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Oxidative Stress Suppresses Cysteinyl Leukotriene Generation by Mouse Bone Marrow-derived Mast Cells*

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Cysteinyl leukotrienes and oxidative stress have both been implicated in bronchial asthma; however, there is no previous study that focused on the ability of oxidative stress to alter cysteinyl leukotriene generation. In this study, treatment of bone marrow-derived mast cells with prostaglandin D_2 reduced their ability to generate leukotriene (LT) C_4 upon calcium ionophore stimulation but had little effect on LTB_4 generation. This effect could be reproduced by a selective agonist of the DP_2 receptor, 15*R*-methyl prostaglandin D_2 (15*R*-D_2). 15*R*-D_2 dose-dependently inhibited LTC_4 generation with an IC_{50} of 2 μM, and the effect was not altered by a DP_2/thromboxane antagonist or by a peroxisome proliferator-activated receptor-γ antagonist. 15*R*-D_2 exerted its suppressive effect via a reduction in intracellular GSH, a mechanism that involved the conjugation of its non-enzymatic breakdown product to GSH. At 10 μM, 15*R*-D_2 reduced LTC_4 generation to 10%, intracellular GSH to 50%, and LTC_4 synthase (LTC_4S) activity to 33.5% of untreated cells without altering immunoreactive LTC_4S protein expression or 5-lipoxygenase activity. The effects of 15*R*-D_2 on LTC_4S activity could be partially reversed by reducing reagent. The sulfhydryl-reactive oxidative agent diamide suppressed LTC_4S activity and induced a reversible formation of covalent dimer LTC_4S. LTC_4S bearing a C56S mutation was resistant to the effect of diamide. Covalent dimer LTC_4S was observed in nasal polyp biopsies, indicating that dimerization and inactivation of LTC_4S can occur at the site of inflammation. These results suggest a cellular redox regulation of LTC_4S function through a post-translational mechanism.

The cysteinyl leukotrienes (LT)[2] LTC_4, LTD_4, and LTE_4 are potent inflammatory lipid mediators. They are implicated in asthma (1–3) and allergic inflammation (4, 5). The biological actions of the cysteinyl leukotrienes as mediators of human asthma include contraction of bronchi (2), stimulation of mucous secretion from human airways *in vitro* (6), and induction of eosinophil infiltration into the airway of asthmatic individuals *in vivo* (7, 8). Biosynthesis of LTC_4 occurs in limited numbers of effector cells, such as mast cell, basophils, eosinophils, and macrophages that express the 5-lipoxygenase (5-LO) pathway of enzymes as well as LTC_4 synthase (LTC_4S). In mast cells, LTC_4 biosynthesis is initiated upon antigen cross-linking of the high affinity IgE receptors, which leads to influx of Ca^{2+} ion. An increase in intracellular Ca^{2+} activates calcium-dependent cytosolic phospholipase A_2, which subsequently translocates to the perinuclear membrane and hydrolyzes the release of arachidonic acid from the membrane phospholipids (9). The released arachidonic acid interacts with the 5-LO-activating protein FLAP, an integral nuclear membrane protein (10), during presentation to 5-LO, which also translocates to the perinuclear membrane from both the cytosol and the nucleoplasm (11, 12). 5-LO converts arachidonic acid to 5-hydroperoxyeicosatetraenoic acid and subsequently to LTA_4 (13). LTA_4 either is hydrolyzed by cytosolic LTA_4 hydrolase to form LTB_4 (14) or is conjugated to GSH to form LTC_4, the parent compound of cysteinyl leukotrienes (15, 16). This conjugation is the function of LTC_4S (17).

LTC_4S is an 18-kDa integral membrane protein located at the nuclear envelope of LTC_4-generating cells. Cloning of the human and mouse LTC_4S cDNAs revealed the highest amino acid identity to FLAP (17) and to bifunctional microsomal GST2 and GST3 (18). These proteins are all members of a superfamily of membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) (18), which also includes microsomal PGE_2 synthase-1 and microsomal GST1. LTC_4 conjugates GSH to LTA_4 to form LTC_4, and in contrast to microsomal GST2 and GST3, LTC_4S does not conjugate GSH to xenobiotics. Recently, it was demonstrated that LTC_4S can also conjugate GSH to 5-oxohydroxyeicosatetraenoic acid to form FOG-7 (19). X-ray crystallography of human LTC_4S with GSH shows that the LTC_4S monomer has four transmembrane α-helices and forms a 3-fold symmetric trimer as a unit with functional domains across each interface with GSH resides in a U-shaped conformation within an interface between adjacent monomers (20, 21). This binding is stabilized by a loop structure at the top of the interface. X-ray crystallographic data suggest that LTA_4 would fit into the interface so that Arg-104 of one monomer activates GSH to provide the thiolate anion that attacks C6 of LTA_4 to form a thioether bond, and Arg-31 of the neighboring monomer would donate a proton to form a hydroxyl group at C5, resulting in the formation of 5*S*-hydroxy-6*R*,*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetra-

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[2] The abbreviations used are: LT, leukotriene; 5-LO, 5-lipoxygenase; LTC_4S, LTC_4 synthase; CDNB, 1-chloro-2,4-dinitrobenzene; PG, prostaglandin; BMMC, bone marrow-derived mast cell; 15*R*-D_2, 15*R*-methyl-PGD_2; 15d-D_2, 15-deoxy-$\Delta^{12,14}$-PGD_2; 15d-J_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; BSO, buthionine sulfoximine; RP, reverse-phase; GS-DNP, glutathionyl-*S*-dinitrophenyl; PPARγ, peroxisome proliferator-activated receptor-γ; β-ME, β-mercaptoethanol.

enoic acid (LTC_4). In addition, X-crystallography shows that Arg-51, Asn-55, Glu-58, Tyr-93, Tyr-97, and Arg-104 of LTC_4S are involved in GSH binding.

Recently, studies on prostaglandin biosynthesis have demonstrated that nitric oxide *S*-nitrosylates cytosolic phospholipase A_2 (22) and COX2 (23), resulting in the activation of these two enzymes and an increase in prostaglandin synthesis. These results suggest that oxidative stress is able to induce post-translational modification of enzymes associated with eicosanoid biosynthesis. Although a decrease in intracellular GSH upon 1-chloro-2,4-dinitrobenzene (CDNB) treatment of human polymorphonuclear leukocytes has been shown to increase 5-LO activity (24) and to modify 5-LO activity by ERK2 and PKA phosphorylation (25, 26) and 5-LO translocation by MAPKAP2 phosphorylation (27), there is no information on the effect of oxidative stress on the post-translational modification of 5-LO or its pathway of enzymes. In this study, we examined the regulation of LTC_4 biosynthesis by oxidative stress. On the basis of preliminary experiments indicating that prostaglandin (PG) D_2, the major cyclooxygenase product of mast cells, could suppress LTC_4 generation when applied exogenously to mouse bone marrow-derived mast cells (BMMCs), we unexpectedly recognized that PGD_2 and the DP_2 receptor agonist 15*R*-methyl-PGD_2 (15*R*-D_2) suppressed LTC_4 generation by BMMCs through a reduction in intracellular GSH and suppression of LTC_4S activity. In addition, diamide, a sulfhydryl-reactive oxidant, induced oxidative stress, suppressed LTC_4S activity, and induced the formation of covalent dimer LTC_4S, normally a noncovalent trimer. Importantly, the effect of oxidative stress was abolished in C56S mutant LTC_4S, and the covalent dimer was present in nasal polyps of asthmatics, implying that LTC_4S activity levels may be modified *in vivo* by oxidation-related dimerization.

