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DISSERTATION

Role of Cytochrome P450 Metabolites and RGS2 in the Regulation of Vascular Tone in Mice

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Abstract

Epoxyeicosatrienoic acids (EETs) serve as endothelial-derived hyperpolarizing factors (EDHF), but may also affect vascular function by other mechanisms. We identified a novel interaction between EETs and endothelial NO release using soluble epoxide hydrolase (sEH) –/– and +/+ mice. Our data indicate that the EDHF response in mice is caused by hydrogen peroxide, but not by P450 eicosanoids. Moreover, P450 eicosanoids are vasodilatory, largely through their ability to activate endothelial NO synthase (eNOS) and NO release. 17,18-Epoxyeicosatetraenoic acid (17,18-EETeTr) stimulates vascular large-conductance K^+ (BK) channels. We performed whole-cell and perforated-patch clamp experiments in freshly isolated cerebral and mesenteric artery vascular smooth muscle cells (VSMC) from Sprague–Dawley rats, BK β1 gene-deficient (–/–), BK α (–/–), RyR3 (–/–) and wild-type mice. 17,18-EETeTr (100 nM) increased tetraethylammonium (1 mM)-sensitive outward K^+ currents in VSMC from wild-type rats and wild-type mice. The effects were not inhibited by the epoxyeicosatrienoic acid (EET) antagonist 14,15-epoxyeicosa-5(*Z*)-enoic acid (10 μM). BK channel currents were increased 3.5-fold in VSMC from BK β1 (–/–) mice, whereas a 2.9-fold stimulation was observed in VSMC from RyR3 (–/–) mice (at membrane voltage 60 mV). The 17,18-EETeTr did not induce outward currents in VSMC BK α (–/–) cells. Vasodilatation was largely inhibited in cerebral and mesenteric arteries isolated from BK α (–/–) mice compared with that observed in wild-type and BK β1 (–/–) arteries. 17,18-EETeTr represents an endogenous BK channel agonist and vasodilator. BK α represents the molecular target for the principal action of 17,18-EETeTr. The action of 17,18-EETeTr is not mediated by changes of the internal global calcium concentration or local SR calcium release events. Angiotensin II (Ang II) activates signalling pathways predominantly through the G-protein-coupled Ang II type 1 receptor (AT_1R). The regulator of G protein signalling 2 (RGS2) is a negative G protein regulator. We showed in this study that Ang II infusion increased BP more in RGS2 –/– than in RGS2 +/+ mice and that myogenic tone and vasoconstrictor responses to Ang II, ET-1 and PE were increased in isolated interlobular arterioles of RGS2 –/– mice. In both RGS2 –/– and *RGS2* +/+ mice treated with Ang II, urinary adrenaline and noradrenaline excretion were similar and profoundly decreased. These findings suggest that Ang II-induced hypertension in RGS2 –/– mice is mediated through vascular mechanisms rather than sympathetic activation.

1.1 Introduction

The endothelium releases nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF) (Urakami-Harasawa L et al. 1997). EETs may be EDHF (Campbell WB et al. 1996). Other candidates include K^+ ions and hydrogen peroxide (H_2O_2) (Matoba T et al. 2000; Edwards G et al. 1998). Endothelial cell hyperpolarization spread through myoendothelial gap junctions (Griffith TM et al. 2004; Dora KA et al. 2003). Calcium-activated potassium channels, most probably the SK4 (IK_{Ca}) and SK3 (SK_{Ca}) expressed on the endothelium, are the end-cellular gateway mediating hyperpolarization, and subsequent EDHF relaxation (Si H et al. 2006; Eichler I et al. 2003; Dora KA et al. 2008). EETs can induce vasodilation in certain vascular beds by increasing the open-state probability of calcium-activated potassium (BK) channels (Oltman CL et al. 1998; Roman RJ. 2002). sEH inhibition could enhance EET activity (Spector AA et al. 2004). sEH inhibition decreased blood pressure in SHR, angiotensin II-induced hypertension, and salt sensitivity (Yu Z et al. 2000; Imig JD et al. 2002). Male sEH gene-deleted (–/–) mice had lower blood pressures than sEH +/+ mice (Sinal CJ et al. 2000). We studied interactions between P450 eicosanoids, EDHF, and NO using sEH –/– and +/+ mice in the first project.

Eicosapentaenoic acid and other (n-3) PUFA possibly compete with AA for enzymatic conversion by CYP enzymes. This competition may lead to reduced formation of vasoactive AA metabolites (e.g. 20-epoxyeicosatrienoic acid (20-HETE), EETs) while alternative metabolites originating from EPA are increased. We showed earlier that rat cerebral artery vascular smooth muscle cells (VSMC) express the CYP isoforms 4A1 and 4A3 (Lauterbach *et al.* 2002). We found that both CYP 4A isoforms, which are known to metabolize AA (Nguyen et al. 1999), also accepted EPA as an efficient substrate. Cytochrome P450 4A1 showed the highest activity and produced 17,18-epoxyeicosatetraenoic acids (17,18-EETeTr), primarily 17(*R*),18(*S*)-EETeTr (Lauterbach *et al.* 2002). Similarly, Cyp4a12a, the mouse 20-HETE synthase, showed significant epoxygenase activity when converting EPA and produced 17(*R*),18(*S*)-EETeTr as a main metabolite (Muller *et al.* 2006). It has been demonstrated that 17(*R*),18(*S*)-EETeTr stimulates the K^+ outward current (Lauterbach *et al.* 2002). Only the 17(*R*),18(*S*)-enantiomer was effective; the 17(*S*),18(*R*)-enantiomer was not. 17,18-EETeTr relaxes and hyperpolarized both human pulmonary artery and bronchial smooth muscle cell. These effects were related to the activation of BKca and K_{ATP} channels in both tissues (Morin *et al*. 2009). These findings suggested that 17(*R*),18(*S*)-EETeTr may be a novel hyperpolarizing factor in the vessel wall, targeting the BK channel. However, the mechanism of channel stimulation remained unknown and vasodilatory effects have not been

