NEW ASPECTS OF ACE INHIBITION

Importance of ACE co-localization with angiotensin and bradykinin receptors

Beril Tom

ISBN 90-6734-060-X

An electronic version of this thesis in Adobe PDF-format is available on the following Internet address:
http://www.eur.nl/fgg/pharm/

NEW ASPECTS OF ACE INHIBITION

Importance of ACE co-localization with angiotensin and bradykinin receptors

Nieuwe aspecten van ACE remming

Het belang van ACE co-localisatie met angiotensine en bradykinine receptoren

Proefschrift

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

De openbare verdediging zal plaats vinden op
woensdag 3 December 2003 om 09.45 uur

door

Beril Tom

geboren te Kırıkkale, Turkije

Promotiecommissie

Promotores:	Prof. dr. A.H.J. Danser
	Prof. dr. P.R. Saxena
Overige leden:	Prof. dr. D.J.G.M. Duncker
	Prof. dr. P.A.B.M. Smits
	Dr. J.W. de Jong

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

Financial support of the following institutions and companies is gratefully acknowledged:
J.E. Jurriaanse Stichting, Sanofi-Synthelabo Recherche, GlaxoSmithKline B.V., Venlo Türk Sosyal Kültürel Merkezi Vakfı (Stichting Turks Sociaal Cultureel Centrum Venlo), Dostluk Vakfı (Stichting), Harlan Nederland B.V.

Voor Peter,
Aan mijn ouders
(Anne ve babama)

TABLE OF CONTENTS

CHAPTER 1: Introduction and Aim

Bradykinin, Angiotensin-(1-7), and ACE Inhibitors: How Do They Interact?

Summary

The beneficial effect of angiotensin-converting enzyme (ACE) inhibitors in hypertension and heart failure may relate, at least in part, to their capacity to interfere with bradykinin metabolism. In addition, recent studies have provided evidence for bradykinin-potentiating effects of ACE inhibitors that are independent of bradykinin hydrolysis, i.e., ACE-bradykinin type 2 (B_2) receptor 'crosstalk', resulting in B_2 receptor upregulation and/or more efficient activation of signal transduction pathways, as well as direct activation of bradykinin type 1 (B_1) receptors by ACE inhibitors. This review critically reviews the current evidence for hydrolysis-independent bradykinin potentiation by ACE inhibitors, evaluating not only the many studies that have been performed with ACE-resistant bradykinin analogues, but also paying attention to angiotensin-(1-7) (Ang-(1-7)), a metabolite of both angiotensin (Ang) I and II, that could act as an endogenous ACE inhibitor. The levels of Ang-(1-7) are increased during ACE inhibition, and most studies suggest that its hypotensive effects are mediated in a bradykinin-dependent manner.

Based on: Tom B., Dendorfer A. & Danser A.H.J. (2003). Bradykinin, angiotensin-(1-7), and ACE inhibitors: How do they interact? *Int. J. Biochem. Cell. Biol.* **35,792-801.**

1.1. Introduction

Bradykinin and Ang II are important peptides involved in the regulation of vascular tone. Ang II is a vasoconstrictor and growth-promoting substance, whereas bradykinin is a potent vasodilator and growth inhibitor. ACE inhibitors, which are now widely used for the treatment of hypertension and heart failure, not only block the generation of Ang II from Ang I, but also prevent the degradation of bradykinin (Figure 1). Although originally it was thought that their beneficial effects were mainly due to blockade of Ang II generation, recent evidence suggests that bradykinin accumulation may be of equal importance. For instance, plasma and tissue Ang II concentrations are not always decreased during chronic ACE inhibition (Campbell *et al.*, 1999; Farquharson *et al.*, 2002; van Kats *et al.*, 2000), possibly due to the presence of alternative Ang I-II converting enzymes like chymase (Maassen van den Brink *et al.*, 1999). Furthermore, combined inhibition of ACE and neutral endopeptidase (NEP), a second bradykinin-metabolising enzyme, is more cardioprotective than ACE inhibition alone (d'Uscio *et al.*, 2001; Rouleau *et al.*, 2000). An interesting observation in this regard is that ACE inhibitors appear to potentiate bradykinin beyond blocking its hydrolysis, either through ACE-B_2 receptor 'cross-talk' (Benzing *et al.*, 1999; Marcic *et al.*, 1999; 2000a; 2000b; Minshall *et al.*, 1997b) or via direct stimulation of B_1 receptors (Ignjatovic *et al.*, 2002).

This review addresses the various ways through which ACE inhibitors potentiate bradykinin, paying attention in particular to Ang-(1-7), a metabolite of both Ang I and Ang II (Ferrario *et al.*, 1997) (Figure 1) that inhibits ACE. Its levels are increased during ACE inhibitor treatment (Campbell *et al.*, 1993b; Yamada *et al.*, 1998), and according to some reports it may also stimulate a new, as yet unidentified, receptor.

1.2. Bradykinin synthesis, metabolism, receptors and function

Bradykinin is generated from kininogen by kallikrein, either directly or via the intermediate kallidin (Figure 1). *In-vivo*, bradykinin is rapidly degraded (half life <0.5 min) (Cyr *et al.*, 2001) by kininases, the most important of which are the metallopeptidases ACE and NEP, aminopeptidase P, and the carboxypeptidases M and N (Dendorfer *et al.*, 1997b; Kokkonen *et al.*, 2000). In view of its short half life, it

is generally assumed that bradykinin, in order to have a local effect, is synthesized at tissue sites. In support of this concept, the various components required to generate bradykinin locally are present in heart and vessel wall (Nolly *et al.*, 1992; 1994), bradykinin is released from tissue sites into the circulation (Baumgarten *et al.*, 1993; Duncan *et al.*, 2000), and tissue bradykinin levels are higher than those in circulating blood (Campbell *et al.*, 1993a).

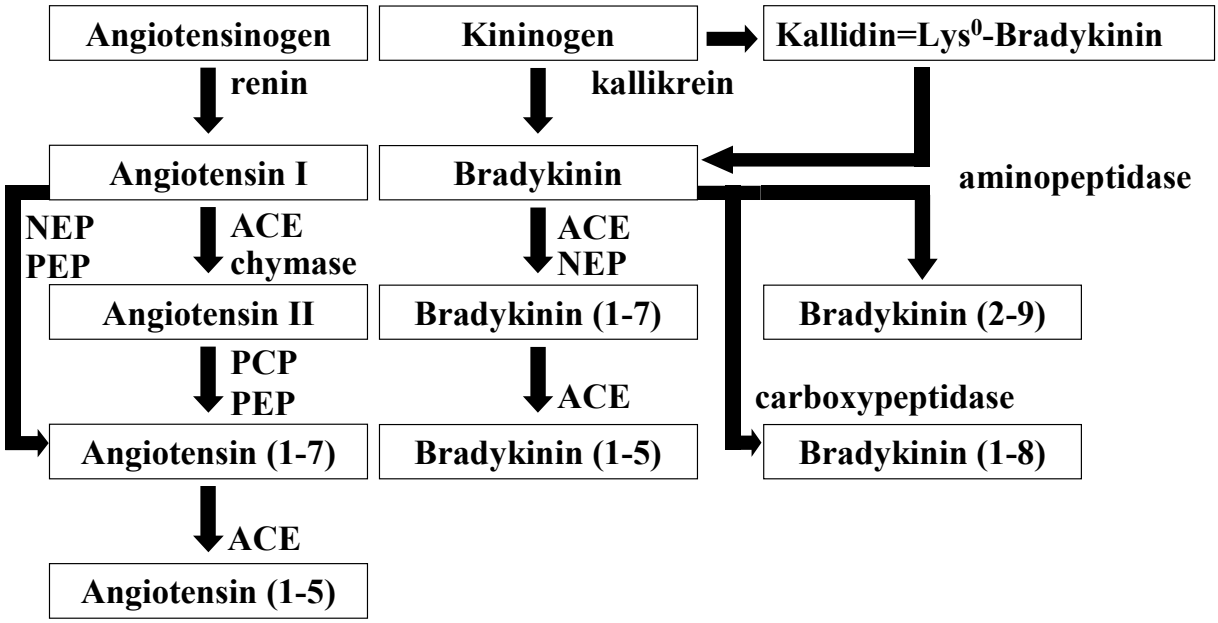


Figure 1. *Synthesis and metabolism of angiotensins and bradykinin. ACE, angiotensin-converting enzyme; NEP, neutral endopeptidase; PEP, prolyl endopeptidase; PCP, prolyl carboxypeptidase.*

Bradykinin exerts its actions via binding to B_1 and B_2 receptors. Both receptors are G protein-coupled receptors (Marceau *et al.*, 1998). B_2 receptors are constitutively expressed in a variety of cells, including endothelial cells, vascular smooth muscle cells (Raidoo *et al.*, 1997), and cardiomyocytes (Minshall *et al.*, 1995). B_1 receptors are weakly detectable under physiological conditions, but strongly expressed in pathological states (Marceau *et al.*, 1998). B_1 receptor upregulation also occurs in B_2 receptor gene knockout mice (Duka *et al.*, 2001).

Activation of endothelial B_2 receptors results in vasorelaxation, an effect that is mediated, at least in part, via stimulation of endothelial nitric oxide synthase (eNOS) and subsequent nitric oxide (NO) generation. Alternative mediators of the B_2 receptor-stimulated vasorelaxation are the so-called endothelium-derived hyperpolarizing factors (EDHF), the identity of which has not yet been established. Putative candidates include prostacyclin, potassium, S-nitrosothiols and epoxyeicosatrienoic acid (Danser *et al.*, 1998; Mombouli *et al.*, 1997). B_2 receptors on vascular smooth muscle cells and cardiomyocytes mediate contractile (Dendorfer *et al.*, 2001a; Gobeil *et al.*, 2002) and inotropic (Tom *et al.*, 2001b) effects, respectively. In addition, B_2 receptor activation has been associated with anti-hypertrophic and/or anti-proliferative effects in cardiomyocytes and fibroblasts (Ishigai *et al.*, 1997; McAllister *et al.*, 1993). B_1 receptors, once induced by inflammatory mediators and tissue damage, assume some of the hemodynamic properties of the B_2 receptor (e.g., vasorelaxation) (Duka *et al.*, 2001; Su *et al.*, 2000). In this regard, it is important to realize that bradykinin and kallidin (Lys-bradykinin) display less affinity for B_1 receptors than for B_2 receptors, and that their carboxypeptidase metabolites, des-Arg^9-bradykinin and in particular des-Arg^{10}-kallidin, might act as the endogenous agonists of B_1 receptors (Marceau *et al.*, 1998).

1.3. Bradykinin potentiation by ACE inhibitors: inhibition of hydrolysis?

ACE is responsible for 45-100 % of kininase activity in blood plasma and tissues (Blais *et al.*, 1997; Dendorfer *et al.*, 2001b; Taylor-McCabe *et al.*, 2001), and it is therefore not surprising that ACE inhibitors potentiate the actions of bradykinin (i.e., cause a leftward shift of the bradykinin concentration-response curve) by a factor of 5-120, both *in-vivo* and *in- vitro* (Bönner *et al.*, 1990; Dendorfer *et al.*, 2000; Mombouli *et al.*, 1992; Tom *et al.*, 2001a). Unexpectedly, this increase in potency was also observed under conditions where bradykinin concentrations were barely affected by degradation, for instance in organ bath experiments (Auch-Schwelk *et al.*, 1993). Moreover, ACE inhibitors also exerted potent relaxant effects when applied on top of subthreshold doses of bradykinin (Auch-Schwelk *et al.*, 1993), or when given in the presence of high bradykinin concentrations designed to desensitize B_2 receptors (Hecker *et al.*, 1994). These findings have provoked hypotheses about degradation-independent mechanisms of kinin potentiation, particularly because the degradation-independent effects were initially confirmed when using bradykinin analogues that

were assumed to be ACE-resistant (Auch-Schwelk *et al.*, 1993; Danser *et al.*, 2000; Minshall *et al.*, 1997a; 2000; Mombouli *et al.*, 2002). However, for two frequently applied analogues, D-Arg-[Hyp^3]-bradykinin and [Hyp^3-Tyr(Me)^8]-bradykinin, this assumption has been proven to be wrong (Dendorfer *et al.*, 2001a; Gobeil *et al.*, 2002). More importantly, the potentiating and resensitizing actions of ACE inhibitors were entirely absent when studied in combination with truly stable B_2 receptor agonists in physiological models such as the perfused rat heart, the rabbit jugular vein, and the porcine coronary artery (Dendorfer *et al.*, 2000; 2001a; Gobeil *et al.*, 2002; Tom *et al.*, 2002). These latter observations permit two possible explanations. Either the stable agonists lack an undefined property that is required to allow ACE inhibitors to potentiate bradykinin in a degradation-independent manner, or potentiation is exclusively related to kinin degradation in those physiological models. The latter hypothesis would imply that ACE co-localizes with B_2 receptors in a compartment in which degradation dramatically impairs bradykinin availability. Application of an ACE inhibitor under such conditions has the potential to increase the bradykinin concentrations in the micro-environment of the B_2 receptor several-fold as well as immediately.

The existence of a tissue compartment with highly effective bradykinin degradation has been demonstrated in the isolated rat heart during bradykinin distribution studies (Dendorfer *et al.*, 1997b). Following intracoronary bolus application of ^{3}H-bradykinin, an early fraction was recovered after intravascular passage in which 72 % of ^{3}H-activity represented intact bradykinin, whereas in a delayed fraction (representing redistribution from a tissue compartment) only 8% of ^{3}H-activity consisted of intact bradykinin (Dendorfer *et al.*, 1997b). The 13-fold reduction of bradykinin availability in the delayed fraction corresponds well with the 19-fold potentiation of bradykinin during combined inhibition of the major kininases in the rat myocardium (ACE and aminopeptidase P) (Dendorfer *et al.*, 2000). Interestingly, the high local efficacy of ACE is not restricted to the inactivation of bradykinin; in vessel segments mounted in organ baths, it also effectively activates Ang I locally, resulting in vascular interstitial Ang II levels that are up to 27-fold higher than the Ang II levels in the organ bath (Schuijt *et al.*, 2002). Since interstitial rather than circulating Ang II was found to determine vasoconstriction, these data suggest that ACE and AT_1 receptors, like ACE and B_2 receptors, co-localize in the

same compartment. Further evidence for the close anatomical localization of ACE, B_2 receptors and AT_1 receptors comes from a recent observation on AT_1-B_2 receptor heterodimerization, resulting in enhanced G-protein activation and altered receptor sequestration (AbdAlla *et al.*, 2000).

Studies in cultured cells confirm that co-localization of B_2 receptors and ACE is a prerequisite for kinin potentiation. When ACE was expressed in Chinese hamster ovary (CHO) cells and directed to lipid rafts by genetic modification of its membrane anchor (Marcic *et al.*, 2000a), the potentiating effect of the ACE inhibitor ramiprilat was abolished. It only returned following restoration of the close sterical relationship between ACE and B_2 receptors by the cholesterol-depleting agent filipin. These results indicate that the proposed micro-environment harboring B_2 receptors and ACE may be constituted by membrane domains, such as caveolae or lipid rafts, the identities of which still need to be clarified.

Finally, there is a quantitative relationship between kinin breakdown and the potentiating effect of ACE inhibitors. For instance, when stimulation is performed with bradykinin analogues that are partially resistant towards ACE, the potentiation of their actions by ACE inhibitors is impaired to about the same extent as their degradation (Dendorfer *et al.*, 2001a). In addition, blockade of only one of the two active sites of ACE (the so-called N- and C-domain) produces bradykinin potentiation that is only half that during blockade of both domains (Tom *et al.*, 2001a). The quantitative relation between bradykinin breakdown and potentiation is further demonstrated by the fact that potentiation can also be provoked by inhibition of other kininases, for instance aminopeptidase P in the rat heart (Dendorfer *et al.*, 2000), and NEP following its overexpression in CHO cells (Deddish *et al.*, 2002). Thus, the phenomenon of kinin potentiation by ACE inhibitors can be mimicked by structurally unrelated inhibitors of other kininases, provided that such kininases are equivalent to ACE in their degradation activities and localizations.

1.4. Bradykinin potentiation by ACE inhibitors: ACE-B_2 receptor crosstalk?

Although studies with ACE-resistant bradykinin analogues did not confirm the hydrolysis-independent bradykinin potentiation/B_2 receptor resensitization in physiological models (Dendorfer *et al.*, 2001a; Gobeil *et al.*, 2002; Tom *et al.*, 2002),

it is still being argued, in view of the fact that the half life of bradykinin in such isolated tissue preparations is >10 minutes (Minshall *et al.*, 2000; Tom *et al.*, 2002), that the ACE inhibitor-induced effects (which occur within seconds) must be independent of blocking bradykinin degradation (Erdös, 2002). In support of this concept, NEP inhibition did not potentiate bradykinin in porcine vessels, nor did it potentiate its substrate atrial natriuretic peptide (Figure 2). Although an alternative explanation for the latter finding might be a low activity of NEP in porcine vessels, a recent study convincingly demonstrated NEP activity in porcine vascular tissue (Miyamoto *et al.*, 2002).

Studies in CHO cells transfected with B_2 receptors and ACE, as well as in endothelial cells that constitutively express these proteins, more convincingly provide evidence for an ACE-B_2 receptor 'crosstalk' that goes beyond blocking hydrolysis (Figure 3). In these cells, ACE inhibitors increase the number of cell surface B_2 receptors, block B_2 receptor desensitization, and decrease B_2 receptor internalization (Minshall *et al.*, 1997b). The latter may relate to the fact that ACE inhibitors prevent bradykinin-activated B_2 receptors from sequestration with caveolin-rich membranes (Benzing *et al.*, 1999). Importantly, these effects are not observed in CHO cells transfected with B_2 receptors alone (Minshall *et al.*, 1997b), thereby arguing against a direct effect of ACE inhibitors on B_2 receptors. Since, as discussed above, ACE inhibitors augment bradykinin only when B_2 receptors and ACE are sterically close (Marcic *et al.*, 2000a), one possibility is that B_2 receptors and ACE form a heterodimer. ACE inhibitors might then affect heterodimer interaction, thus promoting a B_2 receptor conformation that can more efficiently induce signal transduction.

In cultured cells, ACE inhibitors did not resensitize B_2 receptors during blockade of protein kinase C (PKC) or phosphatases (Marcic *et al.*, 2000b). Although this finding could not be reproduced in an intact vessel preparation (Tom *et al.*, 2002), it is in agreement with the concept that B_2 receptor desensitization involves PKC activation and B_2 receptor phosphorylation (Blaukat *et al.*, 1996; Marcic *et al.*, 2000b). It could also relate to the possibility that PKC inhibition affects phosphorylation of an intermediate protein that mediates the interaction between B_2 receptors and ACE. Finally, ACE inhibitors not only induce B_1 receptor upregulation (Marin-Castano *et al.*, 2002), but may also act as B_1 receptors agonists, stimulating NO release at nanomolar concentrations (Ignjatovic *et al.*, 2002).

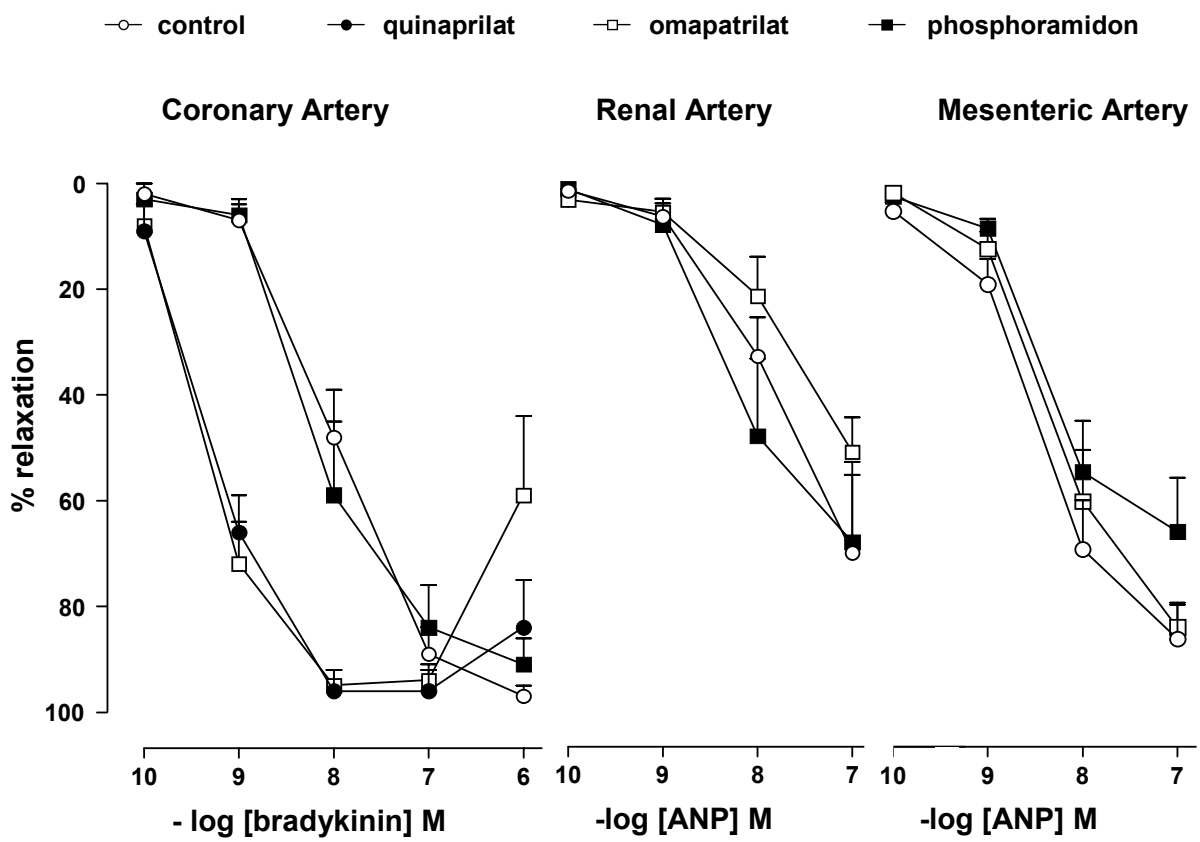


Figure 2. *Relaxations of porcine arteries preconstricted with 1 μM U46619, to bradykinin or atrial natriuretic peptide (ANP) in the absence (control) or presence of 10 μM quinaprilat (ACE inhibitor), 10 μM omapatrilat (combined ACE and NEP inhibitor) or 1 μM phosphoramidon (NEP inhibitor). Data (mean±S.E.M. of 5-10 experiments) are expressed as a percentage of the contraction induced by U46619, and have been obtained from (Tom et al., 2002).*

1.5. Bradykinin and Ang-(1-7)

Ang-(1-7) is one of the metabolites of Ang I that, like the main metabolite of Ang I, Ang II, might have effects of its own, for instance via stimulation of a specific Ang-(1-7) receptor. Ang-(1-7) can be generated both from Ang I (by NEP) and from Ang II (by prolyl endopeptidase, prolyl carboxypeptidase and/or ACE-related carboxypeptidase ('ACE2')) (Figure 1) (Ferrario *et al.*, 1997; Vickers *et al.*, 2002). Its levels, which are normally in the picomolar range, increase during ACE inhibition

(Campbell *et al.*, 1993b), not only because the elevated Ang I levels during ACE inhibition result in enhanced Ang-(1-7) generation by NEP, but also because ACE is one of the main Ang-(1-7)-degrading enzymes (Yamada *et al.*, 1998).

