Posttranslational regulation of cyclooxygenase by tyrosine phosphorylation in cerebral endothelial cells

HELENA PARFENOVA, LILIYA BALABANOVA, AND CHARLES W. LEFFLER

Laboratory for Research in Neonatal Physiology, Department of Physiology and Biophysics, University of Tennessee, Memphis, Tennessee 38163

Parfenova, Helena, Liliya Balabanova, and Charles W. Leffler. Posttranslational regulation of cyclooxygenase by tyrosine phosphorylation in cerebral endothelial cells. Am. J. Physiol. 274 (Cell Physiol. 43): C72–C81, 1998.—Endothelium-derived cyclooxygenase (COX) products regulate cerebral vascular tone in newborn pigs. Both COX-1 and COX-2 are constitutively expressed in endothelial cells from newborn pig cerebral microvessels. We investigated the role of protein phosphorylation in the regulation of COX activity. The protein tyrosine phosphatase (PTP) inhibitors phenylarsine oxide, vanadate, and benzylphosphonic acid rapidly stimulated COX activity, whereas the protein tyrosine kinase inhibitors, genistein and tyrphostins, inhibited it. Protein synthesis inhibitors did not reverse the stimulation of COX activity evoked by PTP inhibitors. Similar changes were observed in other vascular cells from newborn pigs that also express COX-1 and COX-2 (cerebral microvascular smooth muscle cells and aortic endothelial cells) but not in human umbilical vein endothelial cells or Swiss 3T3 fibroblasts that express COX-1 only. Tyrosine-phosphorylated proteins were immunodetected in endothelial cell lysates. COX-2 immunoprecipitated from 32P-loaded endothelial cells incorporated 32P that was increased by PTP inhibitors. COX-2, but not COX-1, was detected in endothelial fractions immunoprecipitated with anti-phosphotyrosine. These data indicate that tyrosine phosphorylation posttranslationally regulates COX activity in newborn pig vascular cells and that COX-2 is a substrate for phosphorylation.

VASCULAR ENDOTHELIUM PLAYS an important role in maintenance of vascular tone by producing a variety of vasorelaxants, including prostanoids. Endothelial cells from newborn pig cerebral microvessels in primary culture make prostanoids, mainly prostacyclin and prostaglandin E2 (PGE2), during basal conditions and culture make prostanoids, mainly prostacyclin and PGE2 from newborn pig cerebral microvessels in primary culture. Endothelial cells release vasodilators, including prostanoids. Endothelial cells maintain vascular tone by producing a variety of vasorelaxants, including prostanoids. Endothelial cells also release vasodilators, mainly prostacyclin and PGE2, which are integral components of the regulation of cerebral hemodynamics in newborn pigs and contribute to the rapid cerebral vascular responses induced by hypercapnia, asphyxia, and hypotension. Therefore, mechanisms that regulate prostanoid production in cerebral vascular endothelium are of great physiological importance.

COX (EC 1.14.99.1), a key enzyme in prostaglandin synthesis, is represented by two immunologically distinct isoforms, COX-1 and COX-2, which share only 60% homology and are encoded by different genes (32). In many cell types, COX-1 provides prostanoid synthesis during basal conditions, whereas COX-2 is transiently induced when stimulated with serum, growth factors, cytokines, and other factors (14, 32). Because COX is irreversibly inactivated following catalysis, it is assumed that COX activity is determined by the amount of the enzyme protein and is regulated exclusively at the levels of transcription and translation (14, 32).

Since the discovery of inducible COX-2, the main focus of investigations has shifted toward mechanisms up-regulating COX-2 gene expression, which is of special importance in cell growth and inflammation (14). Mitogen-activated pathways that induce COX-2 early gene expression may involve protein kinase C (3, 13) and protein tyrosine kinase (PTK; Refs. 1, 6, 29, 30). However, the induction of COX-2 (as mRNA and protein) does not always result in increased prostanoid synthesis. Therefore, posttranslational regulation may be important in determination of COX-2 activity. However, there are no reports in the literature indicating that COX-2 activity could be regulated posttranslationally.

