The presence of prostaglandin (PG) H₂ in the supernatant of human umbilical vein endothelial cells (HUVEC) stimulated by thrombin restores the capacity of aspirin-treated platelets to generate thromboxane (TX) B₂. Induction of cyclooxygenase-2 (Cox-2) by interleukin (IL)-1α or a phorbol ester increases this formation. HUVEC treated with aspirin lost their capacity to generate PGs but recovery occurred after 3- or 6-h induction of Cox-2 with phorbol ester or IL-1α. Enzyme activity of the newly synthesized Cox-2 in aspirin-treated cells, evaluated after immunoprecipitation, was similar to untreated cells but after 18 h of cell stimulation only 50-60% recovery of Cox-1 was observed. The use of SC58125, a selective Cox-2 inhibitor, confirmed these findings in intact cells. Cyclooxygenase activity was related to the amount of Cox proteins present in the cells, but after induction of Cox-2, contribution of the latter to TG production was 6-8-fold that of Cox-1. Aspirin-treated or untreated cells were incubated in the absence or presence of SC58125 and stimulated by thrombin, the ionophore A23187, or exogenous arachidonic acid. The production of endogenous (6-keto-PGF₁₂, PGE₂, PGF₂α) versus transcellular (TXB₂) metabolites was independent of the inducer, the source of arachidonic acid and the Cox isozyme. However, in acetylsalicylic acid-treated cells, after 6-h stimulation with IL-1α, newly synthesized Cox-2 produced less TXB₂ than 6-keto-PGF₁₂ compared to untreated cells. At later times (>18 h), there was no metabolic difference between the cells. These studies suggest that in HUVEC, Cox compartmentalization occurring after short-term activation may selectively affect transcellular metabolism, but not constitutive production, of PGs.

Thromboxane (TX) A₂ and prostaglandin (PG) I₂ (prostaglandin H₂) are the main products of arachidonic acid metabolism via the cyclooxygenase (Cox; also known as PGH synthase (EC 1.14.99.1)) pathway in platelets and endothelial cells, respectively (1). The synthesis of TXA₂ is suppressed by aspirin (ASA), an irreversible Cox inhibitor (2, 3). Some years ago, Marcus and colleagues (4) demonstrated that endoperoxides generated by platelets stimulated by thrombin, collagen, or the Ca²⁺ ionophore A23187 could restore the capacity of ASA-treated endothelial cells to synthesize PGI₂, as hypothesized earlier (5). Because platelets are non-nucleated cells, their potential for producing TXA₂ is irreversibly suppressed in vitro or in vivo during their lifetime. Whereas platelets contain a constitutively expressed Cox (Cox-1), in most other cells the existence of an inducible Cox has been demonstrated. This novel PGH synthase 2, i.e. Cox-2, is highly regulated by external ligands such as cytokines, tumor promoters, or growth factors (6). Cox-2 was identified as a separate product of an early response gene (7, 8). It has been cloned and sequenced in human endothelial cells (9, 10). In addition to rapid renewal of the enzyme, induction of Cox-2 has been regarded as a mechanism by which cells increase their capacity to synthesize PGs in excess of that provided by Cox-1 (11). However, metabolic (12) and immunofluorescence localization experiments (13) suggest that substrate and/or enzyme compartmentalization may constitute an additional important step in regulation of PG production.

Here we show that endothelial cells stimulated by thrombin can restore the production of TX by aspirin-treated platelets via transcellular metabolism. Both Cox isozymes contribute to the capacity of endothelial cells to synthesize PGH₂, from endogenous or exogenous arachidonic acid. The net amount of products synthesized was dependent on the quantity of active cyclooxygenases in cells, and Cox compartmentalization occurring after short-term activation transiently affects transcellular metabolism, but not constitutive production, of PGs.

