Cyclic Stretch Enhances the Expression and Activity of Coronary Endothelium-Derived Hyperpolarizing Factor Synthase

Beate Fisslthaler, Rüdiger Popp, U. Ruth Michaelis, Ladislau Kiss, Ingrid Fleming, Rudi Busse

Abstract—Endothelium-derived hyperpolarizing factor (EDHF) mediates NO/prostacyclin-independent relaxation in the coronary circulation. Because hemodynamic stimuli modulate endothelial gene expression and because coronary arteries are subjected to pronounced variations in vessel distension, we determined the effects of cyclic stretch on the expression and activity of the coronary EDHF synthase/cytochrome P450 (CYP) 2C8/9. In cultured porcine coronary and human umbilical vein endothelial cells, acute application of cyclic stretch (6%, 1 Hz, 10 minutes) elicited the generation of 8,9-epoxyeicosatrienoic acid (EET), 11,12-EET, and 14,15-EET. Prolonged stretch (4 to 36 hours) increased the expression of CYP 2C mRNA and protein 5- to 10-fold and was accompanied by a 4- to 8-fold increase in EET generation. A corresponding increase in CYP 2C mRNA and protein was also observed in pressurized segments of porcine coronary artery perfused under pulsatile conditions (8%, 1 Hz) for 6 hours. Although in cultured endothelial cells, cyclic stretch elicited the rapid activation of tyrosine kinases as well as Akt and the p38 mitogen-activated protein kinase, the mechanism by which cyclic stretch induces the expression of CYP 2C could not be elucidated, because inhibitors of these pathways induced CYP 2C expression in cells maintained under static conditions. These results have identified coronary EDHF synthase/CYP 2C as a novel mechanosensitive gene product in native and cultured endothelial cells. Because this enzyme generates both EETs and superoxide anions, this finding has wide-reaching implications for vascular homeostasis in conditions of manifest endothelial dysfunction. (Hypertension. 2001;38:1427–1432.)

Key Words: endothelium-derived factor ■ cytochrome P450 ■ arteries ■ inositol ■ protein kinases

Humoral stimuli acutely modulate local blood flow by eliciting the production and release of vasoactive autacoids from the endothelium, such as NO, prostacyclin (PGI₂), endothelin-1, prostaglandin H₂, and superoxide anions (O₂⁻). However, hemodynamic stimuli such as fluid shear stress and pulsatile stretch, to which the endothelial lining of blood vessels is continually subjected, are the physiologically most important determinants of the continuous production of these autacoids in vivo. In several vascular beds, an endothelium-derived hyperpolarizing factor (EDHF) represents a third endothelial vasodilator that mediates NO/PGL₁-independent relaxation. Several different EDHFs are now known to exist in different species, but the hyperpolarizing factor produced by coronary and renal arteries from humans, pigs, cows, dogs, rats, and rabbits displays characteristics similar to those of an epoxyeicosatrienoic acid (EET) generated from arachidonic acid by a cytochrome P450 (CYP) epoxygenase.

Pulsatile changes in transmural pressure, causing simultaneous vessel distension, enhance the production of EDHFs/EETs by native porcine coronary endothelial cells, and with the use of a patch-clamp bioassay system, it could be demonstrated that such rhythmic changes in arterial diameter evoke the generation of a CYP inhibitor–sensitive EDHF. Because cyclic stretch elicits the production of a hyperpolarizing factor from porcine coronary arteries and because a CYP 2C epoxygenase homologous to CYP 2C8/9 was recently identified as a putative coronary EDHF synthase, we sought to determine whether this hemodynamic stimulus modulates the expression and activity of CYP 2C.