EXPERIMENTAL PROCEDURES

Materials—15-Deoxy-$\Delta^{12,14}$-PGD_2 (15d-D_2), 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-J_2), Δ^{12}-PGJ_2, 15*R*-D_2, PGB_2, PGD_2, PGE_2, $PGF_{2\alpha}$, GW 9662, Ramatroban, LTA_4 methyl ester, and BW 245C were from Cayman Chemical (Ann Arbor, MI). Ionophore A23187 was from Calbiochem. FuGENE 6 transfection reagent was from Roche Applied Science. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin was from Bio-Rad. Chemiluminescence substrate was from Pierce. Bovine serum albumin, diamide, CDNB, GSH, ionomycin, human GST, and buthionine sulfoximine (BSO) were from Sigma.

Culture of BMMCs—Bone marrow cells were collected from femurs and tibiae of BALB/c mice and cultured for 6–10 weeks in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% culture supernatant from CHO cells expressing mouse interleukin-3. The culture medium for the BMMCs was changed every week, and the cell density was adjusted to 5×10^5/ml at every passage. After 4 weeks, >97% of the cells were BMMCs as assessed by staining with toluidine blue.

Ionophore-stimulated 5-LO Product Generation—BMMCs were first treated with PGD_2, 15*R*-D_2, or vehicle (ethanol) for up to 48 h. Cells were then harvested, washed, and suspended in Hanks' buffer containing calcium, magnesium, and 2 mg/ml bovine serum albumin (buffer A). Stimulation of BMMCs were initiated by the addition of ionophore (2 μM), followed by incubation at 37 °C for 15 min. Reactions were terminated by the addition of 3 volumes of methanol containing 400 ng/ml PGB_2. Samples were analyzed for 5-LO product formation by reverse-phase (RP) HPLC with a Beckman System Gold Model 126 pump and Model 168 diode array UV detector using a programmed gradient and solvent system as described previously (28) and are expressed as picomoles/10^6 cells.

Enzyme Assay—To assay for LTC_4S activity, BMMCs were first pelleted by centrifugation and then resuspended in 200 μl of assay buffer (50 mM HEPES and 20 mM $MgCl_2$, pH 7.6) and lysed by microtip sonication. The cell lysates were incubated with 20 μM LTA_4 methyl ester and 10 mM GSH in assay buffer at room temperature for 10 min. Samples were analyzed by RP-HPLC (17). To assay for 5-LO activity, BMMCs were lysed in 5-LO assay buffer (0.1 M Tris, 30 mM KH_2PO_4, and 1.5 mM EDTA), and the reactions were initiated with the addition of 50 μM arachidonic acid, 2 mM ATP, and 1 mM $CaCl_2$ and incubated at 37 °C for 15 min. Reaction products were analyzed by RP-HPLC.

Western Blot Analysis—Cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.6, 100 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 50 μg/ml PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), boiled in SDS-PAGE sample buffer under reducing and nonreducing conditions, and separated on Tris/glycine-14% SDS-polyacrylamide gel (Novex). After transfer to a PVDF membrane, the blot was blocked with 4% nonfat dry milk and incubated with affinity-purified anti-LTC_4S IgG (1 μg/ml) for 2 h. After washing, the blot was incubated with horseradish peroxidase-linked secondary antibody for 1 h, washed, and developed with chemiluminescence substrate. For nasal polyps, samples were minced in lysis buffer, homogenized, and boiled in SDS-PAGE sample buffer under reducing and nonreducing conditions.

Real-time PCR—To determine the effect of 15*R*-D_2 on LTC_4S mRNA, we examine the time-dependent changes in LTC_4S mRNA by real-time PCR. Cells were treated with 10 μM 15*R*-D_2 for 0, 3, 6, and 24 h and harvested, and total RNA were then isolated with TRIzol reagent (Invitrogen). The relative LTC_4S mRNA level was determined by real-time PCR (GeneDirect, Princeton, NJ) and by comparing the values of the ratio of the LTC_4S gene product to the 18 S housekeeping gene product.

Measurement of Intracellular GSH—Intracellular GSH levels were measured by enzymatic conjugation of CDNB to GSH present in the mast cell lysate to form glutathionyl-*S*-dinitrophenyl (GS-DNP). Mast cells were lysed with 200 μl of 100 mM Tris-HCl, pH 8.0, and then incubated with 200 μM CDNB at room temperature for 30 min in the presence of 1 unit of GST from human placenta. Reactions were terminated by the addition of 10 μl of acetic acid (17.4 M) and 3 volumes of methanol containing 400 ng/ml PGB_2 and then analyzed by RP-HPLC for GS-DNP levels, with the ultraviolet absorbance monitored at 280 and 340 nm (for GS-DNP). Intracellular GSH was calculated by comparing the peak area of GS-DNP with that of a standard curve generated by incubating known amounts of

GSH (125–4000 pmol) with 200 μM CDNB and 1 unit of GST under identical conditions and is expressed as picomoles/10^6 cells.

Effect of BSO and Diamide—To examine the effect of the oxidizing condition on the ability of BMMCs to generate 5-LO products, BMMCs were pretreated with either 200 μM BSO, a GSH synthesis inhibitor, for 24 h or with 300 μM diamide, a sulfhydryl-reactive oxidative agent, for 30 min to deplete their intracellular GSH. Cells were then harvested and separated into three aliquots. One aliquot of cells was suspended in buffer A and stimulated with 2 μM ionophore to determine the ability to generate 5-LO products, and the other two aliquots were lysed and assayed for LTC_4S activity and GSH levels, respectively.

Metabolism of PGD_2 and 15R-D_2—BMMCs were incubated with 10 μM PGD_2 or 10 μM 15*R*-D_2 for 24 h and then analyzed by RP-HPLC for product profiles. For non-enzymatic conversion of these prostanoids, we incubated PGD_2 and 15*R*-D_2 with culture medium alone for 24 h and then analyzed the product profiles.