Cerebral microvascular endothelial cells from newborn pigs provide a suitable model for investigating mechanisms that regulate the contribution of COX-2 to prostanoid synthesis under nonstimulated conditions. We have shown previously that endothelial cells from newborn pig cerebral microvessels at quiescence express COX-2 protein (27). Moreover, endothelial prostanoid production is highly sensitive to NS-398, a COX-2-selective inhibitor, indicating that COX-2 is a major functional isoform in nonstimulated cells (27). In addition to cerebral microvascular endothelial cells, other vascular cells in newborn pigs (smooth muscle cells from cerebral microvessels and aortic endothelium) also express the COX-2 isoform at quiescence (27).

Protein phosphorylation can modulate a variety of functionally important proteins at the transcriptional, translational, and posttranslational levels (17). To elucidate the role of protein phosphorylation in intact cells, a pharmacological approach using protein kinase and/or phosphatase inhibitors has been successfully applied (5, 17). Prostanoid production in different cell types is affected by protein kinase/phosphatase inhibitors in both delayed and acute manners (10, 11, 24). Delayed effects of protein phosphorylation on prostanoid production could be associated with the induction of key enzymes in prostanoid biosynthesis. Induction of COX-2 can be regulated by protein phosphorylation at the levels of transcription and translation. Signal transduction pathways leading to the induction of COX-2 gene expression may involve protein kinase C and PTK (3, 6, 13, 29, 30). Phospholipase A2 activity can also be regulated posttranslationally by direct changes in enzyme phosphorylation (11, 24), which may explain the acute effects of modulators of protein phosphorylation.
on prostanoid synthesis. However, no role of protein phosphorylation in posttranslational regulation of COX activity has been found (33).

Recently, we presented preliminary data demonstrating that in vivo COX-mediated vascular responses in newborn pigs are rapidly affected when modulation of protein tyrosine phosphorylation occurs (26). These data indicate that COX activity could be regulated by tyrosine phosphorylation. Because COX-2 is a major isoform in the newborn cerebral circulation, in the present study we investigated the hypothesis that COX-2 activity is regulated posttranslationally by protein tyrosine phosphorylation. We tested this hypothesis in two groups of prostanoid-producing cells: 1) cerebral microvascular endothelial cells and other vascular cells from newborn pigs that express both COX-1 and COX-2 proteins, and 2) human umbilical vein endothelial cells and Swiss 3T3 fibroblasts that express only COX-1 protein. As pharmacological tools to modulate protein tyrosine phosphorylation in intact cells, a variety of compounds that inhibit PTK and protein tyrosine phosphatases (PTP) by different mechanisms were used. We investigated the effects of these inhibitors on COX activity in intact cells, as well as on COX-1 and COX-2 isoform-specific proteins and tyrosine-phosphorylated proteins in cell lysates. We report here a novel finding indicating that the activity of COX-2, but not COX-1, can be posttranslationally regulated by protein tyrosine phosphorylation.

MATERIALS AND METHODS

Protocols involving animals were approved by the Animal Care and Use Committee at the University of Tennessee (Memphis, TN). All procedures were done using aseptic techniques.

Materials. Cell culture reagents were obtained from Life Technologies (Gaithersburg, MD) and Sigma (St. Louis, MO). Matrigel (growth factor reduced) was purchased from Becton Dickinson, (Bedford, MA). Phenylarsine oxide, benzylphosphonic acid, tyrphostin 47, tyrphostin 25, H-7, and okadaic acid were from Biomol. NS-398 and arachidonic acid were from Cayman Chemical (Ann Arbor, MI). Genistein, sodium orthovanadate, actinomycin D, and cycloheximide, as well as other reagents, were from Sigma. Protein G Sepharose was from Pharmacia (Piscataway, NJ).

Cell cultures. Brain cortex and aortae were extracted from newborn pigs anesthetized with ketamine-acepromazine. Primary cultures of cerebral microvascular endothelial cells from newborn pigs were established as previously described (16, 27). Briefly, endothelial cells were obtained from isolated cerebral microvessels (60–300 µm) using collagenase-dispase (1 mg/ml) treatment for 2 h at 37°C, followed by Percoll density gradient centrifugation. Cells were plated onto Matrigel-coated 12-well cell culture plates and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% fetal bovine serum (FBS), 30 µg/ml endothelial cell growth supplement (ECGS), and 1 U/ml heparin and antibiotic-antimycotic mixture in a 5% CO2-air incubator at 37°C for 5–6 days to reach confluency.