Several lines of evidence suggest that Ang-(1-7) counteracts the effects of Ang II. Ang-(1-7) infusions in spontaneously hypertensive rats (SHR) lower blood pressure (Benter *et al.*, 1995), and monoclonal Ang-(1-7) antibodies as well as NEP inhibitors raise blood pressure during renin-angiotensin system blockade in SHR (Iyer *et al.*, 1998a; 1998b). However, these findings were not uniformly confirmed by others. Abbas et al. (Abbas *et al.*, 1997) observed hypertensive responses to Ang-(1-7) in Sprague-Dawley rats. Similarly, Ang-(1-7) infusion into the forearm of normotensive men resulted in modest vasoconstriction (Ueda *et al.*, 2000), whereas in patients with heart failure treated with an ACE inhibitor, Ang-(1-7) did not affect forearm blood flow (Davie *et al.*, 1999). The doses at which Ang-(1-7) induced vasoconstriction were high (up to 50 nmol/min; for comparison: Ang II exerts maximal vasoconstrictor effects at doses that are more than 1000-fold lower) (Ueda *et al.*, 2000), and the most likely explanation for this contractile response is therefore that Ang-(1-7) at these high doses stimulates AT_1 receptors. This concept simultaneously implies that Ang-(1-7) will antagonize Ang II effects that are mediated via AT_1 receptors. Combined infusions of Ang II and Ang-(1-7) in humans and rats (Mahon *et al.*, 1994; Ueda *et al.*, 2000), as well as *in-vitro* studies in human internal mammary arteries (Roks *et al.*, 1999) and rabbit aortae (Mahon *et al.*, 1994), confirmed this assumption. Thus, Ang-(1-7) binds with low affinity to AT_1 receptors, allowing it to act as an agonist in the absence of Ang II, and as an antagonist in the presence of Ang II.

Pörsti et al. (Pörsti *et al.*, 1994) initially reported potent vasodilator effects of Ang-(1-7) in porcine coronary arteries, but were unable to confirm these findings when using a second batch of Ang-(1-7), leading the authors to suggest that the presence of small amounts of Ang-(1-7) with retro-inverted peptide bonds in the first batch may have caused the relaxant effects (Pörsti *et al.*, 1996). Remarkably, vasorelaxation of isolated arteries did occur when Ang-(1-7) was added to the organ bath following the application of bradykinin (Fernandes *et al.*, 2001; Gorelik *et al.*, 1998; Tom *et al.*, 2001a). An alternative explanation for the discrepant findings of Pörsti et al. might therefore be that the relaxant effects of Ang-(1-7) in their initial

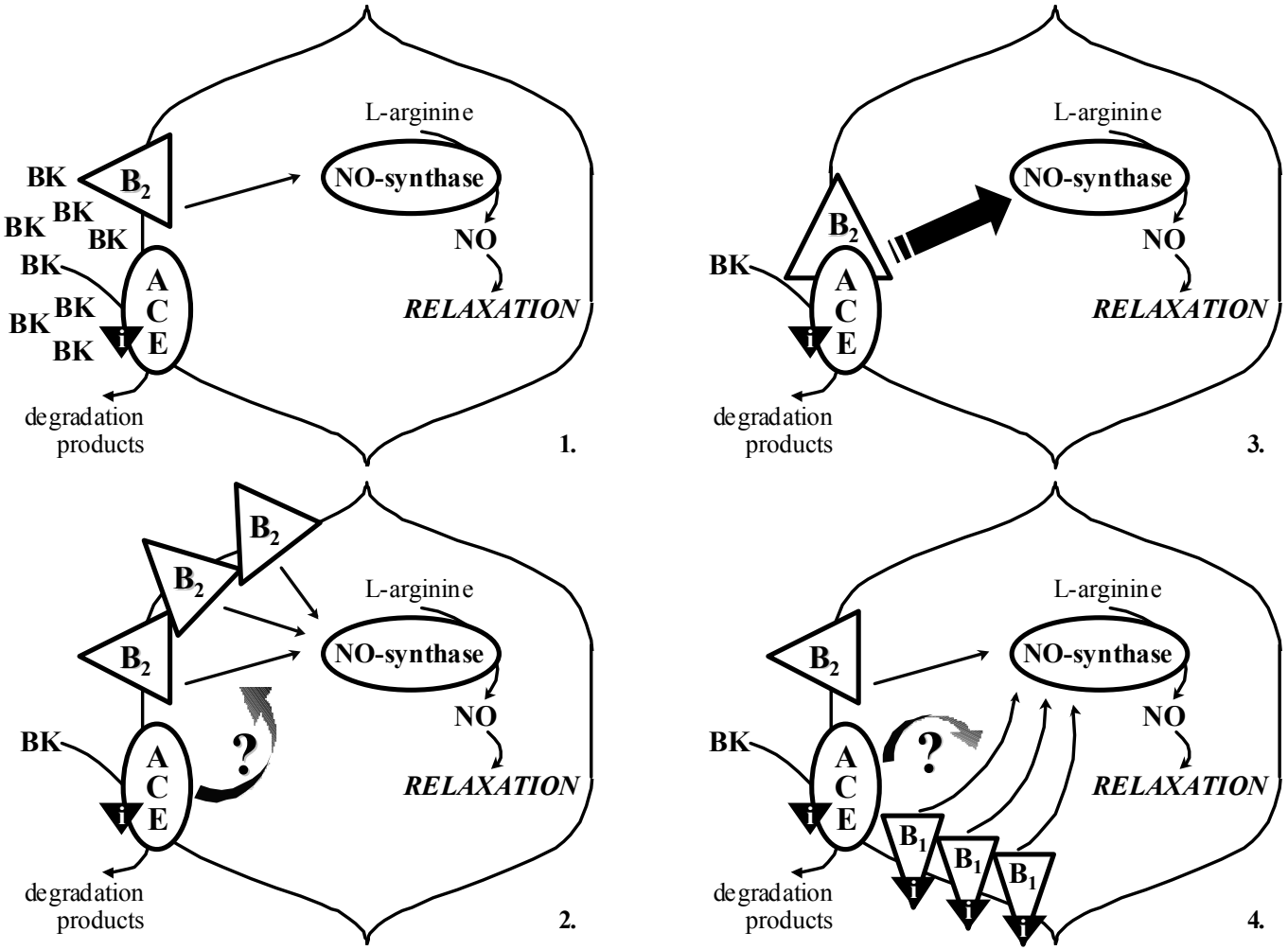


Figure 3. *Possible mechanisms underlying bradykinin potentiation by ACE inhibitors in endothelial cells, where B_2 receptor activation normally results in NO synthase activation and subsequent NO generation. ACE inhibitors (i) either 1) increase the bradykinin (BK) levels in the micro-environment of the B_2 receptor, 2) increase the number of B_2 receptors, 3) affect ACE-B_2 receptor heterodimer interaction, thereby more efficiently inducing signal transduction, or 4) induce B_1 receptor upregulation and act as B_1 receptor agonists.*

report were due to the fact that in this report endothelial integrity had been verified with bradykinin (Pörsti *et al.*, 1994). Co-infusion of Ang-(1-7) with bradykinin *in-vivo* also potentiated the hypotensive effect of bradykinin (Abbas *et al.*, 1997; Ueda *et al.*, 2001), unless an ACE inhibitor had been added before (Davie *et al.*, 1999; Fernandes *et al.*, 2001).

The bradykinin-Ang-(1-7) interaction may involve vasodilatory AT_2 receptors (Schuijt *et al.*, 2001), since the AT_2 receptor antagonist PD123319 partially blocked the Ang-(1-7) + bradykinin-induced relaxation *in-vitro* (Gorelik *et al.*, 1998). Alternatively, Ang-(1-7) may act as an ACE inhibitor (Deddish *et al.*, 1998; Roks *et al.*, 1999; Tom *et al.*, 2001a). Ang-(1-7) inhibits the ACE C-domain more potently than the N-domain (IC_{50} 1-8 μM versus 28-71 μM) (Deddish *et al.*, 1998), and is cleaved to Ang-(1-5) by the N-domain (Chappell *et al.*, 1998). Since ACE is predominantly located on endothelial cells, the ACE inhibitory capacity of Ang-(1-7) not only explains why the relaxant effects of Ang-(1-7) are endothelium-dependent (Brosnihan *et al.*, 1996) and why Ang-(1-7) does not potentiate bradykinin in subjects treated with ACE inhibitors (Davie *et al.*, 1999), but also puts into perspective the existence of a low-affinity (IC_{50} 2.9 μM) binding site for Ang-(1-7) on bovine aortic endothelial cells (Tallant *et al.*, 1997). This binding site may in fact be ACE. It must be realised that the physiological concentrations of Ang-(1-7) are several orders of magnitude below the range required to block ACE (Campbell *et al.*, 1993b), and that beneficial effects of Ang-(1-7) will therefore only be observed when infused in sufficiently high amounts (Loot *et al.*, 2002).

Finally, a high-affinity (IC_{50} 19.3 nM) binding site for Ang-(1-7) has also been reported (Tallant *et al.*, 1997). D-Ala^7-Ang-(1-7) (A779) selectively blocks this site (but not ACE), and some (Fernandes *et al.*, 2001; Iyer *et al.*, 2000; Ren *et al.*, 2002), but not all (Widdop *et al.*, 1999), studies support the concept that Ang-(1-7) induces vasodilation via activation of this 'Ang-(1-7) receptor' in rats and rabbits. Inhibitors of nitric oxide synthase (NOS) and cyclooxygenase, but not the B_2 receptor antagonist Hoe140 (Fernandes *et al.*, 2001; Ferreira *et al.*, 2001; Ren *et al.*, 2002), block the Ang-(1-7) effects mediated via this receptor, suggesting coupling of the receptor to NO and prostaglandins, but not to B_2 receptors and bradykinin. Evidence for the occurrence of Ang-(1-7) receptors in humans is currently not available.

1.6. Conclusion and perspective

The reduction of bradykinin availability in the micro-environment of B_2 receptors due to degradation appears to account in full for the potentiating effects observed with ACE inhibitors, as long as kinin potentiation is defined as a leftward shift of the bradykinin concentration-response relationship. The arousal of bradykinin responses by ACE inhibitors in the presence of high bradykinin concentrations that served to induce B_2 receptor desensitization is mostly addressed as 'receptor resensitization' and may occur more universally. Although this phenomenon is also absent in vascular preparations after stimulation with degradation-resistant bradykinin analogues (Dendorfer *et al.*, 2000; Gobeil *et al.*, 2002; Tom *et al.*, 2002), it may occur in other models, for instance because competitors for alternative bradykinin binding sites displace local kinins and increase their concentrations in a degradation-independent manner. The possibility of such an indirect action prohibits the interpretation of 'resensitization' as a direct interaction of ACE inhibitors with B_2 receptors, so long as the mechanism of such an interaction is not clarified at the molecular level. A further potential for ACE inhibitors to act as kinin-mimetics not requiring ACE-B_2 receptor 'crosstalk' is offered by the recent observation that ACE inhibitors may act as potent B_1 receptor agonists. Finally, the blood pressure-lowering effects of Ang-(1-7) appear to be largely bradykinin-dependent, and most likely reflect its capacity to potentiate bradykinin due to the fact that Ang-(1-7) acts as an ACE inhibitor.

1.7. Aim of the thesis

ACE generates Ang II from Ang I, and inactivates bradykinin. ACE inhibitors are widely used for the treatment of hypertension and heart failure. The beneficial cardiovascular effects of ACE inhibitors may therefore be attributed to both diminished Ang II production and increased bradykinin levels. Both aspects were clarified in this thesis.

First, we investigated ACE inhibitor-induced potentiation of bradykinin (Chapters 2, 4 and 5). Although it seems logical to attribute this to inhibition of bradykinin metabolism, recent studies in isolated cells propose that such potentiation is of non-metabolic origin, and is based on interference of ACE inhibitors with ACE-bradykinin type 2 (B_2) receptor 'crosstalk'. We addressed this issue by studying

bradykinin-induced relaxation in isolated human and porcine coronary arteries, using ACE-resistant bradykinin analogues, ACE inhibitors (captopril and quinaprilat), and the ACE-inhibiting angiotensin metabolite Ang-(1-7). In addition, bradykinin potentiation following inhibition of other bradykinin-metabolizing enzymes (neutral peptidase) was evaluated. Subsequently, we investigated the mediator(s) of bradykinin-induced effects in porcine coronary arteries and isolated trabeculae, focussing in particular on NO and NO-containing factors (Chapters 2 and 3).

Second, we quantified Ang II generation in human coronary arteries (Chapter 6). Ang II levels are not always lowered during chronic ACE inhibitor therapy, and one reason for this absence of Ang II suppression might be that alternative converting enzymes, the most important of which is chymase, contribute to Ang II generation during chronic ACE inhibitor therapy. In this study we made use of both Ang I and the chymase-specific substrate Pro^{11}-D-Ala^{12}-Ang I. By unraveling the site of Ang II generation, we tried to address the physiological importance of Ang II generated by ACE and chymase.

Finally, we studied to what degree superoxide mediates the acute vasoconstrictor effects of Ang II (Chapter 7). A wide range of studies suggests that Ang II stimulates the generation of superoxide through activation of NAD(P)H and/or xanthine oxidase. Since superoxide induces vasoconstriction by inactivating NO, ACE inhibition may result in increased NO release, not only because of bradykinin potentiation, but also due to reduced superoxide generation.

CHAPTER 2

L-NAME-Resistant Bradykinin-Induced Relaxation in Porcine Coronary Arteries is NO-Dependent: Effect of ACE Inhibition

Summary

NOS inhibitors partially block bradykinin-mediated vasorelaxation. Here we investigated whether this is due to incomplete NOS inhibition and/or NO release from storage sites. We also studied the mechanism behind ACE inhibitor-mediated bradykinin potentiation. Porcine coronary arteries were mounted in organ baths, preconstricted, and exposed to bradykinin or the ACE-resistant bradykinin analogue [Hyp^3-$Tyr(Me)^8$]-bradykinin with or without the NOS inhibitor L-NAME (100 μM), the NO scavenger hydroxocobalamin (200 μM), the Ca^{2+}-dependent K^+-channel blockers charybdotoxin + apamin (both 100 nM), or the ACE inhibitor quinaprilat (10 μM). Bradykinin and [Hyp^3-$Tyr(Me)^8$]-bradykinin dose-dependently relaxed preconstricted vessels (pEC_{50} 8.0±0.1 and 8.5±0.2, respectively). pEC_{50}'s were ≈10-fold higher with quinaprilat, and ≈10-fold lower with L-NAME or charybdotoxin + apamin. Complete blockade was obtained with hydroxocobalamin or L-NAME + charybdotoxin + apamin. Repeated exposure to 100 nM bradykinin or [Hyp^3-$Tyr(Me)^8$]-bradykinin, to deplete NO storage sites, produced progressively smaller vasorelaxant responses. With L-NAME, the decrease in response occurred much more rapidly. L-Arginine (10 mM) reversed the effect of L-NAME. Adding quinaprilat to the bath following repeated exposure (with or without L-NAME), at the time bradykinin and [Hyp^3-$Tyr(Me)^8$]-bradykinin no longer induced relaxation, fully restored vasorelaxation, while quinaprilat alone had no effect. Quinaprilat also relaxed vessels that, due to pretreatment with hydroxocobalamin or L-NAME + charybdotoxin + apamin, previously had not responded to bradykinin. In conclusion, L-NAME-resistant bradykinin-induced relaxation in porcine coronary arteries depends on NO from storage sites, and is mediated via stimulation of guanylyl cyclase and/or Ca^{2+}-dependent K^+-channels. ACE inhibitors potentiate bradykinin independent of their effect on bradykinin metabolism.

Based on: Danser A.H.J., Tom B., de Vries R., Saxena P.R. (2000). L-NAME resistant bradykinin-induced relaxation in porcine coronary arteries is NO-dependent: effect of ACE inhibition, *Br. J. Pharmacol.,* **131:195-202**.

2.1. Introduction

Bradykinin induces vasodilation via endothelial bradykinin type 2 (B_2) receptors. This effect can be blocked partly by inhibitors of NOS, suggesting a role for *de-novo* synthesis of NO from L-arginine by NOS (Bjornstad-Ostensen *et al.*, 1997; Gardiner *et al.*, 1990; Mombouli *et al.*, 1992; Palmer *et al.*, 1988; Rees *et al.*, 1990). The relaxant effect of bradykinin that is not blocked by NOS inhibitors is generally attributed to so-called endothelium-derived hyperpolarizing factors, of which the exact identity has not yet been established. Several candidates have been proposed, including prostacyclin, potassium and cytochrome P-450 products of arachidonic acid (Edwards *et al.*, 1998; Fisslthaler *et al.*, 1999; Mombouli *et al.*, 1997). However, NOS inhibitors, even at high concentrations, do not block NO release completely (Cohen *et al.*, 1997). Moreover, *in-vivo* studies in the rat hindlimb (Davisson *et al.*, 1996) and *in-vitro* studies in the isolated perfused rat heart (Danser *et al.*, 1998) have shown that bradykinin also induces release of NO from stores of NO-containing factors, such as S-nitroso-thiols and dinitrosyl iron (II) thiol complexes (Ignarro, 1990; Myers *et al.*, 1990; Vedernikov *et al.*, 1992). Depletion of such stores occurred only after repeated exposure to bradykinin or after prolonged inhibition of NOS, treatments which themselves do not alter the response to NO (Danser *et al.*, 1998; Davisson *et al.*, 1996). Taken together therefore, bradykinin-induced relaxation in the presence of NOS inhibitors might also be due to NO generated by residual NOS activity or to NO released from storage sites.

ACE inhibitors block bradykinin degradation. Accumulation of bradykinin is believed to contribute to the beneficial effects of ACE inhibitors in hypertension and heart failure, although elevated bradykinin levels were not always found during ACE inhibitor treatment (Campbell *et al.*, 1999; Miki *et al.*, 1996). Two recent studies propose that ACE inhibitors potentiate bradykinin beyond blocking its hydrolysis, by inhibiting desensitization of its receptor. These studies were performed in CHO cells transfected with human B_2 receptors and human ACE (Minshall *et al.*, 1997b) and in porcine aortic endothelial cells that naturally express these proteins (Benzing *et al.*, 1999). The mechanism underlying the ACE inhibitor-induced inhibition of B_2 receptor desensitization is currently unknown, but it may involve interference with the translocation of B_2 receptor to caveolin-rich membrane domains (Benzing *et al.*, 1999;

Haasemann *et al.*, 1998; Marcic *et al.*, 1999). Dendorfer *et al.* (Dendorfer *et al.*, 2000) however, using the isolated perfused rat Langendorff heart, found no evidence for ACE inhibitor-induced B_2 receptor upregulation and suggested that the ACE inhibitor-induced potentiation of bradykinin was due to inhibition of bradykinin degradation in the vicinity of B_2 receptors (e.g., in caveolae).

It was the aim of the present study to investigate, in intact porcine coronary arteries (PCAs), 1) whether the NOS inhibitor-resistant bradykinin-induced vasorelaxation involves NO, and 2) whether the ACE inhibitor quinaprilat potentiates bradykinin via blockade of bradykinin metabolism or via other mechanisms. To address the second question, we used the ACE-resistant bradykinin analogue [Hyp^3-$Tyr(Me)^8$]-bradykinin (Minshall *et al.*, 1997b; Rhaleb *et al.*, 1990).

2.2. Methods

Drugs

Bradykinin (acetate salt), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619), substance P (acetate salt), L-arginine HCl, N^{ω}-nitro-L-arginine methyl ester HCl (L-NAME), aminoguanidine, 7-nitroindazole (7-NI), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), hydroxocobalamin (acetate salt), indomethacin, glibenclamide, charybdotoxin, apamin, sulfaphenazole and captopril were from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). [Hyp^3-$Tyr(Me)^8$]-bradykinin was from Calbiochem/Novabiochem AG, Läufelfingen, Switzerland. D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (Hoe140) was a kind gift of dr. W. Linz, Hoechst, Frankfurt, Germany. Quinaprilat was a kind gift of dr. H. van Ingen, Parke-Davis, Hoofddorp, The Netherlands. 7-NI, indomethacin, glibenclamide and quinaprilat were dissolved in dimethylsulfoxide. Sulfaphenazole was dissolved in ethanol. Hydroxocobalamin was dissolved in methanol. All other chemicals were dissolved in saline.

Tissue collection

PCAs were obtained from 31 2-3 month-old pigs (Yorkshire x Landrace, weight 10-15 kg). The pigs had been used in *in-vivo* experiments studying the effects of

α-adrenoceptor and serotonin receptor agonists and antagonists under pentobarbital (600 mg, i.v.) anaesthesia (de Vries *et al.*, 1999; Willems *et al.*, 1999). The Ethics Committee of the Erasmus University Rotterdam dealing with the use of animals for scientific experiments approved the protocol for this investigation. Hearts were explanted at the end of the experiment, and the coronary arteries were removed immediately and stored overnight in a 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. Vessels 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.

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 mM KCl twice. The functional integrity of the endothelium was verified by observing relaxation to 1 nM substance P after preconstriction with 1 μM $PGF_{2\alpha}$. Subsequently, the tissue was exposed to 100 mM KCl to determine the maximal contractile response to KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min. Thereafter, the following experiments were performed.

First, possible mediators of the relaxant effect of bradykinin with and without NOS inhibition were investigated. Vessels were pre-incubated for 30 min in the absence or presence of the non-selective NOS inhibitor L-NAME (100 μM), the inducible NOS inhibitor aminoguanidine (1 mM), the neuronal NOS inhibitor 7-NI (10 μM), the guanylyl cyclase inhibitor ODQ (10 μM), the NO scavenger hydroxocobalamin (200 μM), the cyclooxygenase inhibitor indomethacin (10 μM), the ATP-sensitive K^+-channel inhibitor glibenclamide (1 μM), the large-conductance voltage and Ca^{2+}-activated K^+-channel (BK_{Ca}) blocker charybdotoxin + the small-conductance Ca^{2+}-activated K^+-channel (SK_{Ca}) blocker apamin (both 100 nM), the cytochrome P-450 inhibitor sulfaphenazole (10 μM) or the B_2 receptor antagonist

Hoe140 (1 μM). Vessels were then preconstricted with 10 μM $PGF_{2\alpha}$ or 1 μM U46619 and concentration-response curves (CRCs) to bradykinin (0.1 nM-1 μM) were constructed.

Second, to test whether desensitization of the bradykinin-induced relaxation occurs more rapidly with NOS inhibition (due to more rapid depletion of NO storage sites), preconstricted vessel segments were exposed three times to a concentration of bradykinin (0.1 μM) that is capable of inducing maximal relaxation. Each next exposure was started as soon as the effect of the previous exposure had disappeared, i.e., after approximately 15 min. To investigate whether L-arginine could reverse the effect of L-NAME, the repetitive exposure experiments were repeated in the presence of L-arginine (10 mM), using a concentration of bradykinin (10 nM) that induces submaximal relaxation. To rule out NOS inhibitor-related differences in B_2 receptor desensitization, we constructed two consecutive bradykinin CRCs in a preconstricted vessel segment in the presence or absence of L-NAME.

Third, the effect of ACE inhibition on bradykinin-induced relaxation was investigated. Vessel segments were preconstricted with 10 μM $PGF_{2\alpha}$ or 1 μM U46619 and CRCs were constructed to quinaprilat (1 nM-10 μM) to verify the presence of endogenous bradykinin. Next, in the presence of the highest concentration of quinaprilat, CRCs were constructed to bradykinin and the ACE-resistant bradykinin analogue [Hyp^3-$Tyr(Me)^8$]-bradykinin (0.1 nM-1 μM). In addition, quinaprilat (10 μM) or captopril (100 μM) were added to preconstricted vessels with desensitized B_2 receptors (i.e., vessels that had been exposed three times to a concentration of bradykinin (0.1 μM) that is capable of inducing maximal relaxation; see above). For comparison, quinaprilat (10 μM) was also added to preconstricted vessels that had been exposed three times to another ACE substrate, substance P, at a concentration (1 nM) that is capable of inducing maximal relaxation.

