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 D2 reduced their ability to generate leukotriene (LT) C4 upon calcium ionophore stimulation but had little effect on LTB4 generation. This effect could be reproduced by a selective agonist of the DP2 receptor, 15R-methyl prostaglandin D2 (15R-D2). 15R-D2 dose–dependently inhibited LTC4 generation with an IC50 of 2 μM, and the effect was not altered by a DP2/thromboxane antagonist or by a peroxisome proliferator-activated receptor-γ antagonist. 15R-D2 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, 15R-D2 reduced LTC4 generation to 10%, intracellular GSH to 50%, and LTC4 synthase (LTC4S) activity to 33.5% of untreated cells without altering immunoreactive LTC4S protein expression or 5-lipoxygenase activity. The effects of 15R-D2 on LTC4S activity could be partially reversed by reducing reagent. The sulphydryl-reactive oxidative agent diamide suppressed LTC4S activity and could be partially reversed by reducing reagent. The sulfhydryl-bearing a C56S mutation was resistant to the effect of diamide. Covalent dimer LTC4S was observed in nasal polyph biosynthesis, indicating that dimerization and inactivation of LTC4S can occur at the site of inflammation. These results suggest a cellular redox regulation of LTC4S function through a post-translational mechanism.

The cysteinyl leukotrienes (LT)2 LTC4, LTD4, and LTE4 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 mucus secretion from human airways in vitro (6), and induction of eosinophil infiltration into the airway of asthmatic individuals in vivo (7, 8). Biosynthesis of LTC4 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 LTC4 synthase (LTC4S). In mast cells, LTC4 biosynthesis is initiated upon antigen cross-linking of the high affinity IgE receptors, which leads to influx of Ca2+ ion. An increase in intracellular Ca2+ activates calcium–dependent cytosolic phospholipase A2, 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-hydroxyeicosatetraenoic acid and subsequently to LTA4 (13). LTA4 either is hydrolyzed by cytosolic LTA4 hydrolase to form LTB4 (14) or is conjugated to GSH to form LTC4, the parent compound of cysteinyl leukotrienes (15, 16). This conjugation is the function of LTC4S (17).

LTC4S is an 18-kDa integral membrane protein located at the nuclear envelope of LTC4-generating cells. Cloning of the human and mouse LTC4S 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 PGE2 synthase-1 and microsomal GST1. LTC4 conjugates GSH to LTA4 to form LTC4, and in contrast to microsomal GST2 and GST3, LTC4S does not conjugate GSH to xenobiotics. Recently, it was demonstrated that LTC4S can also conjugate GSH to 5-oxohydroxyeicosatetraenoic acid to form FOG-7 (19). X-ray crystallography of human LTC4S with GSH shows that the LTC4S 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 LTA4 would fit into the interface so that Arg-104 of one monomer activates GSH to provide the thiolate anion that attacks C6 of LTA4 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 5S-hydroxy-6R,S-glutathionyl-7,9-trans-11,14-cis-eicosatetra-

---

*This work was supported, in whole or in part, by National Institutes of Health Grants HL36110, HL082695, and HL090630. 1To whom correspondence should be addressed: Brigham and Women’s Hospital, Smith Bldg., Rm. 628, One Jimmy Fund Way, Boston, MA 02115. Tel.: 617-525-1270; Fax: 617-525-1310; E-mail: blam@rics.bwh.harvard.edu. 2The abbreviations used are: LT, leukotriene; 5-LO, 5-lipoxygenase; LTC4S, LTC4 synthase; CDNB, 1-chloro-2,4-dinitrobenzene; PG, prostaglandin; BMCC, bone marrow–derived mast cell; 15R-D2, 15R-methyl-PGD2; 15d-D2, 15-deoxy-Δ12,14-PGD2; 15d-15j, 15-deoxy-Δ12,14-PGJ2; BSO, buthionine sulfoximine; RP, reverse-phase; GS-DNP, glutathionyl-5-dinitrophenyl; PPARγ, peroxisome proliferator-activated receptor-γ; β-ME, β-mercaptoethanol.
Oxidative Stress Suppresses LTC₄ Generation

enolic acid (LTC₄). In addition, X-crystallography shows that Arg-51, Asn-55, Glu-58, Tyr-93, Tyr-97, and Arg-104 of LTC₄S are involved in GSH binding.