Methods

Cell Culture
Porcine coronary artery endothelial cells and human umbilical vein endothelial cells were prepared as described and seeded on flexible-bottomed 6-well culture plates coated with pronectin (BiolFlex, Flexcell International Corp). After they reached confluence, the cells were loaded into Flexcell FX-3000 strain unit (Flexcell) and placed in an incubator. Cells were stretched with an average strain of 6% at a rate of 1 Hz, and static control experiments were performed on cells on stretch plates not exposed to cyclic strain. After stimulation, endothelial cells were harvested, and CYP-derived eicosanoids were determined as described.

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Cyclic Stretch of Porcine Coronary Artery Segments

Porcine epicardial artery segments (length ~40 mm, mean external diameter 2.4 to 2.8 mm) were excised and cut into 2 segments of equal length. The segments were cannulated at both ends and placed into vessel chambers in which perfusion pressure was continuously monitored. Vessels were pressurized to 40 to 60 mm Hg and perfused (1 mL/min) with minimum essential medium. After stabilization, sinusoidal pressure changes (30 to 40 mm Hg, 1 Hz), were applied to 1 of the coronary artery pairs. The diameter changes induced corresponded to a calculated strain of between 6% and 8%.

Isolation of RNA and RT-PCR

Total RNA was isolated from cultured coronary endothelial cells or coronary arterial segments by intraluminal incubation with either dispase (2.4 U/mL) or guanidine isothiocyanate. Random hexanucleotide primers were used for reverse transcription (RT) of equal amounts of RNA, and the oligonucleotides used for polymerase chain reaction (PCR) were derived from a porcine CYP 2C34 sequence that exhibits a high homology to the human 2C8/9 sequence, as described.6 In some experiments, the expression of CYP 2A, CYP 2J, and the endothelial NO synthase (eNOS) was determined. Elongation factor-2 (EF-2) served as an internal control for the verification of the DNA sequence, as described.6 In some experiments, the expression of CYP 3A, CYP 2J, and the endothelial NO synthase (eNOS) was determined. Elongation factor-2 (EF-2) served as an internal control for cultured endothelial cells, whereas platelet and endothelial cell adhesion molecule-1 (DECAM-1) was used as a control for porcine coronary arteries (for primers, see online data supplement available at http://www.hypertensionaha.org). For the verification of the DNA fragment, the PCR products were transferred to nylon membranes and hybridized with 32P-labeled DNA fragments derived from a plasmid containing the coding sequence of CYP 2C8.

Immunofluorescence Experiments

In some experiments, cells or arterial segments were fixed with formaldehyde and permeabilized with Triton X-100 (0.2% [vol/vol]), and the segments were incubated with a specific CYP 2C antibody (kindly provided by Dr E. Morgan, Emory University, Atlanta, Ga) or antibodies recognizing actin (Sigma), Akt/protein kinase B (PKB) (Cell Signaling), or phosphotyrosine (Transduction Laboratories), as described.7

Statistical Analysis

Data are expressed as mean±SEM, and statistical evaluation was performed by using the Student’s t test for paired or unpaired data, 1-way ANOVA followed by a Bonferroni t test, or ANOVA for repeated measures, where appropriate. Values of P<0.05 were considered statistically significant.

Results

Acute Effect of Cyclic Stretch on the Generation of EETs by Coronary Endothelial Cells

In unstimulated endothelial cells, free arachidonic acid (13.5±1.8 ng per well) and small amounts of 5,6-, 8,9-, 11,12-, and 14,15-EET were detected. Cyclic strain (6%, 1 Hz, 10 minutes) slightly enhanced the liberation of arachidonic acid (to 17.6±1.6 ng per well) as well as the generation of EETs (Figure 1). Although stretch enhanced the production of all of the regioisomers detected, the most pronounced effects were on the generation of 11,12- and 14,15-EET. To enhance endothelial CYP expression, some cell batches were incubated with the Ca2+ antagonist nifedipine. As previously reported,6 nifedipine (0.1 μmol/L, 18 hours) enhanced the expression of CYP 2C protein by ~3-fold (data not shown). In these cells, the amount of free arachidonic acid was virtually unchanged (23.1±8.1 versus 38.3±8.7 ng per well, n=3, P=NS), whereas the basal generation of EETs was greater than that detected in solvent-treated cells. In contrast to solvent-treated cells, nifedipine-treated cells produced greater amounts of 5,6- and 8,9-EET, findings that may indicate that in addition to CYP 2C, nifedipine enhances the expression of at least 1 additional CYP enzyme, with a profile of EET generation slightly different from that of CYP 2C8/9.