Statistical analysis

Data are given as mean±S.E.M. and expressed as a percentage of the contraction in response to $PGF_{2\alpha}$ or U46619. CRCs were analyzed using the logistic function described by de Lean *et al.* (de Lean *et al.*, 1978) to obtain pEC_{50} ($-^{10}\log EC_{50}$) values. The addition of L-NAME, ODQ, hydroxocobalamin, L-NAME + ODQ or L-NAME +

hydroxocobalamin caused an increase in basal tone of 6±1 mN (n=31), 11±2 mN (n=6), 8±1 mN (n=6), 8±1 mN (n=6) and 9±2 mN (n=6), respectively. The $PGF_{2\alpha}$- and U46619-induced preconstrictions were corrected for this increase in baseline. Statistical analysis was by ANOVA, followed by post hoc evaluation (according to Tukey or Dunnett where appropriate). *P* values <0.05 were considered significant.

2.3. Results

Precontractions

The $PGF_{2\alpha}$- and U46619-induced precontractions in control vessels did not differ and amounted to approximately 30% (13±1 mN, *n*=31) of the maximal contraction induced by 100 mM KCl. Precontractions were not affected by aminoguanidine, 7-NI, indomethacin, glibenclamide, charybdotoxin + apamin, sulfaphenazole or Hoe140. In the vessel segments pretreated with L-NAME, ODQ, hydroxocobalamin, L-NAME + ODQ, or L-NAME + hydroxocobalamin, the precontractions (23±1 mN, n=31; 20±2 mN, n=6; 28±2 mN, n=6; 21±3 mN, n=6; and 24±2 mN, n=6, respectively) were approximately 2-fold higher than in control vessel segments ($p<0.01$), which illustrates the importance of endogenous NO generation by eNOS in this preparation.

Mediators of the relaxant effect of bradykinin

Bradykinin caused complete relaxation of preconstricted vessel segments in a concentration-dependent manner (pEC_{50}=8.03±0.05, n=31; Figure 1). The bradykinin CRC was not affected by aminoguanidine (pEC_{50}=8.21±0.11, n=5), 7-NI (pEC_{50}=7.98±0.22, n=5), indomethacin (pEC_{50}=7.58±0.22, n=5), glibenclamide (pEC_{50}=8.35±0.28, n=5) or sulfaphenazole (pEC_{50}=7.63±0.07, n=6). L-NAME (pEC_{50}=6.93±0.07, n=25, $P<0.01$ vs. control) and ODQ (pEC_{50}=7.19±0.30, n=5; $P<0.05$ vs. control) shifted the CRC of bradykinin to the right, while in the presence of hydroxocobalamin (n=6) relaxation was only observed at the highest concentration of bradykinin (Figure 1). Charybdotoxin + apamin also shifted the CRC of bradykinin to the right (pEC_{50}=6.47±0.10, n=6; $p<0.01$ vs. control, Figure 1). Complete blockade of the response was obtained with Hoe140 (Figure 1).

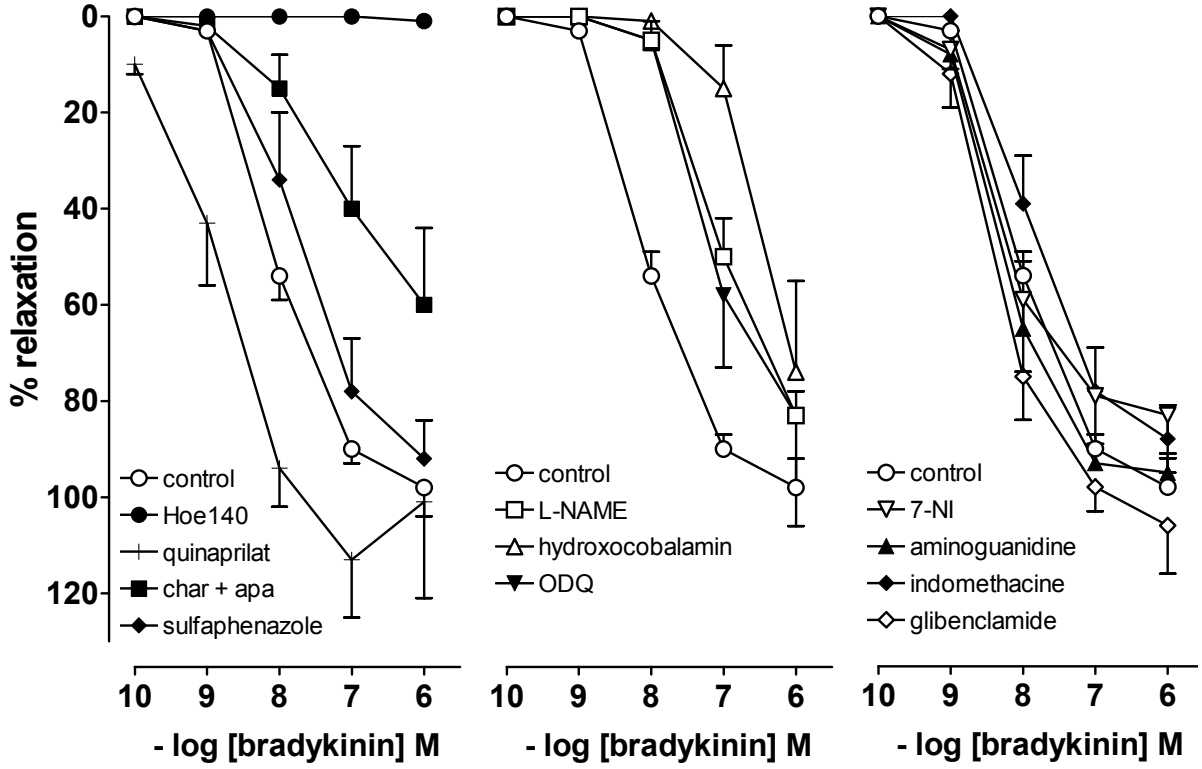


Figure 1. *Relaxations of porcine coronary arteries, preconstricted with 10 µM $PGF_{2\alpha}$ or 1 µM U46619, to bradykinin in the absence (control) or presence of 1 µM Hoe140, 10 µM quinaprilat, 100 nM charybdotoxin (char) + 100 nM apamin (apa), 10 µM sulfaphenazole, 100 µM L-NAME, 200 µM hydroxocobalamin, 10 µM ODQ, 10 µM 7-NI, 1 mM aminoguanidine, 10 µM indomethacin or 1 µM glibenclamide. For the sake of clarity, data have been divided across 3 panels, and the control curve is shown in each panel. Data (mean±S.E.M. of 5-31 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$ or U46619.*

The addition of indomethacin (pEC_{50}=7.19±0.16, n=4) or sulphaphenazole (pEC_{50}=7.08±0.04, n=6) on top of L-NAME did not cause a further rightward shift of the bradykinin CRC as compared to L-NAME alone (data not shown), nor did the addition of ODQ on top of L-NAME (pEC_{50}=7.24±0.12, n=5; Figure 2). Charybdotoxin + apamin in combination with L-NAME completely blocked the response to all bradykinin concentrations tested (Figure 2), whereas in the presence of

L-NAME + hydroxocobalamin (n=6) relaxation was again observed at the highest concentration of bradykinin only (Figure 2).

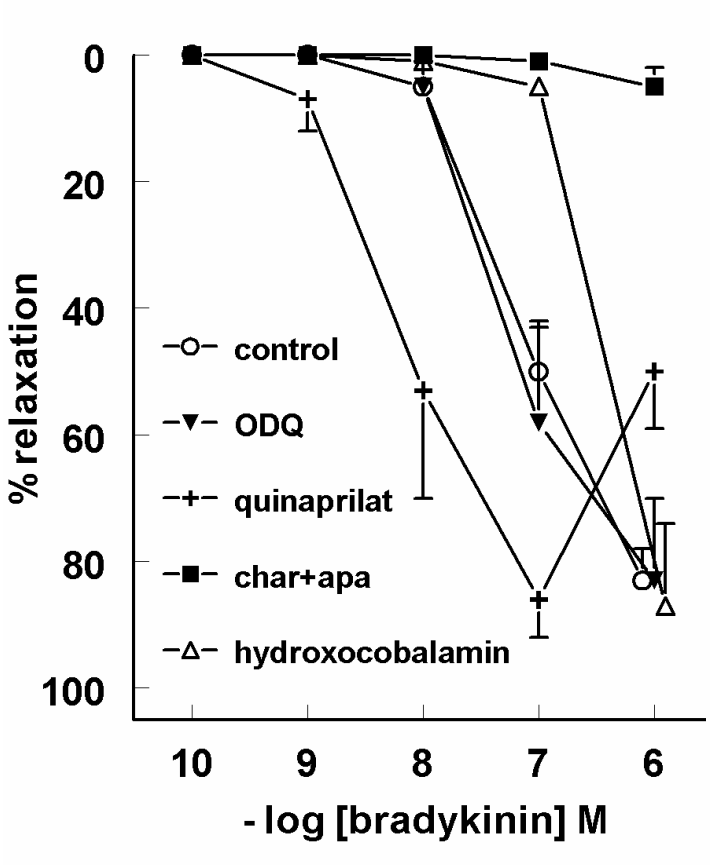


Figure 2. *Relaxations of porcine coronary arteries, following preconstriction with 10 µM $PGF_{2\alpha}$ or 1 µM U46619, to bradykinin in the presence of 100 µM L-NAME without (control) or with 10 µM ODQ, 10 µM quinaprilat, 100 nM charybdotoxin (char) + 100 nM apamin (apa) or 200 µM hydroxocobalamin. Data (mean±S.E.M. of 5-25 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$ or U46619.*

Desensitization of the bradykinin-induced effect

The construction of a bradykinin CRC resulted in B_2 receptor desensitization, as evidenced by the approximate 10-fold rightward shift that was observed when constructing a second bradykinin CRC in the same vessel segment (Figure 3). A similar rightward shift was observed in the presence of L-NAME.

Repeated exposure of preconstricted vessel segments to 0.1 μM bradykinin produced progressively smaller relaxant responses (Figure 4, top panel). The response to the third bradykinin dose was less than 50% of the response to the first bradykinin dose (Figure 4, bottom panel). A similar pattern was observed in the presence of charybdotoxin + apamin, although the relaxation to bradykinin in the presence of these drugs was always smaller than under control conditions (Figure 4, bottom panel). In the presence of L-NAME (Figure 4, top panel) or hydroxocobalamin (Figure 4, bottom panel), the relaxation observed in response to the first dose of bradykinin was significantly smaller than under control conditions, and relaxation was virtually absent in response to the second and third bradykinin dose. Results obtained with L-NAME + hydroxocobalamin were not different from those with hydroxocobalamin alone (data not shown). Charybdotoxin + apamin combined with L-NAME fully prevented all responses to bradykinin (Figure 4, bottom panel).

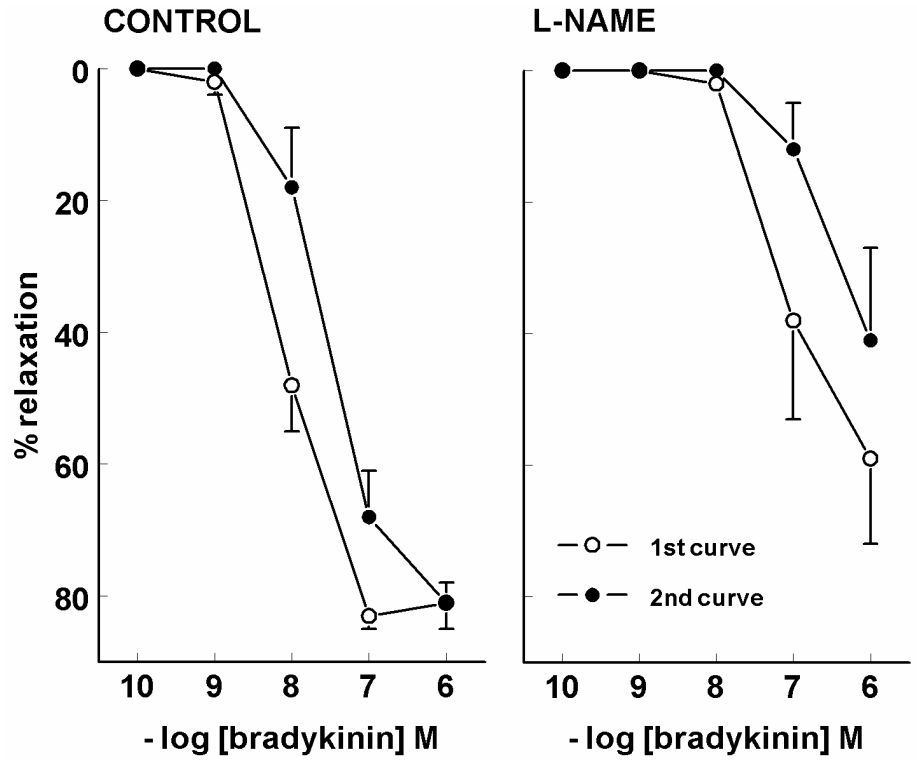
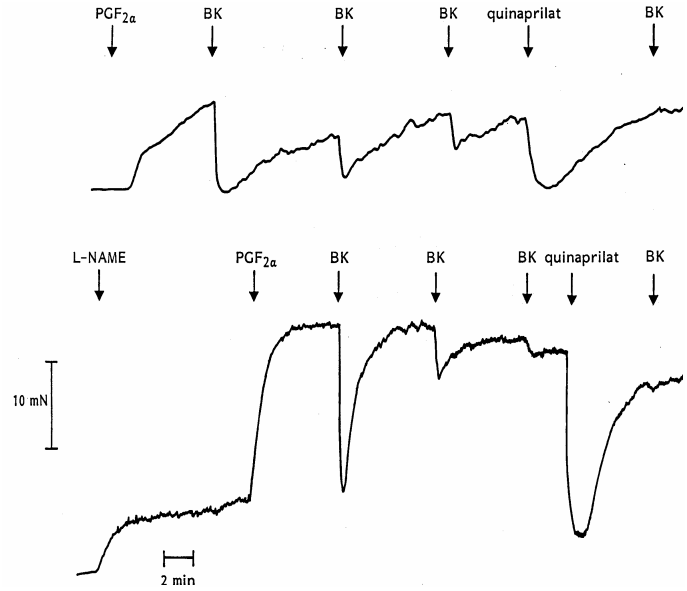
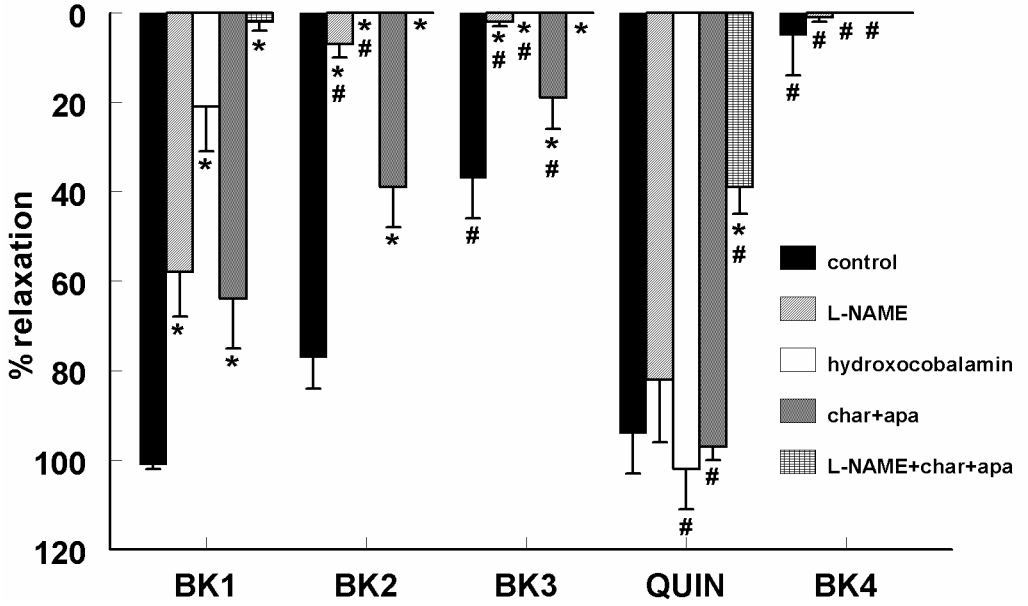


Figure 3. *Two consecutive concentration response curves, obtained in the same porcine coronary artery segment following preconstriction with 10 μM $PGF_{2\alpha}$, to bradykinin in the absence (left panel) or presence (right panel) of 100 μM L-NAME. Data (mean±S.E.M. of 5 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$.*

Figure 4.



Top panel: Original tracings of an experiment in which a porcine coronary artery in the absence (control; top) or presence (bottom) of 100 μM L-NAME was preconstricted with 10 μM $PGF_{2\alpha}$ and subsequently exposed to 0.1 μM bradykinin (BK, three times), 10 μM quinaprilat and 0.1 μM bradykinin.



*Bottom panel: Relaxations of porcine coronary arteries, following preconstriction with 10 μM $PGF_{2\alpha}$ or 1 μM U46619, to three consecutive bradykinin doses (0.1 μM; BK1, BK2, BK3), 10 μM quinaprilat (QUIN) and a fourth bradykinin dose (0.1 μM; BK4) in the absence or presence of 100 μM L-NAME, 200 μM hydroxocobalamin, 100 nM charybdotoxin (char) + 100 nM apamin (apa), or 100 μM L-NAME + 100 nM charybdotoxin (char) + 100 nM apamin (apa). Data (mean±S.E.M. of 6-24 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$ or U46619. * P<0.01 vs. control; # P<0.05 vs. BK1.*

Exposing preconstricted vessels three times to a submaximal concentration of bradykinin (10 nM) revealed that, under control conditions, the relaxation in response to the third bradykinin dose was not significantly different from the response to the first bradykinin dose (Figure 5).

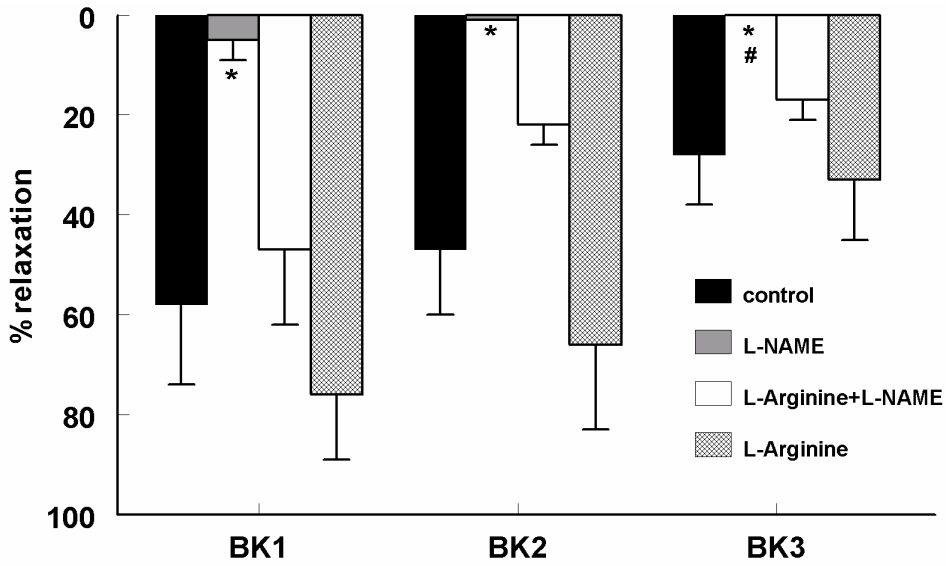


Figure 5. *Relaxations of porcine coronary arteries, following preconstriction with 10 μM $PGF_{2\alpha}$, to three consecutive bradykinin doses (10 nM; BK1, BK2, BK3) in the absence (control) or presence of 100 μM L-NAME, 100 μM L-NAME + 10 mM L-arginine, or 10 mM L-arginine. Data (mean±S.E.M. of 5-7 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$. * P<0.01 vs. control; # P<0.05 vs. BK1.*

In the presence of L-NAME, the first bradykinin dose induced a modest relaxation, and no further response was observed at the second and third exposure to bradykinin. L-Arginine reversed the inhibitory effect of L-NAME, and tended to enhance the effect of bradykinin in the absence of L-NAME (P=NS).

Effect of ACE inhibition on bradykinin-induced relaxation

Quinaprilat alone did not cause relaxation of preconstricted vessel segments (data not shown), thereby ruling out the presence of endogenous bradykinin. In the presence of the ACE inhibitor, the CRC to bradykinin was shifted to the left (pEC_{50}=8.89±0.20,

n=5; P<0.05 vs. control, see Figure 1). This was also the case in vessel segments that had been pre-incubated with L-NAME (pEC_{50}=8.03±0.23, n=5; P<0.05 vs. L-NAME alone, see Figure 2). Quinaprilat caused a similar leftward shift of the CRC to the ACE-resistant analogue [Hyp^3-$Tyr(Me)^8$]-bradykinin (pEC_{50}'s resp. 8.50±0.18 and 9.18±0.06 without and with quinaprilat, n=4; P<0.05, Figure 6). Hoe140 fully blocked the effects of [Hyp^3-$Tyr(Me)^8$]-bradykinin, confirming that this agonist induces relaxation via stimulation of B_2 receptors.

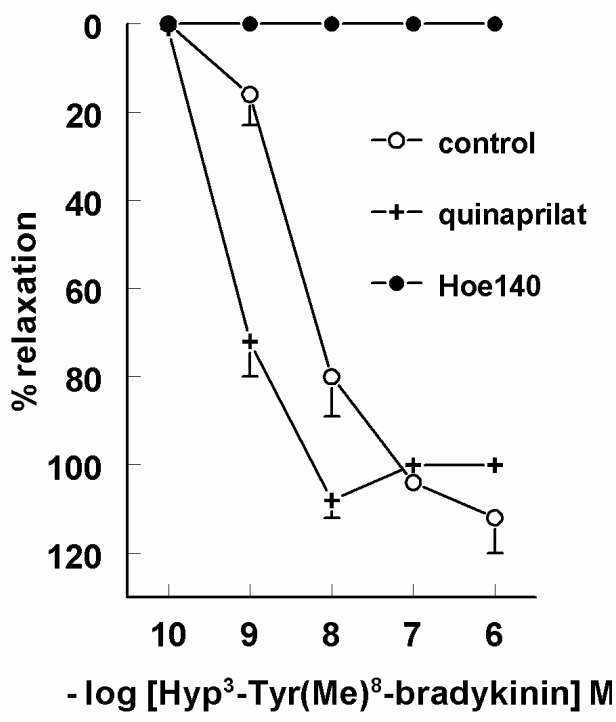


Figure 6. *Relaxations of porcine coronary arteries, preconstricted with 10 µM $PGF_{2\alpha}$, to [Hyp^3-$Tyr(Me)^8$]-bradykinin in the absence (control) or presence of 1 µM Hoe140 or 10 µM quinaprilat. Data (mean±S.E.M. of 4 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$.*

Quinaprilat added to vessel segments that had been exposed three times to 0.1 µM bradykinin, after the effect of the last dose of bradykinin had disappeared, caused complete relaxation, even in L-NAME- or hydroxocobalamin-pretreated vessel segments that previously had not responded to bradykinin (Figure 4). Quinaprilat also induced complete relaxation of vessel segments pretreated with charybdotoxin + apamin, whereas in vessel segments pretreated with L-NAME combined with charybdotoxin + apamin, which previously had not shown any response to bradykinin, the ACE inhibitor induced a modest relaxant response. Similar results were obtained with captopril (n=5, data not shown). A fourth bradykinin dose, added after the effect of quinaprilat or captopril had disappeared, induced no further effect.

Results obtained with bradykinin and quinaprilat in the presence of aminoguanidine (n=5), 7-NI (n=5), indomethacin (n=4), glibenclamide (n=4) and sulfaphenazole (n=6) were not different from those obtained in the absence of these inhibitors (data not shown). Moreover, results obtained with [Hyp^3-$Tyr(Me)^8$]-bradykinin and quinaprilat (n=4) exactly mimicked those with bradykinin and quinaprilat (data not shown). Hoe140 completely prevented the quinaprilat-induced potentiation (data not shown). The effect of quinaprilat was specific for bradykinin, since it was not observed in combination with substance P (Figure 7).