Effect of C56S and C82V Mutations on Diamide-induced Suppression of Enzyme Activity and Covalent Dimer Formation—To determine whether Cys-56 of LTC_4S is involved in covalent dimer formation, we transfected wild-type, C56S, and C82V plasmid cDNAs into CHO cells by lipofection using FuGENE 6 reagent. Two days after transfection, cells were harvested, washed, and then incubated with 300 μM diamide. After 30 min, cells were lysed and assayed for LTC_4S activity and molecular size.

RESULTS

PGD_2 and PGE_2 Inhibit LTC_4 Generation in BMMCs—Like cysteinyl leukotrienes, PGD_2 is abundant in allergic inflammation and a product of activated mast cells. No previous study had addressed whether PGD_2 could modify the generation of LTC_4. In addition, as LTC_4S catalyzes the conjugation of LTA_4 to GSH, we were also interested in determining whether changing intracellular GSH through oxidative stress will affect LTC_4 generation. Because PGD_2 has previously been shown to increase LTC_4 generation by mouse eosinophils (29), and its cyclopentenone metabolite has been proposed as a potential endogenous regulator of redox-sensitive transcription factors (30) and has been shown to deplete intracellular GSH (31), we thus examined the effect of PGD_2 on LTC_4 generation by BMMCs. BMMCs were incubated with 10 μM PGD_2 for 48 h, and for specificity purposes, we also included 10 μM PGE_2 and 10 μM $PGF_{2\alpha}$ for comparison. After harvesting, cells were stimulated with 2 μM calcium ionophore A23187 for 10 min. As shown in Fig. 1, whereas both PGD_2 and PGE_2 inhibited LTC_4 generation by >70 and 65%, respectively, $PGF_{2\alpha}$ had no effect. We concentrated on the effect of PGD_2, as it is also a mast cell-derived product. None of these prostaglandins affected the biosynthesis of 12-hydroxyheptadecatrienoic acid (data not shown).

Inhibitory Effect of PGD_2 Does Not Act through the DP_1 or DP_2 Receptor or Peroxisome Proliferator-activated Receptor-γ (PPARγ)—To investigate the possible receptor involved in the suppression of LTC_4 generation by PGD_2, BMMCs were incubated with the DP_1 agonist BW 245C or the DP_2 agonists

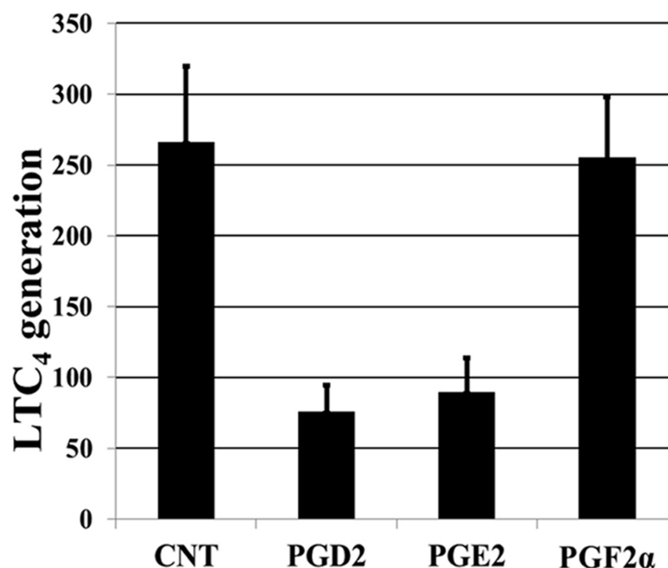

FIGURE 1. **Effect of PGD_2, PGE_2, and $PGF_{2\alpha}$ on ionophore-stimulated LTC_4 generation by BMMCs.** BMMCs were treated with and without (control (*CNT*)) prostaglandins (10 μM) for 48 h, harvested, and stimulated with the calcium ionophore A23187 (2 μM) for 15 min, and LTC_4 levels were analyzed by HPLC and are expressed as picomoles/10^6 cells (mean ± S.E., $n = 4$). *, $p < 0.01$.

15*R*-D_2 and 15-deoxy-$\Delta^{12,14}$-PGD_2 (15d-D_2; also a PPARγ agonist), and we then examined the ionophore-stimulated generation of 5-LO products. As shown in Fig. 2*A*, the effect of PGD_2 could be mimicked by the DP_2 agonists 15*R*-D_2 and 15d-D_2 but not by the DP_1 agonist BW 245C. To further investigate whether the suppressive effect is mediated through the DP_2 receptor or PPARγ, we examined the effect of the DP_2 antagonist Ramatroban and the PPARγ antagonist GW 9662 using 15*R*-D_2 as an agonist. As shown in Fig. 2*B*, the effect of 15*R*-D_2 on LTC_4 generation was not inhibited by the DP_2 antagonist Ramatroban or by the PPARγ antagonist GW 9662, suggesting that the inhibitory effect of PGD_2 or 15*R*-D_2 is not mediated through DP_1, DP_2, or PPARγ. Because the effect of PGD_2 could be reproduced by 15*R*-D_2, we performed subsequent experiments using 15*R*-D_2 as an agonist.

Time-dependent Effect of 15R-D_2 on the Ability of BMMCs to Generate LTC_4 and LTC_4S Protein or mRNA Expression—When 15*R*-D_2 was incubated with BMMCs, it caused a time-dependent decrease in LTC_4 generation when stimulated with the calcium ionophore. When BMMCs were treated with 15*R*-D_2 for 24 h, there was a >75% reduction in ionophore-stimulated LTC_4 generation (Fig. 3*A*), with little additional reduction when treated for 48 h (data not shown). Thus, we examined the dose-dependent effect of 15*R*-D_2 over 24 h of treatment.

To determine whether 15*R*-D_2 reduced cellular LTC_4S activity through reduced LTC_4S transcription and protein expression, we incubated BMMCs with 10 μM 15*R*-D_2 for 0, 3, 6, and 24 h. LTC_4S mRNA expression was then analyzed by real-time PCR, and protein expression was examined by immunoblot analysis. As shown in Fig. 3 (*B* and *C*), 15*R*-D_2 did not alter the expression of either the mRNA or immunoreactive LTC_4S protein. This result indicates that 15*R*-D_2 does not reduce the cellular LTC_4 activity of BMMCs through a reduction in LTC_4S