**EXPERIMENTAL PROCEDURES**

Materials—Recombinant human IL-1α was from Genzyme (Cambridge, MA). Furegrelate was from Cayman Chemical (Ann Arbor, MI). PMA, bovine thrombin, bovine serum albumin (fraction V), arachidonic acid, PGE₂, hematin, n-octyl-β-D-glucopyranoside (n-octyl glucoside), acetylsalicylic acid (ASA), salicylic acid (SA), monoclonal anti-β-actin, and sheep polyclonal anti-mouse IgG coupled to peroxidase were from Sigma. Arachidonic acid was purified by silicic acid column chromatography and stored at −70 °C. Enhanced chemiluminescence substrates, [¹H]thromboxane B₂ (4.4 TBq/mmol), and [¹H]arachidonic acid (7.99 TBq/mmol) were from Amersham (Les Ulis, France). Electrophoresis reagents were from Bio-Rad (Richmond, CA), and chemical products from Prolabo (Paris, France) and Carlo Erba (Farmatilia, Milan, Italy). The IgGs against 9,11-azo-15-hydroxy-prosta-5,13-diene acid, a stable PGH₂ mimic, were a kind gift from Dr. Frank Fitzpatrick, the Upjohn Co. (Kalamazoo, MI). Protein A-Sepharose CL-4B was from Pharmacia.
Platelet-Endothelial Cell Interactions

(Bromma, Sweden). SC58125 was kindly provided by Dr. Peter Isakson (Searle Monsanto, St. Louis, MO).

Platelets—Venous blood was obtained from normal donors who had not received any medication for at least 1 week prior to collection at the blood bank in the Hôpital Lariboisière. Blood (9 volumes) was anticoagulated with 1 volume of ACD-C, NHH, formula A (130 mM citric acid, 124 mM sodium phosphate, and 110 mM containing ASA, 7.72 mM concentration) and centrifuged at room temperature for 15 min at 100 × g to obtain platelet-rich plasma. After further addition of ASA (1 mM), platelet-rich plasma was centrifuged for 15 min at 1,000 × g. Platelets were washed using a citrate buffer, pH 6.5, containing 10−5 M PGE2, according to the method described by Patscheke (14). The platelet pellet was finally resuspended in Hanks’ buffer and washed twice in the same buffer and the cell concentration was adjusted to 106/ml unless otherwise indicated. Platelet microsomes from ASA-treated cells were prepared as described (15) and stored at −70 °C until use. TX synthase activity was verified by the platelet microsomes’ capacity to convert PGH2 into TX. For incubations, microsomes were centrifuged at 150,000 × g for 60 min at 4 °C and suspended in Hanks’ buffer prior to addition to endothelial cells.

Cell Culture and Stimulation—Human umbilical vein endothelial cells (HUVEC) were prepared according to the method of jf et al. (16) with some modifications. Culture conditions were described previously (17). Passage cells were subcultured on gelatin-coated plates (Costar), or in 100-mm2 culture dishes (Nunc, Roskilde, Denmark); they were seeded at 7.5 × 104 cells/ml and allowed to grow to confluence. Heparin and endothelial cell growth supplement were removed 48 h before stimulation. Cells at first passage were incubated in culture medium containing 5% heat-inactivated normal human serum (NHS). Cultures were activated with IL-1α or PMA for different times prior to co-incubations with platelets, as described in the figure legends.

Cell Incubations—Platelets in Hanks’ buffer were incubated alone, with HUVEC activated with 20 nM PMA or 25 units/ml IL-1α, or with endothelial cells maintained in 5% NHS. Conditions are indicated in the figure legends. After activation, the medium was changed, and after 1-min preincubation at 37 °C individual cells or co-incubates were stimulated by thrombin (2 units/ml) for 2 min unless otherwise indicated in the figure legends. The reaction was quenched by transferring the supernatant to citric acid-containing tubes (final concentration, 50 mM). After neutralization with 1 mM KH2PO4, the supernatants were analyzed by EIA.

In some experiments, endothelial cells were incubated for 30 min with 100 μM ASA, SA, or vehicle (i.e., ethanol). After removal of the supernatant, cells were washed 3 times and maintained for 30 min in culture medium in order to remove any residual ASA or SA. The medium was changed and cells were activated with PMA (25 nM), IL-1α (25 units/ml), or medium alone (containing 5% NHS), for different times. Cells were either lysed for Western blot analysis (see below) or incubated with aspirin-treated platelets and stimulated by thrombin, arachidonic acid, or the Ca2+ ionophore A23187 in the absence or presence of a specific Cox-2 inhibitor (18). The supernatant was assayed for TXB2 and 6-keto-PGF1α.

Prostaglandin Production—The stable metabolite of prostacyclin, 6-keto-PGF1α, TXB2, PGE2, and PGF2α were determined in the supernatants using EIA with acetylcholinesterase-labeled 6-keto-PGF1α, TXB2, PGE2, or PGF2α as tracers (19). Results are expressed in nanograms/ml and the cell concentration is indicated in the figure legends. Verification of cell counts revealed no major variation in number for a given setting throughout the experimental period.