In nifedipine-treated cells, cyclic stretch enhanced only the production of 11,12- and 14,15-EET, although the effect was no longer statistically significant (Figure 1).

Effect of Cyclic Stretch on the Expression of CYP 2C in Cultured Endothelial Cells

Low levels of CYP 2C mRNA were expressed in porcine coronary endothelial cells cultured under static conditions. Cyclic stretch time-dependently increased the expression of CYP 2C, an effect that was first evident after 4 to 8 hours and was highly significant after 18 to 24 hours (Figure 2). CYP 2C mRNA was maintained at an elevated level (~10-fold greater than in unstimulated endothelial cells) as long as the stimulus was applied (maximally, 36 hours). In the same experiments, cyclic stretch did not significantly affect the expression of either CYP 3A, CYP 2J, or eNOS mRNA (Figure 2).

In primary cultures of porcine coronary endothelial cells, immunofluorescence experiments revealed that not all endothelial cells expressed CYP 2C. When present, the enzyme was generally localized to a perinuclear region (Figure 3). Exposing these cells to cyclic stretch increased the overall intensity of the signal as well as the number of positively stained cells.

Human umbilical vein endothelial cells express markedly lower levels of CYP 2C protein than coronary endothelial cells, but the expression of CYP mRNA and protein was also significantly increased by exposure to cyclic stretch over 9 to 18 hours (Figure 4).

Chronic Effect of Cyclic Stretch on the Generation of EETs by Coronary Endothelial Cells

The increased expression of CYP 2C was accompanied by an increase in endothelial EET production (Figure 5). Stimulation of endothelial cells with ionomycin to elevate intracel-
lular Ca\(^{2+}\) and activate phospholipase A\(_2\) enhanced the production of these 3 regioisomers 2- to 3-fold (Figure 5). Exposure to cyclic stretch (6%, 1 Hz) for 18 hours increased the generation of 11,12-EET and 14,15-EET by 4-fold and induced an 8-fold increase in 8,9-EET production. Subsequent treatment of these stretch-conditioned cells with ionomycin did not significantly increase EET production (Figure 5).

**Effect of Cyclic Stretch on the Expression of CYP 2C in Native Endothelial Cells**

In porcine coronary artery segments, CYP 2C9 is expressed exclusively in the endothelial cell layer (Figure 6). No CYP 2C immunostaining was detected in either smooth muscle cells or in the adventitia. Exposure of coronary segments to cyclic stretch (6%, 1 Hz) for 6 hours markedly increased the CYP 2C fluorescent signal compared with that observed in vessels that were perfused under nonpulsatile conditions (Figure 2).

**Figure 2.** Effect of stretch on the expression of CYP 2C mRNA in cultured endothelial cells. Cultured porcine coronary artery endothelial cells were either incubated under static conditions or exposed to cyclic stretch (6%, 1 Hz) for up to 24 hours. Total RNA was isolated for RT-PCR, and CYP 2C, CYP 2J, CYP 3A, eNOS, and EF-2 PCR products were detected as described in the text. The bar graph provides a statistical summary of the data obtained in 4 separate experiments. *P<0.05 vs control.

