# Angiotensin II-induced vasodilation

## Role of bradykinin, NO and endothelium-derived hyperpolarizing factors

W.W. Batenburg

**Angiotensin II-induced vasodilation.**
**The role of bradykinin, NO and endothelium-derived hyperpolarizing factors**
**Thesis, Erasmus University, Rotterdam. With summary in Dutch**
**ISBN 90-8559-058-2**



An electronic version of this thesis is available in Adobe(R) PDF format on the following internet address: www.eur.nl/fgg/pharm/

Printed by [Optima] Grafische Communicatie, Rotterdam

# Angiotensin II-Induced Vasodilation

Role of bradykinin, NO and
endothelium-derived hyperpolarizing factors

Angiotensine II-geïnduceerde vasodilatatie.
De rol van bradykinine, NO en endotheliale hyperpolariserende factoren

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaats vinden op
donderdag 30 juni 2005 om 11.00 uur

door

**Wendy Wilhelmina Batenburg**
geboren te Sliedrecht

**Promotiecommissie**

| | |
|---|---|
| Promotor | : Prof.dr. A.H.J. Danser |
| Overige leden | : Prof.dr. P.R. Saxena |
| | Prof.dr. J.M.J. Lamers |
| | Prof.dr. P.W. de Leeuw |

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Financial support by the following companies and foundations is gratefully acknowledged:
J.E. Jurriaanse Stichting
Jacques H. de Jong Stichting
Glaxo Smith Kline B.V.
Pfizer B.V.

*Voor Marco*

## Table of contents

**Chapter 1** 9
General introduction

**Chapter 2** 23
Angiotensin II type 2 receptor-mediated vasodilation in human coronary microarteries

**Chapter 3** 37
Mediators of bradykinin-induced vasorelaxation in human coronary microarteries

**Chapter 4** 49
Bradykinin-induced relaxation of coronary microarteries: *S*-nitrosothiols as EDHF?

**Chapter 5** 69
L-*S*-nitrosothiols: endothelium-derived hyperpolarizing factors in porcine coronary arteries?

**Chapter 6** 85
Carvedilol-induced antagonism of angiotensin II: a matter of $\alpha_1$-adrenoceptor blockade

**Chapter 7** 99
Summary and general discussion

Nederlandse samenvatting 107

Publications 110

Dankwoord 113

Curriculum vitae 115

Abbreviations 116

References 117

# Chapter 1

## General introduction

**Based on:**
Angiotensin II type 2 receptor-mediated vasodilation. Focus on bradykinin, NO and endothelium-derived hyperpolarizing factor(s).
*Vascular Pharmacology* 2005, 42:109-118.

## Summary

Angiotensin (Ang) II type 1 ($AT_1$) receptors account for the majority of the cardiovascular effects of Ang II, including vasoconstriction and growth stimulation. Recent evidence, mainly obtained in animals, suggests that Ang II type 2 ($AT_2$) receptors counteract some or all of these effects. This review summarizes the current knowledge on the vasodilator effects induced by $AT_2$ receptors in humans and animals, focussing not only on the mediators of this effect, but also on the modulatory role of age, gender, and endothelial function. It is concluded that $AT_2$ receptor-mediated vasodilation most likely depends on the bradykinin – bradykinin type 2 ($B_2$) receptor – NO – cGMP pathway, although evidence for a direct link between $AT_2$ and $B_2$ receptors is currently lacking. If indeed $B_2$ receptors are involved, this would imply that, in addition to NO, also the wide range of non-NO 'endothelium-derived hyperpolarizing factors' (EDHFs) that are released following $B_2$ receptor activation (e.g., $K^+$, cytochrome P450 products from arachidonic acid, $H_2O_2$ and *S*-nitrososothiols), could contribute to $AT_2$ receptor-induced vasodilation.

## Introduction

The octapeptide angiotensin (Ang) II mediates its biological actions by activating multiple intracellular pathways following its binding to membrane-bound receptors. Two Ang receptor subtypes are present in humans: Ang II type 1 ($AT_1$) and type 2 ($AT_2$) receptors.[1-3] Most of the known cardiovascular effects induced by Ang II (e.g., vasoconstriction, water and salt retention, aldosterone synthesis and release, growth and remodelling) are mediated via $AT_1$ receptors. Recent studies emphasize the importance of the $AT_2$ receptor in the cardiovascular system.[4-10] $AT_2$ receptor-mediated effects will become apparent especially during $AT_1$ receptor blockade, because the increased Ang II levels that accompany such blockade will predominantly result in $AT_2$ receptor stimulation. It has even been suggested that this mechanism underlies the beneficial effects of $AT_1$ receptor blockade.[11,12]

### *$AT_2$ receptors and vasodilation: results from animal studies*

In vitro experiments in cultured cells and isolated vessels, as well as in vivo studies in rats and mice, including studies in transgenic animals, have shown that $AT_2$ receptor stimulation counteracts some or all of the above-mentioned effects mediated via $AT_1$ receptors.[4,13-21] The vasodilator effects have been investigated most intensely,[4,22-26] although not all studies agree on this matter.[27,28]

$AT_2$ receptors are highly expressed during fetal development,[2,29,30] and re-appear in large numbers under pathological conditions.[26,31] This does not mean that $AT_2$ receptor are absent in healthy adult animals. In fact, $AT_2$ receptor-induced vasodilation can be observed in normal adult animals.[4,32,33]

$AT_2$ receptors are G-protein-coupled receptors,[32] and their stimulation results in the activation of protein phosphatases,[34] thereby directly reversing the effects mediated by protein kinases in response to $AT_1$ receptor stimulation. Alternatively, as $AT_1$ and $AT_2$ receptors also form heterodimers,[35] $AT_2$ receptor-mediated effects may occur through a direct interaction with $AT_1$ receptors, possibly even in a ligand-independent manner.[36]

Many studies support a link between $AT_2$ receptor stimulation and the NO-cGMP pathway, either directly or via bradykinin and subsequent $B_2$ receptor activation.[4,18,22,33,37-39]

In support of a role for NO, the $AT_2$ receptor-mediated vasodilator effects of Ang II in the rat heart *in vivo*[26] were reversed by the NO synthase inhibitor L-NAME, but not by the cyclo-oxygenase inhibitor indomethacin (Figure 1).

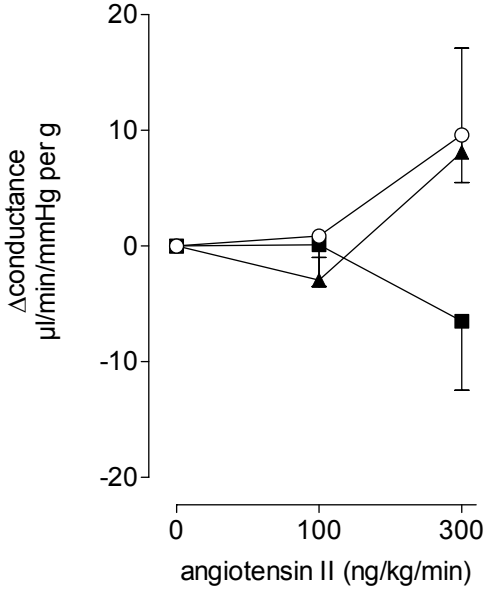



**Figure 1.** *Effects of 10 min-intravenous infusions of angiotensin (Ang) II on myocardial conductance (=myocardial blood flow/mean arterial pressure) in rats pretreated with saline (open circles), L-NAME (10 mg/kg, followed by a continuous infusion of sodium nitroprusside to restore blood pressure to pre-L-NAME level; squares) or indomethacin (triangles). Data (mean±SEM) are obtained from Schuijt et al., 2001a and 2001b[26,40]. Note that the increase in conductance (indicative of vasodilation, and blocked by PD123319[26] is reversed into a decrease by L-NAME (P=0.06) but not indomethacin.*

The exact location of vasodilator $AT_2$ receptors in the vessel wall is still a matter of debate. The most likely site, in view of the stimulation of the bradykinin-$B_2$ receptor-NO-cGMP pathway, appears to be the endothelium. Heterodimerisation with vasoconstrictor $AT_1$ receptors however would require the presence of $AT_2$ receptors on vascular smooth muscle cells. Thus, perhaps $AT_2$ receptors occur at multiple sites in the vessel wall.

### *$AT_2$ receptor-mediated effects in humans*

Data on $AT_2$ receptor-mediated effects in humans are scarce. Gene variants of the $AT_2$ receptor are associated with left ventricular mass index in both young hypertensive males[41] and females with hypertrophic cardiomyopathy,[42] thereby supporting the antitrophic effect of $AT_2$ receptors. $AT_2$ receptors are upregulated in the human heart with interstitial fibrosis, and cardiac fibroblasts appear to be the major cell type for their expression.[31,43]

Intrabrachial infusion of the $AT_2$ receptor antagonist PD123319 increased forearm vascular resistance in elderly women treated with the $AT_1$ receptor antagonist candesartan,[44] whereas Ang II tended to induce vasodilation in the forearm of healthy volunteers during $AT_1$ receptor blockade.[45] These data indirectly support the concept that $AT_2$ receptors mediate vasodilation in human forearm resistance vessels.

In large human coronary arteries (HCAs, diameter ≈ 4-5 mm), obtained from subjects who had died of non-cardiovascular causes, Ang II induced contractile responses that were fully blocked by the $AT_1$ receptor antagonist irbesartan (Figure 2).[46] PD123319 modestly increased the contractile response to Ang II, but the difference was not significant. In contrast, in human coronary microarteries (HCMAs, diameter ≈ 200-300 μm), PD123319 significantly increased the constrictor, $AT_1$ receptor-mediated effects of Ang II (Figure 2).[47] Moreover, Ang II relaxed preconstricted HCMAs in the presence of irbesartan, and this relaxation was prevented by PD123319. To the best of our knowledge, these data are the first to directly demonstrate $AT_2$ receptor-induced vasorelaxation in humans.

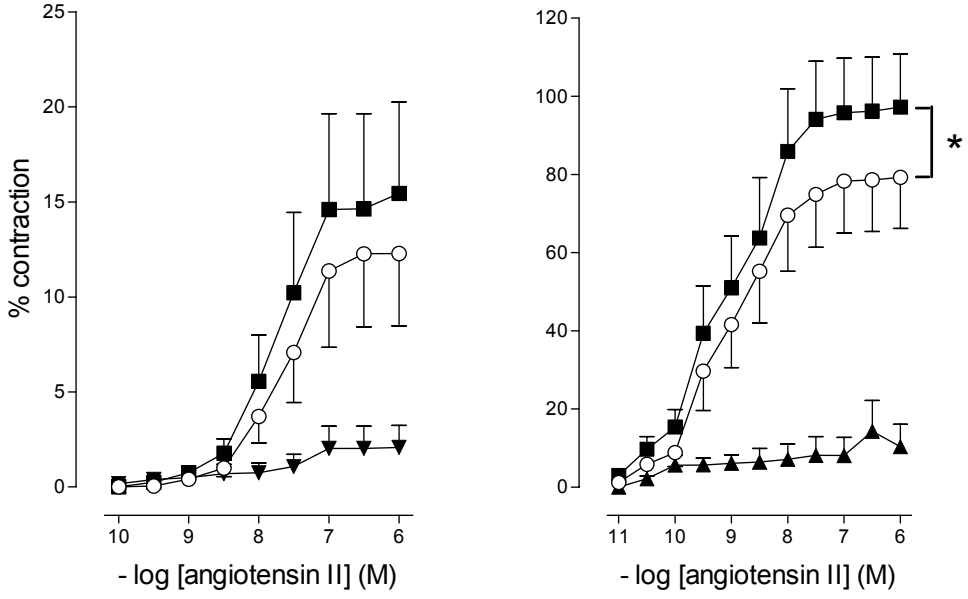



**Figure 2.** *Contractions of large human coronary arteries (left panel) and human coronary microarteries (right panel) to angiotensin II in the absence (control, circles) or presence of irbesartan (triangles) or PD123319 (squares). Data (mean±SEM) are expressed as a percentage of the response to 100 mM $K^+$ and have been obtained from MaassenVanDenBrink et al. (1999) and Batenburg et al. ( 2004b). Note the difference in scale. * P<0.05 vs. control*

Radioligand binding studies and RT-PCR support the expression of $AT_2$ receptors in HCMAs, HCAs and human coronary endothelial cells.[47-49] The presence of $AT_2$ receptors in HCAs, despite the non-significant effect of PD123319 on Ang II-induced vasoconstriction in these vessels, suggests either that their density in these arteries is lower, or that they mediate other (non-dilatory) effects, e.g., effects on vascular growth and remodelling.

### *Mediators of $AT_2$ receptor-mediated vasodilation in humans and the role of age, gender and endothelial function*

Endothelium removal, NOS blockade and $B_2$ receptor blockade fully prevented the PD123319-induced potentiation of Ang II in HCMAs.[47] Thus, $AT_2$ receptor-mediated vasodilation in HCMAs depends on the activation of endothelial $B_2$ receptors and NO, thereby fully supporting the animal data on this subject.[7,22,37] The PD123319-induced effects correlated positively with age.[47] Since endothelial function decreases with age (Figure 3), this could point to increased $AT_2$ receptor expression in the face of decreased endothelial function, in agreement with the concept that $AT_2$ receptor density increases under pathological conditions.[31]

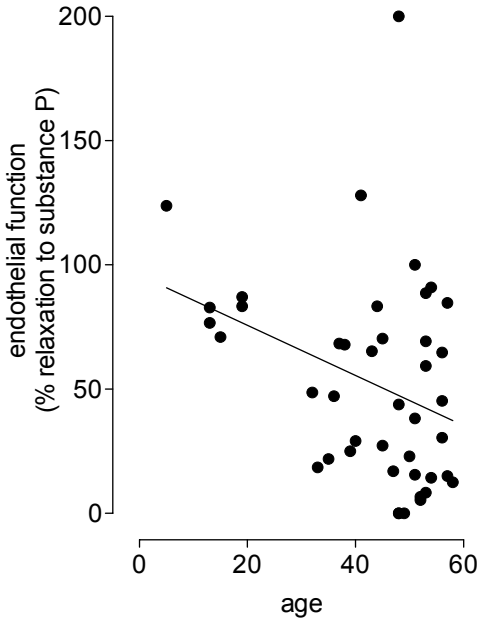



**Figure 3.** *Correlation between age and endothelial function (defined as the substance P-induced relaxation of preconstricted vessels) of human coronary arteries (r=0.33, P<0.05). Data are obtained from MaassenVanDenBrink et al. (1999), Schuijt et al. (2003) and Tom et al. (2003).*

The maximal contractile response ($E_{max}$) to Ang II was larger in women than in men (Figure 4), but this did not affect the effect of PD123319. There are at least two explanations for the increased $E_{max}$ in women. Men display higher renin levels[50] and this could result, through increased Ang II levels, in $AT_1$ receptor downregulation. Secondly, estrogens modulate $AT_1$ receptor expression, which will directly affect Ang II-induced vasoconstriction.[51]

A multivariate regression analysis of all (n=43) Ang II concentration-response curves in HCAs,[46,52,53] entering age (range 5-58 years, mean±SEM 43±2.1 years), sex (21 women, 22 men), cause of death (cerebrovascular accident, trauma, hypoxia), maximal contractile response (i.e., the response to 100 mmol/L $K^+$, 49±3 mN) and endothelial function (i.e., substance P-induced relaxation of preconstricted vessels, 53±6%) as variables, confirmed that Ang II efficacy was larger in women than in men (21±3.1 vs. 14±2.1% of the response to 100 mmol/L $K^+$, $P<0.05$), independently of all the above parameters. Ang II potency did not differ between men and women ($pEC_{50}$ 7.8±0.1 vs. 7.7±0.1), and correlated positively with endothelial function ($P<0.02$) independently of age, sex, cause of death and maximal contractile response. Thus, the more dysfunctional the endothelium, the more Ang II is needed to exert a certain vasoconstrictor effect. This observation is in agreement with a counterbalancing ($AT_2$ receptor-mediated) effect under pathological conditions, even in vessels where the PD123319-induced effect is of modest proportion (Figure 2).

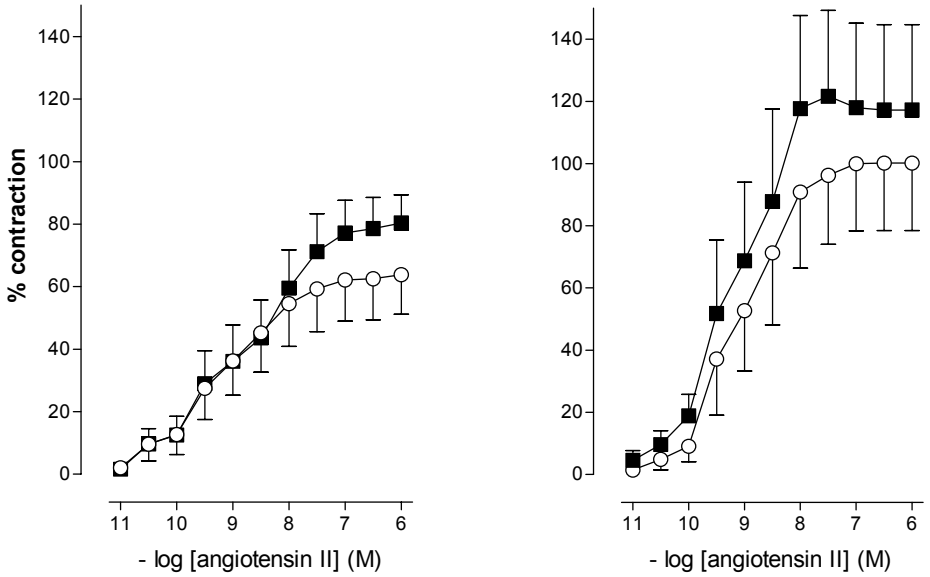



**Figure 4.** *Contractions of human coronary microarteries obtained from men (left panel, n=12) and women (right panel, n=10) to angiotensin II in the absence (control, circles) or presence of PD123319 (squares). Data (mean±SEM) are expressed as a percentage of the response to 100 mM $K^+$ and have been obtained from Batenburg et al. (2004b). Note that the maximum contraction is larger in women than in men (P=0.05), but that the effect of PD123319 is observed in both sexes.*

### *Bradykinin and vasodilatation: role of NO and EDHF*

The interaction between $AT_2$ receptors and $B_2$ receptor activation is not yet fully understood. Tsutsumi et al. (1999) proposed that Ang II decreases the intracellular pH in endothelial cells, which subsequently activates kininogenases that cleave bradykinin from intracellularly stored kininogens. It is difficult to conceive how this mechanism explains the $AT_2$ receptor-mediated dilatation of isolated vessels mounted in organ baths, since ACE inhibitors do not induce vasodilation in such vessels, thereby arguing against the presence of endogenous bradykinin in isolated vessels.[54-56] One possibility is that $AT_2$ receptors and $B_2$ receptors heterodimerize and interact in a bradykinin-independent manner.

$B_2$ receptor activation results in NO synthesis by endothelial NOS, and NO relaxes vascular smooth muscle cells through guanylyl cyclase activation and subsequent cGMP generation.[54,57,58] Thus, if $B_2$ receptors are indeed involved in the relaxant effect mediated by $AT_2$ receptors, it is not surprising that $AT_2$ receptor activation results in cGMP accumulation. NOS inhibitors however do not completely block bradykinin-induced

vasorelaxation, suggesting the existence of either NO-storage sites[59,60] or a non-NO 'endothelium-derived hyperpolarizing factor' (EDHF).[61]

EDHF-mediated responses in different arteries have been linked to $K^+$, cytochrome-P450 products from arachidonic acid (epoxyeicosatrienoic acids (EETs), in particular 5,6-EET, 11,12-EET and 14,15-EET), prostacyclin and $H_2O_2$.[62-67] The identity of EDHF and its contribution to overall relaxation appear to differ between species, between vascular beds and between vessels of different sizes.[68] In general, de novo synthesized NO is of greater importance in large arteries than in microarteries (Figure 5).

Busse et al.[61] recently summarized all currently available data on EDHF and proposed that EDHF-mediated relaxation depends on the activation of endothelial intermediate- and small-conductance $Ca^{2+}$-activated $K^+$-channels ($IK_{Ca}$, $SK_{Ca}$).[69,70] Such activation results in the release of $K^+$ into the myoendothelial space, which subsequently induces smooth muscle hyperpolarization by activating inwardly rectifying $K^+$ channel ($K_{IR}$) channels and/or $Na^+$-$K^+$-ATPase (Figure 6). According to this concept, EETs regulate endothelial hyperpolarization as well as the spread of this hyperpolarization to the adjacent smooth muscle cells through myoendothelial gap junctions. In addition, EETs directly activate large-conductance $Ca^{2+}$-activated $K^+$-channels ($BK_{Ca}$) on smooth muscle cells.[71] With regard to the latter, it is important to note that NO itself is capable of inducing hyperpolarization via activation of $Ca^{2+}$-activated $K^+$ channels in vascular smooth muscle.[72] Interestingly, $AT_2$ receptor-induced relaxation of rat mesenteric microvessels has also been reported to depend on opening of $BK_{Ca}$ channels.[24]

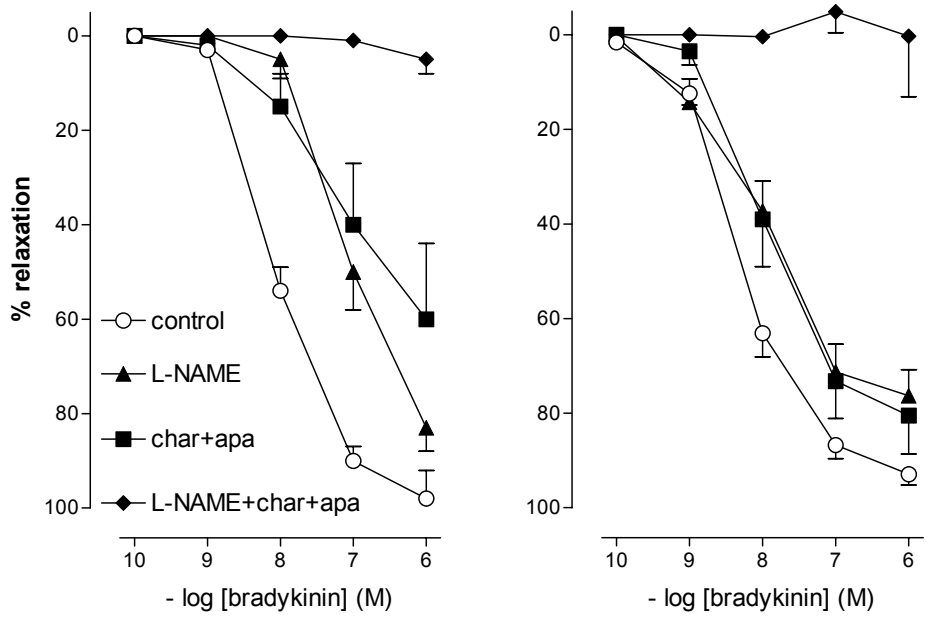



**Figure 5.** *Relaxations of large porcine coronary arteries (left panel) and porcine coronary microarteries (right panel), following preconstriction with prostaglandin $F_{2\alpha}$ or U46619, to bradykinin in the absence (control) or presence of 100 $\mu$M L-NAME and/or 100 nM charybdotoxin (char) + 100 nM apamin (apa). Data (mean$\pm$SEM) are expressed as a percentage of preconstriction and have been obtained from Danser et al. (2000) and Batenburg et al. (2004c). Note that the rightward shift induced by L-NAME is much larger in large coronary arteries, supporting a more important role for NO in these arteries.*

Data obtained in human and porcine coronary microarteries fully agree with the unifying EDHF concept proposed by Busse et al..[57,58] Bradykinin-induced relaxation of these vessels was found to depend on 1) endothelial $IK_{Ca}$ and $SK_{Ca}$ channels, and 2) the activation of guanylyl cyclase, $K_{IR}$ channels and $Na^+/K^+$ ATPase. Activation of $Ca^{2+}$-activated $K^+$-channels occurred by a factor other than NO. In HCMAs, this factor was not a cytochrome P450 epoxygenase product or $H_2O_2$. Since NO scavengers blocked bradykinin-induced relaxations of coronary arteries to a greater degree than NOS inhibitors,[54,58,73] whereas increasing the concentration of the NOS inhibitor did not yield additional blocking effects,[58,73] one possibility is that this factor is a NO-containing factor from a source that does not depend on the acute conversion of L-arginine by NOS.

NO-containing factors are thought to mediate light-induced photorelaxation of vascular smooth muscle cells.[43,74] Nitrosothiol-depleting agents reduce photorelaxation responses,[74] and *S*-nitrosothiols have therefore been proposed to mediate this phenomenon.

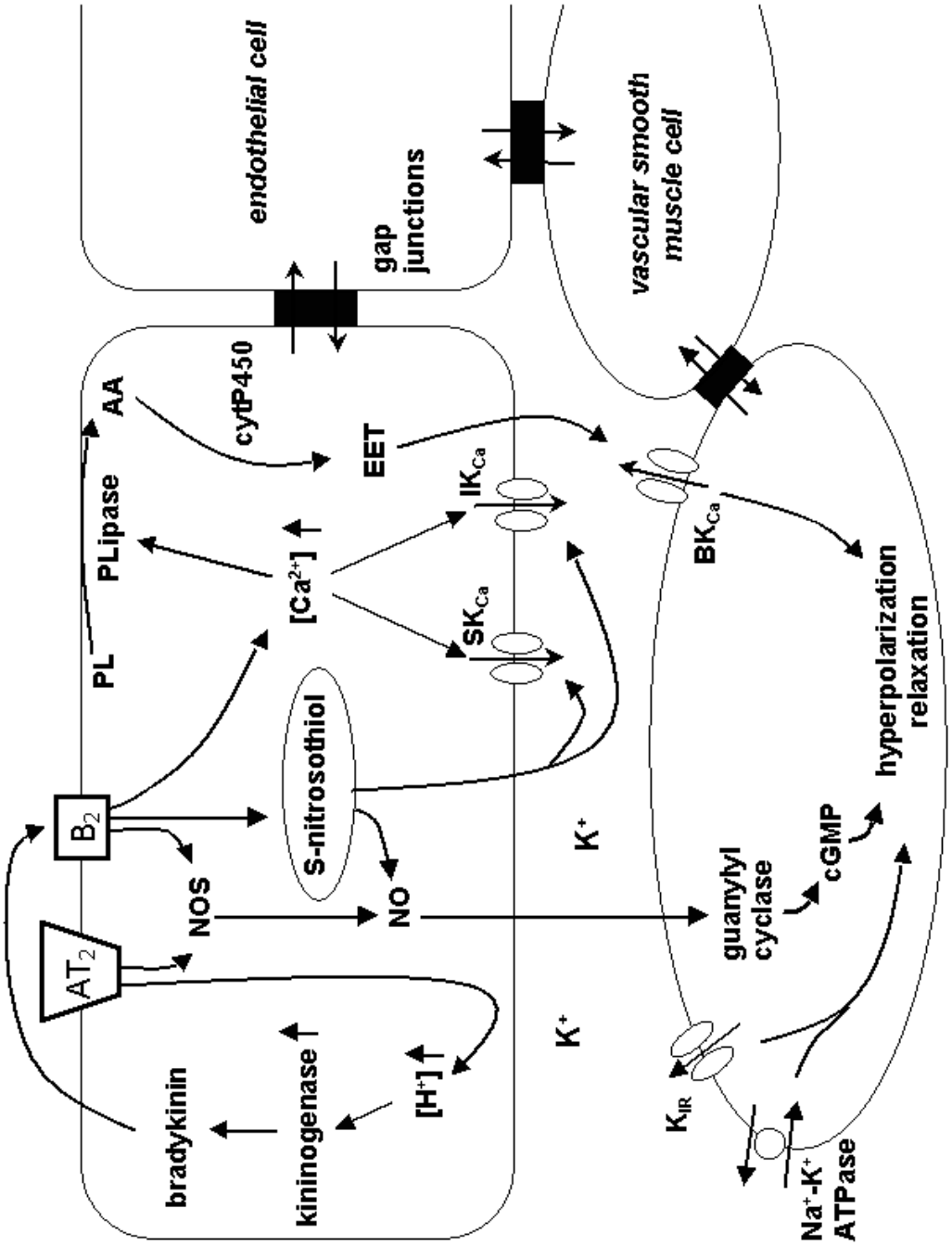



**Figure 6**. *Unifying scheme linking $AT_2$ receptors to bradykinin, $B_2$ receptors, NO and the EDHF pathway. Modified according to Busse et al. (2002). AA, arachidonic acid; $BK_{Ca}$, $IK_{Ca}$ and $SK_{Ca}$, $Ca^{2+}$-activated $K^+$-channels of large, intermediate and small conductance; EET, epoxyeicosatrienoic acid; $K_{IR}$, inwardly rectifying $K^+$ channel; NOS, NO synthase; PL, phospolipase. See text for explanation.*

*S*-nitrosothiols induce relaxation not only through their decomposition to NO,[75] but also by activating stereoselective recognition sites.[76] Recently, the cysteine residues within the α subunit of the $BK_{Ca}$ channel were identified as a *S*-nitrosothiol binding site.[77] In support of the concept that *S*-nitrosothiols mediate bradykinin-induced, EDHF-dependent relaxations, the nitrosothiol-depleting agent p-hydroxymercurobenzoic acid reduced the relaxant effects of bradykinin in porcine coronary arteries.[78] Furthermore, the *S*-nitrosothiol L-*S*-nitrosocysteine activated endothelial $IK_{Ca}$ and $SK_{Ca}$ channels (Figure 7) and hyperpolarized smooth muscle cells.[58]

The effect of NO blockade on bradykinin-induced relaxation of HCMAs was larger in men than in women (Figure 8). This suggests that the contribution of EDHF is larger in women. Similar observations were made in rat arteries, where it was simultaneously demonstrated that estrogens directly upregulate the EDHF pathway, without altering NOS expression.[79,80] The reduced efficacy of Ang II in coronary arteries of men compared to women (Figure 4) may also relate to this phenomenon.

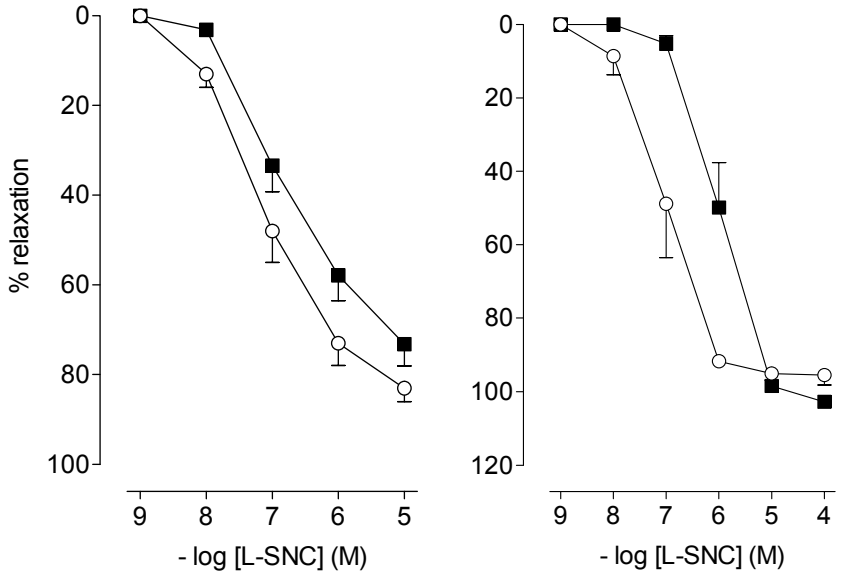



**Figure 7.** *Relaxations of large porcine coronary arteries (left panel) and porcine coronary microarteries (right panel), following preconstriction with U46619, to the S-nitrosothiol L-S-nitrosocysteine (L-SNC) in the absence (control, circles) or presence of 100 nM charybdotoxin + 100 nM apamin (squares). Data (mean±SEM) are expressed as a percentage of preconstriction and have been obtained from Batenburg et al. (2004d, c).*

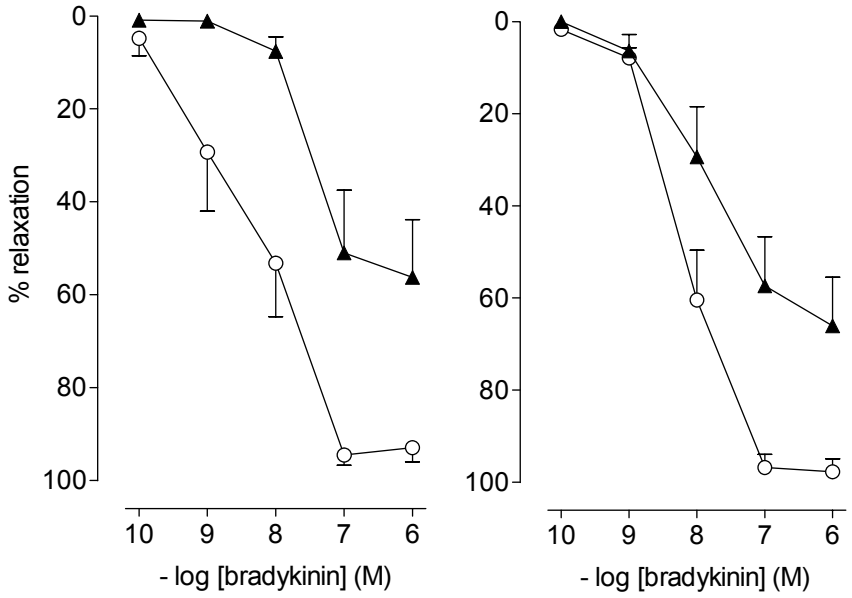



**Figure 8.** *Relaxations of human coronary microarteries, following preconstriction with U46619, to bradykinin in men (left panel, n=8) and women (right panel, n=12) in the presence (control, circles) or absence of NO (triangles). Data (mean±SEM) are expressed as a percentage of preconstriction and have been obtained from Batenburg et al. (2004a). Note that the rightward shift induced by NO removal (with either L-NAME or hydroxocobalamin) is larger in men (P=0.003) than in women (P=0.01).*

## Aim of this thesis

The release of paracrine factors from endothelial cells is a critical determinant of vascular tone. Endothelium-dependent vasodilators such as acetylcholine and bradykinin stimulate endothelial cells to release the vasodilators NO and/or endothelium-derived hyperpolarizing factor (EDHF). The relative contribution of each of these mediators to endothelium-dependent vasodilation is related to vessel size: NO-mediated responses predominate in conduit arteries, whereas EDHF is more prominent in resistance vessels.[68] The latter vessels, with a diameter <400 μm, are the main contributors to peripheral vascular resistance.

Animal studies have recently shown that angiotensin (Ang) II, in addition to being a potent vasoconstrictor, is also capable of causing vasorelaxation, most likely in an endothelium-dependent manner (involving both bradykinin and NO).[81] Consequently, the net effect of Ang II depends on the ratio of the Ang II receptors mediating constriction (Ang II type 1, $AT_1$) and relaxation (Ang II type 2, $AT_2$). Possibly, these receptors

interact as a consequence of heterodimerization,[35] similar to the interaction that has been reported previously between $AT_1$ receptors and other vasoconstrictor receptors, e.g., endothelin-1 receptors[82] and $\alpha_1$-adrenoceptors.[83]

It was the aim of the current thesis to investigate Ang II-dependent vasodilatation in humans, focussing in particular on the $AT_2$ receptor – bradykinin – NO/EDHF pathway (Chapter 2). We also tried to obtain more detailed information on the nature of EDHF, by studying bradykinin-induced relaxation during NO synthase (NOS) blockade in both human and porcine coronary (micro-)arteries (Chapters 3-5). Finally, we investigated the (heterodimeric) interaction between $AT_1$ and $\alpha_1$-adrenoceptors in human microarteries, in order to obtain a better understanding of the mechanism of action of the Ang II-inhibiting properties of the non-selective β- and $\alpha_1$-adrenoceptor antagonist carvedilol (Chapter 6).

All human studies were performed in coronary microarteries (diameter <500 μm) obtained from heart valve donors, and mounted in Mulvany myographs. The porcine vessels that were used in this thesis were either large coronary arteries or coronary microarteries. All studies were complemented by simultaneous biochemical and molecular-biological measurements in vascular segments, in order to determine the contributing second messengers and receptor subtypes.

# Chapter 2

## Angiotensin II type 2 receptor-mediated vasodilation in human coronary microarteries

Circulation 2004; 109: 2296-2301

## Summary

Angiotensin (Ang) II type 2 ($AT_2$) receptor stimulation results in coronary vasodilation in the rat heart. In contrast, $AT_2$ receptor-mediated vasodilation could not be observed in large human coronary arteries. Here we studied Ang II-induced vasodilation of human coronary microarteries (HCMAs). HCMAs (diameter 160-500 μm) were obtained from 49 heart valve donors (age 3-65 years) and mounted in Mulvany myographs. Ang II constricted HCMAs in a concentration-dependent manner ($pEC_{50}$ 8.6±0.2; maximal effect ($E_{max}$) 79±13 % of the contraction to 100 mmol/L $K^+$). The Ang II type 1 receptor antagonist irbesartan prevented this vasoconstriction, whereas the $AT_2$ receptor antagonist PD123319 increased $E_{max}$ to 97±14% ($P<0.05$). The increase in $E_{max}$ was larger in older donors (correlation $\Delta E_{max}$ vs. age $r=0.47$, $P<0.05$). The PD123319-induced potentiation was not observed in the presence of the NO synthase inhibitor L-NAME, the bradykinin type 2 ($B_2$) receptor antagonist Hoe140 or after removal of the endothelium. Ang II relaxed U46619-preconstricted HCMAs in the presence of irbesartan by maximally 48±16%, and PD123319 prevented this relaxation. Finally, radioligand binding studies and RT-PCR confirmed the expression of $AT_2$ receptors in HCMAs. In conclusion, $AT_2$ receptor-mediated vasodilation in the human heart appears to be limited to coronary microarteries, and is mediated via $B_2$ receptors and NO. Most likely, $AT_2$ receptors are located on endothelial cells and their contribution increases with age.

