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Human Microvascular Endothelial Cell Prostaglandin E1 Synthesis During In Vitro Ischemia–Reperfusion

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Abstract Ischemia–reperfusion injury is a microvascular event documented in numerous in vivo animal models. In animal models, prostaglandin and prostaglandin analogues have been found to ameliorate reperfusion injury. These studies were undertaken to evaluate human microvascular endothelial PGE_1 synthesis during in vitro ischemia followed by reperfusion. Human (neonatal) microvascular endothelial cell (MEC) cultures ($n = 6$) were subjected to sequential 2 h periods of normoxia (20% O_2), ischemia (1.5% O_2), and reperfusion (20% O_2). Prostaglandin E_2 synthesis in conditioned media was determined by ELISA. Steady state levels of MEC prostaglandin H synthase (PGHS)-1 and -2 mRNA were assessed at the end of each 2-h period using RT-PCR and a quantitative mRNA ELISA. MEC PGHS protein levels were analyzed using an ELISA. PGE_1 release increased significantly during the initial 30 min of ischemia, but rapidly fell below normoxic levels by 90 and 120 min. During reperfusion, PGE_1 release returned to normoxic levels at 30, 60, and 90 min, and exceeded normoxic levels at 120 min. PGHS-1 mRNA levels were undetectable during all experimental conditions. PGHS-2 mRNA levels were unchanged by ischemia, but were decreased by reperfusion. In contrast, PGHS-2 protein levels increased 3-fold during ischemia, and remained elevated during reperfusion. Human MEC do not express PGHS-1 mRNA in vitro. Prolonged ischemia decreases MEC PGE_1 synthesis, and stimulates increased PGHS-2 protein levels without altering the steady state levels of COX-2 mRNA. During reperfusion, increased PGHS-2 protein levels persist and are associated with stimulated PGE_2 secretion, despite relative decreases in PGHS-2 mRNA. J. Cell. Biochem. 92: 472–480, 2004. Published 2004 Wiley-Liss, Inc.†

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Tissue ischemia followed by reperfusion is common to clinical conditions such as trauma, organ transplantation, and surgical reconstructive procedures. Microscopic evaluation of parenchyma subjected to ischemia demonstrates paradoxical worsening of tissue perfusion when flow is restored, and this condition, first noted in cerebral tissue has been termed the "no-reflow phenomenon" [Ames et al., 1968]. The no-reflow phenomenon is characterized by tissue edema, platelet aggregation, adhesion of inflammatory cells and platelets to the microvascular endothelial cells (MEC) [Verrier, 1996; Duran et al., 1998]. Animal models of ischemia–reperfusion and studies of ischemic human skeletal muscle confirm substantial MEC damage, but no endothelial cell death [Gildof et al., 1982, 1988; Formigli et al., 1995]. In order to understand the biosynthetic abnormalities that might be present in human endothelium as a consequence of decreased oxygen tension, in vitro studies of macrovascular endothelial cell function on static tissue culture plates have been employed by numerous investigators. These investigators have consistently reported abnormalities in macrovascular EC synthesis of

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prostacyclin [Madden et al., 1986; Farber and Barnett, 1991], nitric oxide [McQuillan et al., 1994], and endothelin [Kourembanas et al., 1991]. These powerful endothelial autacoids may influence the microvascular milieu during ischemia and reperfusion through their action on vascular smooth muscle, platelets, and neutrophils. Evidence to suggest that abnormalities in eicosanoid biosynthesis may play a role in the development of the no-reflow phenomenon is provided by in vivo evidence that prostacyclin and prostacyclin analogues limit tissue injury following reperfusion [Belkin et al., 1989, 1990; Blebea et al., 1990]. Since prostaglandins have been shown to modulate the no-reflow phenomenon in animal models, these studies were designed to determine whether human EC synthesis of prostaglandin E_1 was altered by in vitro ischemia–reperfusion. Prostaglandin E_1, a potent inhibitor of platelet aggregation, is the predominant vasodilating eicosanoid synthesized by MEC. Prostaglandin H synthase (PGHS) is a key enzyme involved in the synthesis of prostaglandins, which are important regulators of vascular function. The constitutive (PGHS-1) and its inducible form (PGHS-2) have differential regulation, and may be influenced by ischemia and reperfusion. PGHS-1 and -2 have very similar cyclooxygenase active sites, catalytic mechanisms, products and kinetics, but the structural differences between the two may have important biological and therapeutic consequences [Smith et al., 2002].