Culture and [3H]Arachidonic Acid Labeling of HUVEC—HUVEC in T-162 flasks (6 × 105 cells) were labeled for 18 h at 37 °C with 7,400 kBq of [3H]arachidonic acid. The medium was changed and the cells were incubated with NHS for 6 h in the absence or presence of 20 nM PMA. After washing, the cells were incubated alone or with platelets (ratio of endothelial cell/platelet ratio, 1/125) and stimulated by thrombin. The reaction was quenched with citric acid and the sample neutralized by addition of K2HPO4. After addition of methanol (2 volumes), the samples were kept overnight at −20 °C for protein precipitation. The supernatant was subjected to solid-phase extraction (C18, Bakerbond, Baker, Phillipsburg, N.J.) and analyzed by HPLC.

Chromatographic Analysis—After solid-phase extraction, the different compounds formed from [3H]arachidonic acid-labeled endothelial cells were resolved by HPLC using a C18 column (Ultrisilapex Si, 5 μm, 250 × 4.6 mm, Beckman, Palo Alto, CA) with a flow rate of 1 ml/min. Elution was performed using a nonlinear gradient between solvent A (water/acetonitrile/acetic acid, 96/10/0.1, v/v) and solvent B (acetonitrile/acetic acid, 100/0.1, v/v). The column was eluted in 100% solvent A and the percentage of solvent B was 20% at 2 min, 45% at 12 min, 80% at 20 min, and 100% between 22 and 26 min. After 7-min re-equilibration in 100% solvent A, the system was ready for a new run. Fractions were collected every 20 s. Radioactivity was counted in each fraction by liquid scintillation counting (Packard 6000 SC). Each fraction was also analyzed by EIA for its content in 6-keto-PGF1α, PGE2, and TXB2.

In preliminary experiments, the specificity of the TXB2 determination by EIA was verified by purifying the samples by thin-layer chromatography or HPLC. In these experiments, [3H]TXB2 was added before solid-phase extraction to estimate the losses occurring during the various steps of purification. HPLC was done as described above. The fractions corresponding to TXB2 were pooled, evaporated to dryness, and the A23187 was performed after developing the plates in chloroform/methanol/acetic acid/water (90:8:1.8, v/v). Authentic TXB2 (1–2 μg) was applied in a separate lane and visualized using a 3.5% phosphomolybdic acid spray. The corresponding lane was scraped and compounds were eluted by adding the silica to EIA buffer (0.1 M phosphate, pH 7.4, containing 0.15 M NaCl, 0.1% bovine serum albumin, and 0.01% sodium azide). Results after ether purification technique corresponded to those obtained by direct EIA.

Western Blot Analysis—After incubation, monolayers of HUVEC in 6-well plates (3–4 × 105 cells/well) were washed twice in phosphate-buffered saline and lysed for 30 min in ice-cold buffer (50 mM Tris/HCl, pH 7.4, containing 1 mM EDTA and 1 mM benzamidine hydrochloride), spiked with a rubber policeman, and centrifuged at 4 °C for 10 min at 10,000 × g. Protein content was determined by a micro-biornichoninic acid assay (Pierce) with bovine serum albumin as standard. SDS-PAGE analysis was performed as described previously (17, 20, 21). Monoclonal antibodies Cox-2 29 (1/10,000) and 3 specific monoclonal antibodies against Cox-1 (20, 10, 11, at 5 μg/ml) were used for Cox-2 and Cox-1 analysis, respectively (17, 22, 23). Monoclonal antibody directed against β-actin was used as internal standard for control of protein load. Blots were further incubated with sheep antirabbit IgG conjugated with horseradish peroxidase at 1/2,000 (0.1 ml/cm2) for 1 h at room temperature. ECL substrates were used to reveal positive bands according to the manufacturer’s instructions and bands were visualized after exposure to Hyperfilm™ ECL (Amersham). Protein bands were quantified on the films by densitometry using an LKB Ultrascan XL laser densitometer (Pharmacia).

