATP loss. BSO treatment resulted in decreased GSH concentration, as expected (Fig. 3A). This was accompanied by a complete loss of protection by TBHQ against ATP depletion (Fig. 3B).

DISCUSSION

This study used ADP-stimulated Ca^{2+} signaling as a cellular process that served as a sensitive measure of tBOOH toxicity. ADP, like ATP, is a potent purinergic agonist that triggers elevation in intracellular Ca^{2+} in various tissues [22–25]. ADP is suggested to stimulate P_{2y} receptors [25, 26]. In type II cells from which L2 cells are derived, purinergic stimulation leads to surfactant secretion [26–30]. P_{2y} receptors are coupled to G-proteins that activate phospholipase C, whose products, diacylglycerol and IP_3, lead to the activation of protein kinase C and Ca^{2+} release from IP_3-sensitive stores [25, 31, 32].

Previously, we have shown that purinoceptor-mediated Ca^{2+} signaling is inhibited by both tBOOH and H_2O_2 in rat alveolar macrophages [33]. In this study, we demonstrated for the first time that L2 cells retained the purinergic Ca^{2+} response of type II cells and that this process was inhibited concentration dependently by tBOOH in these cells as well. tBOOH has been shown to inhibit IP_3 production, which may explain the observed decrease in the Ca^{2+} response [34–36]. The inhibitory effect of tBOOH could not be attributed to the loss of cell viability since we did not detect any change in trypan blue exclusion or adherence to the coverslips during the calcium measurements and because it took at least 2 hr for even the highest concentration of tBOOH employed (200 μM) to result in ATP depletion. Interestingly, pre-exposure to 50–200 μM tBOOH resulted in a depressed Ca^{2+} response which was followed by Ca^{2+} falling below the baseline (Fig. 1). A recent report by Gailly *et al.* [37] showed that in the absence of extracellular Ca^{2+}, IP_3 stimulation led to a transient elevation in $[Ca^{2+}]_i$, which eventually dropped to a

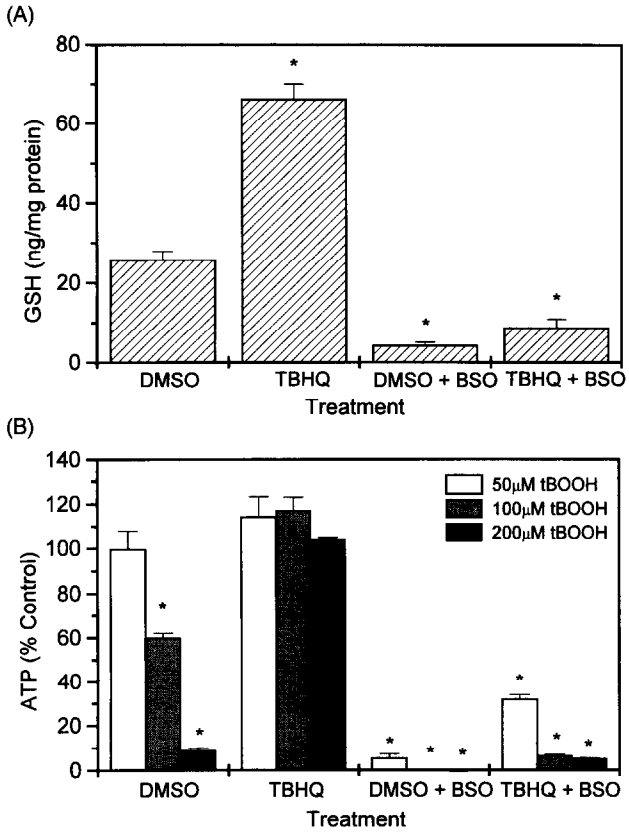

FIG. 3. Effect of BSO on the GSH and ATP content in L2 cells. (A) L2 cells were pretreated with 0.1% DMSO or 50 μM TBHQ, with and without 20 μM BSO as indicated for 16 hr. (B) L2 cells were pretreated with 0.1% DMSO or 50 μM TBHQ, with and without 20 μM BSO, as indicated for 16 hr before 2-hr incubations with various concentrations of tBOOH. The control ATP levels in (B) were 13.97 ± 1.27, 13.90 ± 0.20, 13.56 ± 0.31, and 15.32 ± 0.13 nmol ATP/mg protein for DMSO, TBHQ, DMSO + BSO, and TBHQ + BSO treatments, respectively. Values are means ± SEM, N = 4 in (A) and N = 3 in (B). Key (*) statistically significant difference ($P < 0.05$) from the control.