These studies were also undertaken with the understanding that the no-reflow phenomenon appears to be primarily a microvascular [Duran et al., 1998; Blaisdell, 2002], rather than a macrovascular event. EC are a heterogeneous population of cells [Gerritsen, 1987] with specialized function depending on the organ site from which they originate (i.e., skin vs. brain). In vitro techniques are now available which allow growth of MEC for prolonged periods of time with retention of their specialized functions [Kraling and Bischoff, 1998]. These recent advances in tissue culture methodologies prompted the experiments reported in this article. Studies were designed to evaluate human MEC viability and function under rapidly changing oxygen tensions. Furthermore, in order to attempt to reflect the in vivo condition more closely than standard tissue culture protocols, EC were studied under mechanical agitation (stirring of microcarrier beads) [Sinskey et al., 1981] during control and reperfusion conditions to include mechanical stress as a variable in the experimental milieu. Conversely, ischemia was designed to include a relative decrease in mechanical agitation to simulate in vivo conditions of stasis. The microcarrier bead model was selected to allow measurement of indices of human vascular cell function under simultaneous, rapidly changing oxygen tensions and mechanical stress [Cherry and Papoutsakis, 1988; Cherry and Kwon, 1990]. While it is clear that the physiologic milieu of EC grown on microcarrier beads will never approximate in vivo conditions precisely, by including these subtle variables in the experimental protocol, novel data regarding MEC indices of cellular viability, metabolism, gene expression, and biosynthetic capacity in response to in vitro ischemia–reperfusion are reported.

MATERIALS AND METHODS

Cell Cultures

Frozen cultures of neonatal human dermal MEC (2–4 cumulative population doublings) were obtained in frozen vials (BioWhittaker, Inc., Walkersville, MD). According to the manufacturer, these cells were derived from the subcutaneous tissue of infant human foreskin, screened for mycoplasma, HIV, hepatitis C, and had their EC identity confirmed using indirect immunofluorescence for factor VIII, and di-I-acetylated LDL. In our laboratory, the EC identity was confirmed via their polygonal monolayer morphology, and the presence of von Willebrand factor in conditioned medium/cellular protein extracts (ELISA, passage 3 and 6 cells, Diagnostica Stago, Asinieres-Sur-Siene, France). The cells were thawed, and grown in complete medium which contained: medium 199 supplemented with 10% (v/v) defined fetal calf serum, sodium bicarbonate (2.2 g/L) recombinant human basic fibroblast growth factor (bFGF, 5 ng/ml, Peprotech, Rocky Hill, NJ) penicillin (50 U/ml), streptomycin (50 mg/ml), and fungizone (5 μg/ml), and then plated on 100 mm tissue culture plates. The cells were kept in a 37°C CO_2 (5%) incubator, fed every 48 h with complete medium and passaged at 1:4 ratios using 0.05% Trypsin/0.125% EDTA upon reaching confluence. Passage 4–6 EC were seeded (1:4–5 split ratio) onto Cytodex 3 microcarrier beads in complete medium. The microcarrier bead cultures were fed every 48 h with

complete medium and kept in a 5% CO_2 37°C incubator. At confluence (visual confirmation) the cells were fed with experimental medium for 24 h prior to the experiment. Experimental medium was identical to complete medium except as follows: M199 was supplemented with 1% defined fetal calf serum, and 10 μM tissue culture tested fatty acid free albumin (Sigma, St. Louis, MO). No phenol red or bFGF was added to experimental medium. Confluent cultures contained approximately 10^8 cells per 6.5 ml microcarrier bead volume.

