Nifedipine Increases Cytochrome P4502C Expression and Endothelium-Derived Hyperpolarizing Factor–Mediated Responses in Coronary Arteries

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Abstract—In addition to NO and prostacyclin, endothelial cells release a factor that elicits vasodilatation by hyperpolarizing the underlying vascular smooth muscle cells. In some vascular beds, this so-called endothelium-derived hyperpolarizing factor (EDHF) displays the characteristics of a cytochrome P450 (CYP)-derived arachidonic acid metabolite, such as an epoxyeicosatrienoic acid. Native porcine and cultured human coronary artery endothelial cells were screened for CYP epoxygenases, and CYP2B, CYP2C, and CYP2J were detected with reverse transcription–polymerase chain reaction. The CYP inducer β-naphthoflavone and the Ca\(^{2+}\) antagonist nifedipine significantly increased CYP2C mRNA but did not change the expression of CYP2J or CYP2B. To determine the relationship between CYP2C expression and EDHF production in native endothelial cells, we incubated porcine coronary arteries with nifedipine. Nifedipine enhanced endothelial CYP2C protein expression, as well as the generation of 11,12-epoxyeicosatrienoic acid. In organ bath experiments, pretreatment with nifedipine enhanced bradykinin-induced, EDHF-mediated relaxations as well as the concomitant hyperpolarization of smooth muscle cells. The specific CYP2C9 inhibitor sulfaphenazole, on the other hand, significantly attenuated EDHF-mediated hyperpolarization and relaxation. These results demonstrate that in porcine coronary arteries, the elevated expression of a CYP epoxygenase, homologous to CYP2C8/9, is associated with enhanced EDHF-mediated hyperpolarization in response to bradykinin. Therefore, we propose that an isozyme of CYP2C is the most likely candidate for the CYP-dependent EDHF synthase in porcine coronary arteries. (Hypertension. 2000;36:270-275.)

Key Words: endothelium-derived hyperpolarizing factor ■ cytochrome P450 ■ nifedipine ■ arteries ■ potassium channels

The existence of an NO/prostacyclin (prostaglandin [PG]I\(_2\))-independent component of endothelium-dependent relaxation has been convincingly demonstrated in various conduit and resistance arteries. Because this NO/PGI\(_2\)-independent vasodilatation was associated with vascular smooth muscle hyperpolarization, the term “endothelium-derived hyperpolarizing factor” (EDHF) was coined.\(^1\)

On the basis of the experimental data published to date, it is evident that several EDHFs exist in different species.\(^1\) However, the majority of reports that characterize EDHF in the coronary vascular bed support the concept that this EDHF is a cytochrome P450 (CYP)-related product. This hypothesis was based on the observation that CYP inhibitors and a number of anesthetic agents markedly attenuate EDHF-mediated responses.\(^1\) However, these conclusions are limited by the fact that these CYP inhibitors cannot discriminate between the different CYP isoforms and can directly interfere with Ca\(^{2+}\)-dependent K\(^+\) channels,\(^2\) which are thought to be the main targets of EDHF in vascular cells.\(^3,4\)

Therefore, the aim of the present study was to identify a candidate EDHF synthase in endothelial cells by screening for the expression of CYP epoxygenases and to determine the effects of clinically relevant antihypertensive agents (ie, ramiprilat, nifedipine) on the induction of CYP enzymes and CYP product formation, as well as the bradykinin-induced EDHF-mediated hyperpolarization and relaxation.

Methods

Cultured Endothelial Cells

Human umbilical vein endothelial cells were isolated and cultured as described previously,\(^5\) and human coronary artery endothelial cells (HCAECs) were obtained from CellSystems and cultured in SmGM-2 (CellSystems). In some experiments, cGMP levels were determined.\(^5\)

Preparation of Porcine Coronary Arteries

Porcine epicardial artery segments (~40 mm long, mean external diameter 2.4 to 2.8 mm) were excised, side branches were sealed...
with surgical clips, and the segment was cannulated at both ends and
placed into vessel chambers. After equilibration, coronary arteries
were perfused with MEM containing β-naphthoflavone (3 μmol/L,
48 hours), or nifedipine (0.1 μmol/L, 18 hours). Half of each
segment was then cut into rings for organ chamber studies and
precontracted with U46619 (0.1 to 1 μmol/L), and relaxation in
response to sodium nitroprusside (SNP) were determined. After pre-
treatment with either solvent or nifedipine, and relaxant
responses to sodium nitroprusside (SNP) were determined.