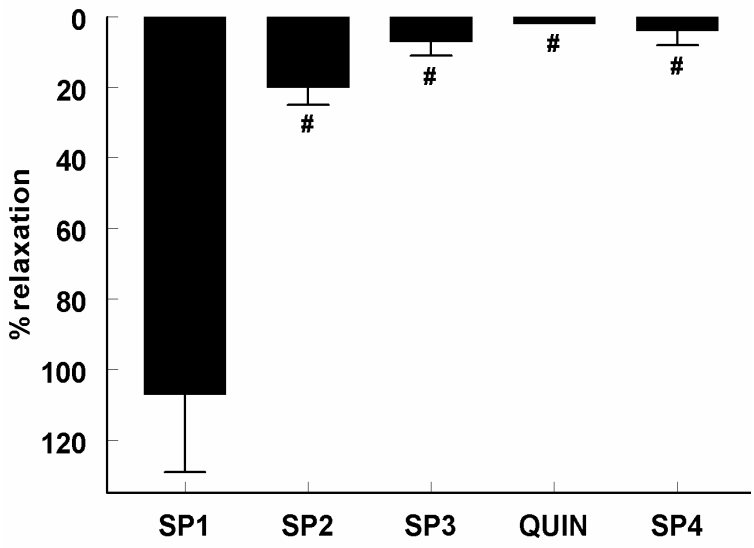


Figure 7. *Relaxations of porcine coronary arteries, following preconstriction with 10 µM $PGF_{2\alpha}$, to three consecutive doses of substance P (1 nM; SP1, SP2, SP3), 10 µM quinaprilat (QUIN) and a fourth dose of substance P (1 nM; SP4). Data (mean±S.E.M. of 5 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$. # P<0.01 vs. SP1.*

2.4. Discussion

NO is responsible for bradykinin-induced vasorelaxation in porcine coronary arteries

The results of the present study show that the B_2 receptor-mediated relaxant effects of bradykinin in PCAs, at doses up to 0.1 µM, depend on NO, either synthesized *de-novo*

by eNOS or derived from NO storage sites. These data fully support the interaction between eNOS and B_2 receptors that was recently described by Golser *et al.* (Golser *et al.*, 2000). We found no evidence for a role of NO synthases other than eNOS in PCAs, since neither aminoguanidine, a preferential inhibitor of inducible NOS (Boulanger *et al.*, 1998; Joly *et al.*, 1994), nor 7-NI, a preferential inhibitor of neuronal NOS (Boulanger *et al.*, 1998; Moore *et al.*, 1993) affected basal tone or the bradykinin CRC. Furthermore, neither prostaglandins, ATP-sensitive K^+-channels nor cytochrome P-450 products appeared to be involved in the bradykinin-induced vasodilation in PCAs. The latter contrasts with a recent observation in PCAs showing that bradykinin-induced relaxation in the presence of the NOS inhibitor N^{ω}nitro-L-arginine was due to the release of 11,12-epoxyeicosatrienoic acid, an arachidonic acid metabolite formed by cytochrome P-450 (Fisslthaler *et al.*, 1999). However, the EC_{50} of this effect was approximately 1 μM (Fisslthaler *et al.*, 1999), and we did not test bradykinin concentrations above 1 μM.

The 10-fold rightward shift of the bradykinin CRC induced by both the non-selective NOS inhibitor L-NAME and the guanylyl cyclase inhibitor ODQ contrasts with the much more complete blockade observed in the presence of the NO scavenger hydroxocobalamin. Hydroxocobalamin did not block the effect of 1 μM bradykinin. This is not due to the formation of relaxant prostaglandins or cytochrome P-450 products at this concentration of bradykinin, since indomethacin and sulfaphenazole did not affect the bradykinin CRC in the presence of L-NAME. The most likely explanation is that, at the concentration used in the present study (200 μM), hydroxocobalamin did not completely scavenge all NO (Li *et al.*, 1999). The maximum solubility of hydroxocobalamin in methanol (10 mg/mL) prevented us from reaching higher concentrations in the organ bath. Taken together therefore, NO release is responsible for the bradykinin-induced relaxation in PCAs, at least at bradykinin concentrations up to 0.1 μM, and NOS blockade as well as guanylyl cyclase blockade cannot prevent this relaxation completely.

The modest effect of ODQ might be due to the fact that this drug inhibits guanylyl cyclase reversibly (Garthwaite *et al.*, 1995). Alternatively, NO-induced relaxation may occur independently of guanylyl cyclase. In support of the first possibility, we observed that ODQ fully inhibited the relaxant effects of low (<1 μM)

but not of high (≥1 μM) concentrations of the NO donor S-nitroso-N-acetylpenicillamine (Danser *et al.*, 1999, unpublished observations). In support of the second possibility, NO has been described to induce hyperpolarization directly via activation of Ca^{2+}-dependent K^+-channels (Bolotina *et al.*, 1994), and bradykinin is known to activate these channels through stimulation of tyrosine kinase (Lee *et al.*, 1993; Ogiwara *et al.*, 1995). Indeed, in the present study the BK_{Ca} blocker charybdotoxin and the SK_{Ca} blocker apamin partially blocked the bradykinin-induced relaxation when given together, and fully blocked the bradykinin-induced effects when given in combination with L-NAME. Charybdotoxin plus apamin also blocked the bradykinin-induced hyperpolarization of PCA rings (Quignard *et al.*, 1999). Thus, the most likely explanation for our findings is that the bradykinin-induced NO release causes vasodilation via stimulation of guanylyl cyclase and/or via direct activation of Ca^{2+}-dependent K^+-channels.

Release of NO from storage sites?

The limited effect of NOS blockade on NO release has been described before in rabbit carotid arteries (Cohen *et al.*, 1997), and may involve either residual NOS activity or release of NO from storage sites (Danser *et al.*, 1998; Davisson *et al.*, 1996). To investigate the latter, we exposed coronary arteries repeatedly to the same concentration of bradykinin. Previous studies have shown that repetitive exposure to bradykinin or acetylcholine will cause depletion of NO storage sites, and that this will occur more rapidly in the presence of NOS inhibitors (Colombari *et al.*, 1998; Danser *et al.*, 1998; Davisson *et al.*, 1996). In the present study, exposure to bradykinin, both with and without L-NAME, resulted in a relaxant effect which lasted approximately 10-15 minutes. The disappearance of the relaxation is suggestive for bradykinin B_2 receptor desensitization and/or bradykinin metabolism. Desensitization was similar with and without L-NAME (Figure 3). Subsequent exposures to bradykinin initiated progressively smaller relaxant effects, and the decrease in response was much more rapid in the presence of L-NAME. L-Arginine reversed the rapid decrease in response to consecutive bradykinin doses in the presence of L-NAME. Taken together, these findings support the concept of bradykinin-coupling to NO storage sites. With NOS activity intact, the NO pools are continuously supplied with fresh NO and relaxation can be obtained multiple times, even when B_2 receptors are desensitized. During NOS

inhibition the NO storage sites will become depleted, especially during exposure to drugs that cause release of NO from these sites. In an earlier study in isolated perfused rat hearts, we found that a 30 minute-exposure to L-NAME was sufficient to cause depletion of all existing NO pools (Danser *et al.*, 1998). Depletion may also occur during exposure to high levels of superoxide anion (Arnal *et al.*, 1996), although in PCAs superoxide anions scavengers did not affect the response to bradykinin (Pomposiello *et al.*, 1999).

The nature and localization of NO pools is currently unknown. Although NO pools have been demonstrated in vascular smooth muscle cells (Venturini *et al.*, 1993), the pools in the present study are most likely localized in endothelial cells, in view of the fact that we (Danser *et al.*, 1999, unpublished results) and others (Mombouli *et al.*, 1992) have found that bradykinin-induced relaxations are virtually absent following endothelium removal. The long half life of NO present at storage sites, evidenced by the fact that bradykinin was still capable of inducing relaxation after the vessels had been exposed to L-NAME for more than 30 minutes, is compatible with the idea that NO pools consist of stable NO-containing compounds, such as S-nitroso-thiols and dinitrosyl iron (II) thiol complexes (Ignarro, 1990; Myers *et al.*, 1990; Vedernikov *et al.*, 1992).

ACE inhibitors potentiate bradykinin independently of their effect on bradykinin metabolism

Finally, the ACE inhibitor quinaprilat, added at a time when the relaxant effect of bradykinin had disappeared, immediately restored the vasorelaxation, both with and without L-NAME. The effect of quinaprilat could be mimicked by captopril, and did not occur in combination with the B_2 receptor antagonist Hoe140 or without prior exposure to bradykinin. The latter finding suggests that not all bradykinin has been metabolized at the time the ACE inhibitor is added to the organ bath. When added prior to bradykinin, quinaprilat shifted the bradykinin CRC approximately tenfold to the left, both with and without L-NAME. It is unlikely that the potentiating effects of ACE inhibition are due simply to inhibition of bradykinin metabolism, as suggested by Dendorfer *et al.* (Dendorfer *et al.*, 2000), because 1) the effect of quinaprilat was also observed in combination with the ACE-resistant bradykinin analogue [Hyp^3-$Tyr(Me)^8$]-bradykinin, 2) the effect of quinaprilat was not observed in relationship

with the vasorelaxant ACE substrate substance P, and 3) quinaprilat even induced complete relaxation in vessel segments that had previously not responded to bradykinin, i.e., vessel segments that had been pre-incubated with hydroxocobalamin or charybdotoxin + apamin + L-NAME. Taken together therefore, our data support the concept of ACE inhibitor-induced bradykinin potentiation independently of the effect of these drugs on bradykinin metabolism. The mechanism underlying this phenomenon is currently unknown, but it may involve the ACE inhibitor-induced resensitization of desensitized B_2 receptors that has been described in isolated cells (Benzing *et al.*, 1999; Minshall *et al.*, 1997b). Resensitized B_2 receptors may still cause relaxation via coupling to remaining NO pools, even after exposure to L-NAME and hydroxocobalamin, since we do not know whether these drugs, combined with repetitive exposure to bradykinin, have resulted in complete depletion of all existing NO pools. Alternatively, non-NO-related mechanisms may have come into play. These mechanisms do not involve prostaglandins, ATP-sensitive K^+-channels or cytochrome P-450 products, since indomethacin, glibenclamide and sulfaphenazole did not affect the quinaprilat-induced relaxation.

2.5. Conclusions and possible clinical implications

In conclusion, the L-NAME-resistant bradykinin-induced relaxation, at least at physiological bradykinin concentrations (i.e., concentrations up to 0.1 μM; Campbell *et al.*, (Campbell *et al.*, 1993a)), is NO-dependent, and is mediated via stimulation of guanylyl cyclase and/or Ca^{2+}-dependent K^+-channels. NO is either synthesized *de-novo* by eNOS or released from storage sites. Depletion of such sites or a decrease in their number might be involved in the impaired endothelium-dependent vasodilatory response observed in subjects with hypertension or atherosclerosis (Hirooka *et al.*, 1992; Zeiher *et al.*, 1993). ACE inhibitors potentiate bradykinin-induced vasorelaxation independently of their effect on bradykinin metabolism. Such potentiation has also been observed *in-vivo* in human subjects (Hornig *et al.*, 1997; Kuga *et al.*, 1997). Since elevated bradykinin levels have not been found consistently during ACE inhibitor treatment (Campbell *et al.*, 1999; Miki *et al.*, 1996), our findings might explain, at least in part, the beneficial effects of ACE inhibitors in hypertension and heart failure, as well as the ACE inhibitor-induced reversal of the impaired endothelium-dependent vasorelaxation in hypertensive patients (Hirooka *et al.*, 1992).

CHAPTER 3

Negative Inotropic Effect of Bradykinin in Porcine Isolated Atrial Trabeculae: Role of NO

Summary

We investigated whether bradykinin affects cardiac contractility independently of its effects on coronary flow and noradrenaline release, and whether such inotropic effects, if present, are mediated via NO. Right atrial trabeculae were obtained from 35 pigs, suspended in organ baths and attached to isometric transducers. Resting tension was set at approximately 750 mg and tissues were paced at 1.5 Hz. Tissue viability was checked by constructing a concentration response curve to noradrenaline. Next, concentration response curves were constructed to bradykinin (1 nM-1 μM), either under baseline conditions or after pre-stimulation with the positive inotropic agent forskolin (1 or 10 μM), in the absence or presence of the B_2 receptor antagonist Hoe 140 (1 μM), the NOS inhibitor L-NAME (100 μM) and/or the NO scavenger hydroxocobalamin (200 μM). Bradykinin exerted a negative inotropic effect, both with and without forskolin pre-stimulation, reducing contractility by maximally 22±3.6% (mean±S.E.M.) and 23±3.6%, respectively (pEC_{50} 8.37±0.23 and 8.62±0.22, respectively). L-NAME reduced this effect in pre-stimulated, but not in unstimulated, trabeculae. Hoe 140 and hydroxocobalamin fully blocked the inotropic effect of bradykinin. In conclusion, bradykinin induces a modest negative inotropic effect in porcine atrial trabeculae that is mediated via B_2 receptors and NO. The inconsistent results obtained with L-NAME suggest that it depends on *de-novo* synthesised NO and/or NO from storage sites.

Based on: Tom B., de Vries R., Saxena P.R. and Danser A.H.J., (2001). Negative inotropic effect of bradykinin in porcine isolated atrial trabeculae: role of NO, *J. Hypertension,* **19(7): 1289-1293.**

3.1. Introduction

Accumulation of bradykinin is believed to contribute to the beneficial cardiac effects of ACE inhibitors, not only during myocardial ischaemia but also during the development of left ventricular hypertrophy (Gohlke *et al.*, 1994; 1997; Kitakaze *et al.*, 1995; McDonald *et al.*, 1995; Tio *et al.*, 1991). The mechanism behind these beneficial effects has not yet been clarified, but most likely involves stimulation of B_2 receptors. In support of the importance of cardiac B_2 receptors, Emanueli *et al.* (Emanueli *et al.*, 1999) have recently shown that disruption of the B_2 receptor in mice results in left ventricular remodelling and cardiac functional impairment.

Cardiac B_2 receptors are localized on endothelial cells, sympathetic nerve endings and myocytes, and their respective stimulation leads to NO synthesis and release (Danser *et al.*, 2000), facilitation of noradrenaline release (Minshall *et al.*, 1994; Rump *et al.*, 1997; Seyedi *et al.*, 1997), and hydrolysis of phosphatidylinositol 4,5-biphosphate into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (Clerk *et al.*, 1996; Minshall *et al.*, 1995). Noradrenaline and IP_3 will cause a rise in cardiac contractility, whereas NO, depending on its concentration may both increase and decrease contractility (Kojda *et al.*, 1999; Vila-Petroff *et al.*, 1999). Moreover, NO affects cardiac inotropy indirectly through its effects on coronary flow (Minshall *et al.*, 1997c; Munch *et al.*, 1991). In this light, it is of no surprise that both positive and negative inotropic responses to bradykinin have been reported (Anning *et al.*, 1995; Cheng *et al.*, 1998; Kasel *et al.*, 1996; Minshall *et al.*, 1997c; Munch *et al.*, 1991).

In the present study, we set out to investigate the flow- and catecholamine-independent inotropic effects of bradykinin, using isolated porcine atrial trabeculae. We also studied whether these effects are mediated via NO.

3.2. Methods

Drugs

Bradykinin (acetate salt), N^{ω}-nitro-L-arginine methyl ester HCl (L-NAME), hydroxocobalamin (acetate salt), forskolin and carbachol were from Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands. L(-)-noradrenaline bitartrate was from

Research Biochemicals International, Zwijndrecht, The Netherlands. D-Arg [Hyp^3-Thi^5, D-Tic^7, Oic^8]-bradykinin (Hoe 140) was a kind gift of Dr. W. Linz, Hoechst, Frankfurt, Germany. Hydroxocobalamin was dissolved in methanol. Forskolin was dissolved in dimethylsulfoxide. All other chemicals were dissolved in saline.

Tissue collection

Trabeculae were obtained from 35 female 3 month-old pigs (Yorkshire x Landrace; weight 10-15 kg). The pigs had been used in *in-vivo* experiments, studying the effects of α-adrenergic and/or serotonergic (ant)agonists under pentobarbital anaesthesia (de Vries *et al.*, 1999). The Ethics Committee of the Erasmus University Rotterdam dealing with the use of animals for scientific experiments approved the protocol for this investigation. At the end of the experiments, cardiac arrest was induced by pouring ice-cold saline in the thorax. Right atrial tissue was rapidly removed and stored in a cold, oxygenated, high KCl-containing Krebs bicarbonate solution modified according to HTK-Bretschneider (composition in mM: NaCl 113.7, KCl 9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, D-glucose 8.3; pH 7.4). After overnight storage, trabeculae (1 mm thickness; 6-12 per atrium) were carefully dissected free, mounted in 15 mL organ baths containing Krebs bicarbonate solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, D-glucose 8.3; pH 7.4) and attached to Harvard isometric transducers (South Natick, MA, USA). The baths were kept at 37°C and continuously aerated with 95% O_2/ 5% CO_2. Resting tension was set at approximately 750 mg and tissues were paced at 1.5 Hz using field stimulation (5 ms, voltage 20% above threshold for contractile response) (Schoemaker *et al.*, 1992).

Experimental protocol

After a stabilization period of 60 min, during which the organ bath fluid was refreshed every 15 min, tissue viability was tested by constructing a concentration response curve (CRC) to noradrenaline (10 nM-10 μM). Each next noradrenaline dose was added as soon as a maximum contraction to the previous dose had been reached (usually within 5 min). Tissue viability was not affected by overnight storage, since the noradrenaline response of stored trabeculae was indistinguishable from that of

freshly obtained trabeculae (n=4, data not shown). After finalization of the noradrenaline CRC, the baths were washed and the trabeculae were allowed to stabilise for 30 min.

Next, bradykinin CRCs (1 nM-1 μM) were constructed, both under baseline conditions and after pre-stimulation with forskolin (1 or 10 μM), in the presence or absence of the B_2 receptor antagonist Hoe 140 (1 μM), the NOS inhibitor L-NAME (100 μM), the NO scavenger hydroxocobalamin (200 μM) (Danser *et al.*, 2000; Li *et al.*, 1999) or L-NAME (100 μM) + hydroxocobalamin (200 μM). In the forskolin pre-stimulated trabeculae, at the end of each bradykinin CRC, the negative inotropic response to carbachol (1 μM) was studied.

Data analysis

Data are expressed as mean±S.E.M. and represent percent change, either from baseline contractile force or from the contractile force obtained after pre-stimulation with forskolin. Tissues with less than 25 mg contractile response to noradrenaline as well as trabeculae showing less than 5% relaxation to bradykinin in the absence of inhibitors were excluded from further analysis. CRCs were analysed using the logistic function described by de Lean *et al.* (de Lean *et al.*, 1978) to obtain pEC_{50} ($-^{10}\log EC_{50}$) values. Statistical analysis was by Student's paired or unpaired t-test. P values < 0.05 were considered significant.

3.3. Results

Forskolin-induced pre-stimulation and the effect of inhibitors

Baseline contractility ranged from 35 mg to 260 mg. In the absence of inhibitors, forskolin increased contractility from 78±11 mg to 162±14 mg (n=13, $p<0.01$). This increase in contractility is comparable to the increase in contractility observed in response to 10 μM noradrenaline. The forskolin-induced contraction remained stable for at least 45 min. Hoe 140 and L-NAME did not effect baseline contractility or the response to forskolin. Hydroxocobalamin reduced baseline contractility from 123±46 mg to 61±30 mg (n=4, $p<0.05$). This reduction in baseline contractility also occurred when applying the hydroxocobalamin solvent, methanol (reduction in

contractility from 182±71 mg to 102±60 mg, n=4, $p<0.05$), indicating that it was due to methanol rather than to hydroxocobalamin itself. However, following forskolin, contractility in methanol-pretreated (210±56 mg) and methanol+hydroxocobalamin-pretreated (193±34 mg) trabeculae was indistinguishable from control.

Effects of bradykinin

Bradykinin decreased contractile force both in unstimulated (n=8) and in forskolin pre-stimulated (n=13) trabeculae in a concentration-dependent manner (pEC_{50} of 8.62±0.22 and 8.37±0.23, respectively; Figure 1). Relaxations were maximal within 1 min and contractile force returned to baseline after approximately 3 min (Figure 1). No differences in bradykinin-induced responses were observed between trabeculae obtained from untreated pigs and trabeculae from pigs exposed to α-adrenergic and/or serotonergic (ant)agonists (data not shown). Hoe 140 fully blocked the bradykinin-mediated effects (Figure 1). L-NAME did not affect the bradykinin-induced effects in unstimulated trabeculae (n=8; Figure 1). In forskolin pre-stimulated trabeculae L-NAME reduced the maximal negative inotropic effect of bradykinin by approximately 50% (n=13, $p<0.05$; Figure 1).

Full blockade of the bradykinin-induced negative inotropic effects was obtained in the presence of hydroxocobalamin, with or without L-NAME (Figure 2). Methanol did not affect the response to bradykinin (pEC_{50} of 8.51±0.11; P=NS vs. control).

Carbachol, added to the organ bath following the finalization of the bradykinin CRC, reduced contractile force by 88±1.4% (n=4) in the absence of inhibitors and by 87±1.5%, 86±2.0%, 85±2.0%, 83±3.5% (n=4 each) in the presence of L-NAME, hydroxocobalamin, L-NAME+ hydroxocobalamin, and methanol respectively.