## Introduction

Angiotensin (Ang) II type 2 ($AT_2$) receptors are believed to mediate vasodilation, although data to support this concept in humans are not available. Neither in vitro studies investigating Ang II-induced vasoconstriction in isolated human coronary arteries[46] and saphenous veins,[84] nor in vivo studies investigating Ang II-induced vasoconstriction in the forearm vascular bed of healthy volunteers,[45,85] provided evidence for $AT_2$ receptor-mediated vasodilation. In contrast, both in vitro and in vivo studies in rats and mice support this notion.[5,20,24,26,86,87] One explanation for the discrepancy between the lack of $AT_2$ receptor-mediated vasodilation in human coronary arteries,[46] and the occurrence of such dilation in the rat coronary vascular bed,[26] is that $AT_2$ receptors are located in coronary microarteries only. In the present study we therefore investigated $AT_2$ receptor-induced vasodilation in human coronary microarteries (HCMAs) mounted in Mulvany myographs. We also investigated whether endothelial NO and/or bradykinin type 2 ($B_2$) receptors mediate such vasodilation in HCMAs, since studies in animals support this possibility.[5,22,33,88] Finally, we verified, both through radioligand binding studies and RT-PCR, whether HCMAs express $AT_2$ receptors.

## Methods

### *Human tissue collection*

HCMAs were obtained from 49 heart-beating organ donors (22 men, 27 women, age 3-65 years, mean 45 years), who died of non-cardiac causes (3 cerebrovascular accident, 9 head trauma, 21 subarachnoid bleeding, 4 post-anoxic encephalopathy, 12 intracranial bleeding) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the heart valves for transplantation purposes. The study was approved by the Ethics Committee of the Erasmus MC. The hearts were stored in an ice-cold sterile organ-protecting solution after circulatory arrest. After arrival at the laboratory, a tertiary branch of the left anterior descending coronary artery (diameter 160-500 μm, mean 360 μm) was removed and stored overnight in a cold (4°C), oxygenated Krebs bicarbonate solution of the following composition (mmol/L):

NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. In addition, HCMAs, right epicardial coronary arteries and/or pieces of left ventricular tissue from 29 hearts were frozen in liquid nitrogen for mRNA determinations or radioligand binding studies.

### *Myograph studies*

Following overnight storage, HCMAs were cut into segments of approximately 2 mm length and mounted in a Mulvany myograph (J.P. Trading) with separated 6-mL organ baths containing oxygenated Krebs at 37°C. The Krebs was continuously aerated with 95% $O_2$ and 5% $CO_2$, and tissue responses were measured as changes in isometric force, using a Harvard isometric transducer. Following a 30-min stabilization period, the optimal internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure as described by Mulvany and Halpern.[89] In some vessels, the endothelium was removed by gently rubbing a hair through the lumen of the mounted artery. Endothelial integrity or removal was verified by observing relaxation (or lack thereof) to 10 nmol/L substance P after preconstriction with 10 nmol/L of the thromboxane $A_2$ ($TxA_2$) analogue U46619 (Sigma). Subsequently, to determine the maximum contractile response, the tissue was exposed to 100 mmol/L KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min. Next, segments were pre-incubated for 30 min with the Ang II type 1 ($AT_1$) receptor antagonist irbesartan (1 µmol/L, a gift of Bristol-Myers Squibb),[46] the $AT_2$ antagonist PD123319 (1 µmol/L, a gift of Parke-Davis),[13] the $B_2$ receptor antagonist Hoe140 (1 µmol/L, a gift of Hoechst)[54] and/or L-NAME (100 µmol/L, Sigma). Thereafter, concentration-response curves (CRCs) were constructed to Ang II, either directly or following preconstriction with 10 nmol/L U46619 to 60% of the maximum contractile response. A higher concentration of U46619 (30 nmol/L) was required in segments that had been pre-incubated with irbesartan, because irbesartan antagonizes $TxA_2$ receptors.[90] The cyclo-oxygenase inhibitor indomethacin (5 µmol/L) was present during the entire experiment to suppress spontaneously occurring contractions and relaxations.

### *Cyclic GMP measurement*

To study Ang II-induced cGMP production, vessel segments (5-10 mg) were exposed to 1 μmol/L Ang II in 10 mL oxygenated Krebs bicarbonate solution for 1 min at 37ºC in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (100 μmol/L), following a 30-min preincubation without (control) or with 1 μmol/L PD123319 or irbesartan. Tissues were then frozen in liquid nitrogen, and stored at -80ºC. To determine cGMP, frozen tissues were homogenized in 0.5 mL 0.1 mol/L HCl using a stainless steel ultraturrax (Polytron). Homogenates were centrifuged at 3300 g, and cGMP was measured in 300 μL supernatant by ELISA following acetylation (R&D Systems). Results are expressed as pmol/mg protein. The lower limit of detection was 0.1 pmol/mg protein.

### *Radioligand binding studies*

Sarcolemmal membrane fractions were prepared from HCMAs and porcine adrenal glands as described before.[91] The adrenals were obtained from three 2-3 month old pigs that had been used in in-vivo experiments investigating the effects of calcitonin gene-related peptide receptor (ant)agonists.[92] $^{125}$I-Ang II, prepared with the chloramine T-method (specific activity 2200 Ci/mmol),[93] was used as the radioligand. Assays were run for 60 minutes at 18°C in 30 μL Tris buffer (50 mmol/L), 40 μL membrane fraction (containing 100 μg protein, determined by the Bradford assay as described before[13]) and 30 μL radioligand (final volume: 100 μL). Non-specific binding, $AT_1$ receptor-specific binding and $AT_2$ receptor-specific binding were determined by repeating the experiment in the presence of Ang II (at a concentration 100 times the concentration of $^{125}$I-Ang II), irbesartan (0.3 pmol/L-0.3 mmol/L) and PD123319 (0.3 pmol/L-0.3 mmol/L), respectively. Incubation was stopped by adding 4 mL ice-cold phosphate-buffered saline (PBS), pH 7.4. Samples were then filtered through a Whatman GF/B filter. Filters were washed twice with 4 mL ice-cold PBS, and filter-bound radioactivity was measured in a gamma-counter.

### *$AT_1$ and $AT_2$ receptor mRNA*

Total RNA was isolated from HCMAs, right epicardial coronary arteries and left ventricular tissue using the Trizol reagent (Gibco-BRL). RT-PCR was performed according to standard procedures and 35 cycles of amplification, using primer sequences as follows: $AT_1$ receptor sense 5'-CTT TTC CTG GAT TCC CCA C-3', and antisense 5'-CTT CTT GGT GGA TGA GCT TAC-3', $AT_2$ receptor sense 5'-GTG ACC AAG TCC TGA AGA TG-3' and antisense 5'-CAC AAA GGT CTC CAT TTC TC-3', resulting in amplification products of 304 and 335 bp, respectively. Positive and negative controls were mRNAs extracted from human liver, a human breast carcinoma cell line (MCF7) and a human colon carcinoma cell line (SW480).[94] The absence of non-specific amplification was verified by running RT-PCR and PCR amplifications without adding tissue extracts. As controls for RNA quality, amplification reactions were performed using pairs of primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[95] Amplified transcripts were analyzed on 2% agarose gels.

### *Data analysis*

Data are given as mean±SEM. Contractile or relaxant responses are expressed as a percentage of the contraction to 100 mmol/L $K^+$ or U46619. CRCs were analyzed as described to obtain $pEC_{50}$ ($-^{10}\log EC_{50}$) values.[46] Statistical analysis was by paired t-test, once one-way ANOVA, followed by Dunnett's post-hoc evaluation, had revealed that differences existed between groups. $P<0.05$ was considered significant.

## Results

### *Myograph studies*

Ang II constricted HCMAs in a concentration-dependent manner ($pEC_{50}$ 8.6±0.2, n=22) with a maximal response ($E_{max}$) of 79±13% (Figure 1). Irbesartan nearly completely blocked the Ang II-mediated constriction. PD123319 increased $E_{max}$ to 97±14% ($P<0.05$). PD123319 did not affect the potency of Ang II ($pEC_{50}$=8.7±0.2, n=22), although in 11 experiments a leftward shift of the Ang II CRC (i.e., an increase in the $pEC_{50}$ value of 0.2 or more) was observed in the presence of the $AT_2$ receptor antagonist.

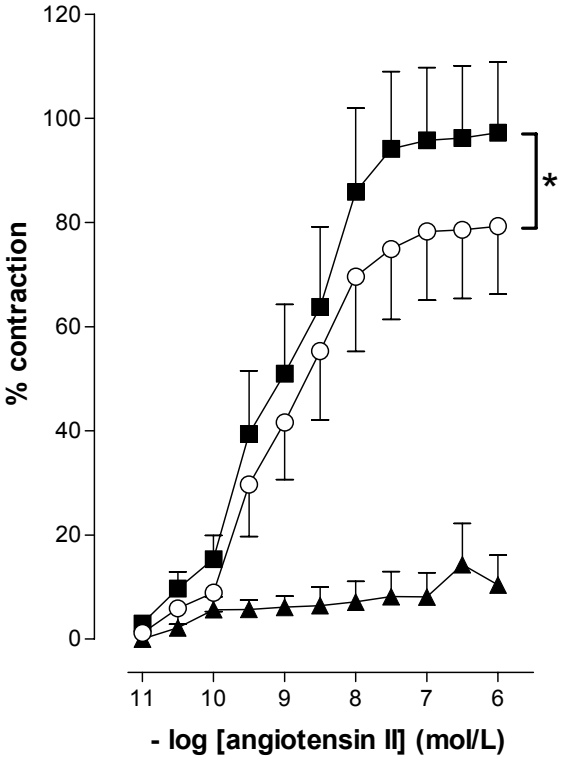



**Figure 1.** *Contractions of HCMAs to Ang II in the absence (control; circles) or presence of irbesartan (triangles) or PD123319 (squares). Contractions (mean±SEM; n=5-22) are expressed as a percentage of the response to 100 mmol/L $K^+$. * P<0.05 vs control.*

The PD123319-dependent increase in $E_{max}$ was larger in older donors (r=0.47, P<0.05; Figure 2). The increase in $E_{max}$ was largest in the 11 experiments where PD123319 induced a leftward shift of the Ang II CRC: +34±10% vs. +2.2±8.4% in the experiments where PD123319 induced either no (i.e., $\Delta pEC_{50}$ <0.2; n=7) or a rightward (i.e., $pEC_{50}$ decreased by 0.2 or more; n=4) shift of the Ang II CRC.

L-NAME increased baseline contraction to 20-30% of the maximum response to 100 mmol/L $K^+$ and prevented the PD123319-induced potentiation of Ang II (Figure 3). Potentiation was also not observed following removal of the endothelium and in the presence of Hoe140 (Figure 3).

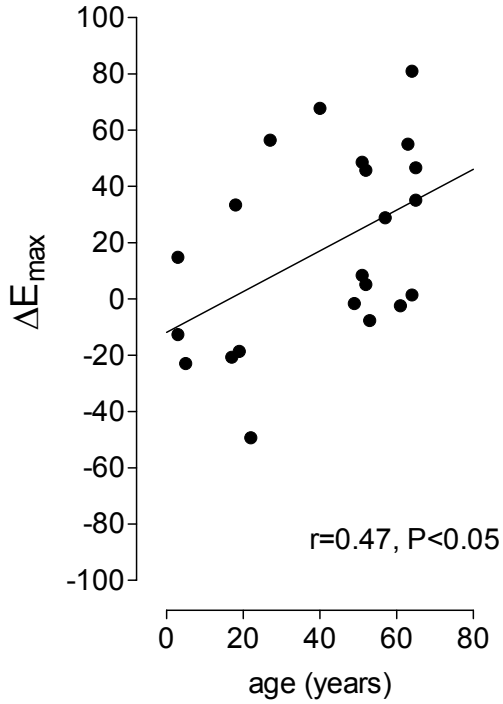



**Figure 2.** *Correlation between donor age and the change in $E_{max}$ ($\Delta E_{max}$) of the Ang II CRC observed following the addition of PD123319 to the organ bath (n=22).*

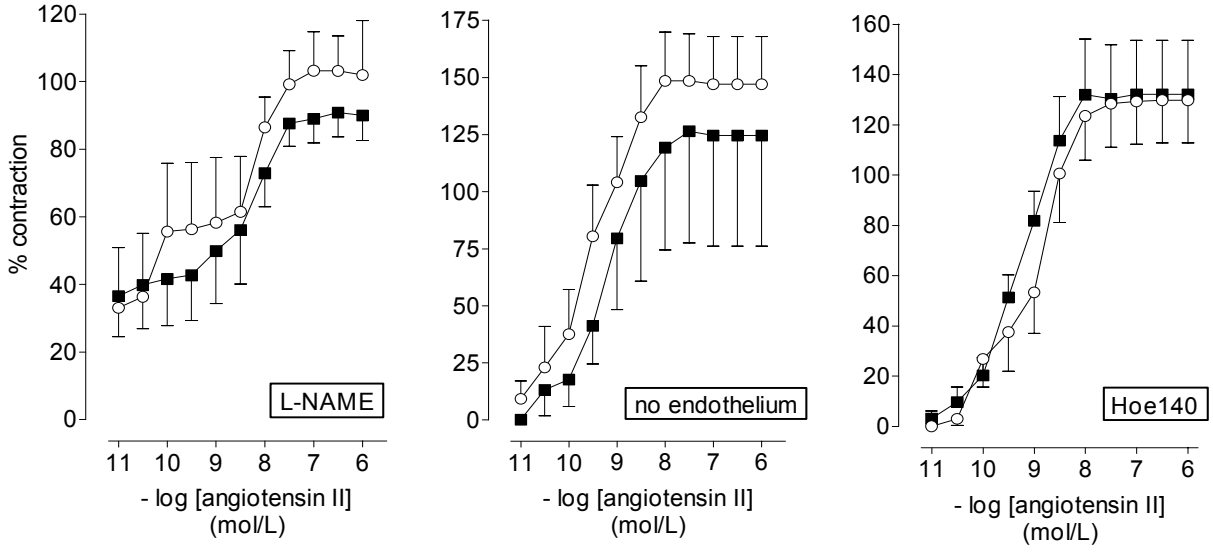



**Figure 3.** *Contractions of HCMAs to Ang II in the absence (circles) or presence of PD123319 (squares) following pretreatment with L-NAME, endothelium removal, or pretreatment with Hoe140. Contractions (mean±SEM; n=3-7) are expressed as a percentage of the response to 100 mmol/L $K^+$.*

Following preconstriction with U46619 (to approximately 60% of the maximum response to 100 mmol/L $K^+$), Ang II caused a marginal further increase (P=NS) in contraction (Figure 4). This response was unaltered by PD123319 and reversed into a relaxation (by maximally 49±16%) in the presence of irbesartan. PD123319 fully prevented the latter relaxation. Without Ang II, U46619-induced preconstrictions in the presence of irbesartan remained stable for at least 60 min (data not shown). Thus, the Ang II-induced relaxations under these conditions cannot be attributed to $TxA_2$ receptor antagonism by irbesartan.[90]

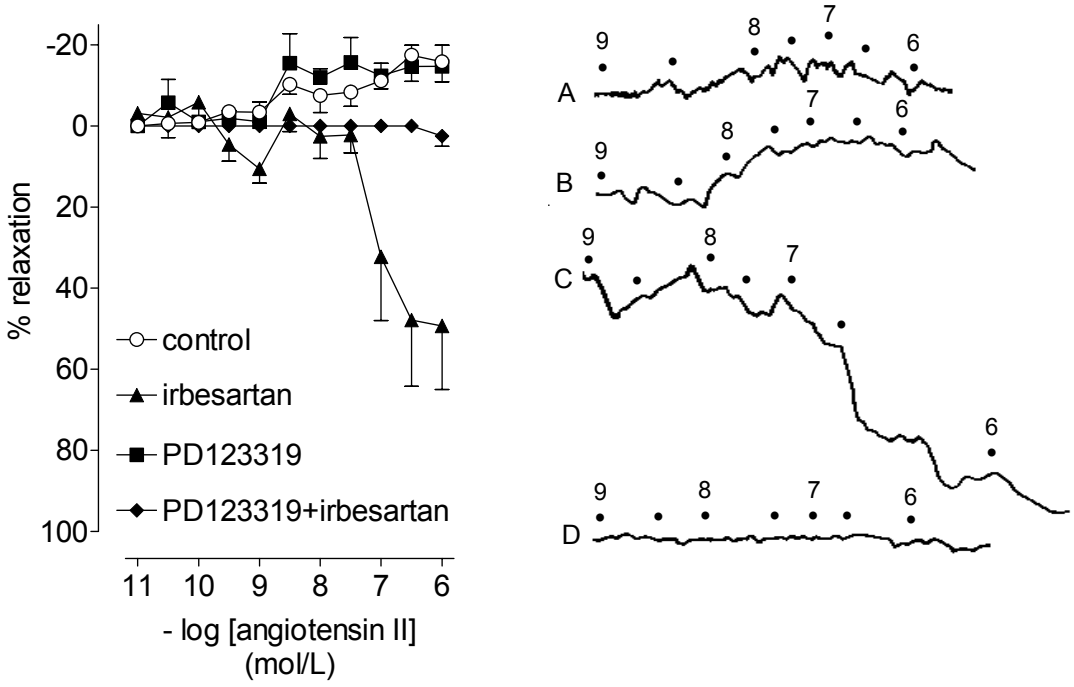



**Figure 4.** *Left, response of U46619-preconstricted HCMAs to Ang II in the absence or presence of irbesartan, PD123319 or irbesartan + PD123319. Data (mean±SEM; n=2-5) are expressed as a percentage of the response to U46619. Right, original tracing of an experiment in which an U46619-preconstricted HCMA was exposed to Ang II under control conditions (A), or following pre-incubation with PD123319 (B), irbesartan (C), or irbesartan + PD123319 (D). Ang II concentrations were increased with half log steps, starting at 1 nmol/L (9) and ending at 1 µmol/L (6).*

### *Cyclic GMP measurement*

Ang II did not significantly increase microvascular cGMP (Figure 5; n=8, P=0.11 vs. control), neither alone, nor in the presence of PD123319 or irbesartan.

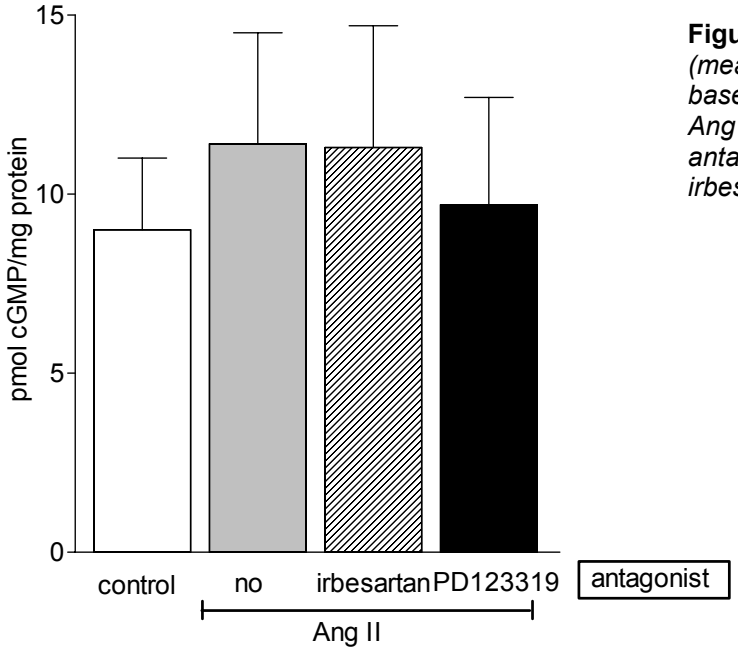



**Figure 5.** *Cyclic GMP levels (mean±SEM; n=8) in HCMAs at baseline and after a 1-min exposure to Ang II under control conditions (no antagonist) and in the presence of irbesartan or PD123319.*

### *Radioligand binding studies*

The total amount of protein in the HCMA sarcolemmal membrane fraction (≈500 µg), prepared from vessel segments of 19 subjects, was too small to study a wide range of conditions. We therefore used sarcolemmal membrane fractions prepared from 6 porcine adrenal glands to obtain the most optimal conditions to demonstrate the presence of $AT_2$ receptors in HCMAs. Following a 1-hour incubation with $^{125}$I-Ang II (final concentration in the incubation mixture: 0.5 nmol/L), total and non-specific $^{125}$I-Ang II binding to porcine adrenal membranes amounted to 4660±150 and 2100±80 cpm/100 µg protein (n=8), respectively. PD123319 and irbesartan abolished specific binding in a concentration-dependent manner (Figure 6, panel A). The inhibitor concentration required to reduce specific binding by 50% ($IC_{50}$) was 50±1 nmol/L for PD123319. This value mimics the $IC_{50}$ of PD123319 obtained in previous experiments with cells expressing $AT_2$ receptors only.[96] In contrast, the $IC_{50}$

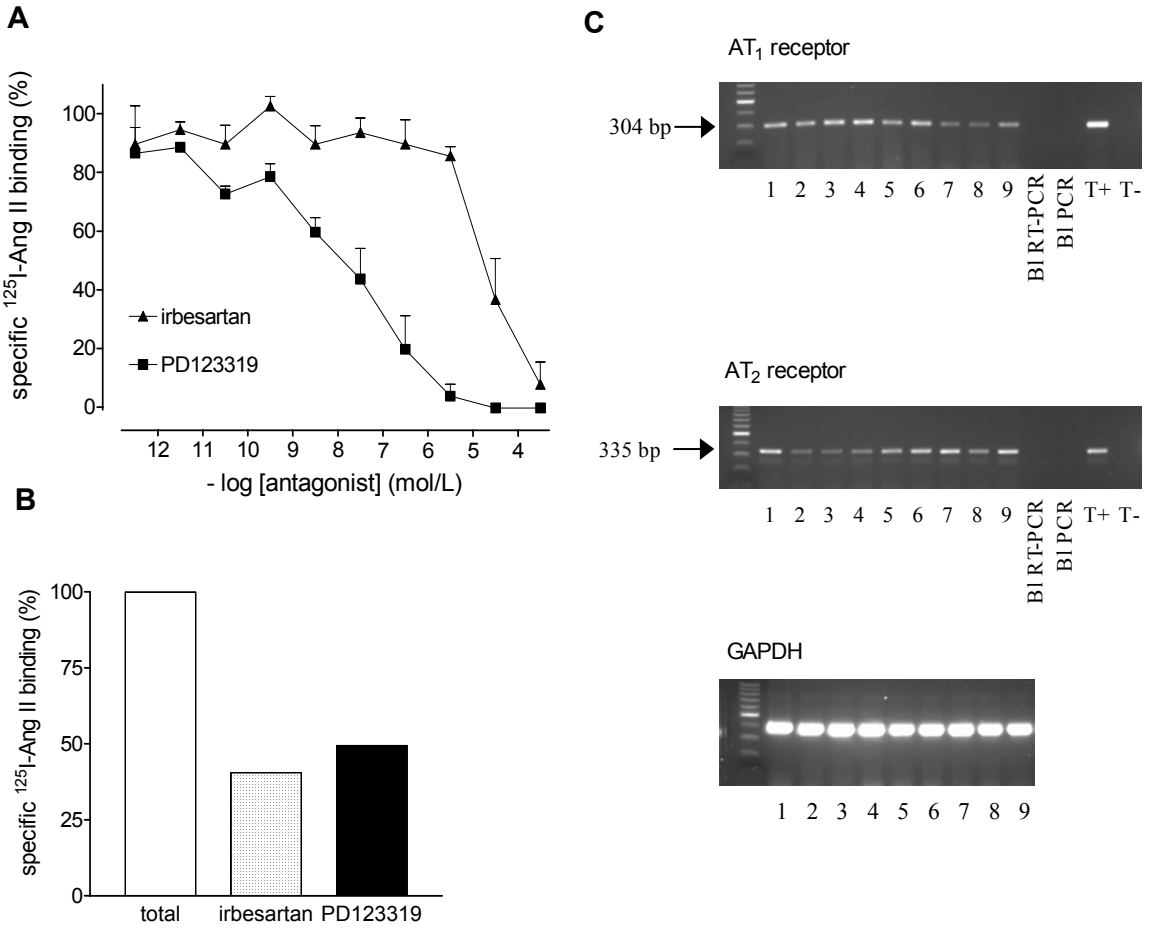



**Figure 6.** *Panels A and B. Displacement of specifically bound $^{125}$I-Ang II by irbesartan or PD123319 in sarcolemmal membrane fractions prepared from 6 porcine adrenal glands (A) and 19 HCMAs (B). Panel C. Results from the RT-PCR amplification of $AT_1$ receptor mRNA (304 bp), $AT_2$ receptor mRNA (335 bp), and GAPDH mRNA in HCMAs (lanes 1-3), large epicardial human coronary arteries (lanes 4-6) and human left ventricular tissue (lanes 7-9) obtained from 5 hearts. Positive controls (T+) for $AT_1$ - and $AT_2$ receptor mRNA are extracts of human liver and human breast carcinoma cells (MCF7), respectively. Negative controls (T-) for $AT_1$ - and $AT_2$ receptor mRNA are extracts of human breast carcinoma cells (MCF7) and colon carcinoma cells (SW480), respectively. Bl RT-PCR and Bl PCR represent the results of RT-PCR or PCR amplifications performed in the absence of added tissue extracts (to exclude contamination).*

of irbesartan in the present study (20±1 µmol/L) exceeded its $IC_{50}$ in cells exclusively expressing $AT_1$ receptors by three orders of magnitude.[97] Taken together, these data suggest that our porcine adrenal membrane fraction contained predominantly $AT_2$ receptors. A PD123319 concentration of 10 µmol/L is required to fully block $^{125}$I-Ang II binding to these receptors.

Based on these findings, as well as on previous studies investigating irbesartan concentrations that selectively block $AT_1$ receptors,[97,98] we incubated HCMA membranes with 0.5 nmol/L $^{125}$I-Ang II, in the absence or presence of 50 nmol/L Ang II, 10 µmol/L PD123319, or 1 µmol/L irbesartan. Ang II reduced $^{125}$I-Ang II binding from 1813 to 1175 cpm/100 µg protein. PD123319 and irbesartan both reduced specific binding by approximately 50%, thereby indicating that HCMAs contain $AT_1$ as well as $AT_2$ receptors (Figure 6, panel B).

### *$AT_1$ and $AT_2$ receptor mRNA*

RT-PCR revealed expression of $AT_1$ and $AT_2$ receptors in HCMAs, large epicardial coronary arteries and/or left ventricular tissue from 5 hearts (Figure 6, panel C). Similar data were obtained in an additional HCMAs from 7 hearts (data not shown).

## Discussion

This study is the first to show $AT_2$ receptor-mediated vasodilation in human blood vessels. Evidence for this effect was obtained in two ways. First, the $AT_2$ receptor antagonist PD123319, at a concentration that has been reported to result in complete blockade of $AT_2$ receptor-mediated effects,[13] increased the maximal contractile response to Ang II, thereby indirectly demonstrating that $AT_2$ receptor stimulation counteracts $AT_1$ receptor-mediated vasoconstriction. Second, during $AT_1$ receptor blockade with irbesartan (allowing selective $AT_2$ receptor stimulation) Ang II relaxed preconstricted HCMAs and this was prevented by PD123319. Such vasodilation was not observed in quiescent HCMAs in the presence of irbesartan, probably because vasodilator responses are more difficult to detect without preconstriction. Based on these data, it is clear that, at least in HCMAs, the net contractile effect of Ang II is determined by the magnitude of the response mediated via $AT_1$ (contraction) and $AT_2$ (relaxation) receptors.

In addition to its effect on $E_{max}$, PD123319 caused a leftward shift of the Ang II CRC in ≈50% of the experiments. Such increased potency of Ang II in the presence of PD123319 is not due to an effect of the $AT_2$ receptor antagonist on Ang II metabolism.[53,98] It could point to more efficient $AT_1$ receptor signal transduction during $AT_2$ receptor blockade.

Furthermore, a recent study has suggested that $AT_1$ and $AT_2$ receptors form heterodimers.[35] An alternative explanation for the increased potency might therefore be that in some donors $AT_1$ receptor-$AT_2$ receptor heterodimers exist which bind Ang II with higher affinity during $AT_2$ receptor blockade. The underlying assumption for this explanation is however that $AT_1$ and $AT_2$ receptors in these donors are located on the same cell.

The increase in $E_{max}$ was larger in older donors, suggesting that the contribution of $AT_2$ receptors increases with age. Although $AT_2$ receptor density increases under pathological conditions,[88] the donors in the present study died of non-cardiac causes and did not use cardiovascular medication. Thus, it is unlikely that the increased $E_{max}$ during $AT_2$ receptor blockade in older donors simply reflects the occurrence of cardiovascular disorders in these subjects. It might reflect a general decrease of vascular function with age.

In an earlier study in large epicardial human coronary arteries we were unable to detect $AT_2$ receptor-mediated vasodilation,[46] whereas vasodilation did occur in the rat coronary vascular bed.[26] The present study solves this discrepancy, by demonstrating that $AT_2$ receptor-mediated vasodilation is limited to coronary microarteries. Importantly, $AT_2$ receptor expression in HCMAs could be demonstrated by both RT-PCR and radioligand binding experiments. Unexpectedly, $AT_2$ receptor mRNA was also detected by RT-PCR in large coronary arteries. This would imply that either the $AT_2$ receptor density in large coronary arteries is too low to allow detection of vasodilation in the organ bath setup, or that $AT_2$ receptors in these arteries mediate other (non-dilatory) effects, e.g., effects on vascular growth and remodeling.[99,100] $AT_2$ receptor expression has been demonstrated before in the human myocardium, including the coronary vascular bed.[31,48]

The mechanism underlying $AT_2$ receptor-mediated vasodilation in HCMAs is currently unknown. $AT_2$ receptors themselves may act as $AT_1$ receptor antagonists, independently of Ang II.[35] This would require their occurrence on the same cell, as discussed above. Furthermore, $B_2$ receptors, NO, cGMP, $Ca^{2+}$-activated $K^+$ channels and/or phosphatases have been implicated in $AT_2$ receptor-induced effects.[5,18,22,24,33,87,88,101] Our data with L-NAME and Hoe140 in HCMAs support a role for $B_2$ receptors and NO. Since the vasodilator effects in HCMAs were observed in the presence of indomethacin,

prostaglandins do not appear to be involved. The lack of effect of PD123319 in de-endothelialized segments confirms the contribution of endothelial $B_2$ receptor-induced NO release, and simultaneously suggests that $AT_2$ receptors in HCMAs are located on endothelial cells. In agreement with this concept, cultured human coronary artery endothelial cells do express $AT_2$ receptors.[49]

Taken together, the most likely scenario to explain our results is that Ang II stimulates endothelial $AT_2$ receptors in HCMAs. This results in endothelial $B_2$ receptor activation and NO release. NO subsequently activates guanylyl cyclase in vascular smooth muscle cells, thereby counteracting the contractile responses mediated via the $AT_1$ receptors on these cells. Guanylyl cyclase generates cGMP, and although the Ang II-induced ($AT_2$ receptor-mediated) increase in the microvascular cGMP content in the present study was not significant, the tendency of PD123319 (but not irbesartan) to block this increase mimics similar observations in rat aorta and rat uterine arteries.[5,18] The lack of significance in the present experiments most likely relates to the modest (≈30%) increase in cGMP content induced by Ang II as compared to other agonists. For instance, in our experimental setup, 1 μmol/L bradykinin increased microvascular cGMP 7±2 fold (n=4, data not shown).

In conclusion, $AT_2$ receptor-mediated vasodilation occurs in the coronary microcirculation of non-diseased human hearts in an endothelium-dependent manner, and is mediated via $B_2$ receptors and NO. This finding could be of clinical relevance, not only because cardiac $AT_2$ receptors are upregulated under pathological conditions,[31] but also because animal studies have shown that the beneficial effects of $AT_1$ receptor antagonists, in contrast to those of ACE inhibitors, depend on $AT_2$ receptor stimulation.[11,12]

# Chapter 3

## Mediators of bradykinin-induced vasorelaxation in human coronary microarteries

Hypertension, 2004; 43: 488-492.

## Summary

To investigate the mediators of bradykinin-induced vasorelaxation in human coronary microarteries (HCMAs), HCMAs (diameter ≈300 μm), obtained from 42 heart valve donors (20 men, 22 women, age 3-65 years, mean 46 years) were mounted in Mulvany myographs. In the presence of the cyclo-oxygenase inhibitor indomethacin, bradykinin relaxed preconstricted HCMAs ($pEC_{50}$ 8.2±0.1). L-NAME and ODQ (inhibitors of NO synthase and guanylyl cyclase, respectively), and the NO scavenger hydroxocobalamin, alone or in combination, shifted the bradykinin concentration-response curve (CRC) to the right. Removal of $H_2O_2$ (with catalase), inhibition of cytochrome P450 epoxygenase (with sulfaphenazole or clotrimazole) or gap junctions (with 18α-glycyrrhetinic acid or carbenoxolone), and blockade of large- ($BK_{Ca}$) and small-conductance ($SK_{Ca}$) $Ca^{2+}$-dependent $K^+$-channels (with iberiotoxin and apamin), either alone or on top of hydroxocobalamin, did not affect bradykinin. In contrast, complete blockade of bradykinin-induced relaxation was obtained when combining the non-selective large- and intermediate-conductance ($IK_{Ca}$) $Ca^{2+}$-dependent $K^+$-channel blocker charybdotoxin and apamin with hydroxocobalamin, whereas charybdotoxin + apamin alone were without effect. Inhibition of inwardly rectifying $K^+$ channels ($K_{IR}$) and $Na^+/K^+$ ATPase (with $BaCl_2$ and ouabain, respectively) shifted the bradykinin CRC 10-fold to the right, but did not exert an additional effect on top of hydroxocobalamin. In conclusion, bradykinin-induced relaxation in HCMAs depends on 1) the activation of guanylyl cyclase, $K_{IR}$, and $Na^+/K^+$ ATPase by NO, and 2) $IK_{Ca}$ and $SK_{Ca}$ channels. The latter are activated by a factor other than NO. This factor is not a cytochrome P450 epoxygenase product or $H_2O_2$, nor does it depend on gap junctions or $BK_{Ca}$.

## Introduction

Endothelium-dependent relaxation induced by bradykinin cannot fully be attributed to the release of nitric oxide (NO). In resistance-sized vessels a large proportion of endothelium-derived relaxation involves the release of endothelium-derived hyperpolarizing factors (EDHFs).[68] Putative EDHF candidates are prostacyclin, S-nitrosothiols, $K^+$, cytochrome-P450 products of arachidonic acid (epoxyeicosatrienoic acids, EETs) and hydrogen peroxide ($H_2O_2$),[54,63-65,67,71,102], and EDHF-dependent responses have been reported to involve large-, intermediate- and/or small-conductance $Ca^{2+}$-activated $K^+$ channels ($BK_{Ca}$, $IK_{Ca}$, $SK_{Ca}$), inwardly rectifying $K^+$ ($K_{IR}$) channels, $Na^+$-$K^+$-ATPase and gap junctions.[61,64,65,71,103]

Busse et al.[61] recently summarized all currently available data on EDHF and proposed that EDHF-mediated relaxation (i.e., relaxation observed in the absence of NO) depends on the activation of endothelial $IK_{Ca}$ and $SK_{Ca}$ channels.[64] Such activation results in the release of $K^+$ into the myo-endothelial space, which subsequently induces smooth muscle hyperpolarization by activating $K_{IR}$ channels, $Na^+$-$K^+$-ATPase and/or $BK_{Ca}$ channels.[64] According to this concept, EETs regulate endothelial hyperpolarization as well as the spread of this hyperpolarization to the adjacent smooth muscle cells through myo-endothelial gap junctions. In addition, EETs may directly activate $BK_{Ca}$ channels on smooth muscle cells.[71]

In the present study we set out to verify the above concept in human coronary microarteries (HCMAs). Bradykinin has already been reported to hyperpolarize smooth muscle cells in human coronary arteries,[104] and this hyperpolarization could not be attributed to NO.[73,105]

## Methods

### *Human tissue collection*

HCMAs were obtained from 42 heart beating organ donors (20 men, 22 women, age 3-65 years, mean 46 years), who died of non-cardiac causes (3 cerebrovascular accident, 11 head trauma, 18 subarachnoidal bleeding, 3 post-anoxic encephalopathy, 7 intracranial

bleeding) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the heart valves for transplantation purposes. The study was approved by the Ethics Committee of the Erasmus MC. The hearts were stored in an ice-cold sterile organ-protecting solution after circulatory arrest. After arrival at the laboratory, a tertiary branch of the left anterior descending coronary artery (diameter 160-600 μm, mean 380 μm) was removed and stored overnight in a cold (4°C), oxygenated Krebs bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4.

### *Myograph studies*

Following overnight storage, HCMAs were cut into segments of approximately 2 mm length and mounted in a Mulvany myograph (J.P. Trading) with separated 6-mL organ baths containing Krebs bicarbonate solution, aerated with 95% $O_2$ and 5% $CO_2$, and maintained at 37 °C. Tissue responses were measured as changes in isometric force, using a Harvard isometric transducer. Following a 30-min stabilization period, the optimal internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure as described by Mulvany and Halpern.[89] Endothelial integrity was verified by observing relaxation to 10 nmol/L substance P after preconstriction with 10 nmol/L of the thromboxane $A_2$ analogue U46619. Subsequently, to determine the maximum contractile response, the tissue was exposed to 100 mmol/L KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min in the absence or presence of one or more of the following inhibitors: the bradykinin type 2 ($B_2$) receptor antagonist Hoe140 (1 μmol/L), the NO synthase inhibitor L-NAME (100 μmol/L), the NO scavenger hydroxocobalamin (200 μmol/L), the guanylyl cyclase inhibitor ODQ (10 μmol/L), the $IK_{Ca}$ + $BK_{Ca}$ channel inhibitor charybdotoxin (100 nmol/L), the $SK_{Ca}$ channel inhibitor apamin (100 nmol/L), the $BK_{Ca}$ channel inhibitor iberiotoxin (100 nmol/L), the inwardly rectifying $K^+$ channel ($K_{IR}$) inhibitor $BaCl_2$ (30 μmol/L), the $Na^+$-$K^+$-ATPase inhibitor ouabain (1 mmol/L), the $H_2O_2$ inhibitor catalase (1000 units/ml), the cytochrome P450 epoxygenase inhibitors sulfaphenazole (10 μmol/L) and clotrimazole (50 μmol/L), or the gap junction inhibitors

18α-glycyrrhetinic acid (10 μmol/L) and carbenoxolone (100 μmol/L). Vessels were then preconstricted with U46619 (3-30 nmol/L), and concentration-response curves (CRCs) were constructed to bradykinin. The cyclo-oxygenase inhibitor indomethacin (5 μmol/L) was present during all experiments to suppress spontaneously occurring contractions and relaxations.