Isolation of RNA and Protein

From the remaining coronary artery segment, endothelial cells or
total RNA was isolated through intraluminal incubation with either
dispase (2.4 U/mL) or guanidine isothiocyanate. Random hexa-
ucleotide 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 CYP2C34
sequence (GenBank accession no. U35843) (upstream primer AGA-
CCAGAGCACCACCTCTG, downstream primer CTTGGGGAT-
GAGGATGT1T) that exhibited a high homology to the human 2C8
sequence (GenBank accession no. Y00498) (CYP2B2 upstream
primer TGGTGGAGGARCTGCGGAATCC, downstream primer
TGCCCTGGCCAAAGACAAAYGCG [GenBank accession no.
M34452]; CYP2J2 upstream primer CCTCAYTTTACAAGAT-
CAACA, downstream primer GCAGATGAGGTTTTCTCTCAT
[GenBank accession no. U37143]; and elongation factor [EF] up-
stream primer GACATCACCAAGGGTGACAG, downstream
primer GCCGTCAGACACTGCGATA). PCR products were separated on a 1.5% TAE agarose gel and
visualized by staining with ethidium bromide. 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 CYP2C8, CYP2B,
or CYP2J2. Phenol-soluble protein or microsomal fractions
(100,000 g pellets) prepared from isolated porcine coronary artery
endothelial cells (PCAECs) were subjected to SDS–polyacrylamide
gel electrophoresis (8%) and Western blotting with the use of 2
different CYP2C11 polyclonal antibodies (kindly provided by Dr E.
Morgan [Atlanta, Ga] or purchased from Daiichi Pure Chemicals):
an endothelial NOS (eNOS) polyclonal antibody (Santa Cruz).

Analysis of CYP-Derived Products

Native PCAECs harvested with the use of dispase, as described
earlier, were allowed to equilibrate in HEPES-modified Tyrode’s
solution containing 300 μmol/L Nω-nitro-l-arginine and 10 μmol/L
diclofenac, were stimulated with 100 μmol/L bradykinin (5 minutes),
and were frozen in liquid nitrogen. Endothelial cells were homo-
genized, and CYP-derived eicosanoids were detected as described
previously.7

Electrophysiological Measurements

After incubation with solvent or nifedipine, coronary artery rings
were slit, mounted in a heated chamber, and maintained in HEPS-
modified Tyrode solution containing 10 μmol/L diclofenac,
300 μmol/L Nω-nitro-l-arginine, and 1 μmol/L U46619 to mimic
conditions in the organ chamber experiments. Smooth muscle
membrane potential was recorded by impaling cells through the
intima as described previously.8

Statistical Analysis

Data are expressed as mean±SEM. Statistical evaluation was per-
formed with Student’s t test for 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.
CYP Induction and Endothelium-Dependent Hyperpolarization

Induction of CYP2C potentiated the bradykinin-induced, endothelium-dependent hyperpolarization of porcine coronary artery smooth muscle. The bradykinin (Bk; 100 nmol/L)-induced hyperpolarization was determined in endothelium-intact porcine coronary artery segments pretreated with either solvent (CTL; 18 hours) or nifedipine (Nif; 0.1 μmol/L; 18 hours). Representative membrane potential recordings (A) and statistical summary of data (B) were obtained in 4 to 6 separate experiments (*P<0.05 vs control). C, Statistical summary of the bradykinin-induced hyperpolarization determined in freshly isolated porcine coronary arteries in the absence (CTL) and presence of charybdotoxin (CbTx; 100 nmol/L)/apamin (Apa; 100 nmol/L) and in the absence and presence of iberiotoxin (IbTx; 100 nmol/L). The results are presented as the mean±SEM of data obtained from 6 different animals (**P<0.01 vs control). All experiments were performed in the presence of 10 μmol/L diclofenac, 300 μmol/L N\textsuperscript{\textalpha}-nitro-L-arginine, and 1 μmol/L U46619 to mimic conditions in organ chamber experiments.

