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Contribution of cytochrome P450 metabolites to bradykinin-induced vasodilation in endothelial NO synthase deficient mouse hearts

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1 We have characterized the contribution of endothelial nitric oxide synthase (eNOS), cyclo-oxygenase (COX) and cytochrome P450 (CYP450) to the bradykinin (BK)- induced vasodilation in isolated hearts from wildtype (WT) and eNOS deficient mice (eNOS−/−).

2 The endothelium-dependent vasodilation by bradykinin (BK, 1 μM) was significantly lower in eNOS−/− hearts than that in WT hearts (+247% and +325% of basal flow, respectively), while there was no difference in the endothelium-independent vasodilation by adenosine.

3 In WT hearts, the BK-induced vasodilation was markedly attenuated following inhibition of NOS with ETU (10 μM) but not after COX inhibition with diclofenac (3 μM) ($P<0.01$). In line with this finding, Bk did not increase the cardiac prostacyclin release as measured by ELISA for 6-keto-PGF1α in the coronary venous effluent. In eNOS−/− hearts, the flow response to BK was insensitive to both NOS and COX inhibition.

4 The NOS/COX-independent vasodilatory factor which remained under L-NMMA+DF application was almost completely eliminated by either clotrimazole (3 μM), miconazole (0.5 μM) or 17-ODYA (1 μM), suggesting that it was a metabolite of CPY450 enzymes. Sulfaphenazole (10 μM), a CYP450 2C inhibitor, exerted only a minimal inhibitory effect.

5 In eNOS−/− hearts the effect of CYP450 inhibitors on the BK response was significantly more pronounced than in WT hearts, indicating an enhanced contribution of CYP450 enzymes.

6 These findings suggest that in isolated mouse hearts the BK-induced vasodilation is mediated by NO and CYP450 metabolites but not by prostaglandins. The CYP450 dependent vasodilator was was functionally up-regulated in eNOS−/− hearts and thus likely to compensate for the loss of eNOS in the coronary circulation.
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Abbreviations: AA, arachidonic acid; Ach, acetylcholin; BK, bradykinin; COX, cyclo-oxygenase; CYP450, cytochrome P450; EDHF, endothelium-derived relaxing factor; EET, epoxyeicosatrienoic acid; eNOS, endothelial nitric oxide synthase; eNOS−/− eNOS-deficient; ETU, 2-Ethyl-2-thiopseudourea; HETE, hydroxyeicosatetraenoic acid; iNOS, inducible NO synthase; 6-keto-PGF1α, 6-keto-prostaglandin-1-α; L-NMMA, N^G-mono-methyl-L-arginine; LVP, left ventricular pressure; nNOS, neuronal NO synthase; 17-ODYA, 17-octadecynoic acid; TMB, 3,3′,5,5′-Tetramethylbenzidine; WT, wildtype

Introduction

Endothelial cells control coronary vascular tone by the release of numerous vasoactive autacoids which act on underlying smooth muscle cells (Garland *et al*., 1995; Puybasset *et al*., 1996). In addition to nitric oxide (NO) and prostacyclin (PGI_2), cytochrome P450 monoxygenase (CYP450) metabolites of arachidonic acid (AA) contribute substantially to the vasorelaxing properties of the endothelium in different vascular beds (Harder *et al*., 1995; McGiff, 1991; Pratt *et al*., 1996), including the coronary circulation (Fisslthaler *et al*., 1999; Gebremedhin *et al*., 1998; Harder *et al*., 1995; Mombouli *et al*., 1996; Nishikawa *et al*., 1999). Two classes of CYP450 isoenzymes exist in the vasculature, namely, epoxygenases which catalyze the formation of epoxyeicosatrienoic acids (EETs), and ω-hydroxylases that produce 20-hydroxyeicosatetraenoic acid (20-HETE) (Harder *et al*., 1995; Kaley, 2000). EETs are known to hyperpolarize the membrane potential of smooth muscle cells by acting on the calcium-dependent K^+ channel (Bauersachs *et al*., 1994; Campbell *et al*., 1996; Harder *et al*., 1995) thereby relaxing coronary vessels (Weintraub *et al*., 1997). They are proposed candidates for the endothelium-derived hyperpolarizing factor (EDHF) (Bauersachs *et al*., 1994; Campbell *et al*., 1996; Fisslthaler *et al*., 1999; Gebremedhin *et al*., 1998; Miura & Gutterman, 1998; Mombouli & Vanhoutte, 1997; Oltman *et al*., 1998; Popp *et al*., 1996; Quilley & McGiff, 2000).