Recovery of Cyclooxygenase Activity in Immunoprecipitates of Cox-1 or Cox-2 from Cells Treated with ASA—HUVEC in a 60-mm2 culture dish were treated with 100 μM ASA, SA, or vehicle as described in the cell incubation section. After 3 (PMA) or 6 (IL-1α) h, control or activated HUVEC were lysed in solubilization buffer (see above). The lysates were immediately frozen and kept at −70 °C. Cyclooxygenase activity was assessed after selective immunoprecipitation of Cox-2 using specific polyclonal anti-Cox-2 peptide antibody coupled to protein A-Sepharose as described previously (17), except that the reaction buffer consisted of Tris/HCl, pH 8.0, containing 1 mM phenol and 1 mM magnesium protamine. After 1 min at 37 °C in the presence of ECL buffer, the reaction was initiated by adding arachidonic acid (25 μM). After 10 min at 37 °C, the reaction was quenched by adding 1 volume of ice-cold EIA buffer and the mixture was centrifuged in a microcentrifuge (5,000 × g, 1 min). PGE2 in the supernatant was measured by EIA (19). Protein A-Sepharose coupled to IgG-bound Cox-2 was washed 5 times with 0.5 ml of the lysis buffer and mixed with Laemmli reagent under reducing conditions. Samples were subjected to SDS-PAGE electrophoresis as described above and Cox-2 protein was revealed under the same conditions.

Statistics—The different parameters were evaluated by means of a specialized software (Sigma-Stat™, J andel, CA).

RESULTS

Co-incubation of Aspirin-treated Platelets with Endothelial Cells—Co-incubation of aspirin-treated platelets with endothelial cells (2 × 105 cells in 12-well dishes, endothelial cell/platelet ratio, 1/25) stimulated by thrombin resulted in significant synthesis of TXB2 (3.9 ± 0.5 ng/ml, n = 4 compared to 0.5 ng/ml for aspirin-treated platelets) (Fig. 1A). An additional 3-fold increase in TX over control HUVEC (10.9 ± 0.7 ng/ml) was obtained with endothelial cells activated for 6 h by PMA prior to co-incubation. The production of 6-keto-PGF1α by endothelial cells was not modified by the presence of platelets, but the synthesis of PGE2 decreased (not shown). Addition of ASA (100 μM) to endothelial cells prior to co-incubation suppressed
Stimulated by Thrombin—

formation of TXB2, different sets of experiments were performed. Dodeperoxides liberated by HUVEC were responsible for the formation of TXB2 in a platelet suspension (0.2 × 10^9 cells/ml) and TXB2 was measured by EIA. In some incubates, EC were activated for 6 h with 20 mM PMA prior to addition of platelets. In all incubations, platelets were pre-treated with ASA (see "Experimental Procedures"), but in the data presented in the last histogram ASA (100 μM) was added to endothelial cells prior to co-incubation. B, effect of platelet concentration. Endothelial cells (as above) activated by PMA were incubated with increasing numbers of platelets and stimulated with thrombin. C, concentration-response effect of thrombin. Co-incubations (same as above) were stimulated with increasing concentrations of thrombin. The supernatants were acidified with citric acid (final concentration, 50 mM), neutralized, and assayed by EIA. Data represent the mean ± S.D. (three to four experiments in replicates of 2).

TX and 6-keto-PGF$_1\alpha$, formation by more than 95%. The time for maximal formation of TXB$_2$ after addition of thrombin was 2 min (not shown); subsequent incubations were performed at this time, unless indicated. HUVEC activated for 6 h by PMA and challenged for 2 min with 2 units/ml thrombin synthesized negligible amounts of TXB$_2$ (0.4 ± 0.07 ng/ml, n = 5) compared to 6-keto-PGF$_1\alpha$ (21.9 ± 4.5 ng/ml, n = 5). The identity of TXB$_2$ was verified by EIA after purification of the samples by thin-layer chromatography or HPLC (see "Experimental Procedures").

The dependence of platelets was tested as a function of the ratio of platelets to endothelial cells. The formation of TXB$_2$, increased as a function of platelet number and a plateau was reached at 0.5–1 × 10^9 platelets/well (i.e. endothelial cell/platelet ratio of 1:250, Fig. 1B). No TX was formed in the absence of thrombin stimulation; TXB$_2$, although increased as a function of thrombin concentration (0–4 units/ml) (Fig. 1C). In subsequent experiments, respective platelet/endothelial cell ratios of 125 and 2 units/ml of thrombin were used.

