Synthesis of 8-epi-prostaglandin $F_{2\alpha}$ by human endothelial cells: role of prostaglandin H_2 synthase

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The experiments described in this paper were designed to determine the mechanism underlying the increase in 8-isoprostaglandin $F_{2\alpha}$ (8-epi-PGF $_{2\alpha}$) production by cultured human endothelial cells during reoxygenation following hypoxia. Human umbilical artery endothelial cells were grown on microcarrier beads and exposed to sequential periods of normoxia, hypoxia, and reoxygenation. The amount of 8-epi-PGF $_{2\alpha}$ in the medium was determined by ELISA. The production of 8-epi-PGF $_{2\alpha}$ decreased by greater than 90% during hypoxia. Upon reoxygenation 8-epi-PGF $_{2\alpha}$ production increased linearly for 90 min reaching nearly 3 times normoxic levels. When added to the medium during reoxygenation, neither superoxide dismutase nor Tiron, a cell-permeable superoxide scavenger, inhibited 8-epi-PGF $_{2\alpha}$ production. However, 8-epi-PGF $_{2\alpha}$ production was inhibited by catalase. The production of 8-epi-PGF $_{2\alpha}$ was also inhibited by indomethacin and aspirin. Exogenous hydrogen peroxide stimulated 8-epi-PGF $_{2\alpha}$ production by normoxic cells,

and aspirin inhibited the hydrogen peroxide-mediated increase in 8-epi-PGF $_{2\alpha}$ production. These results indicate that the reactive oxygen species responsible for 8-epi-PGF $_{2\alpha}$ synthesis during reoxygenation is hydrogen peroxide and that in endothelial cells 8-epi-PGF $_{2\alpha}$ synthesis is mediated by prostaglandin H_2 synthase (PGHS). To verify the role of PGHS in 8-epi-PGF $_{2\alpha}$ synthesis, human PGHS-1 was expressed in COS-7 cells, a PGHS negative cell line that does not synthesize 8-epi-PGF $_{2\alpha}$. In the presence of exogenous arachidonic acid the COS-7 cells expressing human PGHS-1 produced substantial amounts of PGE $_2$ and 8-epi-PGF $_{2\alpha}$. These data indicate that human PGHS-1 can support the synthesis of 8-epi-PGF $_{2\alpha}$ and that 8-epi-PGF $_{2\alpha}$ synthesis by cultured human endothelial cells during reoxygenation is dependent on the activity of PGHS-1.

Key words: endothelium, hypoxia.

INTRODUCTION

The F_2 -isoprostanes are a family of oxygenated arachidonic acid derivatives that are structurally similar to prostaglandins and thromboxanes. One of the more abundant isoprostanes in the plasma and urine of humans is 8-epi-PGF $_{2\alpha}$ [1]. 8-epi-PGF $_{2\alpha}$ is a potent vasoconstrictor [2], potentiator of platelet aggregation [3,4], smooth muscle cell mitogen [5], and a mediator of pulmonary oedema [6]. It is also a weak platelet thromboxane/endoperoxide receptor agonist [2,7]. Because the biological effects of 8-epi-PGF $_{2\alpha}$ are similar to those of thromboxane A_2 , it was initially thought that the effects of 8-epi-PGF $_{2\alpha}$ were mediated through the thromboxane- A_2 receptor. However, it has been shown that 8-epi-PGF $_{2\alpha}$ binds to an as yet unidentified receptor that is distinct from the thromboxane- A_2 receptor [3,5,8,9].

8-epi-PGF $_{2\alpha}$ can be synthesized by both enzymatic and non-enzymatic processes and was initially shown to be formed in ram seminal vesicle microsomes during the synthesis of prostaglandins [10]. 8-epi-PGF $_{2\alpha}$ synthesis has also been shown to be dependent on the activity of PGHS-1 in human platelets [11], and on the activity of PGHS-2 in zymosan [12] or lipopolysaccharide [13] activated human monocytes, cytokine-treated human pulmonary artery segments [14], and rat mesangial cells [15]. The F_2 -isoprostanes can also be formed non-enzymatically by free radicals including reactive oxygen species [7,16,17]. Non-enzymatic 8-epi-PGF $_{2\alpha}$ synthesis is sustained in large part by free

radicals generated by p450 enzymes. Thus, in liver 8-epi-PGF $_{2\alpha}$ synthesis is stimulated by ethanol [18], carbon tetrachloride [16], or diquat [19], compounds that are metabolized by p450 enzymes. F_2 -isoprostanes are also formed in a number of models of oxidative stress, hyperoxia [20], cigarette smoking [21,22], coronary reperfusion [23], hypercholesterolaemia [24], ventricular dilatation (heart failure) [25], and copper catalysed oxidation of LDL [26,27]. Consequently, isoprostanes have been characterized as a marker of oxidative stress.

During reperfusion, endothelial cells are exposed to endogenous [28–30] and exogenous [31,32] reactive oxygen species. The synthesis of 8-epi-PGF $_{2\alpha}$ by reactive oxygen species generated during reperfusion might alter vascular tone, haemostasis and thromboreactivity. The present studies were designed to determine whether human endothelial cells synthesize 8-epi-PGF $_{2\alpha}$ during reoxygenation following hypoxia, to determine which reactive oxygen species is responsible for 8-epi-PGF $_{2\alpha}$ production during reoxygenation, and to provide mechanistic information on the synthesis of 8-epi-PGF $_{2\alpha}$ by human vascular endothelium during reoxygenation.