demonstrated. We have characterized the effects of 17,18-EETeTr on VSMC BK channels and arterial tone. The BK channels are composed of the pore-forming BK_{α} and auxiliary BK β1 subunits that confer an increased sensitivity for changes in membrane potential and calcium to BK channels (Pluger *et al.* 2000; Lohn *et al.* 2001*b*). Ryanodine-sensitive calcium-release channels (RyR3) in the sarcoplasmic reticulum (SR) control the process (Lohn *et al.* 2001*a*). In VSMC, BK β1 deficiency produces an abnormal coupling between local Ca^{2+} signals, such as Ca^{2+} sparks, and BK channels (Pluger *et al.* 2000), whereas RyR3 deficiency produces an increased BK channel activity (Lohn *et al.* 2001*a*). The purpose of the present study was to determine the BK channel subunit requirements for the activity of 17,18-EETeTr on BK currents. Furthermore, we determined whether or not local Ca^{2+} release signals are involved in the principal actions of 17,18-EETeTr. We performed whole-cell and perforated-patch clamp experiments in freshly isolated VSMC from Sprague–Dawley rats, BK β1 gene-deficient (–/–) mice, RyR3 (–/–) mice and wild-type mice. We also performed vascular studies in isolated arteries from BK β1 (–/–) mice and from mice lacking the BK_{α} pore-forming subunit (Sausbier *et al.* 2005).

In our last project, we look at a possible relationship between the regulator G protein signaling 2 (RGS2) and Angiotensin II (Ang II) on the cardiovascular system.

RGS2 accelerates the rate of G protein deactivation by stimulating GTP hydrolysis. The RGS2 is a potent regulator of $G_{\alpha}q$ (Hepler, 1999; Zhong & Neubig, 2001). Ang II activates an array of signalling pathways predominantly through the G-protein-coupled angiotensin II type 1A receptor ($AT_{1A}R$), which is coupled to Gq. The RGS2 is a candidate for regulation of $AT_{1A}R$ signalling (Grant *et al.* 2000; Cho *et al.* 2003). AT_1 receptor blockade elicited a greater depressor response in *RGS2 (-/-)* mice compared with *RGS2 (+/+)* control animals (Heximer *et al.* 2003). Ang II upregulates RGS2 (Li *et al.* 2005), supporting the idea that RGS2 modulates complex signalling resulting from $AT_{1A}R$ activation. We infused Ang II chronically in conscious *RGS2*-deleted (*RGS2 -/-*) and wild-type (*RGS2 +/+*) mice and combined telemetric arterial blood pressure recordings with fast Fourier transformation (FFT) of mean arterial blood pressure (MAP) and heart rate (HR). We also studied the myogenic response and vascular reactivity of interlobar arterioles in *RGS2 (-/-)* and *RGS2 (+/+)* mice.

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Objectives

1) To identify a novel interaction between EETs and endothelial NO release using soluble epoxide hydrolase (sEH) –/– and +/+ mice (Project 1),
2) To demonstrate that 17,18-epoxyeicosatetraenoic acid (17,18-EETeTr) is a novel vasodilator that targets the pore-forming BK-α channel subunit in rodents (Project 2), and,
3) To prove whether RGS2 deletion increases the vascular response to Ang II and blood pressure (Project 3).

Project 1

Interaction Between P450 Eicosanoids and Nitric Oxide in the Control of Arterial Tone in Mice

1.1.1 Summary

EETs serve as endothelial-derived hyperpolarizing factors (EDHF), but may also affect vascular function by other mechanisms. We identified a novel interaction between EETs and endothelial NO release using soluble epoxide hydrolase (sEH) –/– and +/+ mice. Our data indicate that the EDHF response in mice is caused by hydrogen peroxide, but not by P450 eicosanoids. Moreover, P450 eicosanoids are vasodilatory, largely through their ability to activate endothelial NO synthase (eNOS) and NO release.

