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Effect of Intracellular Glutathione on the Production of Prostaglandin D_2 in RBL-2H3 Cells Oxidized by *tert*-Butyl Hydroperoxide

Hikaru Sakamoto, Jun Kitahara, and Yasuhito Nakagawa[1]

School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641

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The liberation of arachidonic acid and the production of prostaglandin D_2 (PGD_2) were significantly influenced by peroxide and the level of intracellular glutathione (GSH). The productions of free arachidonic acid and PGD_2 in RBL-2H3 cells were enhanced considerably by exposure to *tert*-butyl hydroperoxide (t-BHP). The liberation of arachidonic acid induced by t-BHP was not inhibited by EGTA. The productions of PGD_2 and arachidonic acid induced by t-BHP were significantly facilitated by the depletion of intracellular GSH using buthionine sulfoximine (BSO) or diethyl maleate (DEM), although the depletion of GSH had no effect on the production of PGD_2 induced by A23187. t-BHP failed to activate the conversion of free arachidonic acid to PGD_2, since the formation of PGD_2 from exogenously added arachidonic acid was not enhanced by treatment with t-BHP. The level of lipid hydroperoxides in t-BHP-treated cells was significantly elevated by treatment with DEM. These results suggest that hydroperoxides increase the free arachidonic acid available for the synthesis of PGD_2 by activating phospholipase A_2 (PLA_2) and that the depletion of GSH by DEM accelerates the activation of PLA_2 by raising peroxide levels in cells. Thus, the observed alterations in GSH levels are large enough to cause increased PGD_2 synthesis in RBL-2H3 cells exposed to oxidative stress.

Key words: arachidonic acid, glutathione, lipid peroxidation, phospholipase A_2, prostaglandin D_2.

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Prostaglandin D_2 (PGD_2) is one of the major products formed from arachidonic acid by cyclooxygenase (COX) in a variety of tissues and cells (*1*), and modulates cellular functions under various physiological and pathological conditions. PGD_2 released from mast cells functions as the modulator of the anaphylactic process (*2, 3*) and the platelet aggregation process (*4*). PGD_2 is a major COX product in the central nervous system and is involved in brain functions such as hypothermia (*5*), sleep (*6*), and luteinizing hormone secretion (*7*). The production of prostaglandins is initiated by the release of arachidonic acid from membrane phospholipids, which is caused by phospholipase A_2 (PLA_2). The liberated arachidonic acid is peroxidized to prostaglandin G_2 (PGG_2), and subsequent reduction yields prostaglandin H_2 (PGH_2). These sequential reactions are catalyzed by COX (*8*). PGH_2 is further converted to a variety of prostaglandins, such as PGD_2, PGE_2, and $PGF_{2\alpha}$. In the biosynthesis of PGD_2 from PGH_2, two types of PGD_2 synthase have been found (*9-11*). One is the hematopoietic form of PGD_2 synthase, which was purified from rat spleen and requires glutathione (GSH) for its activity. The other is GSH-independent PGD_2 synthase, which was purified from rat brain and localizes in the central nervous system (*12*).

Recent studies have indicated that oxidative stress modulates the production of prostaglandins. Studies *in vitro* using microsomes and homogenates have shown that the activities of prostaglandin synthetic enzymes are modulated by peroxide levels (*13-17*). COX requires low levels of hydroperoxides for activation; however, higher levels of hydroperoxides are inhibitory (*16*). Prostacyclin synthase is very sensitive to destruction by oxidizing agents, while thromboxane synthase is resistant (*14*). In intact cells, the production of prostaglandins is influenced by exposure to exogenously added hydroperoxides. Low levels of hydroperoxides stimulate the production of prostaglandins by activating COX and/or PLA_2 (*18-21*).