Experimental Ischemia and Reperfusion

Human MEC grown on microcarrier beads were exposed to a basal mechanical agitation in the microcarrier flask by stirring at 20 rpm. An experimental protocol developed in our lab [Watkins et al., 1995, 1996] was modified as follows to study the effects of mechanical agitation and oxygen tension on human EC function: EC were allowed to stabilize during a 120 min period of normoxia ($pO_2 = 122 \pm 4$ mmHg, 20 rpm), and the medium was changed with fresh normoxic experimental medium every 30 min. Cells were then exposed to 120 min of ischemia ($pO_2 = 9 \pm 4$ mmHg, 5 rpm). Stirring rate (5 rpm) was selected because it represented the slowest stirring rate whereupon the confluent cultures of microvascular cells grown on the beads did not clump after 2 h. During in vitro ischemia, the slow stirring provided a relatively static condition compared to normoxia and reperfusion, in addition to no addition of fresh medium. Only enough medium (500 μl) was removed during in vitro ischemia to complete the assays for lactate and PGE_1. Following ischemia, EC were exposed to 120 min of in vitro reperfusion ($pO_2 = 118 \pm 6$ mmHg, 20 rpm, fresh medium every 30 min). To confirm the presence of normoxic or hypoxic oxygen tensions, aliquots of medium were removed from the normoxic and hypoxic reservoirs prior to the start of the experiments and from the microcarrier bead flask at the conclusion of each interval. The oxygen tension in the medium was assessed by injecting these aliquots into a AVL 995 Blood Gas analyzer (Roche-AVL Biomedical Instruments, Roswell, GA).

PGE_2 ELISA Assay

The medium aspirated from the microcarrier bead flask was aliquoted into polypropylene tubes, centrifuged at 250 rpm for 10 min at 4°C, snap frozen in dry ice and acetone, then stored at −80°C prior to assay. Aliquots of medium from each experimental interval were frozen in duplicate to avoid the need to thaw and refreeze samples for re-assay of eicosanoids following the experiments. To quantitate EC production of PGE_1, its stable breakdown product, PGE_2 was measured with an ELISA (Amersham, Arlington Heights, IL). Standard curves for PGE_2 were generated using concentrations ranging from 3.9 to 500 pg/ml. Antibody cross-reactivity to related eicosanoids was less than 1% for all eicosanoids according to the manufacturer.

PGHS mRNA ELISA

Total RNA from the cells was extracted [Chomczynski and Sacchi, 1987] from 1 ml aliquots of bead sediments collected at the end of the normoxic, ischemic, and reperfusion intervals. Using the quantikine mRNA ELISA kit (R&D Systems, Minneapolis, MN) 3 μg of total RNA samples was hybridized with biotin labeled—PGHS-1 or -2 capture oligonucleotide probe and digoxigenin-labeled detection probes in a microplate. The samples were transferred to a streptavidin-coated microplate where the RNA/probe hybrid is captured. The PGHS mRNA was detected with an anti-digoxigenin alkaline phosphatase conjugated antibody and color was developed after adding a substrate and amplifier solutions. The optical density of the samples was measured using a microplate reader and the concentration of the gene specific mRNA was calculated against an RNA calibrator curve.

RT-PCR for PGHS-1 and -2

A 5 μg aliquot of LiCl purified total RNA was reverse transcribed using Superscript First-Strand Synthesis Systems, (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions using oligo-dt as primer. The reverse transcription reaction was diluted with 100 μl molecular biology grade water, and 5 μl aliquots were subjected to PCR (25 μl reaction volume) using Amplitaq Gold Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Cycler conditions were: 94°C for 3 min followed by 32 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final 2 min at 72°C. The forward and reverse

primers for PGHS-1 was: [5′-GGGGTACCATGAGCCGGAGTC TCTTGCTC-3′], [5′-GGCCAGGGATGGTGCAGTTGGG-3′] and primers for PGHS-2 were: [5′-TTCAAATGAGATTGTGG GAAAATTGCT-3′], [5′-AGATCATCTCTGCCTGAGTAT CTT-3′] [Topper et al., 1996]. The amplified samples were analyzed by gel electrophoresis under UV light after staining with ethidium bromide.