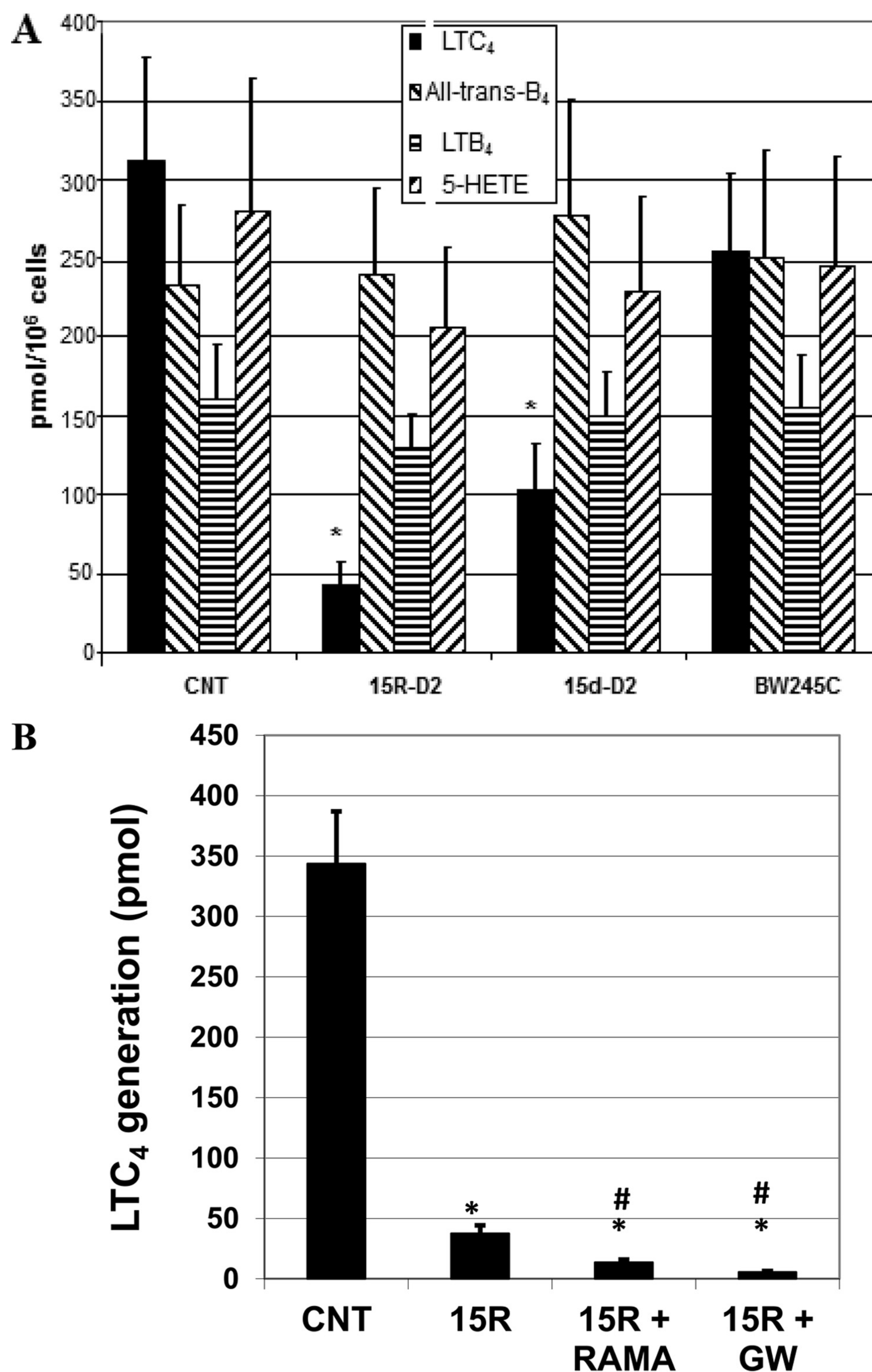

FIGURE 2. **Effect of 15*R*-D_2, BW 245C, and 15d-D_2 (10 μM each) on LTC_4 generation by BMMCs (*A*) and effect of Ramatroban (10 μM) and GW 9662 (10 μM) on the suppressive effect of 15*R*-D_2 (10 μM) on A23187-stimulated LTC_4 generation.** Cells were treated with 10 μM DP agonists for 48 h and then stimulated with A23187 for 15 min (*A*) or treated with 15*R*-D_2 (*15R*) in the absence or presence of Ramatroban (*RAMA*) and GW 9662 (*GW*) for 48 h and then stimulated with 2 μM A23187 for 15 min (*B*). Data are expressed as picomoles/10^6 cells. *, $p < 0.01$ *versus* the control (*CNT*; mean ± S.E., $n = 6$); #, $p < 0.02$ *versus* 15*R*-D_2 (mean ± S.E., $n = 5$).

gene transcription and protein expression, thus suggesting a post-translational modification of LTC_4S.

Dose-dependent Effect of 15R-D_2—To examine the dose-dependent ability of 15*R*-D_2 to suppress LTC_4 generation and its effect on 5-LO activity, we incubated BMMCs with various concentrations of 15*R*-D_2 for 24 h and then performed various assays. As shown in Fig. 4*A*, 15*R*-D_2 dose-dependently reduced ionophore-stimulated LTC_4 generation by BMMCs with >90% inhibition at 10 μM 15*R*-D_2. With 10 μM 15*R*-D_2, the mean LTC_4 generation by BMMCs was 10.0 ± 2.2% (mean ± S.E., n =

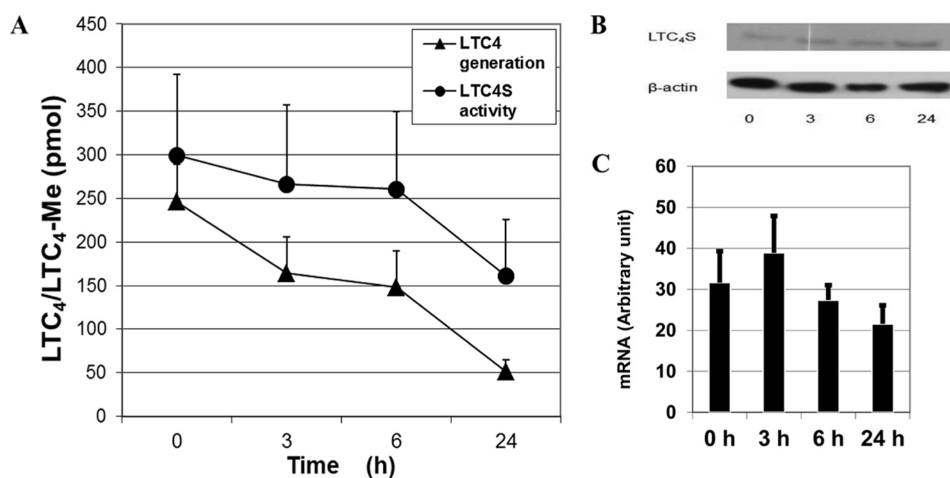

FIGURE 3. **Time-dependent effect of 15*R*-D$_2$.** BMMCs were incubated with 10 μM 15*R*-D$_2$ for the indicated times, washed, and separated into four aliquots. One aliquot was stimulated with A23187 (2 μM) to determine LTC$_4$ generation, and a second aliquot was used for LTC$_4$S activity determination (*A*). One aliquot was used for immunoreactive LTC$_4$S protein expression under reducing conditions (*B*). The last aliquot was used for LTC$_4$S mRNA expression (*C*) (mean ± S.E., $n = 4$). *Me*, methyl ester.