1.1.2 Methods

Animals and Blood Pressure Measurements by telemetry

sEH activity

Vessel experiments by videomicroscopy

Determination of EET and DHET-levels

Enzyme determination

Synthesis of N-adamantyl-N'-dodecylurea

Vessel experiments on isolated mesenteric bed

Isolation of endothelial cells from mouse aorta

Measurement of nitric oxide from laser confocal fluorescent microscopy

Measurements of hydrogen peroxide from endothelial cells

Determination of endogenous EETs and DHETs by LC-MS

1.1.3 Results

1.1.4 sEH Activity in Mesenteric Artery

We found that all four EETs (5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET) were present in the vessel wall of mesenteric arteries. EETs were also detected in red blood cells and blood plasma. The functional expression of sEH in the mesenteric artery was tested with the sEH inhibitor, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (ADU); which efficiently inhibited the sEH activity present in the cytosolic fraction of mouse renal homogenates. Using 10 μmol/L 14,15-EET as the substrate, ADU produced significant inhibition. sEH protein expression was detected by Western blotting in arteries from sEH +/+ mice, but not from –/– mice. Vessels isolated from male sEH +/+ mice, but not from male sEH –/– mice, hydrolyzed ^{14}C-labeled 14,15-EET within 30 minutes. DHET production by the wild-type vessels was significantly reduced by preincubation with ADU at concentrations of 1 and 10 μmol/L. Thus, sEH is present and metabolically active in mesenteric and possibly other arteries of mice, and can be effectively blocked by ADU.

1.1.5 EDHF Response Is Most Probably Caused by H_2O_2, but not by P450 Eicosanoids

We examined whether or not EDHF-dependent vasodilation is affected by inhibiting sEH. Under basal conditions, vasorelaxation by acetylcholine (ACh) was equipotent between sEH +/+ and –/– arteries. Vasoconstriction by 60 mmol/L KCl and vascular reactivity to U46619 expressed as % of KCl showed no differences. ACh-dependent relaxation was significantly reduced in vessels treated with L-NAME alone or L-NAME plus indomethacin. However, the relaxation was not different between sEH +/+ and –/– mice. Note that this relaxation was completely abolished by apamin/ChTx, suggesting that activation of both endothelial small and intermediate-conductance Ca^{2+}-activated K^+ channels (SK3, SK4) is crucial in EDHF-dependent signaling and relaxation in mouse mesenteric arteries (Matoba T et al. 2000; Harrington LS. 2007) In contrast, iberiotoxin was not effective in inhibiting EDHF-dependent relaxation, indicating that large-conductance Ca^{2+}-activated K^+ (BK) channels in arterial smooth muscle cells play no role in the EDHF response in this vascular preparation. EDHF-dependent dilation was completely inhibited by catalase 1000 U/mL or carbenoxolone 100

μmol/L but not by 6-(2-propargyloxyphenyl)hexanoic acid (PPOH, 10 μmol/L, 30 minutes), a selective CYP inhibitor, or by 14,15-epoxyeicosa-5(*Z*)-enoic acid (EEZE), an EET antagonist. H_2O_2 production was measured in endothelial cells of intact mesenteric arterioles using a laser confocal microscope with CM-H_2DCFDA, a peroxide-sensitive fluorescence dye. A significant increase in the dichlorofluorescein fluorescence was observed in endothelial cells stimulated by acetylcholine (ACh 1 μmol/L) as compared with controls or tissues treated with catalase (1000 U/mL). Removal of endothelial cells (-E) abolished the dichlorofluorescein signal in response to acetylcholine. Taken together, these results suggest that H_2O_2, but not EETs, significantly contributes to EDHF-mediated dilation in mouse mesenteric arteries, which is in line with previous findings (Matoba T et al. 2007; Luksha L et al. 2006)

1.1.6 Vasodilatory Effects of EETs and DHETs

Vasodilator responses were tested in the U46619-preconstricted pressurized (60 mm Hg) arteries. All 4 EETs (order of potency: 8,9-EET >14,15-EET≈5,6-EET >11,12-EET) and all 4 DHETs (14,15-DHET≈8,9-DHET≈11,12-DHET >5,6-DHET) produced dose-dependent vasodilation. The 8,9-EET-dependent vasodilations were slightly inhibited by the SK3/SK4 channel blockers apamin/ChTx. DHETs are up to ≈10-fold more potent vasodilators in mouse mesenteric arteries than EETs. EETs/DHETs exhibit their vasodilatory properties without any, or with very little, involvement of SK3/SK4 channels.

1.1.7 EET/DHET-Dependent Dilation Is Mediated by eNOS Activation

To study the mechanisms of P450 eicosanoid-induced vasodilation, we used the most potent metabolites, namely 8,9-EET and 14,15-DHET. 8,9-EET– and 14,15-DHET–induced vasodilations were dependent on intact endothelium. In the presence of SK3/SK4 channel blockers, a prominent residual dilation to 8,9-EETs and 14,15-DHETs occurred indicating the presence of a major additional endothelial vasodilator mechanism. To determine whether or not EETs/DHETs activate eNOS to produce vasodilation, N(omega)-nitro-L-arginine methyl ester (L-NAME) was coadministered with apamin/ChTx. The dilation in response to both 8,9-EET and 14,15-DHET was completely inhibited by L-NAME/Apamin/ChTx. Both 8,9-EET and 14,15-DHET are able to induce NO production in primary mouse aortic endothelial cells. Our results suggest that EETs/DHETs can modulate the bioavailability and/or action of NO to produce vasodilation.