Recently, studies on prostaglandin biosynthesis have demonstrated that nitric oxide S-nitrosylates cystolic phospholipase A₂ (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 oxidant 15R-methyl-PGd₂ (15R-D₂) suppressed LTC₄ generation by BMMCs through a reduction in intracellular GSH and suppression of LTC₄S activity. In addition, diamide, a sulfhydryl-reactive oxidant, induced oxidative stress, suppressed LTC₄S activity, and induced the formation of covalent dimer LTC₄S, normally a noncovalent trimer. Importantly, the effect of oxidative stress was abolished in C56S mutant LTC₄S, and the covalent dimer was present in nasal polyps of asthmatics, implying that LTC₄S activity levels may be modified in vivo by oxidation-related dimerization.

EXPERIMENTAL PROCEDURES

Materials—15-Deoxy-Δ¹²,1⁴-15D₂, 15-deoxy-Δ¹²,1⁴-PGF₁α, 15R-D₂, PGF₄α, PGD₂, PG₂, PGE₂, GW 9662, Ramatroban, LTA₄ methyl ester, and BW 245C were from Cayman Chemical (Ann Arbor, MI). Ionomophore 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 used for 6–10 weeks in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM 1-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. The cell density was adjusted to 5 × 10⁶/ml at every passage.

Ionophore-stimulated 5-LO Product Generation—BMMCs were first pelleted by centrifugation and then resuspended in 200 μl of assay buffer (50 mM HEPES and 20 mM MgCl₂, pH 7.6) and lysed by microtip sonication. The cell lysates were incubated with 20 μM LTA₄, 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 mM Tris, 30 mM KH₂PO₄, and 1.5 mM EDTA), and the reactions were initiated with the addition of 50 μM arachidonic acid, 2 mM ATP, and 1 mM CaCl₂ 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, 10 μM Na₂VO₄, 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-LTC₄S IgG (1 μg/ml) for 2 h. After washing, the blot was incubated with horseradish peroxidase-conjugated 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 15R-D₂ on LTC₄S mRNA, we examine the time-dependent changes in LTC₄S mRNA by real-time PCR. Cells were treated with 10 μM 15R-D₂ for 0, 3, 6, and 24 h and harvested, and total RNA were then isolated with TRIzol reagent (Invitrogen). For each experiment, equal amounts of the ratio of the LTC₄S gene product to the 18 S housekeeping gene product was performed. For nasal polyps, the relative LTC₄S mRNA level was determined by real-time PCR (GeneDirect, Princeton, NJ) and by comparing the values of the ratio of the LTC₄S 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 μl) and 3 volumes of methanol containing 400 ng/ml PGB₂, 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/10⁶ 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 LTC₄S activity and GSH levels, respectively.

**Metabolism of PGD₂ and 15R-D₂**—BMMCs were incubated with 10 μM PGD₂ or 10 μM 15R-D₂ for 24 h and then analyzed by RP-HPLC for product profiles. For non-enzymatic conversion of these prostanooids, we incubated PGD₂ and 15R-D₂ 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 LTC₄S 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 LTC₄S activity and molecular size.