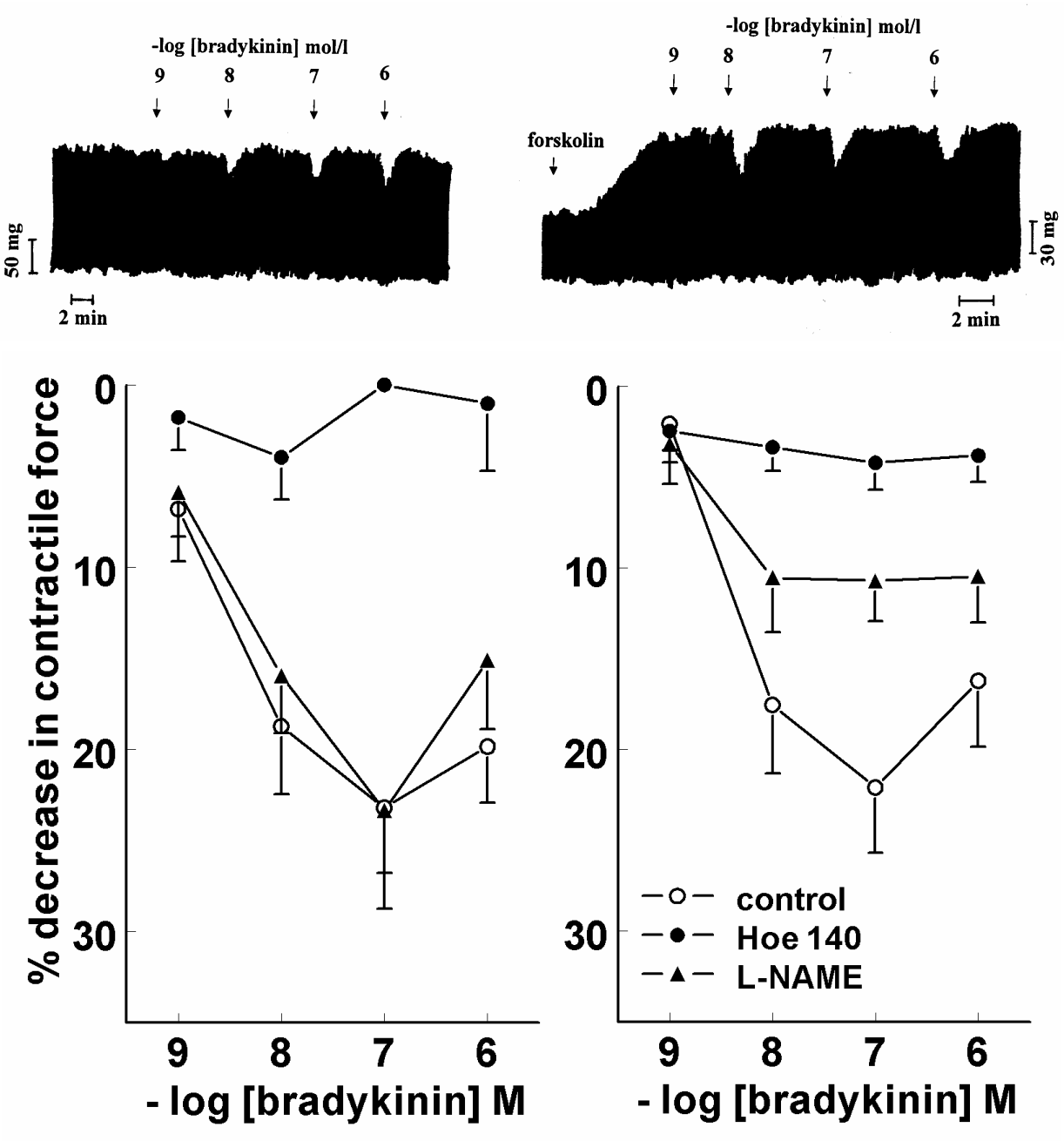


Figure 1. *Bradykinin-induced negative inotropic effect in porcine atrial trabeculae under baseline conditions (left) or after pre-stimulation with 1 or 10 μM forskolin (right) without (control) or with Hoe 140 (1 μM) or L-NAME (100 μM). Top panel: original tracing; bottom panel: percent decrease (mean±S.E.M. of 7-13 exp.) from baseline contractile force or from the contractile force obtained after prestimulation with forskolin.*

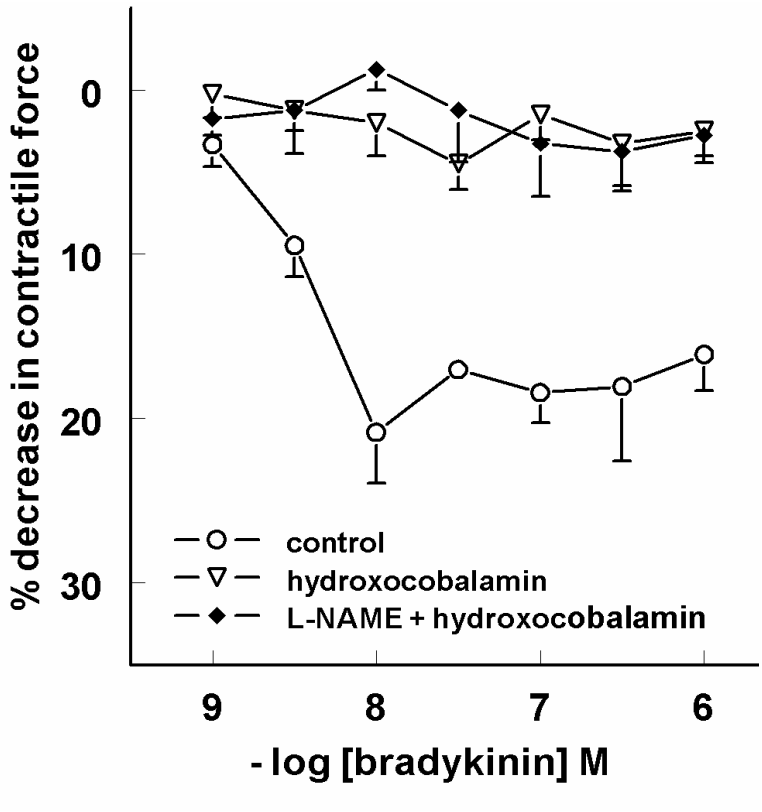


Figure 2. *Bradykinin-induced negative inotropic effect in porcine atrial trabeculae pre-stimulated with 1 or 10 μM forskolin, in the absence (control) or presence of hydroxocobalamin (200 μM) or L-NAME (100 μM) + hydroxocobalamin (200 μM). Data (mean±S.E.M. of 4 experiments) represent percent change from the contractile force obtained after pre-stimulation with forskolin.*

3.4. Discussion

The results of the present study show that bradykinin exerts a modest negative inotropic effect on isolated porcine atrial trabeculae that is mediated exclusively via B_2 receptors and NO. The effect is short lasting and most likely depends on NO from storage sites and/or *de-novo* synthesised NO.

The lack of effect of L-NAME and the NO scavenger hydroxocobalamin on baseline and stimulated contractility contrasts with our earlier findings in isolated porcine coronary arteries, where the addition of these drugs not only resulted in a

strong contractile response but also in an augmentation of the contraction to prostaglandin $F_{2\alpha}$ (Danser *et al.*, 2000). These coronary arteries, like the trabeculae in the present study, had been stored overnight in an organ-protecting solution. Since storage does not affect tissue viability or endothelial function (Maassen van den Brink *et al.*, 1999), it appears that endogenous NO in isolated trabeculae does not contribute importantly to contractility under baseline conditions. The strong NO-independent negative inotropic effect of the muscarinic agonist carbachol reveals that the modest effects of bradykinin in the present study were not due to our inability to detect a negative inotropic response.

NO has been reported to induce a positive inotropic effect in submicromolar concentrations, and a negative inotropic effect in submillimolar concentrations, via activation of adenylyl cyclase and guanylyl cyclase, respectively (Vila-Petroff *et al.*, 1999). We studied the effects of bradykinin both with and without forskolin pre-stimulation, and observed a negative inotropic response of similar magnitude under both conditions. It appears therefore that the amount of NO released in response to bradykinin, even at nanomolar concentrations of the agonist, is in the submillimolar range. Remarkably however, despite the presence of such high NO levels, NO does not seem to play a major role in cardiac contractility.

The limited effect of L-NAME on the bradykinin-induced inotropic response in forskolin pre-stimulated trabeculae, combined with the full blockade of this response by hydroxocobalamin, suggests that L-NAME does not block NOS completely and/or that bradykinin induces NO release from NO storage pools. Incomplete NOS blockade, even at high NOS inhibitor doses, has been demonstrated before (Cohen *et al.*, 1997). NO release from storage sites might also offer an explanation for the lack of effect of L-NAME in unstimulated trabeculae during exposure to bradykinin.

NO pools, which consist of stable NO-containing compounds such as S-nitroso-thiols (Ignarro, 1990), are present in endothelial cells (Danser *et al.*, 1998; 2000; Davisson *et al.*, 1996), and may also exist in other NOS-synthesising cells such as cardiomyocytes (Kojda *et al.*, 1999). From our data, it cannot be concluded whether endothelial cells and/or myocytes are the source of the NO released by bradykinin. Both cell types express B_2 receptors (Minshall *et al.*, 1995; Sung *et al.*, 1988). The short duration of action of bradykinin in trabeculae is in full agreement

with the rapid B_2 receptor desensitization that is known to occur upon bradykinin stimulation (Danser *et al.*, 2000; Minshall *et al.*, 1997b).

Finally, stimulation of B_2 receptors on sympathetic nerve endings and neonatal rat cardiomyocytes, via facilitation of noradrenaline release (Seyedi *et al.*, 1997) and generation of IP_3 (Minshall *et al.*, 1995), respectively, might result in a positive inotropic response independently of NO. No such response was observed in the present study, neither under baseline conditions, nor after forskolin pre-stimulation in the presence of hydroxocobalamin and/or L-NAME. Moreover, in the same experimental set-up, propranolol did not affect baseline contractility (Du *et al.*, 1993). Thus, endogenous noradrenaline release from isolated porcine trabeculae does not appear to occur, and in porcine adult cardiomyocytes either B_2 receptors are not expressed, or their stimulation does not result in sufficient IP_3 formation. Similar species-related differences have been observed for Ang II (Holubarsch *et al.*, 1993; Ishihata *et al.*, 1995).

In conclusion, in the absence of B_2 receptor-mediated coronary vasodilatation and/or facilitation of noradrenaline release, bradykinin decreases cardiac contractility in a NO-dependent manner. These data may explain not only the previously reported conflicting results on bradykinin-induced inotropic effects (Anning *et al.*, 1995; Cheng *et al.*, 1998; Kasel *et al.*, 1996; Minshall *et al.*, 1997c; Munch *et al.*, 1991), but also help to understand the beneficial bradykinin-dependent effects of ACE inhibitors on cardiac function (Gohlke *et al.*, 1994; 1997; Kitakaze *et al.*, 1995; McDonald *et al.*, 1995; Tio *et al.*, 1991).

CHAPTER 4

Bradykinin Potentiation by Angiotensin-(1-7) and ACE Inhibitors Correlates with ACE C- and N- Domain Blockade

Summary

ACE inhibitors block B_2 receptor desensitization, thereby potentiating bradykinin beyond blocking its hydrolysis. Ang-(1-7) also acts as an ACE inhibitor, and, in addition, may stimulate bradykinin release via AT_2 receptors. Here, we compared the bradykinin-potentiating effects of Ang-(1-7), quinaprilat and captopril. Porcine coronary arteries, obtained from 32 pigs, were mounted in organ baths, preconstricted with $PGF_{2\alpha}$, and exposed to quinaprilat, captopril, Ang-(1-7) and/or bradykinin. Bradykinin induced complete relaxation (pEC_{50}=8.11±0.07, mean±S.E.M.), while quinaprilat, captopril and Ang-(1-7) alone were without effect. Quinaprilat shifted the bradykinin curve to the left in a biphasic manner: a 5-fold shift at concentrations that specifically block the C-domain (0.1-1 nM), and a 10-fold shift at concentrations that block both domains. Captopril and Ang-(1-7) monophasically shifted the bradykinin curve to the left, by a factor of 10 and 5, respectively. A 5-fold shift was also observed when Ang-(1-7) was combined with 0.1 nM quinaprilat. Repeated exposure of porcine coronary arteries to 0.1 μM bradykinin induced B_2 receptor desensitization. The addition of 10 μM quinaprilat or Ang-(1-7) to the bath, at a time when bradykinin alone was no longer able to induce relaxation, fully restored the relaxant effects of bradykinin. AT_1 or AT_2 receptor blockade did not affect any of the observed effects of Ang-(1-7). In conclusion, Ang-(1-7), like quinaprilat and captopril, potentiates bradykinin by acting as an ACE inhibitor. Bradykinin potentiation is maximal when both the ACE C- and N-terminal domain are inhibited. The inhibitory effects of Ang-(1-7) are limited to the ACE C-domain, raising the possibility that Ang-(1-7) synergistically increases the blood pressure lowering effects of N-domain-specific ACE inhibitor.

Based on: Tom B., de Vries R., Saxena P.R. and Danser A.H.J., (2001). Bradykinin potentiation by angiotensin-(1-7) and ACE inhibitors correlates with ACE C- and N-domain blockade, *Hypertension,* **38: 95-99.**

4.1. Introduction

Ang-(1-7) is a heptapeptide that is formed endogenously from both Ang I and Ang II (Ferrario *et al.*, 1997). In rats and dogs, Ang-(1-7) exerts direct vasodilatory effects via non-AT_1, non-AT_2 receptors, possibly by stimulating bradykinin and NO release (Brosnihan *et al.*, 1996; Ferrario *et al.*, 1997). In contrast, in humans or pigs, no direct vasodilatory effects of Ang-(1-7) were observed (Davie *et al.*, 1999; Gorelik *et al.*, 1998; Pörsti *et al.*, 1996; Roks *et al.*, 1999; Ueda *et al.*, 2000), although Ang-(1-7) did antagonize the pressor effects of Ang II, suggesting that it may cause vasodilation indirectly, by acting as an AT_1 receptor antagonist (Roks *et al.*, 1999; Ueda *et al.*, 2000). In addition, Ang-(1-7) potentiates bradykinin, either via an AT_2 receptor-dependent mechanism, or through inhibition of ACE (Deddish *et al.*, 1998; Gorelik *et al.*, 1998; Roks *et al.*, 1999). The latter effect is not necessarily based on blockade of bradykinin hydrolysis, since recent studies have shown that ACE inhibitors, including Ang-(1-7), potentiate bradykinin by inhibiting desensitization of its receptor (Benzing *et al.*, 1999; Danser *et al.*, 2000; Minshall *et al.*, 1997b). Somatic ACE has two homologous domains, each containing an active center. According to their position (N- or C-terminal), these domains are designated as the N- and C-domain, respectively. Interestingly, Ang-(1-7) inhibits the C-domain more potently than the N-domain (by one order of magnitude) (Deddish *et al.*, 1998), and is cleaved to Ang-(1-5) by the N-domain (Chappell *et al.*, 1998).

In the present study, we set out to investigate the bradykinin-potentiating effects of Ang-(1-7) in porcine coronary arteries (PCAs), its dependency on ACE, and the possible involvement of AT_1 and/or AT_2 receptors. The effects of Ang-(1-7) were compared with those of quinaprilat and captopril, two ACE inhibitors with preference for the ACE C- and N-domain, respectively (Michaud *et al.*, 1997; Perich *et al.*, 1994). We also verified the effect of Ang-(1-7) in human coronary arteries (HCAs).

4.2. Methods

Tissue collection

HCAs were obtained from 4 ‘heart beating’ organ donors (2 men and 2 women; age 14-38 years, mean±S.E.M. 23±5 years) who died of non-cardiac causes

(1 subarachnoidal bleeding, 3 head trauma) less than 24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank (Bio Implant Services/Eurotransplant Foundation) after removal of the aortic and pulmonary valves for transplantation purposes. The study was approved by the joint Ethics Committee of the Erasmus University Rotterdam and the University Hospital Rotterdam. Immediately after circulatory arrest, the hearts were stored in an ice-cooled sterile organ-protecting solution. After arrival in the laboratory, the HCA was removed and stored overnight in a 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. Vessels 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. Segments containing macroscopically visible atherosclerotic lesions were not used.

PCAs were obtained from 32 2-3 month-old pigs (Yorkshire x Landrace, weight 10-15 kg). The pigs had been used in *in-vivo* experiments studying the effects of α-adrenoceptor and serotonin receptor agonists and antagonists under pentobarbital (600 mg, i.v.) anesthesia (de Vries *et al.*, 1999). The Ethics Committee of the Erasmus University Rotterdam dealing with the use of animals for scientific experiments approved the protocol for this investigation. Hearts were explanted at the end of the experiment, and the coronary arteries were removed immediately and handled in the same way as HCAs.

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 mM KCl twice. The functional integrity of the endothelium was verified by observing relaxation to 1 nM substance P after preconstriction with 1 μM $PGF_{2\alpha}$ (Maassen van den Brink *et al.*, 1999). Subsequently, the tissue was exposed to 100 mM KCl to determine the maximal contractile response to KCl. The segments

were then allowed to equilibrate in fresh organ bath fluid for 30 min., thereafter, the following experiments were performed.

First, Ang-(1-7) concentration response curves (CRCs) (0.1 nM-10 μM) were constructed in HCAs and PCAs, both at baseline and after preconstriction with 10 μM $PGF_{2\alpha}$, in the absence or presence of the AT_1 receptor antagonist irbesartan (1 μM) or the AT_2 receptor antagonist PD123319 (1 μM).

Second, the effects of Ang-(1-7), quinaprilat and captopril on bradykinin-induced vasorelaxation were studied in PCAs. Vessels were pre-incubated for 30 min in the absence or presence of Ang-(1-7) (10 pM-10 μM), quinaprilat (0.1 pM-0.1 mM), captopril (0.1 pM-0.1 mM), 10 μM Ang-(1-7) + 1 μM irbesartan, 10 μM Ang-(1-7) + 1 μM PD123319, 10 μM Ang-(1-7) + 0.1 nM quinaprilat, or 10 μM Ang-(1-7) + 10 μM quinaprilat. Vessels were then preconstricted with 10 μM $PGF_{2\alpha}$ or 1 μM U46619 and CRCs to bradykinin (0.1 nM-1 μM) were constructed.

Third, the effect of quinaprilat and Ang-(1-7) on desensitized B_2 receptors was studied in PCAs. Vessels were pre-incubated for 30 min with or without 1 μM irbesartan or 1 μM PD123319. Vessels were then preconstricted with 10 μM $PGF_{2\alpha}$ and exposed three times to a concentration of bradykinin (0.1 μM) that is capable of inducing maximal relaxation. Each next exposure was started as soon as the effect of the previous exposure had disappeared, i.e., after approximately 15 min. After the third exposure, when bradykinin no longer exerted a vasodilatory effect, quinaprilat (10 μM) or Ang-(1-7) (10 μM) was added to the organ bath. Thereafter, when the effects of quinaprilat and Ang-(1-7) had disappeared, a fourth bradykinin dose (0.1 μM) was added to the organ bath.

Statistical analysis

Data are given as mean±S.E.M. and expressed as a percentage of the contraction in response to $PGF_{2\alpha}$ or U46619. CRCs were analyzed using the logistic function described by de Lean *et al.* (de Lean *et al.*, 1978) to obtain pEC_{50} ($-^{10}\log EC_{50}$) values, EC_{50} representing the concentration at which 50% of the maximal relaxant effect has been reached. Statistical analysis was by ANOVA, followed by post hoc evaluation

(according to Tukey or Dunnett where appropriate). P values <0.05 were considered significant.

4.3. Results

Effect of Ang-(1-7) on human and porcine coronary arteries

Ang-(1-7), at concentrations up to 10 μM, did not exert a response in HCAs (n=4) and PCAs (n=5-12), neither at baseline nor after preconstriction with $PGF_{2\alpha}$, nor in the presence of irbesartan or PD123319.

Effects of Ang-(1-7), quinaprilat and captopril on bradykinin-induced relaxation in porcine coronary arteries

Bradykinin caused complete relaxation of preconstricted PCAs in a concentration dependent-manner (pEC_{50}=8.11±0.07, n=12; Figure 1, left panel). Quinaprilat (10 μM) and captopril (10 μM), like Ang-(1-7) at this concentration, did not exert any effect on preconstricted PCAs. However, in the presence 10 μM Ang-(1-7), the bradykinin CRC was shifted ≈5-fold to the left (pEC_{50} 8.72±0.09, n=12; P<0.05 vs. control), while in the presence of 10 μM quinaprilat or 10 μM captopril the bradykinin CRC was shifted ≈10-fold to the left (pEC_{50} 9.03±0.21 and 8.91±0.09, respectively, n=8; P<0.01 vs. control) (Figure 1, left panel). The effect of Ang-(1-7) on the bradykinin CRC was not affected by irbesartan or PD123319 (Figure 1, right panel).

To study the concentration-dependency of the Ang-(1-7)- and ACE-inhibitor-induced leftward shifts, bradykinin CRCs were also constructed in the presence of a wide range of Ang-(1-7), quinaprilat and captopril concentrations (Figure 2). The leftward shift caused by quinaprilat occurred in a biphasic manner, with a 5-fold shift at concentrations in the subnanomolar range, and a 10-fold shift at concentrations above 1 nM. This biphasic shift is in agreement with earlier studies (Perich *et al.*, 1994) demonstrating different quinaprilat binding affinities of the ACE C- (K_d=7 pM) and N-domain (K_d=1267 pM), and suggests that the 5- and 10-fold shifts represent C-domain inhibition and complete (i.e., C- plus N-domain) ACE inhibition, respectively.

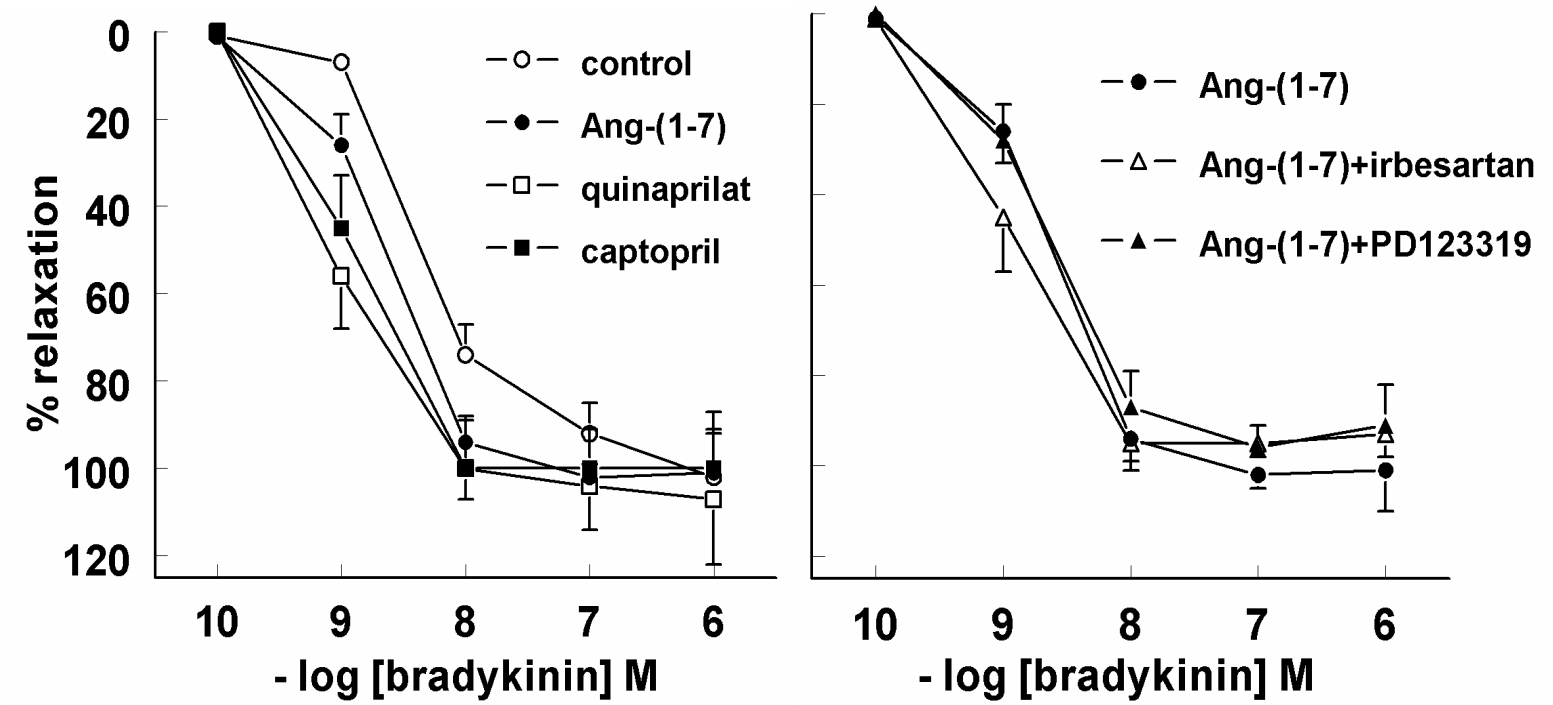


Figure 1. *Left panel: Relaxations of porcine coronary arteries preconstricted with 10 μM $PGF_{2\alpha}$ or 1 μM U46619, to bradykinin in the absence (control) or presence of 10 μM Ang-(1-7), 10 μM quinaprilat or 10 μM captopril. Right panel: The effect of Ang-(1-7) on bradykinin-induced relaxations was also studied in the presence of 1 μM irbesartan or 1 μM PD123319. Data (mean±S.E.M. of 5-12 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$ or U46619. Ang-(1-7), quinaprilat and captopril significantly shifted the bradykinin concentration response curve to the left (P<0.05 vs. control for Ang-(1-7), P<0.01 vs. control for the two ACE inhibitors), and the effect of Ang-(1-7) was not influenced (P=NS) by the presence of irbesartan or PD123319.*

The 10-fold leftward shift caused by captopril occurred monophasically (Figure 2), suggesting that captopril is bound with similar affinity by the two ACE domains. The 5-fold leftward shift caused by Ang-(1-7) also occurred monophasically (Figure 2), suggesting either that Ang-(1-7) inhibits one ACE domain only, or that Ang-(1-7) potentiates bradykinin independently of its effects on ACE. To sort out the latter, the effect of 10 μM Ang-(1-7) on top of quinaprilat, either at a concentration that selectively inhibits the ACE C-domain (0.1 nM) or at a concentration that inhibits both ACE domains (10 μM), was studied.