CYP Induction and EDHF-Mediated Relaxation

In accordance with the effects of nifedipine on the agonist-induced, endothelium-dependent hyperpolarization of porcine coronary artery smooth muscle cells, nifedipine induced a leftward shift in the EDHF-mediated concentration-relaxation response (Figure 2). A >3-fold increase in endothelial EET generation was detected after the incubation of coronary segments with either β-naphthoflavone or nifedipine (Figure 2).

CYP Induction and Epoxyeicosatrienoic Acid Production

To establish a functional link between the expression of CYP2C and the production of EDHF, experiments were performed with isolated endothelium-intact porcine coronary arteries, and epoxyeicosatrienoic acid (EET) levels were measured in endothelial cell homogenates. Intraluminal incubation with nifedipine enhanced the expression of CYP2C protein in endothelial cells (Figure 1C). In endothelial cells harvested from coronary arteries maintained under control conditions for 18 hours, detectable amounts of 11,12-EET were generated in response to bradykinin (Figure 2). A >3-fold increase in endothelial EET generation was detected after the incubation of coronary segments with either β-naphthoflavone or nifedipine (Figure 2).

Figure 2. CYP induction enhances the generation of 11,12-EET in native PCAECs pretreated with either solvent (CTL; 48 hours), β-naphthoflavone (β-NF; 3 μmol/L; 48 hours), or nifedipine (Nif; 0.1 μmol/L; 18 hours). After equilibration in HEPES-modified Tyrode’s solution (37°C, 4 hours) containing 10 μmol/L diclofenac and 300 μmol/L N\textsuperscript{\textalpha}-nitro-L-arginine, the isolated cells were stimulated with 100 nmol/L bradykinin (5 minutes). Arachidonic acid metabolites were analyzed as described. The results (ng of 11,12-EET) are presented as the mean±SEM of data obtained with 4 to 8 porcine coronary arteries, all from different animals. *P<0.05 vs control.

Figure 3. Nifedipine pretreatment enhances the EDHF-mediated hyperpolarization of porcine coronary artery smooth muscle. The bradykinin (Bk; 100 nmol/L)-induced hyperpolarization was determined in endothelium-intact porcine coronary artery segments pretreated with either solvent (CTL; 18 hours) or nifedipine (Nif; 0.1 μmol/L; 18 hours). Representative membrane potential recordings (A) and statistical summary of data (B) were obtained in 4 to 6 separate experiments (*P<0.05 vs control). C, Statistical summary of the bradykinin-induced hyperpolarization determined in freshly isolated porcine coronary arteries in the absence (CTL) and presence of charybdotoxin (CbTx; 100 nmol/L)/apamin (Apa; 100 nmol/L) and in the absence and presence of iberiotoxin (IbTx; 100 nmol/L). The results are presented as the mean±SEM of data obtained with arteries from 6 different animals (**P<0.01 vs control). All experiments were performed in the presence of 10 μmol/L diclofenac, 300 μmol/L N\textsuperscript{\textalpha}-nitro-L-arginine, and 1 μmol/L U46619 to mimic conditions in organ chamber experiments.
Figure 4. Effect of nifedipine on the EDHF- and NO-mediated relaxation of endothelium-intact porcine coronary artery rings. Porcine coronary arteries incubated with either solvent (18 hours) or nifedipine (0.1 μmol/L, 18 hours) were cut into rings, and the relaxation to bradykinin was determined after contraction with U46619 (0.1 to 1 μmol/L). A, EDHF-mediated relaxation in the combined presence of diclofenac and the NO donor (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) in control versus nifedipine (100 μmol/L, 18 hours), the PKC inhibitor Ro-31-8220 (100 μmol/L), or combinations of more than 1 substance. Endothelial proteins were subjected to Western blot analysis with specific antibodies directed against eNOS or CYP2C. To control for the protein loading of each lane, blots were reprobed with the endothelial marker protein PECAM-1. *ve represents the signal generated by a positive control: rat liver for CYP 2C and an endothelial cell lysate for PECAM-1. The blots presented are representative of data obtained in 3 to 5 additional experiments.