**RESULTS**

**PGD₂ and PGE₂ Inhibit LTC₄ Generation in BMMCs**—Like cysteinyl leukotrienes, PGD₂ is abundant in allergic inflammation and a product of activated mast cells. No previous study had addressed whether PGD₂ could modify the generation of LTC₄. In addition, as LTC₄S catalyzes the conjugation of LTA₄ to GSH, we were also interested in determining whether changing intracellular GSH through oxidative stress will affect LTC₄ generation. Because PGD₂ has previously been shown to increase LTC₄ 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 PGD₂ on LTC₄ generation by BMMCs. BMMCs were incubated with 10 μM PGD₂ for 48 h, and for specificity purposes, we also included 10 μM PGE₂ and 10 μM PGE₂ for comparison. After harvesting, cells were stimulated with 2 μM calcium ionophore A23187 for 10 min. As shown in Fig. 1, whereas both PGD₂ and PGE₂ inhibited LTC₄ generation by >70 and 65%, respectively, PGE₂ had no effect. We concentrated on the effect of PGD₂, as it is also a mast cell-derived product. None of these prostanooids affected the biosynthesis of 12-hydroxyheptadecatrienoic acid (data not shown).

**Inhibitory Effect of PGD₂ Does Not Act through the DP₁ or DP₂ Receptor or Peroxisome Proliferator-activated Receptor-γ (PPARγ)**—To investigate the possible receptor involved in the suppression of LTC₄ generation by PGD₂, BMMCs were incubated with the DP₁ agonist BW 245C or the DP₂ agonists 15R-D₂ and 15-deoxy-Δ¹₂,1₄-PGD₂ (15d-D₂; also a PPARγ agonist), and we then examined the ionophore-stimulated generation of 5-LO products. As shown in Fig. 2A, the effect of PGD₂ could be mimicked by the DP₂ agonists 15R-D₂ and 15d-D₂ but not by the DP₁ agonist BW 245C. To further investigate whether the suppressive effect is mediated through the DP₂ receptor or PPARγ, we examined the effect of the DP₂ antagonist Ramatroban and the PPARγ antagonist GW 9662 using 15R-D₂ as an agonist. As shown in Fig. 2B, the effect of 15R-D₂ on LTC₄ generation was not inhibited by the DP₂ antagonist Ramatroban or by the PPARγ antagonist GW 9662, suggesting that the inhibitory effect of PGD₂ or 15R-D₂ is not mediated through DP₁, DP₂, or PPARγ. Because the effect of PGD₂ could be reproduced by 15R-D₂, we performed subsequent experiments using 15R-D₂ as an agonist.

**Time-dependent Effect of 15R-D₂ on the Ability of BMMCs to Generate LTC₄ and LTC₄S Protein or mRNA Expression**—When 15R-D₂ was incubated with BMMCs, it caused a time-dependent decrease in LTC₄ generation when stimulated with the calcium ionophore. When BMMCs were treated with 15R-D₂ for 24 h, there was a >75% reduction in ionophore-stimulated LTC₄ generation (Fig. 3A), with little additional reduction when treated for 48 h (data not shown). Thus, we examined the dose-dependent effect of 15R-D₂ over 24 h of treatment.

To determine whether 15R-D₂ reduced cellular LTC₄S activity through reduced LTC₄S transcription and protein expression, we incubated BMMCs with 10 μM 15R-D₂ for 0, 3, 6, and 24 h. LTC₄S 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), 15R-D₂ did not alter the expression of either the mRNA or immunoreactive LTC₄S protein. This result indicates that 15R-D₂ does not reduce the cellular LTC₄ activity of BMMCs through a reduction in LTC₄S

**Oxidative Stress Suppresses LTC₄ Generation**
gene transcription and protein expression, thus suggesting a post-translational modification of LTC₄S.

Dose-dependent Effect of 15R-D₂—To examine the dose-dependent ability of 15R-D₂ to suppress LTC₄ generation and its effect on 5-LO activity, we incubated BMMCs with various concentrations of 15R-D₂ for 24 h and then performed various assays. As shown in Fig. 4A, 15R-D₂ dose-dependently reduced ionophore-stimulated LTC₄ generation by BMMCs with >90% inhibition at 10 μM 15R-D₂. With 10 μM 15R-D₂, the mean LTC₄ generation by BMMCs was 10.0 ± 2.2% (mean ± S.E., n =...
8) of untreated BMMCs. An IC_{50} of ~2 μM was observed for the inhibition of LTC₄ generation by 15R-D₂. There were no significant changes in all-trans-diastereoisomers of LTB₄ or LTB₄ or 5-hydroxyeicosatetraenoic acid generation (Fig. 2A), suggesting that 15R-D₂ selectively reduces the ability of BMMCs to generate LTC₄ without affecting the 5-LO activity. To confirm that 15R-D₂ did not affect 5-LO enzyme activity, both the 5-LO and LTC₄S activities were measured in BMMC lysates obtained