Intracellular GSH plays a central role in the antioxidant system as the most abundant non-protein thiol *in vivo* and a cofactor of various kinds of glutathione peroxidase. GSH depletion in cells leads to severe oxidative stress and cellular damage. GSH might participate in the modulation of prostaglandin biosynthesis. The activity of purified COX is completely inhibited by the addition of GSH and glutathione peroxidase (*16*). GSH is also required as a cofactor for PGD_2 synthase and PGE_2 isomerase (*22*). Thus, GSH acts not only as a potent regulator of intracellular redox, but also plays a significant role as a modulator of GSH-dependent PGD_2 synthase activity. Few studies, however, have addressed the role of GSH and oxidative stress in

[1] To whom correspondence should be addressed. Tel/Fax: +81-3-3444-4943, E-mail: nakagaway@pharm.kitasato-u.ac.jp

Abbreviations: GSH, glutathione; PGD_2, prostaglandin D_2; BSO, buthionine sulfoximine; DEM, diethyl maleate; PLA_2, phospholipase A_2; COX, cyclooxygenase; t-BHP, *tert*-butyl hydroperoxide; PBS, phosphate buffer saline; PVDF, polyvinylidene difluoride; TBS-T, Tris buffered saline-Tween 20; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid.

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PGD_2 production by intact cells. Rouser *et al.* demonstrated that the biosyntheses of PGE_2 and leukotriene C are inhibited in peritoneal macrophages by the GSH depletion (*23, 24*). In the present study, we examined the effects of t-BHP as an oxidant and DEM as the GSH-depleting agent on the synthesis of PGD_2 in RBL-2H3 cells, which possess a potent GSH-dependent PGD_2 synthase activity and predominantly produce PGD_2.

MATERIALS AND METHODS

Materials—[1-^{14}C]Arachidonic acid (2.22 GBq/mmol) was purchased from New England Nuclear (Boston, MA). The antibody against COX-1 was a kind gift from Dr. M. Murakami (Showa University, Tokyo). BSO and t-BHP were from Sigma Chemical (St. Louis, MO). A23187 and the PGD_2-MOX-Enzyme Immunoassay Kit were from Cayman Chemical (Ann Arbor, MI). Other chemicals used were from Wako Pure Chemical Industries (Osaka).

Cell Culture—Rat basophilic leukemia cells (RBL-2H3) were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin by passage of 1×10^6 cells/ml upon reaching semi-confluence. All experiments were performed on confluent cells.

Liberation of [1-^{14}C]Arachidonic Acid from Oxidant Loaded- or A23187 Stimulated Cells—RBL-2H3 cells (4×10^4 cells) were grown in 24-well plates for 2 days. The cells were then incubated with [1-^{14}C]arachidonic acid (1.85 kBq) for 24 h at 37°C. Labeled cells were washed twice with phosphate-buffered saline (PBS) containing 1 mg/ml fatty acid-free bovine serum albumin. The cells were preincubated for 10 min in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgSO_4$ and then exposed to either 75 μM t-BHP for 1 h or 5 μM A23187 for 10 min. The release of radiolabeled arachidonic acid was determined as described previously (*25*).

To study the effect of Ca^{2+} on the release of arachidonic acid, cells were exposed to t-BHP or A23187 in the presence of 0.5 mM EGTA. To deplete intracellular GSH, cells were pretreated with 0.5 mM BSO and/or 1 mM DEM for 18 and 2 h, respectively, before adding t-BHP or A23187.

Quantitation of Phospholipid Hydroperoxides—Phospholipid hydroperoxides were analyzed by chemiluminescence high-performance liquid chromatography (CL-HPLC), as described previously (*26*). Total lipids were extracted from RBL-2H3 cells by the method of Bligh and Dyer (*27*). An aliquot of total lipids was directly injected into an HPLC system (model 10A, Shimadzu, Kyoto) fitted with a propylamino column (microbondasphere; Nihon Waters, Tokyo). The column was eluted with a mixture of acetonitrile, methanol, and 0.2% triethylamine (pH 6; 82:10:8, v/v/v) at 1 ml/min. The eluate was monitored by a UV detector (SPD-10A, Shimadzu, Kyoto) at 205 nm. After passage through the UV detector, the eluate was mixed with the chemiluminescence reagent consisting of 50 mM borate buffer (pH 10.2), 10 μg/ml cytochrome *c*, and 3 μg/ml luminol. The chemiluminescence reagent was fed into the post-column eluate at a flow rate of 1.2 ml/min. The chemiluminescence of the mixture was measured with a chemiluminescence detector (model 825-CI; Japan Spectroscopic, Tokyo).