FIGURE 4. **Dose-dependent effect of 15*R*-D$_2$.** Cells were treated with the indicated concentrations of 15*R*-D$_2$ (in 2 μl of ethanol) or with an equal volume of vehicle (0 μM 15*R*-D$_2$) for 24 h, washed, and split into four aliquots. One aliquot was stimulated with A23187 (2 μM) to examine LTC$_4$ generation ($n = 8$) (*A*), one for LTC$_4$S activity ($n = 8$) (*B*), one for 5-lipoxygenase activity ($n = 8$) (*C*), and one for intracellular GSH measurement ($n = 5$) (*D*) as described under "Experimental Procedures." *ME*, methyl ester.

8) of untreated BMMCs. An IC$_{50}$ of ~2 μM was observed for the inhibition of LTC$_4$ generation by 15*R*-D$_2$. There were no significant changes in all-*trans*-diastereoisomers of LTB$_4$ or LTB$_4$ or 5-hydroxyeicosatetraenoic acid generation (Fig. 2*A*), suggesting that 15*R*-D$_2$ selectively reduces the ability of BMMCs to generate LTC$_4$ without affecting the 5-LO activity. To confirm that 15*R*-D$_2$ did not affect 5-LO enzyme activity, both the 5-LO and LTC$_4$S activities were measured in BMMC lysates obtained

from BMMCs that were treated with various concentrations of 15*R*-D_2 and from untreated cells. As shown in Fig. 4 (*B* and *C*), 15*R*-D_2 did not affect cellular 5-LO activity but dose-dependently reduced LTC_4S activity. The mean LTC_4S activity of 15*R*-D_2-treated BMMCs was 33.5 ± 3.5% (mean ± S.E., $n = 8$) (Fig. 3*B*) of the control cells, thus confirming that the reduction in LTC_4S activity upon 15*R*-D_2 treatment contributed to the decrease in ionophore-stimulated LTC_4 generation. Furthermore, the effect of 15*R*-D_2 on LTC_4 generation was not restricted to the calcium ionophore A23187, as 15*R*-D_2 also suppressed LTC_4 generation induced by ionomycin and by cross-linking of IgE. 15*R*-D_2-reduced ionomycin (2 μM) stimulated LTC_4 generation to 8.6 ± 2.9% (mean ± S.E., $n = 10$) of the untreated cells. In two experiments, 10 μM 15*R*-D_2 also reduced LTC_4 generation induced by cross-linking of IgE to 10.6 and 11.8% of the untreated cells, respectively.

15R-D_2 Reduces Intracellular GSH Levels in BMMCs—15*R*-D_2 inhibited LTC_4 generation without a significant effect on LTC_4S protein expression or 5-LO activity, suggesting that 15*R*-D_2 may affect LTC_4 generation by altering the intracellular levels of GSH, the second substrate of the LTA_4 conjugation reaction. Thus, we investigated the level of intracellular GSH by assaying the ability of the cell lysate to enzymatically conjugate CDNB to endogenous GSH to form GS-DNP at pH 8.0 by adding excess CDNB and exogenous GST. As shown in Fig. 4*D*, 15*R*-D_2 dose-dependently decreased the intracellular GSH levels as indicated by a reduction in the amount of GS-DNP generated when cell lysates were incubated with CDNB. At 10 μM 15*R*-D_2, the mean GSH level of treated BMMCs was 43.9 ± 5.8% (mean ± S.E., $n = 5$) of the control cells.

BSO Reduces LTC_4 Generation—To confirm that 15*R*-D_2 reduces ionophore-stimulated generation of LTC_4 by BMMCs through a reduction in intracellular GSH, we compared the effect of BSO, a glutathione synthase inhibitor, with that of 15*R*-D_2 on ionophore-stimulated LTC_4 generation. When BMMCs treated with 15*R*-D_2 were stimulated with ionophore, LTC_4S activity was reduced to 15.2 ± 5.9% (mean ± S.E., $n = 3$) of the control BMMCs. Treatment with BSO reduced LTC_4 generation to undetectable levels. Similarly, BSO reduced intracellular GSH levels to 0.57 ± 0% (mean ± S.E., $n = 3$) of the control cells.

Mechanism of Reduction in Intracellular GSH by 15R-D_2—Because cyclopentenone metabolites of PGD_2 have previously been shown to be able to reduce intracellular GSH through conjugation to GSH and because mast cells have been shown to be poor producers of reactive oxygen species (32), we examined the metabolism of 15*R*-D_2 and PGD_2 in BMMCs. When PGD_2 was incubated with BMMCs or with culture medium alone for 24 h and then analyzed by HPLC for its metabolites present in the culture medium, we found three major non-enzymatic metabolites that corresponded to synthetic standards of Δ^{12}-PGJ_2, 15d-J_2, and 15d-D_2, respectively, in both the BMMC culture medium (Fig. 5*A*) and the medium with no BMMCs (Fig. 5*B*). However, there was an additional polar metabolite (compound X) in the HPLC chromatograph of BMMC culture medium that was absent in the medium-alone sample. Furthermore, the relative peak heights of Δ^{12}-PGJ_2 (also monitored at its UV maximum of 244 nm; chromatograph not shown) and 15d-J_2 culture media were nearly identically with or without BMMCs, whereas the peak height of 15d-D_2 in the medium with BMMCs was 24.9 ± 1.82% (mean ± S.E., $n = 3$) lower than that with no BMMCs. Similarly, incubation of 15*R*-D_2 with BMMCs and culture medium alone yielded metabolite profiles identical to those of PGD_2 with three 15*R*-methyl derivatives of the corresponding PGD_2 metabolites (data not shown). We thus examined whether compound X is derived from 15d-D_2 or from 15d-J_2. We first incubated 15d-D_2 and 15d-J_2) with and without GSH for 4 h at 37 °C and then analyzed the polar metabolite formation, as they are readily available commercially. Both 15d-D_2 and 15d-J_2 were conjugated to GSH non-enzymatically to form polar metabolites with a similar retention time in HPLC in the presence of GSH (data not shown). However, only 15d-D_2 was converted to a polar metabolite with a HPLC retention time identical to that of compound X when incubated with BMMCs (Fig. 6, *A* and *B*). There was no metabolism of 15d-J_2 observed when it was incubated with BMMCs (data not shown). These results suggest that compound X is a PGD_2 metabolite derived from the conjugation of 15d-D_2 to GSH and that 15*R*-D_2 spontaneously degrades to form 15*R*-derivatives of 15d-D_2, which are then conjugated to GSH, and lowers intracellular GSH. The chemical structure of compound X is not known and is being elucidated. This result is very similar to that reported for the conjugation of 9-deoxy-$\Delta^{9,12}$(*E*)-PGD_2 to GSH (31).