**Figure 3.** Effect of prolonged cyclic stretch on the expression of CYP 2C protein in cultured porcine endothelial cells. A, Immunohistochemical staining of CYP 2C in human umbilical vein endothelial cells after incubation under static conditions and after exposure to cyclic stretch (6%, 1 Hz). The results presented are representative of data obtained in 3 further experiments, and the bar represents 20 \(\mu\)m. B, Confluent cultures of human endothelial cells were either maintained under static conditions (time 0) or exposed to cyclic stretch (6%, 1 Hz). Thereafter, total RNA was isolated for RT-PCR as described in the text, and in each experiment, a negative control (nc, no reverse transcriptase in the RT reaction) was included. The results presented are representative of data obtained in 2 further experiments.

**Effect of Cyclic Stretch on the Expression of CYP 2C mRNA in Cultured Porcine Endothelial Cells**

In porcine coronary artery segments, CYP 2C9 is expressed exclusively in the endothelial cell layer (Figure 6). No CYP 2C immunostaining was detected in either smooth muscle cells or in the adventitia. Exposure of coronary segments to cyclic stretch (≈8%, 1 Hz) for 6 hours markedly increased the CYP 2C fluorescent signal compared with that observed in vessels that were perfused under nonpulsatile conditions.
Similarly, CYP 2C mRNA expression was increased by 5-fold in samples prepared from intact porcine coronary arteries (Figure 6).

Effect of Kinase Inhibitors on Stretch-Induced Expression of CYP 2C

In unstimulated endothelial cells, Akt/PKB was distributed throughout the cytoplasm and in the nucleus. The application of cyclic stretch induced the time-dependent translocation of Akt/PKB, so that after 60 minutes of stimulation, the kinase was exclusively localized to the nucleus (Figure 7). However, the stretch-induced activation and translocation of Akt/PKB was unrelated to the increase in CYP 2C expression, inasmuch as neither wortmannin, a phosphatidylinositol 3-kinase (PI 3-K) inhibitor, nor rapamycin, an inhibitor of the Akt/PKB substrate the mammalian target of rapamycin (mTOR), affected the stretch-induced CYP expression (Figure 7).

Rapamycin also failed to affect the increase in EET generation induced by cyclic stretch (6%, 1 Hz, 18 hours) (data not shown).

In unstimulated endothelial cells, immunostaining with a phosphotyrosine antibody revealed a punctate signal mostly localized to the cell boundary. In response to cyclic stretch, the pattern rapidly altered so that the cell-cell boundary was less intensely labeled, whereas the phosphotyrosine signal in the nuclear region was markedly increased. The tyrosine kinase inhibitor herbimycin and the Src inhibitor 4-amino-5-(4-methylphenyl)-7-(t-butyl)phrazol[3,4-d]pyrimidine (PP1) both attenuated the stretch-induced alterations in phosphotyrosine staining. To determine the effects of tyrosine kinase inhibition on CYP expression, endothelial cells were exposed to cyclic stretch in the absence and presence of herbimycin. As described before, cyclic stretch increased the expression of CYP 2C protein and mRNA, but herbimycin, on its own, elicited a much more pronounced effect (Figure 8B and 8C). The signal obtained in response to the combination of herbimycin and stretch was not different from that observed with stretch alone.

An additional signaling molecule rapidly activated after the application of cyclic stretch was the p38 mitogen-activated protein (MAP) kinase (data not shown). However, it was not possible to link the activation of this kinase to the stretch-induced increase in CYP 2C expression, because 2 specific inhibitors, SB203580 and SB220025, increased CYP 2C expression under static conditions (by 513% and 461%, respectively; P<0.01; n=5). None of the kinase inhibitors used directly affected the activity of isolated microsomes containing CYP 2C9 (data not shown).

Discussion

In the present study, we have identified an epoxygenase homologous to CYP 2C8/9 as a novel stretch-inducible
protein in native and cultured endothelial cells. The effects of stretch on CYP 2C appear to be 2-fold, inasmuch as the acute application of moderate levels of cyclic stretch enhanced enzyme activity and EET production, whereas chronic stretch enhanced the expression of CYP 2C mRNA and protein.