EXPERIMENTAL

Materials

Human umbilical artery endothelial cells (HUAEC), 2–4 cumulative population doublings, were obtained frozen from Cell

Abbreviations used: 8-epi-PGF $_{2\alpha}$, 8-epi-prostaglandin $F_{2\alpha}$; HUAEC, human umbilical artery endothelial cells; PGHS, prostaglandin H_2 synthase; PGI $_2$, prostacycline; bFGF, basic fibroblast growth factor.

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Systems (Kirkland, WA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) and medium 199 were obtained from Gibco (Grand Island, NY, U.S.A.), defined fetal calf serum was from Hyclone (Orem, UT, U.S.A.), basic fibroblast growth factor (bFGF) was from Peprotech (Rocky Hill, NJ, U.S.A.), Cytodex 3 microcarrier beads were from Pharmacia (Piscataway, NJ, U.S.A.), and tissue culture-tested fatty acid-free albumin, bovine erythrocyte superoxide dismutase, bovine catalase and 4,5-dihydroxy-1,3-benzene disulphonic acid (Tiron) were from Sigma (St. Louis, MO, U.S.A.). Indomethacin, acetylsalicylic acid (aspirin) and ELISA kit for 8-epi-PGF_{2α} were obtained from Cayman (Ann Arbor, MI, U.S.A.). ELISA kit for PGE₂ was obtained from Amersham (Arlington Heights, IL, U.S.A.). Mammalian transfection kit was obtained from Stratagene (La Jolla, CA, U.S.A.). Human PGHS-1 cDNA was a gift of Dr. Timothy Hla (American Red Cross). The coding region for human PGHS-1 was excised and subcloned into the expression vector pRC-CMV (Invitrogen, San Diego, CA, U.S.A.) with *Hind*III and *Xba*I.

HUAEC cultures

The endothelial cells were plated on 100-mm tissue culture plates and grown in 10 ml complete medium which contained medium 199 supplemented with 10% (v/v) defined fetal calf serum, 25 mM Hepes, 5 ng/ml bFGF, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 µg/ml fungizone. The cells were cultured in a humidified 37 °C CO₂ (5%) incubator. The medium was changed every 48–72 h with complete medium and passaged at 1:4 ratios. At passages 5–8 the cells were seeded (1:4–5 split ratio) onto 6.5 ml of Cytodex 3 microcarrier beads and cultured in a Techne (Princeton, NJ, U.S.A.) microcarrier bead flask with 350 ml of complete medium as previously described [33]. The flex-type, bulb-shaped glass impeller of the microcarrier bead flask rotated around the indentation in the base of the flask from a fixed position above the medium level at 20 rev./min. The medium was changed every 48 h with complete medium. At confluence (visual confirmation under phase microscope) the medium was changed to experimental medium and cultured for 24 h prior to the experiment. Experimental medium was identical to complete medium except medium 199 was supplemented with 1% (v/v) defined fetal calf serum and 10 µM fatty acid-free albumin. No bFGF was added to the experimental medium.

The culture flask was transferred to a bench-top incubator (37 °C), the medium was changed with fresh normoxic experimental medium (final volume including beads was 50 ml) and stirring maintained at 20 rev./min. The cells were exposed to conditions of normoxia (O₂/N₂/CO₂, 20%:70%:5%), hypoxia (0%:95%:5%) and reoxygenation (normoxia) as previously described [33]. Hypoxic and normoxic circuits were completely separate and medium was directed to the microcarrier bead flasks through gas impermeable Teflon tubing at 30 min intervals. At the end of each 30 min interval, 70% (i.e. 35 ml) of the medium was exchanged for an identical volume of fresh normoxic or hypoxic medium. The medium was changed with normoxic medium every 30 min for 120 min. The cells were then exposed to hypoxic conditions for 120 min by changing medium to hypoxic medium (fresh hypoxic medium every 30 min). After 120 min of hypoxia, cells were subjected to reoxygenation for 120 min by changing medium to normoxic medium (fresh normoxic medium every 30 min). To confirm the oxygen tension in the normoxic and hypoxic circuits, aliquots of medium were removed from the reservoirs prior to the start of the experiments and from the flasks at the conclusion of each interval. The oxygen tension in the media was determined by an AVL 995 Blood Gas

analyser (AVL Biomedical Instruments, Roswell, GA, U.S.A.). The oxygen tensions in the medium were normoxia ($p_{O_2} = 128.0 \pm 2.4$ mmHg, mean $\pm$ S.E., $n = 14$), hypoxia (14.0 ± 3.5 mmHg, $n = 5$), and reoxygenation (131.5 ± 1.5 mmHg, $n = 5$).

Cell number determination

At the beginning and end of the experiments, an aliquot of beads was removed from the microcarrier bead flask and sedimented with gravity for approx. 2 min. HUAEC were harvested from 100 µl of sedimented beads with trypsinization and mechanical shaking, and the cell number was determined with a haemocytometer. Cell viability was determined by Trypan blue exclusion.