### *Cyclic GMP (cGMP) Measurement*

To study bradykinin-induced cGMP production, vessel segments (5-10 mg) were exposed to 1 μmol/L bradykinin in 10 mL oxygenated Krebs bicarbonate solution for 1 min at 37ºC in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (100 μmol/L) following a 30-min preincubation in the absence (control) or presence of ODQ or L-NAME at the above concentrations. Tissues were then frozen in liquid nitrogen, and stored at -80ºC. To determine cGMP, frozen tissues were homogenized in 0.5 mL 0.1 mol/L HCl using a stainless steel ultraturrax (Polytron). Homogenates were centrifuged at 3300 g, and cGMP was measured in 300 μL supernatant by ELISA following acetylation (R&D Systems). Results are expressed as pmol/mg protein. The lower limit of detection was 0.1 pmol/mg protein.

### *Data Analysis*

Data are given as mean±SEM or median and range. Relaxant responses are expressed as a percentage of the contraction to U46619. CRCs were analyzed as described[46] to obtain $pEC_{50}$ ($-^{10}\log EC_{50}$) values. In experiments where no clear maximum effect ($E_{max}$) was reached, $E_{max}$ was defined as the relaxation obtained at the highest bradykinin concentration tested (1 μmol/L). $pEC_{50}$ values were not calculated when $E_{max}$ was < 50%, and in such cases statistical analysis was performed under the assumption that $pEC_{50}$ equaled 6. The addition of L-NAME, ODQ, hydroxocobalamin, charybdotoxin + apamin, iberiotoxin + apamin, or ouabain + $BaCl_2$ increased basal tone by 20-80%. In such cases the concentration of U46619 was adjusted to obtain a preconstriction corresponding with ≈95 % of the maximal contractile response. Statistical analysis of the relaxant responses ($pEC_{50}$ and $E_{max}$) was by t-test, once one-way ANOVA, followed by Dunnett's post-hoc evaluation, had revealed that differences existed between groups. Statistical analysis of

the cGMP data was by Mann-Whitney U-test, because of non-normal distribution of the cGMP levels. $P<0.05$ was considered significant.

## Results

### *Role of NO and $H_2O_2$*

Bradykinin relaxed preconstricted vessel segments in a concentration-dependent manner ($pEC_{50}$ 8.2±0.1, $E_{max}$ 94±2%, n=32; Figure 1). Relaxation was fully prevented by Hoe140 (n=3, data not shown), confirming that it is mediated via $B_2$ receptors.[106] L-NAME (n=8), ODQ (n=5) and hydroxocobalamin (n=14) shifted the bradykinin CRC to the right ($pEC_{50}$ 7.4±0.4, 7.8±0.1, and 7.1±0.3, respectively), although significance was reached for L-NAME ($P<0.05$) and hydroxocobalamin ($P<0.01$) only. When given on top of hydroxocobalamin, L-NAME did not induce a further rightward shift ($pEC_{50}$ 7.2±0.3, n=7). Both L-NAME and hydroxocobalamin reduced $E_{max}$ (from 95±2% to 69±9% and 60±9%, respectively; $P<0.02$), and a similar reduction ($E_{max}$ 73±11%; $P<0.01$) was observed when the two drugs were combined. ODQ did not affect $E_{max}$ (85±8%). Catalase, either alone ($pEC_{50}$ 7.7±0.5, $E_{max}$ 73±13%; n=5) or on top of hydroxocobalamin ($pEC_{50}$ 6.9±0.3, $E_{max}$ 50±12%; n=7), did not affect the bradykinin CRC.

### *Role of $K^+$ channels*

Charybdotoxin + apamin tended to decrease $E_{max}$ (to 73±15%, P=NS vs. control, n=8; Figure 2) without affecting potency ($pEC_{50}$ 7.7±0.5). When given on top of hydroxocobalamin, charybdotoxin + apamin completely abolished bradykinin-induced relaxations in 6 experiments, whereas a >10-fold rightward shift was observed in 2 experiments (difference vs. hydroxocobalamin for all 8 experiments: $P<0.05$). Iberiotoxin + apamin did not affect the bradykinin CRC ($pEC_{50}$ 8.0±0.4, $E_{max}$ 101±1%; n=5), nor did these drugs exert additional effects on top of hydroxocobalamin ($pEC_{50}$ 7.1±0.4, $E_{max}$ 62±15%; n=6). Moreover, in no experiment did these drugs, in combination with hydroxocobalamin, fully block the effects of bradykinin. Ouabain + $BaCl_2$ decreased $E_{max}$ to 53±14% (n=5; $P<0.05$) and shifted the bradykinin CRC 10-fold to the right ($pEC_{50}$

7.1±0.5; P<0.05), but did not exert additional effects on top of hydroxocobalamin ($pEC_{50}$ 6.4±0.4, $E_{max}$ 26±13%; n=5).

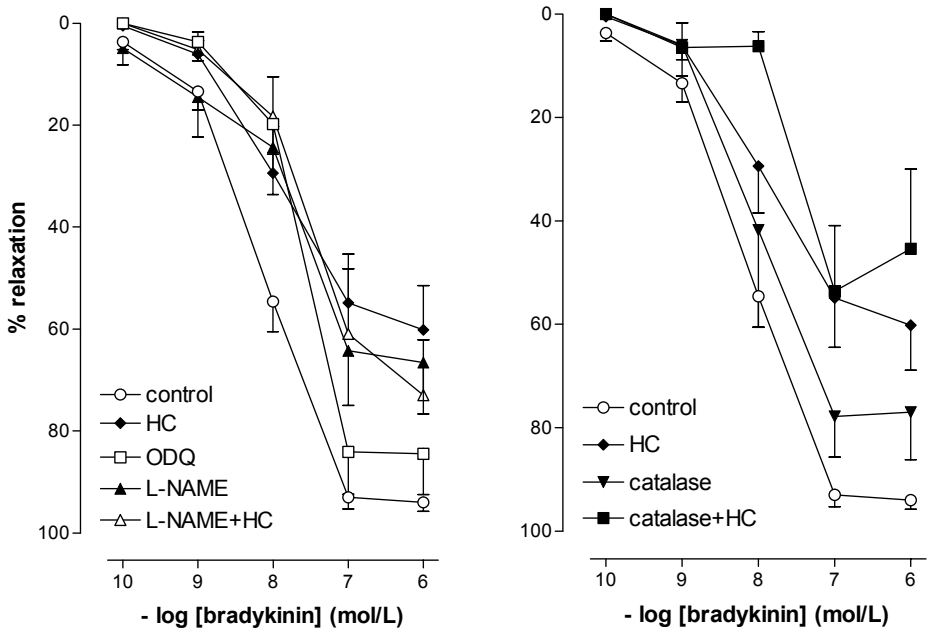



**Figure 1**. *Relaxations of HCMAs, preconstricted with U46619, to bradykinin in absence (control) or presence of one or more of the following inhibitors: 10 µmol/L ODQ, 100 µmol/L L-NAME, 200 µmol/L hydroxocobalamin (HC) or 1000 units/mL catalase. Data (mean±SEM; n=5-32) are expressed as a percentage of the contraction induced by U46619.*

### *Role of EETs and gap junctions*

Clotrimazole (n=5) and sulfaphenazole (n=7) did not affect the bradykinin CRC ($pEC_{50}$ 7.9±0.3 and 8.3±0.2 and $E_{max}$ 89±6% and 85±10%, respectively; Figure 3), nor did clotrimazole exert effects on top of hydroxocobalamin ($pEC_{50}$ 6.7±0.6, $E_{max}$ 63±8%; n=5). Similarly, carbenoxolone (n=5) and 18α-glycyrrhetinic acid (n=5) did not affect bradykinin-induced relaxation when given alone ($pEC_{50}$ 8.2±0.3 and 8.3±0.1 and $E_{max}$ 81±9% and 85±11%, respectively) or on top of hydroxocobalamin ($pEC_{50}$ 6.6±0.5 and 6.7±0.4 and $E_{max}$ 58±13% and 47±17%, respectively; n=5 for each).

*cGMP levels.* Bradykinin increased microvascular cGMP from 3.9 (range 0.1-12.6) to 9.1 (0.7-43) pmol/mg protein (n=11, P<0.01). ODQ and L-NAME reduced cGMP following bradykinin stimulation to 0.1 (0.1-0.5; n=5) and 0.1 (0.1-1.7; n=4) pmol/mg protein, respectively (P<0.001 for both).

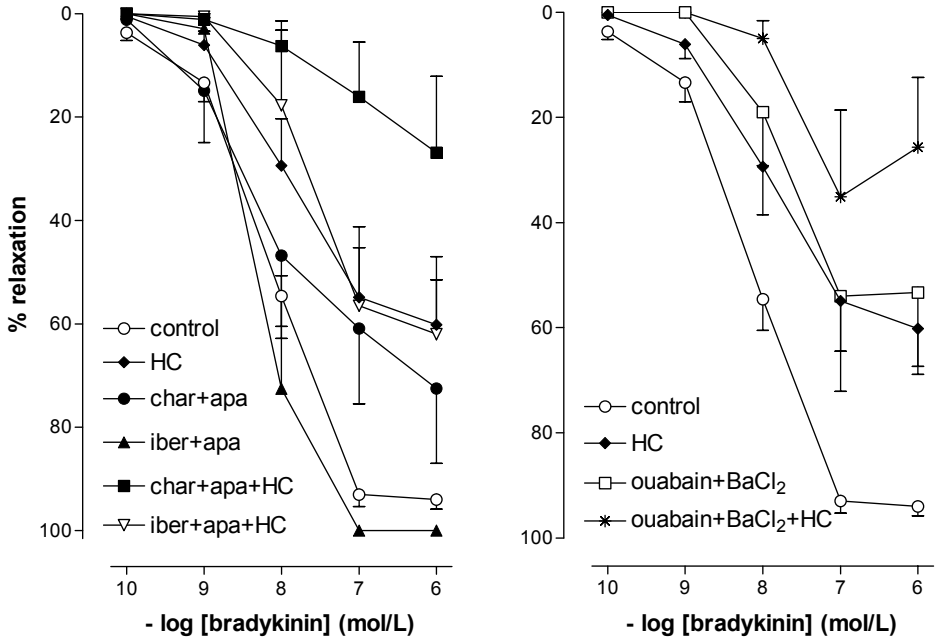



**Figure 2**. *Relaxations of HCMAs, preconstricted with U46619, to bradykinin in absence (control) or presence of 200 µmol/L hydroxocobalamin (HC) with one or more of the following inhibitors: 100 nmol/L charybdotoxin (char), 100 nmol/L apamin (apa), or 100 nmol/L iberiotoxin (iber), 1 mmol/L ouabain + 30 µmol/L $BaCl_2$. Data (mean±SEM; n=5-32) are expressed as a percentage of the contraction induced by U46619.*

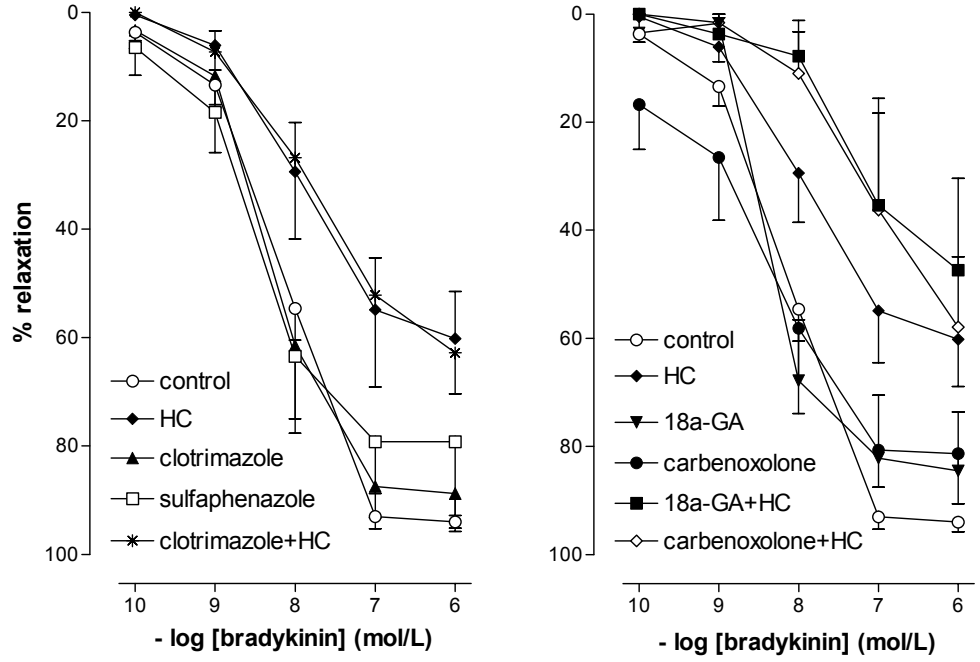



**Figure 3**. *Relaxations of HCMAs, preconstricted with U46619, to bradykinin in absence (control) or presence of 200 µmol/L hydroxocobalamin (HC) with one or more of the following inhibitors: 50 µmol/L clotrimazole, 10 µmol/L sulfaphenazole, 18$\alpha$-glycyrrhetinic acid (18$\alpha$-GA) or 100 µmol/L carbenoxolone. Data (mean±SEM; n=5-32) are expressed as a percentage of the contraction induced by U46619.*

## Discussion

Bradykinin-induced, $B_2$ receptor-mediated relaxation of HCMAs in the presence of indomethacin depends on NO and an EDHF that is not de novo synthesized NO. Both pathways appear to be interchangeable, since inhibiting the EDHF pathway only (with the $K_{Ca}$ channel inhibitors charybdotoxin + apamin) did not significantly shift the bradykinin CRC, whereas combined inhibition of the NO and EDHF pathways (with the NO scavenger hydroxocobalamin and charybdotoxin + apamin) resulted in full blockade of the bradykinin-induced effects in 6 out of 8 experiments. In the two remaining experiments a >10-fold rightward shift of the bradykinin CRC occurred, without an alteration in the maximum effect of bradykinin. The most likely explanation of this latter finding is incomplete scavenging of NO at the hydroxocobalamin concentration that was used in the present study. Its solubility did not allow us to use higher concentrations,[54] and thus, in vessels that release large amounts of NO in response to bradykinin, a rightward shift of the bradykinin CRC rather than complete inhibition of the bradykinin-induced effects will occur at this concentration of hydroxocobalamin. Heterogeneity in bradykinin-induced NO release, as well as the possibility that EDHF replaces NO in vessels where endothelial $B_2$ receptor stimulation no longer results in sufficient NO release, were already predicted in an earlier study investigating the effects of intracoronary Hoe140 application in humans.[106]

In agreement with previous studies in porcine coronary arteries,[54] the rightward shift of the bradykinin CRC in the presence of hydroxocobalamin was larger than the rightward shift in the presence of L-NAME. Similar data were obtained in HCMAs using the NO scavenger HbO and the NOS inhibitor L-NOARG.[73] Taken together, these data suggest the release of NO from a source other than L-arginine, e.g. from NO-containing factors such as *S*-nitrosothiols. Such sources become depleted only upon repeated exposure to bradykinin or following prolonged NOS inhibition.[54,59,107] Interestingly, the guanylyl cyclase inhibitor ODQ did not significantly affect the bradykinin CRC, despite the fact that ODQ fully prevented the 2-3-fold rise in cGMP levels following the exposure of HCMAs to 1 µmol/L bradykinin. This suggests that NO is capable of inducing relaxation through mechanisms other than the guanylyl cyclase-cGMP pathway. Since the blocking

effects of $BaCl_2$ and ouabain towards bradykinin were comparable to the effect of hydroxocobalamin, whereas these drugs did not exert significant additional effects on top of hydroxocobalamin, one possibility is that NO activates $K_{IR}$ channels and/or $Na^+$-$K^+$-ATPase directly. Evidence to support the latter is available.[108,109] Direct activation of $K_{Ca}$ channels by NO,[72,110] seems unlikely, since neither charybdotoxin + apamin, nor iberiotoxin + apamin, affected the bradykinin CRC in the absence of hydroxocobalamin.

With regard to the identity of EDHF, our data confirm the blocking effects of charybdotoxin + apamin, reported by Miura et al.,[105] towards bradykinin in HCMAs in the absence of NO. However, since no significant effects were observed with the selective $BK_{Ca}$ channel inhibitor iberiotoxin on top of hydroxocobalamin, the present results suggest that the effects of the non-selective $IK_{Ca}$ and $BK_{Ca}$ channel inhibitor charybdotoxin are due to blockade of $IK_{Ca}$ channels rather than blockade of $BK_{Ca}$ channels. Thus, the EDHF component of the bradykinin-induced relaxation in HCMAs involves the activation of both $IK_{Ca}$ and $SK_{Ca}$ channels. In endothelial cells, such activation results in the release of $K^+$ in the myo-endothelial space.[64] This $K^+$ subsequently relaxes smooth muscle cells through activation of $K_{IR}$ channels and $Na^+$-$K^+$-ATPase.[111] Although our data are in agreement with this concept, we cannot exclude the possibility that the $IK_{Ca}$ and $SK_{Ca}$ channels are located on smooth muscle cells.

Finally, our data do not support a role for $H_2O_2$, EETs or gap junctions in the bradykinin-induced relaxations of HCMAs, despite previous studies in other human vessels that have demonstrated such a role.[103,112] Apparently, the nature of EDHF differs among vessels from different organs, as it also varies between vessels from different species and even between vessels of different sizes. The lack of effect of the cytochrome P450 epoxygenase inhibitors is in agreement with the non-significant effect of iberiotoxin, since EETs have been reported to exert their effects through activation of $BK_{Ca}$ channels on smooth muscle cells.[71]

In conclusion, bradykinin-induced relaxation in HCMAs depends on 1) the activation of guanylyl cyclase, $K_{IR}$, and $Na^+/K^+$ ATPase by NO, and 2) $IK_{Ca}$ and $SK_{Ca}$ channels. The latter are activated by a factor other than NO. This factor is not a cytochrome P450 epoxygenase product or $H_2O_2$, nor does it depend on gap junctions or $BK_{Ca}$.

## Perspective

Our data are the first to show that the unifying EDHF concept proposed by Busse et al.[61] also applies to human coronary arteries. As such, they form a basis for further investigations on the identity of EDHF, as well as on ways to interfere with EDHF in humans. This is of particular importance in patients with endothelial dysfunction, where $B_2$ receptor-mediated vasorelaxation depends largely on EDHF.[106]

# Chapter 4

## Bradykinin-induced relaxation of coronary microarteries: *S*-nitrosothiols as EDHF?

British Journal of Pharmacology, 2004; 142: 125-135

## Summary

We investigated whether *S*-nitrosothiols, in addition to NO, mediate bradykinin-induced vasorelaxation. Porcine coronary microarteries (PCMAs) were isolated and mounted in Mulvany myographs. Following preconstriction, concentration-response curves (CRCs) were constructed to bradykinin, the NO donors *S*-nitroso-N-penicillamine (SNAP) and diethylamine NONOate (DEA-NONOate) and the *S*-nitrosothiols L-*S*-nitrosocysteine (L-SNC) and D-SNC. All agonists relaxed PCMAs. L-SNC was ≈5-fold more potent than D-SNC. The guanylyl cyclase inhibitor ODQ and the NO scavenger hydroxocobalamin induced a larger shift of the bradykinin CRC than the NO synthase inhibitor L-NAME, although all three inhibitors equally suppressed bradykinin-induced cGMP responses.
Complete blockade of bradykinin-induced relaxation was obtained with L-NAME in the presence of the large- and intermediate-conductance $Ca^{2+}$-activated $K^+$-channel ($BK_{Ca}$, $IK_{Ca}$) blocker charybdotoxin and the small-conductance $Ca^{2+}$-activated $K^+$-channel ($SK_{Ca}$) channel blocker apamin, but not in the presence of L-NAME, apamin and the $BK_{Ca}$ channel blocker iberiotoxin. Inhibitors of cytochrome P450 epoxygenase, cyclo-oxygenase, voltage-dependent $K^+$ channels and ATP-sensitive $K^+$ channels did not affect bradykinin-induced relaxation. SNAP-, DEA-NONOate- and D-SNC-induced relaxations were mediated entirely by the NO-guanylyl cyclase pathway. L-SNC-induced relaxations were partially blocked by charybdotoxin + apamin, but not by iberiotoxin + apamin, and this blockade was abolished following endothelium removal. ODQ, but not hydroxocobalamin, prevented L-SNC-induced increases in cGMP, and both drugs shifted the L-SNC CRC 5-10 fold to the right. L-SNC hyperpolarized intact and endothelium-denuded coronary arteries. Our results support the concept that bradykinin-induced relaxation is mediated via de-novo synthesized NO and a non-NO, endothelium-derived hyperpolarizing factor (EDHF). *S*-nitrosothiols, via stereoselective activation of endothelial $IK_{Ca}$ and $SK_{Ca}$ channels, and through direct effects on smooth muscle cells, may function as an EDHF in porcine coronary microarteries.

## Introduction

Bradykinin relaxes coronary arteries in an endothelium-dependent manner. This effect is mediated via bradykinin type 2 ($B_2$) receptors. $B_2$ receptor activation results in NO synthesis by endothelial NOS, and NO relaxes vascular smooth muscle cells through guanylyl cyclase activation and subsequent cGMP generation.[54] NOS inhibitors however do not completely block bradykinin-induced vasorelaxation, suggesting the existence of either NO-storage sites[59,60] or a non-NO 'endothelium-derived hyperpolarizing factor' (EDHF).[61,113]

EDHF-mediated responses in different arteries have been linked to the release of $K^+$, the generation of cytochrome-P450 products from arachidonic acid (epoxyeicosatrienoic acids, EETs), and to the production of $H_2O_2$.[62-64,66,67,111,114] The identity of EDHF and its contribution to to overall relaxation differs between species, between vascular beds and between vessels of different sizes.[68]

EDHF-mediated relaxation depends on the activation of intermediate- and small-conductance $Ca^{2+}$-activated $K^+$-channels ($IK_{Ca}$, $SK_{Ca}$).[61] These channels are located on endothelial cells,[69,70] and (as a consequence of endothelial hyperpolarization), may be responsible for the subsequent relaxation that is generally attributed to the release of an EDHF.[64,65] This EDHF induces smooth muscle hyperpolarization by activating inwardly rectifying $K^+$ channel ($K_{IR}$) channels, $Na^+$-$K^+$-ATPase and/or large-conductance $Ca^{2+}$-activated $K^+$ ($BK_{Ca}$) channels.[61,71] With regard to the latter, it is important to note that NO itself is capable of inducing hyperpolarization via activation of $Ca^{2+}$-activated $K^+$ channels in vascular smooth muscle.[72]

Bradykinin induces release of NO-containing factors (e.g., *S*-nitrosothiols) from cellular storage sites.[56,59,60] Depletion of NO storage sites occurs only after repeated exposure to bradykinin or following prolonged inhibition of NOS.[59,60] *S*-nitrosothiols induce relaxation through decomposition to NO,[75] or by activating stereoselective recognition sites.[76] These recognition sites could either be cysteine residues within $Ca^{2+}$-activated $K^+$ channels[77] or a novel class of receptors which specifically recognize L-*S*-nitrosocysteine (L-SNC) and structurally similar *S*-nitrosothiols such as L-*S*-nitroso-β,β-dimethylcysteine.[115]

In the present study we set out to investigate the possibility that *S*-nitrosothiols act as an EDHF in porcine coronary microarteries (PCMAs). PCMAs rather than large porcine coronary arteries were used, because the contribution of EDHF to vasorelaxation is larger in smaller vessels.[54,68] We compared the relaxant effects of L-SNC to those of bradykinin and the NO donors *S*-nitroso-N-acetylpenicillamine (N-acetyl-3-(nitrosothio)-D-valine or SNAP) and diethylamine NONOate (DEA-NONOate), both in the absence and presence of an inhibitor of NOS, an inhibitor of guanylyl cyclase, and inhibitors of a wide range of EDHF candidates. To rule out residual NO (i.e., non-EDHF)-mediated effects as much as possible we also made use of the NO scavenger hydroxocobalamin. Guanylyl cyclase activation by NO or NO-containing factors was quantified by measuring cGMP generation. To verify the stereoselectivity of L-SNC-induced effects, parallel experiments were performed with D-*S*-nitrosocysteine (D-SNC). Finally, electrophysiological measurements were performed in intact and endothelium-denuded porcine coronary arteries to verify direct hyperpolarization by bradykinin and L-SNC.

## Methods

### *Drugs*

Bradykinin, SNAP, DEA-NONOate, L-cysteine, D-cysteine, $NaNO_2$, 9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin $F_{2\alpha}$ (U46619), $N^{\omega}$-nitro-L-arginine methyl ester HCl (L-NAME), $N^{\omega}$-nitro-L-arginine (L-NA), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), hydroxocobalamin, indomethacin, diclofenac, glibenclamide, 4-aminopyridine, charybdotoxin, apamin, iberiotoxin, ouabain, $BaCl_2$, sulfaphenazole, miconazole and 3-isobutyl-1-methyl-xanthine were from Sigma-Aldrich (Zwijndrecht, The Netherlands). D-Arg[$Hyp^3$,$Thi^5$,D-$Tic^7$,$Oic^8$]-bradykinin (Hoe140) was a kind gift of Dr. W. Linz, Hoechst, Frankfurt, Germany. Indomethacin, glibenclamide, ouabain and ODQ were dissolved in dimethylsulphoxide. Sulfaphenazole and miconazole were dissolved in ethanol. Hydroxocobalamin was dissolved in methanol. All other chemicals were dissolved in water.

### *Tissue collection*

Pig hearts (n=123) were collected at the local slaughterhouse. Epicardial arteries (diameter ≈1.5 mm) and tertiary branches of the left anterior descending coronary artery (PCMAs; diameter 337±8.4 μm) were removed and either used directly or stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4.

### *Organ bath studies*

PCMAs were cut into segments of ≈2 mm length and mounted in microvascular myographs (J.P. Trading) with separated 6-ml organ baths containing Krebs bicarbonate solution aerated with 95% $O_2$/5% $CO_2$ and maintained at 37°C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30-min stabilization period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure.[89]

The normalised vessel segments were exposed to 30 mM KCl twice. In some vessels, the endothelium was removed by gently rubbing a hair through the lumen of the mounted artery. Endothelial integrity or removal was verified by observing relaxation (or lack thereof) to 10 nM substance P after preconstriction with 10 nM of the thromboxane-$A_2$ analogue U46619. The maximal contractile response to KCl was determined by exposing the tissue to 100 mM KCl. Thereafter, vessels were allowed to equilibrate in fresh organ bath fluid for 30 min in the absence or presence of one or more of the following inhibitors: the NOS inhibitor L-NAME (100 μM), the NO scavenger hydroxocobalamin (200 μM), the guanylyl cyclase inhibitor ODQ (10 μM), the COX inhibitor indomethacin (10 μM), the $IK_{Ca}$ + $BK_{Ca}$ channel inhibitor charybdotoxin (100 nM), the $SK_{Ca}$ channel inhibitor apamin (100 nM), the $BK_{Ca}$ channel inhibitor iberiotoxin (100 nM), the voltage-dependent $K^+$ channel ($K_v$) inhibitor 4-aminopyridine (5 mM), the ATP-sensitive $K^+$ channel ($K_{ATP}$) inhibitor glibenclamide (1 μM), the $K_{IR}$ inhibitor $BaCl_2$ (30 μM), the $Na^+$-$K^+$-ATPase inhibitor ouabain (0.5 mM), the cytochrome P450 epoxygenase inhibitors sulfaphenazole or miconazole (10 μM) or the $B_2$ receptor antagonist Hoe140 (1 μM).

Vessels were then preconstricted with U46619, and concentration-response curves (CRCs) were constructed to bradykinin, SNAP, DEA-NONOate, L-SNC or D-SNC. L-SNC and D-SNC were prepared immediately prior to the experiment and stored in the dark below 0°C. In short, 50 µL of a 0.2 M solution of L-cysteine, D-cysteine was mixed with 50 µL 0.2 M $NaNO_2$. The subsequent addition of 10 µL 1 M HCl resulted in a stable 0.1 M solution (pH ≈ 5) of the respective SNC isomers.[76] Preliminary studies with $NaNO_2$, L-cysteine and D-cysteine (n=3 each) revealed that, separately, these drugs did not exert relaxant effects in preconstricted PCMAs (data not shown).

### *Cyclic GMP measurement*

To study bradykinin- and L-SNC induced cGMP production, vessel segments (5-10 mg) were exposed to bradykinin (1 µM) or L-SNC (10 or 100 µM) in 10 ml oxygenated Krebs bicarbonate solution for 1 min at 37ºC in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (100 µM), following a 30-min preincubation in the absence (control) or presence of hydroxocobalamin, ODQ, Hoe140 and/or L-NAME at the above concentrations. Tissues were subsequently frozen in liquid nitrogen, and stored at -80ºC. To determine cGMP, frozen tissues were homogenized in 0.5 mL 0.1 M HCl using a stainless steel ultraturrax (Polytron). Homogenates were centrifuged at 3300 g, and cGMP was measured in 300 µL supernatant by ELISA following acetylation (R&D Systems). Experiments were performed in quadruplicate, and results are expressed as pmol $mg^{-1}$ protein. The lower limit of detection was 0.1 pmol $mg^{-1}$ protein.

### *Electrophysiological measurements*

Freshly isolated epicardial artery segments (≈40 mm length) were excised, slit and mounted in heated (37°C) chambers and maintained in modified Tyrode's solution (in mM: NaCl 132, KCl 4, $CaCl_2$ 1.6, $MgCl_2$ 1.2, $NaH_2PO_4$ 0.36, $NaHCO_3$ 23.8, $Ca^{2+}$-EDTA 0.05, glucose 10; gassed with 20% $O_2$/5% $CO_2$/75 % $N_2$, pH 7.4) containing the NOS inhibitor L-NA (300 µM), the COX inhibitor diclofenac (10 µM), and U46619 (1 µM) to mimic conditions in the organ chamber experiments as closely as possible.

Both endothelium intact and endothelium-denuded segments were used. Smooth muscle membrane potential was recorded by impaling cells through the intima as described.[63] Bradykinin (100 nM) and L-SNC (50 μM) were applied as bolus injections into the bath.

### *Data analysis*

Data are given as mean±s.e.mean. Contractile responses are expressed as a percentage of the contraction to U46619. CRCs were analysed as described using the logistic function described by de Lean et al.[116] to obtain $pEC_{50}$ ($-^{10}logEC_{50}$) values (Table 1). L-NAME, ODQ, hydroxocobalamin and/or ouabain+$BaCl_2$ increased basal tone by 10-40%, whereas 4-aminopyridine increased basal tone by 80%. In such cases the concentration of U46619 (range 10-30 nM) was adjusted to obtain a preconstriction corresponding to ≈95% of the maximal contractile response in all vessels. Statistical analysis was by paired t-test, once one-way ANOVA, followed by Dunnett's post-hoc evaluation, had revealed that differences existed between groups. $P<0.05$ was considered significant.

## Results

### *Mechanism of Bradykinin-Induced Relaxation*

Bradykinin relaxed preconstricted vessel segments in a concentration-dependent manner ($pEC_{50}$ = 8.2±0.1, n=45; Figure 1). Bradykinin-induced relaxations were unaffected by indomethacin and abrogated by Hoe140. L-NAME shifted the bradykinin CRC ≈5-fold to the right, whereas ODQ and hydroxocobalamin induced a ≈10-fold rightward shift (see Table 1 for $pEC_{50}$ values). Apamin, iberiotoxin and charybdotoxin, separately or in combination, did not significantly affect the bradykinin CRC (Figure 2, Table 1), nor did ouabain + $BaCl_2$ (Figure 1, Table 1).

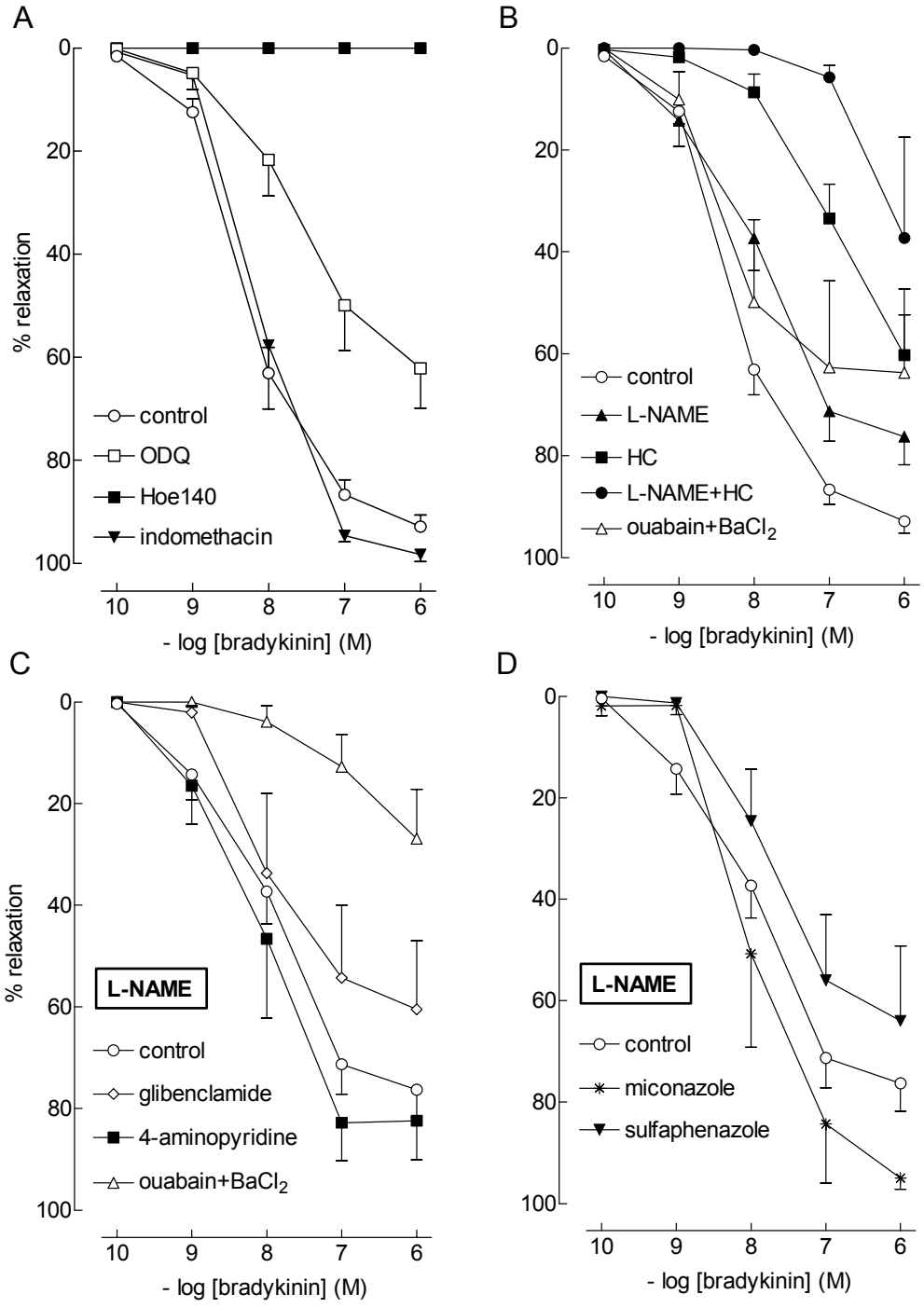



**Figure 1**. *Relaxations of PCMAs, preconstricted with U46619, to bradykinin in absence (control; A, B) or presence of of 100 µM L-NAME (C, D) with one or more of the following inhibitors: 1 µM Hoe140, 10 µM ODQ, 10 µM indomethacin, 100 µM L-NAME, 200 µM hydroxocobalamin (HC), 0.5 mM ouabain, 30 µM $BaCl_2$, 1 µM glibenclamide, 5 mM 4-aminopyridine, 10 µM miconazole or 10 µM sulfaphenazole. Data (mean±s.e.mean; n=5-45) are expressed as a percentage of the contraction induced by U46619.*

**Table 1.** *$pEC_{50}$ values as calculated for the bradykinin, SNAP, L-SNC or D-SNC CRCs in the absence or presence of several inhibitors*

| Inhibitor | $pEC_{50}$ | | | |
|---|---|---|---|---|
| | **Bradykinin** | **SNAP** | **L-SNC** | **D-SNC** |
| none | 8.2±0.1 (40) | 7.1±0.1 (14) | 6.5±0.1 (22)# | 6.0±0.1 (18) |
| Hoe140 | <6 (3)* | | | |
| indomethacin | 8.0±0.1 (5) | | | |
| ODQ | 7.2±0.2 (15)* | 5.7±0.1 (11)* | 5.3±0.1 (9)* | 5.3±0.1 (13)* |
| hydroxocobalamin | 6.8±0.2 (16)* | 6.5±0.2 (11)* | 5.4±0.2 (8)† | 5.5±0.1 (13)* |
| L-NAME | 7.7±0.1 (23)* | | 7.5±0.3 (5) | |
| apamin | 8.5±0.2 (7) | | 7.2±0.1 (4) | |
| charybdotoxin | 8.2±0.2 (8) | | | |
| iberiotoxin | 8.4±0.2 (6) | | 7.1±0.1 (4) | |
| charybdotoxin + apamin | 7.6±0.3 (7) | 7.2±0.1 (6) | 6.0±0.1 (10)* | 5.9±0.1 (13) |
| iberiotoxin + apamin | 8.8±0.2 (6) | | 7.1±0.2 (5) | |
| ouabain+$BaCl_2$ | 8.7±0.1 (6) | | 6.7±0.1 (4)† | |
| glibenclamide | | | 7.2±0.1 (5) | |
| 4-aminopyridine | | | 7.1±0.5 (4) | |
| no endothelium | | | 7.3±0.1 (9)† | |
| no endothelium + charybdotoxin + apamin | | | 7.0±0.1 (4) | |
| ODQ + hydroxocobalamin | | <5 (5)*‡ | 4.6±0.3 (5)*§ | 4.3±0.2 (12)*‡ |
| ODQ + charybdotoxin + apamin | | 5.6±0.2 (5)* | 5.3±0.1 (5)* | 5.2±0.1 (5)* |
| hydroxocobalamin + charybdotoxin + apamin | | 6.3±0.2 (6)† | 5.3±0.2 (5)† | 5.7±0.1 (5)† |
| ODQ + hydroxocobalamin + charybdotoxin + apamin | | <5 (3)*‡ | 4.5±0.2 (5)*‡ | 4.3±0.2 (8)*‡ |

Data are mean±SEM (n value); *P<0.01, †P<0.05 vs none; ‡P<0.01, §P<0.05 vs hydroxocobalamin or ODQ; #P<0.05 vs D-SNC.