Effect of 15R-D_2 on LTC_4S Is Partially Reversible—Because β-mercaptoethanol (β-ME) counters the effect of 15*R*-D_2 on LTC_4 generation, we examined whether β-ME can rescue LTC_4S activity 24 h after 15*R*-D_2 treatment. BMMCs were treated with 10 μM 15*R*-D_2 for 24 h, harvested, washed, and incubated with and without 10 mM β-ME. After 20 min, cells were washed once and then assayed for LTC_4S activity. Treatment of BMMCs with 15*R*-D_2 for 24 h reduced LTC_4S activity to 29.9 ± 8.5% (mean ± S.E., $n = 4$) of the control cells. LTC_4S activity increased to 65.4 ± 9.8% with β-ME treatment. These results suggest that the effect of 15*R*-D_2 on LTC_4S is at least a partially reversible process.

Effect of Diamide on LTC_4S—Our demonstration that a reduction in intracellular GSH suppresses LTC_4S function suggested that oxidative stress may also exert an effect similar to that of 15*R*-D_2. Thus, we utilized the sulfhydryl-reactive oxidant diamide to acutely induce oxidative stress and to examine the enzyme activity of LTC_4S. Incubation of diamide with BMMCs for 30 min reduced LTC_4S activity by >75% (data not shown). To further study the effect of oxidative stress on LTC_4S, we examine the effect of diamide on CHO cells transfected with wild-type and mutant LTC_4S cDNAs. When diamide was incubated with CHO cells transfected with wild-type LTC_4S cDNA, it reduced LTC_4S function to 22.8 ± 3.4% (mean ± S.E., $n = 4$) of the control cells. In addition, diamide induced the formation of covalent dimer LTC_4S in a nonreducing gel (Fig. 7*A*). In contrast, C56S mutant LTC_4S retained >91% of the enzyme activity ($n = 2$) with diamide treatment and no covalent dimer formation (Fig. 7*A*). Mutation of the only other cysteine residue (Cys-82) did not protect enzyme activity from diamide treat-

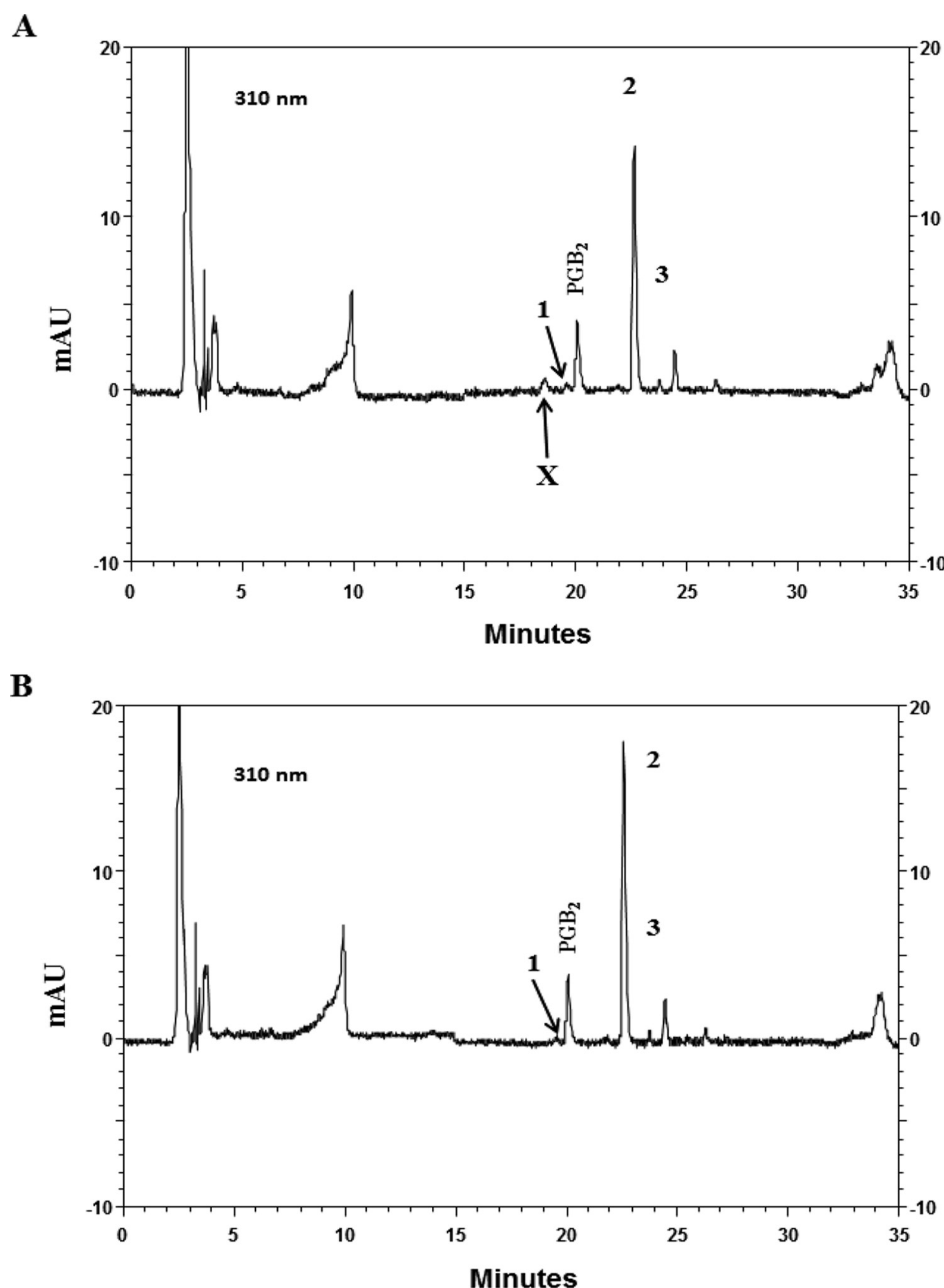

FIGURE 5. **HPLC chromatographs of the culture media obtained from incubations of 10 μM PGD_2 with (*A*) and without (*B*) BMMCs monitored at 310 nm.** The identities of *peaks 1–3* are Δ^{12}-PGJ_2, 15d-D_2, and 15d-J_2, respectively. The unknown polar PGD_2 metabolite is labeled *X*. *mAU*, milliabsorbance units.

ment and formation of a covalent dimer (Fig. 7*A*). The effect of diamide was also reversible, as diamide-treated CHO cells that were subsequently incubated with 10 mM β-ME restored LTC_4S activity to 89.2 $\pm$ 9.1% ($n = 4$) of the control enzyme, and there was no covalent dimer formation.