Treatment with scavengers of reactive oxygen species and inhibitors of PGHS

The media supplemented with either superoxide dismutase (150 units/ml), catalase (1200 units/ml), Tiron (10 mM), aspirin (100 µM), or indomethacin (20 µM) were prepared immediately prior to reoxygenation. These media were then sterilized by filtration and equilibrated with normoxic gas. Previous work in our laboratory showed that filtration does not affect the *ex vivo* activity of the scavengers against reactive oxygen species [29]. After equilibration with normoxic gas for 30 min, the medium was added to the cells at the onset of reoxygenation. In both experiments with scavengers (superoxide dismutase, Tiron and catalase) or PGHS inhibitors (aspirin and indomethacin), the inhibition of 8-epi-PGF_{2α} production during the reoxygenation period was compared to that obtained during reoxygenation in the experimental medium alone (without scavengers or inhibitors) and the results are presented as percent inhibition.

Exposure to hydrogen peroxide

HUAEC were exposed to 120 min (changing medium every 30 min) of normoxic conditions in the experimental medium alone (i.e. control conditions). After the fourth normoxic interval was completed, the medium was replaced with normoxic experimental medium supplemented with hydrogen peroxide (50 µM). Two 30-min intervals of treatment with hydrogen peroxide were followed by a 30-min treatment in experimental medium supplemented with aspirin (100 µM) alone. The cells were then exposed to two additional 30-min treatments in experimental medium supplemented with hydrogen peroxide (50 µM) and aspirin (100 µM). 8-epi-PGF_{2α} production with these treatments was compared to that obtained with experimental medium alone and the results are presented as percent control.

8-epi-PGF_{2α} and PGE₂ analysis by ELISA

The medium aspirated from each experimental interval was centrifuged at 250 *g* for 5 min at 4 °C, an aliquot was transferred to a polypropylene centrifuge tube and stored at –80 °C. The analyses of 8-epi-PGF_{2α} and PGE₂ were performed within 2 weeks. The amount of 8-epi-PGF_{2α} was determined by ELISA. According to the manufacturer, antibody cross-reactivity to PGE₂ and PGF_{2α} was 0.02 and 0.14%, respectively. The validity of the 8-epi-PGF_{2α} ELISA was confirmed by HPLC/MS. PGE₂ was also determined by ELISA. The absolute amount of eicosanoids produced during a 30-min time period was corrected for the amount remaining from the previous interval.

Extraction of 8-epi-PGF_{2α} from culture medium for MS analysis

HUAEC were cultured in the microcarrier bead flask with or without 20 μ M indomethacin as described above except the medium was free of Phenol Red. The medium was collected from four intervals of reoxygenation, butylated hydroxytoluene was added, and centrifuged. The supernatants from each interval were combined and 8-epi-PGF_{2α} was extracted as described by Powell [34]. Briefly, ethanol was added to the combined medium to a final concentration of 15% and acidified to pH 3.0 with formic acid. The sample was applied to Supelclean LC-18 SPE columns (Supelco, Bellefonte, PA, U.S.A.) and washed with water (adjusted to pH 3.0 with formic acid), 15% ethanol in water (pH 3.0), and hexane. 8-epi-PGF_{2α} was then eluted from the tube with ethylacetate containing 1% methanol. The recovery of 8-epi-PGF_{2α} determined by ELISA was 98.7% compared to the original combined media. The eluate was dried and redissolved in 1 ml of ethylacetate. An aliquot was taken for 8-epi-PGF_{2α} analysis by ELISA and 10 ng of deuterated 8-epi-PGF_{2α} was added to the remaining ethylacetate as an internal standard.

HPLC/MS analysis of 8-epi-PGF_{2α}

Online HPLC/MS/MS analysis of 8-epi-PGF_{2α} was performed by negative ion electrospray (ESI) full scan MS/MS using a Finnigan LCQ quadrupole ion trap mass spectrometer. ESI was performed with the sheath gas flow set at 45%, the source voltage at 4.5 kV and the heated capillary at 200 °C. The automatic gain control target was set to 2×10^7 . The maximum ion time was set at 200 ms and 5 microscans were acquired per scan. Alternate collisional activation product ion scans of [M-H]⁻ precursor ions for 8-epi-PGF_{2α} and for deuterated 8-epi-PGF_{2α} (internal standard) were performed on isolated ions at m/z 353.3 and 357.3, respectively. The isolation widths used were 2 u and the collision energy 19%. Product ion spectra were acquired from m/z 100–360. Product ion plots of ions resulting from the loss of three H₂O (M-H-54), and one H₂O plus either C₂H₄O (from the prostane ring) or CO₂ (M-H-62) [35], were used to detect the elution of 8-epi-PGF_{2α} and deuterated 8-epi-PGF_{2α}.