In the presence of L-NAME, apamin and charybdotoxin, when given separately, did not affect the bradykinin CRC ($pEC_{50}$s 7.8±0.3 and 8.2±0.2, respectively, n=6 for each; Figure 2), nor did glibenclamide, 4-aminopyridine, sulfaphenazole and miconazole ($pEC_{50}$s 7.8±0.5, 8.0±0.3, 7.8±0.4 and 7.9±0.6 , respectively, n=5 for each; Figure 1). In contrast, when given in addition to L-NAME, charybdotoxin + apamin fully blocked the bradykinin-induced responses (Figure 2, n=5), whereas hydroxocobalamin (n=5) and ouabain + $BaCl_2$ (n=5) shifted the bradykinin CRC >100-fold ($P<0.01$; Figure 1) to the right. Iberiotoxin, without (n=6) or with (n=6) apamin, reduced the maximum effect of bradykinin in the presence of L-NAME ($P<0.01$; Figure 2), without altering its $pEC_{50}$ (7.8±0.5 and 8.1±0.2, respectively).

Thus, NO and/or NO-containing factors as well as $Ca^{2+}$-activated $K^+$-channels ($BK_{Ca}$, $IK_{Ca}$ and $SK_{Ca}$), $K_{IR}$ channels, and $Na^+$-$K^+$-ATPase are involved in the bradykinin-induced relaxation, and the NO-induced effects are mediated, at least in part, via activation of guanylyl cyclase. No evidence for a role of $K_v$ channels, $K_{ATP}$ channels, COX products, or cytochrome P450 epoxygenase products was obtained.

### *Mechanism of NO-Induced Relaxation*

SNAP and DEA-NONOate relaxed preconstricted coronary microvessels in a concentration-dependent manner (Figures 3 and 4; Table 1). Both hydroxocobalamin and ODQ shifted the SNAP and DEA-NONOate-induced CRC to the right and, in combination, completely blocked SNAP-induced relaxation. Charybdotoxin + apamin, either as combination or together with hydroxocobalamin or ODQ, did not elicit a rightward shift in the SNAP or DEA-NONOate CRC.

Thus, the relaxation induced by exogenous NO depends entirely on activation of guanylyl cyclase, and does not involve $Ca^{2+}$-activated $K^+$-channels.

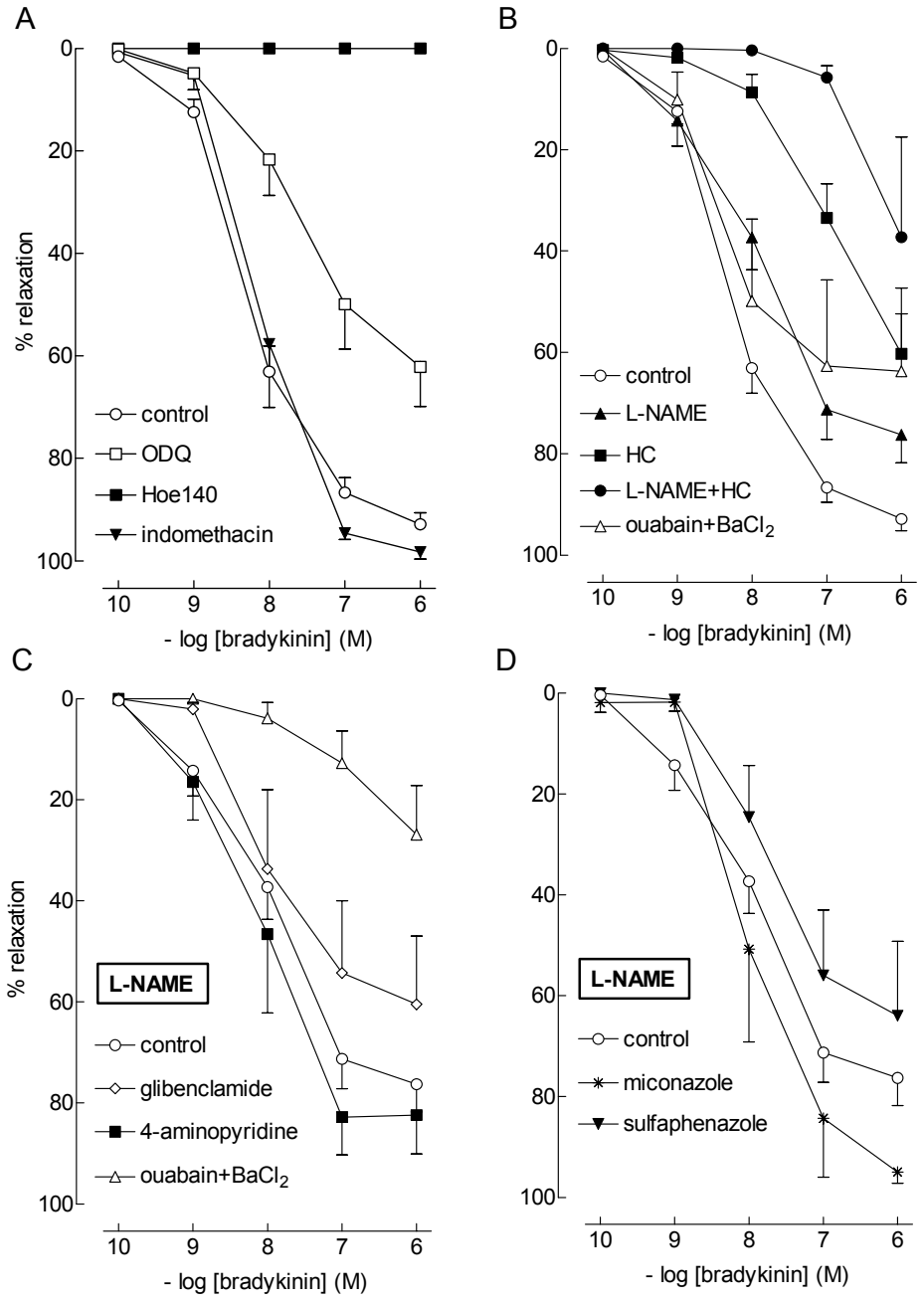



**Figure 2**. *Relaxations of PCMAs, preconstricted with U46619, to bradykinin in absence (control; A, B) or presence of 100 µM L-NAME (C, D) with one or more of the following inhibitors: 100 nM charybdotoxin (char), 100 nM apamin (apa), or 100 nM iberiotoxin (iber). Data (mean±s.e.mean; n=5-45) are expressed as a percentage of the contraction induced by U46619.*

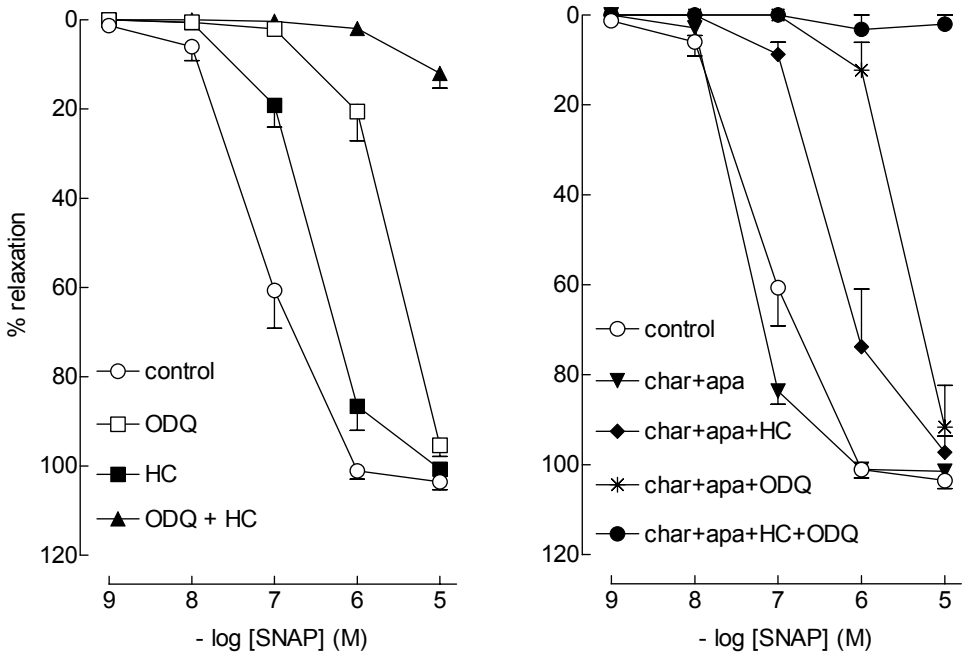



***Figure 3**. Relaxations of PCMAs, preconstricted with U46619, to SNAP in the absence (control) or presence of one or more of the following inhibitors: 10 µM ODQ, 200 µM hydroxocobalamin (HC), 100 nM charybdotoxin (char) or 100 nM apamin (apa). Data (mean±s.e.mean; n=5-14) are expressed as a percentage of the contraction induced by U46619.*

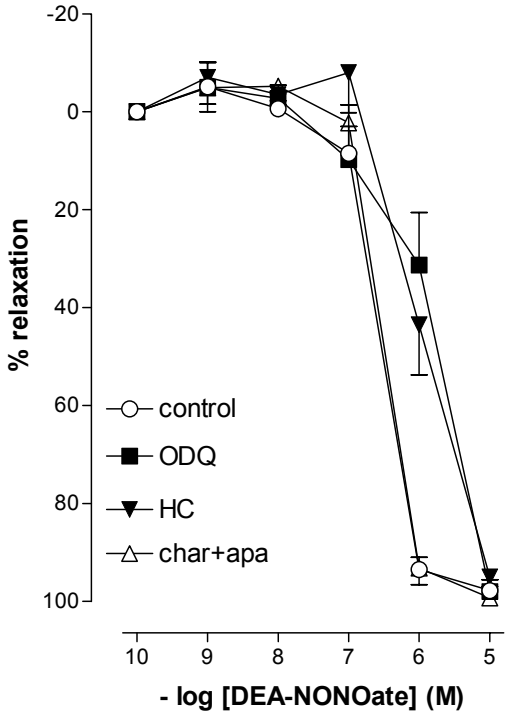



***Figure 4.** Relaxations of PCMAs, preconstricted with U46619, to DEA-NONOate in the absence (control) or presence of one or more of the following inhibitors: 10 µM ODQ, 200 µM hydroxocobalamin (HC), 100 nM charybdotoxin (char) or 100 nM apamin (apa). Data (mean±s.e.mean; n=4) are expressed as a percentage of the contraction induced by U46619.*

### *Mechanism of S-nitrosothiol-induced relaxation*

L-SNC and D-SNC relaxed preconstricted PCMAs in a concentration-dependent manner. L-SNC was 5 times more potent than D-SNC ($P<0.05$; Figure 5, Table 1). ODQ and hydroxocobalamin shifted the CRCs of both L-SNC and D-SNC 5-10-fold to the right (P=NS for the difference in rightward shift between L-SNC and D-SNC) and, when combined, caused a further rightward shift (Table 1).

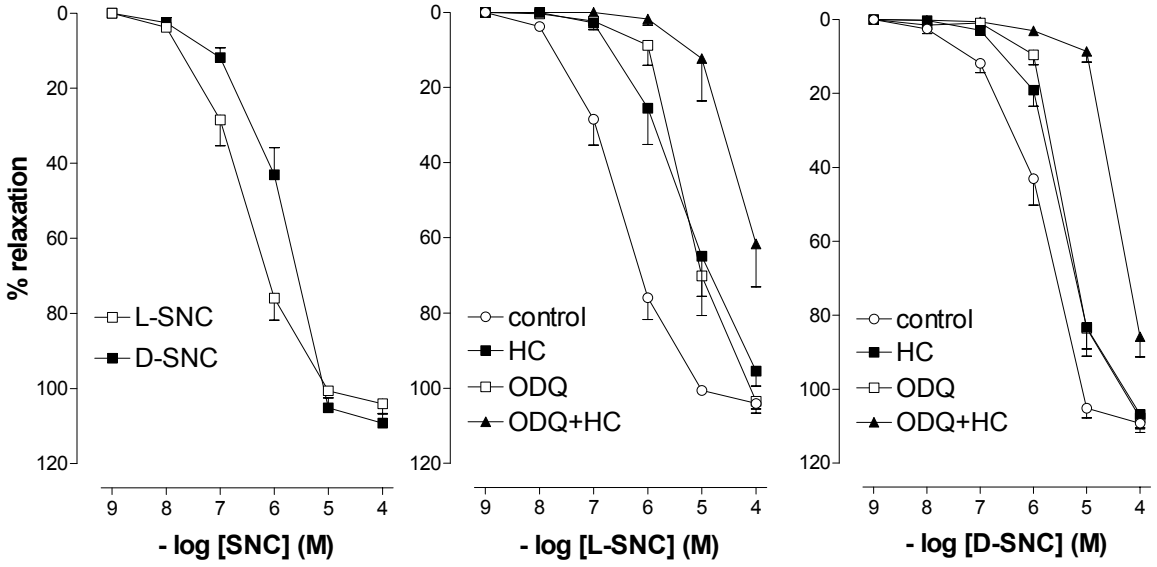



**Figure 5.** *Relaxations of PCMAs, preconstricted with U46619, to L-SNC or D-SNC in the absence (control) or presence of 10 μM ODQ and/or 200 μM hydroxocobalamin (HC). Data (mean±s.e.mean; n=4-18) are expressed as a percentage of the contraction induced by U46619.*

Charybdotoxin + apamin shifted the L-SNC CRC, but not the D-SNC CRC, 5-10 fold to the right (Figure 6, Table 1). Endothelium-denudation shifted the L-SNC CRC 5-10 fold to the left, and abolished the rightward shift induced by charybdotoxin + apamin (Figure 6, Table 1). Charybdotoxin + apamin did not have additional effects on top of ODQ, hydroxocobalamin (Table 1) or ODQ + hydroxocobalamin (Figures 5 and 6, Table 1) with either L-SNC or D-SNC.

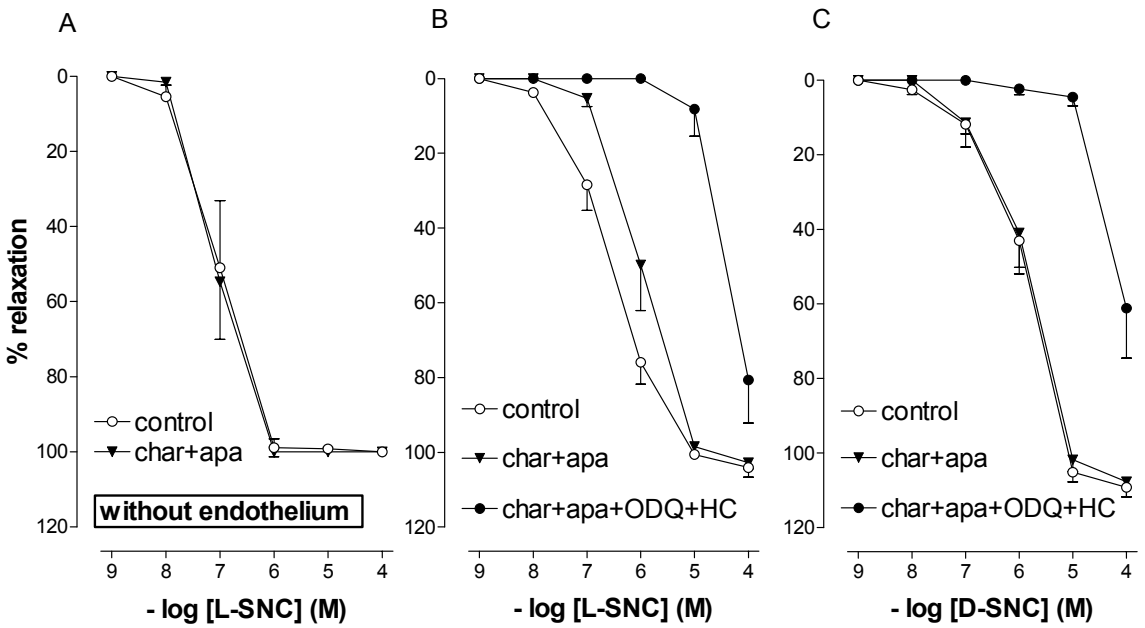



**Figure 6**. *Relaxations of PCMAs without (A) or with (B, C) endothelium, preconstricted with U46619, to L-SNC or D-SNC in the absence (control) or presence of one or more of the following inhibitors: 10 µM ODQ, 200 µM hydroxocobalamin (HC), 100 nM charybdotoxin (char) or 100 nM apamin (apa). Data (mean±s.e.mean; n=4-18) are expressed as a percentage of the contraction induced by U46619.*

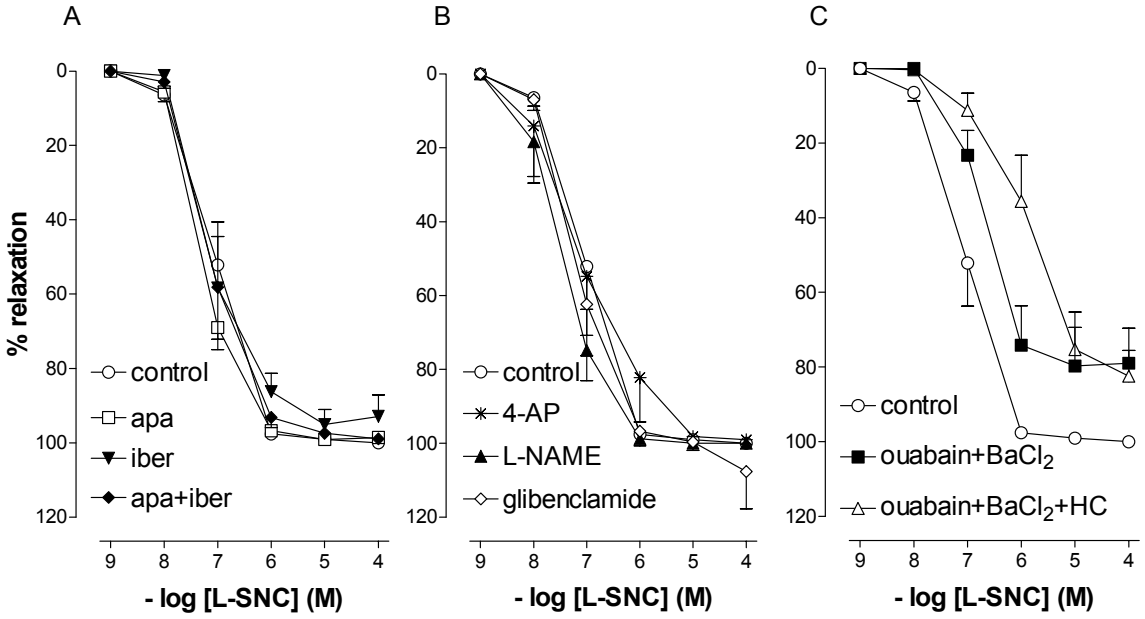



**Figure 7**. *Relaxations of PCMAs, preconstricted with U46619, to L-SNC in the absence (control) or presence of one or more of the following inhibitors: 100 nM iberiotoxin (iber), 100 nM apamin (apa), 5 mM 4-aminopyridine (4-AP), 100 µM L-NAME, 1 µM glibenclamide, 0.5 mM ouabain or 30 µM $BaCl_2$. Data (mean±s.e.mean; n=4-9) are expressed as a percentage of the contraction induced by U46619.*

Glibenclamide, 4-aminopyridine, L-NAME, apamin, iberiotoxin, and iberiotoxin + apamin did not affect the L-SNC CRC (Figure 7, Table 1).

Ouabain + $BaCl_2$ shifted the L-SNC CRC 5-fold to the right (Figure 7, Table 1) but did not exert an additional effect on top of hydroxocobalamin ($pEC_{50}$ 5.9±0.2, n=4; Figures 5 and 7).

Thus, *S*-nitrosothiol-induced relaxation occurs in a stereoselective manner, and is mediated via activation of guanylyl cyclase, endothelial $IK_{Ca}$ and $SK_{Ca}$ channels, $K_{IR}$ channels and the $Na^+$-$K^+$-ATPase. Neither NOS, $K_v$ channels, $K_{ATP}$ channels, nor $BK_{Ca}$ channels appear to mediate this response.

### *Cyclic GMP*

Baseline cGMP levels were 9.4±2.7 pmol $mg^{-1}$ protein (n=16). Bradykinin increased the microvascular cGMP levels 4-5 fold (Figure 8; $P<0.05$ vs. control). Hoe140 largely prevented this increase, whereas L-NAME, hydroxocobalamin and ODQ reduced the microvascular cGMP content following bradykinin stimulation to levels below baseline. The combination of hydroxocobalamin and L-NAME did not further decrease cGMP levels. L-SNC increased cGMP >5-fold ($P<0.05$ vs control). ODQ fully prevented the increase induced by 10 μM L-SNC, and a similar tendency was observed for hydroxocobalamin (P=NS). ODQ did not fully prevent the increase induced by by 100 μM L-SNC.

### *Electrophysiological measurements*

Both bradykinin (n=6) and L-SNC (n=6) hyperpolarized vascular smooth muscle cells ($P<0.01$) in porcine coronary arteries (Figure 9). The effect of L-SNC was not affected by the removal of the endothelium (n=5).

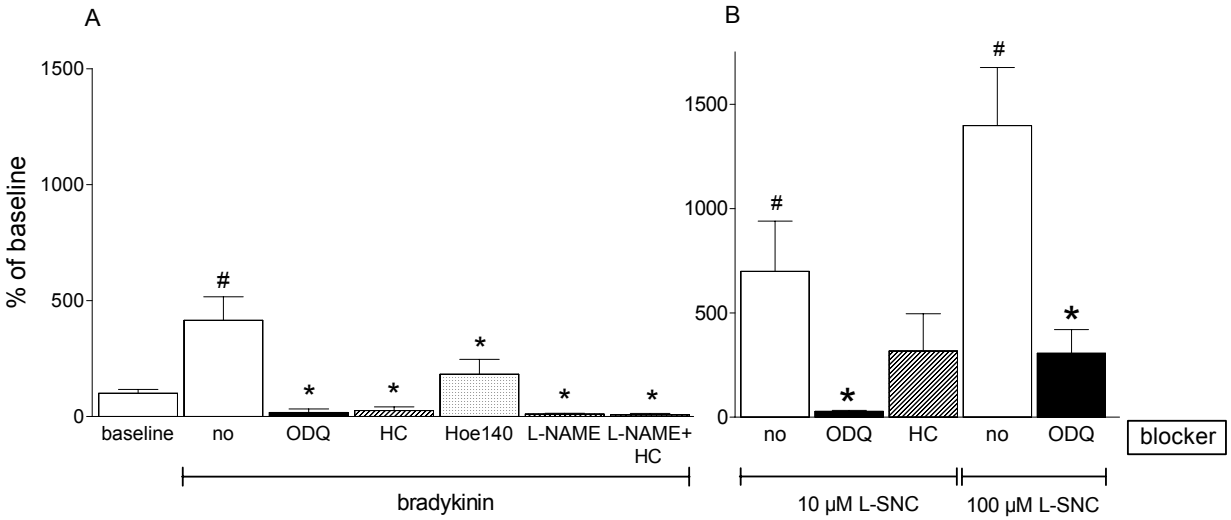



**Figure 8.** *Cyclic GMP levels (expressed as % of baseline) in PCMAs after 1 min exposure to (A) bradykinin (1 µM) or (B) L-SNC (10 µM or 100 µM) under control conditions (no blocker) and in the presence of 10 µM ODQ, 200 µM hydroxocobalamin (HC), 1 µM Hoe140 and/or 100 µM L-NAME. Data are mean±s.e.mean (n=3-10). # P<0.05 vs. control, * P<0.05 vs. no blocker.*

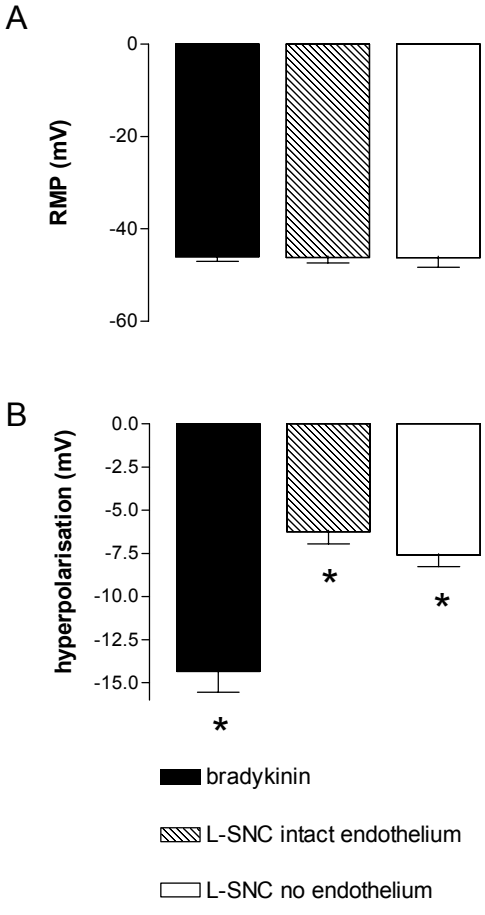



**Figure 9.** *Hyperpolarization of smooth muscle cells by 100 nM bradykinin and 50 µM L-SNC in porcine coronary arteries with or without endothelium. A, resting membrane potential (RMP). B, change in membrane potential. Experiments were performed in the presence of 300 µM L-NA, 10 µM diclofenac and 1 µM U46619. Data are mean±s.e.mean of 5-6 separate experiments; * P<0.01 vs. control. U46619 did not significantly affect RMP (–44.5±1.2 mV vs. –42.7± 1.7 mV, n=6), and in parallel experiments, using arterial rings from the same pig and following preconstriction with the same U46619 concentration (1 µM), 100 nM bradykinin relaxed the arteries by 89±9% (n=6).*

## Discussion

The present study shows that $B_2$ receptor-mediated vasodilation in porcine coronary microarteries involves the NOS/NO/guanylyl cyclase/cGMP pathway and $Ca^{2+}$-activated $K^+$-channels, but not COX products or $K_{ATP}$ channels. This is in full agreement with the concept that both NO and an EDHF that is not de novo synthesized NO determine bradykinin-induced relaxation. The two pathways appear to be interchangeable, since blocking each pathway separately (with L-NAME and charybdotoxin + apamin, respectively) only marginally affected bradykinin-mediated relaxation, whereas blocking both pathways together abrogated the effects of bradykinin. The modest effect of blocking NOS in the present study opposes earlier data in large porcine coronary arteries, where L-NAME alone induced a ≈10-fold rightward shift of the bradykinin CRC.[54] Apparently, as has been suggested before, de novo synthesized NO is of greater importance in large arteries, and the contribution of EDHF is larger in microarteries.[68]

The $BK_{Ca}$ channel inhibitor iberiotoxin, with or without the $SK_{Ca}$ channel inhibitor apamin, reduced the maximum effect but not the potency of bradykinin in the presence of L-NAME. This finding, combined with the lack of effect of the $K_v$ channel inhibitor 4-aminopyridine, suggests that the complete inhibition of bradykinin-induced relaxation obtained with charybdotoxin (a non-selective inhibitor of $BK_{Ca}$, $IK_{Ca}$ and $K_v$ channels) in the presence of apamin and L-NAME can be attributed to the blockade of all three types of $K_{Ca}$ channels.

$BK_{Ca}$ channels are located on vascular smooth muscle cells.[71] Although endothelial EETs are believed to activate these channels,[61,63,64,71,117-119] the lack of effect of the cytochrome P450 epoxygenase inhibitors miconazole and sulfaphenazole excludes this possibility in our experimental setup.

$IK_{Ca}$ and $SK_{Ca}$ channels are expressed in endothelial cells [69,70],[69,70] and their activation results in endothelial hyperpolarization and the accumulation of $K^+$ in the myo-endothelial space. This $K^+$ is believed to subsequently hyperpolarize vascular smooth muscle cells by activating $K_{IR}$ channels and/or the $Na^+$-$K^+$-ATPase.[61,64,65] The inhibitory effect of $BaCl_2$ and ouabain towards bradykinin in the presence of L-NAME confirms this concept in PCMAs.

### *S-Nitrosothiols as EDHF?*

We propose that NO-containing/releasing factors, *S*-nitrosothiols in particular, act as an EDHF in PCMAs. The contribution of such factors is supported by our observations that the guanylyl cyclase inhibitor ODQ and the NO scavenger hydroxocobalamin inhibited the bradykinin-induced effects to a much greater degree than L-NAME, and that, in combination, L-NAME + hydroxocobalamin almost completely prevented bradykinin-induced relaxations. Since *S*-nitrosothiol-induced relaxations occur through activation of stereoselective recognition sites and/or via their decomposition to NO,[76,120] we used both L-SNC and D-SNC to verify this proposal.

L-SNC was ≈5 times more potent than D-SNC. This difference disappeared in the presence of charybdotoxin + apamin (but not in the presence of iberiotoxin with or without apamin), suggesting that L-SNC, but not D-SNC, hyperpolarizes endothelial cells via $IK_{Ca}$ and $SK_{Ca}$ channel activation. The comparable rightward shift of the L-SNC CRC in the presence of ouabain + $BaCl_2$ and charybdotoxin + apamin is in agreement with the concept that such hyperpolarization results in endothelial $K^+$ release and subsequent smooth muscle cell hyperpolarization. In further support of this hypothesis, endothelium-denudation abolished the effect of charybdotoxin + apamin towards L-SNC, and L-SNC reduced the membrane potential of smooth muscle cells in intact porcine coronary arteries.

Unexpectedly however, the removal of the endothelium potentiated L-SNC 5-10 fold. This suggests that L-SNC, like other endothelium-dependent vasodilators, not only hyperpolarizes endothelial cells, but also induces the release of an endothelium-derived contractile factor.[121] Alternatively, endothelial denudation might uncover direct L-SNC-induced effects on smooth muscle cells, as evidenced by the fact that L-SNC also hyperpolarized endothelium-denuded coronary arteries. One such direct effect is $BK_{Ca}$ channel activation via *S*-nitrosylation of cysteine residues.[77] However, the lack of effect of charybdotoxin + apamin towards L-SNC in endothelium-denuded vessels does not support this concept in PCMAs.

Taken together, the relaxant effects of L-SNC, like those of bradykinin, involve $K_{Ca}$ channels, and they occur, at least in part, in a stereoselective manner.

The greater potency of L-SNC versus D-SNC is in agreement with previous in vivo studies,[76,120] and may indicate the existence of binding sites that specifically recognize L-SNC and structurally related *S*-nitrosothiols. These binding sites may either be novel receptors or 'nitrosation motifs' in functional proteins such as receptors and ion channels.[77,115,122,123]

### *NO Release from S-Nitrosothiols?*

The effects of L-SNC, at the concentrations used in the present study, are unlikely to be due entirely to its decomposition to NO, nor do they involve de novo NO generation by NOS. First, NO did not activate $Ca^{2+}$-activated $K^+$ channels in PCMAs, because the dilatory effects of the NO donors SNAP and DEA-NONOate were unaffected by charybdotoxin and apamin. Second, L-NAME did not affect L-SNC-mediated responses. Third, detectable NO production has been reported to occur at *S*-nitrosothiol concentrations above 100 μM only,[124] i.e., at concentrations that are >100 times above the $EC_{50}$ value of L-SNC in the present study. Fourth, the ODQ, but not hydroxocobalamin, fully prevented the L-SNC-induced increases in cGMP. This suggests direct, NO-independent, activation of guanylyl cyclase by L-SNC, in agreement with a previous study in cultured vascular smooth muscle cells.[125] Alternatively, the concentration of hydroxocobalamin used in the present study may have been too low to scavenge all NO generated following L-SNC application.[126]

Taken together, the following mechanisms may underlie L-SNC-induced vasorelaxation: direct activation of endothelial $IK_{Ca}$ and $SK_{Ca}$ channels, direct activation of guanylyl cyclase in smooth muscle cells, and decomposition to NO. Simultaneous inhibition of all mechanisms (with charybdotoxin + apamin, ODQ and hydroxocobalamin, respectively) did not fully prevent the relaxations induced by the highest concentration of L-SNC (100 μM). This could relate to the inability of hydroxocobalamin to scavenge all NO[126] and/or the competitive inhibition of guanylyl cyclase by ODQ,[127] allowing full blockade of the cGMP increases and relaxations induced by 10 μM L-SNC, but not of those induced by a tenfold higher L-SNC concentration (Figures 6 and 8).

### *Release of S-nitrosothiols?*

Finally, despite the fact that L-SNC is capable of exerting EDHF-like effects, direct evidence demonstrating that L-SNC (or a related compound) mediates bradykinin-induced, EDHF-dependent relaxation is currently lacking. Previous studies support the existence of preformed pools of NO-containing factors (such as *S*-nitrosothiols) in endothelial and vascular smooth muscle cells.[54,59,107,128] These pools become depleted after repeated exposure to endothelium-dependent agonists such as acetylcholine and bradykinin, following prolonged NOS inhibition, or after exposure to UV.[54,59,107]

We did not measure *S*-nitrosothiol release following bradykinin stimulation in the present study. Such release may occur in a specific compartment (e.g., the myo-endothelial space, gap junctions, intra-endothelial) that does not allow easy detection. Moreover, since it depends on preformed pools, it cannot be monitored by measuring the vascular *S*-nitrosothiol content following bradykinin stimulation. Similar difficulties exist with regard to EETs,[71,118] and it has therefore been proposed that these cytochrome-P450 products contribute to the activation of endothelial $K^+$ channels as second messengers,[61] rather than being released from endothelial cells in large amounts.

### *Clinical perspective*

*S*-nitrosylated proteins, the most abundant of which is albumin, are present in micromolar concentrations in normal subjects.[122] They are thought to serve both as a source and a sink of NO, thereby buffering the concentration of free NO. A recent in vivo study showed that *S*-nitrosothiols induce dilator responses in human conduit and resistance arteries that are comparable with those of bradykinin and acetylcholine,[129] and it has therefore been suggested[130] that *S*-nitrosothiols provide a new pharmacological route for delivering NO regionally. Our data extend these findings, by implying not only that *S*-nitrosothiols may act by inducing hyperpolarization in microarteries (i.e., exert NO-independent effects), but also by showing that their effects occur in a stereoselective manner.

# Chapter 5

## L-*S*-nitrosothiols: endothelium-derived hyperpolarizing factors in porcine coronary arteries?

Journal of Hypertension, 2004; 22: 1927-1936

## Summary

Bradykinin-induced, endothelium-derived hyperpolarizing factor (EDHF)-mediated responses depend on $Ca^{2+}$-dependent $K^+$-channels ($K_{Ca}$) of small ($SK_{Ca}$) and intermediate ($IK_{Ca}$) conductance, inwardly rectifying $K^+$ ($K_{IR}$) channels and/or $Na^+$-$K^+$-ATPase. Here we investigated in porcine coronary arteries (PCAs) whether *S*-nitrosothiols can act as EDHF. Preconstricted PCAs were exposed to bradykinin, the NO donor *S*-nitroso-N-penicillamine (SNAP), or the *S*-nitrosothiols L-*S*-nitrosocysteine (L-SNC), D-SNC and L-*S*-nitrosoglutathione (L-SNG), with or without KCl, the NO scavenger hydroxocobalamin, the *S*-nitrosothiol-depleting agent p-hydroxymercurobenzoic acid (PHMBA) and/or inhibitors of NO synthase (L-NAME), guanylyl cyclase (ODQ), $SK_{Ca}$ channels (apamin), $K_{Ca}$ channels of large conductance ($BK_{Ca}$) (iberiotoxin), $IK_{Ca}$ + $BK_{Ca}$ channels (charybdotoxin), $K_{IR}$ channels ($BaCl_2$) or $Na^+$-$K^+$-ATPase (ouabain). All agonists concentration-dependently relaxed PCAs. L-NAME, charybdotoxin + apamin, KCl, and ouabain shifted the bradykinin concentration-response curve (CRC) ≈10-fold to the right. $BaCl_2$ did not exert additional effects on top of ouabain. Full blockade of bradykinin was obtained when combining L-NAME with charybdotoxin + apamin, KCl or ouabain + $BaCl_2$. PHMBA reduced the maximum effect of bradykinin. Iberiotoxin + apamin, alone or on top of L-NAME, did not affect bradykinin, SNAP or L-SNC. ODQ and hydroxocobalamin shifted the SNAP, L-SNC, D-SNC, and L-SNG CRCs ≈10-fold to the right, and, in combination, fully blocked SNAP-induced effects. Charybdotoxin + apamin shifted the L-SNC and L-SNG CRCs, but not the D-SNC or SNAP CRCs, ≈5-fold to the right. KCl and ouabain (but not $BaCl_2$) shifted the SNAP, L-SNC and L-SNG CRCs 5-10 fold to the right. In conclusion, L-*S*-nitrosothiols activate $SK_{Ca}$ + $IK_{Ca}$ channels in a stereoselective manner, whereas NO activates $Na^+$-$K^+$-ATPase. Since S-nitrosothiols decompose to NO, stored L-*S*-nitrosothiols may mediate bradykinin-induced, EDHF-dependent relaxation.

## Introduction

Bradykinin-induced relaxation of coronary arteries is thought to depend on NO synthesized de novo by endothelial NO synthase (NOS) as well as a non-NO endothelium-derived hyperpolarizing factor (EDHF). At present, there is no clear consensus on the identity of EDHF or the exact mechanism by which EDHF relaxes smooth muscle cells. According to a recent review,[61] EDHF release from endothelial cells depends on the activation of endothelial intermediate-conductance and small-conductance $Ca^{2+}$-dependent $K^+$-channels ($IK_{Ca}$, $SK_{Ca}$).[64,65,69,70,131] Subsequently, EDHF is assumed to hyperpolarize smooth muscle cells through activation of inwardly rectifying $K^+$ channel ($K_{IR}$) channels, $Na^+$-$K^+$-ATPase and/or large-conductance $Ca^{2+}$-dependent $K^+$ ($BK_{Ca}$) channels.[61,64,71,132] With regard to the latter, it is important to note that NO itself is capable of inducing hyperpolarization via activation of $Ca^{2+}$-dependent $K^+$ channels and $Na^+$-$K^+$-ATPase.[72,108,109] EDHF candidates include prostacyclin, $K^+$, and cytochrome-P450 products of arachidonic acid (epoxyeicosatrienoic acids, EETs).[61,63-65,71]

In previous studies on bradykinin-induced relaxation of porcine coronary arteries (PCAs), we and others were unable to demonstrate a role for either prostacyclin or EETs, both in the absence and presence of the NOS inhibitor $N^{\omega}$-nitro-L-arginine methyl ester HCl (L-NAME).[54,133,134] Interestingly, the NO scavenger hydroxocobalamin blocked the bradykinin-induced relaxation of PCAs to a greater degree than L-NAME.[54] Similar observations were made in human coronary arteries, where it was simultaneously observed that increasing the concentration of the NOS inhibitor did not yield additional effects.[57,73] Taken together, these data support the release of NO or an NO-containing factor from a source that does not depend on the acute conversion of L-arginine by NOS. Repeated bradykinin exposure rapidly depletes such 'NO pools', independently of bradykinin type 2 receptor desensitisation.[54,56,59,60] NO-containing factors are also thought to mediate light-induced photorelaxation of vascular smooth muscle cells.[74,135] Nitrosothiol-depleting agents reduce photorelaxation responses,[74] and *S*-nitrosothiols have therefore been proposed to mediate this phenomenon.