Immunoblot Analysis—Cell homogenates were centrifuged at $1{,}000\times g$ for 5 min to remove nuclei, unbroken cells, and debris. The supernatant proteins were separated by SDS-PAGE on 7.5% acrylamide gels and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane filter (ATTO, Tokyo) at 2 mA/cm^2 for 1 h in 100 mM Tris, 192 mM glycine, and 5 (v/v)% methanol. The PVDF membrane with the blotted protein was blocked by incubation for 2 h with 3 (w/v)% de-fatted milk in 10 mM Tris-HCl (pH 7.4), containing 150 mM NaCl and 0.1% Tween 20 (TBS-T). The membrane was then incubated for 1.5 h with antiserum against COX-1 that had been diluted with TBS-T at an appropriate concentration. After the PVDF membrane was washed twice with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed, CA). The binding of the antibody to the antigen on the PVDF membrane was detected by an enhanced chemiluminescence Western blotting analysis system (Amersham, Buckinghamshire, UK).

Quantitation of PGD_2 by Enzyme Immunoassay—RBL-2H3 cells were preincubated in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgSO_4$ for 10 min at 37°C and then treated with 75 μM t-BHP for 1 h or stimulated with 5 μM A23187 for 10 min. Incubation media were prepared for the quantitation of PGD_2 by the PGD_2-MOX Enzyme Immunoassay Kit (Cayman Chemical, MI).

Quantitation of Proteins—Protein concentrations were determined by the Protein assay reagent (Bio-Rad) with bovine serum albumin as the standard.

RESULTS

Liberation of Arachidonic Acid from RBL-2H3 Cells Exposed to t-BHP—The release of arachidonic acid was determined in RBL-2H3 cells that were pre-labeled with

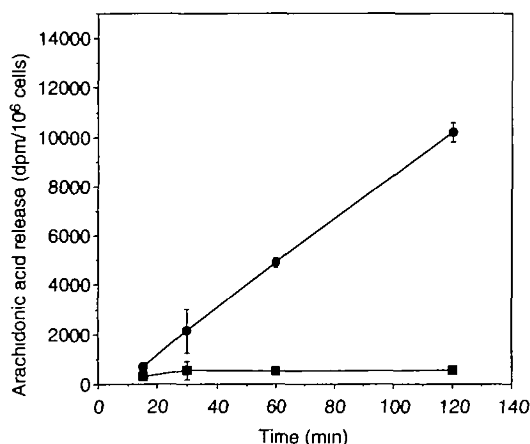

Fig. 1. **t-BHP causes the release of [1-^{14}C]arachidonic acid from RBL-2H3 cells.** Cells were pre-labeled with [1-^{14}C]arachidonic acid for 24 h and then treated with 75 μM t-BHP for the indicated period. The release of radioactive arachidonic acid into the incubation medium was determined as described under "MATERIALS AND METHODS." Closed squares represent control and closed circles represent t-BHP-treated cells. Data are expressed as mean±SD of triplicate measurement in three separate preparations.

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[1-^{14}C]arachidonic acid. The release of arachidonic acid from the membrane phospholipids was initiated by the addition of t-BHP (Fig. 1). A linear rate of free arachidonic acid production was observed over a 2 h interval.

A23187, a Ca-ionophore, caused an increase in intracellular Ca^{2+} by stimulating the influx of extracellular Ca^{2+} into the cells. Stimulation of RBL-2H3 cells with A23187 induced a higher elevation in free arachidonic acid release higher than that seen in oxidant-treated cells (Fig. 2). When the extracellular Ca^{2+} concentration was minimized with EGTA, the liberation of arachidonic acid with A23187 was dramatically reduced; however, the t-BHP-induced release was not influenced. These data suggest that the release of arachidonic acid produced by t-BHP is independent of extracellular Ca^{2+}.

Effect of GSH-Depletion on the Release of Arachidonic Acid—RBL-2H3 cells were pretreated to deplete intracellular GSH with either BSO for 18 h or DEM for 2 h. The liberation of arachidonic acid induced by t-BHP was determined in the GSH-depleted RBL-2H3 cells (Fig. 3). GSH-depletors did not influence the basal level of free arachidonic acid in control cells that were not exposed to t-BHP. The t-BHP-induced liberation of arachidonic acid was significantly enhanced to 130 and 200% that from non-treated cells by the addition of DEM and BSO, respectively.

The amount of the reduced-form of GSH in RBL-2H3 cells treated with DEM, t-BHP, or DEM+t-BHP decreased to 3.1±0.7, 75.4±13.1, or 1.5±0.3% of control values, respectively (data not shown).