The HPLC column was a Hypersil 120 Å ODS (1 $\times$ 100 mm, 3 μ m) (Keystone Scientific, Bellefonte, PA, U.S.A.). Mobile phase A consisted of 10% acetonitrile-methanol (95:5, v/v) and 90% water containing 0.05% acetic acid adjusted to pH 5.7 with ammonium hydroxide; mobile phase B consisted of 50% acetonitrile-methanol (95:5, v/v) and 50% water containing 0.05% acetic acid adjusted to pH 5.7 with ammonium hydroxide [36]. The HPLC was operated at a flow rate of 60 μ l/min with a linear gradient of 0–100% mobile phase B over 20 min. Samples were dried under nitrogen, suspended in the mobile phase A, sonicated in a water bath, and centrifuged. The supernatant was injected onto the HPLC column.

Transfection of COS-7 cells with human PGHS-1

COS-7 cells were cultured on 100-mm plates in 10 ml DMEM containing 10% fetal calf serum. COS-7 cells were transfected with human PGHS-1 in pRC-CMV (20 μ g) or empty vector by the calcium phosphate method [37]. Twenty hours after transfection, the cells were washed twice with PBS and incubated in fresh medium containing fetal calf serum for 48 h. The medium was removed and the cells were washed with DMEM and then incubated in 5 ml DMEM containing 10 μ M fatty acid-free albumin for 60 min. The medium was changed to 5 ml DMEM containing 20 μ M arachidonic acid bound to 10 μ M fatty acid-free albumin and incubated another 60 min. Arachidonic acid

was purified before use with HPLC [38]. Aliquots of medium were removed for 8-epi-PGF_{2α} and PGE₂ analysis. The cells were washed twice with PBS and then scraped into cold saline. The cell pellets obtained by centrifugation were extracted with chloroform-methanol (2:1, v/v) and partitioned with saline by the method of Folch et al. [39]. Lipid phosphorus content was determined in the lower phase [40]. Number of COS-7 cells was estimated from the amount of lipid phosphorus (30 ± 5 nmol/10⁶ cells, $n = 3$).

Lipid analysis of COS-7 cells

Separation and quantitation of phospholipid classes and their molecular species were performed as previously described [38].

Statistical analysis

ANOVA tests employed SAS (SAS Institute, Cary, NC, U.S.A.) with post tests using the general linear method on the absolute values for 8-epi-PGF_{2α} production. Intergroup variation was analysed using INSTAT (GraphPad, San Diego, CA, U.S.A.), ANOVA with Bonferroni post tests, and Mann-Whitney unpaired t tests.

RESULTS

Cell viability

When HUAEC were exposed to 120 min of hypoxia followed by 120 min of reoxygenation in experimental medium alone, there was no significant change in the number of viable cells. The starting cell number was $1.10 \pm 0.15 \times 10^6$ (means $\pm$ S.E., $n = 6$) per 100 μ l sedimented beads, and at the end of reoxygenation cell number was $1.20 \pm 0.07 \times 10^6$ ($P > 0.05$).

Profile of 8-epi-PGF_{2α} release during normoxia, hypoxia and reoxygenation

8-epi-PGF_{2α} production (23 ± 8 pg/10⁶ cells per 30 min, mean $\pm$ S.E.) by HUAEC was essentially constant during normoxia (Figure 1). During the first 30 min of hypoxia, there

Figure 1 8-epi-PGF_{2α} production by HUAEC during normoxia, hypoxia and reoxygenation

HUAEC were exposed to sequential periods of normoxia, hypoxia and reoxygenation as described in the Experimental section. The absolute amount of 8-epi-PGF_{2α} produced during a 30-min interval is corrected for the amount remaining in the medium from the previous interval. The data are means $\pm$ S.E. of six experiments. * $P < 0.05$ vs. normoxia.

Figure 2 Effects of scavengers of reactive oxygen species on 8-epi-PGF_{2α} production during reoxygenation

HUAEC were incubated in medium containing either 150 units/ml superoxide dismutase ($n = 6$, cross-hatched bars), 10 mM Tiron ($n = 6$, open bars), or 1200 units/ml catalase ($n = 5$, solid bars) during reoxygenation. The results are means $\pm$ S.E. and are presented as percent inhibition compared to the cells reoxygenated in experimental medium alone ($n = 6$, 19.9 ± 4.0 pg/ 10^6 cells during first 30 min reoxygenation, 55.3 ± 24.7 during second 30 min, 78.4 ± 32.4 during third 30 min, and 91.1 ± 36.6 during fourth 30 min). * $P < 0.05$.

was a small but insignificant increase in 8-epi-PGF_{2α} production. By 60 min of hypoxia, 8-epi-PGF_{2α} production decreased and remained significantly below normoxic levels at 90 min (5 ± 2 pg) and 120 min (3 ± 1 pg). However, upon reoxygenation, 8-epi-PGF_{2α} production increased nearly linearly for 90 min (59 ± 9 pg) reaching almost three times normoxic levels and remained at that high level at 120 min (62 ± 5 pg).