To determine whether oxidative stress similarly affects purified LTC_4S, we incubated purified LTC_4S with diamide and then assayed its conjugating activity and analyzed the formation of the covalent dimer by Western blotting. Similar to the transfected CHO cells, diamide treatment suppressed the enzyme activities of purified LTC_4S by $>$90% (data not shown) as well as the formation of a covalent dimer (Fig. 7*B*). These results indicate that oxidative stress can reversibly suppress LTC_4S function at least in part through induction of disulfide bridging of Cys-56 from neighboring monomers and the formation of an enzymatically inactive covalent dimer.

To determine whether the covalent dimer exists *in vivo*, we examined nasal polyps from asthmatic subjects, which have been reported to contain large numbers of infiltrated activated eosinophils (33). Western blot analyses demonstrated the presence of a 34-kDa covalent dimer LTC_4S (Fig. 8) under nonreducing conditions, similar to wild-type LTC_4S treated with diamide. In addition, the presence of covalent dimer LTC_4S was also observed in nasal polyps obtained from a normal individual. These results suggest

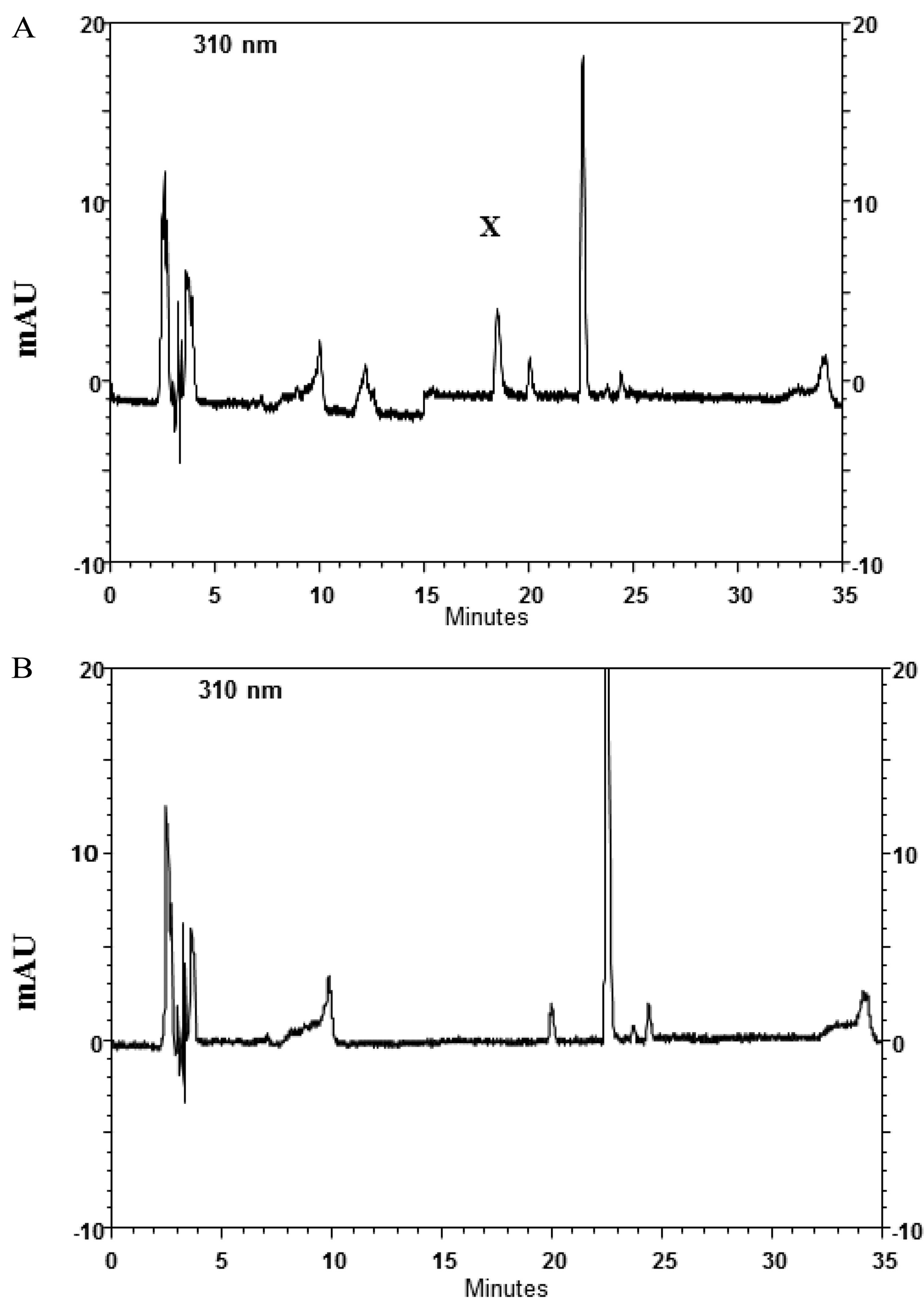

FIGURE 6. **HPLC analysis of the culture media obtained from incubations of 10 μM 15d-D_2 with (*A*) and without (*B*) BMMCs.** The unknown polar metabolite (*X*) corresponds to that obtained from PGD_2 incubation with BMMCs. *mAU*, milliabsorbance units.

that dimerization of LTC_4S monomers can occur *in vivo* under oxidative stress.

DISCUSSION

This study has demonstrated that PGD_2, the DP_2 agonist 15*R*-D_2, and the PPARγ agonist 15d-J_2 suppress ionophore-stimulated LTC_4 generation. The suppressive effect is a receptor-mediated event, as it was not block by either the DP_2 antagonist Ramatroban or the PPARγ antagonist GW 9662 (Fig. 2*B*), and on the contrary, these antagonists significantly enhanced the suppressive effect of 15*R*-D_2 on LTC_4 generation. This potentiation effect has not been investigated further. The effect of 15*R*-D2 is not mediated through an alteration of transcription or protein expression, as neither was affected by 15*R*-D2 (Fig. 3). Because reduction in intracellular GSH has previously been shown to affect various cell functions, including gene transcription and cell proliferation (34, 35), we thus examined if the suppressive effect could be mediated through a reduction in intracellular GSH, a second substrate for LTC_4S. We demonstrated that the DP_2 agonist 15*R*-D_2 reduces intracellular GSH in BMMCs (Fig. 4*D*) and reduces the ability of these cells to synthesize cysteinyl leukotrienes in response to ionophore stimulation as well as suppression of LTC_4S activity through post-translational modification (Fig. 3, *A* and *B*, and Fig. 4). The