Similarly, *S*-nitrosothiols might be the tissue source of non-de novo synthesized NO. *S*-nitrosothiols induce relaxation not only through their decomposition to NO,[75] but also by

activating stereoselective recognition sites.[76] Recently, the cysteine residues within the α subunit of the $BK_{Ca}$ channel were identified as a *S*-nitrosothiol binding site.[77]

It was the aim of the present study to investigate whether *S*-nitrosothiols mediate the bradykinin-induced, EDHF-dependent relaxation in PCAs. First, we verified the exact mechanism of the bradykinin-induced, EDHF-mediated responses in PCAs, and we studied the effects of nitrosothiol-depleting agents on bradykinin-induced relaxation with or without L-NAME. Second, we ruled out the possibility that NO mediates EDHF-like responses, using the NO donor *S*-nitroso-N-penicillamine (SNAP). Third, we investigated the pathways involved in the relaxant effects of S-nitrosothiols, as well as the stereoselectivity of their effects, using the *S*-nitrosothiols L-*S*-nitrosocysteine (L-SNC), D-SNC and L-*S*-nitrosoglutathione (L-SNG).

## Methods

### *Tissue collection*

Coronary arteries were obtained from 92 slaughterhouse pigs. The arteries were removed after the heart had been brought to the laboratory in cold, oxygenated Krebs bicarbonate solution of the following composition (mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. Vessels were stored in cold, oxygenated Krebs bicarbonate solution for 12 or 36 hours. They were then cut into segments of approximately 4 mm length, suspended on stainless steel hooks in 15 ml-organ baths containing Krebs bicarbonate solution, aerated with 95% $O_2$ / 5% $CO_2$, and maintained at 37°C. In some segments, the tips of a pair of watchmakers forceps had been inserted into the lumen to remove the endothelium by gently rolling the ring back and forth over saline-loaded filter paper.

### *Organ bath studies*

All vessel segments were allowed to equilibrate for at least 30 min and the organ bath fluid was refreshed every 15 min during this period. Changes in tissue contractile force were recorded with a Harvard isometric transducer (South Natick, MA, USA). The vessel segments, stretched to a stable force of about 15 mN, were exposed to 30 mmol/l KCl

twice. The functional integrity of the endothelium (or its absence) was verified by observing relaxation to 1 nmol/l substance P after preconstriction with 1 µmol/l U46619. Subsequently, the tissue was exposed to 100 mmol/l KCl to determine the maximal contractile response to KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min in the absence or presence of one or more of the following inhibitors: the NOS inhibitors L-NAME (100 or 300 µmol/l) and Nω-nitro-L-arginine (L-NOARG) (100 µmol/l), the NO scavenger hydroxocobalamin (200 µmol/l), the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 µmol/l), the *S*-nitrosothiol-depleting agents ethacrynic acid (50 µmol/l) and p-hydroxymercurobenzoic acid (PHMBA, 10 µmol/l), the $IK_{Ca}$ + $BK_{Ca}$ channel inhibitor charybdotoxin (100 nmol/l), the $SK_{Ca}$ channel inhibitor apamin (100 nmol/l), the $BK_{Ca}$ channel inhibitors iberiotoxin (100 nmol/l) and tetraethylammonium (1 mmol/l), the two-pore domain $K^+$ channel (TASK-1) inhibitor $ZnCl_2$ (200 µmol/l),[136] the $K_{IR}$ inhibitor $BaCl_2$ (30 µmol/l), the $Na^+$-$K^+$-ATPase inhibitor ouabain (1 mmol/l) or KCl (20 mmol/l). Vessels were then preconstricted with U46619, and concentration-response curves (CRCs) were constructed to bradykinin, SNAP, L-SNC, D-SNC, L-SNG or KCl. In the experiments evaluating the effect of relaxant effects of KCl, KCl in the Krebs buffer was replaced by NaCl. L-SNC, D-SNC and L-SNG were prepared immediately prior to the experiment and stored in the dark on ice. In short, 50 µl of a 0.2 mol/l solution of L-cysteine, D-cysteine or L-glutathione was mixed with 50 µl 0.2 mol/l $NaNO_2$. The subsequent addition of 10 µl 1 mol/l HCl resulted in a stable 0.1 mol/l solution of the respective isomers. Preliminary studies with $NaNO_2$, L-cysteine, D-cysteine and L-glutathione (n=3-6 each) revealed that, separately, these drugs did not exert relaxant effects in preconstricted PCAs (data not shown).

### *Data analysis*

Data are given as mean±SEM. No differences were observed between PCAs that had been stored for 12 hours or 36 hours, and data from all vessels were therefore combined. Relaxant responses are expressed as a percentage of the contraction to U46619. CRCs were analyzed as described using the logistic function described[46] to obtain $pEC_{50}$ ($-{}^{10}\log EC_{50}$) values. L-NAME, ODQ, hydroxocobalamin, $BaCl_2$, ouabain and KCl

increased basal tone to 10-15% of the maximum contractile response when given separately, and to 15-40% of the maximum contractile response when given in combination. U46619-induced preconstrictions were corrected for this increase in baseline. Statistical analysis versus control was by one-way ANOVA, followed by Dunnett's post-hoc evaluation. $P<0.05$ was considered significant.

## Results

### *Bradykinin-induced relaxation*

Bradykinin relaxed preconstricted vessel segments in a concentration-dependent manner ($pEC_{50}$=7.8±0.1, n=21; Figure 1). L-NAME (100 μmol/l) shifted the bradykinin CRC ≈10-fold to the right ($pEC_{50}$=7.1±0.1, n=20; $P<0.01$). Increasing the L-NAME concentration threefold (n=6), or using the NOS inhibitor L-NOARG (n=6) yielded similar results (Figure 2), indicating that NOS inhibition was maximal at a concentration of 100 μmol/l L-NAME. Comparable rightward shifts were obtained with KCl ($pEC_{50}$=6.6±0.3, n=7; P=0.10), charybdotoxin + apamin ($pEC_{50}$=6.5±0.1, n=6; $P<0.01$) and ouabain + $BaCl_2$ ($pEC_{50}$=6.3±0.4, n=8; $P<0.01$), but not with iberiotoxin + apamin ($pEC_{50}$=7.4±0.1, n=8).

Complete blockade of the bradykinin-induced effects was obtained when combining L-NAME with either KCl (n=5), charybdotoxin + apamin (n=6), or ouabain + $BaCl_2$ (n=8). In contrast, iberiotoxin + apamin did not have an additional effect on top of L-NAME (n=10).

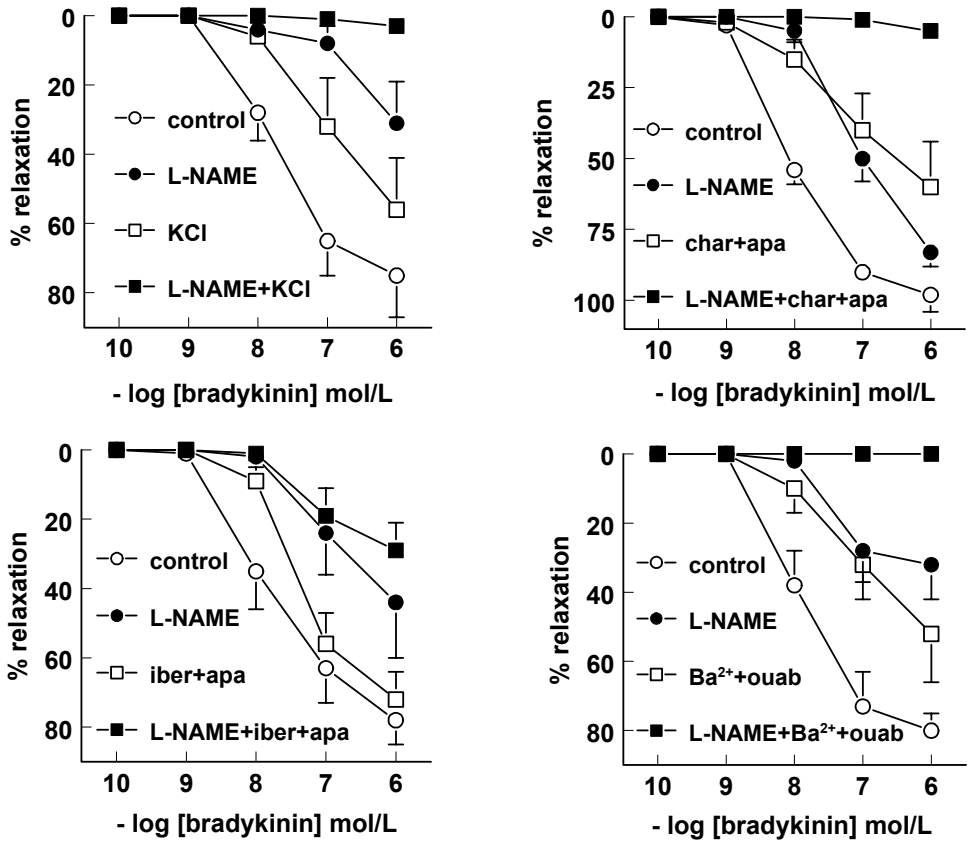



**Figure 1.** *Relaxations of PCAs, preconstricted with U46619, to bradykinin in the absence (control) or presence of 100 µmol/l L-NAME and/or 20 mmol/l KCl, 100 nmol/l charybdotoxin (char) + 100 nmol/l apamin (apa), 100 nmol/l iberiotoxin (iber) + 100 nmol/l apamin, or 1 mmol/l ouabain (ouab) + 30 µmol/l $BaCl_2$ ($Ba^{2+}$). Data (mean±SEM of 5-21 experiments) are expressed as a percentage of the constriction induced by U46619.*

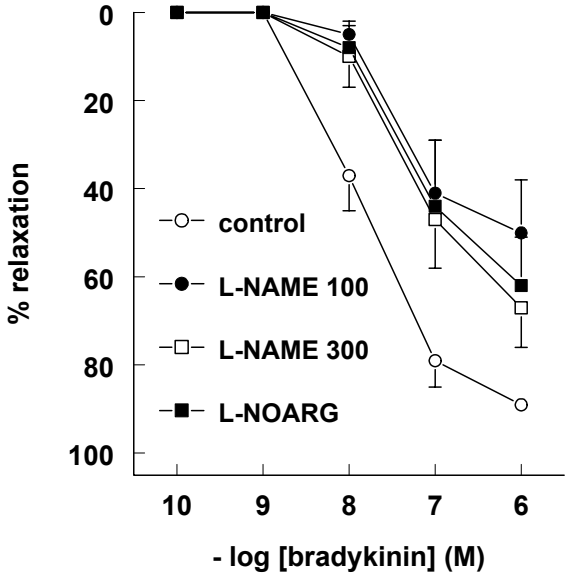



**Figure 2.** *Relaxations of PCAs, preconstricted with U46619, to bradykinin in the absence (control) or presence of 100 µmol/l L-NAME, 300 µmol/l L-NAME or 100 µmol/l L-NOARG. Data (mean±SEM of 6-9 experiments) are expressed as a percentage of the constriction induced by U46619.*

When given separately, apamin, charybdotoxin, iberiotoxin, tetraethylammonium, $BaCl_2$ and $ZnCl_2$ did not affect the bradykinin CRC (Figure 3, n=7 for each). Ouabain shifted the bradykinin CRC to the right ($pEC_{50}$=6.9±0.3, n=7; P<0.01). This shift was not different from the shift observed in the presence of ouabain + $BaCl_2$, suggesting that $BaCl_2$ has no additional effect on top of ouabain.

The *S*-nitrosothiol-depleting agent PHMBA reduced the maximum relaxant effect of bradykinin from 88±2% to 64±7% (n=6, P=0.01; Figure 4), without affecting its potency. Similar results were obtained with PHMBA in the presence of L-NAME (n=6). In contrast, the *S*-nitrosothiol-depleting agent ethacrynic acid (alone or on top of L-NAME) did not affect the bradykinin CRC (n=11, Figure 4).

Thus, NO, *S*-nitrosothiols, $IK_{Ca}$ and $SK_{Ca}$ channels and $Na^+$-$K^+$-ATPase are involved in bradykinin-induced relaxations of PCAs.

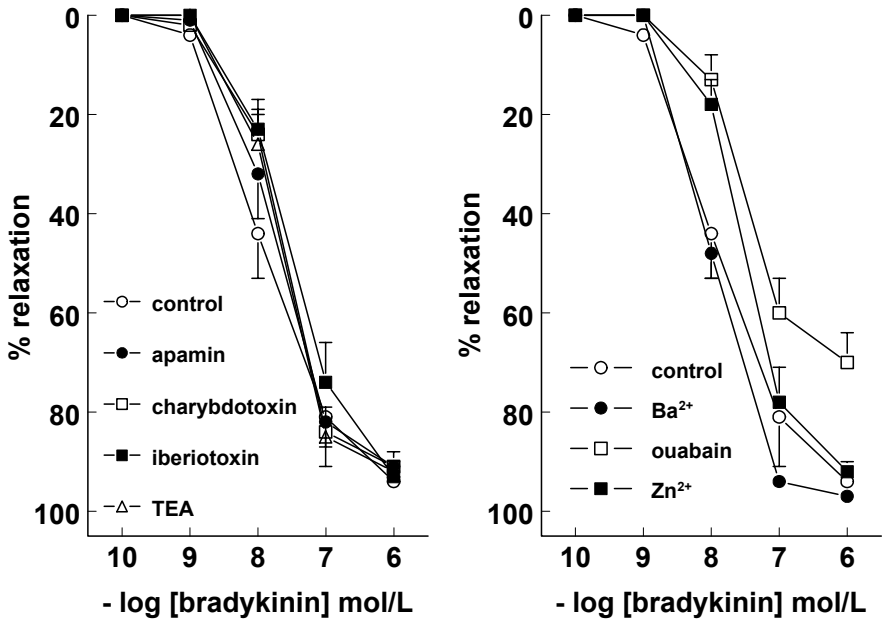



**Figure 3**. *Relaxations of PCAs, preconstricted with U46619, to bradykinin in the absence (control) or presence of 100 nmol/l apamin (apa), 100 nmol/l charybdotoxin (char), 100 nmol/l iberiotoxin (iber), 1 mmol/l tetraethylammonium (TEA), 30 µmol/l $BaCl_2$ ($Ba^{2+}$), 1 mmol/l ouabain or 200 µmol/l $ZnCl_2$ ($Zn^{2+}$). Data (mean±SEM of 7 experiments) are expressed as a percentage of the constriction induced by U46619.*

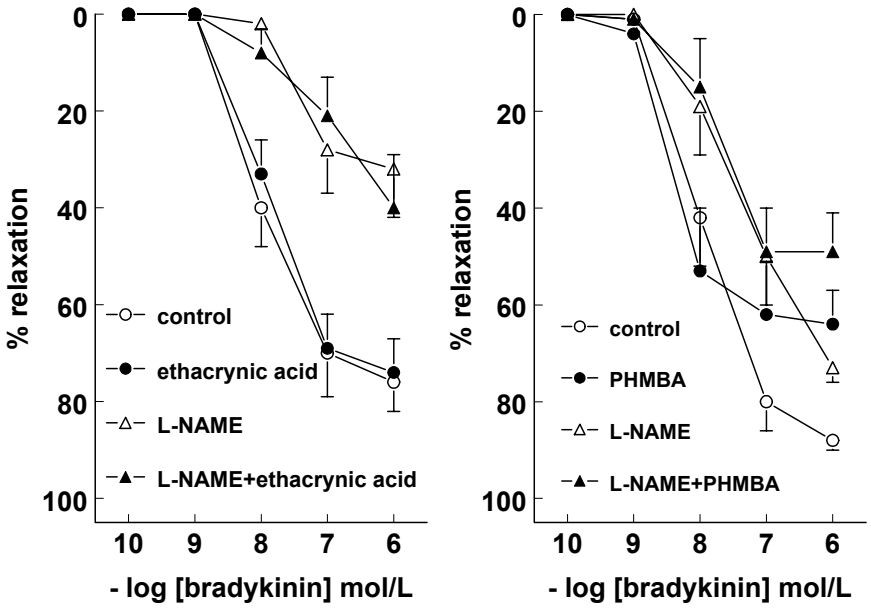



**Figure 4**. *Relaxations of PCAs, preconstricted with U46619, to bradykinin in the absence (control) or presence of 100 µmol/l L-NAME and/or 50 µmol/l ethacrynic acid and 10 µmol/l PHMBA. Data (mean±SEM; n=6-11) are expressed as a percentage of the constriction induced by U46619.*

### *NO-induced relaxation*

SNAP relaxed preconstricted vessel segments in a concentration-dependent manner ($pEC_{50}$=6.4±0.1, n=21; Figure 5). Both hydroxocobalamin ($pEC_{50}$=5.6±0.1, n=14; P<0.005) and ODQ ($pEC_{50}$=5.3±0.1, n=13; P<0.001) shifted the SNAP-induced CRC to the right, and, in combination, completely blocked SNAP-induced relaxation (Figure 5). Charybdotoxin + apamin, as well as iberiotoxin + apamin, either together or on top of hydroxocobalamin or ODQ, did not induce a rightward shift of the SNAP CRC (Figures 5 and 6; n=6-13). KCl ($pEC_{50}$=6.0±0.2, n=10) and ouabain + $BaCl_2$ ($pEC_{50}$=5.7±0.2, n=8) comparably shifted the SNAP CRC to the right, although significance (P=0.02) was reached for ouabain + $BaCl_2$ only. When given separately, $BaCl_2$ (n=8) did not affect the SNAP CRC, whereas ouabain ($pEC_{50}$=6.1±0.3, n=8; P=0.02) induced a similar rightward shift as ouabain + $BaCl_2$ (Figures 5 and 7).

Thus, relaxations induced by exogenous NO depend on guanylyl cyclase and $Na^+$-$K^+$-ATPase.

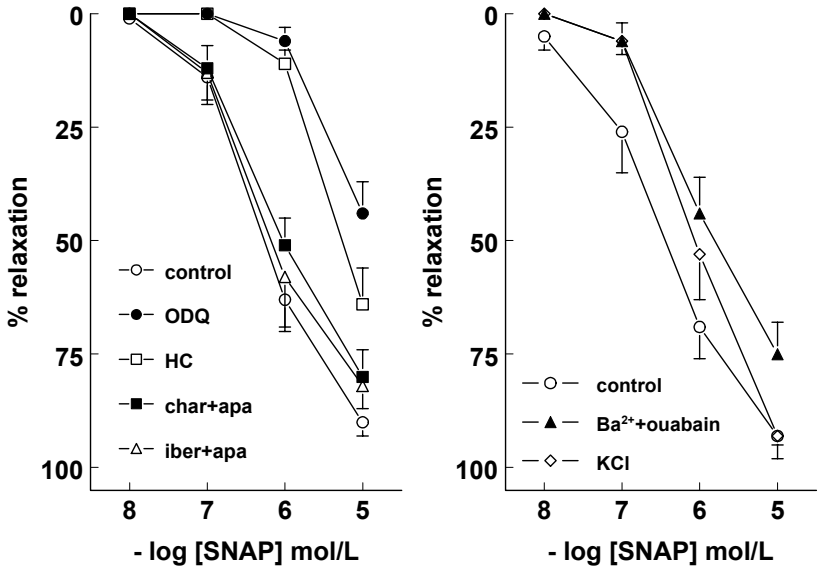



**Figure 5**. *Relaxations of PCAs, preconstricted with U46619, to SNAP in the absence (control) or presence of 10 µmol/l ODQ, 200 µmol/l hydroxocobalamin (HC), 100 nmol/l charybdotoxin (char) + 100 nmol/l apamin (apa), 100 nmol/l iberiotoxin (iber) + 100 nmol/l apamin, 20 mmol/l KCl or 1 mmol/l ouabain + 30 µmol/l $BaCl_2$ ($Ba^{2+}$). Data (mean±SEM; n=6-21) are expressed as a percentage of the constriction induced by U46619.*

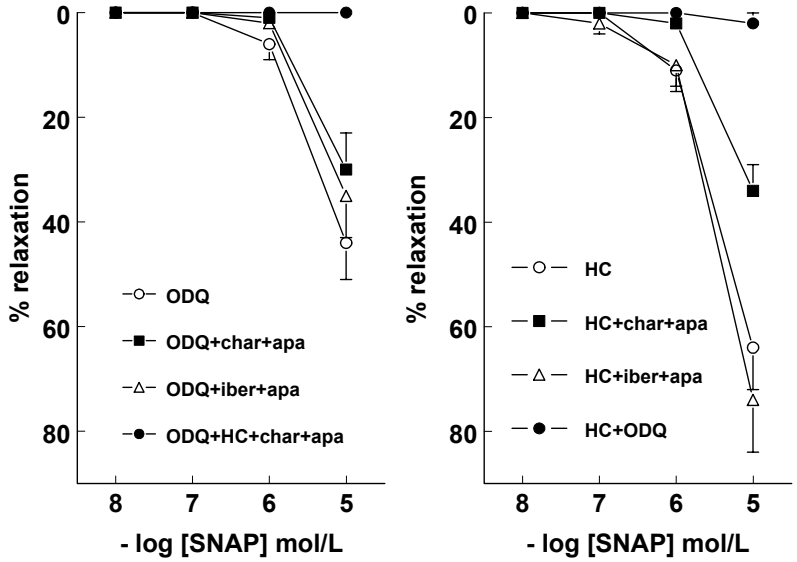



**Figure 6**. *Relaxations of PCAs, preconstricted with U46619, to SNAP in the absence (control) or presence of one or more of the following inhibitors: 10 µmol/l ODQ, 200 µmol/l hydroxocobalamin (HC), 100 nmol/l charybdotoxin (char), 100 nmol/l apamin (apa) and 100 nmol/l iberiotoxin (iber). Data (mean±SEM; n=6-13) are expressed as a percentage of the constriction induced by U46619.*

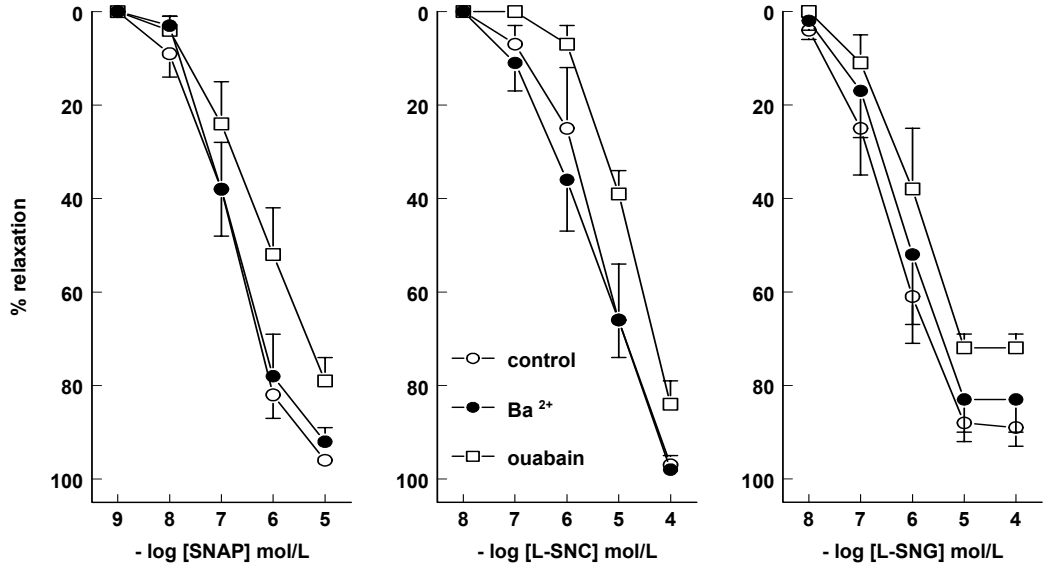



**Figure 7**. *Relaxations of PCAs, preconstricted with U46619, to SNAP, L-SNC or L-SNG in the absence (control) or presence of 30 µmol/l $BaCl_2$ ($Ba^{2+}$) or 1 mmol/l ouabain. Data (mean±SEM; n=6-8) are expressed as a percentage of the constriction induced by U46619.*

### *S-nitrosothiol-induced relaxation*

Both L-SNC ($pEC_{50}$=6.3±0.3, n=20) and D-SNC ($pEC_{50}$=5.9±0.2, n=15) relaxed preconstricted PCAs in a concentration-dependent manner (Figure 8). ODQ and hydroxocobalamin shifted the CRCs of both L-SNC ($pEC_{50}$'s 4.2±0.1 and 4.3±0.1, n=6; P<0.01) and D-SNC ($pEC_{50}$'s 4.6±0.3 and 4.2±0.1, n=5-6; P<0.01) >10 fold to the right and, when combined, nearly completely prevented the L-SNC- and D-SNC-induced effects. Charybdotoxin + apamin shifted the L-SNC CRC ($pEC_{50}$=5.5±0.2, n=14; P=0.02), but not the D-SNC CRC, 5-10 fold to the right. Endothelium-denudation (n=7) did not prevent L-SNC-induced relaxations, nor did iberiotoxin + apamin (n=7) affect the L-SNC CRC (Figure 9). KCl ($pEC_{50}$=5.0±0.1, n=7; P<0.01) and ouabain + $BaCl_2$ ($pEC_{50}$=5.0±0.3, n=7; P=0.04) shifted the L-SNC CRC to the right. When given on top of ODQ and hydroxocobalamin, ouabain + $BaCl_2$, but not charybdotoxin + apamin, iberiotoxin + apamin or KCl, fully blocked the L-SNC-induced effects (Figure 9, n=9 for all). When given separately, $BaCl_2$ (n=6) did not affect the L-SNC CRC, whereas ouabain ($pEC_{50}$=4.8±0.1, n=6; P=0.03) induced a similar rightward shift as ouabain + $BaCl_2$ (Figures 7 and 9).

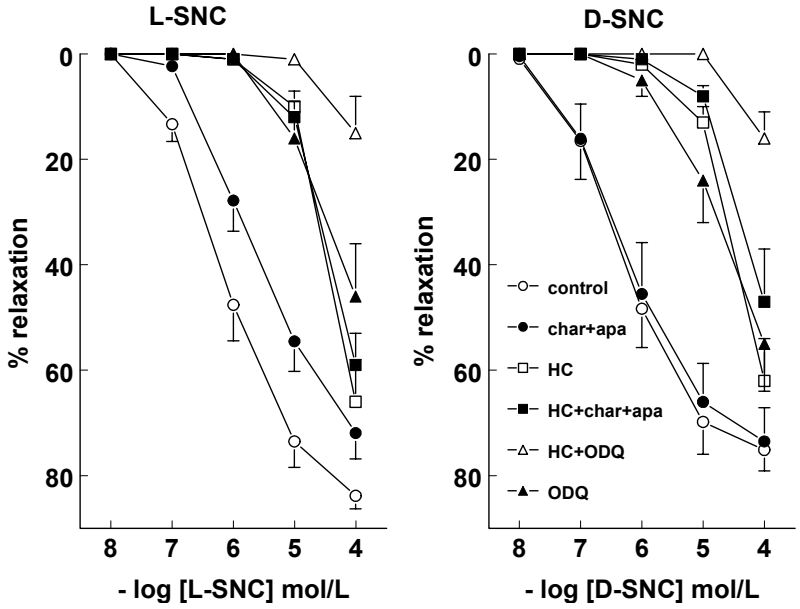



**Figure 8**. *Relaxations of PCAs, preconstricted with U46619, to L-SNC or D-SNC in the absence (control) or presence of one or more of the following inhibitors: 10 µmol/l ODQ, 200 µmol/l hydroxocobalamin (HC), 100 nmol/l charybdotoxin (char) and 100 nmol/l apamin (apa). Data (mean±SEM; n=5-20) are expressed as a percentage of the constriction induced by U46619.*

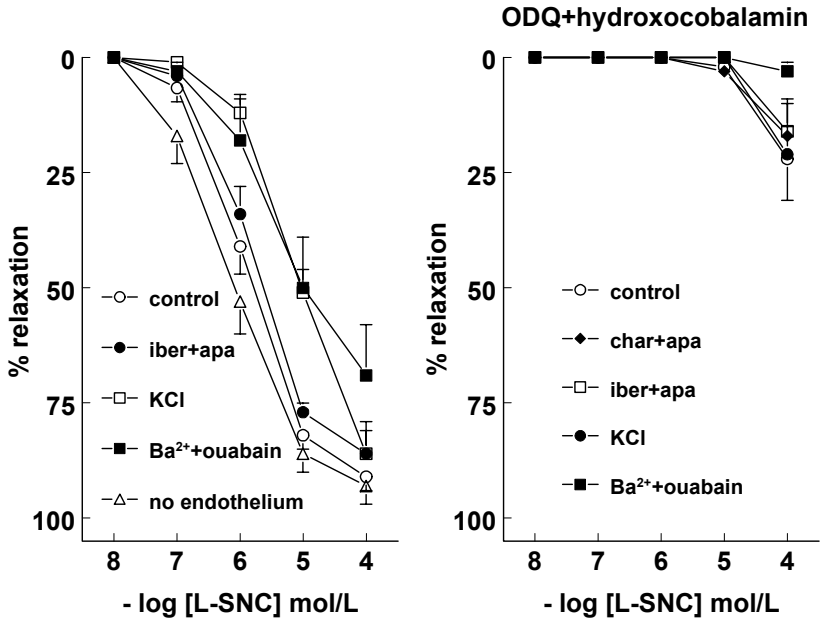



**Figure 9**. *Relaxations of intact or endothelium-denuded PCAs, preconstricted with U46619, to L-SNC without (left panel) or with 10 µmol/l ODQ and 200 µmol/l hydroxocobalamin (right panel) in the absence (control and no endothelium) or presence of 100 nmol/l charybdotoxin (char) + 100 nmol/l apamin (apa), 100 nmol/l iberiotoxin (iber) + 100 nmol/l apamin, 20 mmol/l KCl or 1 mmol/l ouabain + 30 µmol/l $BaCl_2$ ($Ba^{2+}$). Data (mean±SEM; n=7-20) are expressed as a percentage of the constriction induced by U46619.*

Results obtained with L-SNG ($pEC_{50}$=6.6±0.2, n=9; Figure 10) were identical to those with L-SNC, in that ODQ ($pEC_{50}$=5.0±0.1, n=9; P<0.001 vs. control), hydroxocobalamin ($pEC_{50}$=5.4±0.1, n=9; P<0.001), charybdotoxin + apamin ($pEC_{50}$=6.0±0.2, n=9; P=0.04) and ouabain + $BaCl_2$ ($pEC_{50}$=4.8±0.3, n=9; P<0.01) all shifted the L-SNG CRC to the right. In addition, ouabain + $BaCl_2$ reduced the maximal relaxant effect of L-SNG from 95±3% to 46±11% (P<0.001). Results with ouabain alone ($pEC_{50}$=5.6±0.3, n=6; P=0.04) were identical to those with ouabain + $BaCl_2$ (Figures 7 and 10). $BaCl_2$ alone (n=6) did not affect L-SNG (Figure 7).

Thus, *S*-nitrosothiol-induced relaxation occurs in a stereoselective manner and depends on guanylyl cyclase, $IK_{Ca}$ and $SK_{Ca}$ channels and $Na^+$-$K^+$-ATPase.

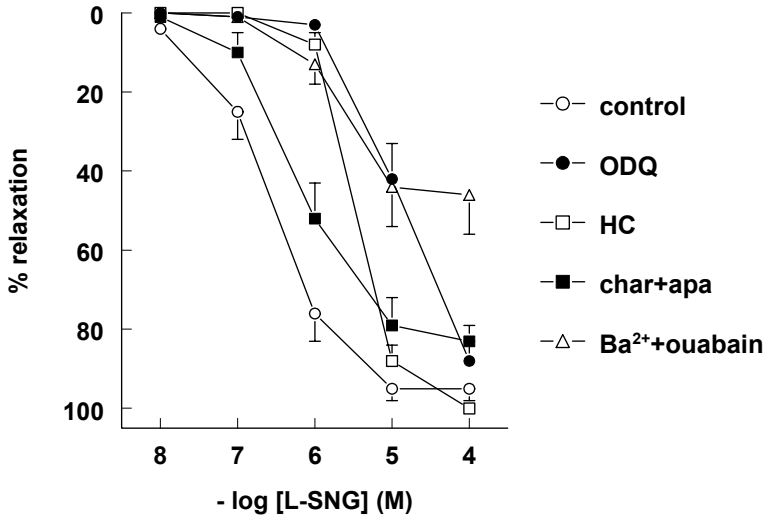



**Figure 10.** *Relaxations of PCAs, preconstricted with U46619, to L-SNG without (control) or with 10 µmol/l ODQ, 200 µmol/l hydroxocobalamin, 100 nmol/l charybdotoxin (char) + 100 nmol/l apamin (apa) or 1 mmol/l ouabain + 30 µmol/l $BaCl_2$ ($Ba^{2+}$). Data (mean±SEM; n=9) are expressed as a percentage of the constriction induced by U46619.*

### *KCl-induced relaxation*

KCl relaxed preconstricted PCAs in a concentration-dependent manner. At a concentration of 2.5 mmol/l KCl, relaxation amounted to 30±4% of the U46619-induced preconstriction (n=7), and 7.5 mmol/l KCl relaxed preconstricted vessels by 45±7%. In contrast, 17.5 mmol/l KCl induced a constrictor response (corresponding with 18±1% of the maximum contraction) on top of the U46619-induced preconstriction.

Thus, low concentrations of KCl induce relaxation, and high (depolarizing) concentrations induce constriction.

## Discussion

In the present study, bradykinin-induced, EDHF-mediated vasodilation in PCAs was found to involve $IK_{Ca}$ and $SK_{Ca}$ channels and $Na^+$-$K^+$-ATPase, in agreement with a recent proposal by Busse et al..[61] It does not involve $BK_{Ca}$ channels, two-pore domain $K^+$ channels (TASK-1), $K_{IR}$ channels or (as shown in a previous study[54]) ATP-dependent $K^+$ channels. The NO donor SNAP, in addition to its well-known effects on guanylyl cyclase, also activated $Na^+$-$K^+$-ATPase. SNAP did not affect $Ca^{2+}$-dependent $K^+$-channels, thereby confirming that, in PCAs, NO is not EDHF. Finally, the nitrosothiols L-SNC and L-SNG, but not D-SNC, on top of their relaxant effects via the NO-guanylyl cyclase-cGMP pathway, activated $IK_{Ca}$ and $SK_{Ca}$ channels, and $Na^+$-$K^+$-ATPase. This finding, combined with our observation that the nitrosothiol-depleting agent PHMBA reduced the maximum relaxant effect of bradykinin, supports the concept that *S*-nitrosothiols, in a stereoselective manner, mediate bradykinin-induced, EDHF-dependent effects in PCAs.

Despite reports on EET- (a putative EDHF candidate) and NO-induced activation of $BK_{Ca}$ channels,[63,71,72] the selective $BK_{Ca}$ channel inhibitor iberiotoxin did not affect the relaxant response to any of the agonists applied in the present study. Tetraethylammonium, at a concentration that selectively blocks $BK_{Ca}$ channels,[137] also did not affect the relaxant response to bradykinin. Thus, in PCAs, $BK_{Ca}$ channels do not contribute to bradykinin-induced relaxation. In addition, based on the lack of effect of inhibitors of the cytochrome-P450 metabolism of arachidonic acid towards bradykinin in porcine and human coronary arteries,[54,57,58] an EET is unlikely to be EDHF in these coronary arteries.

Because of the lack of effect of $BK_{Ca}$ channel blockade, the inhibitory effects of the non-selective $IK_{Ca}$ and $BK_{Ca}$ channel inhibitor charybdotoxin in this study must have been due to blockade of $IK_{Ca}$ channels. Charybdotoxin-induced blockade became apparent only in the presence of the $SK_{Ca}$ channel inhibitor apamin, in agreement with the idea that both

$IK_{Ca}$ and $SK_{Ca}$ channels contribute to the EDHF response, and that blockade of one channel offers an 'escape' via the other.[57,58,69,70,131] $IK_{Ca}$ and $SK_{Ca}$ channels reside in porcine coronary artery endothelial cells,[70] and $SK_{Ca}$ channels have also been demonstrated in smooth muscle cells.[124] Thus, the $IK_{Ca}$ and $SK_{Ca}$ channel-dependent effects of L-SNC and L-SNG may have resulted in hyperpolarization of both endothelial and smooth muscle cells. In support of the latter, L-SNC also relaxed de-endothelialized PCAs. However, this cannot be taken as unequivocal evidence for the activation of $Ca^{2+}$-dependent $K^+$-channels on smooth muscle cells by *S*-nitrosothiols, since it may well be explained by the activation of guanylyl cyclase through the decomposition of L-SNC and L-SNG to NO.

Endothelial $IK_{Ca}$ and $SK_{Ca}$ channel activation results in modest $K^+$ accumulation in the myo-endothelial space. This $K^+$ is believed to subsequently hyperpolarize vascular smooth muscle cells by activating $Na^+$-$K^+$-ATPase and/or $K_{IR}$ channels.[61,64,65,132] We tested this hypothesis by studying the relaxant effects of KCl in PCAs. Indeed, in agreement with a study in porcine renal arteries,[132] low (<10 mmol/l) KCl concentrations relaxed preconstricted vessels, whereas higher (depolarizing) concentrations induced constriction. $K_{IR}$ channels did not contribute to the relaxations observed in our experimental setup, as $BaCl_2$ (alone or on top of ouabain) did not exert a blocking effect towards bradykinin, SNAP, L-SNC or L-SNG. Since NO itself was also found to activate $Na^+$-$K^+$-ATPase, both endothelial $IK_{Ca}$ and $SK_{Ca}$ channel activation and decomposition to NO may underlie the *S*-nitrosothiol-induced activation $Na^+$-$K^+$-ATPase.

