0026-895X/03/6305-1075–1081
MOLECULAR PHARMACOLOGY
U.S. Government work not protected by U.S. copyright
Mol Pharmacol 63:1075–1081, 2003

Vol. 63, No. 5
2061/1058559
Printed in U.S.A.

Regulation of Activator Protein-1 by 8-iso-Prostaglandin E_2 in a Thromboxane A_2 Receptor-Dependent and -Independent Manner

THOMAS J. WEBER and LYE M. MARKILLIE

Cell Biology, Pacific Northwest National Laboratory, Richland, Washington

Received August 7, 2002; accepted January 30, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The thromboxane (TX) A_2 receptor (TP) encompasses two alternatively spliced forms, termed the platelet/placental (TP-P) and endothelial (TP-E) type receptors. Experimental evidence suggests that TP activity may be modulated by novel ligands, termed the isoprostanes, that paradoxically act as TP agonists in smooth muscle and TP antagonists in platelet preparations. Here we have investigated whether prototypical isoprostanes 8-iso-prostaglandin (PG)$F_{2\alpha}$ and 8-iso-PGE_2 regulate the activity of TP isoforms expressed in Chinese hamster ovary (CHO) cells using activator protein-1 (AP-1)-luciferase activity as a reporter. AP-1–luciferase activity was increased by a TP agonist [9,11-dideoxy-9α,11α-methanoepoxy $PGF_{2\alpha}$ (U46619)] in CHO cells transfected with the human TP-P and TP-E receptors, and this response was fully inhibited by TP antagonists [1*S*-[1α,2β(*Z*),3α,5α]]-7-[3-[[4-iodophenyl)sulfonyl]amino]-6,6-dimethylbicyclo[3.1.1]hept-2-yl]-5-heptenoic acid (I-SAP) and [1S-[1α,2α(*Z*),3α,4α]]-7-[[2-[(phenylamino) carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ 29,548)]. AP-1–luciferase activity was potently (nanomolar concentrations) increased by 8-iso-PGE_2 in CHO TP-P and TP-E cells, and this response was partially inhibited by cotreatment of cells with TP antagonists, whereas 8-iso-$PGF_{2\alpha}$ was without effect. Cyclooxygenase inhibitors did not abolish 8-iso-PGE_2 mediated AP-1–luciferase activity, indicating that this response is not dependent on de novo TXA_2 biosynthesis. Interestingly, 8-iso-PGE_2-mediated AP-1–luciferase activity was near maximal in naive cells between 1 and 10 nM concentrations, and this response was not inhibited by TP antagonist or reproduced by agonists for TP or EP_1/EP_3 receptors. These observations 1) support a role for novel ligands in the regulation of TP-dependent signaling, 2) indicate that TP-P and TP-E couple to AP-1, 3) provide further evidence that isoprostanes function as TP agonists in a cell-type specific fashion, and 4) indicate that additional targets regulated by 8-iso-PGE_2 couple to AP-1.

Oxygen free radicals have been implicated in the pathophysiology of a number of human diseases, including cancer, atherosclerosis, neurodegenerative disorders, and aging. Lipid peroxidation is a central feature associated with oxidative stress, and various methods have been used to quantify lipid damage (Halliwell and Grootveld, 1987). In particular, a unique series of prostaglandin-like compounds, termed the isoprostanes, have been identified as products of the peroxidation of arachidonic acid catalyzed by oxygen free radicals (Longmire et al., 1994; Morrow and Roberts, 1997; Rokach et al., 1997). Since their initial discovery, numerous reports have demonstrated the formation of isoprostanes in vivo and in vitro (for review, see Longmire et al., 1994; Morrow and Roberts, 1997; Rokach et al., 1997).

The isoprostanes are structurally similar to the prostaglandins but differ in the orientation of the fatty acid side chains; prostaglandins have *trans*-oriented side chains and isoprostanes have *cis*-oriented side chains (Morrow and Roberts, 1997). Although a number of isoprostane chemistries have been defined, research interests have primarily focused on prototypical isoprostanes, whose levels are significantly increased in response to oxidant injury. One of the abundant detectable isoprostanes is similar to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and is termed 15-F(2t)-isoprostane [more commonly referred to as 8-iso-$PGF_{2\alpha}$]. There are also several stereochemistries related to 8-iso-$PGF_{2\alpha}$ that are broadly termed the F-series isoprostanes (Morrow and Roberts, 1996). In addition, E-series isoprostanes (containing the E-prostane

This research was supported by a grant from the Low Dose Radiation Research Program, Office of Biological and Environmental Research, United States Department of Energy (DOE) (to T.J.W.). Pacific Northwest National Laboratory is operated for the DOE by Battelle Memorial Institute under Contract DE-AC06-76RLO 1830.

ABBREVIATIONS: PG, prostaglandin; TX, thromboxane; TP, thromboxane A_2 receptor; TP-E, endothelial thromboxane A_2 receptor; PKC, protein kinase C; AP-1, activator protein-1; TPA, 12-O-tetradecanoyl phorbol-13-acetate; U46619, 9,11-dideoxy-9a,11a-methanoepoxy prostaglandin $F_{2\alpha}$; I-SAP, [1*S*-[1α,2β(*Z*),3α,5α]]-7-[3-[[4-iodophenyl)sulfonyl]amino]-6,6-dimethylbicyclo[3.1.1]hept-2-yl]-5-heptenoic acid; SQ 29,548, [1*S*-[1α,2α(*Z*),3α,4α]]-7-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo(2.2.1)hept-2-yl]-5-heptenoic acid; CHO, Chinese hamster ovary; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium.

rings) are also formed in vivo and in vitro in response to an oxidative stress (Morrow et al., 1998) and 8-iso-PGE_2 is used as a prototypical E-series isoprostane. It is noteworthy that 8-iso-$PGF_{2\alpha}$ and 8-iso-PGE_2 are potent renal vasoconstrictors (Habig et al., 1974; Takabashi et al., 1992) and stimulate endothelial cell proliferation (Yura et al., 1999), raising the possibility that these products of lipid peroxidation may actively participate in biological processes.

Prostanoid receptors (i.e., heptahelical G-protein coupled receptors for prostaglandins and thromboxanes) have been implicated as candidate isoprostane receptors (Fukunaga et al., 1993; Fukunaga et al., 1997; Janssen and Tazzeo, 2002; Tintut et al., 2002), and pharmacological evidence suggests that a unique isoprostane receptor may also exist (Fukunaga et al., 1993, 1997). Within the context of prostanoid receptors, significant effort has been directed at understanding the regulation of thromboxane (TX) A_2 receptor (TP) activity by isoprostanes. Interestingly, isoprostanes seem to paradoxically function as agonists for smooth muscle TP and as antagonists for platelet TP (Longmire et al., 1994). The mechanisms underlying this contradictory activity are not known.