Several reports have demonstrated an interaction among endothelium-derived vasodilators (Kosonen *et al*., 1998; Nishikawa *et al*., 2000; Puybasset *et al*., 1996; Yajima *et al*.,

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1999). For instance, NOS inhibition augmented the contribution of CYP450 metabolites acting most likely as EDHF to vasodilation in carotid and coronary arteries (Bauersachs *et al*., 1996) as well as in mesenteric vessels (Randall & March, 1998), suggesting that EDHF may function as a 'backup' mechanism when NO synthesis is impaired. The cyclooxygenase (COX) pathway was also found to be upregulated in coronary circulation after sustained NOS inhibition in conscious dogs (Puybasset *et al*., 1996), in gracilis muscle arterioles (Sun *et al*., 1999), and in mesenteric arteries as well as coronary arteries (Chataigneau *et al*., 1999) from eNOS−/− mice. In this context it is interesting to note that basal coronary flow is not compromised in isolated eNOS−/− mouse hearts (Gödecke *et al*., 1998). This led us to hypothesize that the cardiovascular system might adapt to the chronic lack of eNOS and maintain a normal regulation of vascular tone by upregulation of other mediators.

In order to elucidate the mechanism regulating the coronary vasomotor tone in mice that have a targeted disruption of the eNOS gene, we analysed the contribution of NOS, COX, and CYP450 pathway to the bradykinin (BK)-induced vasodilation in isolated mouse hearts from WT and eNOS−/− hearts.

Methods

Mouse strains

Endothelial NOS deficient mice (eNOS−/−) have been generated as described (Gödecke *et al*., 1998). In this study, eNOS−/− mice which have been backcrossed for seven generations into the C57BL/6 background were used. C57BL/6 mice were used as WT control.

Isolation of mouse hearts

Mice were anaesthetized by intraperitoneal application of urethane (1.5 g kg^{-1}). Heparin (250 U, i.p.) was given simultaneously which efficiently inhibited blood coagulation and prevented intracoronary clot formation. After thoracotomy, hearts were rapidly excised and transferred for preparation of the aortic trunk to warm, oxygenated Krebs-Henseleit buffer. The aorta was then cannulated with a 20-gauge stainless steel cannula, and hearts were perfused in a nonrecirculating Langendorff mode at constant pressure (100 mmHg) with a modified Krebs-Henseleit buffer containing (in mmol l^{-1}) NaCl 116, KCl 4.6, $MgSO_4$ 1.1, $NaHCO_3$ 24.9, $CaCl_2$ 2.5, KH_2PO_4, 1.2, glucose 10, EDTA, 0.5 and pyruvate 2.0 equilibrated with 95% O_2 and 5% CO_2 (pH = 7.4, 37°C). Coronary flow was measured with an ultrasonic flowmeter (Transonic) located above the aortic cannula. Isovolumetric left ventricular pressure (LVP) was measured with a pressure transducer (Statham P23XL) which was connected to a polyethylene catheter (PE-50) with a fluid-filled balloon at the tip. The balloon was placed into the cavity of the left ventricle through the opening of the pulmonary vein *via* the left atrium. Balloon volume was adjusted to a diastolic pressure of about 5–8 mmHg. Heart rate and dP/dt were derived from the pressure signal. Hearts were paced at 600 beats min^{-1} by electrodes fixed at the right atrium and the heart apex. All physiological parameters were simultaneously recorded by an 8-channel MacLab system (AD instruments).

Experimental protocols

Hearts were allowed to equilibrate for at least 30 min until coronary flow and LVP attained constant values. Only hearts exhibiting a normal reactive hyperemia (peak flow >2 fold basal flow after 20 s of coronary occlusion) were included in this study.

Pharmacological interventions were performed by intracoronary infusion of inhibitors and agonists through a Y-connector in the aortic perfusion line at the flow rate of 1/1000 to 1/100 of the coronary flow to generate the following final concentrations showing maximal inhibition of target enzymes: 10 μM 2-Ethyl-2-thiopseudourea (ETU) for NOS inhibition; 3 μM diclofenac for COX inhibition (Gödecke *et al*., 1998). Four structurely different CYP450 inhibitors are employed in the present experiments: 1 μM 17-ODYA, 3 μM clotrimazole, 0.5 μM miconazole and 10 μM sulfaphenazole according to published data. All chemicals were infused for a period of >10 min until a response plateau of coronary flow had been reached.

Bradykinin (BK) was administrated at the concentration of 1 μM until a plateau of the flow response had been reached. Adenosine (1 μM), an endothelium-independent vasodilator was employed to test the viability of vessels after application of inhibitors. In a first series of experiments, we assessed the BK response after inactivation of NOS and COX achieved by N^G-monomethyl-L-arginine (L-NMMA, 100 μM) or ETU (10 μM) and diclofenac (3 μM) in both WT and eNOS−/− hearts.

In a second series of experiments, the involvement of CYP450 metabolites in BK-induced vasodilation was analysed.