Generation of PGH$_2$ in the Supernatant of Endothelial Cells Stimulated by Thrombin—In order to demonstrate that endoperoxides liberated by HUVEC were responsible for the formation of TXB$_2$, different sets of experiments were performed. In the first, PMA-treated endothelial cells were stimulated by thrombin for 2 min. The supernatant was transferred either to a platelet suspension (0.2 × 10^9 cells/ml) or to Hank’s buffer. After 2 min, the reaction was quenched by citric acid and TXB$_2$ was measured by EIA (Fig. 2A). Significant amounts of TXB$_2$ were detected only in the supernatant transferred to the tube containing platelets (3 ± 0.2 versus 0.3 ± 0.04 ng/ml, mean ± S.E. n = 3). These results demonstrate that direct cell contact is not necessary for this reaction, although the net amount of TXB$_2$ formed under these conditions was reduced 3–5-fold compared to co-incubates (compare Fig. 2A to Fig. 1, A or B).

another set of experiments, endothelial cells were stimulated by thrombin and the supernatant was transferred to a tube. Aliquots were added at intervals to tubes containing platelets. There was a rapid time-dependent decrease in the capacity of the supernatant added to platelets to generate TXB$_2$, which disappeared at later times (i.e. 30 min) (Fig. 2B). The apparent half-life of the unstable intermediate contained in the supernatant of endothelial cells stimulated by thrombin was calculated by plotting TXB$_2$ values as a function of time. The half-life, calculated from five separate experiments, was t$_{1/2}$ = 92 ± 17 s, which is very similar to that described previously for PGH$_2$ (24). In order to confirm the identity of PGH$_2$, we used IgGs directed against a PGH$_2$ mimic, previously shown to decrease platelet aggregation and TXA$_2$ generation after addition of PGH$_2$ (25). Addition of specific IgGs to platelet/endothelial cell co-incubations (25–150 μg/ml) resulted in a concentration-dependent inhibition in the formation of TXB$_2$, with a weaker effect on 6-keto-PGF$_1\alpha$ (Fig. 2C).

In contrast, nonspecific IgGs did not modify the production of TX compared to controls, even at the highest concentration (150 μg/ml). Addition of furegrelate, a TX synthase inhibitor (26), to the co-incubation mixture concentration dependently inhibited the synthesis of TX (Fig. 2D). In the same samples, there was a symmetric increase in PGE$_2$, whereas the synthesis of 6-keto-PGF$_1\alpha$, was unchanged (not shown). These results support the involvement of a TX
synthase catalyzing the transformation of PGH2 into TX. Finally, endothelial cells, labeled with [3H]arachidonic acid and activated by PMA, were stimulated by thrombin in the absence or presence of platelets. HPLC analysis of the supernatant of [3H]arachidonic acid-labeled endothelial cells in the absence (A) or presence (B) of aspirin-treated platelets. PMA-activated HUVEC (6 × 10^5 cells) grown in T-150 flasks were labeled with [3H]arachidonic acid as described under "Experimental Procedures." Cells were stimulated for 2 min by 2 units/ml thrombin in the absence or presence of platelets and analysis of samples by HPLC was performed as described under "Experimental Procedures." Each fraction (0.33 min) was counted for radioactivity, and immunoreactivity was analyzed by EIA.

Relation between the Amount of TXA2 Formed by Transcellular Biosynthesis and Expression of Cox-2—In order to verify the importance of Cox-2 in the increased capacity of endothelial cells to generate PGH2, cells were activated under conditions known to modify the expression of Cox-2 but not that of Cox-1 (e.g. various concentrations of PMA or IL-1α for different times) (23). In some experiments, Western blot analysis of HUVEC was performed (not shown) to assess the relation between the level of expression of Cox-2 and the amount of 6-keto-PGF1α formed upon thrombin stimulation (17). The good correlation between 6-keto-PGF1α and the amount of TX generated in the co-incubate (r = 0.82, p < 0.001, n = 22) reflects the fact that the generation of PGH2 for constitutive and transcellular metabolism is related to the net cyclooxygenase activity of endothelial cells (Fig. 4).