Radioligand-binding studies have suggested the existence of multiple TP subtypes (Takahara et al., 1990; Ko et al., 1995; Ko, 1997). Consistent with binding studies, TP cloning efforts have identified a single gene (Abramovitz et al., 1995) but two alternative splice variants, termed the platelet/placental (TP-P or TPα) and -endothelial (TP-E or TPβ) type receptors (Raychowdhury et al., 1994; Parent et al., 1999). Alternative splicing occurs selectively in the carboxyl terminus and confers association with different G-proteins, supporting the experimental finding that these receptors couple to both common and unique signaling pathways (Hirata et al., 1996). TP-related signal transduction is consistently associated with calcium mobilization, inositol phospholipid turnover, and activation of protein kinase C (PKC) (Armstrong and Wilson, 1995; Sachinidis et al., 1995; Ohkubo et al., 1996; Karim et al., 1997). We have demonstrated that a renal proximal tubular TP couples to redox-responsive transcription factors, including activator protein-1 (AP-1) and nuclear factor κB (Weber et al., 1997, 2000). Renal AP-1 activity was also increased by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), a PKC activator, and a PKC inhibitor abolished both phorbol ester- and TP-dependent AP-1 activity (Weber et al., 1997, 2000). Collectively, these observations suggest that the renal TP regulates AP-1 via phorbol ester-sensitive PKC isoforms.

Emerging clinical evidence raises the possibility that TP activity may be modulated by novel agonists. Specifically, increased nonenzymatic formation of isoprostanes was suggested to provide a biochemical link between altered oxidant/antioxidant balance and the synthesis of a TXA_2-like activity that was not inhibited by aspirin but was inhibited by vitamin E (Cipollone et al., 2002). Furthermore, a TXA_2-like activity that induces contraction of the human saphenous vein through interaction with the TP, but does not seem to be synthesized through the conventional cyclooxygenase pathway, seems to contribute to temperature-dependent basal tone (Simonet et al., 2002). In this study, we examined whether prototypical isoprostanes (8-iso-$PGF_{2\alpha}$ or 8-iso-PGE_2) regulate AP-1 activity via the cloned human TP receptor alternative splice variants to determine whether oxidized lipids may provide a biochemical link to novel TP-dependent signaling in physiological and pathophysiological processes.

Materials and Methods

Materials. U46619, I-SAP, SQ 29,548, sulprostone, 8-iso-PGE_2, and 8-iso-$PGF_{2\alpha}$ were obtained from Cayman Chemical (Ann Arbor, MI). LipofectAMINE was purchased from Invitrogen (Carlsbad, CA). pTRE-luciferase was obtained from Stratagene (La Jolla, CA). Luciferase assay kit was from Promega (Madison, WI). All other chemicals were from Sigma Chemical (St. Louis, MO).

Cell Culture. Chinese hamster ovary (CHO) cells were maintained in DMEM/Ham's F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals; Norcross, GA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml amphotericin B in 5% CO_2/95% air at 37°C. CHO cells transfected with the human TP-P and TP-E were the kind gift of Dr. Anthony Ware (Beth Israel Hospital, Harvard Medical School, Boston MS). Vector control groups represent cells that were stably transfected with pcDNA1/Neo (Invitrogen). Cells were subcultured by trypsinization.

Transient Transfection Assay. CHO cells were seeded in 24-well plates (3×10^4 cells/well) and maintained for 24 h. Cells were then serum-starved (0.1% FBS/DMEM/Ham's F12) for 24 h, transfected with the pTRE-luciferase reporter vector using LipofectAMINE reagent (0.2 μg DNA/well; 1 μl of LipofectAMINE/well in a total volume of 140 μl/well) in serum-free media for 3 h and subsequently maintained in 0.1% FBS DMEM/Ham's F12 for an additional 16 h. Cells were then treated with test agents at the indicated concentrations for 6 h and luciferase activity measured using a luciferase assay kit and a microplate luminometer (Luminoskan Ascent; LabSystems, Helsinki, Finland). Luciferase activity was normalized to protein and results are expressed as fold induction.

Statistics. Individual comparisons were made using the Student's *t* test or analysis of variance with a post hoc Student-Newman-Keul test, as appropriate. The $p < 0.05$ level was accepted as significant.

Results

Experimental evidence suggests that the TP couples to AP-1 in renal proximal tubule epithelial cells via PKC-related signal transduction (Weber et al., 1997, 2000). Initially, we characterized AP-1–luciferase activity in CHO cells stably transfected with the TP-P and TP-E receptors (henceforth referred to as CHO TP-P or TP-E cells) to determine 1) whether TXA_2-related pharmacology was behaving in a predictable fashion and 2) whether both TP isoforms coupled to AP-1 in the CHO model. AP-1–luciferase activity was significantly increased in CHO TP-P and TP-E cells treated with 100 nM U46619 (TP agonist), and this response was fully inhibited by a TP antagonist (SQ 29,548; Fig. 1). Similar results were observed when a structurally distinct TP antagonist (I-SAP) was substituted for SQ 29,548 (data not shown). Therefore, TXA_2-related pharmacology is predictable in the CHO model and both TP isoforms seem to couple to AP-1.

AP-1–luciferase activity was significantly increased in CHO TP-P and TP-E cells treated for 6 h with 100 nM 8-iso-PGE_2, but not 8-iso-$PGF_{2\alpha}$ (Fig. 2). Similar results were observed over a range of 8-iso-$PGF_{2\alpha}$ concentrations (1–1000 nM; data not shown). Importantly, 8-iso-PGE_2-mediated AP-1–luciferase activity was clearly detected at low (nanmolar) concentrations in CHO TP-P and TP-E cells (Fig. 3). A weak but dose-dependent increase of AP-1–luciferase activity

(ranging from 1- to 2-fold) was observed in stable vector control cells treated with 1–100 nM 8-iso-PGE_2 but not 8-iso-$PGF_{2\alpha}$ (data not shown). This weak increase of AP-1 will be discussed within the context of 8-iso-PGE_2-mediated AP-1 activity detected in naive CHO cells (shown in Figs. 6 and 7). To determine whether 8-iso-PGE_2-mediated AP-1 luciferase activity was dependent on the TP, cells were pretreated with a TP antagonist (1 μM SQ 29,548) for 30 min, subsequently cotreated with 10 nM 8-iso-PGE_2 and 1 μM SQ 29,548 for 6 h, and the cells processed for measurements of AP-1–luciferase activity as described under *Materials and Methods*. SQ 29,548 treatment significantly reduced but did not fully inhibit the AP-1 luciferase response to 8-iso-PGE_2 in CHO TP-P and TP-E cells (Fig. 4). Similar results were observed using the TP antagonist I-SAP (data not shown).