Four structurally different CYP450 inhibitors are used in the present study. In control experiments prior to the experimental series it was tested which concentrations affected the endothelium-dependent vasodilation without effect on vascular smooth muscle cells or contractile function. Based on this analysis and in line with literature data reporting inhibition of CYP450 enzymes the following concentrations were chosen: 17-octadecynoic acid (17-ODYA, 1 μM; Zou *et al*., 1994; Harder *et al*., 1995), clotrimazole (3 μM, Popp *et al*., 1996; Huang *et al*., 2000), and sulfaphenazole (10 μM; Schmider *et al*., 1997; Fisslthaler *et al*., 2000). Miconazole at published concentrations of 2–10 μM (Zou *et al*., 1994; Harder *et al*., 1995), induced a slight increase in cardiac enddiastolic pressure and was therefore used at a concentration of 0.5 μM. In WT mouse hearts, combined inhibition of NOS and COX was achieved by combination of L-NMMA (100 μM) and diclofenac (3 μM) which have been demonstrated to completely inhibit the NO- and prostaglandin-induced vasodilation (Cable *et al*., 1997; Gödecke *et al*., 1998). All inhibitors at the concentrations given above did not affect the adenosine mediated vasodilation.

Measurement of 6-keto-PGF_1-α release

Coronary effluent perfusate (1 ml) was collected under basal conditions and during the flow response plateau to BK (1 μM) and to ACh (330 nM). Samples were stored at −70°C. 6-keto-PGF1-α was determined by ELISA (Amersham, Braunschweig) according to the manufacturer's instructions.

Fifty microlitres of coronary venous effluent and standards (0.5–64 pg) diluted in Krebs-Henseleit buffer were transferred to the wells of a microtiter plate precoated with donkey anti-rabbit antibodies. Then, 50 μl of rabbit anti-6-keto-PGF1α antiserum was added and the mixture incubated at room temperature for 30 min. Subsequently, 50 μl of a 6-keto-PGF1α-peroxidase complex were added and the mixture incubated for another 60 min. After washing, 150 μl of the peroxidase substrate 3,3′,5,5′-Tetramethylbenzidine (TMB)/H_2O_2 were added and the reaction allowed to proceed for 15 min. Reactions were stopped by addition of 100 μl 1 M sulphuric acid and the absorption read at 450 nm in a microplate reader.

Materials

Diclofenac was purchased from ICN. L-NMMA, miconazole, clotrimazole, sulfaphenazole, 17-ODYA and BK were from Sigma, and ETU from Aldrich. All the substances were dissolved in Krebs-Henseleit solution except 17-ODYA, miconazole, clotrimazole and sulfaphenazole which were dissolved in DMSO.

Statistical analysis

Coronary flow was normalized to heart weight and presented as mean$\pm$s.d. Statistical evaluation was done by using paired Student's *t*-test in comparing flow before and in the presence of inhibitors, whereas the unpaired Student's *t*-test was used to evaluate differences between WT and eNOS$-/-$ hearts. The effects of inhibitors on BK-induced vasodilation were tested by two-way ANOVA. Statistical analysis was performed using Statview 5.0 software (Abacus Concepts, Inc., Berkeley, U.S.A.). Means were considered to differ significantly when $P<0.05$.

Results

We first tested whether repeated application of vasodilators results in reproducible flow responses or whether tachyphylaxis is observed in isolated mouse hearts. Figure 1 shows coronary vasodilation induced by the endothelium-dependent agonist, BK (1 μM) and by the endothelium-independent vasodilator, adenosine (1 μM). When applied repeatedly with a time interval of 28–35 min, both vasodilators elicited similar vasodilator response. Although not statistically significant, the BK response tended to decrease after repeated application by 10%.

Role of NO and prostaglandins

Next the contribution of NO, and prostaglandins to the vasodilator response of BK in isolated WT and eNOS$-/-$ hearts was analysed. In WT hearts, acute inhibition of NOS with ethylisothiourea (ETU, 10 μM) reduced basal coronary flow by -2.6 ± 0.6 ml min^{-1} g^{-1} (-25%) and the BK-induced vasodilation by 12.7 ± 0.8 ml min^{-1} g^{-1} (-51%) ($n=6$) (Figure 2A). In contrast, basal coronary flow and the BK-induced vasodilation in eNOS$-/-$ hearts were not affected by ETU application. However, the BK-induced vasodilation was significantly lower than in WT hearts (18.4 ± 2.7 vs 32.4 ± 7.6 ml min^{-1} g^{-1}, $n=5$, $P<0.05$) and was not affected by NOS inhibition.