Figure 5. The role of NO and PKC in nifedipine-mediated CYP induction. Confluent primary cultures of human umbilical vein endothelial cells were incubated with either solvent (CTL; 18 hours), nifedipine (Nif; 0.1 μmol/L, 18 hours), or combinations of more than 1 substance. Endothelial proteins were subjected to Western blot analysis with specific antibodies directed against eNOS or CYP2C. To control for the protein loading of each lane, blots were reprobed with the endothelial marker protein PECAM-1. *ve represents the signal generated by a positive control: rat liver for CYP 2C and an endothelial cell lysate for PECAM-1. The blots presented are representative of data obtained in 3 to 5 additional experiments.

Role of NO and Protein Kinase C in Nifedipine-Mediated CYP Induction

The nifedipine-induced attenuation of NO-mediated relaxation could be correlated with a slight decrease in the expression of eNOS. In cultured endothelial cells, 0.1 μmol/L nifedipine (18 hours) decreased the expression of eNOS (Figure 5) and attenuated the basal production of cGMP (1.2±0.5 versus 0.7±0.2 pmol/well in the absence and presence of nifedipine, P=0.38, n=8, NS), as well as the bradykinin-stimulated increase in cGMP levels (15.5±1.4 versus 12.3±0.3 pmol/well in the absence and presence of nifedipine, n=8, P<0.05).

To determine whether the effect of nifedipine on CYP2C expression was a consequence of its effect on eNOS expression, experiments were performed with Nω-nitro-L-arginine and the NO donor (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO). In 3 of 6 experiments, 300 μmol/L Nω-nitro-L-arginine (18 hours) enhanced CYP2C expression, whereas 500 μmol/L DETA-NO (18 hours) consistently attenuated CYP2C expression. Moreover, in the presence of DETA-NO, nifedipine did not increase CYP2C expression (Figure 5).

Because nifedipine has been suggested to inhibit PKC in endothelial cells,10 we compared the effects of the Ca2+-antagonist with those of an inhibitor of Ca2+-dependent and -independent protein kinase C (PKC) isoforms. Ro-31-8220 (100 nmol/L, 18 hours) slightly depressed the expression of CYP2C in human endothelial cells and completely prevented its induction by nifedipine (Figure 5).
**Discussion**

The results presented here demonstrate that EDHF-mediated hyperpolarization and relaxation elicited by bradykinin in porcine coronary arteries can be modulated by enhancement of the expression of CYP2C with nifedipine or by inhibition of CYP2C9 activity with the use of sulfaphenazole. This supports the concept that a CYP-dependent process is crucial for the generation of EDHF-mediated responses in porcine coronary arteries.

Previously, indirect evidence has suggested that a CYP epoxygenase-derived product mediates NO/PGL-1-independent relaxation in the human, porcine, bovine, canine, and rat coronary circulations. One hindrance to the identification of the CYP isozyme or isozymes that may generate a hyperpolarizing factor in endothelial cells has been the fact that these enzymes are expressed at extremely low levels in cultured cells. Thus, it was necessary to screen both cultured and native coronary endothelial cells to determine which CYP enzymes may be involved in the generation of EDHF. Previous reports demonstrated that 2 CYP epoxygenases (CYP2C8/9 and CYP2J2) can be expressed in human endothelial cells, and we were able to detect homologs of both enzymes in the endothelium removed from porcine coronary arteries. Because we previously reported that a CYP-derived hyperpolarizing factor can be physically transferred from cultured endothelial to smooth muscle cells after the induction of CYP enzymes with β-naphthoflavone, we took the sensitivity to this inducer as a criterion for the identification of the putative coronary EDHF synthase. Only the CYP2C enzymes were consistently induced after incubation with β-naphthoflavone. Moreover, the results of the present investigation, which show the generation of EETs and the amplitude of EDHF-mediated smooth muscle hyperpolarization and relaxation were potentiated after the induction of CYP2C, suggest that a homolog of CYP2C8/9 is involved in the generation of EDHF in porcine coronary arteries. Indeed, we have recently shown that antisense oligonucleotides against CYP2C selectively attenuate the EDHF-mediated hyperpolarization and relaxation elicited by bradykinin in porcine coronary arteries.