Direct activation of $Ca^{2+}$-dependent $K^+$-channels by *S*-nitrosothiols has been demonstrated before.[77,124] Such activation most likely involves 'nitrosation motifs' within the ion channels.[115] For instance, in the $BK_{Ca}$ channel, the cysteine residues within the $\alpha$ subunit have been identified as S-nitrosothiol binding sites.[77] The stereoselectivity of this effect (Figure 8) confirms previous in vivo studies with *S*-nitrosothiols in rats.[76]

The contribution of $IK_{Ca}$ and $SK_{Ca}$ channels to the relaxant effects of L-*S*-nitrosothiols appears to be more modest than the NO-related contribution. Complete blockade of the L-SNC-induced effects was obtained only in the presence of ODQ, hydroxocobalamin and ouabain. This supports the idea that, in the absence of NO, $IK_{Ca}$ and $SK_{Ca}$ channel activation results in $K^+$ release and subsequent activation of $Na^+$-$K^+$-ATPase. However,

in contrast with this concept, $IK_{Ca}$ and $SK_{Ca}$ channel inhibition did not yield additional effects on top of blockade of the NO-guanylyl cyclase-cGMP pathway. This discrepancy may relate to incomplete or reversible inhibitory actions of both ODQ and hydroxocobalamin,[54,58,127] which could become apparent at high agonist concentrations and/or during particular inhibitor combinations only.

Although bradykinin-induced *S*-nitrosothiol release has not been demonstrated so far, our data with the *S*-nitrosothiol-depleting compound PHMBA (which reduced the relaxant effect of bradykinin by ≈30%, both in the presence and absence of NO) fully support this concept. Furthermore, endothelial and smooth muscle cells have been reported to contain preformed pools of NO-containing factors such as *S*-nitrosothiols.[54,59,107,128] These pools become depleted after repeated exposure to endothelium-dependent agonists (e.g., bradykinin), following prolonged NOS inhibition, and after exposure to light.[54,59,60,107] The thiol alkylating agent ethacrynic acid did not affect the bradykinin-induced relaxations in PCAs, possibly because this agent inhibits regeneration of the stores rather than directly inducing depletion.[138] Both NOS and non-NOS sources are believed to contribute to the maintenance of NO stores.[107,135,138]

In conclusion, our data support the concept that L-*S*-nitrosothiols mediate bradykinin-induced, EDHF-dependent relaxation in PCAs. Moreover, they raise the possibility that L-*S*-nitrosothiols are EDHFs themselves. Thus, in addition to providing a new pharmacological route for delivering NO regionally,[129] *S*-nitrosothiols could simultaneously exert EDHF-like actions.

# Chapter 6

## Carvedilol-induced antagonism of angiotensin II: A matter of $\alpha_1$-adrenoceptor blockade

Submitted, 2005

## Summary

Recent trials have suggested favourable effects on diabetes onset and control of the non-selective β+$\alpha_1$-adrenoceptor blocker carvedilol as compared to the selective $\beta_1$-adrenoceptor blocker metoprolol. Similar metabolic effects have been established for renin-angiotensin-system (RAS) blockers. Angiotensin (Ang) II type-1 ($AT_1$)-$\alpha_1$-receptor crosstalk may provide a mechanism by which carvedilol affects the RAS. Accordingly, we examined the effects of selective versus non-selective β-adrenoceptor blockade on $AT_1$ receptor-mediated vasoconstrictor responses to Ang II in human coronary microarteries (HCMAs), simulating both the hyperadrenergic and activated RAS states present in cardiovascular disease. Therefore, HCMAs, obtained from 23 heart valve donors, were mounted in myographs. Ang II and the $\alpha_1$-adrenoceptor agonist phenylephrine constricted HCMAs to maximally 57±15 and 56±25% of the contraction to 100 mmol/L $K^+$. Neither carvedilol, metoprolol, the nonselective β-adrenoceptor antagonist propranolol, nor the $\alpha_1$-adrenoceptor antagonist prazosin affected the constrictor response to Ang II. Thus, $\alpha_1$- and β-adrenoceptors are not involved in the direct constrictor effects of Ang II, nor do the anti-oxidant and anti-endothelin-1 properties of carvedilol antagonize Ang II. Ang II, when added to the organ bath at a subthreshold concentration, greatly amplified the response to phenylephrine. Both carvedilol and the $AT_1$ receptor antagonist irbesartan inhibited this Ang II-induced potentiation. Furthermore, carvedilol blocked the Ang II-induced amplification of phenylephrine-induced inositolphosphate accumulation in cardiomyocytes. In conclusion, $AT_1$-$\alpha_1$-receptor crosstalk, involving inositolphosphates, sensitizes HCMAs to $\alpha_1$-adrenoceptor agonists. Our results suggest that, in the presence of an increased sympathetic tone, carvedilol provides $AT_1$ receptor blockade via its $\alpha_1$-adrenoceptor blocking effects. Herein lies an explanation for the favourable metabolic effects of carvedilol versus metoprolol.

## Introduction

Diabetes mellitus is a co-morbidity with adverse prognostic impact in subjects with cardiovascular disease and its incidence and progression is favorably affected by blockade of the renin-angiotensin system (RAS) in a dose-dependent fashion.[139-141] Recent evidence obtained in subjects with chronic heart failure (CHF)[142] as well as hypertension[143] further suggests that the incidence and progression of diabetes mellitus may be differentially affected by selective (metoprolol) versus non-selective (carvedilol) β-adrenoceptor blockade in subjects already treated with RAS-blocking agents. The precise mechanism for this effect of carvedilol remains to be elucidated. Interestingly, it has recently been demonstrated that subjects with CHF treated chronically with carvedilol are less sensitive to angiotensin (Ang) II than subjects treated with metoprolol.[144] It is conceivable that this potentiation of RAS blockade impacts metabolic control in a similar manner as an increase in the dose of a RAS blocker.[141]

Several mechanisms could underlie the carvedilol-induced antagonism of Ang II: First, $\alpha_1$-Ang II type 1 ($AT_1$)-receptor crosstalk (e.g., due to heterodimerization) greatly sensitizes $\alpha_1$-adrenoceptor-mediated responses in the presence of subthreshold concentrations of Ang II.[145,146] Carvedilol, being an $\alpha_1$-adrenoceptor antagonist, will block such Ang II-dependent potentiation. Second, carvedilol inhibits the synthesis of both endothelin-1 (ET-1) and superoxide,[147,148] two mediators of Ang II-induced responses.[82,149] Third, carvedilol stimulates NO release from endothelial cells through ATP efflux[150] and might thus counteract Ang II-induced hypertensive responses.[40] Finally, Barki-Harrington et al.[151] recently reported a direct interaction between β-adrenoceptors and $AT_1$ receptors in mouse cardiomyocytes, allowing dual inhibition of both receptors by either the non-selective β-adrenoceptor antagonist propranolol or the $AT_1$ receptor antagonist valsartan.

In the present study we investigated the effect of selective versus non-selective β-adrenoceptor blockade on Ang II-induced vasoconstriction in human coronary microarteries (HCMAs). We also addressed the precise mechanism of this effect, using both HCMAs and isolated cells.

## Methods

### *Human tissue collection*

HCMAs were obtained from 23 heart-beating organ donors (9 men, 14 women, age 13-61 years, mean 45 years), who died of non-cardiac causes (1 cerebrovascular accident, 8 head trauma, 7 subarachnoid bleeding, 1 post-anoxic encephalopathy, 6 intracranial bleeding) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the heart valves for transplantation purposes. The study was approved by the Ethics Committee of the Erasmus MC. The hearts were stored in an ice-cold sterile organ-protecting solution after circulatory arrest. After arrival at the laboratory, a tertiary branch of the left anterior descending coronary artery (diameter, 240-500 μm; mean 320 μm) was removed and stored overnight in a cold (4°C), oxygenated Krebs bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4.

### *Myograph studies*

Following overnight storage, HCMAs were cut into segments of ≈2 mm length and mounted in a Mulvany myograph (J.P. Trading) with separated 6-mL organ baths containing oxygenated Krebs at 37°C. The Krebs was continuously aerated with 95% $O_2$ and 5% $CO_2$, and tissue responses were measured as changes in isometric force, using a Harvard isometric transducer. After a 30-minute stabilization period, the optimal internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure as described before.[47] Endothelial integrity was verified by observing relaxation to 10 nmol/L substance P after preconstriction with 10 nmol/L of the thromboxane $A_2$ analogue U46619 (Sigma). Subsequently, to determine the maximum contractile response, the tissue was exposed to 100 mmol/L KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min. Next, segments were pre-incubated for 30 minutes with carvedilol (a gift of Roche), metoprolol (Sigma), propranolol (Sigma), prazosin (Sigma), irbesartan (a gift of Bristol-Myers Squibb) or eprosartan (a gift of SmithKline-Beecham). Thereafter, concentration-

response curves (CRCs) were constructed to Ang II, endothelin-1 (ET-1) and/or phenylephrine. The cyclo-oxygenase inhibitor indomethacin (5 μmol/L) was present during the entire experiment to suppress spontaneously occurring contractions and relaxations.

### *Inositolphosphate generation in cardiomyocytes*

Primary cultures of neonatal rat ventricular cardiomyocytes were prepared as described before.[13] Cells were labelled with 2 μCi myo-[2-$^3$H]inositol/mL for 24 hours, washed, and preincubated for 10 minutes with vehicle, carvedilol or irbesartan. Next, the cells were stimulated for 30 minutes with phenylephrine and/or Ang II in the presence of 10 mmol/L LiCl to inhibit inositolmonophosphatase activity. Intracellular water-soluble inositolphosphates ($InsP_n$) were then separated and quantified as described before.[13]

### *Bioluminescence Resonance Energy Transfer (BRET)*

Enhanced yellow fluorescent protein (eYFP) was appended to the C-terminal tail of the human $AT_1$ receptor from which the stop codon was eliminated, and the human $\alpha_{1B}$-adrenoceptor was modified by C-terminal, in-frame addition of *Renilla* luciferase. The $\alpha_{1B}$-adrenoceptor was chosen because it represents the major $\alpha_1$-adrenoceptor subtype in human arteries.[152] In addition, carvedilol inhibits $\alpha_{1B}$-adrenoceptor-mediated effects with high potency ($pK_i$=8.6).[153] HEK293 cells were transfected with the two receptors and harvested after 48 hours as described before.[154] Cells were then suspended in phosphate-buffered saline and mixed (1:1) with 10 μmol/L h-coelenterazine. eYFP will act as a resonance energy transfer acceptor for light emitted by the oxidation of h-coelenterazine by *Renilla* luciferase only if the two receptors are within 50-100 Å.[155] Immediately after the addition of h-coelenterazine, using a Spex fluorolog spectrofluorimeter, the emission spectrum (400-600 nm) was acquired with the excitation lamp turned off (slit width=10 nm, 2 sec/increment). For comparisons between experiments, emission spectra were normalized with the peak emission from *Renilla* luciferase in the region of 480 nm being defined as an intensity of 1.00. Energy transfer signal was calculated by measuring the area under the curve between 500 nm and 550 nm. Background was taken as the area of this region of the spectrum when examining emission of cells expressing only the energy

donor. Energy acceptor to energy donor expression ratios were measured as described previously.[154]

### *Data analysis*

Data are given as mean±SEM. Contractile responses are expressed as a percentage of the contraction to 100 mmol/L $K^+$. CRCs were analyzed as described to obtain $pEC_{50}$ ($-^{10}log50$) values.[46] In order to compare the effects of the various agents, the amounts of $InsP_n$ were expressed as a percentage of the total cellular amount of [2-$^3$H]inositol-labeled products. Statistical analysis was by paired t-test or ANOVA, followed by post hoc evaluation according to Dunnet. $P<0.05$ was considered significant.

## Results

### *Myograph Studies*

Ang II (n=9), ET-1 (n=5) and phenylephrine (n=9) constricted HCMAs in a concentration-dependent manner ($pEC_{50}$'s: 8.5±0.1, 8.2±0.1 and 4.8±0.2; $E_{max}$ 57±15, 121±16 and 56±25% of the contraction to 100 mmol/L $K^+$; Figure 1). Carvedilol (n=6), metoprolol (n=6), propranolol (n=7), and prazosin (n=8), all at a concentration of 10 µmol/L, did not affect the Ang II CRC (Figure 2). Prior application of 1 nmol/L Ang II to the organ bath greatly amplified the maximum response to phenylephrine ($E_{max}$ 113±28 vs. 56±25%, n=6; $P<0.01$), without altering the $pEC_{50}$. No such amplification was observed following prior application of 1 nmol/L ET-1 ($E_{max}$ 64±17 vs 58±36%, n=4;

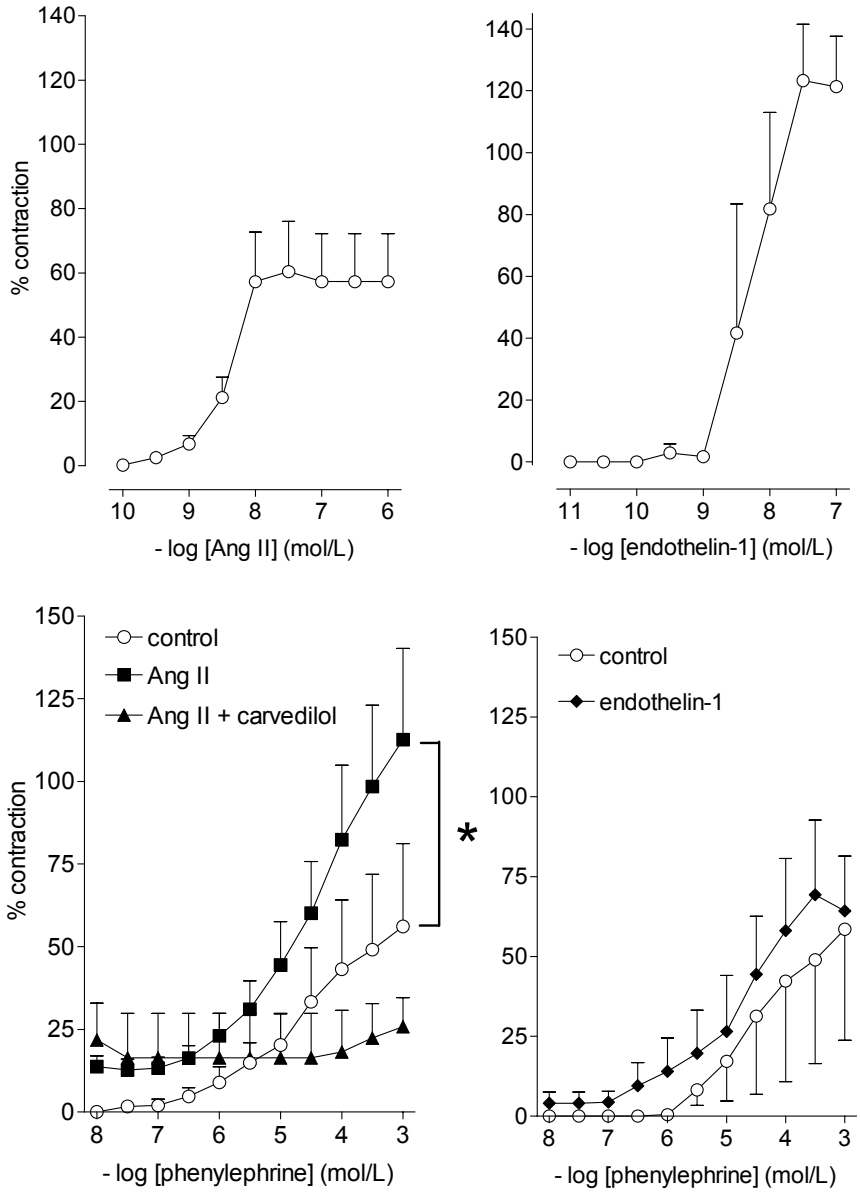



**Figure 1**. *Top panels, contractions of HCMAs to Ang II or endothelin-1. Bottom panels, contractions of HCMAs to phenylephrine in the absence (control) or presence of 1 nmol/L Ang II, 1 nmol/L Ang II + 10 µmol/L carvedilol, or 1 nmol/L endothelin-1. Contractions (mean±SEM, n=3-9) are expressed as a percentage of the response to 100 mmol/L $K^+$. *, P<0.01.*

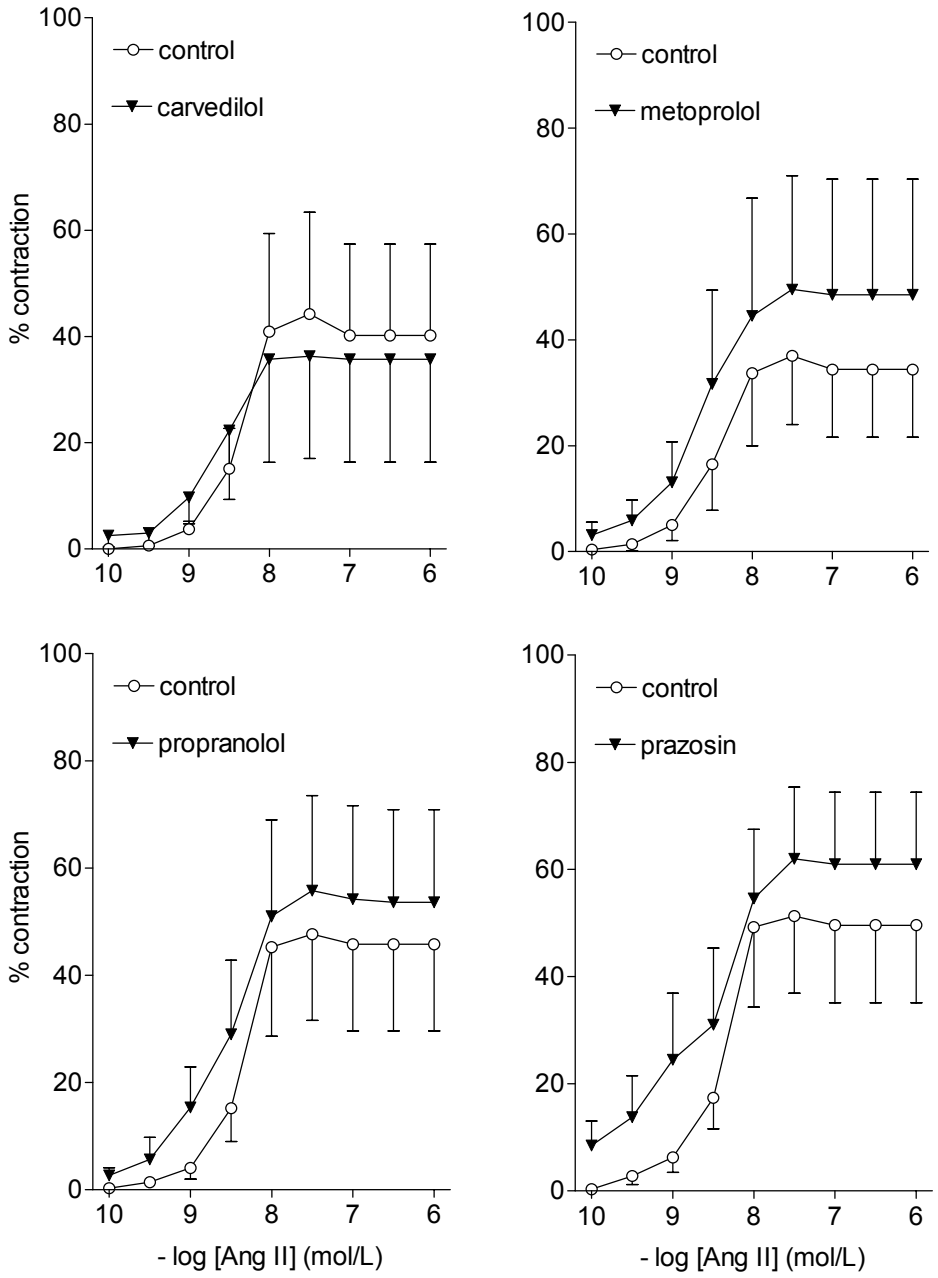



**Figure 2.** *Contractions of HCMAs to Ang II in the absence (control) or presence of carvedilol, metoprolol, propranolol, or prazosin, all at a concentration of 10 µmol/L. Contractions (mean±SEM, n=5-8) are expressed as a percentage of the response to 100 mmol/L $K^+$.*

P=NS), although the vasoconstriction induced by this concentration of ET-1 (4.0±3.4%) was not significantly different from that induced by 1 nmol/L Ang II (13.6±3.3%). Carvedilol (10 μmol/L; n=3) fully prevented the phenylephrine-induced effects in the presence of Ang II (Figure 1).

Irbesartan (n=5) concentration-dependently inhibited the Ang II-induced increase in phenylephrine efficacy, fully reversing the potentiation at a concentration of 100 nmol/L and higher (Figure 3). The inhibitory profile of irbesartan towards phenylephrine in the presence of 1 nmol/L Ang II was indistinguishable from that towards Ang II (Figure 3). Furthermore, neither 1 μmol/L irbesartan (n=5) nor 1 μmol/L eprosartan (n=5) affected the phenylephrine-induced responses in the absence of Ang II (data not shown).

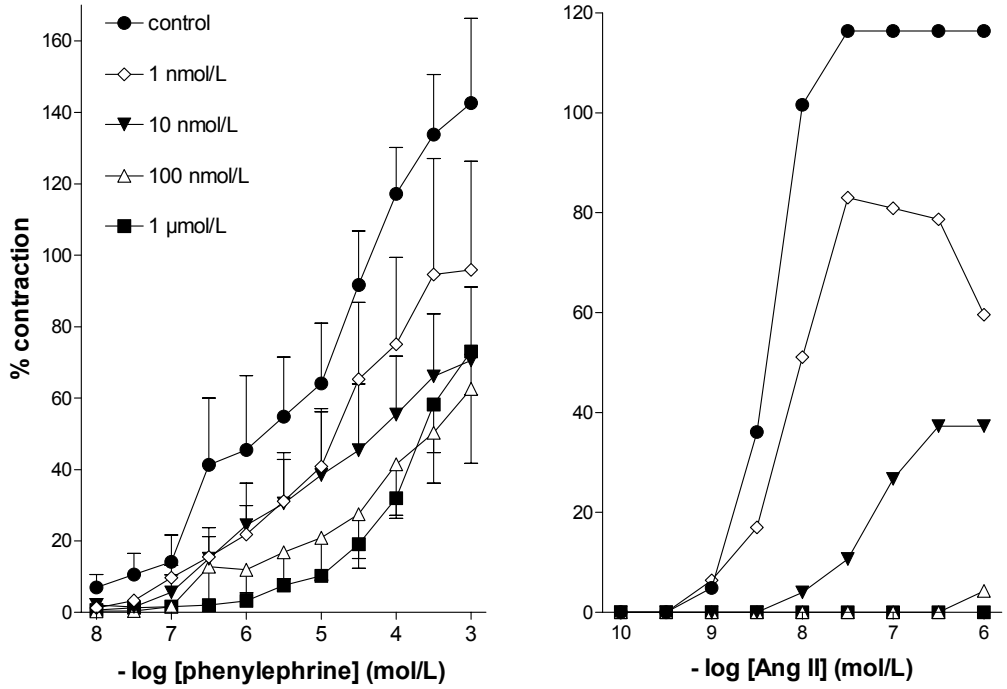



**Figure 3.** *Contractions of HCMAs to phenylephrine in the presence of 1 nmol/L Ang II (left) and to Ang II (right) at increasing concentrations of irbesartan. Contractions (mean±SEM; n=4-5) are expressed as a percentage of the response to 100 mmol/L $K^+$. The phenylephrine CRCs in the presence of the two highest irbesartan concentrations are indistinguishable from the phenylephrine CRC in the absence of this inhibitor (see Figure 2). Note that the inhibitory profile of irbesartan towards the Ang II-dependent component is identical in the two graphs.*

### *Inositolphosphate generation in cardiomyocytes*

Phenylephrine stimulated $InsP_n$ accumulation in neonatal rat cardiomyocytes in a concentration-dependent manner (Figure 4, n=3). Carvedilol (10 μmol/L) fully blocked this effect (n=3). In agreement with a previous study,[13] 10 nmol/L Ang II (n=3) did not significantly affect $InsP_n$ accumulation. However, this concentration of Ang II amplified the response to 1 μmol/L phenylephrine, an effect that was abolished by 10 μmol/L carvedilol (n=3 for all conditions).

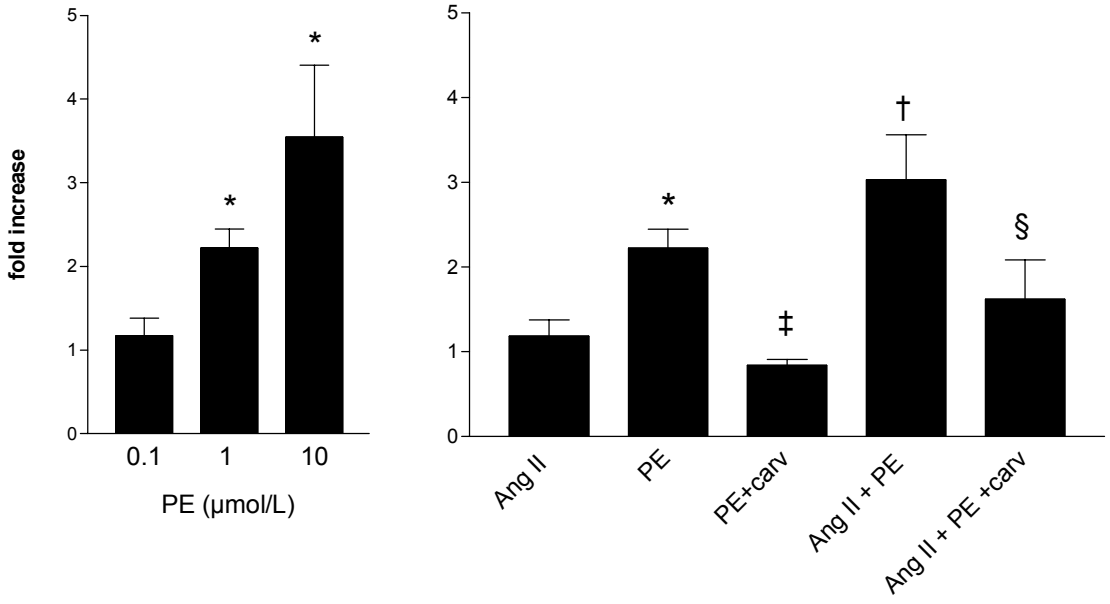



**Figure 4.** *Left panel, concentration-dependent effect of phenylephrine (PE) on inositolphosphate ($InsP_n$) accumulation in neonatal rat cardiomyocytes. Right panel, effect of 1 μmol/L phenylephrine and/or 10 nmol/L angiotensin II (Ang II) in the absence or presence of 10 μmol/L carvedilol (carv) on $InsP_n$ accumulation in neonatal rat cardiomyocytes. Data are mean±SEM of 3 experiments each. Phenylephrine increased $InsP_n$ accumulation in all 3 experiments at concentrations ≥ 1 μmol/L (*). Ang II increased the effect of phenylephrine in all 3 experiments (†), and carvedilol reduced the effect of phenylephrine both without (‡) and with Ang II (§) in all 3 experiments.*

### *BRET*

Co-expression of the $\alpha_{1B}$-adrenoceptor C-terminally tagged with *Renilla* luciferase and the $AT_1$ receptor with eYFP in HEK293 cells, followed by the addition of h-coelenterazine, generated a major peak in the region of 480 nm (representing light output from the oxidation of the substrate by the luciferase) and a second peak (representing resonance energy transfer between luciferase and eYFP), centred at 527 nm (Figure 5). This is consistent with the hypothesis that the two receptors are able to form a constitutive oligomeric complex. The signal generated was of similar magnitude to that reported previously for the interaction between the $AT_1$ receptor and a second G-protein-coupled receptor, the Mas proto-oncogene.[156]

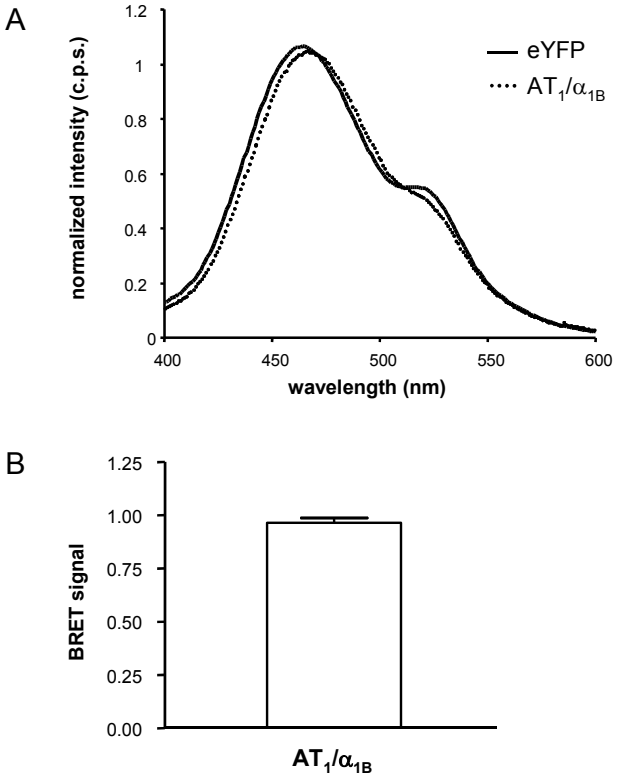



**Figure 5.** *Panel A, Hetero-oligomeric interaction following co-expression of the $\alpha_{1B}$-adrenoceptor-Renilla luciferase and the $AT_1$ receptor-eYFP in HEK293 cells. A fusion protein incorporating the sequences of both Renilla luciferase and eYFP serves as a BRET-positive control.(Ramsay et al., 2002) c.p.s., counts per second. Panel B, Quantitation of the area under the peak from 500-550 nm. Data are mean±SEM of 3 experiments.*

## Discussion

Ang II stimulates the sympathetic nervous system in a number of ways, including a central action to improve sympathetic outflow,[157,158] together with a facilitation of the release of catecholamines from peripheral sympathetic neurons via ganglionic and axonal presynaptic receptors.[159,160] In addition, Ang II increases the vascular sensitivity to $\alpha_1$-adrenoceptor agonists.[146] Indirect confirmation of this concept in humans has come from a study in which the ACE inhibitor perindoprilat attenuated the forearm blood flow response to exogenous norepinephrine.[161] The present study is the first to directly confirm Ang II-induced potentiation of $\alpha_1$-adrenoceptor agonists in HCMAs. Potentiation was not observed after application of a subthreshold concentration of ET-1 to the organ bath, and thus, this effect is specific for Ang II. Furthermore, the lack of effect of prazosin towards Ang II excludes the possibility that release of endogenous norepinephrine (through stimulation of presynaptic $AT_1$ receptors) contributed to the vasoconstrictor effect of Ang II in this preparation.

Irbesartan blocked the Ang II-induced potentiation of phenylephrine in a concentration-dependent manner. The inhibitory profile of the $AT_1$ receptor antagonist towards the potentiating effects of Ang II was indistinguishable from that towards the direct constrictor effects of Ang II. At irbesartan concentrations of 100 nmol/L and higher, the effects of phenylephrine were identical to those without Ang II, and neither irbesartan nor eprosartan interfered with phenylephrine in the absence of Ang II. Thus, the potentiating effects of Ang II depend on $AT_1$ receptor activation, and do not involve direct $\alpha_1$-adrenoceptor stimulatory effects. Consequently, $AT_1$ receptors and $\alpha_1$-adrenoceptors may interact physically (e.g., through heterodimerization), and/or the Ang II-induced amplification results from changes in a common signaling process regulating contractility or from crosstalk between different signaling processes. Our data support both possibilities. BRET confirms the potential for constitutive physical association between the two receptor types, and the inositolphosphate data are in full agreement with the fact that both receptors couple to $G_{q/11}$, and, through activation of phospholipase Cβ, promote the hydrolysis of phosphatidylinositol 4,5- bisphosphate into inositol 1,4,5-trisphosphate

and *sn-1-2* diacylglycerol.[162,163] The latter molecule activates isoforms of protein kinase C, which have already been shown to contribute to the Ang II-induced amplification of $\alpha_1$-adrenoceptor agonists in rabbits.[164]

Carvedilol inhibited both the enhanced vasoconstriction to phenylephrine and the increased inositolphosphate accumulation in the presence of Ang II. It did not block the direct constrictor effects of Ang II in HCMAs. This implies that ET-1 and superoxide (which are both blocked by carvedilol[147,148]) do not mediate the effects of Ang II, in agreement with a previous study in human coronary arteries,[52] and that the carvedilol-induced release of NO from endothelial cells,[150] if occurring in HCMAs, is insufficient to counteract the Ang II-induced vasoconstriction. Taken together therefore, particularly in view of the increased sympathetic tone in chronic heart failure, the $\alpha_1$-adrenoceptor blocking effects of carvedilol most likely underlie its capacity to antagonize angiotensin II. Such antagonism may be expected to exert additional effects on top of RAS blockade in chronic heart failure, since the ACE inhibitors and/or $AT_1$ receptor antagonists that are currently being used do not fully suppress the RAS. Either they are dosed too low[165] or their effects are overcome, at least in part, by the reactive rise in renin release,[166] ACE upregulation[167] and/or alternative converting enzymes like chymase.[53] Thus, carvedilol blocks the RAS in two ways: it inhibits renin release through blockade of renal $\beta_1$-adrenoceptors, and it prevents the functional consequences of $\alpha_1$-$AT_1$-receptor interaction. As the latter does not apply to the selective $\beta_1$-adrenoceptor antagonist metoprolol, this additional property could underlie the favourable metabolic profiles in subjects with heart failure or hypertension treated with carvedilol as compared to metoprolol.[142,143]

Our data do not support a direct interaction between β-adrenoceptors and $AT_1$ receptors in HCMAs. Such interaction has been reported in mouse cardiomyocytes,[151] allowing propranolol and the $AT_1$ receptor antagonist valsartan to block both Ang II- and isoproterenol-induced contractile responses. At least two explanations for this discrepancy may be put forward. First, the interaction may be limited to $\beta_1$-adrenoceptors, whereas HCMAs contain $\beta_2$-adrenoceptors.[168] Second, unlike the comparable (positive inotropic) effects of β-adrenoceptors and $AT_1$ receptors in cardiomyocytes, $\beta_2$-adrenoceptors and $AT_1$ receptors exert opposite effects in

HCMAs,[47,168] and thus, if anything, propranolol would be expected to enhance Ang II-induced vasoconstriction. For instance, blockade of vasodilator $AT_2$ receptors (which also heterodimerize with $AT_1$ receptors[35]) increases the Ang II-induced constriction in HCMAs.[47] The fact that $\beta_2$-adrenoceptor blockade did not enhance Ang II-induced vasoconstriction confirms the absence of endogenous catecholamines in this preparation.

In summary, we report for the first time that $\alpha_1$-$AT_1$-receptor crosstalk is of functional importance in human coronary arteries. Our findings provide a mechanism that could explain the results of clinical studies comparing metoprolol and carvedilol in cardiovascular diseases states.

# Chapter 7

## Summary and general discussion

## Summary


### *Introduction and aim (Chapter 1)*

Angiotensin (Ang) II induces vasorelaxation via activation of Ang II type 2 ($AT_2$) receptors. This effect most likely depends on endothelial bradykinin type 2 ($B_2$) receptor-induced NO release, suggesting that $AT_2$ receptors either stimulate bradykinin release and/or couple directly (e.g., through heterodimerization) to $B_2$ receptors in a bradykinin-independent manner. $B_2$ receptor stimulation also results in the release of a wide range of non-NO endothelium-derived hyperpolarizing factors (EDHFs) like $K^+$, prostacyclin, epoxyeicosatrienoic acids (EETs), $H_2O_2$ and *S*-nitrososothiols. Consequently, some or all of these EDHFs might simultaneously contribute to the $AT_2$ receptor-mediated vasorelaxation. It was the aim of the present thesis to study $AT_2$ receptor-induced vasodilation in humans, and to focus further on the identity of EDHF. In addition, the interaction between $AT_1$ and $\alpha_1$-adrenergic receptors was studied in order to obtain an explanation for the Ang II-antagonistic effects of the non-selective β- and $\alpha_1$-adrenoceptor antagonist carvedilol.

### *$AT_2$ receptors and vasorelaxation in humans (Chapter 2)*

Ang II constricted human coronary microarteries (HCMAs) through activation of $AT_1$ receptors. Pre-incubation of HCMA segments with the $AT_2$ receptor antagonist PD123319 increased the constrictor effect of Ang II, a phenomenon that was particularly present in older subjects and that was abolished after endothelium removal and during blockade of either $B_2$ receptors or NO synthase (NOS). Exposure of preconstricted HCMAs to Ang II in the presence of the $AT_1$ receptor antagonist irbesartan (allowing selective $AT_2$ receptor activation) resulted in relaxation. PD123319 fully abolished this effect. Radioligand binding studies and RT-PCR confirmed the expression of $AT_1$ and $AT_2$ receptors in HCMAs. Taken together, these data are the first to support $AT_2$ receptor-mediated vasodilation in human coronary arteries. This effect depends on endothelial $B_2$ receptors and NO, and appears to increase with age.

### *The identity of EDHF (Chapters 3, 4 and 5)*

To obtain further insight into the identity of EDHF, bradykinin-induced vasorelaxation was studied in HCMAs, porcine coronary microarteries (PCMAs) and large porcine coronary arteries (PCAs), both in the absence and presence of inhibitors of NO and/or EDHF.

As expected, the contribution of de novo synthesized NO was more important in large arteries than in microarteries. In all vessels, the NO scavenger hydroxocobalamin induced a greater degree of inhibition towards bradykinin than the NOS inhibitor L-NAME. This suggests that NO can also be released from a source other than L-arginine, e.g., from NO-containing factors like *S*-nitrosothiols.

In the absence of NO, full inhibition of the bradykinin response was obtained during combined blockade of intermediate- and small-conductance $Ca^{2+}$-dependent $K^+$-channels ($IK_{Ca}$, $SK_{Ca}$), and in the presence of the $Na^+/K^+$-ATPase inhibitor ouabain. Ouabain also diminished the response to the NO donor SNAP, whereas combined $IK_{Ca}$ and $SK_{Ca}$ channel blockade did not. Furthermore, inhibitors of catalase, cyclooxygenase (COX), cytochrome P450 epoxygenase, gap junctions and large-conductance $Ca^{2+}$-activated $K^+$-channels ($BK_{Ca}$) did not interfere with bradykinin-mediated responses.