To analyse whether COX metabolites were involved in the BK-induced vasodilation, we blocked COX activity with

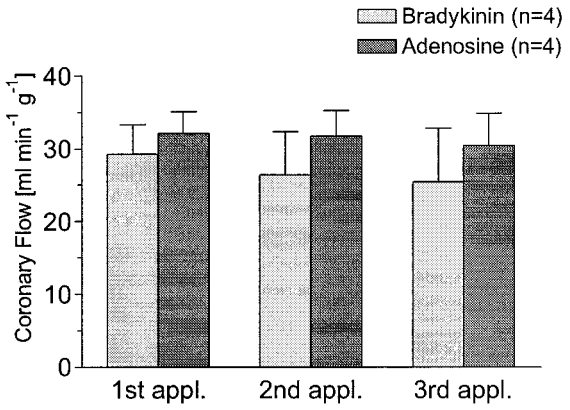

Figure 1 Coronary vasodilation induced by BK (1 μM; light grey) and adenosine (1 μM dark grey). When applied repeatedly with time intervals of 28–35 min, both vasodilators elicited similar vasodilatory responses.

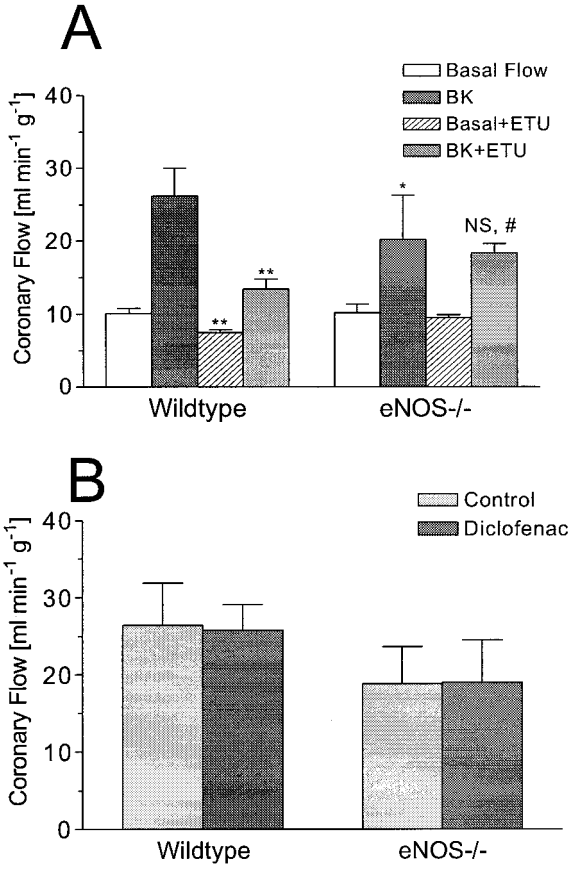

Figure 2 Effects of NOS (A) and COX (B) inhibition on BK-induced vasodilation. (A) acute NOS inhibition by ETU (10 μM) significantly reduced basal and the BK-induced flow response in WT hearts ($n=6$) but had no effects in eNOS$-/-$ hearts ($n=5$). (B) COX inhibition by diclofenac (3 μM) did not alter the BK-induced flow response both in WT ($n=6$) and eNOS$-/-$ hearts ($n=5$). $^{**}P<0.01$ vs control; $\#P<0.05$ vs WT.

3 μM diclofenac. As shown in Figure 2B, coronary vasodilation induced by BK was not significantly influenced by COX inhibition either in WT nor in eNOS−/− hearts. The combined inhibition of NOS and COX in WT hearts did not further reduce coronary vasodilation evoked by BK when compared to single NOS inhibition (data not shown). The apparent lack of prostaglandins in mediating the BK-induced coronary vasodilation could be directly demonstrated by measurement of 6-keto-PGF1-α the stable end product of prostacyclin in the coronary effluent perfusate (Table 1). As can be seen, BK (1 μM) did not increase 6-keto-PGF1-α release. In contrast, ACh (330 nM) augmented PGI_2 release about 20 fold ($P<0.01$).

Role of CYP450 metabolites

Commonly the part of the BK-mediated vasodilation which is resistant to NOS/COX inhibition is attributed to EDHF, which was proposed to be a CYP450 metabolite of arachidonic acid (Fisslthaler *et al*., 1999). To characterize the role of the BK mediated flow response in mouse hearts, three chemically different CYP450 inhibitors (17-ODYA, clotrimazole, and miconazole) were employed in the present experiments. As shown in Figure 3A, combination of NOS and COX inhibition substantially attenuated the BK response in WT hearts (control) and subsequent addition of clotrimazole (3 μM) almost abolished the BK-induced vasodilation. Similar results were obtained when miconazole (0.5 μM), or 17-ODYA (1 μM) was applied in separate experiments (Figure 3B,C).