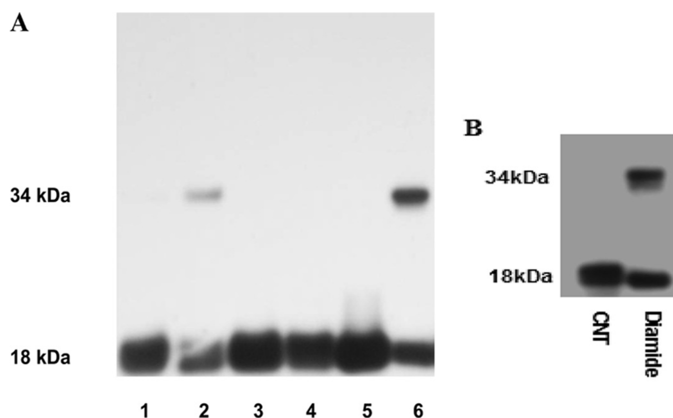

FIGURE 7. **SDS-PAGE immunoblot analysis of cell lysates obtained from CHO cells transfected with wild-type (*lanes 1* and *2*), C56S mutant (*lanes 3* and *4*), and C82V mutant (*lanes 5* and *6*) LTC_4S cDNAs treated with diamide (*lanes 2*, *4*, and *6*) and untreated controls (*lanes 1*, *3*, and *5*) (*A*) and of purified LTC_4S treated with and without diamide (*B*).** SDS-PAGE was performed under nonreducing conditions. *CNT*, control.

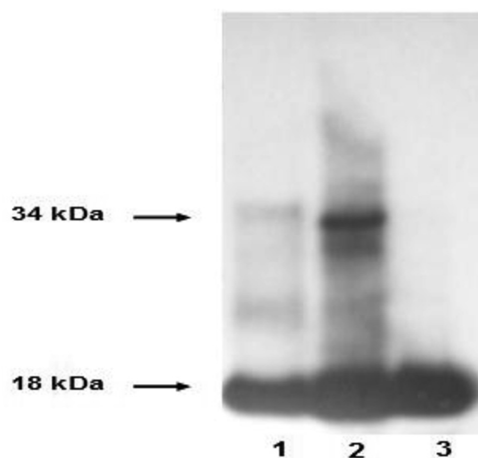

FIGURE 8. **Immunoblot analysis of the molecular size of LTC_4S.** Shown are a nasal polyp biopsy obtained from an aspirin-sensitive asthmatic patient under nonreducing (*lane 2*) and reducing (*lane 3*) conditions and a LTC_4S plasmid-transfected COS cell (*lane 1*).

mechanism by which 15*R*-D_2 reduces intracellular GSH is at least partially through conjugation of GSH to its non-enzymatic breakdown product and does not involve any known PGD_2 receptor (Figs. 5 and 6). When 15*R*-D_2 was incubated with BMMCs, it non-enzymatically converted to 15*R*-derivatives of Δ^{12}-PGJ_2, 15d-J_2, and 15d-D_2. These determinations were based on the identical product profiles when PGD_2 was utilized (Fig. 5) and identical HPLC retention times compared with available authentic standards of these PGD_2 metabolites. Of these three cyclopentenone metabolites, both 15d-J_2 and 15d-D_2 conjugated to GSH non-enzymatically, with 15d-J_2 being a better substrate for conjugation (data not shown). When incubated with BMMCs, however, a GSH-conjugated metabolite was observed in only incubations of 15d-D_2 with BMMCs (Fig. 6). No metabolism was observed with 15d-J_2 (data not shown), suggesting that the unknown polar metabolite X is a conjugated product of 15d-D_2. The reason for the difference in metabolism of 15d-D_2 and 15d-J_2 by BMMCs is not known. It could be that uptake of these eicosanoids requires a membrane carrier and that there is an uptake carrier in BMMCs (36) for 15d-D_2 but not for 15d-J_2. The ability to non-enzymatically break down to cyclopentenone metabolites may account for the observed ability of PGD_2 and PGE_2 to suppress LTC_4 generation, whereas $PGF_2\alpha$ did not (Fig. 1). Furthermore, the ability of PGD_2 to reduce intracellular GSH did not alter the biosynthesis of 12-hydroxyheptadecatrienoic acid, indirectly suggesting that there is no change in thromboxane synthesis, as 12-hydroxyheptadecatrienoic acid is an equal molar byproduct of thromboxane synthesis (37). We also examined if the reduction in intracellular GSH could be a result of an increase in reactive oxygen species generation, but our experiments showed that BMMCs produced negligible amounts of reactive oxygen species upon A23187 and phorbol 12-myristate 13-acetate stimulation (data not shown), an observation that is in agreement with that of Swindle *et al.* (32). In addition, the NADPH oxidase inhibitor diphenyleneiodonium sulfate did not overcome the effect of 15*R*-D_2 (data not shown), thus confirming that the effect of 15*R*-D_2 is not mediated through an increase in reactive oxygen species generation.

Both 15*R*-D_2 and oxidative stress induced by diamide caused a possible post-translational modification of LTC_4S, as they suppressed LTC_4S activity without affecting protein expression (Figs. 3 and 7). Diamide also induced the formation of inactive covalent dimer LTC_4S (Fig. 7). The suppressive effect of 15*R*-D_2 on LTC_4S activity can only be partially reversed with reducing agent, whereas the effect of diamide is totally reversible. Although the rationale for the difference in reversibility between 15*R*-D_2 and diamide is not known, one possible explanation may be that the cyclopentenone metabolite of 15*R*-D_2 can directly modify Cys-56 of LTC_4S, an effect that has been reported for the p50 subunit of NFκB using 15d-J_2 (38). Unlike the disulfide bridge of the covalent dimer, the metabolite-modified Cys-56 cannot be reduced by reducing agent, and therefore, that portion of LTC_4S is irreversibly inhibited.

Importantly, our experiments suggest that cellular LTC_4S may exist in two different forms, an enzymatically active form and an inactive form, and that the relative ratio of these two forms will depend on the oxidative state of the cells. Thus, our findings that 15*R*-D_2 alters cell function through a reduction in cellular GSH further suggest that the cellular redox state may control the homeostasis of inflammatory cell function and subsequently the inflammatory processes. In a preliminary experiment with one nasal polyp, we also observed a 3-fold increase in LTC_4S activity after it was treated with the reducing agent β-ME (data not shown). This observation, together with the demonstration of the presence of inactive covalent dimer LTC_4S in a nasal polyp biopsy (Fig. 8), suggests that the cellular oxidation/reduction state of the tissue can modify its ability to generate 5-LO products and that tissue immunohistochemistry alone is not sufficient in determining the true capacity of the tissue in leukotriene biosynthesis *in situ*.

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