Recovery of Cox Activity by Endothelial Cells Treated with ASA—As shown previously (see Fig. 1A), endothelial cells treated with ASA lose their metabolic capacity to produce PGH2 and PGF2α (i.e. constitutive metabolism), but also TX by transcellular metabolism. The synthesis of Cox-2 induced by IL-1α was similar in endothelial cells treated with either ASA or SA as analyzed by Western blot (Fig. 5). Densitometric analysis of Cox-2 protein in cells pretreated with SA or ASA indicated respective values of 103 ± 12% and 130 ± 11% of those noted with control cells (n = 4). Similar results were obtained with PMA (not shown). The rapid recovery of PG formation was tested directly on cells pretreated for 30 min with SA or ASA and activated by PMA or IL-1α. Enzyme activity was measured in the immunoprecipitates of Cox-2 or Cox-1 from lysates using specific antibodies (17). Immediately after ASA treatment, activities of Cox-1 or Cox-2 were inhibited by more than 97% compared to SA (Fig. 6). Three hours after activation of endothelial cells by PMA (or 6 h with IL-1α), activity in the immunoprecipitate of Cox-2 from aspirin-treated cells was similar to that obtained from cells treated with SA (Fig. 6). In the absence of cell activation (i.e. cells incubated with NHS alone), immunoprecipitates from ASA- or SA-treated cells showed a lower, but similar activity (Fig. 6). The presence of 5% NHS was likely responsible for the induction of Cox-2 (17). In contrast, no activity was detected in the immunoprecipitate of Cox-1 obtained from aspirin-treated cells (not shown). These results demonstrate that the rapid recovery of PGH2 generation in ASA-treated endothelial cells was due to de novo synthesis of Cox-2.

However, in intact cells less TX was formed after activation of aspirin-treated endothelial cells (Fig. 7A). The specific Cox-2 inhibitor SC58125 was used in order to identify the enzyme responsible for the recovery in activity after treatment with ASA. This compound totally suppressed the synthesis of TX in cells treated with ASA, prior to activation. There was no difference in TXB2 formation in SA-treated and untreated cells (Fig. 7B). These results demonstrate that: 1) after ASA treatment, the rapid recovery of enzyme activity responsible for TX synthesis is due exclusively to Cox-2; 2) in untreated cells (or cells treated with SA), Cox-1 and Cox-2 are involved in transcellular biosynthesis; 3) in control cells (incubated in NHS), a significant amount of TX originates from Cox-2 (50–70% of total) (Fig. 7B, left panel). This last result indicates that induction of Cox-2 increases 6–8-fold the basal capacity (i.e. provided by Cox-1) of endothelial cells to generate PGs. Aspirin-treated cells stimulated for 18 h with IL-1α recovered 92 ± 20% of TX synthesis compared to untreated cells (n = 3) (not shown). Incubation of SC58125 with these cells indicated 50–60% recovery of Cox-1 activity in ASA-treated cells after this prolonged incubation. This is in agreement with a slower turnover of Cox-1 protein compared to Cox-2 found previously using [35S]methionine-labeled cells (17).

Influence of Cox-1 Isotype and/or Substrate Source for the Production of Constitutive versus Transcellular Metabolism—A recent paper (12) suggests that arachidonic acid, released in response to ligand stimulation or added exogenously, is differentially used by Cox-1 or Cox-2. Our experimental setting provides conditions in which PG production originates mostly from Cox-2 (i.e. ASA-treated cells activated by PMA) or Cox-1 (i.e. untreated cells, incubated with the specific Cox-2 inhibitor...
The generation of TXB₂ in endothelial cells stimulated by thrombin in the presence of microsomes rose from 0.5 ng/ml to 6.9 ng.

There was no variation in 6-keto-PGF₁α or PGF₂α (25 versus 24 and 17 versus 18 ng/ml, respectively). In contrast, PGE₂ decreased in the presence of microsomes (13 versus 6 ng/ml), probably due to the shift in metabolism of PGH₂ into TXB₂. Addition of 5 μM arachidonic acid to the system increased the synthesis of all compounds: 13.5 versus 6.8 ng/ml TXB₂; 23 versus 23 ng/ml 6-keto-PGF₁α; 31 versus 17 ng/ml PGF₂α, and 11.5 versus 6 ng/ml PGE₂. These results suggest that exogenous substrate contributes equally to the increment of synthesis of all compounds. Using the same conditions of isozyme activities, no difference in 6-keto-PGF₁α production was observed in the absence or presence of platelets (n = 4, not shown), suggesting that these cells do not influence endothelial cell metabolism. Upon activation of endothelial cells, Cox-1 and Cox-2 are differently compartmentalized (13) and may selectively provide PGH₂ for conversion into transcellular (i.e. TX) or constitutive metabolites (e.g. PGI₂). The amount of TX was compared to that of 6-keto-PGF₁α, under conditions of Cox-1 or Cox-2 activities (see above). The 6-keto-PGF₁α/TX ratio did not vary significantly, irrespective of the nature of the isozymes or the inducers (Table II). However, this ratio was higher (approximately 2-fold) in ASA-treated cells activated by PMA in which only Cox-2 contributes to the formation of products. The increased synthesis of endogenous 6-keto-PGF₁α over TX reflects a lesser capacity of newly synthesized Cox-2 to export PGI₂ from transcellular metabolism. This result is consistent with a lower recovery in TX formation found in intact aspirin-treated endothelial cells after short-term activation (Fig. 7).