To further analyse whether EDHF is constitutively active in the coronary vasculature or becomes functional only after elimination of NO, we tested the effect of four CYP450 inhibitors on BK-induced vasodilation in the presence (WT) and absence of endogenous NO (eNOS−/−). As shown in Figure 4A, coronary vasodilation induced by BK in WT hearts was significantly reduced by clotrimazole (3 μM) (maximal flow reduction: 7.6 ± 3.7 ml min^{-1} g^{-1}, $P<0.01$). In eNOS−/− hearts, the BK-induced vasodilation was almost completely eliminated by clotrimazole leading to a flow decrease of -10.2 ± 4.1 ml min^{-1} g^{-1}. The effect of clotrimazole was only partially reversible after 30 min. Miconazole (0.5 μM) irreversibly caused significant reduction in the BK-response by 7.7 ± 1.99 ml min^{-1} g^{-1} ($P<0.05$) in WT hearts (Figure 4B). In eNOS−/− hearts, miconazole reduced the BK-induced vasodilation by 9.4 ± 5.9 ml min^{-1} g^{-1}. Inactivation of P450 enzymes by 17-ODYA (1 μM) also attenuated the BK response in WT hearts (flow reduction 7.0 ± 4.4 ml min^{-1} g^{-1}) and almost abolished the BK-mediated flow response in eNOS−/− hearts (Figure 4C, -12.1 ± 4.5 ml min^{-1} g^{-1}). The inhibitory effect of 17-ODYA on BK-induced flow response was reversible. It is important to note that the effect of clotrimazole, miconazole and ODYA on the BK-induced vasodilation was significantly higher in eNOS−/− hearts than in WT hearts ($P<0.01$).

However, sulfaphenazole (10 μM), an inhibitor considered to be specific for CYP450/2C enzymes, did not significantly

Table 1 Cardiac release of 6-keto-PGF1-α in response to BK and ACh in the isolated mouse heart

Agonist	*6-keto-PGF1-α Release* (pmol min^{-1} g^{-1})
Control	51.9 ± 14.1
Bradykinin (1 μM)	87.7 ± 19.0
Acetylcholin (330 nM)	105.2 ± 678.3**

Results represent means $\pm$ s.d. for $n=6$ experiments. **$P<0.01$ vs control and bradykinin.

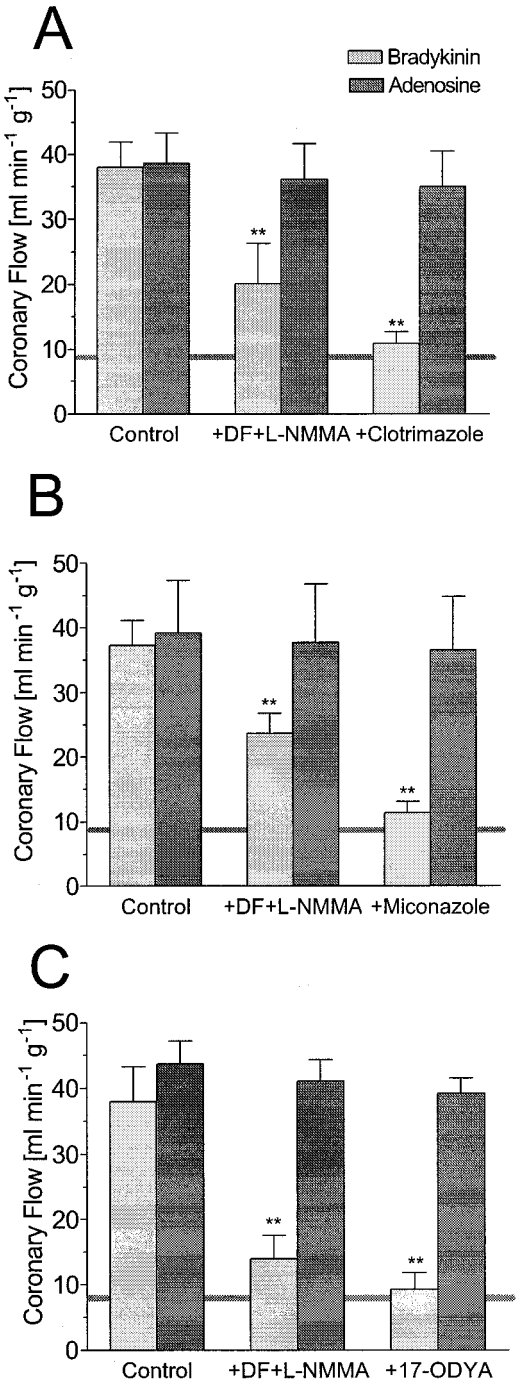

Figure 3 Effects of CYP450 inhibitors on BK-induced vasodilation. The L-NMMA+DF insensitive vasodilation induced by BK was completely eliminated by each of the three CYP450 inhibitors (clotrimazole, $n=4$; miconazole, $n=5$ or 17-ODYA, $n=5$), while the endothelium-independent vasodilation to adenosine was not affected. The horizontal lines indicate the average of basal coronary flow. **$P<0.01$ vs control.