In some experimental systems, 8-iso-$PGF_{2\alpha}$ has been reported to modulate TP-dependent signaling via the up-regulation of de novo TXA_2 biosynthesis (Hou et al., 2002; Opere et al., 2002). We therefore investigated whether this regulation also occurred in response to 8-iso-PGE_2 treatment. TXA_2 biosynthesis requires cyclooxygenase activity, and we have used aspirin and indomethacin to inhibit cyclooxygenase activity in prior studies (Towndrow et al., 2000). CHO TP-E cells were pretreated for 30 min with 10 μM indomethacin or 100 μM aspirin, subsequently treated with 10 nM 8-iso-PGE_2 for 6 h, and processed for measurements of AP-1–luciferase activity as described under *Materials and Methods*. 8-iso-PGE_2 treatment increased AP-1–luciferase activity, and this response was not inhibited by aspirin or indomethacin (Fig. 5). These observations suggest that 8-iso-PGE_2 does not modulate TP-dependent signaling via the up-regulation of de novo TXA_2 biosynthesis.

Interestingly, treatment of naive CHO cells with 100 nM 8-iso-PGE_2, but not 8-iso-$PGF_{2\alpha}$ or U46619, was associated with a significant increase of AP-1–luciferase activity, and this response was not inhibited by SQ 29,548 (Fig. 6). 8-iso-PGE_2-mediated AP-1–luciferase activity was at an apparent maximum between 1 and 10 nM concentrations in naive cells (Fig. 7), suggesting that a second cellular activity coupled to AP-1 may be activated at lower concentrations of 8-iso-PGE_2, relative to the TP-dependent activation of AP-1 (compare with Fig. 3). Sulprostone is a prostaglandin E_2 receptor agonist with selectivity for the EP_1/EP_3 receptor subtypes. Treatment of naive and CHO TP-P and TP-E cells for 6 h with 100 nM sulprostone did not increase AP-1–luciferase activity (data not shown), suggesting that the TP antagonist-insensitive regulation of AP-1 by 8-iso-PGE_2 does not correlate with EP_1/EP_3 receptor pharmacology.

We have correlated TP-dependent AP-1 activity with phorbol ester-sensitive signal transduction in renal proximal tubule epithelial cells (Weber et al., 1997, 2000). We therefore determined whether phorbol ester increased AP-1 activity in CHO cells. Treatment of CHO cells with 10 ng/ml TPA for 6 h was associated with a marginal increase of AP-1–luciferase activity in CHO TP-P, TP-E, and naive cells (Fig. 8). As a positive and negative control, cells were treated with a TP agonist (U46619), which resulted in a dramatic increase of AP-1–luciferase activity in CHO TP-P and TP-E but not naive cells. TPA-mediated AP-1–luciferase activity was not increased further by higher TPA concentration (100 ng/ml; data not shown).

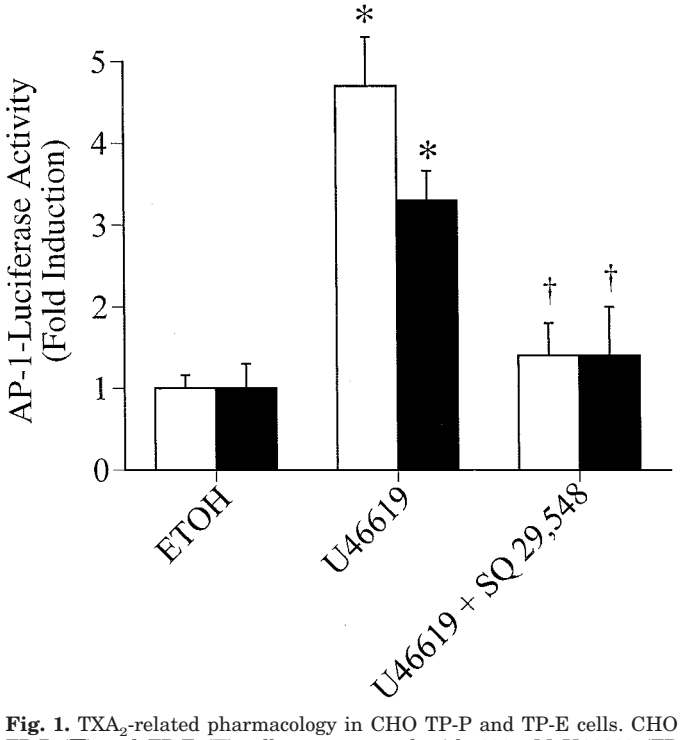

Fig. 1. TXA_2-related pharmacology in CHO TP-P and TP-E cells. CHO TP-P (□) and TP-E (■) cells were treated with 100 nM U46619 (TP agonist) in the presence and absence of 1 μM SQ 29,548 (TP antagonist) for 6 h and processed for measurements of AP-1–luciferase activity as described under *Materials and Methods*. Values represent the mean ± S.E. ($n = 3$). *, Significantly different from respective vehicle control, $p < 0.05$. †, Significantly different from respective U46619-treated cells, $p < 0.05$. Similar results were observed in two separate experiments.

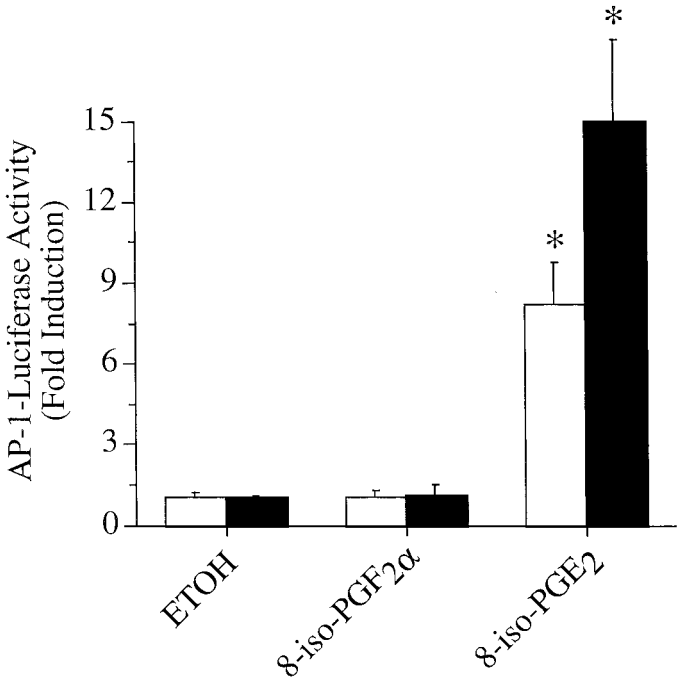

Fig. 2. AP-1–luciferase activity is increased by 8-iso-PGE_2, but not 8-iso-$PGF_{2\alpha}$ in CHO TP-P and TP-E cells. CHO TP-P (□) and TP-E (■) cells were treated with ETOH, 100 nM 8-iso-PGE_2, or 100 nM 8-iso-$PGF_{2\alpha}$ for 6 h and processed for measurements of AP-1–luciferase activity as described under *Materials and Methods*. Values represent the mean ± S.E. ($n = 3$). *, Significantly different from respective vehicle control, $p < 0.05$. Similar results were observed in two separate experiments.