PGHS-2 Protein Levels

Four hundred microliters of sedimented cells on beads were subjected to cell lysis for quantitation of PGHS-2 level via enzyme immunometric assay (Assay Designs, Inc., Ann Arbor, MI). Following control, 2 h ischemia and 2 h of reperfusion the bead sediment was briefly rinsed in PBS (25°C), then lysed in RIPA buffer consisting of 1% NP-40 (Sigma), 50 mM Tris HCl, (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, and 1% SDS. Five cycles of 30 s sonication bursts on ice at 1 min intervals were used to lyse the cells. After 5 min centrifugation at 16,100 rpm, 4°C, and the supernatant was collected for assay. A standard curve was generated between 2.15 through 137.5 ng/ml. Data was normalized to nanograms PGHS-2/mg total protein.

Lactate Assay

Samples of medium were centrifuged at 250 rpm for 10 min at 4°C and kept at 4°C until assay within 4 h of the experiment completion. Samples were deproteinized in 10% trichloroacetic acid. Lactate concentration in these aliquots was measured indirectly as the absolute change in absorbance (ΔA, 340 nm) during the conversion of NAD to NADH in the presence of our sample and added LDH. A standard solution of L-lactic acid in M199 supplemented with 0.5% serum and 10 μM fatty acid free albumin was used in serial dilutions to construct a linear standard curve. Lactate concentration was calculated directly from linear regression parameters for the standard curve.

Cell Counts

Duplicate 100 μl aliquots of sedimented beads were removed for cell counts at the beginning and end of the experiments to document endothelial cell viability as previously described [Watkins et al., 1995].

Statistical Analysis

The cells' first normoxic 30-min interval was treated as a stabilization period in the bench top incubator, and the PGE_2 and lactate production during that period was not included in the statistical analysis. All data are expressed as mean ± SE. During all experimental intervals, the absolute amount of PGE, and lactate produced during a 30-min time period is corrected for the amount remaining from the previous period. Levels of PGHS mRNA after ischemia and reperfusion were compared to normoxic, pre-ischemic levels for all experiments. Intracellular levels of PGHS-2 protein during normoxia, ischemia, and reperfusion were normalized to per milligram total protein. Results for ANOVA (Tukey post-test) and paired *t*-tests (cell counts) were computed with Instat (GraphPad, San Diego, CA).

RESULTS

Cell Viability

There was no significant alteration in cell viability following 120 min of in vitro ischemia and 120 min of in vitro reperfusion ($1.44 \pm 0.29 \times 0^6$ cells/100 μl beads, ($n = 6$)) compared to pre-ischemic normoxia ($1.46 \pm 0.23 \times 10^6$ cells/100 μl beads, $n = 6$, $P > 0.05$).

Lactate Synthesis

The rate of lactate release into the pre-ischemic normoxic intervals was stable and averaged 12.5 nm/10^6 cells (Fig. 1). By 60 min of ischemia there was a significant increase in the rate of lactate release, and the rate continued to increase throughout the ischemic period, reaching a peak of 30.7 ± 4.4 nm/10^6 cells after 120 min of ischemia. Upon reperfusion, the rate of lactate release rapidly returned to pre-ischemic levels.

Prostaglandin E_2 Levels

The rate of PGE_2 synthesis during the pre-ischemic normoxic period was essentially constant, and averaged 65.1 ± 5.5 pg/10^6 cells, 30 min (Fig. 2). During the first 30 min of ischemia the rate of PGE_2 synthesis doubled to 131.4 ± 13.5 pg/10^6 cells, 30 min ($n = 6$, $P < 0.001$ vs. normoxia). By 60 min of ischemia the rate of PGE_2 synthesis was significantly decreased (21.8 ± 13.8 pg/10^6 cells, 30 min, $P < 0.05$ vs. normoxia) and remained near that