---

**FIGURE 3. Time-dependent effect of 15R-D₂.** BMMCs were incubated with 10 μM 15R-D₂ for the indicated times, washed, and separated into four aliquots. One aliquot was stimulated with A23187 (2 μM) to determine LTC₄ generation, and a second aliquot was used for LTC₄S activity determination (A). One aliquot was used for immunoreactive LTC₄S protein expression under reducing conditions (B). The last aliquot was used for LTC₄S mRNA expression (C) (mean ± S.E., n = 4). Me, methyl ester.

**FIGURE 4. Dose-dependent effect of 15R-D₂.** Cells were treated with the indicated concentrations of 15R-D₂ (in 2 μl of ethanol) or with an equal volume of vehicle (0 μM 15R-D₂) for 24 h, washed, and split into four aliquots. One aliquot was stimulated with A23187 (2 μM) to examine LTC₄ generation (n = 8) (A), one for LTC₄S 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.
from BMMCs that were treated with various concentrations of 15R-D2 and from untreated cells. As shown in Fig. 4 (B and C), 15R-D2 did not affect cellular 5-LO activity but dose-dependently reduced LTC4S activity. The mean LTC4S activity of 15R-D2-treated BMMCs was 33.5 ± 3.5% (mean ± S.E., n = 8) (Fig. 3B) of the control cells, thus confirming that the reduction in LTC4S activity upon 15R-D2 treatment contributed to the decrease in ionophore-stimulated LTC4 generation. Furthermore, the effect of 15R-D2 on LTC4 generation was not restricted to the calcium ionophore A23187, as 15R-D2 also suppressed LTC4 generation induced by ionomycin and by cross-linking of IgE. 15R-D2-reduced ionomycin (2 μM) stimulated LTC4 generation to 8.6 ± 2.9% (mean ± S.E., n = 10) of the untreated cells. In two experiments, 10 μM 15R-D2 also reduced LTC4 generation induced by cross-linking of IgE to 10.6 and 11.8% of the untreated cells, respectively.

15R-D2 Reduces Intracellular GSH Levels in BMMCs—15R-D2 inhibited LTC4 generation without a significant effect on LTC4S protein expression or 5-LO activity, suggesting that 15R-D2 may affect LTC4 generation by altering the intracellular levels of GSH, the second substrate of the LTA4 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. 4D, 15R-D2 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 15R-D2, the mean GSH level of treated BMMCs was 43.9 ± 5.8% (mean ± S.E., n = 5) of the control cells.

BSO Reduces LTC4 Generation—To confirm that 15R-D2 reduces ionophore-stimulated generation of LTC4 by BMMCs through a reduction in intracellular GSH, we compared the effect of BSO, a glutathione synthase inhibitor, with that of 15R-D2 on ionophore-stimulated LTC4 generation. When BMMCs treated with 15R-D2 were stimulated with ionophore, LTC4S activity was reduced to 15.2 ± 5.9% (mean ± S.E., n = 3) of the control BMMCs. Treatment with BSO reduced LTC4 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-D2—Because cyclopentenone metabolites of PGD2 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 15R-D2 and PGD2 in BMMCs. When PGD2 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 Δ12-PGJ2, 15d-I2, and 15d-D2, respectively, in both the BMMC culture medium (Fig. 5A) and the medium with no BMMCs (Fig. 5B). 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 Δ12-PGJ2 (also monitored at its UV maximum of 244 nm; chromatograph not shown) and 15d-I2 culture media were nearly identically with or without BMMCs, whereas the peak height of 15d-D2 in the medium with BMMCs was 24.9 ± 1.82% (mean ± S.E., n = 3) lower than that with no BMMCs. Similarly, incubation of 15R-D2 with BMMCs and culture medium alone yielded metabolite profiles identical to those of PGD2, with three 15R-methyl derivatives of the corresponding PGD2 metabolites (data not shown). We thus examined whether compound X is derived from 15d-D2 or from 15d-I2. We first incubated 15d-D2 and 15d-I2 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-D2 and 15d-I2 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-D2 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-I2 observed when it was incubated with BMMCs (data not shown). These results suggest that compound X is a PGD2 metabolite derived from the conjugation of 15d-D2 to GSH and that 15R-D2 spontaneously degrades to form 15R-derivatives of 15d-D2, 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-Δ9,12(E)-PGD2 to GSH (31).