Primary cultures of cerebral microvascular smooth muscle cells were grown as explants from isolated cerebral microvessels plated onto Matrigel-coated 12-well cell culture plates and maintained in DMEM containing 20% FBS and antibiotic-antimycotic mixture (16, 27). Smooth muscle cells were grown in a 5% CO2-air incubator at 37°C for 12–14 days until they reached confluency.

For primary culture of aortic endothelial cells, aortae were cut longitudinally and placed for 2 h at 37°C in isolation medium M199 containing collagenase-dispase (1 mg/ml). Media with dissociated aortic endothelial cells were aspirated; cells were collected by centrifugation, plated onto Matrigel-coated cell culture plates, and maintained for 5–6 days as described above for cerebral microvascular endothelial cells until confluency.

Human umbilical vein endothelial cells (HUVECs) from American Type Culture Collection (Rockville, MD) were plated onto Matrigel-covered 12-well cell culture plates (105 cells/well) and grown in DMEM containing 30 µg/ml ECGS and 20% FBS in a 5% CO2-air incubator at 37°C for 6–7 days to reach confluency.

Stock cultures of Swiss 3T3 fibroblasts from American Type Culture Collection were maintained in DMEM supplemented with 10% bovine serum albumin in a CO2-air incubator at 37°C. For experimental purposes, cells were plated onto 12-well cell culture plates (105 cells/well) and grown in DMEM with 20% FBS in a 5% CO2-air incubator at 37°C for 4–5 days to reach confluency.

All experiments were performed on confluent quiescent cells. To achieve quiescence, cells were exposed to a serum-depleted medium (0% FBS) for 15–24 h before the experiment.

COX activity. To evaluate COX activity in intact cells, we determined prostanoid production (using prostacyclin and PGE2) from exogenous substrate (arachidonic acid). Cells were washed twice with phosphate-buffered saline (PBS) and incubated with 30 or 100 µM arachidonic acid in 1 ml of medium similar to cortical cerebrospinal fluid (in mM: 3.0 KCl, 1.5 MgCl2, 1.5 CaCl2, 132 NaCl, 6.6 urea, 3.7 dextrose, and 24.6 NaHCO3 equilibrated with 5% CO2-7% O2-92% N2 to pH 7.4–7.5; Pco2 of 32–36 mmHg; Po2 of 100–120 mmHg) for 15 min at 37°C. To investigate the functional contribution of COX-2 to total COX activity, cells were pretreated with the COX-2-selective inhibitor NS-398 (10−5–10−4 M) for 30 min. At the end of the incubation period, incubation media were aspirated and stored at −20°C for prostanoid determination.

Prostanoid assay. Concentrations of 6-ketoprostaglandin F1α (6-keto-PGF1α, the stable hydrolysis product of prostacyclin) and PGE2 in the cell incubation medium were determined by radioimmunoassay as described (16). The prostanoid concentration was normalized to cell protein.

COX-2 immunoprecipitation. Quiescent cerebral microvascular endothelial cells were loaded with 200 µCi/ml [32P]orthophosphate (Amersham) in a N-2-hydroxyethylpiperazin-N’-2-ethanesulfonic acid (HEPES)-buffered saline (in mM: 20 HEPES, 130 NaCl, 5 KCl, 1.5 MgCl2, 1.5 CaCl2; pH 7.4) for 1 h at 37°C. The incubation media were discarded, and 32P-loaded cells were thoroughly washed with ice-cold HEPES-buffered saline. All subsequent procedures were performed on ice. Cells were solubilized by sonication in an immunoprecipitation buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) in PBS] containing prote-
ase/phosphatase inhibitors (200 µM leupeptin, 40 µg/ml aprotinin, 200 µM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 1 mM NaEDTA, 1 mM sodium fluoride, and 100 µg/ml trypsin inhibitor). Aliquots of the supernatant were preincubated with protein G Sepharose (50% vol/vol) for 1 h. To immobilize COX-2 antibodies, 1 ml of protein G Sepharose was preincubated for 1 h with 50 µl COX-2 (human) polyclonal antiserum (Oxford Biomedical Research, Oxford, MI). For COX-2 immunoprecipitation, 0.5 ml samples (300 µg protein) were incubated for 4 h with 200 µl of Sepharose-immobilized anti-COX-2. The Sepharose-bound immunoprecipitated fraction was washed five times with the immunoprecipitation buffer containing protease/phosphatase inhibitors, solubilized in the Laemmli sample buffer (0.12 M tris(hydroxymethyl)aminomethane·HCl, 10% glycerol, 2.5% SDS, and 0.006% bromphenol blue; pH 6.8) and resolved by 7.5% polyacrylamide SDS gel electrophoresis. Gels were stained with Coomassie blue, and 32P was quantified using a phosphor mager screen.