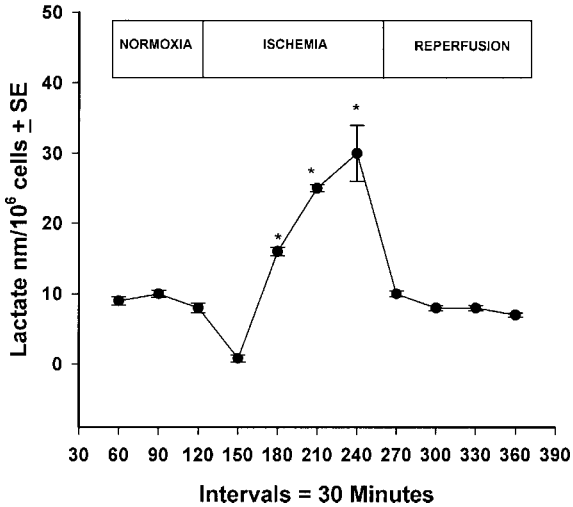

Fig. 1. Microvascular endothelial cells (MEC) release of lactate. During normoxia, MEC released stable amounts of lactate. During ischemia, lactate levels significantly increased by 60 min, and remained elevated throughout the ischemic period (*$P < 0.05$ vs. normoxia, n = 6). Following ischemia, MEC lactate release promptly returned to basal levels.

rate throughout the rest of the ischemic period. Within 30 min of reperfusion, the rate of PGE_2 synthesis returned to pre-ischemic normoxic levels, and continued to increase throughout the reperfusion period. By 120 min of reperfusion PGE_2 synthesis was significantly higher than all of the normoxic levels (114 ± 9.5 pg/10^6 cells, 30 min, $P < 0.05$ vs. all normoxic intervals, 60, 90, and 120 min of ischemia).

PGHS mRNA Levels

PGHS-1 mRNA levels were not detectable by RT-PCR (data not shown) under normoxic, ischemia, or reperfusion conditions. In contrast, PGHS-2 mRNA was easily detected under normoxic, ischemic, and reperfusion conditions and quantitated using an ELISA. Two hours of ischemia did not alter steady state levels of PGHS-2 mRNA ($102 \pm 10\%$ of normoxic levels). In contrast to the results with ischemia, reperfusion decreased PGHS-2 levels to $67 \pm 13\%$ of normoxic levels (n = 6, $P < 0.05$ vs. ischemia, Fig. 3).

PGHS-2 Protein Levels

After 2 h of normoxia, PGHS-2 protein levels were stable at 15 ± 8 ng/mg protein. Following 2 h of ischemia, PGHS-2 levels increased significantly to 60 ± 14 ng/mg protein ($P < 0.05$ vs. normoxia, n = 4) and remained increased after 2 h of reperfusion (57 ± 12 ng/mg protein, $P < 0.05$ vs. normoxia, n = 4).

DISCUSSION

The data presented in this article demonstrates the molecular and biosynthetic profile of isolated human MEC under conditions of in vitro ischemia followed by in vitro reperfusion. Using human microvascular endothelium grown on microcarrier beads, we have produced an environment whereupon stasis