level below the baseline. The authors had attributed such an observation to the inability of the cells to replete intracellular Ca^{2+} by capacitative Ca^{2+} entry. Similarly, when we stimulated L2 cells with ADP in the absence of extracellular Ca^{2+}, Ca^{2+} once again fell below the basal level. Therefore, tBOOH pre-exposure was similar in effect to having zero extracellular Ca^{2+}. Although this may be a mere association, it is possible that tBOOH blocks Ca^{2+} entry from outside. We have reported previously a similar inhibition of stimulated Ca^{2+} entry in alveolar macrophages exposed to hyperoxia [38] and, in fact, tBOOH has been shown to inhibit capacitative Ca^{2+} entry in vascular endothelial cells [39]. tBOOH could not have inhibited Ca^{2+}-ATPase here since that would have led to an increased, rather than a decreased, cytosolic Ca^{2+} level. It may be speculated, however, that dysregulation of Ca^{2+} regulation mechanisms at multiple levels somehow led to a greater Ca^{2+} efflux or sequestration by the organelles, resulting in a lowered intracellular Ca^{2+} concentration. Regardless of what the true targets were, they were clearly protected by TBHQ pretreatment.

We have shown here that a model hydroperoxide induces a sustained decrease in ATP concentration. An oxidant-mediated decrease in ATP concentration independent of cell disruption has been documented extensively in the literature [40, 41]. Possible mechanisms include increased intracellular catabolism such as cellular ATPases, release to extracellular medium and subsequent hydrolysis or decreased synthesis [40, 42, 43–47]. Both tBOOH and H_2O_2 have been suggested to disrupt both mitochondrial and glycolytic ATP production [46, 48–52]. However, when we selectively inhibited glycolysis by incubating L2 cells with 2-deoxyglucose, the effect of tBOOH and TBHQ on the ATP level did not change (data not shown). Similarly, when mitochondrial ATP synthesis was inhibited with oligomycin, the cells fully compensated for the loss in oxidative phosphorylation by up-regulating glycolysis, but the effect of tBOOH and TBHQ on the ATP level persisted (data not shown). This suggested that the observed changes in ATP level did not depend upon modulation of ATP synthesis. TBHQ also failed to protect L2 cells from mitochondrial membrane depolarization induced by tBOOH (data not shown). Thus, it may well be that catabolism or degradation of ATP was increased by tBOOH, such as by cellular ATPases, to result in a decreased ATP level. This hypothesis awaits further investigation.

Exogenous thiols have been shown to counteract oxidant-induced effects on ATP content and intracellular Ca^{2+} [16, 17, 53]. However, in this study, we present a unique means of protecting cells against oxidative stress which is via TBHQ, a redox-cycling quinone, that elevates the intracellular GSH level. As sublethal oxidative stress exerted by the quinone seems to serve as the signal to up-regulate GSH in L2 cells [13], TBHQ may trigger adaptive responses in cells such that the same cells would be protected against subsequent oxidative stress. Since BSO-coadministration completely removes TBHQ-induced protection against ATP loss, the protection appears to be dependent on intracellular GSH. Other redox-cycling quinones such as DMNQ and menadione induce GSH to a similar extent and, thus, are also expected to display a similar protective effect [2, 3]. Therefore, prior exposure to quinones may protect cells against subsequent injury from exposure to physiologically relevant level of oxidants, most likely by increasing the GSH pool.

In summary, we have demonstrated the functional significance of a previously reported increase in intracellular GSH by TBHQ pretreatment in L2 cells. TBHQ pretreatment protected purinoceptor-mediated Ca^{2+} signaling against inhibition by a subsequent greater oxidative stress. Also, the same pretreatment protected the cells against the drop in ATP level that occurs after prolonged exposure to tBOOH; the protection depended upon intracellular GSH. Therefore, quinones may prove their therapeutic potential against oxidant damage via selective up-regulation of cellular antioxidant capacity at a molecular level.

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