8-iso-PGE_2 seems to function as a TP agonist in rat vascular smooth muscle cells but as a TP antagonist in rat and human platelets (Longmire et al., 1994). Because prostaglandins and related compounds can exhibit nonspecific activities in vitro, particularly at elevated concentrations, we chose to validate our primary observation that 8-iso-PGE_2 was acting as an agonist by cotreating CHO TP-P and TP-E cells with submaximal concentrations of test agent (10 nM U46619 and 1–10 nM 8-iso-PGE_2). The concentrations of 8-iso-PGE_2 chosen are within the linear range of the AP-1 assay as shown in Fig. 3 and are well below concentrations (i.e., ≥ 1 μM) capable of eliciting nonspecific actions. Treatment of cells with U46619 was associated with increased AP-1–luciferase activity as previously observed (compare 0 concentration groups in Fig. 9), and cotreatment of cells with 1 to 10 nM 8-iso-PGE_2 further increased AP-1–luciferase activity. These observations support the notion that 8-iso-PGE_2 is behaving as a TP agonist in CHO TP-P and TP-E cells.

Discussion

We have examined whether prototypical isoprostanes modulate TP-dependent signal transduction. The data indicate that 8-iso-PGE_2 is a TP agonist for both TP-P and TP-E isoforms expressed in CHO cells, whereas 8-iso-$PGF_{2\alpha}$ is not a TP agonist. Collectively, these observations 1) support a role for novel ligands in the regulation of TP-dependent signaling, 2) indicate that both the TP-P and TP-E couple to AP-1, 3) provide further evidence that isoprostanes function as TP agonists in a cell-type specific fashion, and 4) suggest that additional targets regulated by 8-iso-PGE_2 couple to AP-1.

8-iso-PGE_2 seems to function as a TP agonist in rat vascular smooth muscle cells but as a TP antagonist in rat and human platelets (Longmire et al., 1994). Experimental evidence indicating that 8-iso-PGE_2 is a TP agonist in CHO cells include: 1) 8-iso-PGE_2 robustly increased AP-1 activity only in cells expressing the cloned TP isoforms (Figs. 3 and 7), 2) the regulation of AP-1 by 8-iso-PGE_2 in CHO TP-P and TP-E, but not naive cells, was inhibited by TP antagonists (Fig. 4), 3) 8-iso-PGE_2, rather than inhibiting U46619-mediated AP-1–luciferase activity, enhanced this response (Fig. 9), and 4) 8-iso-PGE_2-mediated AP-1–luciferase activity could not be accounted for by de novo TXA_2 biosynthesis (Fig. 5). Therefore, the CHO model is useful for investigating the agonist activity of 8-iso-PGE_2 for TP isoforms and has the advantage that the TP-independent regulation of AP-1 can be cleanly dissociated from the apparent TP-dependent regulation of AP-1 observed in naive cells (Fig. 6).

There is speculation that the TP in platelets and smooth muscle is different and this unique cell type-specific property may account for the paradoxical agonist/antagonist activities of prototypical isoprostanes (Longmire et al., 1994). In CHO cells, the dose-response curve for the regulation of AP-1 by 8-iso-PGE_2 was comparable for both TP isoforms, suggesting that alternative splicing does not afford a competitive advantage to a particular splice variant. TP isoforms are associated with unique properties (e.g., differential receptor desensitization; Yukawa et al., 1997); therefore, the potential exists that differences in the ratio of TP-P to TP-E expression patterns could contribute to cell-type specific differences. In

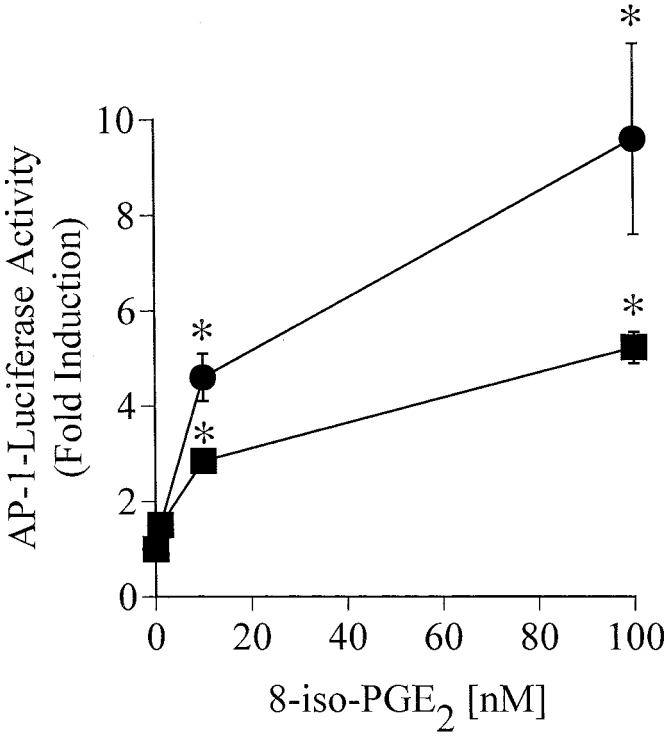

Fig. 3. Dose-dependent regulation of AP-1–luciferase activity by 8-iso-PGE_2. CHO TP-P (●) and TP-E (■) cells were treated with 1 to 100 nM 8-iso-PGE_2 for 6 h and processed for measurements of AP-1–luciferase activity as described under *Materials and Methods*. *, $p < 0.05$, significantly different from respective vehicle control. Values represent the mean ± S.E. ($n = 3$). Similar results were observed in three separate experiments.

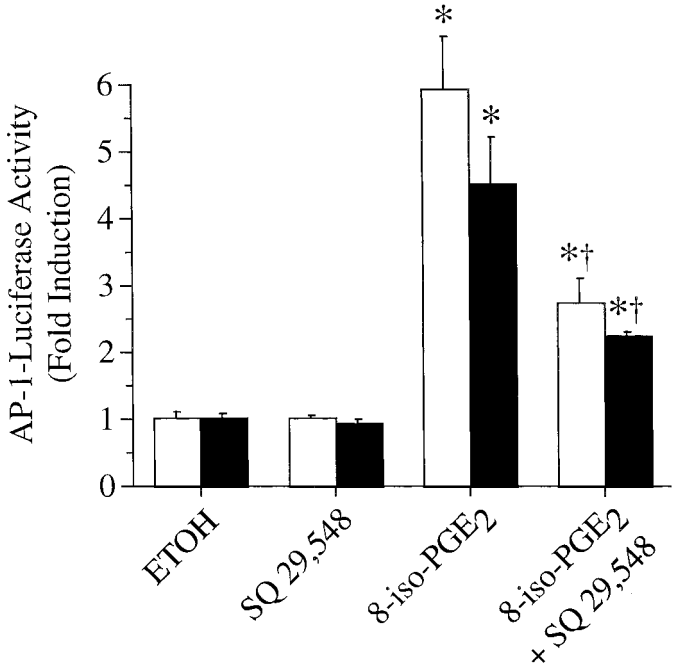

Fig. 4. Inhibition of 8-iso-PGE_2-mediated AP-1–luciferase activity by a TP antagonist (SQ 29,548). CHO TP-P (□) and TP-E (■) cells were pretreated for 30 min with 1 μM SQ 29,548, subsequently cotreated with 10 nM 8-iso-PGE_2 and 1 μM SQ 29,548 for 6 h, and processed for measurements of AP-1–luciferase activity as described under *Materials and Methods*. Values represent the mean ± S.E. ($n = 3$). *, $p < 0.05$, significantly different from respective vehicle control. †, $p < 0.05$, significantly different from respective 8-iso-PGE_2-treated cells. Similar results were observed in two separate experiments.

our studies, however, both TP isoforms coupled to AP-1; therefore, it seems unlikely that overexpression of a particular TP isoform would confer a response diametrically opposed to that of a target ligand, unless the molecular readout of TP activity used here (AP-1) does not accurately reflect TP activity in other model systems.