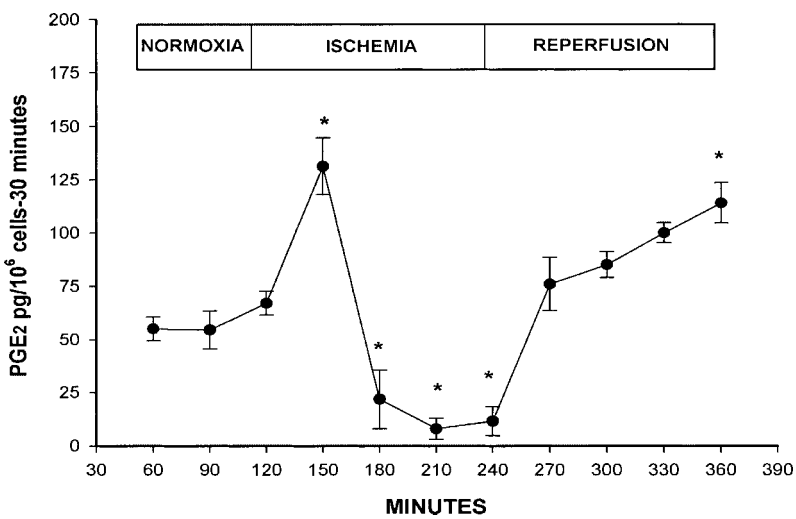

Fig. 2. PGE_2 levels in conditioned medium during normoxia, ischemia, and reperfusion. PGE_1 release was briefly stimulated during the initial 30 min of ischemia (*$P < 0.05$ vs. normoxia). Thereafter, ischemia markedly inhibited PGE_1 synthesis. After reperfusion, PGE_1 synthesis promptly returned to baseline levels, and by 120 min, superceded baseline normoxic levels ($P < 0.05$ vs. normoxia, n = 6).

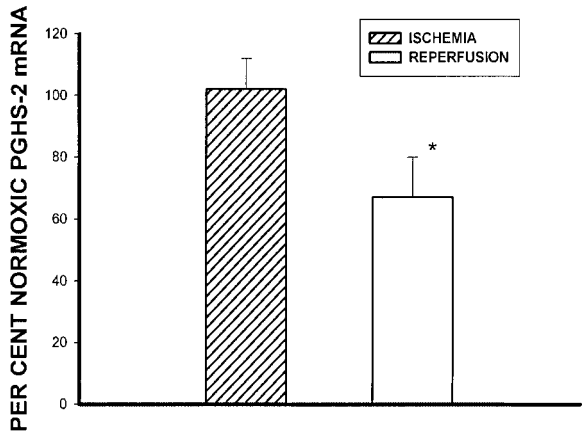

Fig. 3. Steady state levels of prostaglandin H synthase (PGHS)-2 mRNA. PGHS-2 mRNA levels decreased to 67% of normoxic levels after 2 h of reperfusion (*$P < 0.05$ vs. normoxia, n = 6).

(decreased mechanical agitation) and accumulation of metabolites also accompany decreased oxygen tension. Furthermore, during in vitro normoxia and reperfusion, the MEC in this study were subjected to mechanical agitation, washout of metabolites in addition to baseline oxygen tension. The sequential in vitro conditions of ischemia and reperfusion did not result in a significant decrease in endothelial cell viability. The absence of endothelial cell death is consistent with histopathologic examination of ischemic and reperfused tissues, which showed little evidence of EC necrosis [Gildof et al., 1982, 1988]. These results suggest that in the in vitro setting of decreased oxygen tension and mechanical stress, human MEC exhibited an appropriate and reversible metabolic response to the reduction of oxygen tension, exhibiting a

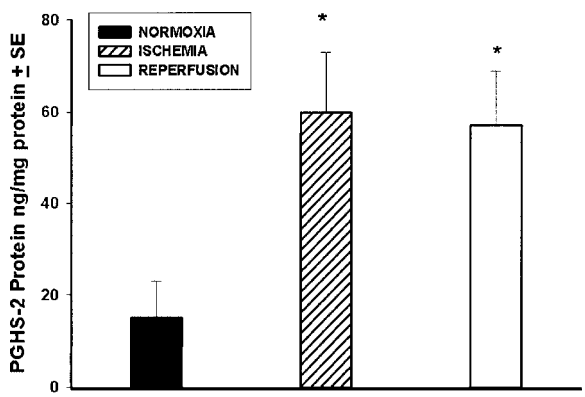

Fig. 4. PGHS-2 protein levels during normoxia, ischemia, and reperfusion. PGHS-2 protein levels markedly increased (3-fold) during ischemia and remained increased during reperfusion (*$P < 0.01$ vs. normoxia, n = 4).

sustained increase in lactate release during ischemia (Fig. 1). Similarly, during in vitro reperfusion, lactate levels returned promptly to baseline levels indicating a rapidly reversible response to the in vitro environment.