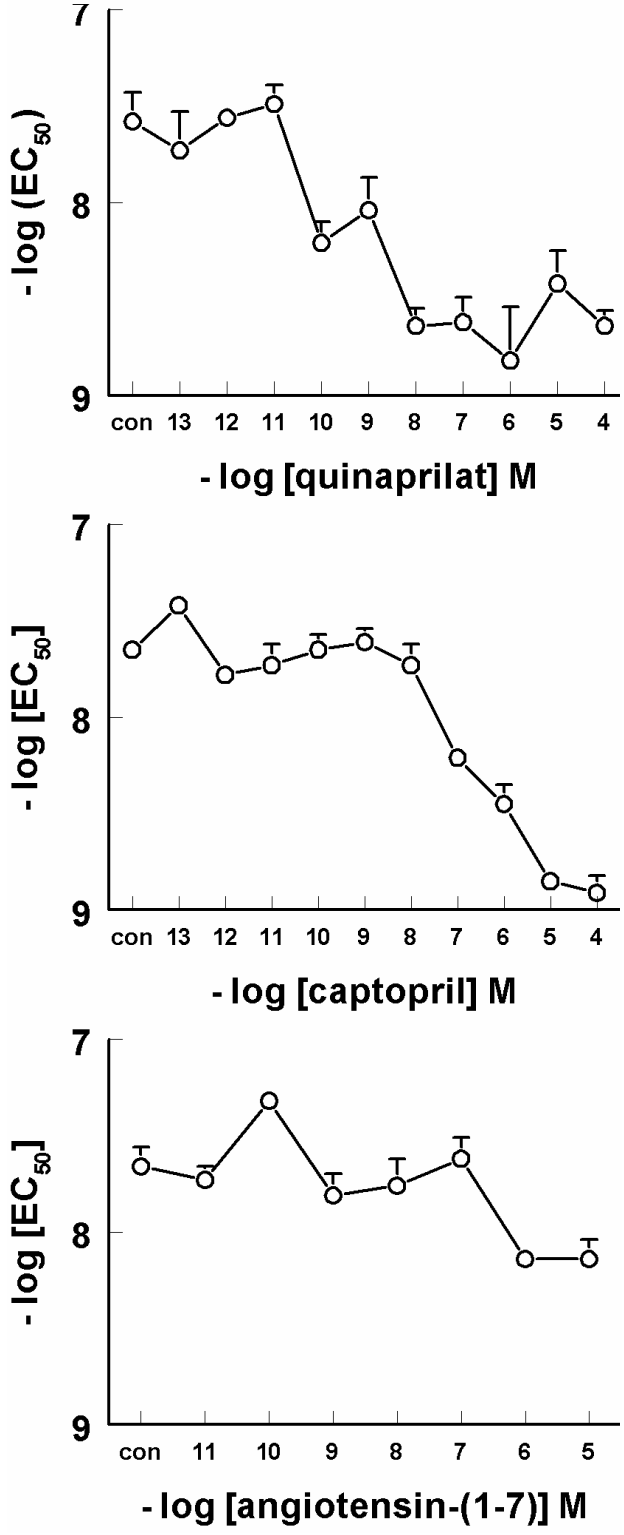


Figure 2. *Change in -log(EC_{50}) of the bradykinin concentration response curve in the presence of increasing concentrations of quinaprilat (top panel), captopril (middle panel) or Ang-(1-7) (bottom panel). An increase in -log(EC_{50}) represents a leftward shift of the bradykinin concentration response curve. Data (mean±S.E.M. of 6-8 experiments) were obtained in porcine coronary arteries preconstricted with 1 μM U46619. Con, control. Significant differences ($P<0.05$) vs. control were obtained for quinaprilat, captopril and Ang-(1-7) at concentrations ≥ 0.1, 100 and 1000 nM, respectively.*

Ang-(1-7) affected neither the 5-fold leftward shift of the bradykinin CRC at 0.1 nM quinaprilat (pEC_{50} 8.59±0.39 and 8.68±0.29 with and without Ang-(1-7), n=13; P=NS) nor the 10-fold shift at 10 μM quinaprilat (pEC_{50} 9.06±0.20 and 9.03±0.21 with and without Ang-(1-7), n=11; P=NS) (Figure 3). Most likely therefore, Ang-(1-7), at concentrations up to 10 μM, acts as a selective inhibitor of the ACE C-domain.

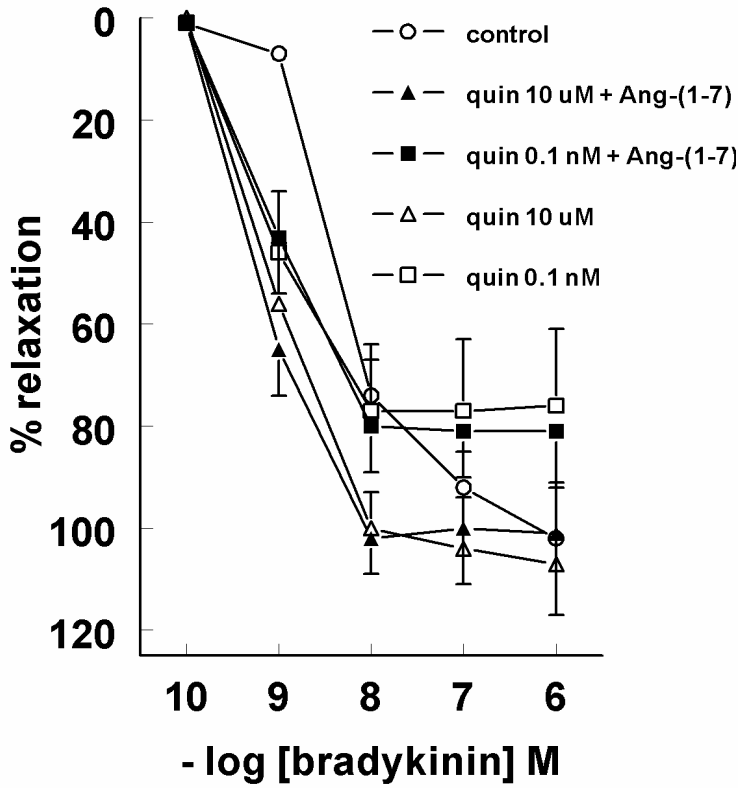


Figure 3. *Relaxations of porcine coronary arteries preconstricted with 10 μM $PGF_{2\alpha}$ or 1 μM U46619, to bradykinin in the absence (control) or presence of 0.1 nM quinaprilat or 10 μM quinaprilat, with or without 10 μM Ang-(1-7). Data (mean± S.E.M. of 11-13 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$ or U46619. The addition of Ang-(1-7) on top of quinaprilat did not have a significant effect.*

Effect of Ang-(1-7) and quinaprilat on desensitized B_2 receptors in porcine coronary arteries

Repeated exposure of preconstricted vessel segments to 0.1 μM bradykinin produced progressively smaller responses (Figure 4, top panel). The response to the third bradykinin dose was less than 50% of the response to the first bradykinin dose.

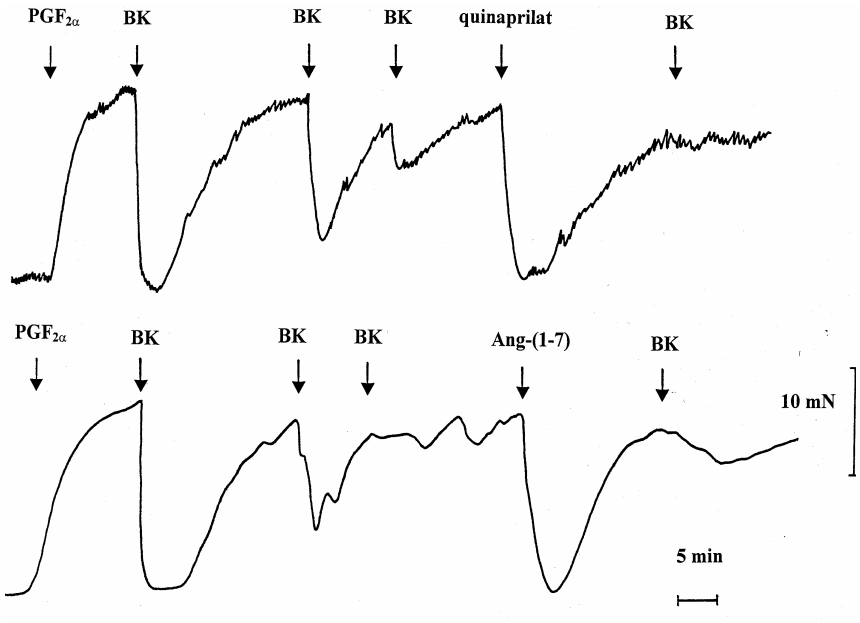
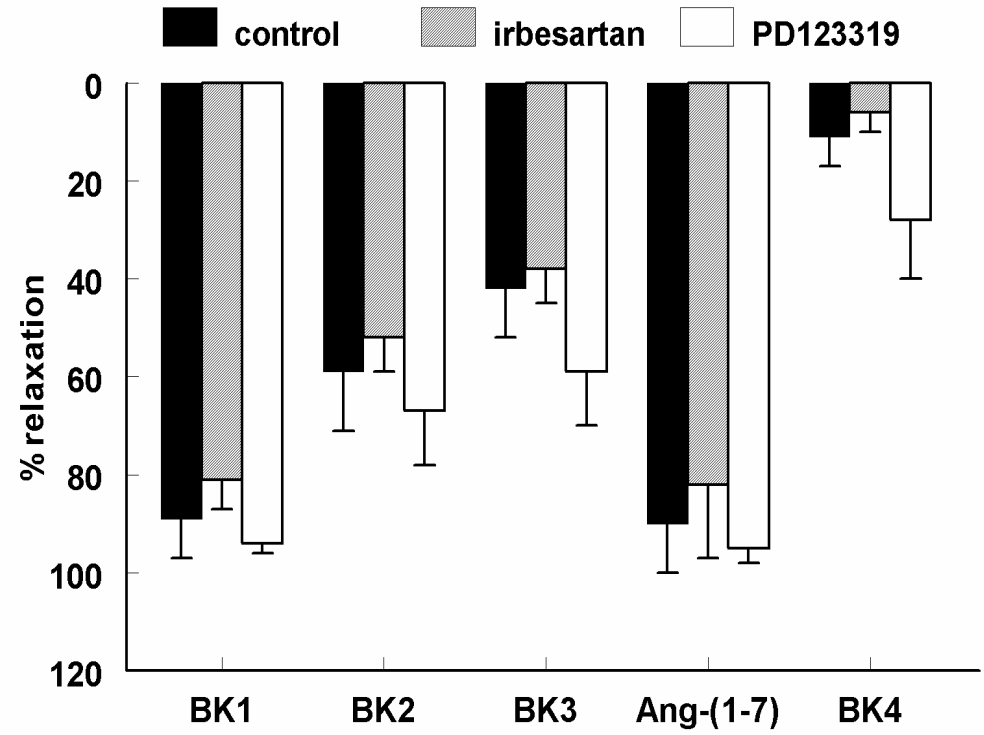


Figure 4.

Top panel: Original tracings of an experiment in which a porcine coronary artery, following preconstriction with 10 μM $PGF_{2\alpha}$, is subsequently exposed to 0.1 μM bradykinin (BK, three times), 10 μM quinaprilat (top) or 10 μM Ang-(1-7) (bottom), and 0.1 μM bradykinin.



Bottom panel: Relaxations of porcine coronary arteries, following preconstriction with 10 μM $PGF_{2\alpha}$, to three consecutive bradykinin doses (0.1 μM; BK1, BK2, BK3), 10 μM Ang-(1-7), and a fourth bradykinin dose (0.1 μM; BK4) in the absence or presence of 1 μM irbesartan or 1 μM PD123319. Data (mean±S.E.M. of 4-8 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$.

Quinaprilat or Ang-(1-7), added to the organ bath after the effect of the third bradykinin dose had disappeared, both at a concentration of 10 μM, completely restored the relaxant effect of bradykinin (90±10% and 83±8% relaxation, respectively, n=8; P=NS). A fourth bradykinin dose, added after the effect of quinaprilat or Ang-(1-7) had disappeared, induced no further effect. The effect of Ang-(1-7) was not different in the presence of irbesartan or PD123319 (Figure 4, bottom panel).

4.4. Discussion

The present study shows that Ang-(1-7) potentiates the vasodilator effects of bradykinin in PCAs through inhibition of the ACE C-domain. AT_1 or AT_2 receptors are not involved in this effect, nor did Ang-(1-7) exert direct (i.e., independently of bradykinin) relaxant effects in either HCAs or PCAs. Furthermore, the maximal potentiating effect of Ang-(1-7) was smaller than that of the ACE inhibitors quinaprilat and captopril, suggesting that full potentiation will only be obtained when both ACE domains are inhibited.

The concentrations of Ang-(1-7) required to obtain ACE C-domain inhibition (≥1 μM) are in agreement with previously reported values (Deddish *et al.*, 1998; Roks *et al.*, 1999). These concentrations exceed the *in-vivo* tissue and plasma Ang-(1-7) concentrations in rats and humans by at least four orders of magnitude. Ang-(1-7) levels increase during ACE inhibition (Campbell *et al.*, 1993b; 1999; Lawrence *et al.*, 1990; Yamada *et al.*, 1998), because ACE inhibitors block the ACE N-domain-mediated degradation of Ang-(1-7) (Deddish *et al.*, 1998; Ferrario *et al.*, 1997). However, the fact that most ACE inhibitors also block the ACE C-domain will mask any potential C-domain-blocking effect of the high Ang-(1-7) levels during ACE inhibition. Indeed, Ang-(1-7) did not enhance bradykinin-induced vasodilation in the forearm in human subjects treated with an ACE inhibitor (Davie *et al.*, 1999). Therefore, the effects of Ang-(1-7) described in the present study may only be of physiological importance if sufficiently high concentrations of Ang-(1-7) are reached in the immediate vicinity of ACE (the levels measured per g tissue do not exclude this possibility) and/or in combination with ACE inhibitors that specifically block the ACE N-domain.

Studies with the N-domain-specific substrate *N*-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) have revealed that the ACE inhibitor captopril inhibits AcSDKP hydrolysis 16 times more potently than Ang I hydrolysis (Michaud *et al.*, 1997). Since the ACE N- and C-domains exhibit similar catalytic activities towards Ang I (Jaspard *et al.*, 1993), these data suggest that captopril, at low concentrations, preferentially inhibits the ACE N-domain. Using captopril, however, we were unable to make a clear distinction between the effects of ACE N- and C-domain inhibition on the relaxant effects of bradykinin. In contrast, using the ACE inhibitor quinaprilat, which binds to the ACE C-domain with almost 200-fold greater affinity than to the ACE N-domain (Perich *et al.*, 1994), we could make such a distinction: quinaprilat shifted the bradykinin CRC to the left in a biphasic manner, with a ≈5-fold shift occurring at concentrations that selectively block the C-domain, and a ≈10-fold shift at concentrations that block both domains. Our inability to find a similar biphasic shift in the presence of increasing concentrations of captopril most likely relates to the limited selectivity of this ACE inhibitor for the ACE N-domain.

Ang-(1-7), like captopril, induced a monophasic leftward shift of the bradykinin CRC. At the highest Ang-(1-7) concentration that was tested (10 μM), however, the shift was only half of that caused by similar concentrations of quinaprilat or captopril. Adding quinaprilat, at a concentration that selectively blocks the ACE C-domain, to 10 μM Ang-(1-7) did not cause a further leftward shift, thereby demonstrating that Ang-(1-7), at least at this concentration, blocks the ACE C-domain only. Since a concentration of 10 μM is already many orders of magnitude above the measured concentrations of Ang-(1-7) *in-vivo* (Campbell *et al.*, 1993b; 1999; Lawrence *et al.*, 1990; Yamada *et al.*, 1998), we did not evaluate whether even higher concentrations of Ang-(1-7) resulted in ACE N-domain inhibition.

Does ACE inhibition result in bradykinin potentiation by blocking its hydrolysis? Since bradykinin, like Ang I, is cleaved equally well by the two ACE domains (Jaspard *et al.*, 1993), our finding that blockade of both domains results in a twice as large leftward shift as blockade of one domain, appears to support this possibility. However, the long half life of bradykinin in this preparation (30 minutes) (Gorelik *et al.*, 1998), as well as our previous finding in PCAs that the quinaprilat-induced leftward shift of the ACE-resistant bradykinin analogue [Hyp^3-$Tyr(Me)^8$]-

bradykinin was not different from that of bradykinin (Danser *et al.*, 2000), argue against this concept. Moreover, under conditions of B_2 receptor desensitization (induced by repeated exposure to a concentration of bradykinin that causes maximal relaxation), when bradykinin itself was no longer active, both quinaprilat and Ang-(1-7) immediately restored the effect of bradykinin. Taken together therefore, a more likely explanation for the Ang-(1-7)-, quinaprilat- and captopril-induced bradykinin potentiation is the upregulation (or resensitization) of B_2 receptors that has recently been described in CHO cells transfected with the human B_2 receptor and human ACE (Deddish *et al.*, 1998; Marcic *et al.*, 1999; 2000a; Minshall *et al.*, 1997b). This effect only occurs when ACE and B_2 receptors are sterically closely associated, probably forming a heterodimer (Marcic *et al.*, 2000a). ACE inhibitors are believed to alter the heterodimer interaction, thereby promoting a conformation in the B_2 receptor that affects its sequestration and coupling to second messengers (Marcic *et al.*, 2000a). Our findings in intact PCAs show that ACE-B_2 receptor crosstalk is not limited to transfected CHO cells, and, in addition, suggest that the conformational changes underlying bradykinin potentiation correlate directly with inhibition of the ACE C- and N-domain.

CHAPTER 5
Bradykinin Potentiation by ACE Inhibitors: A Matter of Metabolism

Summary

Studies in isolated cells overexpressing ACE and B_2 receptors suggest that ACE inhibitors potentiate bradykinin by inhibiting B_2 receptor desensitization, via a mechanism involving protein kinase C and phosphatases. Here we investigated, in intact porcine coronary arteries, endothelial ACE/B_2 receptor 'crosstalk' as well as bradykinin potentiation through neutral endopeptidase (NEP) inhibition. NEP inhibition with phosphoramidon did not affect the bradykinin concentration response curve, nor did combined NEP/ACE inhibition with omapatrilat exert a further leftward shift on top of the ≈10-fold leftward shift of the bradykinin concentration response curve observed with ACE inhibition alone. In arteries that, following repeated exposure to 0.1 μM bradykinin, no longer responded to bradykinin ('desensitized' arteries), the ACE inhibitors quinaprilat and Ang-(1-7) both induced complete relaxation, without affecting the organ bath fluid levels of bradykinin. This phenomenon was unaffected by inhibition of protein kinase C or phosphatases (with calphostin C and okadaic acid, respectively). When using bradykinin analogues that were either completely or largely ACE-resistant ([$Phe^8 \Psi(CH_2\text{-}NH)Arg^9$]-bradykinin and [$\Delta Phe^5$]-bradykinin, respectively), the ACE inhibitor-induced shift of the bradykinin concentration response curve was absent, and its ability to reverse desensitization was absent or significantly reduced, respectively. Caveolar disruption with filipin did not affect the quinaprilat-induced effects. Filipin did however reduce the bradykinin-induced relaxation by ≈25-30%, thereby confirming that B_2 receptor-eNOS interaction occurs in caveolae. In conclusion, in porcine arteries, in contrast to transfected cells, bradykinin potentiation by ACE inhibitors is a metabolic process, that can only be explained on the basis of ACE-B_2 receptor co-localization on the endothelial cell membrane. NEP does not appear to affect the bradykinin levels in close proximity to B_2 receptors, and the ACE inhibitor-induced bradykinin potentiation precedes B_2 receptor coupling to eNOS in caveolae.

Based on: Tom B., Dendorfer A., de Vries R., Saxena P.R. and Danser A.H.J., (2002). Bradykinin potentiation by ACE inhibitors: A matter of metabolism, *Br. J. Pharmacol.,* **137:276-284**.

5.1. Introduction

Bradykinin accumulation is believed to contribute to the beneficial effects of ACE inhibitors in hypertension and heart failure, although elevated bradykinin levels have not always been found during ACE inhibitor treatment (Campbell *et al.*, 1999). Recent studies in isolated cells propose that ACE inhibitors potentiate bradykinin beyond blocking its hydrolysis, by inhibiting desensitization of its receptor (Benzing *et al.*, 1999; Minshall *et al.*, 1997b). The mechanism behind this phenomenon is currently unclear, but may involve 'crosstalk' between ACE and B_2 receptors (Benzing *et al.*, 1999; Marcic *et al.*, 1999; 2000a; 2000b; Minshall *et al.*, 1997b). Although these findings were subsequently confirmed in intact coronary arteries (Danser *et al.*, 2000; Mombouli *et al.*, 2002; Tom *et al.*, 2001a), it has recently been proposed, on the basis of experiments performed in isolated perfused rat Langendorff hearts (Dendorfer *et al.*, 2000) and rabbit jugular veins (Dendorfer *et al.*, 2001a) that inhibition of ACE in the immediate vicinity of B_2 receptors (e.g., in caveolae) is a more likely explanation for the potentiation of bradykinin by ACE inhibitors than a direct interaction between ACE and B_2 receptors. In support of this concept, aminopeptidase P inhibition resulted in a similar leftward shift of the bradykinin concentration response curve (CRC) as ACE inhibition (Dendorfer *et al.*, 2000), and no ACE inhibitor-induced leftward shift was observed when studying B_2 receptor-mediated vasoconstriction in response to ACE-resistant bradykinin analogues (Dendorfer *et al.*, 2001a; Gobeil *et al.*, 2002). However, the latter data were obtained in endothelium-denuded vessels, i.e. a preparation that lacks the B_2 receptor- and ACE-expressing endothelial cells that are responsible for the ACE inhibitor-induced potentiation of B_2 receptor-mediated coronary vasorelaxation (Danser *et al.*, 2000; Mombouli *et al.*, 2002; Tom *et al.*, 2001a).

Neutral endopeptidase 24.11 (NEP) is a membrane-bound metalloprotease with a catalytic unit similar to that of ACE. It is widely distributed in endothelial and vascular smooth muscle cells (Gonzalez *et al.*, 1998; Graf *et al.*, 1993; Llorens-Cortes *et al.*, 1992; Soleilhac *et al.*, 1992; Wang *et al.*, 1994). NEP catalyzes the degradation of a number of endogenous vasoactive peptides, including bradykinin and angiotensin (Blais *et al.*, 2000; Graf *et al.*, 1993; Kokkonen *et al.*, 1999; Raut *et al.*, 1999). Recent studies have shown that combined inhibition of ACE and NEP with so-called

vasopeptidase inhibitors is more cardioprotective than ACE inhibition alone (d'Uscio *et al.*, 2001; Rouleau *et al.*, 2000).

It was the aim of the present study to further investigate the possibility that the ACE inhibitor-induced potentiation of bradykinin in intact (i.e., endothelium-containing) porcine coronary arteries (PCAs) has a non-metabolic origin. First, we studied whether bradykinin potentiation also occurs with NEP inhibitors, and whether dual ACE/NEP inhibition results in even further potentiation of bradykinin. Second, we studied the effect of protein kinase C (PKC) and phosphatase inhibition (with calphostin C and okadaic acid, respectively) on the ACE inhibitor-induced bradykinin potentiation, since such inhibition was found to fully eliminate the ACE/B_2 receptor crosstalk in CHO cells (Marcic *et al.*, 2000b). Third, we studied whether potentiation occurs when using truly ACE-resistant bradykinin analogues. This is particularly important, since most studies that investigated ACE-B_2 receptor crosstalk so far made use of a bradykinin analogue ([Hyp^3-$Tyr(Me)^8$]-bradykinin) that was recently shown not to be ACE-resistant at all (Dendorfer *et al.*, 2001a; Gobeil *et al.*, 2002). Finally, in view of the possibility that ACE and B_2 receptors co-localize in caveolae, we studied the effect of caveolar disruption (with filipin, cyclodextrin or nystatin) on the ACE inhibitor-induced bradykinin potentiation.