Taken together, these findings suggest that bradykinin-induced relaxation in human and porcine coronary arteries depends on activation of guanylyl cyclase and $Na^+/K^+$-ATPase by NO, and activation of $IK_{Ca}$ and $SK_{Ca}$ channels by a factor other than NO. This factor is not prostacyclin, a cytochrome P450 product, or $H_2O_2$, nor does it depend on gap junctions and $BK_{Ca}$ channels. The following evidence was obtained to support that this factor may be a *S*-nitrosothiol: 1) L-*S*-nitrosocysteine (L-SNC) relaxed PCMAs and PCAs, and this effect was diminished during combined $IK_{Ca}$ and $SK_{Ca}$ channel blockade in the presence but not in the absence of the endothelium, 2) L-SNC hyperpolarized vascular smooth muscle cells in PCAs, and 3) *S*-nitrosothiol-depleting agents reduced the response to bradykinin. Interestingly, combined $IK_{Ca}$ and $SK_{Ca}$ channel blockade did not affect the response to D-*S*-nitrosocysteine. Thus, *S*-nitrosothiols, via stereoselective activation of endothelial $IK_{Ca}$ and $SK_{Ca}$ channels, may function as EDHF. In addition, due to their decomposition to NO, these factors may provide an alternative source of NO.

### *$AT_1$ and $\alpha_1$-adrenergic receptor interaction (Chapter 6)*

Neither carvedilol, the selective $\beta_1$-adrenoceptor antagonist metoprolol, the nonselective β-adrenoceptor antagonist propranolol, nor the $\alpha_1$-adrenoceptor antagonist prazosin affected the constrictor response to Ang II in HCMAs. Ang II, when added to the organ bath at a subthreshold concentration, greatly amplified the response to the $\alpha_1$-adrenoceptor agonist phenylephrine. Both carvedilol and the $AT_1$ receptor antagonist irbesartan inhibited this Ang II-induced potentiation. Furthermore, carvedilol blocked the Ang II-induced amplification of phenylephrine-induced inositolphosphate accumulation in neonatal rat cardiomyocytes. Thus, $AT_1$-$\alpha_1$-receptor crosstalk, involving inositolphosphates, sensitizes HCMAs to $\alpha_1$-adrenoceptor agonists. Its $\alpha_1$-adrenoceptor blocking effects allow carvedilol to antagonize Ang II in the face of increased sympathetic activity, e.g., in subjects with chronic heart failure.

## General Discussion and Future Studies

### *$AT_2$ receptors and vasodilation in humans*

Animal studies from many groups around the world support the idea that $AT_2$ receptors mediate vasodilation.[5,18,22,24,33,87,88,101] Our in vitro data are the first to confirm that such vasodilation also occurs in humans. The clinical relevance of $AT_2$ receptor stimulation has not yet been fully established. $AT_2$ receptors are upregulated under pathological conditions in the human heart,[31,43] and their stimulation may underlie, at least in part, the beneficial effect of $AT_1$ receptor antagonists in cardiovascular disease.[44,45] The link between $AT_2$ receptors and $B_2$ receptors suggests that the (side-)effects of $AT_1$ receptor antagonists, like those of ACE inhibitors, may depend on $B_2$ receptor activation. Yet many questions remain: does $AT_2$ receptor activation result in bradykinin release or do these receptor directly couple to $B_2$ receptors without intermediate bradykinin synthesis? Our previous studies do not support the idea of bradykinin generation in isolated human vessels.[47] Furthermore, since the net effect of Ang II depends on the $AT_1$/$AT_2$ receptor ratio, it needs to be established how and where these receptors interact. $AT_2$ receptors occur predominantly on endothelial cells, whereas $AT_1$ receptors are believed to be

located mainly on vascular smooth muscle cells. However, the opposite has also been reported,[126] and thus the idea of heterodimerization[35] may be relevant. Future studies in transgenic animals, allowing selective expression of these receptors, could shed light on this issue. Finally, we detected $AT_2$ receptor expression in large human coronary arteries, but could not relate this to an effect on Ang II-induced contractility. Thus, the role of these receptors in large arteries still needs to be determined.

### *The identity of EDHF*

Bradykinin relaxed human and porcine coronary arteries in the absence of de novo synthesized NO, through pathways that did not involve guanylyl cyclase-dependent cGMP generation. Thus, an EDHF that is not de novo synthesized NO contributes to the relaxant effects of bradykinin in a cGMP-independent manner.

A recent study in endothelial NOS (eNOS)/COX-1 double-knockout mice[169] suggests that EDHF is the predominant endothelium-derived relaxing factor in female mice. Our human data support this conclusion, because the effect of NO blockade on bradykinin-induced relaxation of HCMAs was larger in men than in women. Possibly therefore, estrogens upregulate the EDHF pathway.[79,80,170] Further work is needed to unravel this issue.

Combined blockade of $IK_{Ca}$ and $SK_{Ca}$ channels (with charybdotoxin and apamin, respectively) fully inhibited the EDHF-mediated responses in our experimental setup, as well as in the above double-knockout mice.[169] These channels are located in endothelial cells.[61] Their activation results in the release of $K^+$ into the myo-endothelial space. This $K^+$ is capable of hyperpolarizing vascular smooth muscle cells by activating inwardly rectifying $K^+$ ($K_{IR}$) channels and/or $Na^+/K^+$-ATPase.[61,64,65,132] Consequently, combined blockade of $K_{IR}$ channels and $Na^+/K^+$-ATPase (with $Ba^{2+}$ and ouabain, respectively) should also inhibit EDHF-mediated responses. Our data support this contention, although, at least in PCAs, $Ba^{2+}$ did not exert effects on top of ouabain. Moreover, we and others[108] observed that NO donors also activate $Na^+/K^+$-ATPase. Thus, future studies should delineate to what degree bradykinin-induced $Na^+/K^+$-ATPase activation depends on NO and/or EDHF.

Unlike several other groups,[63,71,72] we were unable to obtain evidence supporting a role for EETs (generated from arachidonic acid by cytochrome P450 epoxygenases) as EDHF. A recent study in human coronary arterioles[171] offers an explanation for this discrepancy. It was found that $H_2O_2$ inhibited the bradykinin-induced generation of EETs as well as the $BK_{Ca}$ channels that normally mediate the EET-induced hyperpolarization of vascular smooth muscle cells. Thus, the contribution of EETs in HCMAs needs to be re-addressed on top of hydroxocobalamin and catalase. A complicating factor in this regard is that cytochrome P450 epoxygenases also generate superoxide, the precursor of $H_2O_2$.[172]

We propose that *S*-nitrosothiols act as EDHF in coronary (micro-)arteries. NO reacts with thiols in proteins such as albumin and hemoglobin to form *S*-nitrosoalbumin and *S*-nitrosohemoglobin. These *S*-nitrosothiols are believed to provide a reservoir of NO bioactivity, although controversy exists with regard to their concentrations in blood. As a consequence of differences in sample handling and detection methods, plasma levels of *S*-nitrosothiols range from 10 nmol/L to 10 μmol/L.[173] Perhaps more important than level is the *S*-nitrosothiol throughput.[174] Depletion of ascorbate reduces the release of NO from *S*-nitrosothiols, and this may explain why the higher circulating levels of *S*-nitrosoalbumin in women with preeclampsia (who have 50% lower ascorbate levels than normal pregnant controls) do not result in more NO release.[174] However, *S*-nitrosothiols induce relaxation not only through their decomposition to NO,[75] but also by activating stereoselective recognition sites.[120] Recently, the cysteine residues within the α subunit of the $BK_{Ca}$ channel were identified as a *S*-nitrosothiol binding site.[77] Our data extend these observations by providing evidence for stereoselective activation of endothelial $IK_{Ca}$ and $SK_{Ca}$ channels by L-*S*-nitrosocysteine and L-*S*-nitrosoglutathion and (subsequent) vascular smooth muscle cell hyperpolarization.

Electrophysiological studies should now further substantiate these findings, investigating in particular which $Ca^{2+}$-activated $K^+$ channel(s) in which cell(s) can be activated by *S*-nitrosothiols. Simultaneously, it should be studied to what degree NO release determines *S*-nitrosothiol-induced coronary vasorelaxation, and how this can be influenced by vitamin C. Furthermore, in view of the findings in eNOS knockout mice,[169] the *S*-nitrosothiol levels in such animals should be measured, considering the fact that inducible NOS and/or neuronal NOS may also contribute to the generation of *S*-

nitrosothiols.[107,175] It is also of importance to demonstrate bradykinin-induced release of *S*-nitrosothiols. This may prove to be difficult, because such release could be limited to a specific compartment (e.g., the myo-endothelial space) that does not allow easy sampling. Theoretically, it is even possible that *S*-nitrosothiols are not released at all and exert their effects on endothelial $IK_{Ca}$ and $SK_{Ca}$ channels entirely through intracellular pathways.

Finally, if $B_2$ receptors are involved in $AT_2$ receptor-induced vasodilation, this would imply that inhibitors of the EDHF pathway will also interfere with $AT_2$ receptor-induced vasodilation. This should be investigated in future experiments. Confounding factors in this regard are the upregulation of $AT_2$ receptors and the EDHF pathway under pathological conditions, the existence of $AT_1$ receptor-$AT_2$ receptor heterodimers, and the gender-related differences in the expression of components of the renin-angiotensin system (RAS) and the EDHF pathway. These aspects should therefore be taken into consideration when designing new studies.

### *$AT_1$ and $\alpha_1$-adrenergic receptor interaction*

The RAS and the sympathetic nervous system interact in several ways. Ang II increases the vascular sensitivity to $\alpha_1$-adrenoceptor agonists,[146] improves central sympathetic outflow,[157,158] and facilitates the release of catecholamines from peripheral sympathetic neurons via ganglionic and axonal presynaptic receptors.[159,160] Vice versa, catecholamines stimulate renin release via renal $\beta_1$-adrenoceptors.

Our study is the first to directly demonstrate $AT_1$-$\alpha_1$-receptor interaction in humans. The interaction may occur both at the receptor level (i.e., physical interaction through heterodimerization) and at the second messenger level (inositol phosphate generation). Future studies should delineate to what degree the enhanced second messenger generation is the consequence of constitutive physical association. Such studies should also verify the $\alpha_1$-adrenoreceptor subtype(s) (A, B and/or D) that contribute(s) to this phenomenon in HCMAs. Furthermore, other second messenger(s) that mediate the interaction should be considered, in particular 20-hydroxyeicosatetraeonic acid (20-HETE). Ang II stimulates 20-HETE production,[176] and 20-HETE has been reported to increase the sensitivity to phenylephrine in renal interlobar arteries.[177] Finally, the possibility of Ang II potentiation by phenylephrine should be investigated.

The current data explain why the non-selective β- and $\alpha_1$-adrenoceptor antagonist carvedilol antagonizes Ang II. In addition, this Ang II-antagonizing capacity offers an explanation for the beneficial metabolic effects of carvedilol versus metoprolol.[143] RAS blockade prevents the onset of diabetes in hypertensive patients and reduces cardiovascular and renal disease progression in diabetic patients with hypertension.[178-180] Most likely, this relates to the fact that Ang II reduces insulin sensitivity.[181] Alternatively, the antidiabetic effects might be explained on the basis of the peroxisome proliferator-activated receptor-γ (PPARγ) activity of (some) $AT_1$ receptor antagonists,[182,183] and/or the bradykinin-potentiating effects of ACE inhibitors.[184] Thus, to fully understand the metabolic effects of carvedilol on top of RAS blockade, future studies should unravel the respective role(s) of Ang II, bradykinin and PPARγ in this regard.

# Nederlandse samenvatting

### *Introductie and doel (Hoofdstuk 1)*

Het renine-angiotensine systeem (RAS) reguleert de bloeddruk en de water- en zout huishouding in het lichaam. Angiotensine (Ang) II is het actieve eindproduct van het RAS. Ang II verhoogt de bloeddruk door binding aan Ang II type 1 ($AT_1$) receptoren in de vaatwand. Verschillende studies naar de werking van Ang II type 2 ($AT_2$) receptoren berichten dat de $AT_2$ receptor dienst doet als tegenpool van de $AT_1$ receptoren. Veel is hier nog niet over bekend en zeker niet in de mens.

Het effect van vaatverwijding via de $AT_2$ receptoren wordt gemedieerd door bradykinine type 2 ($B_2$) receptoren, die vervolgens zorgen voor stimulatie van de afgifte van stikstofmonoxide (NO). Dit proces vindt waarschijnlijk plaats in de endotheelcellen van de bloedvatwand. $AT_2$ receptoren kunnen mogelijk de afgifte van bradykinine stimuleren of een directe interactie met de $B_2$ receptoren aangaan zonder dat bradykinine hier zelf bij betrokken is. Stimulatie van de $B_2$ receptor resulteert naast NO afgifte ook in de afgifte van een groot aantal endotheliale hyperpolarizerende factoren (EDHF's), zoals $K^+$, prostacycline, waterstofperoxide and *S*-nitrososothiolen. Zodoende kunnen wellicht een aantal of alle EDHF's bijdragen aan de vaatverwijding via de $AT_2$ receptor. Het doel van dit proefschrift is om de vaatverwijding via de $AT_2$ receptor verder te bestuderen in de mens en daarbij aandacht te besteden aan de identiteit van EDHF. Daarnaast is de interactie tussen $AT_1$ en $\alpha_1$-adrenerge receptoren bestudeerd, om een verklaring te vinden voor de Ang II-remmende effecten van de niet-selectieve β- en $\alpha_1$-adrenoceptor antagonist carvedilol.

### *$AT_2$ receptoren en vasorelaxatie in de mens (Hoofdstuk 2)*

Ang II contraheert humane coronaire microarteriën (HCMA's) door activatie van $AT_1$ receptoren. Pre-incubatie van HCMA segmenten met de $AT_2$ receptor antagonist PD123319 verhoogde het constrictoire effect van Ang II, een fenomeen dat vooral aanwezig was bij oudere mensen. Dit effect verdween na verwijdering van het endotheel en tijdens blokkade van $B_2$ receptoren of NO synthase (NOS).

Blootstelling aan Ang II van vooraf gecontraheerde HCMA's in de aanwezigheid van de $AT_1$ receptor antagonist irbesartan (zodat selectieve $AT_2$ receptor activatie mogelijk is) resulteerde in relaxatie. PD123319 blokkeerde dit effect volledig. Radioligand bindingsstudies and RT-PCR bevestigden dat $AT_1$ and $AT_2$ receptoren in HCMA's aanwezig zijn. Samenvattend zijn deze data de eerste die vaatverwijding via de $AT_2$ receptor in humane coronairen bevestigen. Dit effect is afhankelijk van endotheliale $B_2$ receptoren en NO, en neemt waarschijnlijk toe met de leeftijd.

### *De identiteit van EDHF (Hoofdstukken 3, 4 en 5)*

Om verder inzicht te krijgen in de identiteit van EDHF werd door bradykinine geïnduceerde vaatrelaxatie bestudeerd in HCMA's, coronaire microarteriën van varkens (PCMA's) en grote coronaire arteriën van varkens (PCA's), in de afwezigheid en aanwezigheid van remmers van NO en/of EDHF.

In overeenstemming met de literatuur was tijdens de relaxatie het aandeel van door NOS gevormd NO belangrijker in grote arteriën dan in microarteriën. In alle vaten induceerde de NO scavenger hydroxocobalamin een grotere mate van remming van de respons op bradykinine dan de NOS remmer L-NAME. Dit suggereert dat er ook NO wordt afgegeven uit een andere bron dan L-arginine, bijvoorbeeld uit NO-bevattende *S*-nitrosothiolen.

In afwezigheid van NO werd de respons op bradykinine volledig geblokkeerd door een combinatie van remmers van intermediate- en small-conductance $Ca^{2+}$-geactiveerde $K^+$-kanalen ($IK_{Ca}$, $SK_{Ca}$) en door de $Na^+/K^+$-ATPase remmer ouabaïne. Ouabaïne verminderde ook de respons op de NO donor SNAP, terwijl gecombineerde blokkade van $IK_{Ca}$ and $SK_{Ca}$ kanalen dit niet deed. De door bradykinine geïnduceerde reacties werden niet beïnvloed door remmers van catalase, cyclooxygenase, cytochroom P450 epoxygenase, gap junctions en large-conductance $Ca^{2+}$-geactiveerde $K^+$-kanalen ($BK_{Ca}$). Samengevat suggereren deze resultaten dat de door bradykinine geïnduceerde relaxatie in humane en varkens coronairen afhankelijk is van de activatie van guanylyl cyclase en $Na^+/K^+$-ATPase door NO, en van de activatie van $IK_{Ca}$ and $SK_{Ca}$ kanalen door een factor die niet *de novo* gevormd NO is. Aanvullende studies maakten duidelijk dat deze factor waarschijnlijk een *S*-nitrosothiol is.

### *Interactie tussen $AT_1$ en $\alpha_1$-adrenerge receptoren (Hoofdstuk 6)*

Carvedilol, de selectieve $\beta_1$-adrenoceptor antagonist metoprolol, de niet-selectieve β-adrenoceptor antagonist propranolol, en de $\alpha_1$-adrenoceptor antagonist prazosin beïnvloedden geen van allen de constrictoire respons van HCMA's op Ang II.

Ang II, na toevoeging aan het orgaan bad in een lage (non-constrictoire) concentratie, versterkte de respons op de $\alpha_1$-adrenoceptor agonist fenylefrine enorm. Zowel carvedilol als de $AT_1$ receptor antagonist irbesartan remden deze door Ang II geïnduceerde potentiatie. Carvedilol remde eveneens de door Ang II versterkte ophoping van inositolfosfaten onder invloed van fenylefrine in hartspiercellen. Samenvattend kan gesteld worden dat $AT_1$-$\alpha_1$-receptor 'crosstalk', mogelijk via inositolfosfaten, HCMA's gevoeliger maakt voor $\alpha_1$-adrenoceptor agonisten. De $\alpha_1$-adrenoceptor blokkerende effecten van carvedilol zorgen er voor dat carvedilol dit potentiërende effect van Ang II tegen kan gaan. Dit verklaart waarom het bloeddrukverhogende effect van Ang II bij patiënten met hartfalen die behandeld worden met carvedilol kleiner is dan bij patiënten die behandeld worden met metoprolol

# Publications

## *Full papers*

Batenburg WW, Garrelds IM, van Kats JP, Saxena PR, Danser AHJ, Mediators of bradykinin-induced vasorelaxation in human coronary microarteries.
*Hypertension.* 2004; 43: 488-492.

Marnewick JL, Batenburg W, Swart P, Joubert E, Swanefelder S, Gelderblom WCA, Ex vivo modulation of chemical-induced mutagenesis by subcellular liver fractions of rats treated with rooibos (*Aspalathus linearis*) tea, honeybush (*Cyclopia intermedia*) tea, as well as green and black (*Camellia sinensis*) teas.
*Mutation Research.* 2004; 558: 145-154.

Batenburg WW, Rüdiger Popp, Ingrid Fleming, de Vries R, Saxena PR, Danser AHJ, Bradykinin-induced vasorelaxation in porcine microcoronaries: *S*-nitrosothiols as EDHF?
*British Journal of Pharmacology*. 2004; 142: 125-135.

Batenburg WW, Garrelds IM, Chapuis Bernasconi C, Juillerat-Jeanneret L, van Kats JP, Saxena PR, Danser AHJ, Angiotensin II Type 2 Receptor-Mediated Vasodilation in Human Coronary Microarteries.
*Circulation.* 2004; 109: 2296-2301.

Batenburg WW, de Vries R, Saxena PR, Danser AHJ, L-*S*-nitrosothiols: endothelium-derived hyperpolarizing factors in porcine coronary arteries?
*Journal of Hypertension.* 2004; 22: 1927-1936.

Van Esch JH, Tom B, Batenburg WW, Georgiadis D, Yiokatis A, van Gool JM, de Bruin RJ, de Vries R, Danser AHJ, Selective Angiotensin-Converting Enzyme C-Domain Inhibition Is Sufficient to Prevent Angiotensin I-Induced Vasoconstriction.
*Hypertension.* 2005; 45: 120-125.

Batenburg WW, Danser AHJ, Angiotensin II type 2 receptor-mediated vasodilation. Focus on bradykinin, NO and endothelium-derived hyperpolarizing factor(s).
*Vascular Pharmacology*. 2005, 42:109-118.

Batenburg WW, van Esch JHM, Garrelds IM, Jorde U, Lamers JMJ, Dekkers DHW, Walther T, Kellett E, Milligan G, van Kats JP, Danser AHJ, Carvedilol-induced antagonism of Angiotensin II: a matter of $\alpha_1$-adrenoceptor blockade.
Submitted, 2005.

## *Abstracts*

Batenburg WW, de Vries R, Saxena PR, Danser AHJ. Bradykinin-induced vasorelaxation in porcine microcoronaries: importance of NO and *S*-nitrosothiols.
*Journal of Hypertension.* 2002; 20 (suppl 4): S286.

Batenburg WW, Schuijt MP, Saxena PR, Danser AHJ. $AT_2$ receptor-mediated vasodilation in human coronary microarteries. Role of NO.
*Hypertension.* 2002; 40: 398.

Batenburg WW, de Vries R, Saxena PR, Danser AHJ. Bradykinin-induced vasorelaxation in porcine microcoronaries: are S-nitrosothiols endothelium-derived hyperpolarizing factors (EDHF)?
*Hypertension.* 2002; 40: 417.

Batenburg WW, Schuijt MP, Saxena PR, Danser AHJ. $AT_2$ receptor-mediated vasodilation in human coronary microarteries. Role of NO.
*Hypertension.* 2002; 40: 571.

Batenburg WW, Schuijt MP, Saxena PR, Danser AHJ. $AT_2$ receptor-mediated vasodilation in human and rat coronary arteries. Role of nitric oxide.
*British Journal of Pharmacology.* 2003; 138: 227P.

Batenburg WW, Saxena PR, Danser AHJ. Angiotensin II type 2 receptor-mediated vasodilation in human coronary microarteries. Role of NO.
*Journal of Hypertension.* 2003; 21 (suppl 4): S44.

Danser AHJ, de Vries R, Batenburg WW, Saxena PR. L-*S*-Nitrocysteine: an endothelium-derived hyperpolarizing factor (EDHF) in porcine coronary arteries?
*Journal Hypertension.* 2003; 21 (suppl 4): S122.

Batenburg WW, Saxena PR, Danser AHJ. Mediators of bradykinin-induced vasorelaxation in human coronary microarteries.
*Journal of Hypertension.* 2003; 21 (suppl 4): S224.

Batenburg WW, Saxena PR, Danser AHJ. Bradykinin-induced vasorelaxation in human coronary microarteries.
*Hypertension.* 2003; 42: 440.

Danser AHJ, Batenburg WW, de Vries R, Saxena PR. L-*S*-Nitrocysteine: an endothelium-derived hyperpolarizing factor (EDHF) in porcine coronary arteries?
*Hypertension.* 2003; 42: 440.

Batenburg WW, Jorde U, Saxena PR, Danser AHJ. Carvedilol-induced antagonism of angiotensin II: a matter of $\alpha_1$-adrenoceptor blockade?
*Journal of Hypertension.* 2004; 22 (suppl 1): S113.

Tom B, Batenburg WW, Garrelds IM, van Gool JMG, de Bruijn RJA, de Vries R, Saxena PR, Danser AHJ. Low ACE inhibitor doses block tissue but not plasma ACE-mediated angiotensin I-II conversion.
*Journal of Hypertension.* 2004; 22 (suppl 1): S173.

Batenburg WW, van Esch JHM, Garrelds IM, Jorde U, van Kats JP, Saxena PR, Danser AHJ. Carvedilol-induced antagonism of angiotensin II: focus on $AT_1$ receptor-$\alpha_1$ adrenoceptor crosstalk.
*Hypertension.* 2004; 44: 498.

Batenburg WW, van Esch JHM, Danser AHJ. Carvedilol-induced antagonism of angiotensin II: focus on $AT_1$ receptor-$\alpha_1$-adrenoceptor crosstalk.
*Naunyn-Schmiedebergs Archives for Pharmacology*. 2005; in press.

## Dankwoord

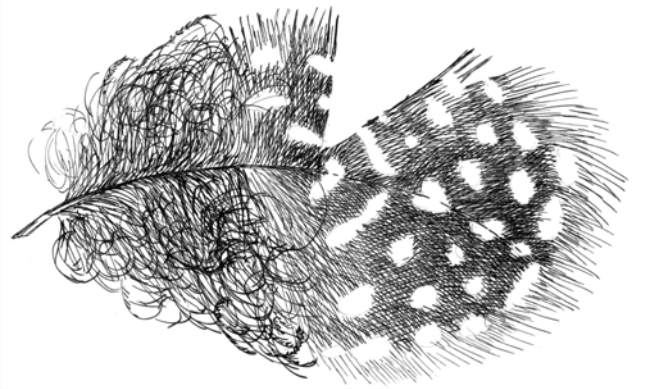
Nooit gedacht dat ik zover zou komen! Natuurlijk is het onmogelijk om dit in je eentje voor mekaar te krijgen en daarom wil ik dit gedeelte van mijn proefschrift wijden aan iedereen die me hierin gesteund heeft.

Als eerste wil ik mijn promotor bedanken. Jan, in de laatste vier jaar heb ik veel van je geleerd en je gaf me alle kans om me verder te ontwikkelen, ook je nieuwsgierigheid over wat er gebeurde op het lab en de tijd die je altijd voor me vrij maakte waardeer ik enorm. Je gaf me de gelegenheid om naar Berlijn te gaan en daar ontzettend veel nieuwe dingen te leren. Berlijn was een enorme uitdaging voor me en mijn tijd daar was geweldig.
Prof. Saxena wil ik bedanken voor de altijd aanwezige intresse in mij en mijn onderzoek. Ik wil Prof.dr. Jos Lamers, Prof.dr. Peter de Leeuw, Prof.dr. Maarten Schalekamp en dr. Jacqueline Witteman bedanken voor hun bijdrage in het beoordelen van mijn proefschrift en het plaatsnemen in mijn commissie. Prof.dr. Michael Bader bedank ik voor zijn komst naar Rotterdam, erg leuk dat u na onze samenwerking in Berlijn nu aanwezig wilt zijn als lid van mijn promotiecommissie.

Tijdens mijn sollicitatie kreeg ik een rondleiding van Martin. Toen we even stilstonden bij de Mulvany myographs vertelde hij me dat deze apparatuur nooit meer gebruikt werd, niet wetende dat uiteindelijk het grootste gedeelte van mijn proefschrift tot stand zou komen door middel van deze techniek. De Mulvany's werden al snel erg gewild en al gauw moest ik vechten om de orgaanbadjes met Saurabh. Saurabh, thanks for the great time in and outside the lab and for supporting me as my paranymph. Ook René bedankt voor je hulp en gezelligheid op het lab en de experimenten die je voor me hebt gedaan. Ook heb ik met veel plezier samengewerkt met René en Jeanette van Interne Geneeskunde. Ik wil de Hartkleppenbank en zijn medewerkers bedanken voor de prettige samenwerking en het mogelijk maken van mijn experimenten in humane kransslagaders.
Mijn kamergenoten Erik, Wenxia, Sue, Inge, Ingrid en Uday bedankt voor de gezellige tijd op nummer 1424. Niet te vergeten de rest uit 'Jan's groep', Martin, Beril, Jasper, Aloys, Mark en Joep. En de rest van de (ex-)collega's, Antoinette, Birgitte, Magda, Ria,

Anna, Vijay, Hari, Regien, Richard, Jan, Rémon, Andor, Roeland, Suneet, Pankaj, Edwin, Jailin, Wang Yong, Silvia, Thomas, Florian, Michaela en iedereen die ik vergeten ben.

Pap en mam bedankt voor jullie steun en vertrouwen in alles wat ik deed ook al was dat iets verder weg dan jullie eigenlijk leuk vonden. Peter, het is zo handig dat je zoveel van computers af weet! Je hebt me gered in Berlijn. Dennis, ik ben heel erg blij dat je me bijstaat als paranimf, we hebben altijd veel lol samen en ik weet zeker dat jij, Saurabh en ik een goed team vormen tijdens mijn promotie.
Ik heb er sinds kort een hele gezellige familie bij. Arie, Corrie, Sonja, Willem en de kids, jullie zijn altijd zo geïnteresseerd in wat ik doe en al lijkt het af en toe moeilijk te begrijpen, het valt allemaal wel mee!
Lieve Marco, hoe snel kan alles gaan. Je steunt me in alles wat ik doe en hebt zoveel vertrouwen in me, super dat je in mijn leven bent gekomen. Ik kijk ontzettend uit naar de tijd samen met jou in ons eigen huisje.



## Curriculum vitae

De auteur van dit proefschrift werd geboren op 18 oktober 1976 te Sliedrecht. Nadat zij het VWO diploma behaalde aan de Lage Waard te Papendrecht in 1995, begon ze aan de opleiding Fundamentele Biomedische Wetenschappen aan de Universiteit van Utrecht om deze vervolgens in 2000 met het doctoraalexamen af te ronden. Tijdens de doctoraalfase liep zij haar eerste stage in Utrecht bij de afdeling Veterinaire Farmacie Farmacologie en Toxicologie. Onder leiding van prof.dr. J. Fink-Gremmels bestudeerde ze de mogelijke rol van haem oxygenase en xanthine oxidase bij de afbraak van cytochroom P450 in *in vitro* celsystemen. Ze vervolgde haar studie met haar tweede stage bij Programme on Mycotoxins and Experimental Carcinogenesis, Medical Research Council, te Kaapstad, Zuid-Afrika onder begeleiding van dr. W. Gelderblom. Hier was ze betrokken bij het bestuderen van de mogelijke anti-mutageniteit van twee inheemse theesoorten, rooibos (*Aspalathus Linearis*) en honeybush thee (C*yclopia Intermedia*) en hun invloed op de enzymen glutathione-S-transferase en cytochroom P450. In haar afstudeerscriptie richtte zij zich op de drugsresistentie van patienten met hepatitis C. Sinds mei 2001 is ze werkzaam geweest als assistent in opleiding op de afdeling Farmacologie van het Erasmus MC te Rotterdam onder supervisie van prof.dr. A.H.J. Danser. Tijdens deze periode heeft ze een half jaar Rotterdam verruild voor Berlijn waar ze onder leiding van dr. D.N. Müller en prof.dr. M. Bader deelnam aan het onderzoek naar de renine receptor, om mogelijk via siRNA downregulatie van de receptor te bewerkstelligen. Haar onderzoek onder directe leiding van haar promotor prof.dr. A.H.J. Danser resulteerde in dit proefschrift.

## Abbreviations

| | |
|---|---|
| ACE | angiotensin converting enzyme |
| Ang | angiotensin |
| apa | apamin |
| $AT_1$ | angiotensin II type 1 |
| $AT_2$ | angiotensin II type 2 |
| $B_2$ | bradykinin type 2 |
| $BK_{Ca}$ | large-conductance $Ca^{2+}$-activated $K^+$ channel |
| cGMP | cyclic guanylylmonophosphate |
| char | charybdotoxin |
| CHF | chronic heart failure |
| CRC | concentration response curve |
| DEA-NONOate | dietylamine NONOate |
| D-SNC | D-S-nitrosocysteine |
| EDHF | endothelium-derived hyperpolarizing factor |
| EET | epoxyeicosatrienoic acid |
| ET-1 | endothelin-1 |
| eYFP | enhanced yellow fluorescent protein |
| HC | hydroxocobalamin |
| HCA | human coronary artery |
| HCMA | human coronary microartery |
| HEK293 | human embryonic kidney cells 293 |
| iber | iberiotoxin |
| $IK_{Ca}$ | intermediate-conductance $Ca^{2+}$-activated $K^+$ channel |
| $InsP_n$ | inositolphospates |
| $K_{IR}$ | inwardly rectifying $K^+$ channel |
| $K_V$ | voltage gated $K^+$ channel |
| L-NAME | $N^{\omega}$-nitro-L-arginine methyl ester HCl |
| L-NOARG | $N^{\omega}$-nitro-L-arginine |
| L-SNC | L-S-nitrosocysteine |
| L-SNG | L-S-nitrosoglutathione |
| NO | nitrix oxide |
| NOS | nitrix oxide synthase |
| PCA | porcine coronary artery |
| PCMA | porcine coronary microartery |
| PHMBA | p-hydroxymercurobezoic acid |
| PE | phenylephrine |
| RAS | renin angiotensin system |
| RMP | resting membrane potential |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| $SK_{Ca}$ | small-conductance $Ca^{2+}$-activated $K^+$ channel |
| SNAP | S-nitroso N-penicillamine |
| $TxA_2$ | thromboxane $A_2$ |
| U46619 | 9,11-dideoxy-11$\alpha$,9$\alpha$-epoxymethano-prostaglandin $F_{2\alpha}$ |

## References


1. Brink M, Erne P, de Gasparo M, Rogg H, Schmid A, Stulz P, Bullock G. Localization of the angiotensin II receptor subtypes in the human atrium. *J Mol Cell Cardiol*. 1996;28:1789-1799.
2. Grone HJ, Simon M, Fuchs E. Autoradiographic characterization of angiotensin receptor subtypes in fetal and adult human kidney. *Am J Physiol*. 1992;262:F326-F331.
3. Regitz-Zagrosek V, Neuss M, Warnecke C, Holzmeister J, Hildebrandt AG, Fleck E. Subtype 2 and atypical angiotensin receptors in the human heart. *Basic Res Cardiol*. 1996;91:73-77.
4. Widdop RE, Matrougui K, Levy BI, Henrion D. AT2 receptor-mediated relaxation is preserved after long-term AT1 receptor blockade. *Hypertension*. 2002;40:516-520.
5. Widdop RE, Jones ES, Hannan RE, Gaspari TA. Angiotensin AT2 receptors: cardiovascular hope or hype? *Br J Pharmacol*. 2003;140:809-824.
6. Zhu YC, Zhu YZ, Lu N, Wang MJ, Wang YX, Yao T. Role of angiotensin AT1 and AT2 receptors in cardiac hypertrophy and cardiac remodelling. *Clin Exp Pharmacol Physiol*. 2003;30:911-918.
7. Kurisu S, Ozono R, Oshima T, Kambe M, Ishida T, Sugino H, Matsuura H, Chayama K, Teranishi Y, Iba O, Amano K, Matsubara H. Cardiac angiotensin II type 2 receptor activates the kinin/NO system and inhibits fibrosis. *Hypertension*. 2003;41:99-107.
8. Xu J, Carretero OA, Liu YH, Shesely EG, Yang F, Kapke A, Yang XP. Role of AT2 receptors in the cardioprotective effect of AT1 antagonists in mice. *Hypertension*. 2002;40:244-250.
9. Yang Z, Bove CM, French BA, Epstein FH, Berr SS, DiMaria JM, Gibson JJ, Carey RM, Kramer CM. Angiotensin II type 2 receptor overexpression preserves left ventricular function after myocardial infarction. *Circulation*. 2002;106:106-111.
10. Carey RM, Wang ZQ, Siragy HM. Role of the angiotensin type 2 receptor in the regulation of blood pressure and renal function. *Hypertension*. 2000;35:155-163.
11. van Kats JP, Duncker DJ, Haitsma DB, Schuijt MP, Niebuur R, Stubenitsky R, Boomsma F, Schalekamp MADH, Verdouw PD, Danser AHJ. Angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade prevent cardiac remodeling in pigs after myocardial infarction: role of tissue angiotensin II. *Circulation*. 2000;102:1556-1563.
12. Liu YH, Yang XP, Sharov VG, Nass O, Sabbah HN, Peterson E, Carretero OA. Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. Role of kinins and angiotensin II type 2 receptors. *J Clin Invest*. 1997;99:1926-1935.
13. van Kesteren CAM, van Heugten HAA, Lamers JMJ, Saxena PR, Schalekamp MADH, Danser AHJ. Angiotensin II-mediated growth and antigrowth effects in cultured neonatal rat cardiac myocytes and fibroblasts. *J. Mol. Cell. Cardiol.* 1997;29:2147-2157.
14. Katada J, Majima M. AT(2) receptor-dependent vasodilation is mediated by activation of vascular kinin generation under flow conditions. *Br J Pharmacol*. 2002;136:484-491.
15. Munzenmaier DH, Greene AS. Opposing actions of angiotensin II on microvascular growth and arterial blood pressure. *Hypertension*. 1996;27:760-765.
16. Widdop RE, Gardiner SM, Kemp PA, Bennett T. Inhibition of the haemodynamic effects of angiotensin II in conscious rats by AT2-receptor antagonists given after the AT1-receptor antagonist, EXP 3174. *Br J Pharmacol*. 1992;107:873-880.
17. Widdop RE, Gardiner SM, Kemp PA, Bennett T. Central administration of PD 123319 or EXP-3174 inhibits effects of angiotensin II. *Am J Physiol*. 1993;264:H117-H125.
18. Gohlke P, Pees C, Unger T. $AT_2$ receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism. *Hypertension*. 1998;31:349-355.