Effect of scavengers of reactive oxygen species on 8-epi-PGF_{2α} production during reoxygenation

To determine whether the increase in 8-epi-PGF_{2α} production during reoxygenation was due to formation of reactive oxygen species, cells were treated with scavengers of reactive oxygen species during reoxygenation (Figure 2). During reoxygenation in the presence of superoxide dismutase (150 units/ml), 8-epi-PGF_{2α} production was not significantly inhibited when compared to cells reoxygenated in experimental medium alone. Since there is no evidence that superoxide dismutase can penetrate mammalian cells, endothelial cells were then reoxygenated in the presence of Tiron, a cell-permeable low-molecular-weight free radical scavenger of intracellular superoxide and hydroxyl radicals [41,42]. Tiron (10 mM) also did not inhibit 8-epi-PGF_{2α} production during reoxygenation. Increasing the concentration of superoxide dismutase (300 units/ml) and Tiron (50 mM) did not change the pattern or extent of 8-epi-PGF_{2α} production during reoxygenation (data not shown). In contrast, catalase (1200 units/ml) significantly inhibited 8-epi-PGF_{2α} production during all reoxygenation intervals. Specifically, 8-epi-PGF_{2α} production was completely inhibited at 30 and 60 min, and remained significantly inhibited even at 120 min ($81 \pm 15\%$, mean $\pm$ S.E.). These results suggest a role for hydrogen peroxide in 8-epi-PGF_{2α} synthesis during reoxygenation.

Figure 3 Effect of PGHS inhibitors on 8-epi-PGF_{2α} production during reoxygenation

HUAEC were incubated in medium containing either 100 μ M aspirin (open bars) or 20 μ M indomethacin (solid bars) during reoxygenation. The results are means $\pm$ S.E. of four separate experiments and are presented as percent inhibition compared to the cells reoxygenated in the experimental medium alone (see Figure 2 legend). * $P < 0.05$ vs. medium alone; + $P < 0.05$ vs. aspirin.

Effects of inhibitors of PGHS on 8-epi-PGF_{2α} production during reoxygenation

Since hydrogen peroxide [43] and hydroperoxides [44] are known to stimulate PGHS activity, the cells were treated with inhibitors of this enzyme during reoxygenation. Both aspirin and indomethacin inhibited 8-epi-PGF_{2α} production during all intervals of reoxygenation (Figure 3). There was a significant difference ($P < 0.01$) in the extent of the inhibition of 8-epi-PGF_{2α} production between indomethacin ($73 \pm 7\%$, mean $\pm$ S.E.) and aspirin ($40 \pm 6\%$, mean $\pm$ S.E.) during the first 30 min of reoxygenation, but both aspirin and indomethacin inhibited 8-epi-PGF_{2α} production equally ($\sim 75\%$) by 60 min of reoxygenation while at 90 and 120 min of reoxygenation, 8-epi-PGF_{2α} production was inhibited significantly more by indomethacin ($> 97\%$) than by aspirin ($\sim 85\%$).

Effect of exogenous hydrogen peroxide and aspirin on 8-epi-PGF_{2α} production

Since experiments with scavengers of reactive oxygen species and inhibitors of PGHS suggest an interaction between hydrogen peroxide and PGHS activity during reoxygenation, experiments were designed to determine if exogenously added hydrogen peroxide could stimulate 8-epi-PGF_{2α} production independent of hypoxia and reoxygenation. Hydrogen peroxide (50 μ M) progressively increased 8-epi-PGF_{2α} production (Figure 4, solid bars). 8-epi-PGF_{2α} production increased $392 \pm 52\%$ (mean $\pm$ S.E.) during the first 30 min of exposure to hydrogen peroxide and $536 \pm 70\%$ (mean $\pm$ S.E.) by 60 min when compared to 8-epi-PGF_{2α} production in experimental medium alone. When the same concentration of hydrogen peroxide was added to the cells in the presence of aspirin (100 μ M), 8-epi-PGF_{2α} production decreased to $123 \pm 24\%$ (mean $\pm$ S.E., cross-hatched bars) and after an additional 30 min period of exposure to both aspirin and hydrogen peroxide decreased further ($70 \pm 17\%$, mean $\pm$ S.E.). The addition of hydrogen peroxide to the medium was not

Figure 4 Effect of exogenous hydrogen peroxide and aspirin on 8-epi-PGF_{2α} production under normoxic conditions

Endothelial cells were incubated in medium containing 50 μ M hydrogen peroxide (solid bars) for two 30-min intervals and then 50 μ M hydrogen peroxide and 100 μ M aspirin (cross-hatched bars) for two 30-min intervals. The results are means $\pm$ S.E. of four separate experiments and are presented as percent control, i.e. by cells incubated in experimental medium alone (23.4 ± 8.3 pg/ 10^6 cells per 30 min). * $P < 0.01$ vs. control; ** $P < 0.001$ vs. hydrogen peroxide.

coincident with cellular cytotoxicity. The number of viable cells prior to exposure to hydrogen peroxide was $1.04 \pm 0.05 \times 10^6$ (means $\pm$ S.E., $n = 5$) per 100 μ l sedimented beads and after exposure to hydrogen peroxide for 60 min was $1.06 \pm 0.08 \times 10^6$ ($P > 0.05$).