Phosphotyrosine immunoprecipitation. All procedures were performed on ice. Quiescent cerebral microvascular endothelial cells (control or pretreated with kinase/phosphatase inhibitor) were washed twice with PBS and solubilized by sonication in the immunoprecipitation buffer containing protease/phosphatase inhibitors as indicated above. Aliquots of the supernatant were preincubated with protein G Sepharose (50% vol/vol) for 1 h. To immobilize antibodies, 1 ml of protein G Sepharose was preincubated for 1 h with 50 µl of monocl- onal anti-phosphotyrosine 4G10, (Upstate Biotechnology, Lake Placid, NY) in the immunoprecipitation buffer. For phosphotyrosine immunoprecipitation, 0.5-ml samples (300 µg protein) were incubated with 200 µl of protein G Sepharose-immobilized anti-phosphotyrosine for 4 h. The Sepharose-bound immunoprecipitated fraction was washed five times with the immunoprecipitation buffer containing protease/phosphatase inhibitors and used for Western blotting.

Western blotting. Quiescent cells (control or treated with PTK/PTP inhibitors) and immunoprecipitated fractions were solubilized with the Laemmli sample buffer (10 min, 100°C). Samples (20–50 µg protein/lane) were separated by 7.5% polyacrylamide SDS gel electrophoresis. For molecular weight standards, high-molecular-weight biotinylated protein standards (Bio-Rad) and prestained protein standards (Sigma) were used. For the specific standards, we used COX-2 from sheep placenta (Cayman Chemical) and COX-1 from ram seminal vesicles (Oxford Biomedical Research). The separated proteins were transferred to polyvinylidene difluoride-Plus transfer membranes (Micron Separations) as described earlier (27) and probed for COX and phosphotyrosine. For COX isozyme detections, the membrane was probed with COX-2 (human) polyclonal antiserum (PG27 from Oxford Biomedical Research, at 1:5,000 dilution) or COX-1 polyclonal antiserum (PG16 from Oxford, at 1:1,000 dilution), followed by peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG; dilution 1:10,000) (Jackson Immunoresearch Laboratories, West Grove, PA). Streptavidin-biotin-horseradish peroxidase complex (Amersham, at 1:1,000 dilution) was added to the second antibody incubation medium to develop biotinylated standards. For phosphotyrosine detection, the membrane was probed with monoclonal antibodies to phosphotyrosine, 4G10 (Upstate Biotechnology), followed by peroxidase-conjugated donkey anti-mouse IgG (dilution 1:10,000) (Jackson Immunoresearch Laboratories). Blots were developed using a chemiluminescence detection system (DuPont).

RESULTS

Effects of PTP inhibitors on COX activity in cerebral microvascular endothelial cells. We investigated rapid effects of PTP inhibitors on COX activity in quiescent endothelial cells from newborn pigs. Phenylarsine oxide (1–20 µM) dose-dependently stimulated prostacyclin (as 6-keto-PGF1α) and PGE2 production from 30 µM arachidonic acid [50% effective concentration (EC50) = 3 µM; maximal stimulation, 3- to 4-fold] (Fig. 1A) within 10–30 min. Another PTP inhibitor, sodium orthovanadate, also rapidly (10–30 min) stimulated prostacyclin and PGE2 production from 30 µM arachidonic acid (EC50 = 1 mM; maximal stimulation, 3- to 4-fold) (Fig. 1B). Phenylarsine oxide and vanadate increased production of both prostacyclin and PGE2, indicating that COX, rather than downstream enzymes of prostanoid biosynthesis, is the target of PTP inhibitors. Benzyolphosphonic acid (2 mM), another PTP inhibitor, also rapidly stimulated prostanoid production from arachidonic acid (6-keto-PGF1α production increased from 20 ± 3 to 54 ± 6 pg/µg protein; PGE2 production increased from 38 ± 5 to 115 ± 18 in control and treated cells). In contrast, okadaic acid (300–600
nM), a serine/threonine phosphatase inhibitor, had no rapid effects on endothelial COX activity (6-keto-PGF$_{1a}$ production from 30 µM arachidonic acid was 15 ± 2 and 14 ± 3 pg/µg protein, respectively, in control and okadaic acid-treated cells).