Native and cultured endothelial cells express several CYP isozymes, including the hydroxylases CYP 3A and CYP 2B and the arachidonic acid epoxygenases CYP 2J and CYP 2C. Of these isozymes, cultured endothelial cells constitutively express CYP 3A and CYP 2J, whereas CYP 2C levels are highly variable, and often no signal is detectable with the use of RT-PCR. For example, although native porcine coronary artery endothelial cells clearly express CYP 2C protein, CYP 2C mRNA and protein are barely detectable in primary cultures of these cells when maintained under static conditions. Because pulsatile stretch has been reported to activate the EDHF synthase/CYP 2C in porcine coronary arteries, we postulated that cyclic stretch may regulate the activity and expression of CYP 2C in a manner analogous to the regulation of eNOS activity and expression by fluid shear stress. The results obtained clearly indicate that cyclic stretch regulates the expression of CYP 2C mRNA and protein in both native and cultured endothelial cells. Thus, it is tempting to speculate that cyclic stretch maintains the expression of EDHF synthase in vivo and that its decreased expression in vitro is attributable to the lack of this continuous hemodynamic stimulus. Although endothelium-dependent hyperpolarization of coronary artery smooth muscle cells was not investigated in the present study, we have previously demonstrated that the generation of EETs by endothelial cells pretreated with either nifedipine or β-naphthoflavone, which were similar to those measured in stretch-stimulated endothelial cells, was sufficient to potentiate the endothelium-dependent hyperpolarization of porcine coronary artery smooth muscle cells.

The application of cyclic stretch to a variety of different cells is reported to activate numerous signal transduction cascades, including the Janus kinase/signal transducer(s) and activator(s) of transcription and Ras/Raf/extracellular signal–regulated kinase 1/2 pathways, as well as the epidermal growth factor receptor tyrosine kinase, the c-Jun NH2-terminal, and p38 MAP kinases. The mechanism by which pulsatile stretch increases CYP expression in endothelial cells remains to be fully elucidated. In the present study, we observed that the PI 3-K/Akt/mTOR signaling pathway was rapidly activated in stretched endothelial cells, but neither wortmannin nor rapamycin was found to significantly inhibit the induction of CYP 2C protein or the stretch-induced generation of EETs. Additional signaling molecules rapidly activated after the application of cyclic stretch were tyrosine kinases and p38 MAP kinase (authors’ unpublished data, 2001). However, it was not possible to link either of these kinases to the stretch-induced increase in CYP 2C expression. Part of the difficulty in elucidating the pathway determining the stretch-induced expression of CYP 2C was due to the fact that SB203580 and SB220025, specific inhibitors of p38 MAP kinase, and the tyrosine kinase inhibitor herbimycin enhanced the basal expression of CYP 2C in endothelial cells maintained under static conditions. However, each of these inhibitors prevented the stretch-induced activation of the respective kinases in coronary endothelial cells. Because many CYP substrates increase the expression of the enzymes responsible for their metabolism, it is likely that the pharmacological inhibitors may be at least partially metabolized by CYP 2C. Indeed, our previous observation, ie, that the Ca2+ antagonist nifedipine enhances the expression and the activity of the EDHF synthase/CYP 2C in native and cultured endothelial cells.
cultured endothelial cells, may be attributable to a similar phenomenon.

The functional consequences of endothelial CYP 2C/EDHF synthase regulation by cyclic stretch are potentially wide reaching, because in addition to activating Ca\(^{2+}\)-dependent K\(^{-}\) channels and modulating arterial tone, the EETs regulate multiple endothelial and smooth muscle signaling pathways and may promote both endothelial cell proliferation and angiogenesis. Moreover, EDHF synthase has recently been shown to generate physiologically relevant amounts of free radicals, including O\(_2^{-}\); thus, the activation of CYP 2C in endothelial cells may participate in the stretch-induced generation of O\(_2^{-}\), which has, until now, been attributed to the activation of NADPH oxidase.

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