1.1.8 L-NAME–Induced Hypertension Is Resistant to sEH Inhibition

Mice given L-NAME exhibited a prompt increase in mean arterial blood pressure (MAP). The combination of L-NAME with ADU increased blood pressure slightly. In addition, L-NAME induced elevated blood pressure in sEH –/– mice that was not different compared to +/+ mice. In contrast, ADU reduced blood pressure in Ang II–induced hypertension. Mice were given Ang II for 7 days followed by 7 days with additional ADU treatment. After ADU, MAP in these mice was reduced. These values did not reach initial blood pressures; however, the blood pressure reduction was highly significant.

1.1.9 Discussion

In this project, we investigate the vascular interaction between P450 eicosanoids, SEH, and NO in mice. We found that (a) both EETs and DHETs are vasodilatory in mesenteric arteries largely through their ability to activate eNOS and NO release (b) ACh-induced EDHF response is predominantly caused by H_2O_2, but not by P450 eicosanoids. (c) L-NAME hypertension is not affected by sEH inhibition, contrary to angiotensin-II induced hypertension.

1.1.10 EDHF in Mouse Mesenteric Arteries

We found that all four EETs and all four DHETs produced dose-dependent vasodilation of mouse mesenteric arteries. The effects were endothelium-dependent, but not or only slightly inhibited by apamin/ChTx. In contrast, apamin/ChTx completely blocked the EDHF response. These results suggest that EETs/DHETs (alone or in combination) do not function as an EDHF in mice, which should be solely dependent on SK4/SK3 channels. Moreover, we observed that EDHF responses in mesenteric arteries were not affected by any measures that influence EET generation or action, including CYP inhibition by PPOH, sEH inhibition by ADU or by gene deletion, or EET antagonism by EEZE. Instead, our data show that the EDHF response is completely inhibited by catalase and accompanied by H_2O_2 production, which is in line with previous findings (Matoba T et al. 2007; Luksha L et al. 2006) Taken together, our results present evidence that P450 eicosanoids do not significantly contribute to EDHF-mediated dilation in mesenteric arteries of mice. Our data strongly support the notion that H_2O_2 is an EDHF in this vessel, (Matoba T et al. 2007; Luksha L et al. 2006; Pannirselvam M et al. 2006) which causes hyperpolarization, most probably via activation of

endothelial SK3/SK4 channels in endothelial cells, which spreads to adjacent vascular smooth muscle cells (VSMC) through myo-endothelial gap junctions and produces subsequent EDHF relaxation (Busse R et al. 2002; Fisslthaler B et al. 1999; Taylor MS et al. 2003). Specifically, the gap junction components Cx40, Cx43, and Cx37 have been recently implicated in the EDHF signal spread from endothelial to smooth muscle cells in mouse mesenteric arteries (Saliez J et al. 2008).

1.1.11 Prominent Role of NO in Vasodilation by EETs/DHET

Red blood cells have been suggested to serve as a potential reservoir for epoxides which on release may act in a vasoregulatory capacity (Jiang H et al. 2007; Jiang H et al. 2005). Our results are the first to demonstrate that EETs/DHETs can modulate the bioavailability of NO via eNOS to produce vasodilation. We observed that the vasodilatory effects of the most potent DHET (i.e. 14,15-DHET) and EET (8,9-EET) in mouse mesenteric arteries are endothelium-dependent and inhibited by L-NAME. Moreover, both eicosanoids induced NO production in primary mouse aortic endothelial cells. Interestingly, 5,6-EET, but not 11,12- and 14,15-EET, produced relaxations in rabbit superior mesenteric arterial ring preparations, which were completely inhibited by removal of the endothelium and partially inhibited by L-NAME (Hutcheson IR. 1998). Nitric oxide and prostaglandins have been suggested to mediate vasodilation by 5,6-EET in rabbit lung (Tan JZ et al. 1997). Thus far, two major pathways of interaction between endothelium-derived relaxing factors (EDRFs) have been described. First, an inhibitory interaction has been described between NO and EET, in which NO inhibits CYP-mediated production of EET from AA (Bauersachs J et al. 1996). Second, an inhibitory interaction between EETs and H_2O_2 has been identified, in which CYP epoxygenases are directly inhibited by H_2O_2 (Larsen BT et al. 2008). The present study reveals a third way of interaction among substances proposed as EDRFs, namely an interaction between EETs/DHETs and NO, in which EETs/DHETs can induce endothelial NO release to modulate vascular tone.

1.1.12 Role of sEH in Blood Pressure Regulation

sEH inhibition has been reported to lower blood pressure in several forms of hypertension. In this study we show that L-NAME-induced hypertension is insensitive to pharmacological or genetic sEH inhibition. These data suggest that the role of sEH in blood pressure regulation depends on the type of secondary hypertension. We propose that sEH inhibition is ineffective

in lowering blood pressure in L-NAME hypertension for the following reasons: (1) L-NAME hypertension is primarily attributable to increased vascular tone and diminished NO release in vessels (2) EETs and DHETs are both potent vasodilators which primarily rely on intact eNOS activity (3) EDHF responses in L-NAME–treated mice are not modified by sEH inhibition, but remain sensitive to catalase. The mechanism of how sEH inhibition ameliorates Ang II–induced hypertension in mice is largely unclear, but may involve renal and cardiac mechanisms rather than changes in peripheral arterial resistance (Jung O et al. 2005).