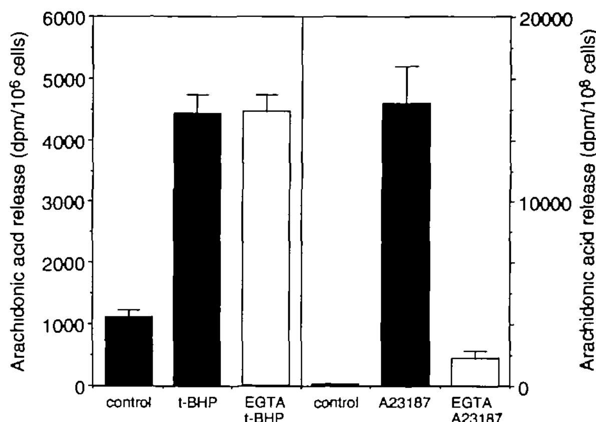

Fig. 2. **Effects of Ca^{2+} chelation on the release of arachidonic acid.** Pre-labeled cells were treated with either 75 μM t-BHP for 1 h or stimulated with 5 μM A23187 for 10 min in the presence of 1 mM $CaCl_2$ (hatched bars) or 0.5 mM EGTA (open bars). Data are expressed as mean±SD of triplicate measurement in three separate preparations.

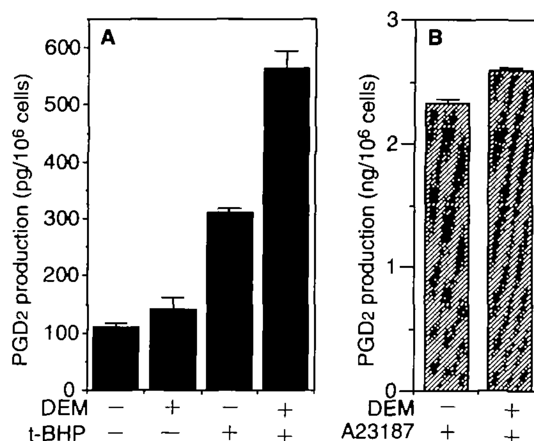

Fig. 4. **Effects of DEM on the production of PGD_2 induced by t-BHP.** Non-pretreated cells or cells preincubated with 1 mM DEM for 2 h were treated with either 75 μM t-BHP for 1 h (A) or 5 μM A23187 for 10 min (B). The levels of PGD_2 released into the incubation medium were determined by EIA. Data are expressed as mean± SD of triplicate measurements in three separate preparations.

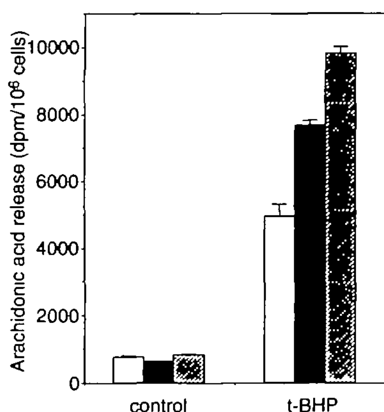

Fig. 3. **Effects of GSH depletion on the oxidative stress-induced release of arachidonic acid.** Cells were pre-labeled with [1-^{14}C]arachidonic acid as described under "MATERIALS AND METHODS." Pre-labeled cells were treated with either 0.5 mM BSO for 18 h or 1 mM DEM for 2 h, and then exposed to 75 μM t-BHP. The radioactivity released into the incubation medium was determined. Open bars represent non-treated cells. Closed and hatched bars represent DEM- and BSO-treated cells, respectively. Data are expressed as mean±SD of triplicate measurements in three separate preparations.

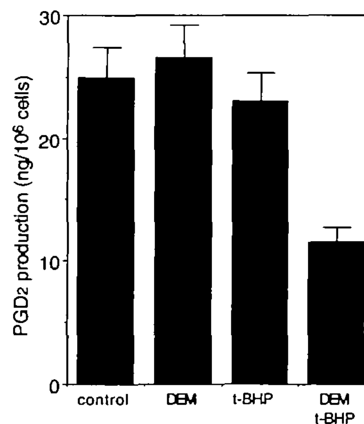

Fig. 5. **Effect of GSH depletion on the production of PGD_2 from exogenous arachidonic acid.** Non-pretreated cells or cells preincubated with 1 mM DEM for 2 h were treated with 100 μM t-BHP. The cells were then washed and incubated with 20 μM arachidonic acid for 30 min. The amount of PGD_2 released into the incubation medium was determined by EIA. Data are expressed as mean±SD of triplicate measurements in three separate preparations.