Alternatively, the TP agonist activity of 8-iso-PGE_2 in vascular smooth muscle preparations may be attributed to the expression of a unique isoprostane receptor, thereby dissociating the actions of 8-iso-PGE_2 from the TP (Longmire et al., 1994). Because the putative isoprostane receptor exhibits higher affinity for the prototypical isoprostanes and couples to signal transducers common to the TP (Fukunaga et al., 1993), this receptor would appear as a TP-like receptor but would have a competitive advantage. Although this interpretation is intriguing in light of the apparent TP-independent regulation of AP-1 by 8-iso-PGE_2 (Fig. 6) that occurs at lower concentrations relative to the TP-dependent regulation of AP-1 (Fig. 7), this observation is not cohesive with the observed TP agonist activity of 8-iso-PGE_2 in CHO cells. Therefore, an alternative explanation to account for results observed in the CHO model within the context of isoprostane actions in platelet and smooth muscle preparations is warranted. One possible alternative explanation is that the isoprostanes activate a number of cellular receptors, thereby eliciting cell type-specific responses based on the complement of target receptors in the model under investigation. In support of this possibility, the activity of 8-iso-PGE_2 at the cellular level has been associated with a number of prostanoid receptors, including the inositol phospholipid coupled TP/EP_3 receptors (Janssen and Tazzeo, 2002; Fig. 4), as well as the cyclic AMP-coupled EP_2 receptor (Tintut et al., 2002). In addition, we provide evidence for a TP-independent regulation of AP-1 by 8-iso-PGE_2 in naive CHO cells (Fig. 6) that does not correlate with prostanoid TP or EP receptor pharmacology. Furthermore, isoprostane treatment in some systems is associated with the up-regulation of de novo thromboxane biosynthesis (Hou et al., 2002; Opere et al., 2002), indicating that the isoprostanes also induce secondary effects, which in turn contribute to their associated biological activities. Therefore, the cellular response to isoprostanes probably results from a complex interplay between primary signaling pathways directly activated by the isoprostanes, and the cell type-specific secondary pathways activated via autocrine loops and signal transduction cross-talk. Any number of combinations could account for cell type-specific differences to isoprostane treatment.

As indicated above, we observed a putative TP-independent regulation of AP-1 by 8-iso-PGE_2 in naive cells that was near maximal at 1 nM concentration (Fig. 7). The relative fold induction of this activity was comparable with that of the TP antagonist-insensitive AP-1 activity in CHO TP-P and TP-E cells (compare 8-iso-PGE_2 + SQ 29,548 group in Fig. 4 with that in Fig. 7). Therefore, we speculate that a novel cellular activity coupled to AP-1 is present in CHO cells and is regulated by 8-iso-PGE_2 at concentrations lower than those required for the TP-dependent regulation of AP-1 by this isoprostane. Whether this activity represents the putative isoprostane receptor (Long-

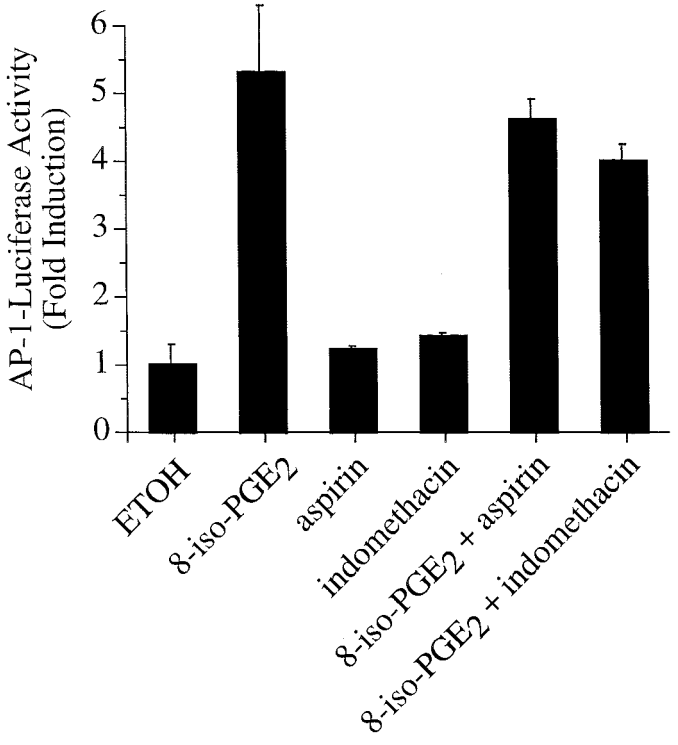

Fig. 5. Effect of cyclooxygenase inhibitors on 8-iso-PGE_2-mediated AP-1–luciferase activity. CHO TP-E cells were pretreated for 30 min with 10 μM indomethacin or 100 μM aspirin and subsequently treated with 10 nM 8-iso-PGE_2 for 6 h. Cells were processed for measurements of AP-1–luciferase activity as described under *Materials and Methods*. Values represent the mean ± S.E. (n = 3). Similar results were observed in two separate experiments.

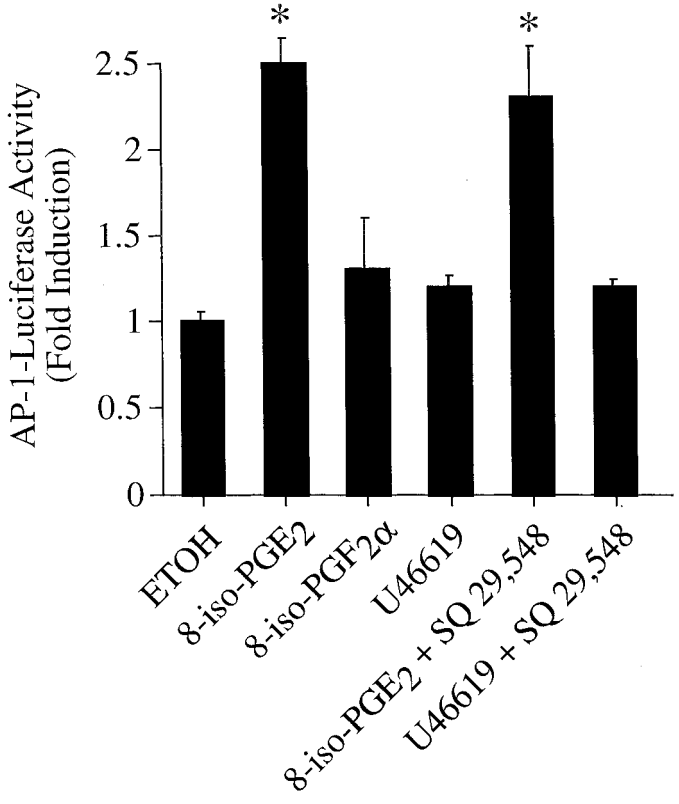

Fig. 6. 8-iso-PGE_2-mediated AP-1–luciferase activity in naive CHO cells. Naive CHO cells were treated with 100 nM 8-iso-PGE_2, 8-iso-$PGF_{2\alpha}$, or U46619 (TP agonist) in the presence and absence of 1 μM SQ 29,548 (TP antagonist) for 6 h and processed for measurements of AP-1–luciferase activity as described under *Materials and Methods*. Values represent the mean ± S.E. (n = 3). *, $p < 0.05$, significantly different from vehicle control. Similar results were observed in three separate experiments.