19. Siragy HM, Senbonmatsu T, Ichiki T, Inagami T, Carey RM. Increased renal vasodilator prostanoids prevent hypertension in mice lacking the angiotensin subtype-2 receptor. *J Clin Invest*. 1999;104:181-188.
20. Ichiki T, Labosky PA, Shiota C, Okuyama S, Imagawa Y, Fogo A, Niimura F, Ichikawa I, Hogan BL, Inagami T. Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. *Nature*. 1995;377:748-750.
21. Hein L, Barsh GS, Pratt RE, Dzau VJ, Kobilka BK. Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. *Nature*. 1995;377:744-747.
22. Tsutsumi Y, Matsubara H, Masaki H, Kurihara H, Murasawa S, Takai S, Miyazaki M, Nozawa Y, Ozono R, Nakagawa K, Miwa T, Kawada N, Mori Y, Shibasaki Y, Tanaka Y, Fujiyama S, Koyama Y, Fujiyama A, Takahashi H, Iwasaka T. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. *J Clin Invest*. 1999;104:925-935.
23. Endo Y, Arima S, Yaoita H, Tsunoda K, Omata K, Ito S. Vasodilation mediated by angiotensin II type 2 receptor is impaired in afferent arterioles of young spontaneously hypertensive rats. *J Vasc Res*. 1998;35:421-427.
24. Dimitropoulou C, White RE, Fuchs L, Zhang H, Catravas JD, Carrier GO. Angiotensin II relaxes microvessels via the $AT_2$ receptor and $Ca^{2+}$-activated $K^+$ ($BK_{Ca}$) channels. *Hypertension*. 2001;37:301-307.
25. Tada H, Egashira K, Yamamoto M, Ueno H, Takemoto M, Shimokawa H, Takeshita A. Glibenclamide, a specific inhibitor of ATP-sensitive K+ channels, inhibits coronary vasodilation induced by angiotensin II-receptor antagonists. *J Cardiovasc Pharmacol*. 1997;30:313-319.
26. Schuijt MP, Basdew M, van Veghel R, de Vries R, Saxena PR, Schoemaker RG, Danser AHJ. $AT_2$ receptor-mediated vasodilation in the heart: effect of myocardial infarction. *Am J Physiol*. 2001a;281:H2590-H2596.
27. Touyz RM, Endemann D, He G, Li JS, Schiffrin EL. Role of AT2 receptors in angiotensin II-stimulated contraction of small mesenteric arteries in young SHR. *Hypertension*. 1999;33:366-372.
28. Schuijt MP, de Vries R, Saxena PR, Danser AHJ. No vasoactive role of the angiotensin II type 2 receptor in normotensive Wistar rats. *J Hypertens*. 1999;17:1879-1884.
29. Yamada H, Akishita M, Ito M, Tamura K, Daviet L, Lehtonen JY, Dzau VJ, Horiuchi M. AT2 receptor and vascular smooth muscle cell differentiation in vascular development. *Hypertension*. 1999;33:1414-1419.
30. Grady EF, Sechi LA, Griffin CA, Schambelan M, Kalinyak JE. Expression of AT2 receptors in the developing rat fetus. *J Clin Invest*. 1991;88:921-933.
31. Tsutsumi Y, Matsubara H, Ohkubo N, Mori Y, Nozawa Y, Murasawa S, Kijima K, Maruyama K, Masaki H, Moriguchi Y, Shibasaki Y, Kamihata H, Inada M, Iwasaka T. Angiotensin II type 2 receptor is upregulated in human heart with interstitial fibrosis, and cardiac fibroblasts are the major cell type for its expression. *Circ Res*. 1998;83:1035-4106.
32. Zhang C, Hein TW, Wang W, Kuo L. Divergent roles of angiotensin II AT1 and AT2 receptors in modulating coronary microvascular function. *Circ Res*. 2003;92:322-329.
33. Siragy HM, Carey RM. The subtype 2 ($AT_2$) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest*. 1997;100:264-269.
34. Nouet S, Nahmias C. Signal transduction from the angiotensin II AT2 receptor. *Trends Endocrinol Metab*. 2000;11:1-6.
35. AbdAlla S, Lother H, Abdel-tawab AM, Quitterer U. The angiotensin II $AT_2$ receptor is an $AT_1$ receptor antagonist. *J Biol Chem*. 2001;276:39721-39726.

36. Jin XQ, Fukuda N, Su JZ, Lai YM, Suzuki R, Tahira Y, Takagi H, Ikeda Y, Kanmatsuse K, Miyazaki H. Angiotensin II type 2 receptor gene transfer downregulates angiotensin II type 1a receptor in vascular smooth muscle cells. *Hypertension*. 2002;39:1021-1027.
37. Hannan RE, Davis EA, Widdop RE. Functional role of angiotensin II AT2 receptor in modulation of AT1 receptor-mediated contraction in rat uterine artery: involvement of bradykinin and nitric oxide. *Br J Pharmacol*. 2003;140:987-995.
38. Siragy HM, Carey RM. The subtype-2 (AT2) angiotensin receptor regulates renal cyclic guanosine 3', 5'-monophosphate and AT1 receptor-mediated prostaglandin E2 production in conscious rats. *J Clin Invest*. 1996;97:1978-1982.
39. Bergaya S, Hilgers RH, Meneton P, Dong Y, Bloch-Faure M, Inagami T, Alhenc-Gelas F, Levy BI, Boulanger CM. Flow-dependent dilation mediated by endogenous kinins requires angiotensin AT2 receptors. *Circ Res*. 2004;94:1623-1629.
40. Schuijt MP, de Vries R, Saxena PR, Danser AHJ. Prostanoids, but not nitric oxide, counterregulate angiotensin II mediated vasoconstriction in vivo. *Eur J Pharmacol*. 2001b;428:331-336.
41. Schmieder RE, Erdmann J, Delles C, Jacobi J, Fleck E, Hilgers K, Regitz-Zagrosek V. Effect of the angiotensin II type 2-receptor gene (+1675 G/A) on left ventricular structure in humans. *J Am Coll Cardiol*. 2001;37:175-182.
42. Deinum J, van Gool JM, Kofflard MJ, ten Cate FJ, Danser AHJ. Angiotensin II Type 2 Receptors and Cardiac Hypertrophy in Women With Hypertrophic Cardiomyopathy. *Hypertension*. 2001;38:1278-1281.
43. Matsumoto T, Ozono R, Oshima T, Matsuura H, Sueda T, Kajiyama G, Kambe M. Type 2 angiotensin II receptor is downregulated in cardiomyocytes of patients with heart failure. *Cardiovasc Res*. 2000;46:73-81.
44. Phoon S, Howes LG. Forearm vasodilator response to angiotensin II in elderly women receiving candesartan: role of AT(2)- receptors. *J Renin Angiotensin Aldosterone Syst*. 2002;3:36-39.
45. Saris JJ, van Dijk MA, Kroon I, Schalekamp MADH, Danser AHJ. Functional importance of angiotensin-converting enzyme-dependent in situ angiotensin II generation in the human forearm. *Hypertension*. 2000;35:764-768.
46. MaassenVanDenBrink A, de Vries R, Saxena PR, Schalekamp MADH, Danser AHJ. Vasoconstriction by in situ formed angiotensin II: role of ACE and chymase. *Cardiovasc Res*. 1999;44:407-415.
47. Batenburg WW, Garrelds IM, Bernasconi CC, Juillerat-Jeanneret L, van Kats JP, Saxena PR, Danser AHJ. Angiotensin II type 2 receptor-mediated vasodilation in human coronary microarteries. *Circulation*. 2004a;109:2296-2301.
48. Wharton J, Morgan K, Rutherford RAD, Catravas JD, Chester A, Whitehead BF, de Leval MR, Yacoub MH, Polak JM. Differential distribution of angiotensin $AT_2$ receptors in the normal and failing human heart. *J Pharmacol Exp Ther*. 1998;284:323-336.
49. Li DY, Zhang YC, Philips MI, Sawamura T, Mehta JL. Upregulation of endothelial receptor for oxidized low-density lipoprotein (LOX-1) in cultured human coronary artery endothelial cells by angiotensin II type 1 receptor activation. *Circ Res*. 1999;84:1043-1049.
50. Danser AHJ, Derkx FHM, Schalekamp MADH, Hense HW, Riegger GAJ, Schunkert H. Determinants of interindividual variation of renin and prorenin concentrations: evidence for a sexual dimorphism of (pro)renin levels in humans. *J Hypertens*. 1998b;16:853-862.
51. Silva-Antonialli MM, Tostes RC, Fernandes L, Fior-Chadi DR, Akamine EH, Carvalho MH, Fortes ZB, Nigro D. A lower ratio of AT(1)/AT(2) receptors of angiotensin II is found in female than in male spontaneously hypertensive rats. *Cardiovasc Res*. 2004;62:587-593.

52. Schuijt MP, Tom B, de Vries R, Saxena PR, Sluiter W, van Kats JP, Danser AHJ. Superoxide does not mediate the acute vasoconstrictor effects of angiotensin II: a study in human and porcine arteries. *J Hypertens*. 2003;21:2335-2344.
53. Tom B, Garrelds IM, Scalbert E, Stegmann AP, Boomsma F, Saxena PR, Danser AH. ACE-versus chymase-dependent angiotensin II generation in human coronary arteries: a matter of efficiency? *Arterioscler Thromb Vasc Biol*. 2003;23:251-256.
54. Danser AHJ, Tom B, de Vries R, Saxena PR. L-NAME resistant bradykinin-induced relaxation in porcine coronary arteries is NO-dependent: effect of ACE inhibition. *Br J Pharmacol*. 2000;131:195-202.
55. Tom B, Dendorfer A, de Vries R, Saxena PR, Jan Danser AHJ. Bradykinin potentiation by ACE inhibitors: a matter of metabolism. *Br J Pharmacol*. 2002;137:276-284.
56. Tom B, de Vries R, Saxena PR, Danser AHJ. Bradykinin potentiation by angiotensin-(1-7) and angiotensin-converting enzyme (ACE) inhibitors correlates with ACE C- and N-domain blockade. *Hypertension*. 2001;38:95-99.
57. Batenburg WW, Garrelds IM, van Kats JP, Saxena PR, Danser AHJ. Mediators of bradykinin-induced vasorelaxation in human coronary microarteries. *Hypertension*. 2004b;43:488-492.
58. Batenburg WW, Popp R, Fleming I, Vries Rd R, Garrelds IM, Saxena PR, Danser AHJ. Bradykinin-induced relaxation of coronary microarteries: S-nitrosothiols as EDHF? *Br J Pharmacol*. 2004c;142:125-135.
59. Davisson RL, Bates JN, Johnson AK, Lewis SJ. Use-dependent loss of acetylcholine- and bradykinin-mediated vasodilation after nitric oxide synthase inhibition. Evidence for preformed stores of nitric oxide-containing factors in vascular endothelial cells. *Hypertension*. 1996a;28:354-360.
60. Danser AHJ, de Vries R, Schoemaker RG, Saxena PR. Bradykinin-induced release of nitric oxide by the isolated perfused rat heart: importance of preformed pools of nitric oxide-containing factors. *J Hypertens*. 1998a;16:239-244.
61. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: bringing the concepts together. *Trends Pharmacol Sci*. 2002;23:374-380.
62. Mombouli JV, Vanhoutte PM. Endothelium-derived hyperpolarizing factor(s): updating the unknown. *Trends Pharmacol Sci*. 1997;18:252-256.
63. Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, Busse R. Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature*. 1999;401:493-497.
64. Edwards G, Dora KA, Gardener MJ, Garland CJ, Weston AH. K+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature*. 1998;396:269-272.
65. Edwards G, Feletou M, Gardener MJ, Glen CD, Richards GR, Vanhoutte PM, Weston AH. Further investigations into the endothelium-dependent hyperpolarizing effects of bradykinin and substance P in porcine coronary artery. *Br J Pharmacol*. 2001;133:1145-1153.
66. Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res*. 1996;78:415-423.
67. Matoba T, Shimokawa H. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in animals and humans. *J Pharmacol Sci*. 2003;92:1-6.
68. Hwa JJ, Ghibaudi L, Williams P, Chatterjee M. Comparison of acetylcholine-dependent relaxation in large and small arteries of rat mesenteric vascular bed. *Am J Physiol*. 1994;266:H952-H958.
69. Bychkov R, Burnham MP, Richards GR, Edwards G, Weston AH, Feletou M, Vanhoutte PM. Characterization of a charybdotoxin-sensitive intermediate conductance Ca2+-activated K+ channel in porcine coronary endothelium: relevance to EDHF. *Br J Pharmacol*. 2002;137:1346-1354.

70. Sollini M, Frieden M, Beny JL. Charybdotoxin-sensitive small conductance K(Ca) channel activated by bradykinin and substance P in endothelial cells. *Br J Pharmacol*. 2002;136:1201-1209.
71. Archer SL, Gragasin FS, Wu X, Wang S, McMurtry S, Kim DH, Platonov M, Koshal A, Hashimoto K, Campbell WB, Falck JR, Michelakis ED. Endothelium-derived hyperpolarizing factor in human internal mammary artery is 11,12-epoxyeicosatrienoic acid and causes relaxation by activating smooth muscle BK(Ca) channels. *Circulation*. 2003;107:769-776.
72. Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*. 1994;368:850-853.
73. Kemp BK, Cocks TM. Evidence that mechanisms dependent and independent of nitric oxide mediate endothelium-dependent relaxation to bradykinin in human small resistance-like coronary arteries. *Br J Pharmacol*. 1997;120:757-762.
74. Lovren F, Triggle CR. Involvement of nitrosothiols, nitric oxide and voltage-gated K+ channels in photorelaxation of vascular smooth muscle. *Eur J Pharmacol*. 1998;347:215-221.
75. Rafikova O, Rafikov R, Nudler E. Catalysis of S-nitrosothiols formation by serum albumin: the mechanism and implication in vascular control. *Proc Natl Acad Sci USA*. 2002;99:5913-5918.
76. Davisson RL, Travis MD, Bates JN, Lewis SJ. Hemodynamic effects of L- and D-S-nitrosocysteine in the rat. Stereoselective S-nitrosothiol recognition sites. *Circ Res*. 1996b;79:256-262.
77. Lang RJ, Harvey JR, Mulholland EL. Sodium (2-sulfonatoethyl) methanethiosulfonate prevents S-nitroso-L-cysteine activation of Ca2+-activated K+ (BK(Ca)) channels in myocytes of the guinea-pig taenia caeca. *Br J Pharmacol*. 2003;139:1153-1163.
78. Batenburg WW, de Vries R, Saxena PR, Danser AHJ. L-S-nitrosothiols: endothelium-derived hyperpolarizing factors in porcine coronary arteries? *J Hypertens*. 2004d;in press.
79. Sakuma I, Liu MY, Sato A, Hayashi T, Iguchi A, Kitabatake A, Hattori Y. Endothelium-dependent hyperpolarization and relaxation in mesenteric arteries of middle-aged rats: influence of oestrogen. *Br J Pharmacol*. 2002;135:48-54.
80. Liu MY, Hattori Y, Sato A, Ichikawa R, Zhang XH, Sakuma I. Ovariectomy attenuates hyperpolarization and relaxation mediated by endothelium-derived hyperpolarizing factor in female rat mesenteric artery: a concomitant decrease in connexin-43 expression. *J Cardiovasc Pharmacol*. 2002;40:938-948.
81. Fukada SY, Tirapelli CR, de Godoy MA, de Oliveira AM. Mechanisms underlying the endothelium-independent relaxation induced by angiotensin II in rat aorta. *J Cardiovasc Pharmacol*. 2005;45:136-143.
82. Dohi Y, Hahn AW, Boulanger CM, Buhler FR, Luscher TF. Endothelin stimulated by angiotensin II augments contractility of spontaneously hypertensive rat resistance arteries. *Hypertension*. 1992;19:131-137.
83. Crespo MJ. Interaction between AT1 and alpha1-adrenergic receptors in cardiomyopathic hamsters. *J Card Fail*. 2000;6:257-263.
84. Borland JAA, Chester AH, Morrison KA, Yacoub MH. Alternative pathways of angiotensin II production in the human saphenous vein. *Br J Pharmacol*. 1998;125:423-428.
85. Baan J, Chang PC, Vermeij P, Pfaffendorf M, van Zwieten PA. Effects of losartan on vasoconstrictor responses to angiotensin II in the forearm vascular bed of healthy volunteers. *Cardiovasc Res*. 1996;32:973-979.
86. Zwart AS, Davis EA, Widdop RE. Modulation of $AT_1$ receptor-mediated contraction of rat uterine artery by $AT_2$ receptors. *Br J Pharmacol*. 1998;125:1429-1436.

87. Carey RM, Howell NL, Jin XH, Siragy HM. Angiotensin type 2 receptor-mediated hypotension in angiotensin type-1 receptor-blocked rats. *Hypertension*. 2001;38:1272-1277.
88. Matsubara H. Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. *Circ Res*. 1998;83:1182-1191.
89. Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res*. 1977;41:19-26.
90. Li P, Fukuhara M, Diz DI, Ferrario CM, Brosnihan KB. Novel angiotensin II $AT_1$ antagonist irbesartan prevents thromboxane $A_2$-induced vasoconstriction in canine coronary arteries and human platelet aggregation. *J. Pharmacol. Exp. Ther.* 2000;292:238-246.
91. Regitz-Zagrosek V, Friedel N, Heymann A, Bauer P, Neuss M, Rolfs A, Steffen C, Hildebrandt A, Hetzer R, Fleck E. Regulation, chamber localization, and subtype distribution of angiotensin II receptors in human hearts. *Circulation*. 1995;91:1461-1471.
92. Kapoor K, Arulmani U, Heiligers JP, Willems EW, Doods H, Villalon CM, Saxena PR. Effects of BIBN4096BS on cardiac output distribution and on CGRP-induced carotid haemodynamic responses in the pig. *Eur J Pharmacol*. 2003;475:69-77.
93. Danser AHJ, van Kats JP, Admiraal PJJ, Derkx FHM, Lamers JMJ, Verdouw PD, Saxena PR, Schalekamp MADH. Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension*. 1994;24:37-48.
94. De Paepe B, Verstraeten VM, De Potter CR, Bullock GR. Increased angiotensin II type-2 receptor density in hyperplasia, DCIS and invasive carcinoma of the breast is paralleled with increased iNOS expression. *Histochem Cell Biol*. 2002;117:13-19.
95. Egidy G, Eberl LP, Valdenaire O, Irmler M, Majdi R, Diserens AC, Fontana A, Janzer RC, Pinet F, Juillerat-Jeanneret L. The endothelin system in human glioblastoma. *Lab Invest*. 2000;80:1681-1689.
96. Brunswig-Spickenheier B, Mukhopadhyay AK. Characterization of angiotensin-II receptor subtype on bovine thecal cells and its regulation by luteinizing hormone. *Endocrinology*. 1992;131:1445-1452.
97. Delisee C, Schaeffer P, Cazaubon C, Chatelain P. Characterization of cardiac angiotensin AT1 receptors by [3H]SR 47436. *Eur J Pharmacol*. 1993;247:139-144.
98. Schuijt MP, de Vries R, Saxena PR, Schalekamp MADH, Danser AHJ. Vasoconstriction is determined by interstitial rather than circulating angiotensin II. *Br J Pharmacol*. 2002;135:275-283.
99. Akishita M, Iwai M, Wu L, Zhang L, Ouchi Y, Dzau VJ, Horiuchi M. Inhibitory effect of angiotensin II type 2 receptor on coronary arterial remodeling after aortic banding in mice. *Circulation*. 2000;102:1684-1689.
100. Suzuki J, Iwai M, Nakagami H, Wu L, Chen R, Sugaya T, Hamada M, Hiwada K, Horiuchi M. Role of angiotensin II-regulated apoptosis through distinct AT1 and AT2 receptors in neointimal formation. *Circulation*. 2002;106:847-853.
101. Nahmias C, Strosberg AD. The angiotensin $AT_2$ receptor: searching for signal-transduction pathways and physiological function. *Trends Pharmacol Sci*. 1995;16:223-225.
102. Matoba T, Shimokawa H, Morikawa K, Kubota H, Kunihiro I, Urakami-Harasawa L, Mukai Y, Hirakawa Y, Akaike T, Takeshita A. Electron spin resonance detection of hydrogen peroxide as an endothelium-derived hyperpolarizing factor in porcine coronary microvessels. *Arterioscler Thromb Vasc Biol*. 2003;23:1224-1230.
103. Kenny LC, Baker PN, Kendall DA, Randall MD, Dunn WR. The role of gap junctions in mediating endothelium-dependent responses to bradykinin in myometrial small arteries isolated from pregnant women. *Br J Pharmacol*. 2002;136:1085-1088.

104. Nakashima M, Mombouli JV, Taylor AA, Vanhoutte PM. Endothelium-dependent hyperpolarization caused by bradykinin in human coronary arteries. *J Clin Invest*. 1993;92:2867-2871.
105. Miura H, Liu Y, Gutterman DD. Human coronary arteriolar dilation to bradykinin depends on membrane hyperpolarization: contribution of nitric oxide and Ca2+-activated K+ channels. *Circulation*. 1999;99:3132-3138.
106. Groves P, Kurz S, Just H, Drexler H. Role of endogenous bradykinin in human coronary vasomotor control. *Circulation*. 1995;92:3424-3430.
107. Andrews KL, McGuire JJ, Triggle CR. A photosensitive vascular smooth muscle store of nitric oxide in mouse aorta: no dependence on expression of endothelial nitric oxide synthase. *Br J Pharmacol*. 2003;138:932-940.
108. Gupta S, Moreland RB, Munarriz R, Daley J, Goldstein I, Saenz de Tejada I. Possible role of Na(+)-K(+)-ATPase in the regulation of human corpus cavernosum smooth muscle contractility by nitric oxide. *Br J Pharmacol*. 1995;116:2201-2206.
109. Prieto D, Simonsen U, Hernandez M, Garcia-Sacristan A. Contribution of K+ channels and ouabain-sensitive mechanisms to the endothelium-dependent relaxations of horse penile small arteries. *Br J Pharmacol*. 1998;123:1609-1620.
110. Bychkov R, Gollasch M, Steinke T, Ried C, Luft FC, Haller H. Calcium-activated potassium channels and nitrate-induced vasodilation in human coronary arteries. *J Pharmacol Exp Ther*. 1998;285:293-298.
111. Edwards G, Gardener MJ, Feletou M, Brady G, Vanhoutte PM, Weston AH. Further investigation of endothelium-derived hyperpolarizing factor (EDHF) in rat hepatic artery: studies using 1-EBIO and ouabain. *Br J Pharmacol*. 1999;128:1064-1070.
112. Coats P, Johnston F, MacDonald J, McMurray JJ, Hillier C. Endothelium-derived hyperpolarizing factor : identification and mechanisms of action in human subcutaneous resistance arteries. *Circulation*. 2001;103:1702-1708.
113. McGuire JJ, Ding H, Triggle CR. Endothelium-derived relaxing factors: a focus on endothelium-derived hyperpolarizing factor(s). *Can J Physiol Pharmacol*. 2001;79:443-470.
114. Randall MD, Alexander SP, Bennett T, Boyd EA, Fry JR, Gardiner SM, Kemp PA, McCulloch AI, Kendall DA. An endogenous cannabinoid as an endothelium-derived vasorelaxant. *Biochem Biophys Res Commun*. 1996;229:114-120.
115. Travis MD, Davisson RL, Bates JN, Lewis SJ. Hemodynamic effects of L- and D-S-nitroso-beta,beta-dimethylcysteine in rats. *Am J Physiol*. 1997;273:H1493-H1501.
116. de Lean A, Munson PJ, Rodbard D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol*. 1978;235:E97-E102.
117. Gauthier KM, Deeter C, Krishna UM, Reddy YK, Bondlela M, Falck JR, Campbell WB. 14,15-Epoxyeicosa-5(Z)-enoic acid: a selective epoxyeicosatrienoic acid antagonist that inhibits endothelium-dependent hyperpolarization and relaxation in coronary arteries. *Circ Res*. 2002;90:1028-1036.
118. Imig JD, Falck JR, Wei S, Capdevila JH. Epoxygenase metabolites contribute to nitric oxide-independent afferent arteriolar vasodilation in response to bradykinin. *J Vasc Res*. 2001;38:247-255.
119. Miura H, Gutterman DD. Human coronary arteriolar dilation to arachidonic acid depends on cytochrome P-450 monooxygenase and Ca2+-activated K+ channels. *Circ Res*. 1998;83:501-507.
120. Davisson RL, Travis MD, Bates JN, Johnson AK, Lewis SJ. Stereoselective actions of S-nitrosocysteine in central nervous system of conscious rats. *Am J Physiol*. 1997;272:H2361-H2368.

121. Sunano S, Nakahira T, Kawata K, Sekiguchi F. Factors involved in the time course of response to acetylcholine in mesenteric arteries from spontaneously hypertensive rats. *Eur J Pharmacol*. 2001;423:47-55.
122. Stamler JS, Singel DJ, Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms. *Science*. 1992;258:1898-1902.
123. Lang D, Mosfer SI, Shakesby A, Donaldson F, Lewis MJ. Coronary microvascular endothelial cell redox state in left ventricular hypertrophy : the role of angiotensin II. *Circ Res*. 2000;86:463-469.
124. Ceron PI, Cremonez DC, Bendhack LM, Tedesco AC. The relaxation induced by S-nitroso-glutathione and S-nitroso-N-acetylcysteine in rat aorta is not related to nitric oxide production. *J Pharmacol Exp Ther*. 2001;298:686-694.
125. Travis MD, Stoll LL, Bates JN, Lewis SJ. L- and D-S-nitroso-beta,beta-dimethylcysteine differentially increase cGMP in cultured vascular smooth muscle cells. *Eur J Pharmacol*. 1996;318:47-53.
126. Li CG, Karagiannis J, Rand MJ. Comparison of the redox forms of nitrogen monoxide with the nitrergic transmitter in the rat anococcygeus muscle. *Br J Pharmacol*. 1999;127:826-834.
127. Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K, Mayer B. Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol Pharmacol*. 1995;48:184-188.
128. Rubanyi GM, Johns A, Wilcox D, Bates FN, Harrison D. Evidence that a S-nitrosothiol, but not nitric oxide, may be identical with endothelium-derived relaxing factor. *J Cardiovasc Pharmacol*. 1991;17 (suppl 3):S41-S45.
129. Rassaf T, Preik M, Kleinbongard P, Lauer T, Heiss C, Strauer BE, Feelisch M, Kelm M. Evidence for in vivo transport of bioactive nitric oxide in human plasma. *J Clin Invest*. 2002;109:1241-1248.
130. Schechter AN, Gladwin MT, Cannon RO. NO solutions? *J Clin Invest*. 2002;109:1149-1151.
131. Zygmunt PM, Hogestatt ED. Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. *Br J Pharmacol*. 1996;117:1600-1606.
132. Büssemaker E, Wallner C, Fisslthaler B, Fleming I. The Na-K-ATPase is a target for an EDHF displaying characteristics similar to potassium ions in the porcine renal interlobar artery. *Br J Pharmacol*. 2002;137:647-654.
133. Tracey A, Bunton D, Irvine J, MacDonald A, Shaw AM. Relaxation to bradykinin in bovine pulmonary supernumerary arteries can be mediated by both a nitric oxide-dependent and -independent mechanism. *Br J Pharmacol*. 2002;137:538-544.
134. Thollon C, Fournet-Bourguignon MP, Saboureau D, Lesage L, Reure H, Vanhoutte PM, Vilaine JP. Consequences of reduced production of NO on vascular reactivity of porcine coronary arteries after angioplasty: importance of EDHF. *Br J Pharmacol*. 2002;136:1153-1161.
135. Megson IL, Holmes SA, Magid KS, Pritchard RJ, Flitney FW. Selective modifiers of glutathione biosynthesis and 'repriming' of vascular smooth muscle photorelaxation. *Br J Pharmacol*. 2000;130:1575-1580.
136. Gurney AM, Osipenko ON, MacMillan D, McFarlane KM, Tate RJ, Kempsill FE. Two-pore domain K channel, TASK-1, in pulmonary artery smooth muscle cells. *Circ Res*. 2003;93:957-964.
137. Langton PD, Nelson MT, Huang Y, Standen NB. Block of calcium-activated potassium channels in mammalian arterial myocytes by tetraethylammonium ions. *Am J Physiol*. 1991;260:H927-934.

138. Megson AC, Dickenson JM, Townsend-Nicholson A, Hill SJ. Synergy between the inositol phosphate responses to transfected human adenosine A1-receptors and constitutive P2-purinoceptors in CHO-K1 cells. *Br J Pharmacol*. 1995;115:1415-1424.
139. Scheen AJ. Prevention of type 2 diabetes mellitus through inhibition of the renin-angiotensin system. *Drugs*. 2004;64:2537-2565.
140. Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med*. 2000;342:145-153.
141. Parving HH, Lehnert H, Brochner-Mortensen J, Gomis R, Andersen S, Arner P. The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. *N Engl J Med*. 2001;345:870-878.
142. Poole-Wilson PA, Swedberg K, Cleland JG, Di Lenarda A, Hanrath P, Komajda M, Lubsen J, Lutiger B, Metra M, Remme WJ, Torp-Pedersen C, Scherhag A, Skene A. Comparison of carvedilol and metoprolol on clinical outcomes in patients with chronic heart failure in the Carvedilol Or Metoprolol European Trial (COMET): randomised controlled trial. *Lancet*. 2003;362:7-13.
143. Bakris GL, Fonseca V, Katholi RE, McGill JB, Messerli FH, Phillips RA, Raskin P, Wright JT, Jr., Oakes R, Lukas MA, Anderson KM, Bell DS. Metabolic effects of carvedilol vs metoprolol in patients with type 2 diabetes mellitus and hypertension: a randomized controlled trial. *JAMA*. 2004;292:2227-2236.
144. Vittorio TJ, Lang CC, Katz SD, Packer M, Mancini DM, Jorde UP. Vasopressor response to angiotensin II infusion in patients with chronic heart failure receiving beta-blockers. *Circulation*. 2003;107:290-293.
145. Qiu HY, Henrion D, Levy BI. Endogenous angiotensin II enhances phenylephrine-induced tone in hypertensive rats. *Hypertension*. 1994;24:317-321.
146. Purdy RE, Weber MA. Angiotensin II amplification of alpha-adrenergic vasoconstriction: role of receptor reserve. *Circ Res*. 1988;63:748-757.
147. Ohlstein EH, Arleth AJ, Storer B, Romanic AM. Carvedilol inhibits endothelin-1 biosynthesis in cultured human coronary artery endothelial cells. *J Mol Cell Cardiol*. 1998;30:167-173.
148. Nakamura K, Kusano K, Nakamura Y, Kakishita M, Ohta K, Nagase S, Yamamoto M, Miyaji K, Saito H, Morita H, Emori T, Matsubara H, Toyokuni S, Ohe T. Carvedilol decreases elevated oxidative stress in human failing myocardium. *Circulation*. 2002;105:2867-2871.
149. Griendling KK, Ushio-Fukai M. Reactive oxygen species as mediators of angiotensin II signaling. *Regul Pept*. 2000;91:21-27.
150. Kalinowski L, Dobrucki LW, Szczepanska-Konkel M, Jankowski M, Martyniec L, Angielski S, Malinski T. Third-generation beta-blockers stimulate nitric oxide release from endothelial cells through ATP efflux: a novel mechanism for antihypertensive action. *Circulation*. 2003;107:2747-2752.
151. Barki-Harrington L, Luttrell LM, Rockman HA. Dual inhibition of beta-adrenergic and angiotensin II receptors by a single antagonist: a functional role for receptor-receptor interaction in vivo. *Circulation*. 2003;108:1611-1618.
152. Giessler C, Wangemann T, Silber RE, Dhein S, Brodde OE. Noradrenaline-induced contraction of human saphenous vein and human internal mammary artery: involvement of different alpha-adrenoceptor subtypes. *Naunyn Schmiedebergs Arch Pharmacol*. 2002;366:104-109.
153. Koshimizu TA, Tsujimoto G, Hirasawa A, Kitagawa Y, Tanoue A. Carvedilol selectively inhibits oscillatory intracellular calcium changes evoked by human alpha1D- and alpha1B-adrenergic receptors. *Cardiovasc Res*. 2004;63:662-672.

154. Ramsay D, Kellett E, McVey M, Rees S, Milligan G. Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences. *Biochem J*. 2002;365:429-440.
155. Milligan G. Applications of bioluminescence- and fluorescence resonance energy transfer to drug discovery at G protein-coupled receptors. *Eur J Pharm Sci*. 2004;21:397-405.
156. Kostenis E, Milligan G, Christopoulos A, Sanchez-Ferrer CF, Heringer-Walther S, Sexton P, Gembardt F, Kellet E, Martini L, Vanderheyden P, Schultheiss HP, Walther T. The G protein-coupled receptor Mas is a physiological antagonist of the angiotensin II $AT_1$ receptor. *Circulation*. 2005;In press.
157. Ohlstein EH, Brooks DP, Feuerstein GZ, Ruffolo RR, Jr. Inhibition of sympathetic outflow by the angiotensin II receptor antagonist, eprosartan, but not by losartan, valsartan or irbesartan: relationship to differences in prejunctional angiotensin II receptor blockade. *Pharmacology*. 1997;55:244-251.
158. Zucker IH, Wang W, Pliquett RU, Liu JL, Patel KP. The regulation of sympathetic outflow in heart failure. The roles of angiotensin II, nitric oxide, and exercise training. *Ann N Y Acad Sci*. 2001;940:431-443.
159. Dendorfer A, Thornagel A, Raasch W, Grisk O, Tempel K, Dominiak P. Angiotensin II induces catecholamine release by direct ganglionic excitation. *Hypertension*. 2002;40:348-354.
160. Balt JC, Mathy MJ, Pfaffendorf M, van Zwieten PA. Inhibition of facilitation of sympathetic neurotransmission and angiotensin II-induced pressor effects in the pithed rat: comparison between valsartan, candesartan, eprosartan and embusartan. *J Hypertens*. 2001;19:2241-2250.
161. Lyons D, Roy S, O'Byrne S, Swift CG. ACE inhibition: postsynaptic adrenergic sympatholytic action in men. *Circulation*. 1997;96:911-915.
162. Guimaraes S, Moura D. Vascular adrenoceptors: an update. *Pharmacol Rev*. 2001;53:319-356.
163. Touyz RM, Schiffrin EL. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev*. 2000;52:639-672.
164. Henrion D, Laher I, Laporte R, Bevan JA. Angiotensin II amplifies arterial contractile response to norepinephrine without increasing Ca++ influx: role of protein kinase C. *J Pharmacol Exp Ther*. 1992;261:835-840.
165. Jorde UP, Ennezat PV, Lisker J, Suryadevara V, Infeld J, Cukon S, Hammer A, Sonnenblick EH, Le Jemtel TH. Maximally recommended doses of angiotensin-converting enzyme (ACE) inhibitors do not completely prevent ACE-mediated formation of angiotensin II in chronic heart failure. *Circulation*. 2000;101:844-846.
166. van Kats JP, Danser AHJ, van Meegen JR, Sassen LM, Verdouw PD, Schalekamp MADH. Angiotensin production by the heart: a quantitative study in pigs with the use of radiolabeled angiotensin infusions. *Circulation*. 1998;98:73-81.
167. Farquharson CA, Struthers AD. Gradual reactivation over time of vascular tissue angiotensin I to angiotensin II conversion during chronic lisinopril therapy in chronic heart failure. *J Am Coll Cardiol*. 2002;39:767-775.
168. Sun D, Huang A, Mital S, Kichuk MR, Marboe CC, Addonizio LJ, Michler RE, Koller A, Hintze TH, Kaley G. Norepinephrine elicits beta2-receptor-mediated dilation of isolated human coronary arterioles. *Circulation*. 2002;106:550-555.
169. Scotland RS, Madhani M, Chauhan S, Moncada S, Andresen J, Nilsson H, Hobbs AJ, Ahluwalia A. Investigation of vascular responses in endothelial nitric oxide synthase/cyclooxygenase-1 double-knockout mice. Key role for endothelium-derived

hyperpolarizing factor in the regulation of blood pressure in vivo. *Circulation*. 2005;111:796-803.

170. Nawate S, Fukao M, Sakuma I, Soma T, Nagai K, Takikawa O, Miwa S, Kitabatake A. Reciprocal changes in endothelium-derived hyperpolarizing factor- and nitric oxide-system in the mesenteric artery of adult female rats following ovariectomy. *Br J Pharmacol*. 2005;144:178-189.
171. Miura H, Gutterman DD. Interaction of two distinct endothelium-derived hyperpolarizing factors in the human coronary microcirculation. *Circulation*. 2004;110:181.
172. Fichtlscherer S, Dimmeler S, Breuer S, Busse R, Zeiher AM, Fleming I. Inhibition of cytochrome P450 2C9 improves endothelium-dependent, nitric oxide-mediated vasodilatation in patients with coronary artery disease. *Circulation*. 2004;109:178-183.
173. Foster MW, Pawloski JR, Singel DJ, Stamler JS. Role of circulating s-nitrosothiols in control of blood pressure. *Hypertension*. 2005;45:15-17.
174. Gandley RE, Tyurin VA, Huang W, Arroyo A, Daftary A, Harger G, Jiang J, Pitt B, Taylor RN, Hubel CA, Kagan VE. S-nitrosoalbumin-mediated relaxation is enhanced by ascorbate and copper: effects in pregnancy and preeclampsia plasma. *Hypertension*. 2005;45:21-27.
175. Andrews KL, McGuire J, Triggle CR. Characterization of vascular smooth muscle photorelaxation in thoracic aorta from NOS knockout mice. *Pharmacologist*. 2002;44 (suppl. 1):A214.
176. Croft KD, McGiff JC, Sanchez-Mendoza A, Carroll MA. Angiotensin II releases 20-HETE from rat renal microvessels. *Am J Physiol Renal Physiol*. 2000;279:F544-551.
177. Kaide J, Zhang F, Wei Y, Wang W, Gopal VR, Falck JR, Laniado-Schwartzman M, Nasjletti A. Vascular CO counterbalances the sensitizing influence of 20-HETE on agonist-induced vasoconstriction. *Hypertension*. 2004;44:210-216.
178. Dahlof B, Devereux RB, Kjeldsen SE, Julius S, Beevers G, de Faire U, Fyhrquist F, Ibsen H, Kristiansson K, Lederballe-Pedersen O, Lindholm LH, Nieminen MS, Omvik P, Oparil S, Wedel H. Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet*. 2002;359:995-1003.
179. Pitt B, Poole-Wilson PA, Segal R, Martinez FA, Dickstein K, Camm AJ, Konstam MA, Riegger G, Klinger GH, Neaton J, Sharma D, Thiyagarajan B. Effect of losartan compared with captopril on mortality in patients with symptomatic heart failure: randomised trial--the Losartan Heart Failure Survival Study ELITE II. *Lancet*. 2000;355:1582-1587.
180. Sleight P, Yusuf S, Pogue J, Tsuyuki R, Diaz R, Probstfield J. Blood-pressure reduction and cardiovascular risk in HOPE study. *Lancet*. 2001;358:2130-2131.
181. Motley ED, Eguchi K, Gardner C, Hicks AL, Reynolds CM, Frank GD, Mifune M, Ohba M, Eguchi S. Insulin-induced Akt activation is inhibited by angiotensin II in the vasculature through protein kinase C-alpha. *Hypertension*. 2003;41:775-780.
182. Schupp M, Janke J, Clasen R, Unger T, Kintscher U. Angiotensin type 1 receptor blockers induce peroxisome proliferator-activated receptor-gamma activity. *Circulation*. 2004;109:2054-2057.
183. Benson SC, Pershadsingh HA, Ho CI, Chittiboyina A, Desai P, Pravenec M, Qi N, Wang J, Avery MA, Kurtz TW. Identification of telmisartan as a unique angiotensin II receptor antagonist with selective PPARgamma-modulating activity. *Hypertension*. 2004;43:993-1002.
184. Shiuchi T, Iwai M, Li HS, Wu L, Min LJ, Li JM, Okumura M, Cui TX, Horiuchi M. Angiotensin II type-1 receptor blocker valsartan enhances insulin sensitivity in skeletal muscles of diabetic mice. *Hypertension*. 2004;43:1003-1010.