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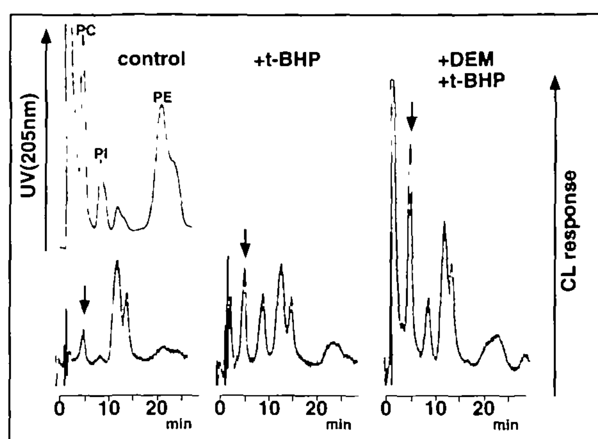

Fig. 6. **Chromatograms of phospholipids and hydroperoxides from membranes.** Cells were preincubated with 1 mM DEM for 2 h, and then treated with 100 μM t-BHP. Total lipids in the cells were extracted by the method of Bligh and Dyer as described previously. Compounds were fractionated by HPLC on a propylamino column. Each chromatogram shows the eluent monitored by absorbance at 205 nm and by the intensity of chemiluminescence. The peaks indicated by the arrows are phosphatidylcholine hydroperoxide. PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

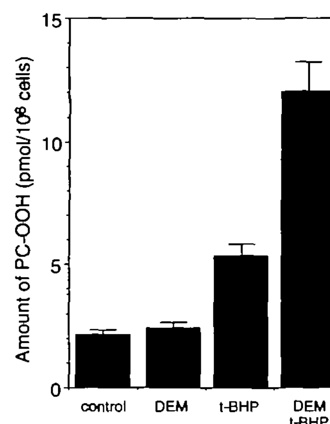

Fig. 7. **Effect of GSH depletion on the production of phosphatidylcholine hydroperoxide in cells treated with t-BHP for 1 h.** Cells were preincubated with 1 mM DEM for 2 h, and then treated with 100 μM t-BHP. Total lipids in the cells were extracted by the method of Bligh and Dyer as described previously. Phosphatidylcholine hydroperoxide (PC-OOH) was quantitated by CL-HPLC as described previously.

Effect of t-BHP and DEM on the Generation of PGD_2—The effect of DEM on the generation of PGD_2, the major product of COX metabolism in RBL-2H3 cells, was examined in RBL-2H3 cells exposed to t-BHP or A23187. PGD_2 production was 3 times greater than in control cells when the cells were exposed to t-BHP (Fig. 4A). DEM and t-BHP treatment facilitated the production of PGD_2, which was approximately 5 times greater than in non-treated cells. A23187 significantly enhanced the production of PGD_2 while DEM had no effect (Fig. 4B).

Production of PGD_2 from Exogenously-Added Arachidonic Acid—In order to estimate the effect of t-BHP and/or DEM on the activities of enzymes involved in the synthesis of PGD_2, the production of PGD_2 from exogenously added arachidonic acid was examined (Fig. 5). The production of PGD_2 was greatly enhanced by the addition of 20 μM arachidonic acid into the culture medium (compare Fig. 4 and Fig. 5), but the formation of PGD_2 from exogenous arachidonic acid was not altered by either peroxidation with t-BHP or depletion of GSH with DEM. These results clearly indicate that the enhancement of PGD_2 formation by t-BHP is not due to the activation of enzymes involved in the synthesis of PGD_2 from arachidonic acid; instead, activation of the release of available arachidonic acid for PGD_2 synthesis by PLA_2 seems to be involved. The synthesis of PGD_2 from exogenous arachidonic acid was, in part, suppressed by the addition of t-BHP and DEM together, indicating that the activity of GSH-dependent PGD_2 synthase was partially inhibited.