Figure 5 Selected ion monitoring chromatogram of 8-epi-PGF_{2α} in HUAEC culture medium

Reoxygenation media were processed as described in the Experimental section. The ions resulting from the loss of three H₂O (54 u) and one H₂O plus either C₂H₄O (from the prostane ring) or CO₂ (62 u) from parent ion (m/z 353) were used to monitor the elution of 8-epi-PGF_{2α} (upper panel) and from m/z 357 were used to monitor the elution of the deuterated 8-epi-PGF_{2α} (lower panel). The peak at 12.06 min is probably PGF_{2α} [15].

Figure 6 Ion spectra of 8-epi-PGF_{2α}

(A) The 8-epi-PGF_{2α} containing peak from reoxygenation media (11.46 min, Figure 5 upper panel). (B) Authentic 8-epi-PGF_{2α}.

LC/MS analysis of 8-epi-PGF_{2α}

To verify that the ELISA was measuring 8-epi-PGF_{2α}, the amount of 8-epi-PGF_{2α} in the media after 2 h of reoxygenation was determined by both ELISA and LC/MS/MS as described in the Experimental section. Based on the ELISA the media sample injected onto the HPLC column contained 0.63 ng of 8-epi-PGF_{2α}. As shown in Figure 5, LC/MS/MS analysis of ion plot peak area indicated that the sample contained approx. 1 ng of 8-epi-PGF_{2α} (peak at 11.46 min). Thus, although ion spectra indicated that this peak contained another component (Figure 6), the LC/MS/MS analysis confirmed the presence of 8-epi-PGF_{2α} and the amount roughly agrees with the amount determined by ELISA. When indomethacin was added to the medium during reoxygenation, LC/MS/MS analysis did not show any product ion spectrum indicative of F₂-isoprostanes (data not shown).

8-epi-PGF_{2α} and PGE₂ production by COS-7 cells transfected with human PGHS-1

On the basis of the experiments with scavengers of reactive oxygen species and inhibitors of PGHS, it appeared that the activity of PGHS was responsible for most 8-epi-PGF_{2α} synthesis by cultured human endothelial cells. To address the involvement of PGHS-1 in 8-epi-PGF_{2α} synthesis, human PGHS-1 was transiently expressed in COS-7 cells, a cell line that does not constitutively express PGHS [45]. Analysis of cell lipids indicated that COS-7 cells contained substantial amounts of arachidonic acid ($\sim 17\%$ of total phospholipid molecular species) (Table 1). Twenty hours after transfection, the cells were washed and refed with fresh medium. After 48 h an aliquot of the cells and medium was analysed for PGE₂ and 8-epi-PGF_{2α} content. Neither the cells transfected with empty vector nor with PGHS-1 secreted

Table 1 Lipid composition of major glycerophospholipid classes in COS-7 cells

Values are average of two determinations. Lipids were determined in non-transfected COS-7 cells incubated in DMEM supplemented with 10% fetal calf serum. The cells (~80% confluence) were washed twice with PBS and harvested by scraping with saline and centrifugation. Lipids were extracted from the cell pellets and lipid phosphorus content was used to determine cell number determination as described in the Experimental section. PS and CL were collected together and not separated further. Sphingomyelin was not determined because this phospholipid contains virtually no arachidonyl molecular species. COS-7 cell lipids also contained alkylacyl-PC (0.9 nmol/10⁶ cells) and alkenylacyl-PC (0.4 nmol/10⁶ cells). Alkylacyl-PE was not detected. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin.

	PC (diacyl)	PE (diacyl)	PE (alkenyl)	PI	PS/CL
nmol/10 ⁶ cells	16.4	8.2	2.4	4.4	2.9
Molecular species			(mol %)		
16:0–22:6	3.7	1.7	7.1	0.7	0.4
16:0–16:1, 18:1–22:6	9.6	3.2	3.0	1.6	0.7
16:1–18:1	6.7	2.3	1.0	4.0	0.4
16:0–18:2	3.6	1.7	5.9	1.8	0.5
18:1–18:2	3.6	4.0	4.8	7.0	0.9
16:0–16:0, 18:0–22:6	2.4	3.9	8.8	1.5	7.9
16:0–18:1	29.4	8.1	4.9	6.8	6.2
18:1–18:1	13.5	14.9	1.6	11.5	6.6
18:0–18:2	1.8	4.1	3.3	4.0	10.2
16:0–18:0	2.1	—	—	—	—
18:0–18:1	4.6	10.8	1.7	5.8	42.7
16:0–20:4	2.2	3.0	20.6	1.8	0.2
18:1–20:4	1.5	5.5	11.9	7.0	0.3
18:0–20:4	1.5	20.6	11.1	28.8	4.7
Other	13.9	16.4	13.6	17.8	18.5

significant amounts of PGE₂ or 8-epi-PGF_{2α} (Table 2). However, the cells transfected with PGHS-1 contained seven times more 8-epi-PGF_{2α} esterified in cell lipids than the cells transfected with empty vector (Table 2). After the 48-h incubation, the remaining cells were washed, refed in fresh medium, and incubated for 60 min to determine secretion rates for PGE₂ and 8-epi-PGF_{2α}. As expected from the analysis of the 48-h media, neither cell secreted significant amounts of PGE₂ or 8-epi-PGF_{2α} (Table 3). The cells were then incubated for 60 min in fresh medium containing 20 μM arachidonic acid (bound to 10 μM albumin). The cells transfected with empty vector did not secrete significant amounts of PGE₂ or 8-epi-PGF_{2α} (Table 3). The cells transfected with PGHS-1 secreted massive amounts of both PGE₂ and 8-epi-PGF_{2α}. There was also a modest increase in the amount of 8-epi-PGF_{2α} esterified in cell lipids (Table 3). Further incubation