mire et al., 1994) or some other target cannot be determined from the present studies. It is important to note that the weak increase of AP-1–luciferase activity by 8-iso-PGE_2 in naive cells is probably under-represented. Specifically, the regulation of AP-1 transcriptional complexes by PKC isoforms is widely recognized (Curran, 1992; Forrest and Curran, 1992). PKC, in turn, is activated by products of inositol phospholipid metabolism such as diacylglycerol, for which phorbol esters are used as surrogates (Nishizuka, 1992). Because 10 to 100 ng/ml TPA is typically associated with a robust activation of PKC and AP-1 in diverse cell types (Weber et al., 1997; Chang et al., 2002), the weak increase of AP-1 activity by TPA suggests that the CHO model is suboptimal for investigating inositol phospholipid- and phorbol ester-sensitive AP-1 activity. This is significant because 1) the TP-dependent regulation of AP-1 in renal epithelial cells correlates with phorbol ester-sensitive signal transduction (Weber et al., 1997, 2000), 2) the TP is known to activate phorbol ester-sensitive PKC isoforms (Ko, 1997; Yukawa et al., 1997), and 3) the isoprostanes increase inositol phospholipid turnover (Kunapuli et al., 1998; Yura et al., 1999; Leitinger et al., 2001). Alternatively, because AP-1 activity was significantly increased by TP agonist despite the weak response to phorbol ester, it seems that the TP can couple to AP-1 via multiple signaling pathways in different cell types.

8-iso-$PGF_{2\alpha}$ did not increase AP-1 activity in CHO TP-P and TP-E cells (Fig. 2), and this observation is consistent with pharmacological evidence demonstrating that 8-iso-

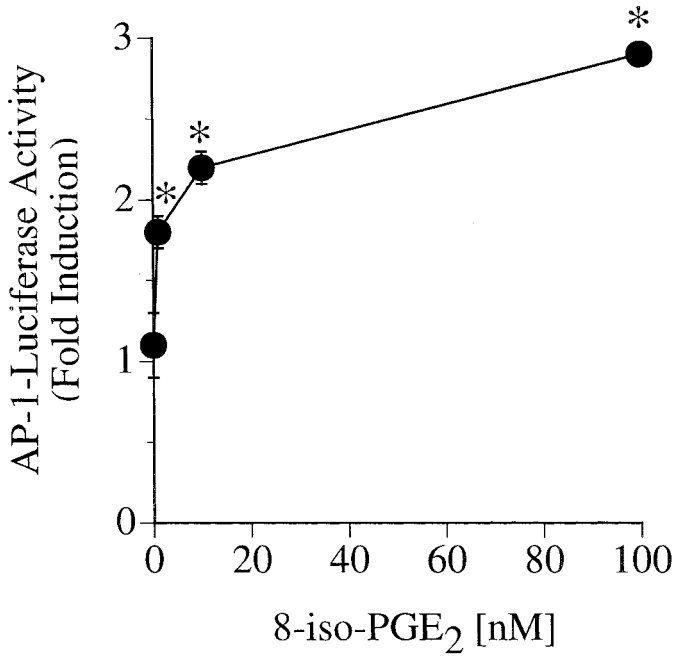

Fig. 7. Dose-dependent regulation of AP-1–luciferase activity by 8-iso-PGE_2 in naive CHO cells. Naive CHO cells were treated with 1 to 100 nM 8-iso-PGE_2 for 6 h and processed for measurements of AP-1–luciferase activity as described under *Materials and Methods*. *, $p < 0.05$, significantly different from vehicle control. Values represent the mean ± S.E. ($n = 3$). Similar results were observed in two separate experiments.

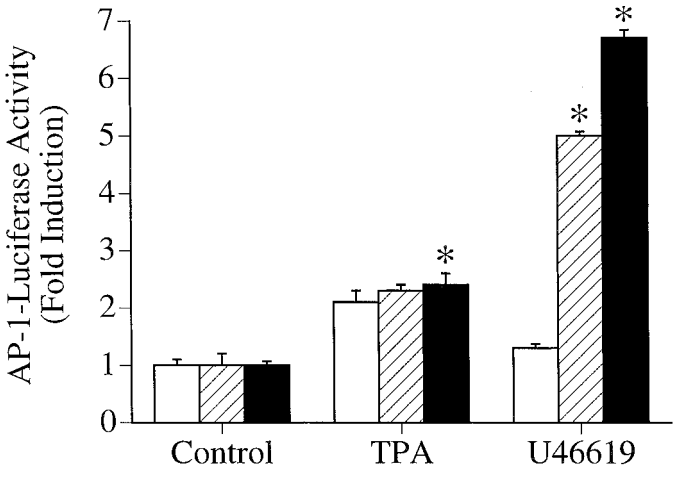

Fig. 8. Regulation of AP-1–luciferase activity by U46619 and TPA. Naive (□) TP-P (▨), and TP-E (■) CHO cells were treated with vehicle, 100 nM U46619, or 10 ng/ml TPA for 6 h and processed for measurements of AP-1–luciferase activity as described under *Materials and Methods*. *, $p < 0.05$, significantly different from vehicle control. Values represent the mean ± S.E. ($n = 3$). Similar results were observed in two separate experiments.