The Formation of Phospholipid Hydroperoxide in RBL-2H3 Cells Treated with t-BHP and/or DEM—Phospholipid hydroperoxides were determined in RBL-2H3 cells by CL-HPLC in order to estimate the level of peroxidized lipids in cells treated with t-BHP and/or DEM (Fig. 6). Small amounts of phospholipid hydroperoxides were detected in non-treated RBL-2H3 cells; however, significant peaks of hydroperoxides appeared in the phosphatidylcholine fraction in t-BHP-treated cells. The height of this peak increased considerably by pre-treatment with DEM. The amount of phosphatidylcholine hydroperoxides in t-BHP-treated cells was twice as high as in control cells (Fig. 7). The accumulation of phosphatidylcholine hydroperoxides was more pronounced in the case of treatment with t-BHP and DEM together, which induced hydroperoxide production that was 6 times greater than in non-treated cells.

Expression of COX-1—The expression of COX-1 in RBL-2H3 cells after exposure to t-BHP or stimulation with A23187 was examined by SDS-PAGE/Immunoblotting with a specific antibody (Fig. 8). The expression of COX-1 protein did not alter after treatment with t-BHP or A23187 in comparison with control cells.

DISCUSSION

The present study addresses the effect of intracellular GSH and oxidative stress on the production of PGD_2 in the basophilic cell line RBL-2H3, which generates predominantly PGD_2 with small amounts of other cyclooxygenase products such as PGE_2, 6-keto-$PGF_{1\alpha}$, and thromboxane B_2 (*28*). The level of intracellular GSH declines when cells are treated with GSH-depleting agents such as BSO or DEM. BSO decreases cellular GSH by the selective inhibition of γ-glutamyl cysteine synthase, the initial enzyme in GSH synthesis (*29*). DEM causes a decrease in GSH levels by inducing the conjugation of glutathione S-transferase with GSH or by direct chemical reaction with GSH (*30*). Treatment of RBL-2H3 cells with either 0.5 mM BSO for 18 h or 1 mM DEM for 2 h results in a decrease in the total content of intracellular GSH by 98 and 95%, respectively (data not shown). Neither the BSO nor DEM treatment used had a cytotoxic effect on RBL-2H3 cells or caused a change in cellular protein levels. GSH functions not only as an antioxidant molecule, but also as a co-factor for glutathione peroxidase, a major antioxidant enzyme in mammalian

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cells. The depletion of GSH in cells would shift the oxidative state and lead an elevated sensitivity to oxidative stress. DEM lacks the ability to induce the production of phospholipid hydroperoxides. However, the production of phospholipid hydroperoxide induced by t-BHP is increased by pretreatment with DEM (Fig. 7). These results indicate that the level of intracellular GSH is determined by the oxidative state of cells suffering oxidation.

t-BHP stimulates the activity of PLA_2 and the liberation of arachidonic acid from RBL-2H3 cells. Previous studies have shown an increase in free arachidonic acid in cells subjected to oxidative stresses such as t-BHP (*31-33*), tumor necrosis factor (*34*), or ultraviolet irradiation (*35*). Several mechanisms that might be involved in this up-regulation of phospholipase have been proposed. The presence of phospholipid hydroperoxides in the membrane is known to enhance the hydrolysis of phospholipids by PLA_2 *in vitro* (*36*). The elevation of intracellular Ca^{2+}, which is supposed to activate PLA_2 in cells exposed to free radicals (*37*) or oxidative stress cause the phosphorylation of $cPLA_2$ (*35*). The PLA_2 that responds to t-BHP is the extracellular Ca^{2+}-independent type, since EGTA fails to inhibit the activation of PLA_2 by t-BHP (Fig. 2). The pathway for the activation of PLA_2 is not defined in RBL-2H3 cells exposed to t-BHP.