Effect of Diamide on LTC4S—Our demonstration that a reduction in intracellular GSH suppresses LTC4S function suggested that oxidative stress may also exert an effect similar to that of 15R-D2. Thus, we utilized the sulfhydryl-reactive oxidant diamide to acutely induce oxidative stress and to examine the enzyme activity of LTC4S. Incubation of diamide with BMMCs for 30 min reduced LTC4S activity by >75% (data not shown). To further study the effect of oxidative stress on LTC4S, we examine the effect of diamide on CHO cells transfected with wild-type and mutant LTC4S cDNAs. When diamide was incubated with CHO cells transfected with wild-type LTC4S cDNA, it reduced LTC4S function to 22.8 ± 3.4% (mean ± S.E., n = 4) of the control cells. In addition, diamide induced the formation of covalent dimer LTC4S in a nonreducing gel (Fig. 7A). In contrast, C56S mutant LTC4S retained >91% of the enzyme activity (n = 2) with diamide treatment and no covalent dimer formation (Fig. 7A). Mutation of the only other cysteine residue (Cys-82) did not protect enzyme activity from diamide treat-
ment and formation of a covalent dimer (Fig. 7A). The effect of diamide was also reversible, as diamide-treated CHO cells that were subsequently incubated with 10 mM β-ME restored LTC₄S activity to 89.2 ± 9.1% (n = 4) of the control enzyme, and there was no covalent dimer formation.

To determine whether oxidative stress similarly affects purified LTC₄S, we incubated purified LTC₄S 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 LTC₄S by >90% (data not shown) as well as the formation of a covalent dimer (Fig. 7B). These results indicate that oxidative stress can reversibly suppress LTC₄S 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 LTC₄S (Fig. 8) under nonreducing conditions, similar to wild-type LTC₄S treated with diamide. In addition, the presence of covalent dimer LTC₄S was also observed in nasal polyps obtained from a normal individual. These results suggest
that dimerization of LTC₄S monomers can occur in vivo under oxidative stress.

DISCUSSION

This study has demonstrated that PGD₂, the DP₂ agonist 15R-D₂, and the PPARγ agonist 15d-J₂ suppress ionophore-stimulated LTC₄ generation. The suppressive effect is a receptor-mediated event, as it was not blocked by either the DP₂ antagonist Ramatroban or the PPARγ antagonist GW 9662 (Fig. 2B), and on the contrary, these antagonists significantly enhanced the suppressive effect of 15R-D₂ on LTC₄ generation. This potentiation effect has not been investigated further. The effect of 15R-D₂ is not mediated through an alteration of transcription or protein expression, as neither was affected by 15R-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 LTC₄S. We demonstrated that the DP₂ agonist 15R-D₂ reduces intracellular GSH in BMMCs (Fig. 4D) and reduces the ability of these cells to synthesize cysteinyl leukotrienes in response to ionophore stimulation as well as suppression of LTC₄S activity through post-translational modification (Fig. 3, A and B, and Fig. 4). The unknown polar metabolite (X) corresponds to that obtained from PGD₂ incubation with BMMCs. mAU, milliabsorbance units.
mechanism by which 15R-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 15R-D_2 was incubated with BMMCs, it non-enzymatically converted to 15R-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 LTC4 generation, whereas PGE_2 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 15R-D_2 (data not shown), thus confirming that the effect of 15R-D_2 is not mediated through an increase in reactive oxygen species generation.