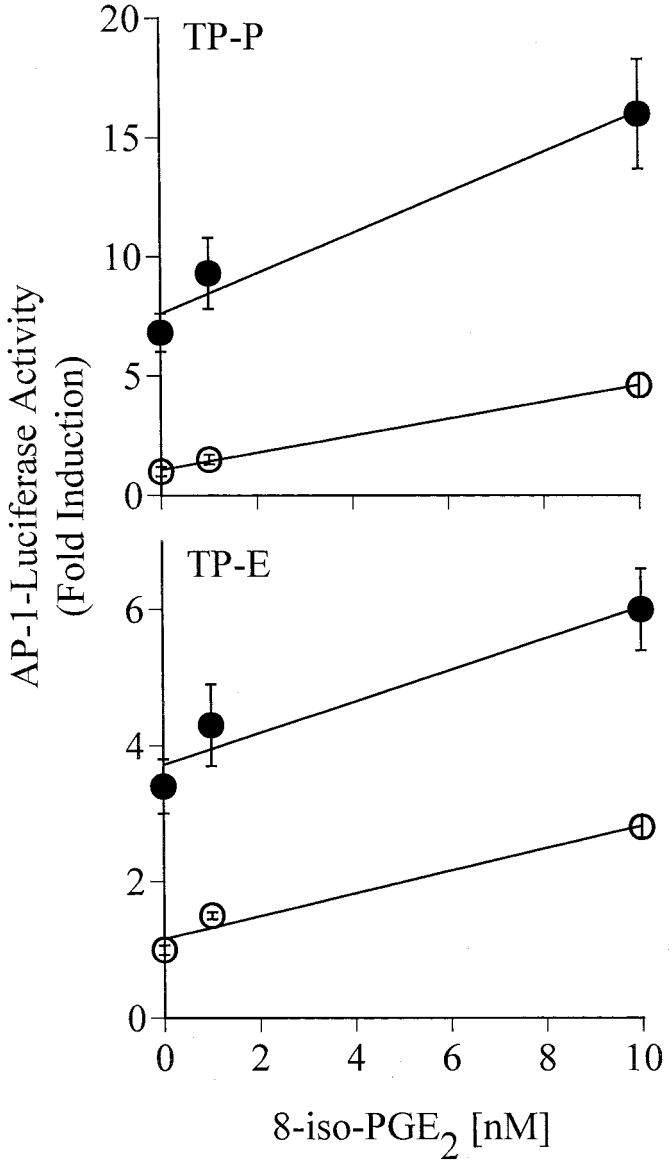

Fig. 9. Effect of U46619 and 8-iso-PGE_2 cotreatment on AP-1–luciferase activity. CHO TP-P and TP-E cells were treated with ETOH (○) or 10 nM U46619 (●) in the presence of 0, 1, and 10 nM 8-iso-PGE_2 for 6 h and processed for measurements of AP-1–luciferase activity as described under *Materials and Methods*. Values represent the mean ± S.E. ($n = 3$). Similar results were observed in two separate experiments.

$PGF_{2\alpha}$ does not compete for binding at TP sites (Pratico et al., 1996). Independent investigators have provided evidence that 8-iso-$PGF_{2\alpha}$ may modulate TP-dependent signaling in some systems through the up-regulation of de novo TXA_2 biosynthesis (Hou et al., 2002; Opere et al., 2002). However, the up-regulation of TXA_2 biosynthesis by prototypical isoprostanes does not occur in all model systems (Takabashi et al., 1992). Furthermore, 8-iso-$PGF_{2\alpha}$ metabolic breakdown products may be biologically active and induce de novo TXA_2 biosynthesis via the same receptor as 8-iso-$PGF_{2\alpha}$ (Hou et al., 2002). Because the metabolic breakdown products of 8-iso-$PGF_{2\alpha}$ seem to interact with the 8-iso-$PGF_{2\alpha}$ receptor with comparable potency, it is unlikely that differences in cellular metabolic activities can account for the lack of an effect of 8-iso-$PGF_{2\alpha}$ on AP-1 activity in CHO cells, assuming that 8-iso-$PGF_{2\alpha}$ is degraded to similar breakdown products in the CHO model. It seems more likely that CHO cells are simply deficient in the signaling pathway that is sensitive to 8-iso-$PGF_{2\alpha}$, which, in turn, is associated with the up-regulation of TXA_2 biosynthesis, consistent with data presented here (Fig. 5). As discussed above, this deficiency could occur at multiple levels ranging from differences in the expression of primary receptors as well as the effectors and autocrine loops associated with these receptors.

In summary, we have shown that 8-iso-PGE_2 increases AP-1–luciferase activity in CHO TP-P and TP-E cells in a TP-dependent and -independent manner, suggesting that multiple targets for 8-iso-PGE_2 may be coupled to AP-1. In contrast, 8-iso-$PGF_{2\alpha}$ does not seem to be a TP agonist, consistent with work from an independent laboratory (Pratico et al., 1996). Because the increase of AP-1 activity by 8-iso-PGE_2 was not sensitive to cyclooxygenase inhibitors, our data suggest that 8-iso-PGE_2 is a TP agonist in the CHO model. This suggestion is further supported by studies demonstrating that 8-iso-PGE_2 enhances rather than antagonizes the AP-1 response to a TP agonist (U46619). Because the apparent TP-dependent and -independent regulation of AP-1 by 8-iso-PGE_2 was potent (nanomolar concentrations), our data suggest that these pathways may play active roles in biological processes.

References

Abramovitz M, Adam M, Boie Y, Grygorczyk R, Rushmore TH, Nguyen T, Funk CD, Bastien L, Sawyer N, and Rochette C (1995) Human prostanoid receptors: cloning and characterization. *Adv Prostaglandin Thromboxane Leukotriene Res* **23:**499–504.

Armstrong RA and Wilson NH (1995) Aspects of the thromboxane receptor system. *Gen Pharmacol* **26:**463–472.

Chang PL, Tucker MA, Hicks PH, and Prince CW (2002) Novel protein kinase C isoforms and mitogen-activated kinase kinase mediate phorbol ester-induced osteopontin expression. *Int J Biochem Cell Biol* **34:**1142–1151**.**

Cipollone F, Ciabattoni G, Patrignani P, Pasquale M, Di Gregorio D, Bucciarelli T, Davi G, Cuccurullo F, and Patrono C (2002) Oxidant stress and aspirin-insensitive thromboxane biosynthesis in severe unstable angina. *Circulation* **102:**1007–1113**.**

Curran T (1992) Fos and Jun: oncogenic transcription factors. *Tohoku J Exp Med* **168:**169–174.

Forrest D and Curran T (1992) Crossed signals: oncogenic transcription factors. *Curr Opin Genet Dev* **2:**19–27.

Fukunaga M, Makita N, Roberts LJ, Morrow JD, Takahashi K, and Badr KF (1993) Evidence for the existence of F_2-isoprostane receptors on rat vascular smooth muscle cells. *Am J Physiol* **264:**C1619–C1624.

Fukunaga M, Yura T, Grygorczyk R, and Badr KF (1997) Evidence for the distinct nature of F_2-isoprostane receptors from those of thromboxane A_2. *Am J Physiol* **272:**F477–F483.

Habig WH, Pabst MJ, and Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249:**7130–7139.

Halliwell B and Grootveld M (1987) The measurement of free radical reactions in humans. Some thoughts for future experimentation. *FEBS Lett* **213:**9–14.

Hirata T, Ushikubi F, Kakizuka A, Okuma M, and Narumiya S (1996) Two thromboxane A_2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. *J Clin Investig* **97:**949–956.

Hou X, Roberts LJ, Taber DF, Morrow JD, Kanai K, Gobeil F Jr, Beauchamp MH, Bernier SG, Lepage G, Varma DR, et al. (2002) 2,3-Dinor-5,6-dihydro-15-F(2t)-isoprostane: a bioactive prostanoid metabolite. *Am J Physiol* **281:**R391–R400.