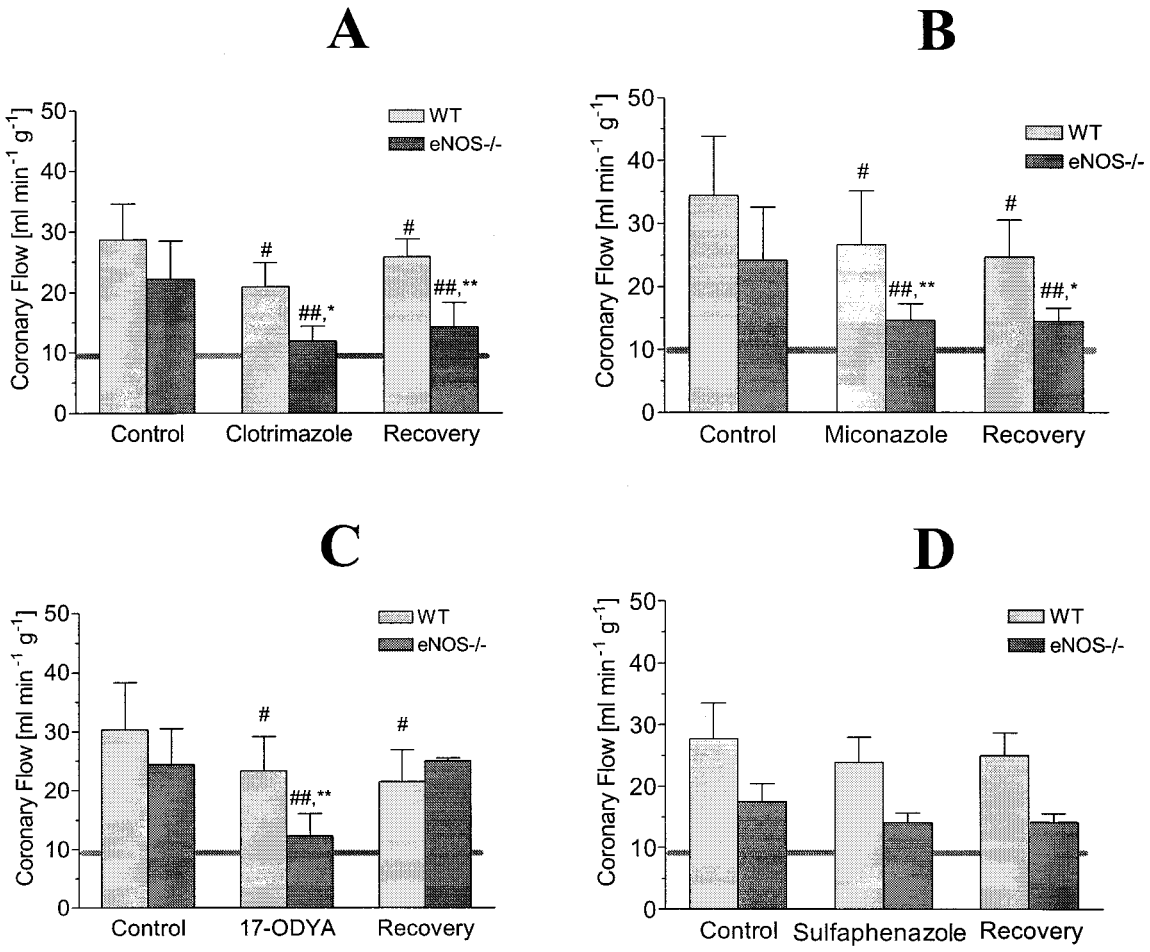

Figure 4 Effects of CYP450 inhibitors on BK-induced vasodilation in WT and eNOS−/− hearts. Clotrimazole (3 μM, A), Miconazole (0.5 μM, B), 17-ODYA (1 μM, C) and sulphaphenazole (10 μM) were tested in WT and in eNOS−/− hearts ($n=5-9$). ##$P<0.01$ vs control; #$P<0.05$ vs control; **$P<0.01$ and *$P<0.05$ vs flow reduction in wildtype, two-way ANOVA.

affect the BK-induced vasodilation both in WT and eNOS−/− hearts (Figure 4D).

The four CYP450 enzyme inhibitors did not alter the endothelium-independent vasodilation elicited by 1 μM of adenosine (Table 2).