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A full presentation of the results of Project 1 has been published in Hercule et al., ***Arterioscler Thromb Vasc Biol*. 2009;29:54-60.** A copy of this paper follows as **Supplement** .

Project 2

The vasodilator 17,18-epoxyeicosatetraenoic acid targets the pore-forming BK-α channel subunit in rodents.

2.1.1 Summary

17,18-epoxyeicosatetraenoic acid (17,18-EETeTr) stimulates vascular large-conductance K^+ (BK) channels. We performed whole-cell and perforated-patch clamp experiments in freshly isolated cerebral and mesenteric artery vascular smooth muscle cells (VSMC) from Sprague–Dawley rats, BK β1 gene-deficient (–/–), BK α (–/–), RyR3 (–/–) and wild-type mice. 17,18-EETeTr (100 nM) increased tetraethylammonium (1 mM)-sensitive outward K^+ currents in VSMC from wild-type rats and wild-type mice. The effects were not inhibited by the epoxyeicosatrienoic acid (EET) antagonist 14,15-epoxyeicosa-5(*Z*)-enoic acid (10 μM). BK channel currents were increased 3.5-fold in VSMC from BK β1 (–/–) mice, whereas a 2.9-fold stimulation was observed in VSMC from RyR3 (–/–) mice (at membrane voltage 60 mV). The 17,18-EETeTr did not induce outward currents in VSMC BK α (–/–) cells. Vasodilatation was largely inhibited in cerebral and mesenteric arteries isolated from BK α (–/–) mice compared with that observed in wild-type and BK β1 (–/–) arteries. 17,18-EETeTr represents an endogenous BK channel agonist and vasodilator. BK α represents the molecular target for the

principal action of 17,18-EETeTr. The action of 17,18-EETeTr is not mediated by changes of the internal global calcium concentration or local SR calcium release events.

2.1.2 Methods

Whole-cell and perforated-patch clamp experiments

Videomicroscopy of small isolated arteries

2.1.3 Results

2.1.4 Effects of 17,18-EETeTr on Rat Cerebral Artery BK Currents and Role of $[Ca^{2+}]$

We investigated the effects of 17,18-EETeTr using the whole-cell configuration of the patch clamp technique in order to explore a possible role of $[Ca^{2+}]_i$. The 17,18-EETeTr (100 nmol l^{-1}) induced large, relatively noisy, non-inactivating currents. As reported for experiments in the perforated-patch configuration (Lauterbach *et al.* 2002), the outward K^+ current is mainly carried by BK channels. Ca^{2+} influx through L-type channels had no effect on the 17,18-EETeTr-induced BK channel current in perforated-patch recordings. We tested the effects of 17,18-EETeTr on BK currents in the presence of different values of extracellular $[Ca^{2+}]$. These results suggest that neither L-type channels nor other Ca^{2+} influx pathways are involved in BK channel stimulation by 17,18-EETeTr. The effects of 17,18-EETeTr were not reduced by pretreatment of cells with the epoxyeicosatrienoic acid antagonist 14,15-epoxyeicosa-5(*Z*)-enoic acid (14,15-EEZE, 10 μM). The 14,15-EEZE had no effect on control BK currents. These findings rule out a major role of receptors for epoxyeicosatrienoic acid in the action of 17,18-EETeTr on BK channels.

2.1.5 Effects of 17,18-EETeTr on Mouse BK β1 (–/–) and RyR3 (–/–) VSMC

Wild-type, BK β1 (–/–) and RyR3 (–/–) cerebral artery VSMC exhibited large BK channel currents with outward rectification and relatively large noise at membrane potentials positive to +60. The current amplitudes were not different among wild-type, BK β1 (–/–) and RyR3 (–/–) cells. Addition of 17,18-EETeTr (100 nmol l^{-1}) increased the K^+ outward current amplitude in wild-type, β1 (–/–) VSMC. The stimulatory effect was not statistically different compared with wild-type cells, suggesting that BK α but not the auxiliary BK β1 subunit is the target of principal action of 17,18-EETeTr.

17,18-EETeTr (100 nmol l^{-1}) did not increase the K^+ current amplitude in cerebral artery VSMCs of BK α(–/–) mice, compared with VSMCs of littermate wild-type mice. In RyR3 (–/–) cerebral artery cells, 17,18-EETeTr (100 nmol l^{-1}) increased the K^+ current amplitude. The reversal potential was not affected by 17,18-EETeTr. 17,18-EETeTr-mediated stimulation of BK channels is independent of local SR Ca^{2+} release controlled by RyR3 in cerebral artery smooth muscle cells.

2.1.6 Effects of 17,18-EETeTr in Pressurized Arteries of BK α(–/–), BK β1 (–/–), and Wild-type Mice

We tested the vasodilatory effects of 17,18-EETeTr in second- and third-order mesenteric arteries of BK α (–/–), BK β1 (–/–) and wild-type mice pressurized to 60 mmHg. The arteries were preconstricted with U46619. 17,18-EETeTr produced dose-dependent vasodilatations. At 0.01, 0.1 and 1 μM, 17,18-EETeTr induced vasodilatation in BKβ1 (–/–) and wild-type arteries. However, vasodilatation was almost completely absent in BK α (–/–) arteries. Similar results were obtained in cerebral arteries. These results indicate that the BK α subunit is crucial in 17,18-EETeTr-induced vasodilatation.