Janssen LJ and Tazzeo T (2002) Involvement of TP and EP_3 receptors in vasoconstrictor responses to isoprostanes in pulmonary vasculature. *J Pharmacol Exp Ther* **301:**1060–1106**.**

Karim S, Berrou E, Levy-Toledano S, Bryckaert M, and MacLouf J (1997) Regulatory role of prostaglandin E_2 in induction of cyclooxygenase-2 by a thromboxane A_2 analogue (U46619) and basic fibroblast growth factor in porcine aortic smooth-muscle cells. *Biochem J* **326:**593–599.

Ko FN, Yu SM, Kang YF, and Teng CM (1995) Characterization of the thromboxane (TP-) receptor subtype involved in proliferation in cultured vascular smooth muscle cells of rat. *Br J Pharmacol* **116:**1801–1808.

Ko FN (1997) Low-affinity thromboxane receptor mediates proliferation in cultured vascular smooth muscle cells of rats. *Arterioscler Thromb Vasc Biol* **17:**1274–1282.

Kunapuli P, Lawson JA, Rokach JA, Meinkoth JL, and Fitzgerald GA (1998) Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and the isoprostane, 8,12-iso-isoprostane $F_{2\alpha}$-III, induce cardiomyocyte hypertrophy. Differential activation of downstream signaling pathways. *J Biol Chem* **273:**22442–22452.

Leitinger N, Huber J, Rizza C, Mechtcheriakova D, Bochkov V, Koshelnick Y, Berliner JA, and Binder BR (2001) The isoprostane 8-iso-PGF_{2alpha} stimulates endothelial cells to bind monocytes: differences from thromboxane-mediated endothelial activation. *FASEB J* **15:**1254–1256**.**

Longmire AW, Roberts LJ, and Morrow JD (1994) Actions of the E_2-isoprostane, 8-iso-PGE_2, on the platelet thromboxane/endoperoxide receptor in humans and rats: additional evidence for the existence of a unique isoprostane receptor. *Prostaglandins* **48:**247–256.

Morrow JD and Roberts LJ (1996) The isoprostanes. Current knowledge and directions for future research. *Biochem Pharmacol* **51:**1–9.

Morrow JD, Roberts LJ, Daniel VC, Awad JA, Mirochnitchenko O, Swift LL, and Burk RF (1998) Comparison of formation of D_2/E_2-isoprostanes and F_2-isoprostanes in vitro and in vivo–effects of oxygen tension and glutathione. *Arch Biochem Biophys* **353:**160–171.

Morrow JD and Roberts LJ (1997) The isoprostanes: unique bioactive products of lipid peroxidation. *Prog Lipid Res* **36:**1–21.

Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science (Wash DC)* **258:**607–614.

Ohkubo S, Nakahata N, and Ohizumi Y (1996) Thromboxane A_2 stimulates mitogen-activated protein kinase and arachidonic acid liberation in rabbit platelets. *Prostaglandins* **52:**403–413.

Opere CA, Awe SO, Harris LC, LeDay AM, and Ohia SE (2002) Potentiation of sympathetic neurotransmission in bovine isolated irides by isoprostanes. *Free Radic Res* **35:**257–264.

Parent JL, Labrecque P, Orsini MJ, and Benovic JL (1999) Internalization of the TXA_2 receptor α and β isoforms. Role of the differentially spliced COOH terminus in agonist-promoted receptor internalization. *J Biol Chem* **274:**8941–8948.

Pratico D, Smyth EM, Violi F, and Fitzgerald GA (1996) Local amplification of platelet function by 8-epi-prostaglandin $F_{2\alpha}$ is not mediated by thromboxane receptor isoforms. *J Biol Chem* **271:**14916–14924.

Raychowdhury MK, Yukawa M, Collins LJ, McGrail SH, Kent KC, and Ware JA (1994) Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A_2 receptor. *J Biol Chem* **269:**19256–19261.

Rokach J, Khanapure SP, Hwang SW, Adiyaman M, Lawson JA, and Fitzgerald GA (1997) The isoprostanes: a perspective. *Prostaglandins* **54:**823–851.

Sachinidis A, Flesch M, Ko Y, Schror K, Bohm M, Dusing R, and Vetter H (1995) Thromboxane A_2 and vascular smooth muscle cell proliferation. *Hypertension* **26:**771–780.

Simonet S, Bonhomme E, Fabiani JN, and Verbeuren T (2002) Temperature-dependent basal tone in isolated human saphenous veins: implication of TP-receptors. *Fundam Clin Pharmacol* **14:**461–467.

Takabashi K, Nammour TM, Fukunaga M, Ebert J, Morrow JD, Roberts LJ, Hoover RL, and Badr KF (1992) Glomerular actions of a free radical-generated novel prostaglandin, 8-epi-prostaglandin F_{2alpha}, in the rat. Evidence for interaction with thromboxane A_2 receptors. *J Clin Investig* **90:**136–141.

Takahara K, Murray R, Fitzgerald GA, and Fitzgerald DJ (1990) The response to thromboxane A_2 analogues in human platelets. Discrimination of two binding sites linked to distinct effector systems. *J Biol Chem* **265:**6836–6844.

Tintut Y, Parhami F, Tsingotjidou A, Tetradis S, Territo M, and Demer LL (2002) 8-isoprostaglandin E_2 enhances receptor-activated NFκB ligand (RANKL)-dependent osteoclastic potential of marrow hematopoietic precursors via the cAMP pathway. *J Biol Chem* **277:**14221–14226.

Towndrow KM, Mertens JJ, Jeong JK, Weber TJ, Monks TJ, and Lau SS (2000) Stress- and growth-related gene expression are independent of chemical-induced prostaglandin E_2 Synthesis in renal epithelial cells. *Chem Res Toxicol* **13:**111–117.

Weber TJ, Monks TJ, and Lau SS (1997) PGE_2-mediated cytoprotection in renal epithelial cells: evidence for a pharmacologically distinct receptor. *Am J Physiol* **273:**F507–F515.

Weber TJ, Monks TJ, and Lau SS (2000) DDM-PGE_2-mediated cytoprotection in renal epithelial cells by a thromboxane A_2 receptor coupled to NF-kappaB. *Am J Physiol* **278:**F270–F278.

Yukawa M, Yokota R, Eberhardt RT, von Andrian L, and Ware JA (1997) Differential desensitization of thromboxane A_2 receptor subtypes. *Circ Res* **80:**551–556.

Yura T, Fukunaga M, Khan R, Nassar GN, Badr KF, and Montero A (1999) Free-radical-generated F_2-isoprostane stimulates cell proliferation and endothelin-1 expression on endothelial cells. *Kidney Int* **56:**471–478.

Address correspondence to: Dr. Thomas Weber, Cell Biology, Pacific Northwest National Laboratory, 790 6th Street, P7-56, Richland, WA 99352. E-mail: thomas.weber@pnl.gov