**DISCUSSION**

We have shown that induction of Cox-2 in human endothelial cells contributes considerably to the synthesis of PGI₂ which can be metabolized by ASA-treated platelets via transcellular biosynthesis. Demonstration of substrate transfer for the synthesis of TXA₂ is supported by several arguments: (i) after stimulation of endothelial cells with thrombin, only early addition of supernatant to ASA-treated platelets generates TX;
(ii) an unstable intermediate is present in the supernatant of stimulated endothelial cells with an apparent half-life compatible with that of PGH₂; (iii) TX formation is reduced when an antibody directed against a PGH₂ mimic is added to the cell co-incubate; (iv) TX synthesis was inhibited in the presence of a TX synthase inhibitor; (v) H-labeled TX is formed when using [³H]arachidonic acid-labeled endothelial cells in the presence of aspirin-treated platelets. The release from the cell of a transient metabolic intermediate, transformed by vicinal acceptor cells, has been reported in other situations (27). Results in Fig. 2A suggest that PGH₂ released in the supernatant of stimulated cells is available for conversion into TX. Although a report claimed that only unidirectional transfer of substrate from platelets to endothelial cells could occur (28), these results are not supported by our studies. This discrepancy could be explained either by low levels of cyclooxygenase in these cells and/or by the selective presence of one isozyme.

The discovery that endothelial cells, like other nucleated cells, contain a primary response gene responsible for the expression of Cox-2 upon cell activation calls for careful evaluation of such reactions. Increased presence of Cox-2 has been explained either by low levels of cyclooxygenase in these cells or "input" or "output" of substrates (9, 10, 23). In the present experiments, induction of Cox-2 also correlates with the greater capacity of the cell co-incubate to generate TX (Fig. 4).

An important aspect of the studies reported here concerns the respective contributions of endothelial cell Cox-1 and Cox-2 to the "input" or "output" of substrates (i.e. arachidonic acid and PGH₂, respectively). The irreversible inhibition of PG synthesis by the 30-min treatment of endothelial cells with ASA is rapidly overcome after induction of Cox-2 by different stimuli. It has been suggested that ASA or other non-steroidal anti-inflammatory drugs may influence de novo synthesis of Cox (29, 30). Although this could be due to inhibition of the transcription factor NF-κB by ASA (31), concentrations were 10–50 times higher (>1 μM) than those used here. Western blot analysis (Fig. 5) shows that pretreatment of cells with ASA or SA does not interfere with de novo synthesis of Cox-2. In addition, the rapid (2–6 h) recovery in activity is due to induction of Cox-2: (i) in cell lysates of ASA-treated cells, enzyme activity is only present in the immunoprecipitates of Cox-2 but not of Cox-1; (ii) in intact cells, the specific Cox-2 inhibitor, SC58125 suppresses totally the activity in ASA-treated cells stimulated by PMA or IL-1α but not that of untreated cells; (iii) Cox-1 activity is partially recovered after longer periods of stimulation (>10 h). In fact, non-activated HUVEC contain a primary response gene responsible for the expression of Cox-2 in endothelial cells and a selective production of PGs related to an exogenous or endogenous source of substrates. Comparable results were obtained in the presence of microsomes from aspirin-treated platelets, and the synthesis of 6-ko-to-PGF₁α, was similar in endothelial cells alone and in the presence of platelets. We suggest that the selectivity of Cox isozymes with respect to the source of arachidonic acid and enzyme compartmentalization may also depend on the biological system (cell source, species, inducer, etc.) as most other examples have been obtained on cell lines (12, 32–34). Moreover, reasons inherent to enzyme activities have been presented to explain a differential control of PG synthesis by the two isoforms when present in the same cell (35).