Liberated arachidonic acid is converted mainly to PGD_2 by catalysis by COX and PGD_2 synthase. The generation of PGD_2 and arachidonic acid increases in RBL-2H3 cells upon treatment with t-BHP (Fig. 4). The production of PGD_2 from exogenously added arachidonic acid is not influenced by treatment with t-BHP (Fig. 5), indicating that t-BHP does not activate the enzymes involved in the conversion of arachidonic acid to PGD_2. The enhancement of PGD_2 synthesis caused by t-BHP is further accelerated by the depletion of intracellular GSH with either DEM (Fig. 4) or BSO (data not shown). It seems unlikely that DEM and BSO could activate COX or PGD_2 synthase directly, since either BSO or DEM alone does not affect PGD_2 synthesis. In mast cells, the rapid, early phase of PGD_2 synthesis is mediated by COX-1, and the delayed phase is required for the induction of COX-2 (*38*). Under these experimental conditions, COX-2 would not be induced. The expression of the COX-1 protein does not change compared to the control after treatment with t-BHP or A23187 (Fig. 8). These results suggest that the mechanism for the enhancement of PGD_2 production in RBL-2H3 cells treated with t-BHP is not the activation of the formation of PGD_2 from arachidonic acid, but an enhancement of the available arachidonic acid for the synthesis of PGD_2 by PLA_2. PLA_2 could be activated by the hydroperoxides generated by t-BHP. The depletion of GSH facilitates the generation of lipid hydroperoxides in t-BHP-treated cells and causes a pronounced activation of PLA_2. Thus, the activation of PLA_2 appears to be the key step in initiating the enhancement of PGD_2 by t-BHP and DEM.

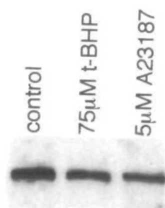

Fig. 8. **Cellular levels of COX-1 in control and stimulated cells.** Cells were treated with either 75 μM t-BHP for 1 h or stimulated with 5 μM A23187 for 10 min. Cell lysates (40 μg of protein) from RBL-2H3 cells were subjected to SDS-PAGE and the proteins were blotted onto a PVDF membrane. Immunoblotting was performed using the polyclonal antibody against COX-1 as described under "MATERIALS AND METHODS."

It is known that the biosynthesis of PGD_2 is catalyzed by at least two different types of PGD_2 synthase. One is the hematopoietic form of PGD_2 synthase, which localizes in the spleen, thymus, bone marrow, intestine, skin, and stomach, and requires GSH for its activity (*9, 11*). The other is GSH-independent PGD_2 synthase, which is found in the brain, epididymis, and spinal cord (*9, 10*). RBL-2H3 cells have hematopoietic PGD_2 synthase (*39*). Murakami *et al.* (*39*) demonstrated that PGD_2 synthase activity *in vitro* is absolutely dependent on the presence of GSH. However, the requirement of GSH is unclear in whole cells. The present study shows that PGD_2 is synthesized in DEM-treated RBL-2H3 cells at low levels of GSH (1.4 nmol/mg) in comparison with non-treated cells (28 nmol/mg). The relatively low K_m value of PGD_2 synthase for GSH (300 μM) (*11*) means that low levels of intracellular GSH can be effectively used, even though intracellular GSH levels are reduced. The production of PGD_2 from exogenously added arachidonic acid is not affected by treatment with DEM or t-BHP alone, but the production is significantly suppressed by treatment with both DEM and t-BHP (Fig. 5). t-BHP treatment results in a decrease in intracellular GSH levels, although its effectiveness is less than DEM. Treatment with DEM and t-BHP causes a further decrease in intracellular GSH. Thus, the inhibition of PGD_2 production by treatment with both DEM and t-BHP is caused by the depletion of the intracellular reduced-form of GSH which is required for the full activation of PGD_2 synthesis. Parker *et al.* have demonstrated that after RBL-1 cells are depleted of GSH by treatment with DEM or 2-cyclohexen-1-one, stimulation by A23187 results in a decrease in 5-HETE production and an increase in 5-HPETE, whereas GSH depletion does not affect PGD_2 generation (*40*). It has been shown that an 85% reduction in intracellular GSH is insufficient to inhibit PGE_2 isomerase activity, which requires GSH as a co-factor in endothelial cells (*41*). Thus, it seems likely that, despite low GSH concentrations, PGD_2 biosynthesis can proceed in intact cells.

We demonstrated that GSH depletion enhances both arachidonic acid release and PGD_2 in RBL-2H3 cells oxidized by t-BHP. The elevation of hydroperoxide levels by decreasing cellular GSH results in the production of arachidonic acid through the activation of PLA_2 and a subsequent increase in PGD_2 synthesis. Under conditions of oxidative stress, prostaglandin synthesis may be influenced by the level of antioxidants such as GSH.

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