Table 2 PGE₂ and 8-epi-PGF_{2α} in medium (48 h) and 8-epi-PGF_{2α} in cell lipids of COS-7 cells (pg/10⁶ cells)

The data are mean ± S.D. of separate experiments presented in parentheses. Each experiment was performed in duplicate or triplicate. COS-7 cells were transfected with pCMV-PGHS-1 or empty vector. After 20 h, the cells were washed with PBS and then incubated in DMEM containing 10% fetal calf serum for 48 h.

	PGE ₂		8-epi-PGF _{2α}	
	Vector	Vector-PGHS-1	Vector	Vector-PGHS-1
Medium	92 ± 30 (3)	112 ± 68 (4)	27 ± 4 (3)	27 ± 8 (4)
Cell lipids	—	—	25 ± 15 (3)	176 ± 49 (4)

Table 3 PGE₂ and 8-epi-PGF_{2α} in medium (60 min) and 8-epi-PGF_{2α} in cell lipids of COS-7 cells (pg/10⁶ cells)

The results are mean ± S.D. of separate experiments presented in parentheses. Each experiment was performed in duplicate or triplicate. After COS-7 cells were incubated for 48 h (Table 2), the cells were washed with DMEM and then sequentially incubated for 60 min in DMEM containing 10 μM fatty acid-free albumin followed by another 60 min in DMEM containing 20 μM arachidonic acid (20:4) bound to 10 μM fatty acid-free albumin.

	PGE ₂		8-epi-PGF _{2α}	
	Vector	Vector-PGHS-1	Vector	Vector-PGHS-1
Medium (–20:4)	28 ± 29 (3)	109 ± 65 (8)	6 ± 3 (3)	29 ± 17 (8)
Medium (+20:4)	28 ± 18 (3)	221 997 ± 62 000 (8)	10 ± 4 (7)	2252 ± 718 (8)
Cell lipids (+20:4)	—	—	16 ± 9 (3)	394 ± 57 (4)

Table 4 Successive production of 8-epi-PGF_{2α} in medium by PGHS-1 transfected COS-7 cells (pg/10⁶ cells)

The medium containing 20 μM arachidonic acid was changed after each 60 min incubation. The results are mean ± S.D. of five separate experiments. Each experiment was performed in duplicate.

First 60 min	2880 ± 1033
Second 60 min	2200 ± 867
Third 60 min	6 ± 9

of cells in the presence of arachidonic acid showed that the secretion of 8-epi-PGF_{2α} virtually ceased after 2 h (Table 4).

DISCUSSION

The present studies show that cultured HUAEC produce substantial amounts of 8-epi-PGF_{2α}; that human endothelial cell synthesis of 8-epi-PGF_{2α} increases in response to endogenous reactive oxygen species generated during reoxygenation; and that the increase in 8-epi-PGF_{2α} synthesis can be abolished by inhibitors of PGHS. We have previously shown that in HUAEC the levels of PGHS-1 do not change during hypoxia and reoxygenation and that under these experimental conditions PGHS-2 cannot be detected by Western blot [46]. Thus, the results of the present *in vitro* study suggest that 8-epi-PGF_{2α} can be synthesized by PGHS-1 and that PGHS-1 is the major source of 8-epi-PGF_{2α} in cultured human endothelial cells. In that study we also demonstrated that HUAEC synthesize 131 ± 10 pg/10⁶ cells per 30 min of PGI₂ under normoxic conditions (compared to 23 ± 8 pg/10⁶ cells per 30 min of 8-epi-PGF_{2α}), that the secretion of PGI₂ decreases during hypoxia to 11 ± 5 pg/10⁶ cells per 30 min, and that it returns to normoxic levels upon reoxygenation [46]. Thus, during normoxia and hypoxia PGI₂ production was approx. six times that of 8-epi-PGF_{2α}. However, during reoxygenation 8-epi-PGF_{2α} synthesis increased to twice normoxic levels. Consequently, during reoxygenation the ratio of 8-epi-PGF_{2α} to PGI₂ decreases to approx. 1:3. By comparison, the ratio of 8-epi-PGF_{2α} to PGH₂ synthesized by PGHS-1 in human platelets *in vitro* is approx. 1:1000 [11] and by PGHS-2 in activated monocytes *in vitro* is approx. 1:250 [12].