2.1.7 Discussion

17,18-EETeTr is a potent vasodilator and stimulates the outward BK channel current in VSMCs of rats and mice. Only the 17(*R*),18(*S*)-enantiomer was effective, while the 17(*S*),18(*R*)-enantiomer was not (Lauterbach *et al.* 2002). 17,18 epoxyeicosatetraenoic acid shares these properties with 11,12-epoxyeicosatrienoic acid, the compound that has been proposed as endothelium-derived hyperpolarizing factor (EDHF) in a number of vascular beds (Campbell & Harder, 1999; Fisslthaler *et al.* 1999; Quilley & McGiff, 2000; Roman *et al.* 2000) and other regioisomeric epoxides derived from n-3 PUFAs in coronary arterioles (Zhang *et al.* 2001; Ye *et al.* 2002). Our findings suggest that 17,18-EETeTr may be a novel hyperpolarizing factor in the vessel walls of rats, mice and probably other mammals. The effects of 17,18-EETeTr were not affected by 14,15-EEZE, suggesting receptor signalling mechanisms distinct from AA epoxides (Gauthier *et al.* 2002) and a number of other fatty acids that have been supposed to directly bind and interact with the BK channel protein (Denson *et al.* 2000). The BK channel stimulation is independent of intracellular calcium and may be explained by direct activation of the pore-forming BK α-channel subunit.

We studied the putative role of intracellular SR calcium signals that control BK channel activity via local, subcellular Ca^{2+} release events such as calcium sparks and other unitary events (Gollasch *et al.* 1998; Lohn *et al.* 2001*a*). The RyR3 is part of the SR calcium release unit and is required to tune the release of SR calcium signals specifically to the needs of arterial smooth muscle cells, thereby enabling BK channel regulation of arterial tone (Lohn *et al.* 2001*a*). 17,18-EETeTr was found to stimulate BK channel currents in RyR3 (–/–) cells. The current stimulation was not significantly different from that observed in wild-type cells. Our data suggest that local Ca^{2+} release signals are not involved in the principal action of 17,18-EETeTr.

We determined the BK channel subunit requirements for the activity of 17,18-EETeTr on BK currents. These experiments were conducted in BK β1 (–/–) cells, which lack the auxiliary β subunit of vascular BK channels (Pluger *et al.* 2000). As reported previously, the BK α subunit in BK β1 (–/–) arterial VSMC is functional, and the cells exhibit voltage-dependent outward K^+ currents. In symmetrical 140 mM K^+ solution, lack of BK β1 decreases the apparent Ca^{2+}/voltage sensitivity of BK channels (Brenner *et al.* 2000). These currents were also sensitive to 17,18-EETeTr. In contrast, 17,18-EETeTr did not induce outward K^+ currents in BK α (–/–) VSMC. Therefore, the BK α subunit probably represents the molecular target for the principal action of 17,18-EETeTr. However, since the epoxyeicosatrienoic acid antagonist 14,15-EEZE had no effect on 17,18-EETeTr-dependent channel stimulation, we believe that 17,18-EETeTr does not use the same putative binding site or signalling molecules mediating epoxyeicosatrienoic acid-induced activation of the BK channels, such as G_s proteins and cyclic adenosine diphosphate-ribose (cADPR) (Li *et al.* 2002). Although the present study clearly indicates that BK α represents the molecular target for the principal action of 17,18-EETeTr, it remains unknown whether or not 17,18-EETeTr binds directly to BK α or activates it via a signalling pathway after binding to a specific receptor. Further studies are needed to clarify this point.

We also evaluated the vasodilatory effects of 17,18-EETeTr with respect to presence or absence of BK α or BK β1 subunits. In both wild-type and BK β1 (–/–) arteries, 17,18-EETeTr induced similar dose-dependent dilatations. However, in BK α (–/–) vessels, the vasodilator effects of 17,18-EETeTr were markedly reduced, suggesting that the α subunit is required for activation. The fact that we observed some residual vasodilatation in the BK α (–/–) vessels may indicate heterogeneity of mechanisms associated with EETs; although here we

report that the main molecular target for 17,18-ETeTr is the BK α subunit. The residual dilatation may be through ATP-sensitive K^+ (K_{ATP}) channels (Ye *et al.* 2005), which play important roles in the regulation of vascular tone (Nelson & Brayden, 1993; Standen & Quayle, 1998; Miki *et al.* 2002).

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A full presentation of the results of Project 2 has been published in Hercule et al., ***exp Physiol.* 2007;92.6:1067-1076** A copy of this paper follows as **Supplement**.

Project 3

RGS2 deletion increases the vascular response to Angiotensin II and blood pressure.