The results of these experiments also demonstrate that prolonged in vitro ischemia inhibits human MEC synthesis of PGE_1 (Fig. 2), the major vasodilating eicosanoid synthesized by human MEC. This decrease in PGE_1 synthesis was not due to a decrease in MEC viability based on the cell counts. It is also unlikely that the decrease in PGE_1 synthesis was due to generalized cellular dysfunction during prolonged ischemia since MEC did simultaneously increase lactate release at the same time PGE_1 synthesis decreased. During the first 30 min of ischemia, MEC did not increase their release of lactate, but did transiently increase their synthesis of PGE_1 (Fig. 2). These findings suggest that during early ischemia, MEC did not shift to anaerobic metabolism (lactate synthesis) for intracellular energy demands. Thus, during the transition from normoxia to early in vitro ischemia, MEC may have enough intracellular oxygen for use in oxygen-dependent cell functions, such as prostaglandin synthesis. This fleeting increase in PGE_1 synthesis during early ischemia may coincide with a transient hyperemic response to hypoxia, which has been described in in vivo models. Frisbee et al. [2001] were able to block hypoxic induced hyperemia in vivo with inhibitors of prostaglandin H_2 synthase, but not nitric oxide synthase. In a similar study, transient increase in prostacyclin synthesis was observed in macrovascular endothelial cells exposed to in vitro ischemia [Soler et al., 1997]. It is also possible that the transient increase in PGE_1 synthesis may be due to liberation of arachidonic acid from membrane phospholipids during the decrease in oxygen tension associated with in vitro ischemia. Acute and chronic hypoxia has been shown to influence endothelial cell phospholipid metabolism, particularly with respect to arachidonic acid stores [Tretyakov and Farber, 1993]. In studies of pulmonary artery endothelium, 24 h of hypoxia did result in increased membrane liberation of free arachidonic acid, but this increase in free arachidonic acid was not associated with increased activity of phospholipase A_2, lysophosphatidylcholine acyltransferase nor diacylglycerol acyltransferase. These studies implicate deacylation pathways and inhibition

of reacylation via ATP-dependent arachidonyl CoA synthetase as the primary mechanisms arachidonic acid liberation from membrane phospholipids [Bhat and Block, 1992].

The finding of an overall decrease in PGE_1 synthesis during in vitro ischemia contrasts with the report of Farber and Barnett [1991]. These investigators reported modest increases in macrovascular endothelial prostacyclin synthesis during hypoxia. Madden et al. [1986] have reported that hypoxia decreases macrovascular EC synthesis of PGI_2 after 4 h. The differences between the findings of these previous investigations and the results in this article are likely explained by the different cell type (i.e., microvascular rather than macrovascular cells), the origin of the cells (neonatal vs. adult human) as well as mechanical agitation provided by stirring the microvascular bead suspension. Also, the use of microcarrier beads facilitated a rapid change in oxygen tension during these experiments. In the microcarrier bead system, the oxygen tension inside the flask and the dissolved oxygen tension in the medium are changed rapidly and simultaneously. The oxygen tension inside tissue culture dishes placed in hypoxic incubators does not change until the normoxic medium inside the dish equilibrates with the hypoxic gas within the incubator.

During reperfusion, PGE_1 levels rapidly returned to basal levels, and by 120 min, exceeded basal levels. To evaluate some of the underlying causes of the rapid changes in PGE_1 synthesis, we measured the change in the mRNA levels for prostaglandin H_2 synthase, a key enzyme involved in prostaglandin biosynthesis. Since PGE_1 synthesis is dependent in part on levels of this enzyme [Smith et al., 2002], we analyzed the levels of its mRNA during normoxia, ischemia, and reperfusion. Using RT-PCR, we were unable to demonstrate the presence of mRNA for PGHS-1 during any experimental interval. In contrast, our data demonstrates that the normally inducible PGHS-2 mRNA is constitutively expressed by neonatal human MEC in vitro. This finding is in stark contrast to human macrovascular endothelium, which when cultured under similar conditions, did not express PGHS-2 protein [Soler et al., 1997]. To some degree, the lack of PGHS-1 in the human microvascular endothelium used in these studies may be related to differences in tissue origin between macro- and microvascular endothelium [Craig et al., 1998]. However, these results are consistent with previous studies using neonatal piglet microvessel preparations. In these neonatal tissues, PGHS-2 is believed to have a dominant role in eicosanoid biosynthesis [Peri et al., 1995; Parfenova et al., 1997, 2002]. It is possible that prevalence of the PGHS-2 isoform in the human microvascular endothelium used in these studies is a reflection of their neonatal origin, which appears to be preserved even after freeze-thawing, and serial propagation in vitro.