Novel protein synthesis is not involved in stimulation of COX activity by PTP inhibitors. Although effects of PTP inhibitors on prostanooid production in endothelial cells were observed within 30 min of application, we could not exclude the contribution of new protein synthesis to the stimulation of prostanooid synthesis. Therefore, we compared the effects of sodium vanadate (1 mM) and phenylarsine oxide (100 µM) in three groups of cerebral microvascular endothelial cells: 1) control, 2) pretreated with actinomycin D (5 µg/ml) for 3 h, and 3) pretreated with cycloheximide (2 × 10^{-6} M) for 3 h. Our data demonstrate that protein synthesis inhibitors did not attenuate the stimulation of COX activity evoked by vanadate or phenylarsine oxide (Fig. 2), indicating that modulation of COX activity by PTP inhibitors is not due to novel protein synthesis.

Effects of PTK inhibitors on COX activity in cerebral microvascular endothelial cells. Incubation of endothelial cells with the PTK inhibitor genistein (20–370 µM) for 10–30 min resulted in dose-dependent inhibition of prostacyclin and PGE$_2$ production from arachidonic acid (EC$_{50}$ = 50 µM, maximal inhibition 50–60%) (Fig. 3A). Tyrphostin 47 (20–370 µM), another PTK inhibitor, also rapidly inhibited COX activity (EC$_{50}$ = 70 µM; maximal inhibition, 40–50%) (Fig. 3A). Tyrphostin 25, a structural analog of tyrphostin 47, was less potent in inhibiting endothelial COX activity (EC$_{50}$ = 90 µM, maximal inhibition was 30–40%, data not shown). Production of both prostacyclin and PGE$_2$ was inhibited to the same extent in cells treated with PTK inhibitors, indicating that the observed changes were due to inhibition of COX activity. In contrast, H-7 (150 µM), which is known to inhibit a variety of protein kinases other than PTK, had no effects on endothelial COX activity (6-keto-PGF$_{1a}$ production from 30 µM arachidonic acid was 22 ± 3 and 19 ± 2 pg/µg protein, respectively, in control cells and cells pretreated with H-7 for 30 min).

Effect of PTP/PTK inhibitors on COX activity in cerebral microvascular smooth muscle cells and aortic endothelial cells. Because no data have been reported that indicated rapid modification of COX activity by compounds that affect protein tyrosine phosphorylation, we addressed the issue of cell specificity of the observed phenomena. Quiescent cells from newborn pig cerebral microvessels (both endothelial and smooth muscle cells) and aortic endothelial cells in primary culture express COX-2 protein under basal conditions (constitutive COX-2; Fig. 4A). In these cells, NS-398 (10^{-5}–10^{-4} M), a COX-2 inhibitor, inhibited prostanooid production by 70–80% (Fig. 4A), indicating a large contribution of the COX-2 isoenzyme to total prostanooid synthesis. We investigated the effects of PTP/PTK inhibitors on COX activity in cerebral microvascular smooth muscle cells and aortic endothelial cells from newborn pigs. Our data demonstrate that in cerebral microvascular smooth muscle cells (Fig. 5A), as well as
in aortic endothelial cells (Fig. 5B), the PTP inhibitors sodium orthovanadate (2 mM), benzyolphosphonic acid (2 mM), and phenylarsine oxide (10 µM) rapidly (in 30 min) stimulated prostacyclin production from arachidonic acid two- to fourfold. However, okadaic acid (300 nM), which inhibits a variety of protein phosphatases other than PTP, had no effect (Fig. 5, A and B). Alternatively, in these cells, the PTK inhibitors genistein, tyrphostin 47, and tyrphostin 25 (150 µM) rapidly inhibited prostacyclin production from arachidonic acid by 60–80%, whereas the protein kinase A and protein kinase C inhibitor H-7 was ineffective (Fig. 5, A and B). Cells treated with PTP/PTK inhibitors showed changes in PGE2 synthesis that were parallel to prostacyclin production (data not shown). Therefore, in newborn pig vascular cells, compounds that purportedly stimulate protein tyrosine phosphorylation rapidly increase prostanoid production from exogenous arachidonic acid, whereas compounds that decrease protein tyrosine phosphorylation inhibit prostanoid production from exogenous arachidonic acid.