3.1.1 Summary

Angiotensin II (Ang II) activates signalling pathways predominantly through the G-protein-coupled Ang II type 1 receptor (AT_1R). The regulator of G protein signalling 2 (RGS2) is a negative G protein regulator. We showed in this study that Ang II infusion increased BP more in RGS2 –/– than in RGS2 +/+ mice and that myogenic tone and vasoconstrictor responses to Ang II, ET-1 and PE were increased in isolated interlobular arterioles of RGS2 –/– mice. In both RGS2 –/– and *RGS2* +/+ mice treated with Ang II, urinary adrenaline and noradrenaline excretion were similar and profoundly decreased. These findings suggest that Ang II-induced

hypertension in RGS2 –/– mice is mediated through vascular mechanisms rather than sympathetic activation.

3.1.2 Methods

Telemetry

Urine catecholamine levels

Spectral analysis and baroreflex sensitivity

Myogenic tone and vascular reactivity

Gene expression analyses (AT_{1A}, AT_{1B} and AT_2 receptors)

3.1.3 Results

3.1.4 Blood Pressure and Heart Rate in RGS2 mice

We measured mean arterial pressure (MAP) in RGS2 –/– and RGS2 +/+ mice, before and during Ang II infusion. Under baseline conditions without Ang II, MAP values in RGS2 –/– and RGS2 +/+ mice stabilized at similar values during the day and at significantly higher value during the night. Angiotensin II increased MAP in RGS2 –/– mice both during the day and night more than in RGS2 +/+ mice. Heart rate was not different between the groups, was not affected by Ang II. Ang II increased BP more in RGS2 –/– than in RGS2 +/+ mice without changing HR. Blood pressure and HR amplitudes were not different in RGS2 –/– and RGS2 +/+ mice and were not affected by Ang II infusion.

3.1.5 Myogenic Tone in RGS2 mice

We directly monitored the effect of intravascular pressure on the diameter of interlobar arterioles from RGS2 –/– and RGS2 +/+ mice. The pressure at which myogenic dilatation reversed into myogenic constriction owing to the Bayliss effect was significantly shifted to the left, towards lower values, in RGS2 –/– vessels compared with RGS2 +/+ arterioles. Interlobar arterioles from RGS2 –/– mice developed an increased myogenic tone in response to a transmural pressure of 90 mmHg.

3.1.6 Agonist-induced vascular reactivity in RGS2 mice

Vasoconstrictory responses were measured in isolated interlobar arterioles of $RGS2^{+/+}$ and RGS2 –/– mice to Ang II, ET-1 and PE. The arterioles showed increased responsiveness to

Ang II. The dose–response curve for Ang II was markedly shifted towards the left in RGS2 –/– arterioles compared with RGS2 +/+ arterioles. Similar effects were observed for ET-1 and PE.

3.1.7 Gene expression analyses (AT_{1A}, AT_{1B} and AT_2 receptors)

The $AT_{1A}R$ gene expression of interlobar arteries in RGS2 –/– mice and in RGS2 +/+ mice were insignificant as well as the $AT_{1B}R$ gene expression. Similarly, the AT_2R gene expression was not different between the strains.

3.1.8 Urinary adrenaline and noradrenaline levels

Urine volume was not different between RGS2 –/– and RGS2 +/+ mice. In both RGS2 –/– and RGS2 +/+ mice treated with Ang II, urinary adrenaline excretion and noradrenaline excretion were similar.

3.1.9 Heart rate variability and baroreflex function

The absolute LF and HF power values were not different between Ang II-treated RGS2 –/– and Ang II-treated RGS2 +/+ mice, which resulted in similar LF/HF ratios of about 3. The RMSSD, which describes HR variability in the time domain, stabilized at 4 ms in both groups. Baroreflex sensitivity calculated by cross-spectral analysis in the LF band (BRS-LF) was not different and stabilized in RGS2 –/– mice at 3 ± 0.4 ms $mmHg^{-1}$ and in RGS2 +/+ mice at 4 ± 0.4 ms $mmHg^{-1}$.

3.1.10 Discussion

As found in earlier studies (Gross *et al.* 2005; Obst *et al.* 2006), the differences of MAP between RGS2 –/– and RGS2 +/+ mice were small. Moreover, in this study the difference in MAP between the strains did not reach significance during the day.

The physiological actions of Ang II occur through its binding to AT_1 and AT_2 receptors. The AT_1 receptor, the dominant receptor for the vascular effects of Ang II (Ardaillou, 1999), exists in two isoforms in rodents, AT_{1A} and AT_{1B} (Iwai & Inagami, 1992; Guimaraes & Pinheiro, 2005), whereby the AT_{1A} receptor is the predominant subtype in most tissues involved with blood pressure regulation (Burson *et al.* 1994; Du *et al.* 1995) and mediates Ang II-induced changes in vascular reactivity (Ryan *et al.* 2004). It has been reported that Ang II infusion is