Discussion

The present study analyses the interaction of nitric oxide (NO) in relation to arachidonic acid (AA) metabolites catalyzed by cyclo-oxygenase (COX) and cytochrome P450 (CYP450) in the regulation of coronary flow in the isolated mouse heart. Major findings are: (1) In the mouse heart, the bradykinin (BK)-induced coronary vasodilation is mediated largely by NO, while COX metabolites are not involved. (2) The NOS/COX independent component of the BK-induced vasodilation is mediated by a CYP450 metabolite which is active both in WT and eNOS−/− hearts. (3) The CYP450 metabolism is up-regulated in mice lacking eNOS, suggesting that this component might be involved in the compensation of the loss of endogenous NO in eNOS−/− hearts.

Coronary vascular resistance is set by the balanced release of vasoconstrictor and vasodilator metabolites from cardiac myocytes and vascular cells. The most important endothelium derived vasodilators include NO, PGI_2, and EDHF, while endothelium-derived vasoconstriction is mainly attributed to endothelin and vasoconstrictor prostanoids (Boulanger & Vanhoutte, 1997). NO is an important mediator, not only regulating basal coronary vascular tone (Kelm & Schrader, 1990), but also mediating vasodilaton in response to agonists like BK (Kuga *et al*., 1997; Persson & Andersson, 1998) and ACh (Chataigneau *et al*., 1999; Nishikawa *et al*., 1999). Surprisingly, genetic disruption of eNOS did not reduce basal coronary flow in mice (Gödecke *et al*., 1998), suggesting that other vasodilator mechanisms were functionally induced to compensate for the lack of endothelium-derived NO in hearts. A possible mechanism might be the up-regulation of other NOS isoforms, i.e. inducible NOS (iNOS) and/or neuronal NOS (nNOS). This seemed likely since it has been shown that nNOS may compensate for the absence of endothelial NO in pial arterioles of mice (Meng *et al*., 1996) and in isolated coronary arteries (Lamping *et al*., 2000). However, our results revealed no effect of acute NOS inhibition on basal and BK-induced flow response in eNOS−/− hearts, indicating that NOS isoforms were not involved in the compensation of eNOS deficiency at the level of coronary resistance vessels.

Table 2 Effect of Cyp450 inhibitors on adenosine induced vasodilation in WT and eNS−/− mouse hearts

	Genotype	*Control* (ml min^{-1} g^{-1})	*Application* (ml min^{-1} g^{-1})	*Recovery* (ml min^{-1} g^{-1})
Clotrimazole	WT	34.64±8.03	34.45±10.33	37.41±8.42
	eNOS−/−	43.47±5.52	42.39±7.88	42.39±9.82
Miconazole	WT	40.36±4.69	38.46±5.74	40.44±9.95
	eNOS−/−	35.67±9.25	32.71±8.53	30.93±9.42
17-ODYA	WT	32.87±11.33	30.29±9.27	28.57±10.32
	eNOS−/−	37.31±3.93	28.31±9.02	31.63±2.46
Sulfaphenazole	WT	34.97±8.09	38.99±6.75	39.00±5.03
	eNOS−/−	44.04±8.34	39.72±10.33	41.36±7.32

The influence of CYP450 inhibitors on smooth muscle cell function was assessed by measurement of adenosine induced vasodilation before (Control), during (Application) and after (Recovery) inhibitor application. Data represent means of coronary flow±s.d. of $n=5-9$ experiments which were performed as controls in parallel to the experiments presented in Figure 4.

Inhibition of COX activity by diclofenac did not alter the vasodilator response evoked by BK either in WT or in eNOS−/− hearts, suggesting that metabolites of the COX pathway did not contribute to the BK-induced vasodilation and were not upregulated in eNOS−/− hearts. The finding that vasodilator COX metabolites did not compensate for the eNOS deficiency is perhaps surprising since COX activity has been shown to be attenuated by NO (Gambone *et al*., 1997; Molina-Holgodo *et al*., 1995), and sustained NOS inhibition led to an up-regulation in canine coronary arteries (Puybasset *et al*., 1996). Genetic inactivation of eNOS in mice leads to a stronger contribution of COX metabolites to ACh induced vasodilation in mesenteric (Chataigneau *et al*., 1999) and coronary arteries (Lamping *et al*., 2000). Similarly, in gracilis muscle arterioles of eNOS−/− mice, the role of COX metabolites (prostaglandins) in the flow-induced vasodilation was enhanced (Sun *et al*., 1999). Taken together, eNOS deficiency may up-regulate different mechanisms which compensate for the lack of eNOS in different vascular beds. In coronary resistance vessels, our results clearly show that neither NOS isoforms nor vasodilator prostaglandins (PGI_2, PGE_2) are functionally up-regulated. Instead we found that CYP450 metabolites are most likely involved.