Effects of PTP/PTK inhibitors on COX activity in HUVECs and Swiss 3T3 fibroblasts. HUVECs and Swiss 3T3 fibroblasts at quiescence express only COX-1 isoform (as mRNA and protein) (3, 19). Our data demonstrate that, in both serum-starved HUVECs and Swiss 3T3 fibroblasts, prostanoid production from exogenous substrate is not sensitive to NS-398 (Fig. 4B), further indicating that COX-1 is responsible for overall basal prostanoid synthesis. In these cell lines, we examined the effects of protein kinase inhibitors (genistein, tyrphostin 47, tyrphostin 25, and H-7) and protein phosphatase inhibitors (sodium vanadate, benzyolphosphonic acid, phenylarsine oxide, and okadaic acid) on COX activity. The inhibitors were used at concentrations that were maximally effective in cerebral microvascular endothelial cells. Neither in HUVECs nor in Swiss 3T3 fibroblasts were significant changes in COX activity (as prostacyclin production from 30 µM arachidonic acid) observed following 30-min preincubation with most of the kinase or phosphatase inhibitors.
Genistein was the only compound that decreased prostanoid production in HUVECs and Swiss 3T3 fibroblasts (20–30% inhibition following treatment with 150 µM genistein).

Detection of phosphorylated proteins in cerebral vascular endothelial cells. In control 32P-loaded endothelial cells, the major phosphorylated protein bands were 220, 160–180, 115, 90, 70, 58, 45, 40, 36, and 30 kDa (Fig. 7). In cells treated with sodium orthovanadate, a significant increase in 32P incorporation was observed in protein bands of 220, 160–180, 115, and 90 kDa, including a band with the molecular mass of COX (∼70 kDa). Phenylarsine oxide caused a much slighter increase in selected phosphorylated proteins, including 115- to 180-kDa proteins. These data indicate that a number of endothelial proteins are phosphorylated on tyrosine residues. However, the PTK inhibitors genistein (150–300 µM) and tyrphostin 47 (150–300 µM) did not change the phosphorylated protein spectrum (Fig. 7).

The COX-2-immunoprecipitated fraction of endothelial cells demonstrated significant 32P incorporation in a major band of ∼70 kDa, as well as in smaller proteins (50–55 and 40–42 kDa), that may correspond to proteolytic fragments of COX-2 (Fig. 8A). No changes in phosphorylation of the COX-2 immunoprecipitated fraction were observed in cells pretreated for 30 min with genistein (300 µM) or tyrphostin (300 µM) (Fig. 8, A and B). However, in endothelial cells pretreated with vanadate or phenylarsine oxide, a two- to threefold higher 32P incorporation into the COX-2-immunoprecipitated fraction was observed (Fig. 8, A and B), indicating that endothelial COX-2 could be phosphorylated on tyrosine residues.

Tyrosine-phosphorylated proteins in cerebral microvascular endothelial cells. Several tyrosine-phosphorylated protein bands were identified in quiescent endothelial cells using monoclonal phosphotyrosine antibodies (180, 130, 105–120, 70, 58, 48, and 40 kDa) (Fig. 9, A and B). Sodium orthovanadate (2 mM, 30 min) increased overall tyrosine phosphorylation (Fig. 9A), whereas genistein (300 µM) did not affect phosphorylated proteins (Fig. 9B).

Although some data in the literature indicate that arachidonic acid may increase protein phosphorylation...
we did not detect any changes in the spectrum of endothelial phosphorylated proteins after exposing cells to 30 µM arachidonic acid for 15 min (Figs. 7–9).

In the fraction of endothelial proteins immunoprecipitated with phosphotyrosine antibodies, we detected a COX-2-immunoreactive protein (Fig. 9C), indicating that COX-2 is tyrosine phosphorylated under basal conditions. No quantitative changes in COX-2 protein were observed in cells treated with PTK/PTP inhibitors (Fig. 9C), confirming our functional data that changes in COX activity are not due to changes in COX protein. Exposure to arachidonic acid also did not alter the amount of COX-2 immunoreactive protein (Fig. 9).