5.2. Methods

Chemicals

Ang-(1-7), bradykinin, calphostin C, captopril, cyclodextrin, 9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619), filipin, S-nitroso-N-acetylpenicillamine (SNAP), nystatin, okadaic acid, phosphoramidon, and substance P were purchased from Sigma (Zwijndrecht, The Netherlands). Quinaprilat was a kind gift of dr. H. van Ingen, Parke-Davis, Hoofddorp, The Netherlands. Omapatrilat was a kind gift of dr. N.C. Trippodo, Bristol-Myers Squibb, Princeton, NJ, USA. [$Phe^8\Psi(CH_2\text{-}NH)Arg^9$]-bradykinin (PA-bradykinin) was bought from Calbiochem (Breda, The Netherlands). [ΔPhe^5]-bradykinin (DP-bradykinin) was synthesized by a solid-phase method by means of the Boc-strategy (Reissmann *et al.*, 1996). All chemical were dissolved in water, with the exception of calphostin C and phosphoramidon, which were dissolved in dimethylsulfoxide, and of filipin, which was dissolved in ethanol.

Tissue collection

PCAs were obtained from 5 2-3 month-old pigs (Yorkshire x Landrace, weight 10-15 kg) that had been used in *in-vivo* experiments studying the effects of α-adrenoceptor and calcitonin-gene related peptide receptor (ant)agonists or capsaicin under pentobarbital (600 mg, i.v.) anesthesia (Willems *et al.*, 2001), and from 38 pigs at the local slaughtherhouse. The Ethics Committee of the Erasmus MC dealing with the use of animals for scientific experiments approved the protocol for this investigation. Arteries were either removed at the end of the experiment, or after the heart from the slaughtherhouse had been brought to the laboratory in cold 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. Vessels were stored overnight in cold, oxygenated Krebs bicarbonate solution, 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.

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 mM KCl twice. The functional integrity of the endothelium was verified by observing relaxation to 1 nM substance P after preconstriction with 1 μM U46619. Subsequently, the tissue was exposed to 100 mM KCl to determine the maximal contractile response to KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min. Thereafter, the following experiments were performed.

First, vessels were preincubated for 30 min in the presence or absence of 10 μM quinaprilat (ACE inhibitor) and/or 10 μM captopril (ACE inhibitor), 10 μM omapatrilat (vasopeptidase inhibitor), 1 μM phosphoramidon (NEP inhibitor), 10 μM Ang-(1-7) (inhibitor of ACE C-domain) (Tom *et al.*, 2001a), 4 μg/mL filipin, 2% cyclodextrin, or 20 μg/mL nystatin. Next, vessels were preconstricted with 1 μM U46619, and CRCs were constructed to bradykinin, PA-bradykinin, or DP-bradykinin.

Second, vessels were pre-incubated for 30 min with or without 1 μM calphostin C, 0.5 μM okadaic acid, 4 μg/mL filipin, 2% cyclodextrin, or 20 μg/mL nystatin. Vessels were then preconstricted with 1 μM U46619 and exposed multiple times (to induce desensitization) to 0.1 μM bradykinin, 0.1 μM PA-bradykinin or 0.03 μM DP-bradykinin. Each next exposure was started as soon as the effect of the previous exposure had disappeared. When bradykinin no longer exerted a vasodilatory effect, 10 μM quinaprilat, 10 μM omapatrilat, or 1 μM phosphoramidon was added to the organ bath.

Finally, to study the metabolism of bradykinin under our experimental conditions, 1 mL fluid samples were taken from the organ bath at 0, 30, 60 and 120 minutes after the addition of the highest bradykinin concentration (1 μM) in the absence or presence of 10 μM quinaprilat. The samples were immediately supplemented with 1% trifluoroacetic acid, and stored until analysis at -70°C. Bradykinin was determined by high performance liquid chromatography and photometric detection at 210 nm (Dendorfer *et al.*, 1997a).

Statistical analysis

Data are given as mean±S.E.M. and expressed as a percentage of the contraction in response to U46619. Differences between arteries obtained from experimental pigs and from slaugtherhouse pigs were not observed (data not shown), and data for the two groups were therefore combined. CRCs were analyzed using the logistic function described by de Lean et al. (de Lean *et al.*, 1978) to obtain pEC_{50} ($-^{10}\log EC_{50}$) values, EC_{50} representing the concentration at which 50% of the maximal relaxant effect has been reached. Statistical analysis was performed by Analysis of Variance (ANOVA), followed by post hoc evaluation according to Dunnett. P values <0.05 were considered significant.

5.3. Results

Potentiation of bradykinin by inhibitors of ACE and/or NEP

Bradykinin relaxed preconstricted PCAs in a concentration-dependent manner (pEC_{50}=7.95±0.03, n=5; Figure 1). Quinaprilat and omapatrilat, but not

phosphoramidon, shifted the bradykinin CRC approximately 10-fold to the left (pEC_{50}'s respectively 9.22±0.05 (P<0.05 vs. control), 9.28±0.08 (P<0.05 vs. control) and 8.06±0.13, n=5 for each condition). These drugs were without effect in the absence of bradykinin, indicating that PCAs do not contain endogenous bradykinin.

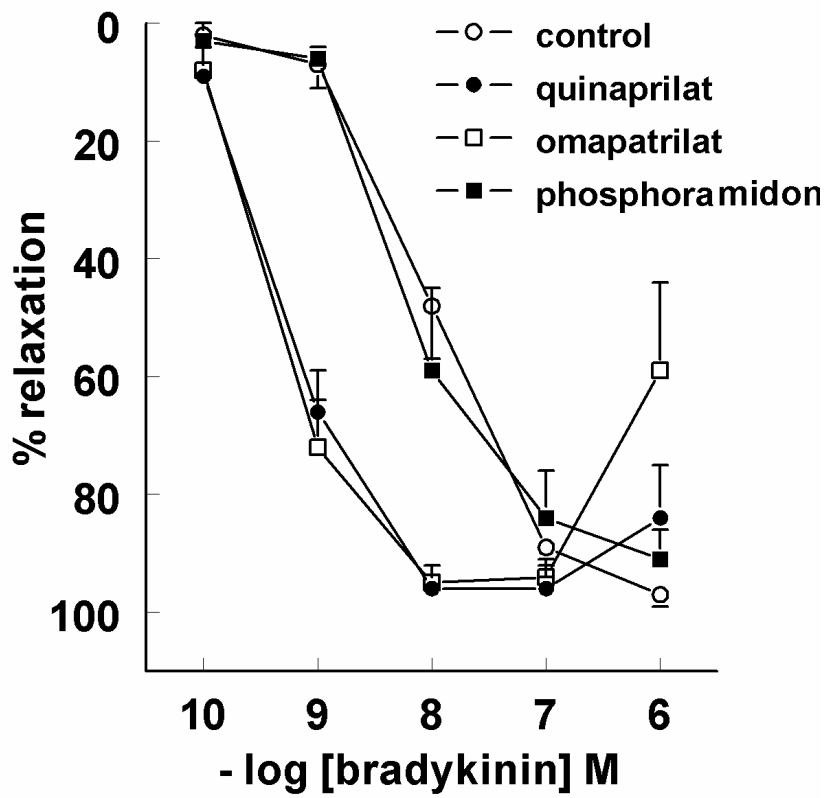


Figure 1. *Relaxations of porcine coronary arteries preconstricted with 1 μM U46619, to bradykinin in the absence (control) or presence of 10 μM quinaprilat, 10 μM omapatrilat or 1 μM phosphoramidon. Data (mean± S.E.M. of 5 experiments) are expressed as a percentage of the contraction induced by U46619.*

Repeated exposure of preconstricted PCAs to bradykinin produced progressively smaller responses (Figure 2, n=5). Its effects lasted less than 10 min, and contractility returned to preconstriction level within 15-20 min. The response to the third bradykinin dose was <50% of the response to the first bradykinin dose. Quinaprilat or omapatrilat, added to the organ bath after the effect of the third bradykinin dose had disappeared, completely restored the relaxant effect of bradykinin, whereas phosphoramidon was without effect (n=5 for each inhibitor). Bradykinin added to the organ bath after the effect of the enzyme inhibitors had disappeared exerted no effect (data not shown).

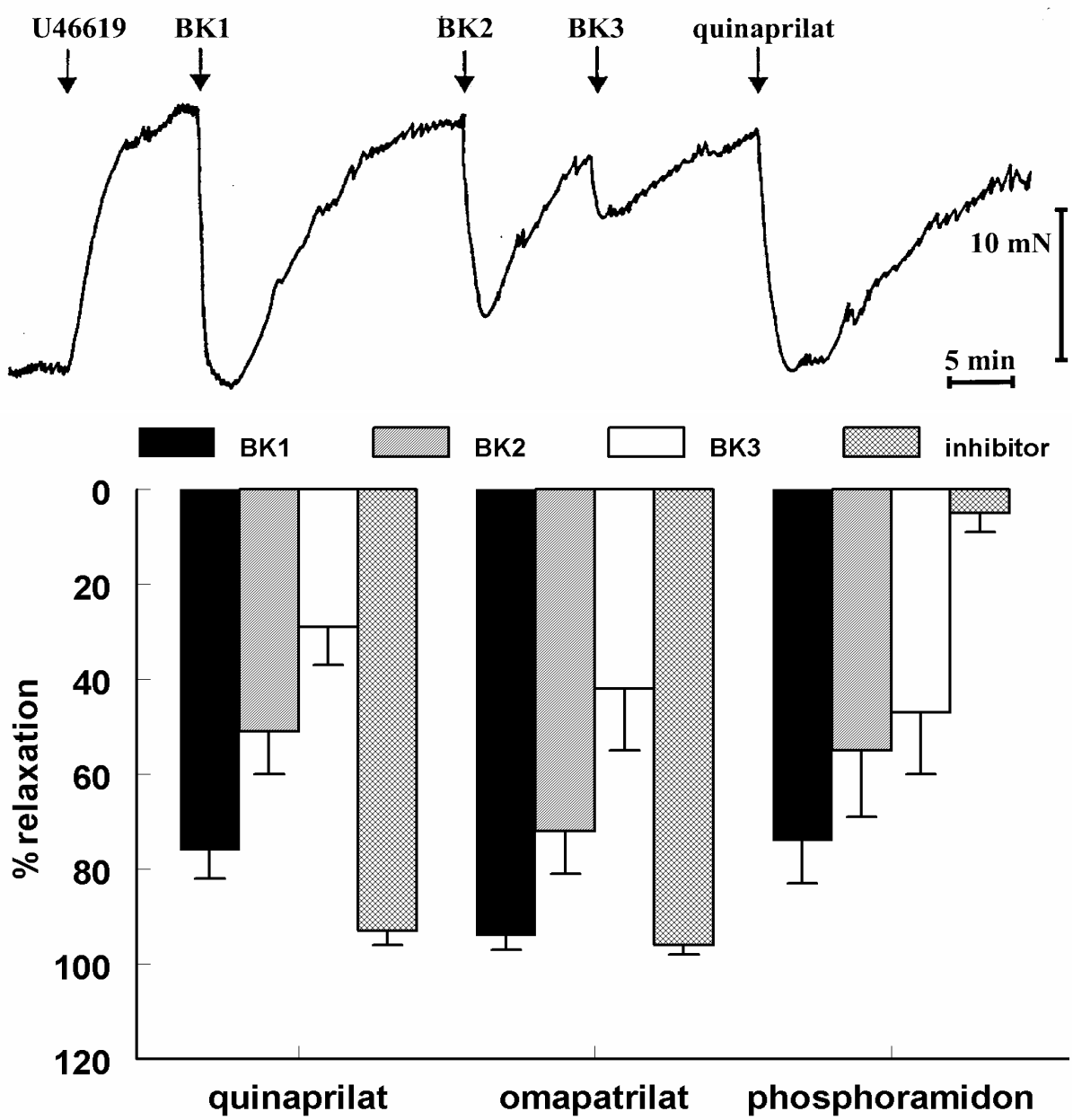


Figure 2. *Relaxations of porcine coronary arteries, following preconstriction with 1 μM U46619, to three consecutive bradykinin doses (0.1 μM; BK1, BK2, BK3), followed by 10 μM quinaprilat, 10 μM omapatrilat, or 1 μM phosphoramidon. Top panel: original tracing; Bottom panel: Mean±S.E.M. of 5 experiments (Data are expressed as a percentage of the contraction induced by U46619).*

Does inhibition of phosphatases or protein kinase C block the effect of quinaprilat in desensitized vessels?

The relaxant effect of quinaprilat in desensitized PCAs was not altered in the presence of calphostin C or okadaic acid (Figure 3).

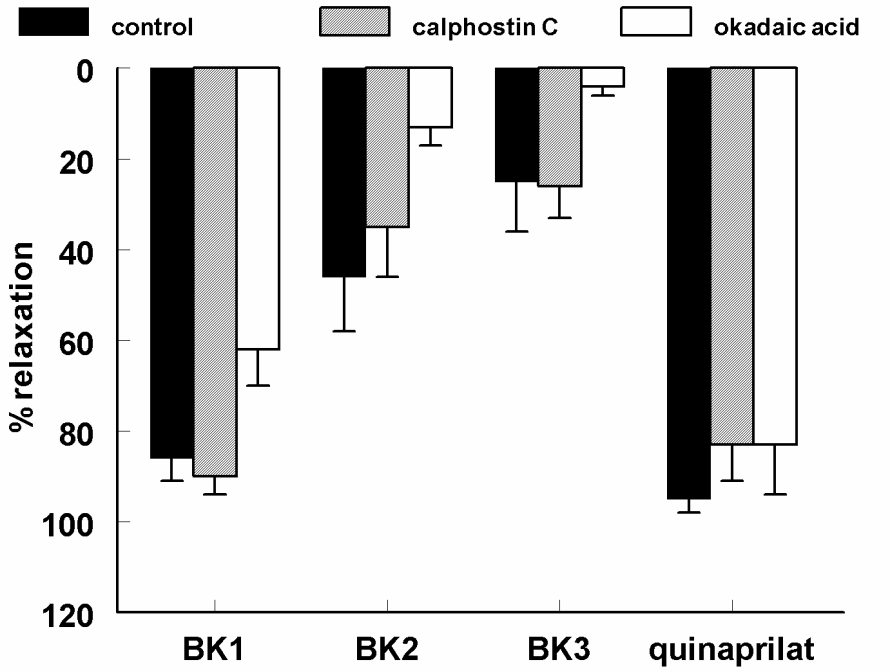


Figure 3. *Relaxations of porcine coronary arteries, following preconstriction with 1 μM U46619, to three consecutive bradykinin doses (0.1 μM; BK1, BK2, BK3), followed by 10 μM quinaprilat, in the absence (control) or presence of 1 μM calphostin C or 0.5 μM okadaic acid. Data (mean±S.E.M. of 5 experiments) are expressed as a percentage of the contraction induced by U46619.*

Do ACE inhibitors potentiate ACE-resistant bradykinin analogues?

In preconstricted PCAs, Ang-(1-7), like quinaprilat, shifted the bradykinin CRC to the left (Figure 4), although its effect was smaller than that of quinaprilat (pEC_{50}'s 8.40±0.09 (n=7) and 8.85±0.06 (n=23) vs. 7.94±0.05 for control (n=28); $P<0.05$). This

relates to the fact that Ang-(1-7) inhibits the ACE C-domain only, whereas quinaprilat inhibits both the C- and N-domain of ACE (Tom *et al.*, 2001a). PA-bradykinin (pEC_{50} 8.05±0.05; n=23) and DP-bradykinin (pEC_{50} 8.64±0.19; n=6) relaxed preconstricted PCAs to a similar degree as bradykinin (Figure 4). Their relaxant effects were not affected by either Ang-(1-7), quinaprilat, or captopril (Figure 4).

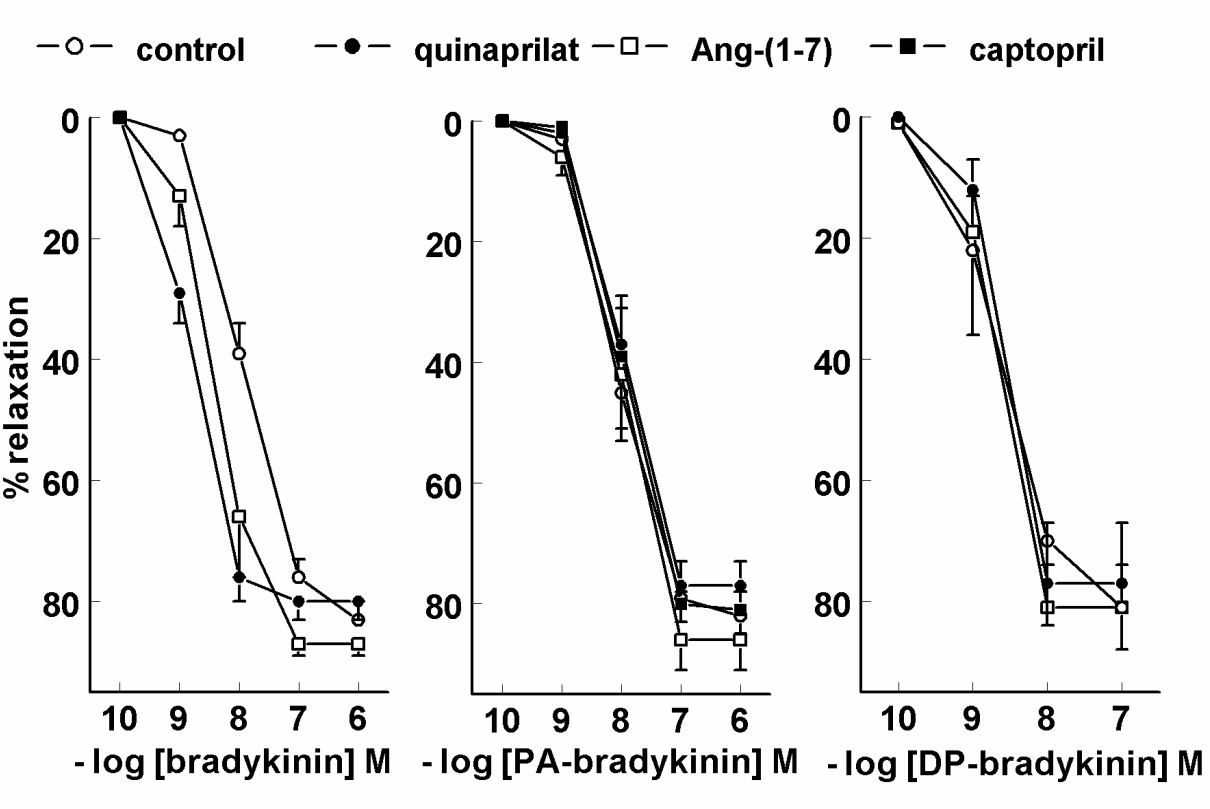


Figure 4. *Relaxations of porcine coronary arteries, preconstricted with 1 μM U46619, to bradykinin in the absence (control) or presence of 10 μM quinaprilat, 10 μM captopril or 10 μM Ang-(1-7). Data (mean±S.E.M. of 5-28 experiments) are expressed as a percentage of the contraction induced by U46619.*

Moreover, in the repeated exposure experiments, in contrast with bradykinin, PA-bradykinin induced relaxation only once, and the application of subsequent doses of PA-bradykinin (Figure 5), quinaprilat (Figure 5) or Ang-(1-7) (data not shown) exerted no effect. DP-bradykinin, like bradykinin, was capable of exerting multiple relaxations. However, the effect of quinaprilat in DP-bradykinin-desensitized vessels

was significantly smaller than in bradykinin-desensitized preparations (Figure 5). Finally, adding 10 µM bradykinin to vessels that no longer responded to 0.1 µM bradykinin induced a vasorelaxation (74±10%, n=5) that was as large as that induced by quinaprilat.

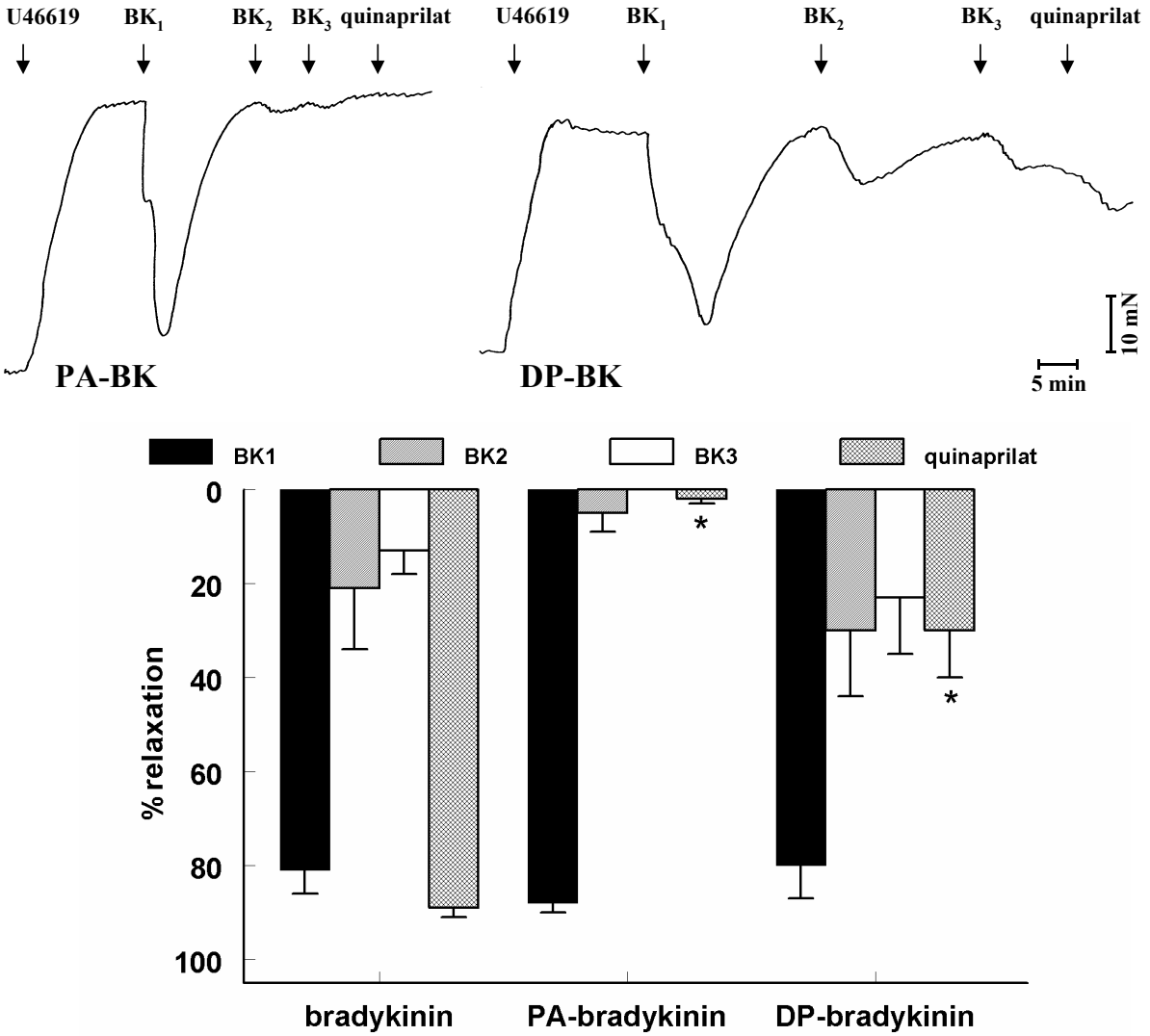


Figure 5. *Relaxations of porcine coronary arteries, following preconstriction with 1 µM U46619, to three consecutive bradykinin, PA-bradykinin or DP-bradykinin doses (0.1 µM, 0.1 µM and 0.03 µM, respectively; BK1, BK2, BK3), followed by 10 µM quinaprilat. Top panel: Original tracings of experiments with PA-bradykinin and DP-bradykinin. Bottom panel: Mean±S.E.M. of 5-12 experiments (Data are expressed as a percentage of the contraction induced by U46619). *P<0.01 vs bradykinin.*

Bradykinin metabolism during incubation with porcine coronary arteries

Bradykinin disappeared mono-exponentially from the organ bath fluid (Figure 6), with a half-life of 96±8 min (n=5). Quinaprilat marginally (P=NS) increased the half-life to 126±12 min.