In spite of the fact that PGHS-2 is frequently transcriptionally regulated in human MEC (where the enzyme is constitutively expressed in vitro), there was no relationship between the steady state level of PGHS-2 mRNA levels and the rate of PGE_2 synthesis (Figs. 2 and 3). Specifically, there is no change in PGHS-2 mRNA during ischemia, although the rate of PGE_1 synthesis is first increasing and then dramatically decreasing. Likewise, during reperfusion, when the rate of PGE_1 synthesis is rapidly increasing, the amount of PGHS-2 mRNA is actually decreasing. To understand the biosynthetic relevance of these paradoxical findings, we evaluated the intracellular levels of PGHS-2 protein. After 2 h of ischemia, there was a 3-fold increase in PGHS-2 protein levels despite stable levels of PGHS-2 mRNA. The increase in PGHS-2 protein may be responsible in part for the transient increase in PGE synthesis during early ischemia. The overall decrease in PGE_2 levels in conditioned medium during ischemia despite a 3-fold increase in PGHS-2 protein level implicates at least a deficiency of oxygen as one limiting factor in human MEC eicosanoid synthesis during ischemia. This increase in PGHS-2 protein levels during ischemia is consistent with the findings of Schmedtje et al. [1997]. These investigators showed that human macrovascular endothelium increased PGHS-2 protein levels 4-fold after 24 h of 1% hypoxia. In their studies, the increase in PGHS-2 protein was not evident after 8 h of hypoxia. However, by 24 h of hypoxia, Schmedtje et al. [1997] showed that the relative increase in PGHS-2 protein was accompanied by a 3-fold increase in the steady state levels of PGHS-2 mRNA levels. While our protein data quantitatively replicates the findings of Schmedtje et al. [1997], the temporal increase in PGHS-2 protein in human neonatal MEC was much faster than the increase

observed in macrovascular cells (2 h vs. 24 h). Furthermore, the steady state levels of PGHS-2 mRNA were not changed in the human MEC during ischemia, thus suggesting either a marked increase in protein translation, or a decrease in PGHS-2 protein degradation.

During reperfusion, the increased PGHS-2 protein levels (which initially increased during ischemia) remained elevated, likely contributing to the prompt return to basal PGE levels. The prompt return and stimulated PGE synthesis during reperfusion might be related to the fact that the cyclooxygase activity of PGHS-2 requires 10-fold lower concentrations of hydroperoxide for activation when compared to PGHS-1 [Chen et al., 1999; Lu et al., 1999; Yada et al., 2003]. Vascular endothelium is known to produce hydroperoxides in response to in vitro mechanical [Hsieh et al., 1998] and oxidative stresses [Michiels et al., 1992]. In addition, PGHS-2 is known to produce eicosanoids at extremely low levels of arachidonate [Spencer et al., 1998]. In contrast to in vitro ischemia where PGHS-2 mRNA levels did not change when compared to normoxia, during in vitro reperfusion the level of PGHS-2 protein levels remained increased despite a 33% decrease in PGHS-2 mRNA. The reasons for this mismatch are unclear and deserve further investigation.

This article describes an in vitro model, which is useful for studying vascular endothelium, or other attachment dependent cells response to rapid simultaneous changes in oxygen tension and mechanical stress. We report rapid temporal alterations in eicosanoid biosynthesis, the steady state levels of PGHS-2 mRNA and PGHS-2 enzyme. The observed changes may have significant implications for therapeutic strategies in humans suffering from ischemic complications.

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