Although COX-1 is detectable in cerebral microvascular endothelial cells from newborn pigs (27), we did not find a COX-1-immunoreactive protein among the antiphosphotyrosine-precipitated endothelial proteins (data not shown).

Together, these data indicate that, in endothelial cells from newborn pig cerebral microvessels, COX-2, but not COX-1, is phosphorylated on tyrosine residues under basal conditions.

**DISCUSSION**

The major novel finding in the present study is that COX-2 activity is regulated posttranslationally by tyrosine phosphorylation. This conclusion is based on the following findings in serum-deprived endothelial cells from newborn pig cerebral microvessels that constitutively express COX-2 as a major functional isoform: 1) PTK inhibitors rapidly decrease prostacyclin and PGE₂ production from arachidonic acid, 2) PTP inhibitors rapidly increase prostacyclin and PGE₂ production from arachidonic acid, and 3) actinomycin D and cycloheximide do not reverse the stimulation of prostaglandin synthesis by PTP inhibitors. Similar results were obtained in other vascular cells from the newborn pig (cerebral microvascular smooth muscle cells and aortic endothelial cells) that also constitutively express COX-2.

In contrast, PTK/PTP inhibitors did not affect prostaglandin biosynthesis in HUVECs and Swiss 3T3 fibroblasts, which express the COX-1 isoform only, thus indicating that COX-1 activity is not regulated by...
tyrosine phosphorylation. 32P-loaded cerebral microvascular endothelial cell immunoprecipitated with COX-2 antiserum showed a vanadate- and phenylarsine oxide-dependent 32P incorporation into a major 70-kDa protein band. COX-2, but not COX-1, was detected in fractions of endothelial cell lysates immunoprecipitated with phosphotyrosine antibodies, indicating that COX-2 itself is a substrate for tyrosine phosphorylation.

We have shown previously (27) and confirm in this study that COX-2 is constitutively expressed and largely contributes to a total prostaglandin synthesis in endothelial cells from cerebral microvessels as well as in other vascular cells (cerebral microvascular smooth muscle cells and aortic endothelial cells) from newborn pigs. These conclusions are based on findings in serum-starved nonstimulated cells: 1) COX-2 immunoreactive protein is expressed, and 2) COX activity in intact cells is greatly inhibited by NS-398, a COX-2-selective inhibitor. Selectivity of NS-398 is confirmed by our present data that this inhibitor did not affect prostaglandin production in HUVECs and Swiss 3T3 fibroblasts, which constitutively express only COX-1 (3, 19). COX-2 is also expressed in freshly isolated cerebral microvessels and aortic endothelium (27), indicating that this isoform is of functional importance in the newborn pig vasculature. Therefore, primary cultures of vascular cells from newborn pigs provide a physiological model to investigate function, enzymatic properties, and regulation of constitutive COX-2 in its natural intracellular environment.

To investigate the role of tyrosine phosphorylation in the regulation of COX activity, we used several pharmacological compounds that inhibit PTK or PTP via different mechanisms (5, 17). In quiescent cerebral microvascular endothelial cells, the PTK inhibitors genistein, tyrphostin 47, and tyrphostin 25 caused a rapid (in 10–30 min) dose-dependent decrease in prostacyclin and PGE2 production from exogenous arachidonic acid. The PTP inhibitors phenylarsine oxide, sodium orthovanadate, and benzylphosphonic acid rapidly (within 30 min) increased prostacyclin and PGE2 production from arachidonic acid three- to fourfold. We observed parallel changes in both major prostanooids produced by endothelial cells (prostacyclin and PGE2), suggesting that effects of PTK/PTP inhibitors are due to modulation of COX activity rather than downstream enzymes of prostanooid biosynthesis. Inhibitors of protein synthesis at the level of transcription/translation (actinomycin D and cycloheximide) did not attenuate the stimulation of endothelial COX activity evoked by PTP inhibitors, indicating that the observed increase in COX activity is due to posttranslational modifications rather than de novo protein synthesis. The effects of PTK/PTP inhibitors on endothelial COX activity appear to be specific because inhibitors of other types of protein kinases/protein phosphatases (the protein kinase C and protein kinase A inhibitor H-7 and the serine/threonine phosphatase inhibitor okadaic acid) did not alter COX activity. Therefore, in intact cerebral microvascular endothelial cells, pharmacological compounds that inhibit protein tyrosine phosphorylation rapidly decrease COX activity, whereas compounds that increase tyrosine phosphorylation elevate COX activity. Similar effects of modulators of protein tyrosine phosphorylation on COX activity were observed in other vascular cells that also constitutively express COX-2 and produce prostanooids in the NS-398-sensitive manner (newborn pig cerebral microvascular smooth muscle cells and aortic endothelial cells).