### Table I

| Role of Cox-1 versus Cox-2 in the production of TX from endogenous or exogenous arachidonic acid in endothelial cells/platelets co-incubates (TXB₂, ng/ml) |
| Control cells incubated in NHS alone and treated with SC58125 (5 μM) represent a source of Cox-1-derived metabolites (Cox-1), whereas in ASA-treated cells activated for 3 h by PMA, Cox-2 accounts for most activity (Cox-2). Cells were treated with aspirin described under "Experimental Procedures." Platelets (10⁷/ml) were added, and after 5 min stimulation by the inducers the reaction was quenched by addition of citric acid and TXB₂ was analyzed by EIA. The results are in ng/ml and represent the mean ± S.D. of 5 different experiments. Comparison of data in either Cox-1 or Cox-2 settings was done using one-way analysis of variance: arachidonic acid versus A23187 versus thrombin. |
| Arachidonic acid (10 μM) | A23187 (2 μM) | Thrombin (2 units/ml) |
|--------------------------|----------------|------------------------|
| Cox-1 (control cells + SC) | 2.1 ± 1.1 | 1.9 ± 1.8 | 3.1 ± 1.8 |
| Cox-2 (ASA-treated cells + PMA) | 4.8 ± 1.7 | 4.3 ± 0.8 | 3.9 ± 1.8 |

*NS, not significant.

### Table II

Production of transcellular (TXB₂) and constitutive (6-keto-PGF₁α) metabolites by Cox-1 or Cox-2 from endothelial cells incubated in the presence of ASA-treated platelets (TXB₂, ng/ml) and 6-keto-PGF₁α, ng/ml)

Endothelial cell/platelets were incubated as described in the legend to Table I. After stimulation with the different inducers, the supernatants were analyzed for TXB₂ and 6-keto-PGF₁α by EIA. Results represent the mean ± S.D. of 4–6 different experiments.

| Control cells | ASA-treated cells + PMA, 3 h |
|----------------|-----------------------------|
| Arachidonic acid (10 μM) | A23187 (2 μM) | Thrombin (2 units/ml) |
| TXB₂ | 6-Keto | 6k/TX | TXB₂ | 6-Keto | 6k/TX | TXB₂ | 6-Keto | 6k/TX |
|--------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 2.7 ± 0.8 | 3.2 ± 2 | 1.1 | 5.8 ± 1.6 | 9.7 ± 4.2 | 1.9 | 9.4 ± 2.6 | 12.3 ± 7.4 | 1.3 |
| 3.1 ± 2.2 | 4.5 ± 2.6 | 1.4 | 4.8 ± 1.3 | 11.6 ± 5.3 | 2.4 | 16.6 ± 7.2 | 155.7 ± 7.8 | 0.9 |
| 4.5 ± 2.1 | 5.1 ± 2 | 1.1 | 4.3 ± 1.8 | 10.2 ± 4.9 | 2.4 | 12.4 ± 7.8 | 12.3 ± 5.1 | 1.0 |
Another aspect of these studies concerns the release of PGH₂ into the external milieu for its conversion into TXA₂ by platelets. Although Cox-1 or Cox-2 can contribute to constitutive and transcellular metabolisms, a different response was observed after treatment of cells with ASA. PGH₂ generated from newly synthesized Cox-2 produced a relative increase in intracellular 6-keto-PGF₁α over transcellular TXB₂ (Table II, “Cox-2” column). This suggests that PGH₂ is more readily metabolized, by intracellular enzymes at early times of protein synthesis (i.e. 3 h of induction), than released outside the cell for transcellular biosynthesis. Short-term synthesis of Cox-2 coincides with lo-

cation of the enzyme around the nucleus (13). These findings reconcile data from Figs. 6 and 7 since, after 2 h activation, aspirin-treated cells recovered >80% of Cox-2 activity evaluated by 6-keto-PGF₁α, but only 35–50% based on TXB₂ production. After 18 h, 90% of 6-keto-PGF₁α and TXB₂ synthesis had been recovered.

In summary, in endothelial cells, enzyme compartmentalization occurring after short-term activation may selectively affect transcellular metabolism but not constitutive production of PGs. The quantity of PGs is clearly related to the amount of Cox (mainly type 2) present in cells under our conditions. Because Cox-2 expression can be regulated rapidly and significantly, its level can significantly affect the capacity of endothelial cells to generate PGs. In addition, when Cox-1 is irreversibly inactivated, induction of Cox-2 provides cells with a rapid de novo capacity to generate PGs.

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