CYP450 enzymes synthesize from arachidonic acid four isomers of EETs by epoxygenation as well as 19- and 20-HETEs by ω-hydroxygenation (McGiff, 1991). Because EETs are able to hyperpolarize and thus relax smooth muscle cells, they are candidate compounds to constitute endothelium-derived hyperpolarizing factor (EDHF) (Bauersachs *et al*., 1994; Campbell *et al*., 1996; Fisslthaler *et al*., 1999; Gebremedhin *et al*., 1998; Miura & Gutterman, 1998; Mombouli & Vanhoutte, 1997; Oltman *et al*., 1998; Popp *et al*., 1996; Quilley & McGiff, 2000). EDHF has been postulated to play a major role in the regulation of coronary resistance vessels in response to agonists like ACh (Nishikawa *et al*., 1999) or BK (Mombouli *et al*., 1992; Quilley *et al*., 1997) or to pressure oscillations (Busse & Fleming, 1998). By the use of various inhibitors we consistently found that CYP450 enzymes mediate the residual BK-induced vasodilation remaining after NOS/COX inhibition in WT hearts and they accounted for the whole BK-induced flow response in eNOS−/− hearts. Furthermore, we were able to demonstrate in WT hearts that these inhibitors caused a slight, but significant, flow reduction even in the presence of NO. These data suggest that CYP450 enzyme metabolites do not only play an important role in the BK-induced flow response when NO is absent but also contribute to the BK-induced vasodilation in the presence of endogenous NO.

The chemical nature of EDHF is still not unambiguously defined. This may be due to the fact that the chemical identity of EDHF is regionally different in vascular beds or varies among species. Evidence for a CYP450 derived EDHF has been presented in isolated gracilis vessels of eNOS−/− mice (Huang *et al*., 2000). In contrast, Brandes *et al*. (2000) showed that the EDHF mediated vasodilation of the murine hindlimb was not affected by CYP450 inhibitors but was sensitive to gap junction uncoupling or CB1 receptor agonists. In addition, Fisslthaler *et al*. (1999) demonstrated that 11,12 EET is an EDHF in the porcine coronary artery and that CYP450/2C8 represents the corresponding EDHF synthase. We also analysed the role of CYP450/2Cs function by the use of sulfaphenazole, which is claimed to be a specific inhibitor of this class of enzyme (Mancy *et al*., 1996). However, sulfaphenazole did not inhibit the BK-induced vasodilation of WT and eNOS−/− hearts, suggesting either that CYP450/2Cs does not play a role in the mouse coronary circulation, or that the pharmacological properties of the mouse enzyme differs from that in porcine coronary arteries.

It has been proposed that NO negatively modulates the CYP450 activity by binding to its heme groups (Alonso-Galicia *et al*., 1997) and therefore CYP450 metabolites may serve as backup vasodilators in case of suppressed NO bioavailability (Bauersachs *et al*., 1996; Nishikawa *et al*., 2000). The present experiments suggest that such a compensation takes place in mouse hearts when eNOS is genetically disrupted. This possibility is supported by the finding that CYP450 inhibitors reduced the BK-induced vasodilation to a significantly larger extent in eNOS−/− hearts than in the presence of NO (i.e. WT hearts). These data therefore suggest that the CYP450 pathway is functionally up-regulated in eNOS−/− hearts and perhaps to compensate in part for the eNOS deficiency. The direct demonstration of a hyperpolarization in the isolated hearts was not possible because all interventions aimed at inhibition of K^+ channels also affect cardiac myocytes and thereby modulate coronary flow secondary to changes in contractile force development. However, several reports on the interaction of NO and EDHF suggest that as in the present work an EDHF like factor is involved in compensation of eNOS deficiency. Thus, renal arteries from cholesterol-fed spontaneously hypertensive rats show an increased importance of EDHF-like mediators when the NO response was attenuated

(Kagota *et al*., 1999). Furthermore, an up-regulation of CYP450 metabolism in various vascular beds from eNOS knockout mice, e.g. in gracilis muscle arterioles (Huang *et al*., 2000) and mesenteric arteries (Thorin *et al*., 1998; Waldron *et al*., 1999) has been observed. In contrast to the data presented in this study, Chataigneau *et al*. (1999) did not detect the existence of EDHF in conduit vessels from eNOS−/− hearts. The discrepancy may reside in the fact that EDHF predominates in small resistance arteries (Garland *et al*., 1995; Widmann *et al*., 1998) and lessens with increase of vessel size (Shimokawa *et al*., 1996). The conclusion that the impact of EDHF may change with vessel size is also supported by Brandes *et al*. (2000) who analysed the contribution of EDHF to flow regulation in the hindlimb of eNOS−/− mice. These authors found no contribution of EDHF to the ACh-induced vasodilation in isolated conduit vessels, but analysis of resistance vessels of the perfused hindlimb revealed a major contribution of EDHF.

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