Both 15R-D_2 and oxidative stress induced by diamide caused a possible post-translational modification of LTC4_S, as they suppressed LTC4_S activity without affecting protein expression (Figs. 3 and 7). Diamide also induced the formation of inactive covalent dimer LTC4_S (Fig. 7). The suppressive effect of 15R-D_2 on LTC4_S 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 15R-D_2 and diamide is not known, one possible explanation may be that the cyclopentenone metabolite of 15R-D_2 can directly modify Cys-56 of LTC4_S, an effect that has been reported for the p50 subunit of NF-kB 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 LTC4_S is irreversibly inhibited.

Importantly, our experiments suggest that cellular LTC4_S 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 15R-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 LTC4_S 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 LTC4_S 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.

Acknowledgment—We are thankful to Dr. Joshua Boyce for helpful comments on the manuscript.
Oxidative Stress Suppresses LTC₄ Generation

REFERENCES

1. Samuelsson, B. (1983) Science 220, 568–575
2. Dahlén, S. E., Hedqvist, P., Hammarström, S., and Samuelsson, B. (1980) Nature 288, 484–486
3. Weiss, J. W., Drazen, J. M., Coles, N., McFadden, E. R., Jr., Weller, P. F., Corey, E. J., Lewis, R. A., and Austen, K. F. (1982) Science 216, 196–198
4. Henderson, W. R., Jr., Lewis, D. B., Albert, R. K., Zhang, Y., Lamm, W. J., Chiang, G. K., Jones, F., Vickers, P., Tien, Y. T., Jonas, M., and Chi, E. Y. (1996) J. Exp. Med. 184, 1483–1494
5. Henderson, W. R., Jr., Tang, L. O., Chu, S. J., Tsao, S. M., Chiang, G. K., Jones, F., Jonas, M., Pae, C., Wang, H., and Chi, E. Y. (2002) Am. J. Respir. Crit. Care Med. 165, 108–116
6. Marom, Z., Shelhamer, J. H., Bach, M. K., Morton, D. R., and Kaliner, M. (1982) Am. Rev. Respir. Dis. 126, 449–451
7. Laitinen, L. A., Laitinen, A., Haahtela, T., Vilkka, V., Spur, B. W., and Lee, T. H. (1993) Lancet 341, 989–990
8. Diamant, Z., Hiltermann, J. T., van Rensen, E. L., Callenbach, P. M., Velicer-Charvat, M., van der Veen, H., Sont, I. J. K., and Sterk, P. J. (1997) Am. J. Respir. Crit. Care Med. 155, 1247–1253
9. Dennis, E. A. (1994) J. Biol. Chem. 269, 13057–13060
10. Dixon, R. A., Diehl, R. E., Opas, E., Rands, E., Vickers, P. J., Evans, J. F., Gillard, J. W., and Miller, D. K. (1990) Nature 343, 282–284
11. Broek, T. G., Paine, R., 3rd, and Peters-Golden, M. (1994) J. Biol. Chem. 269, 22059–22066
12. Ford-Hutchinson, A. W., Gresser, M., and Young, R. N. (1994) Annu. Rev. Biochem. 63, 383–417
13. Rouzer, C. A., Matsumoto, T., and Samuelsson, B. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 857–861
14. Funk, C. D., Rådmark, O., Fu, J. Y., Matsumoto, T., Jörnvall, H., Shimizu, T., and Samuelsson, B. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 6677–6681