accompanied by an increase of AT_2 receptor expression (Bonnet *et al.* 2001; Ryan *et al.* 2004), which may reflect a compensatory mechanism, given that AT_2R activation has vasodilatory effects. Gene expression of the AT_{1A}, AT_{1B} and AT_2 receptors in interlobar arterioles was similar in RGS2 –/– and RGS2 +/+ mice. The interaction of RGS2 predominantly with the AT_{1A} receptor may explain the more pronounced BP increase in Ang II-treated RGS2 –/– mice compared with wild-type animals. Stimulation of the AT_1 receptor causes vasoconstriction and release of aldosterone, thereby modulating renal sodium reabsorption (Aguilera, 1992; Ito *et al.* 1995; Masilamani *et al.* 1999). Activation of the AT_1 receptor in the brain raises BP through sympathetic activation (Averill *et al.* 1994). In the kidney, activation of AT_1 receptors induces vasoconstriction and antinatriuresis (Ichikawa & Brenner, 1980; Navar *et al.* 1987). Our results show an increased myogenic tone of interlobular arterioles and an increased sensitivity of interlobular arterioles to Ang II in RGS2 –/– mice. Therefore, we suggest that increased AT_1 receptor signalling in the vasculature directly contributed to the elevated BP in RGS2 –/– mice compared with RGS2 +/+ mice. These direct vasomotor effects may have aggravated Ang II-induced hypertension in RGS2 –/– mice by increasing peripheral vascular resistance.

The increased BP produced by Ang II infusion was not associated with a bradycardia, which we observed in L-NAME-induced hypertension (Gross *et al.* 2002; Obst *et al.* 2004, 2006). In addition to its peripheral vasoconstrictor effects, Ang II induced a resetting of the HR baroreflex to higher pressures. During Ang II infusion, BP increased to a greater degree in RGS2 –/– animals, while HR was unaffected. Thus, baroreflex resetting was more pronounced in RGS2 –/– compared with wild-type animals. To describe the baroreflex further, we used cross-spectral analysis in the low-frequency band (BRS-LF), which most probably reflects baroreflex sensitivity under resting conditions in humans (deBoer *et al.* 1987) but has not been validated for mice. Nevertheless, baroreflex sensitivity was not affected by Ang II and not different between the groups. Together, these observations suggest that RGS2 attenuates Ang II-mediated resetting of baroreflex heart rate regulation. The mechanism by which Ang II chronically resets the arterial baroreflex depends on structures within the central nervous system such as the area postrema. Hence, Ang II modulates vagal and sympathetic activity (Bishop & Sanderford, 2000; Xue *et al.* 2003) via central pathways. In Ang II-induced hypertension, the decrease in sympathetic activity seems to dominate direct sympathoadrenergic facilitating effects of Ang II. The profoundly decreased urinary noradrenaline excretion rates in Ang II-treated RGS2 –/– and RGS2 +/+ mice compared with untreated RGS2 –/– and RGS2 +/+ mice (Gross *et al.* 2005) strongly suggest a substantially

reduced sympathetic activity (Henriksen *et al.* 1985; Cox & Bishop, 1991; Lohmeier *et al.* 2005).

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A full presentation of the results of Project 3 has been published in Hercule et al., ***Exp Physiol*. 2007;92.6:1014-1022.** A copy of this paper follows as **Supplement**.

Anteilserklärung

Die **Anteilserklärung** muss den Anteil des Promovenden/der Promovendin an den Publikationen ausweisen und von ihm/ihr und dem betreuenden Hochschullehrer/der betreuenden Hochschullehrerin unterschrieben sein:

Hantz Hercule hatte folgenden Anteil an den vorgelegten Publikationen:

Publikation 1: [Hantz C. Hercule; Wolf-Hagen Schunck; Volkmar Gross; Jasmin Seringer; Fung Ping Leung; Steven M. Weldon; Andrey Ch. da Costa Goncalves; Yu Huang; Friedrich C. Luft; Maik Gollasch], [Interaction Between P450 Eicosanoids and Nitric Oxide in the Control of Arterial Tone in Mice], [Arteriosclerosis Thrombosis, and Vascular Biology], [2009]
80 Prozent

Beitrag im Einzelnen: performing vasoreactivity experiments, Western blots, performing EET measurements, sEH activity and animal treatment, data analyses, writing the manuscript, preparing of graphs, and preparation of figures and figure legends, designing of experiments

Publikation 2: [Hantz C. Hercule, Birgit Salanova, Kirill Essin, Horst Honeck, John R. Falck, Matthias Sausbier, Peter Ruth, Wolf-Hagen Schunck, Friedrich C. Luft and Maik Gollasch], [The Vasodilator 17,18-epoxyeicosatetraenoic acid targets the pore-forming BKα channel subunit in rodents], [Experimental Physiology], [2007]
70 Prozent
Beitrag im Einzelnen: performing vascular reactivity experiments, writing the abstract and partly text of the MS of introduction, help in patch clamp experiments, handling of animals

Publikation 3: [Hantz C. Hercule, Jens Tank, Ralph Plehm, Maren Wellner, Andrey C. da Costa Goncalves, Maik Gollasch, André Diedrich, Jens Jordan, Friedrich C. Luft and Volkmar Gross], [Regulator of G protein signalling 2 ameliorates angiotensin II-induced hypertension in mice], [Experimental Physiology], [2007]
55 Prozent
Beitrag im Einzelnen: performing myogenic studies, data analysis, preparing the MS text

Hantz C. Hercule

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Datum Unterschrift

16.07.2009

Erklärung

„Ich, [Hantz C. Hercule], erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: Role of cytochrome P450 metabolites and RGS2 in the regulation of vascular tone in mice selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum Unterschrift

16.07.2009

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Dedication

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