We investigated the effects of PTK/PTP inhibitors on COX activity in cells that express only the COX-1 isoform at quiescence [HUVECs (3) and Swiss 3T3 fibroblasts (19)]. Serum-deprived HUVECs and Swiss 3T3 fibroblasts actively produce prostanooids from arachidonic acid, reflecting high COX activity. We found that NS-398, even at high concentrations (10−4 M), did not inhibit prostanooid biosynthesis, further indicating that COX-2 is not expressed in these cells. In both HUVECs and Swiss 3T3 fibroblasts, neither PTK inhibitors (genistein, tyrphostin 47, tyrphostin 25), PTP inhibitors (sodium orthovanadate, phenylarsine oxide, benzylphosphonic acid), nor other modulators of protein phosphorylation (H-7 and okadaic acid) rapidly affected COX activity. Among all inhibitors used, only genistein had any effect on prostanooid synthesis in both HUVECs and Swiss 3T3 fibroblasts (30–50% inhibition). However, genistein may exert effects other than inhibition of protein phosphorylation (2). Direct inhibition of COX activity by genistein has also been demonstrated (18).

What is the mechanism(s) by which modulation of protein tyrosine phosphorylation affects COX activity? The COX molecule itself is a potential substrate for tyrosine phosphorylation. It has been shown that several tyrosine groups located close to the active site of the COX molecule are important in the mechanism of COX reaction (12, 20) and that chemical modification of tyrosine residues by nitration abolishes the enzyme catalytic activity (31). Therefore, modification of tyrosine residues in the active center could be important in the regulation of the enzyme activity. Our data demonstrate that COX activity in cells that constitutively express COX-2 isoform is rapidly increased with activation of protein tyrosine phosphorylation. The COX-2, but not COX-1, protein is identified in the endothelial cell fraction immunoprecipitated with phosphotyrosine antibodies. Moreover, COX-2 immunoprecipitated from 32P-loaded endothelial cells demonstrates a significant incorporation of 32P under basal conditions that is greatly increased by PTP inhibitors. These data indicate that COX-2 itself could be a substrate for tyrosine phosphorylation. These data may also indicate that tyrosine residues, which are targets for phosphorylation, are located in the active site of the COX-2 molecule. Effective inhibition of both COX-1 and COX-2 isoforms by indomethacin, ibuprofen, and aspirin indicates a structural similarity between the active sites of the enzyme (21, 25). However, certain differences in the active sites of COX isoforms provide a structural basis for selective inhibition of COX-2 by SC-558 and NS-398 (12, 20). Because two tyrosine residues, Tyr-371 and Tyr-341, are situated in the active site of COX-2 near the NS-398 binding site, we may speculate that these
tyrosine residues are among other potential targets for posttranslational phosphorylation that increase the enzyme activity.

We investigated possible physiological contributions of protein tyrosine phosphorylation to COX-mediated changes in cerebral microcirculation. As we have shown previously in newborn pigs, arachidonic acid topically applied to the cerebral surface causes dose-dependent production of prostacyclin and PGE₂, and the vasodilatation of cerebral arterioles, both of which are prevented by indomethacin (23). Therefore, arachidonic acid-induced dilation of cerebral arterioles could be used as a model of a COX-mediated vascular response in vivo. We reported that phenylarsine oxide greatly potentiates the COX-mediated dilation of cerebral microvessels in response to arachidonic acid (26). These effects of phenylarsine oxide on cerebral vascular responses were abolished by indomethacin (26). These data provide physiological evidence that increasing protein tyrosine phosphorylation potentiates COX-mediated responses in vascular beds that constitutively express COX-2.

In conclusion, this study describes a novel mechanism of regulation of prostanooid production by post-translational phosphorylation of COX-2 via PTK- and PTP-dependent pathways. This mechanism may be of special importance in cells and tissues expressing COX-2 constitutively, especially in the newborn circulation. This novel mechanism could provide a basis for a new approach to pharmacological correction of circulatory disorders in newborns.

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