15. Penrose, I. F., Gagnon, L., Goppelt-Stuwe, M., Myers, P., Lam, B. K., Jack, R. M., Austen, K. F., and Soberman, R. J. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 11603–11606
16. Nicholson, D. W., Ali, A., Vaillancourt, J. P., Calaycay, J. R., Mumford, R. A., Zamboni, R. J., and Ford-Hutchinson, A. W. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2015–2019
17. Lam, B. K., Penrose, J. F., Freeman, G. J., and Austen, K. F. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 7663–7667
18. Jakobsson, P. J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A., and Persson, B. (1999) Protein Sci. 8, 689–692
19. Hevko, J. M., and Murphy, R. C. (2002) J. Biol. Chem. 277, 7037–7043
20. Ago, H., Kanaoka, Y., Irikura, D., Lam, B. K., Shimamura, T., Austen, K. F., and Miyano, M. (2007) Nature 448, 609–612
21. Martinez Molina, D., Wetterholm, A., Kohl, A., McCarthy, A. A., Niegoswki, D., Ohlson, E., Hammarberg, T., Eshaghi, S., Haeggström, J. Z., and Nordlund, P. (2007) Nature 448, 613–616
22. Xu, L., Han, C., Lim, K., and Wu, T. (2008) J. Biol. Chem. 283, 3077–3087
23. Kim, S. F., Huri, D. A., and Snyder, S. H. (2005) Science 310, 1966–1970
24. Hatzelmann, A., and Ullrich, V. (1987) Eur. J. Biochem. 169, 175–184
25. Werz, O., Bürkert, E., Fischer, L., Szelas, D., Dishart, D., Samuelsson, B., Rådmark, O., and Steinihilber, D. (2002) FASEB J. 16, 1441–1443
26. Luo, M., Jones, S. M., Phare, S. M., Coffey, M. J., Peters-Golden, M., and Brock, T. G. (2004) J. Biol. Chem. 279, 41512–41520
27. Werz, O., Klemm, J., Samuelsson, B., and Rådmark, O. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 5261–5266
28. Kanaoka, Y., Maekawa, A., Penrose, J. F., Lam, B. K., and Austen, K. F. (2001) J. Biol. Chem. 276, 22608–22613
29. Mesquita-Santos, F. P., Vieira-de-Abreu, A., Calheiros, A. S., Figueiredo, I. H., Castro-Faria-Neto, H. C., Weller, P. F., Bozza, P. T., Diaz, B. L., and Bandeira-Melo, C. (2006) J. Immunol. 176, 1326–1330
30. Kim, E. H., and Surh, Y. J. (2006) Biochem. Pharmacol. 72, 1516–1528
31. Atsmon, J., Freeman, M. L., Meredith, M. J., Sweetman, B. J., and Roberts, L. J., 2nd (1992) Cancer Res. 52, 1879–1885
32. Swindle, E. J., Hunt, J. A., and Coleman, J. W. (2002) J. Immunol. 169, 5866–5873
33. Adamjee, J., Suh, Y. J., Park, H. S., Choi, J. H., Penrose, J. F., Lam, B. K., Austen, K. F., Cazaly, A. M., Wilson, S. J., and Sampson, A. P. (2006) J. Pathol. 209, 392–399
34. Fratelli, M., Goodwin, L. O., Ørom, U. A., Lombardi, S., Tonelli, R., Mengozzi, M., and Ghezzi, P. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 13998–14003
35. Hadzic, T., Li, L., Cheng, N., Walsh, S. A., Spitz, D. R., and Knudson, C. M. (2005) J. Immunol. 175, 7965–7972
36. Banu, S. K., Lee, J., Satterfield, M. C., Spencer, T. E., Bazer, F. W., and Arosh, J. A. (2008) Endocrinology 149, 219–231
37. Okuno, T., Iizuka, Y., Okazaki, H., Yokomizo, T., Taguchi, R., and Shimizu, T. (2008) J. Exp. Med. 205, 759–766
38. Cernuda-Morollón, E., Pineda-Molina, E., Cañada, F. J., and Pérez-Sala, D. (2001) J. Biol. Chem. 276, 35530–35536