Since treatment of HUAEC with superoxide dismutase and Tiron did not significantly inhibit 8-epi-PGF_{2α} production

(Figure 2), it is unlikely that free superoxide ion plays a significant role in 8-epi-PGF_{2α} synthesis by human endothelial cells. That Tiron did not inhibit 8-epi-PGF_{2α} synthesis also indicates that production of hydroxyl radicals (via the Fenton reaction) is not the mechanism of hydrogen peroxide-stimulated 8-epi-PGF_{2α} synthesis. In contrast, treatment of the cells with catalase (hydrogen peroxide scavenger) during reoxygenation resulted in prompt and near total inhibition of 8-epi-PGF_{2α} production. Thus, these experiments implicate hydrogen peroxide as a dominant stimulus for 8-epi-PGF_{2α} production during reoxygenation. Since hydrogen peroxide is known to stimulate the activity of PGHS [43], production of 8-epi-PGF_{2α} during reoxygenation was measured while PGHS was inhibited by aspirin and indomethacin. Both aspirin and indomethacin inhibited 8-epi-PGF_{2α} production (Figure 3). Aspirin effects a time dependent acetylation of PGHS which could account for the initial low level of inhibition (~40%) during the first 30 min interval. Maximal inhibition of 8-epi-PGF_{2α} production by aspirin was approx. 85% while for indomethacin it was greater than 97%. Exogenous hydrogen peroxide was also found to stimulate 8-epi-PGF_{2α} synthesis in the absence of hypoxia and reoxygenation (Figure 4). The hydrogen peroxide mediated increase in 8-epi-PGF_{2α} production was also inhibited by aspirin indicating that hydrogen peroxide stimulated 8-epi-PGF_{2α} production by endothelial cells is largely dependent on PGHS activity. The residual 8-epi-PGF_{2α} production during incubation with aspirin and hydrogen peroxide could be due either to non-enzymatic synthesis or to the approx. 15% of PGHS activity not inhibited by aspirin (Figure 3).

PGHS has two activities, a cyclooxygenase activity (formation of the cyclic hydroperoxide PGG₂) and a peroxidase activity that reduces PGG₂ to PGH₂. Aspirin and indomethacin inhibit the cyclooxygenase activity of PGHS by blocking access of the substrate (free arachidonic acid) to the active site, but they do not inhibit the peroxidase activity of PGHS [47]. That aspirin and indomethacin inhibit PGHS dependent 8-epi-PGF_{2α} synthesis indicates that PGHS dependent 8-epi-PGF_{2α} synthesis is not due to soluble free radicals generated during the peroxidase reaction. However, it is possible that 8-epi-PGF_{2α} is generated during subsequent steps in the synthesis of thromboxanes/prostaglandins. To confirm a direct role for human PGHS-1 *per se* in the synthesis of 8-epi-PGF_{2α}, human PGHS-1 was expressed in COS-7 cells. COS-7 cells do not constitutively express PGHS and when transfected with PGHS-1 they do not synthesize thromboxanes or prostaglandins beyond PGH₂ [45]. PGH₂ is chemically unstable and non-enzymatically breaks down to the relatively stable product, PGE₂.

The rate-limiting step in prostaglandin synthesis is generally believed to be the release of arachidonic acid from endogenous stores by a phospholipase-A₂ or diacylglyceride lipase. Thus, the failure of COS cells transfected with PGHS-1 to release PGE₂/8-epi-PGF_{2α} in the absence of exogenous arachidonic acid probably reflects the absence of arachidonic acid release by a phospholipase-A₂ or diacylglyceride lipase (Table 2). When arachidonic acid is made available exogenously, the cells transfected with PGHS-1 but not with empty vector synthesize large amounts of both PGE₂ and 8-epi-PGF_{2α} (Table 3). PGHS-1 self inactivates after approx. 1300 catalytic cycles [48]. Thus, when stimulated with excess arachidonic acid, the capacity for prostaglandin synthesis declines as PGHS-1 is autoinactivated. Therefore, the inability of COS cells to sustain 8-epi-PGF_{2α} synthesis in the presence of excess arachidonic acid (Table 4) is consistent with 8-epi-PGF_{2α} synthesis by PGHS-1.

There are conflicting reports in the literature regarding the effects of PGHS inhibitors on human urinary and plasma levels

of 8-epi-PGF_{2α} *in vivo*. Wang et al. [49] have shown that urinary levels of 11-dehydro-thromboxane B₂, but not 8-epi-PGF_{2α} determined by ELISA, were reduced by aspirin, indomethacin, or ibuprofen administration while plasma levels of 8-epi-PGF_{2α} and 11-dehydro-thromboxane B₂ were both drastically reduced (>90%) by aspirin administration. These authors concluded that their data supported the concept that 8-epi-PGF_{2α} levels in human urine were a result of non-enzymatic pathways. More recent work has shown that human urinary levels of 8-epi-PGF_{2α} determined by GC/MS are modestly decreased by indomethacin administration [15]. Several studies suggest that there is a specific receptor for 8-epi-PGF_{2α} [3,5,8,9], implying a role for 8-epi-PGF_{2α} in normal physiology and thus also implying regulated (i.e. enzymatic) synthesis. However, unlike the other major products of the cyclooxygenase pathway (thromboxane-A₂ and PGI₂), which have a very short chemical half life (*t*_{1/2} is less than 1 min) [50], 8-epi-PGF_{2α} is chemically stable and remains in the circulation until it is cleared by the kidney [51]. Since our data indicate that human endothelial cells synthesize 8-epi-PGF_{2α} through enzymatic pathways, the normal function of 8-epi-PGF_{2α} in human vascular biology deserves further evaluation.

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