To demonstrate that CYP2C expression and EDHF-mediated responses could be induced by a completely different class of compounds to β-naphthoflavone, experiments were performed with the Ca<sup>2+</sup>-antagonist nifedipine. From a theoretical point of view, there was no reason to expect that nifedipine would be able to induce CYP2C, because this compound is known to be metabolized in the liver by CYP3A4. Moreover, the nifedipine-specific element, described in the CYP3A4 promoter, does not appear to be present in the CYP2C gene family. Because nifedipine has been reported to inhibit PKC in endothelial cells, we compared the effects of nifedipine on CYP2C expression with those of Ro-31-8220, which inhibits Ca<sup>2+</sup>-dependent and -independent isoforms of PKC. However, in contrast to nifedipine, Ro-31-8220 attenuated endothelial CYP2C expression, suggesting that although the activity of PKC may modulate endothelial CYP2C expression, the mechanism that underlies the upregulation of CYP2C expression by nifedipine is not related to its inhibitory action on PKC.

The prolonged incubation of cultured HCAECs with nifedipine downregulated eNOS protein levels. We therefore speculated that NO may regulate CYP/EDHF synthase expression. Indeed, in some experiments, the inhibition of basal NO production enhanced CYP2C expression. The inconsistency in this observation may be related to variations in basal NO production in different cell batches. High concentrations of NO, on the other hand, consistently decreased the expression of CYP2C. It is therefore tempting to suggest that the expression and activity of the EDHF synthase are attenuated by NO. Caution should be exerted in the interpretation of data that relate to the induction of CYP expression by pharmacological substances because these compounds may be partially metabolized by endothelial cells and thus induce the expression of CYP enzymes independent of any effects related to the production of NO.

The list of candidate EDHFs is relatively long, and in most vascular preparations in which EDHF clearly is not a CYP metabolite (eg, the guinea pig carotid artery and the rat hepatic artery), EDHF-mediated hyperpolarizations are insensitive to iberiotoxin but sensitive to the combination of charybdotoxin and apamin. In the porcine and canine coronary arteries, as well as the rat renal artery, in which EDHF also appears to be a CYP metabolite, EDHF-mediated hyperpolarizations are equally sensitive to iberiotoxin and charybdotoxin/apamin. Thus, because the CYP2C product 11,12-EET, which is generated by coronary artery endothelial cells, activates iberiotoxin-sensitive K<sub>Ca</sub>-channels, the evidence accumulated to date strongly suggests that a CYP-derived product plays a crucial role in the bradykinin-induced generation of EDHF-mediated responses in the coronary artery. The reason for the caution in stating that the coronary EDHF is an EET is that it is currently unclear how endothelium-derived EETs are able to access smooth muscle cells to elicit hyperpolarization. EETs are by nature lipophilic, and simple diffusion from the endothelium to smooth muscle cells down a concentration gradient would be too slow to account for the rapidly initiated EDHF-mediated hyperpolarization and relaxation. One possibility that would also link many of the observations made in different vessels is that EETs are able to either pass through gap junctions or influence gap junctional communication of a second hyperpolarizing agent.

Taken together, the results of the present study demonstrate that nifedipine enhances the bradykinin-induced generation of EDHF-mediated responses in porcine coronary artery segments. These effects appear to be directly attributable to the upregulation of an enzyme homologous to CYP2C8/9 and the generation of the CYP metabolite 11,12-EET.

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