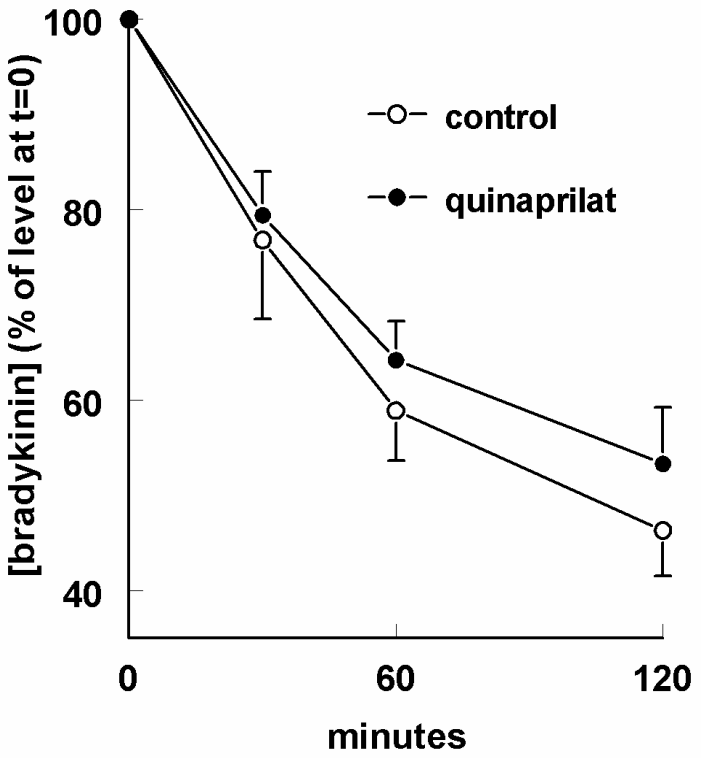


Figure 6. *Metabolism of bradykinin by porcine coronary artery rings during incubation of 15 mL organ bath fluid at 37°C with 1 μM bradykinin in the absence (control) or presence of 10 μM quinaprilat. Data are mean±S.E.M. of 5 experiments and have been expressed as a percentage of the level at t=0 (0.70±0.04 and 0.82±0.04 μM without and with quinaprilat).*

Effect of caveolar disruption on bradykinin potentiation by quinaprilat

Pretreatment with filipin reduced the maximal relaxant effect of bradykinin and PA-bradykinin by approximately 25-30% (Figure 7; $P<0.05$), but did not alter their potencies (7.63±0.19 (n=6) and 7.91±0.20 (n=7); $P>0.05$ vs. without filipin). Filipin did not affect the response to the endothelium-independent relaxant SNAP (Figure 8). Neither cyclodextrin, nor nystatin (20 μg/mL), affected the bradykinin, PA-bradykinin or SNAP CRCs, although there was a tendency for nystatin at this concentration to reduce (P=NS) the maximal relaxant effect of SNAP. At a concentration of 50 μg/mL, nystatin did significantly reduce the SNAP-induced relaxation (data not shown).

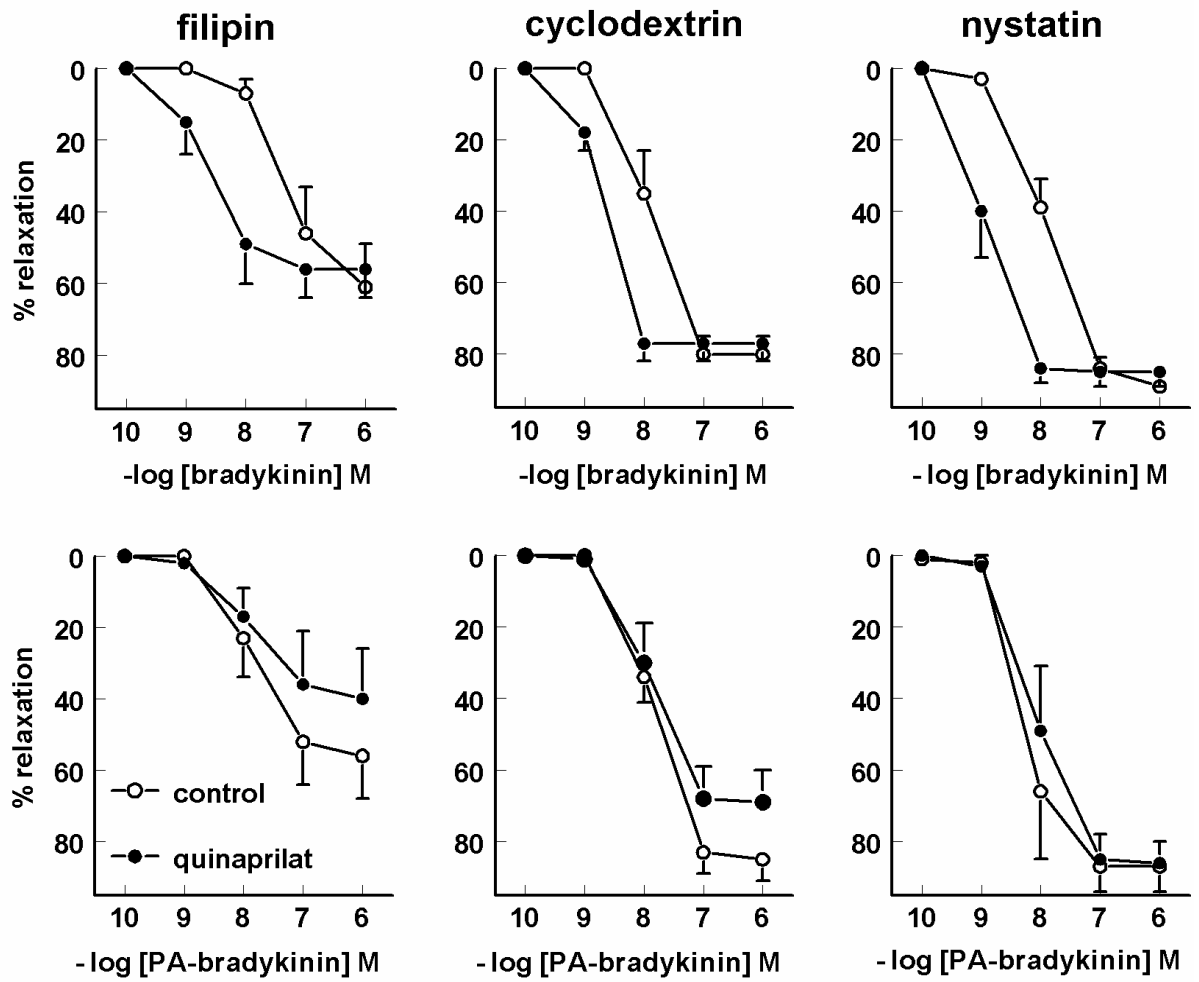


Figure 7. *Relaxations of porcine coronary arteries, preconstricted with 1 μM U46619, to bradykinin (top panels) or PA-bradykinin (bottom panels) in the absence (control) or presence of 10 μM quinaprilat, following pretreatment with 4 μg/mL filipin, 2% cyclodextrin or 20 μg/mL nystatin. Data (mean±S.E.M. of 4-10 experiments) are expressed as a percentage of the contraction induced by U46619.*

Quinaprilat caused a leftward shift of the bradykinin CRC in the presence of all caveolae-disrupting agents, and the shift was fully comparable to that observed in the absence of these agents (Figures 4 and 7). Quinaprilat did not affect the PA-bradykinin CRC in the presence of filipin, cyclodextrin and nystatin.

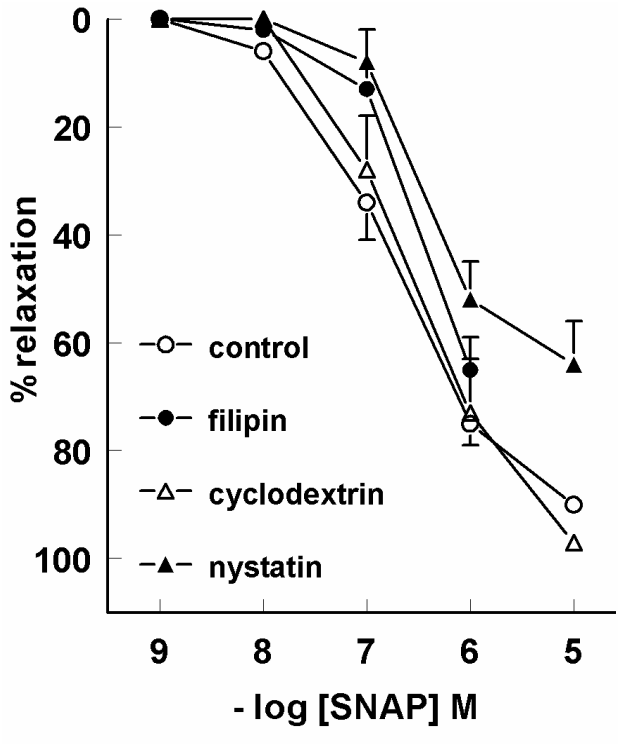


Figure 8. *Relaxations of porcine coronary arteries, preconstricted with 1 μM U46619, to SNAP in the absence (control) or presence of 4 μg/mL filipin, 2% cyclodextrin or 20 μg/mL nystatin. Data (mean±S.E.M. of 5-21 experiments) are expressed as a percentage of the contraction induced by U46619.*

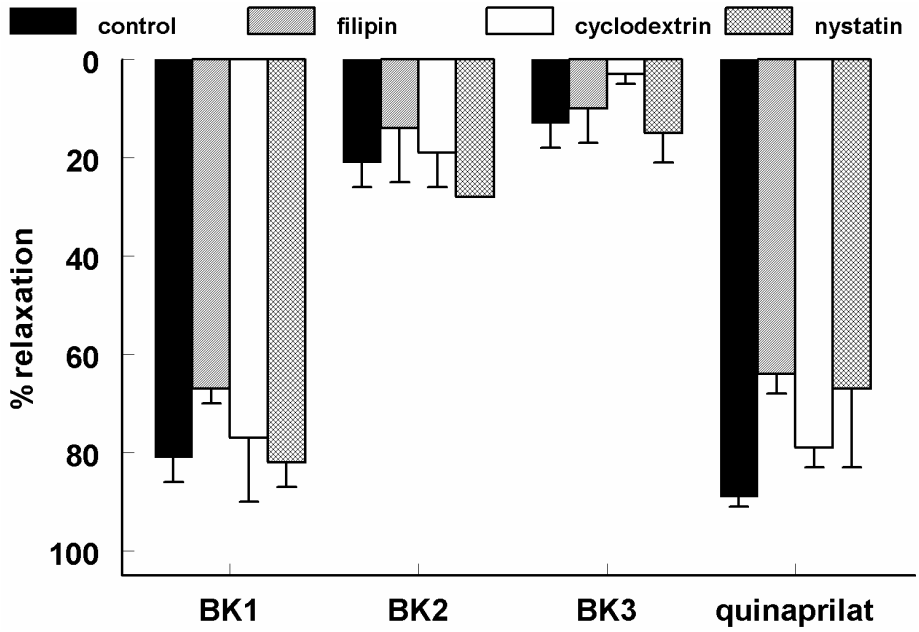


Figure 9. *Relaxations of porcine coronary arteries, following preconstriction with 1 μM U46619, to three consecutive bradykinin doses (0.1 μM; BK1, BK2, BK3), followed by 10 μM quinaprilat, in the absence (control) or presence of 4 μg/mL filipin, 2% cyclodextrin or 20 μg/mL nystatin. Data (mean±S.E.M. of 5-12 experiments) are expressed as a percentage of the contraction induced by U46619.*

Finally, none of the caveolar disrupting agents prevented the relaxant effect of quinaprilat in desensitized preparations (Figure 9).

5.4. Discussion

The present study shows that the ACE inhibitor-induced potentiation of bradykinin in intact PCAs has a metabolic origin. The explanation for this potentiation is therefore not that ACE and B_2 receptors physically interact, as proposed on the basis of studies in isolated cells overexpressing ACE and B_2 receptors (Benzing *et al.*, 1999; Marcic *et al.*, 2000a; 2000b; Minshall *et al.*, 1997b), but rather that ACE is located in close proximity of B_2 receptors, thereby directly determining the bradykinin concentration in the micro-environment of the B_2 receptor. As a consequence, the bradykinin concentrations seen by the receptor will only approach those in the organ bath when ACE is inhibited, a phenomenon that can be achieved instantaneously by adding an ACE inhibitor to the organ bath.

An extensive series of experiments, both in transfected cells (Benzing *et al.*, 1999; Marcic *et al.*, 2000a; 2000b; Minshall *et al.*, 1997b) and in intact vessel preparations (Danser *et al.*, 2000; Mombouli *et al.*, 2002; Tom *et al.*, 2001a), supports the idea that bradykinin potentiation by ACE inhibitors is an effect beyond hydrolysis. The data suggest that ACE inhibitors, in a PKC- and phosphatase-dependent manner (Marcic *et al.*, 2000b), increase the number of cell surface B_2 receptors, thereby preventing/reversing the rapid B_2 receptor desensitization that normally occurs upon exposure to bradykinin (Bachvarov *et al.*, 2001; Marcic *et al.*, 1999; 2000a; 2000b; Minshall *et al.*, 1997b). In order to draw this conclusion, the studies depended on the use of 'ACE-resistant' bradykinin analogues, in particular [Hyp^3-$Tyr(Me)^8$]-bradykinin. However, in a recent investigation Dendorfer et al. (Dendorfer *et al.*, 2001a) showed that rabbit ACE cleaves [Hyp^3-$Tyr(Me)^8$]-bradykinin at 71% of bradykinin degradation activity, and this finding was confirmed by Gobeil et al. (Gobeil *et al.*, 2002).

Potentiation of ACE-resistant bradykinin analogues by ACE inhibitors?

In the present study we therefore re-investigated the ACE inhibitor-induced bradykinin potentiation in PCAs, using 2 bradykinin analogues, one (PA-bradykinin)

of which was truly ACE-resistant, whereas the other (DP-bradykinin) was cleaved at 9% of bradykinin degradation activity (Dendorfer *et al.*, 2001a; Gobeil *et al.*, 2002). We followed two approaches, 1) comparison of the bradykinin CRCs in the absence and presence of an ACE inhibitor, and 2) the application of an ACE inhibitor to desensitized coronary arteries (i.e., arteries that no longer relaxed in response to 0.1 μM bradykinin, a concentration that normally causes complete relaxation). In the latter experimental setup, the addition of the ACE inhibitor immediately results in full relaxation, an effect that depends on the prior addition of bradykinin, since it does not occur in the absence of bradykinin (Danser *et al.*, 2000). Apparently, at the time the ACE inhibitor is added (i.e., approximately 10 min after the last bradykinin dose), the organ bath still contains sufficient bradykinin to allow full relaxation. Our measurement of the bradykinin half-life (>90 min) in the current study confirms this assumption. The quinaprilat-induced relaxation of desensitized preparations is not due to unspecific properties of this drug, since similar data have been obtained with a whole range of ACE inhibitors (Danser *et al.*, 2000; Gobeil *et al.*, 2002; Marcic *et al.*, 2000a).

The results of both experimental approaches indicate that the effect of ACE inhibition is absent or greatly reduced when using ACE-resistant bradykinin analogues. First, the 10-fold leftward shift of the bradykinin CRC that normally occurs in the presence of the ACE inhibitors quinaprilat (Danser *et al.*, 2000), captopril (Tom *et al.*, 2001a) or perindoprilat (Mombouli *et al.*, 2002) was absent when using PA-bradykinin or DP-bradykinin. Moreover, the leftward shift that we (Tom *et al.*, 2001a) and others (Gobeil *et al.*, 2002) observed earlier in the presence of Ang-(1-7), also did not occur in combination with these agonists, indicating that this shift, too, has a metabolic origin. The Ang-(1-7)-induced shift is smaller than the shift induced by quinaprilat and captopril, because Ang-(1-7), at the high concentration that was applied in this study (10 μM), blocks the active center of only one (the C-terminal domain) of the two homologous domains of the ACE molecule, whereas quinaprilat and captopril at this same concentration block both domains (Tom *et al.*, 2001a). These data therefore strongly argue against the idea that a non-ACE related effect of Ang-(1-7) (e.g., an effect mediated via the putative Ang-(1-7) receptor) underlies its bradykinin-potentiating capabilities (Fernandes *et al.*, 2001).

Second, the above described quinaprilat-induced relaxation of desensitized coronary arteries did not occur following PA-bradykinin-induced desensitization, and was significantly reduced after DP-bradykinin-induced desensitization. Complete desensitization was already obtained after the application of 0.1 μM PA-BK, since the addition of subsequent PA-BK doses (increasing the PA-BK concentrations 2- and 3-fold) did not exert further effects. In contrast, desensitization induced by 0.1 μM bradykinin or 0.03 μM DP-bradykinin was less complete, as the application of a second and third dose of these agonists still induced modest relaxations. Desensitization is due to a reduction in the number of B_2 receptors on the endothelial cell surface, and/or to exhaustion of post-receptor mechanisms (e.g., NO depletion). In support of the latter, desensitization occurred much more rapidly in the presence of an NOS inhibitor (Danser *et al.*, 2000). The lack of effect of quinaprilat after PA-bradykinin suggests that the B_2 receptor resensitization that has been detected upon ACE inhibitor administration in isolated cells (Benzing *et al.*, 1999; Marcic *et al.*, 1999; 2000a; 2000b; Minshall *et al.*, 1997b), either does not occur or is insufficient in intact coronary arteries.

How then should the ACE inhibitor-induced relaxation following bradykinin desensitization be explained? Quinaprilat does not increase the organ bath fluid levels of bradykinin. In the absence of significant B_2 receptor resensitization, this leaves the possibility that quinaprilat affects the bradykinin levels that are seen by the receptor. This explanation implies that ACE is located in close proximity of B_2 receptors, and thus, that normally the bradykinin levels in the vicinity of the receptor are below those in the organ bath. In view of the ≈10-fold leftward shift of the bradykinin CRC in the presence of quinaprilat, it seems reasonable to assume that the bradykinin levels in the micro-environment of the B_2 receptor are also ≈10-fold lower than the levels in the organ bath. For a fully ACE resistant bradykinin analogue (PA-bradykinin) such a difference will not exist, whereas for an analogue that is degraded by ACE at <10% of its bradykinin degrading activity (DP-bradykinin) the difference will be much smaller than 10-fold. This explains why we did not observe a significant leftward shift of the DP-bradykinin CRC, and only a modest relaxation following the addition of quinaprilat to DP-bradykinin desensitized vessels. It also explains why desensitization was already complete after one PA-BK dose (resulting in a concentration of 0.1 μM in the organ bath as well as in the micro-environment of B_2 receptors), whereas

desensitization remained incomplete after the addition of 3 subsequent bradykinin doses (resulting in a final concentration of 0.3 μM in the organ bath, and of ≈0.03 μM in the micro-environment of B_2 receptors). In fact, incomplete desensitization is a prerequisite for the quinaprilat-induced relaxation, since in completely desensitized preparations a 10-fold (or more) rise of the bradykinin levels in the micro-environment of the receptor will of course have no effect. In agreement with this concept, exposure of bradykinin-desensitized arteries to a 100-fold higher bradykinin concentration (10 μM) resulted in a similar relaxation as the addition of quinaprilat.

Blockade of phosphatases and PKC did not interfere with the quinaprilat-induced relaxation, whereas these agents did prevent the ACE inhibitor-induced bradykinin potentiation in transfected CHO cells that overexpress ACE and B_2 receptors (Marcic *et al.*, 2000b). One reason for this discrepancy might be that overexpression itself leads to interactions which do not occur at low expression levels.

ACE inhibitor-induced potentiation of B_2 receptor-mediated vasoconstriction

Our study is the first to demonstrate a metabolic background for the ACE inhibitor-induced potentiation of bradykinin-mediated vasodilation in intact arteries, i.e, for an interaction at the level of the endothelial cells, the same cells that were used to demonstrate ACE-B_2 receptor crosstalk in cell culture studies (Marcic *et al.*, 1999; 2000b). The present data are in full agreement with two previous studies, using similar experimental protocols, on the lack of potentiation of PA-bradykinin-mediated vasoconstriction in endothelium-denuded rabbit jugular veins by either ramiprilat (Dendorfer *et al.*, 2001a) or captopril (Gobeil *et al.*, 2002). Thus, the concept of B_2 receptor-ACE co-localization may also apply to vascular smooth muscle cells.

Bradykinin potentiation by NEP inhibitors?

NEP inhibition, in contrast with ACE inhibition, did not potentiate bradykinin in isolated porcine arteries, neither alone, nor on top of ACE inhibition. These findings contrast with reports on the wide presence of NEP in the vascular wall (Dussaule *et al.*, 1993; Gonzalez *et al.*, 1998; Graf *et al.*, 1993; Llorens-Cortes *et al.*, 1992; Soleilhac *et al.*, 1992; Wang *et al.*, 1994) and its important contribution to bradykinin metabolism *in-vivo* (Kentsch *et al.*, 1999; McClean *et al.*, 2000; Rouleau *et al.*, 2000).

Thus, either NEP is not present in PCAs and/or its contribution to bradykinin metabolism in this *in-vitro* model is of limited importance. Earlier studies in porcine vessels oppose the former explanation (Krassoi *et al.*, 2000; Miyamoto *et al.*, 2002). The most likely explanation is therefore that NEP in intact PCAs, unlike ACE, does not co-localize with B_2 receptors, and thus that NEP inhibition does not increase the bradykinin levels in the micro-environment of B_2 receptors. In support of this concept, bradykinin potentiation did occur following NEP inhibition when co-localization had been artificially induced by transfecting CHO cells with both NEP and B_2 receptors (Deddish *et al.*, 2002).

Co-localization of ACE and B_2 receptors in caveolae?

Both ACE and B_2 receptors have been demonstrated in caveolae (Benzing *et al.*, 1999; Haasemann *et al.*, 1998). Caveolae are small micro-invaginations of the plasma membrane enriched with caveolin that are involved in the compartmentalization of signaling molecules. For instance, B_2 receptors interact with eNOS in this compartment (Ju *et al.*, 1998). The structural integrity of caveolae depends on cholesterol, and sterol-binding agents such as filipin, cyclodextrin and nystatin are therefore capable of disrupting caveolae (Neufeld *et al.*, 1996; Rothberg *et al.*, 1992; Schnitzer *et al.*, 1994). Interestingly, a recent study demonstrated that caveolar disruption mimics endothelial dysfunction in atheromatous vessels (Darblade *et al.*, 2001).

To address the possibility of ACE-B_2 receptor co-localization in caveolae, we studied the bradykinin-potentiating effects of quinaprilat in coronary arteries that had been exposed to the above sterol-binding agents. Our data confirm that caveolar disruption results in endothelial dysfunction, since filipin reduced the maximal relaxant effect of both bradykinin and PA-bradykinin by ≈25-30%, without affecting the relaxations induced by the endothelium-independent agent SNAP. Cyclodextrin and nystatin did not affect the CRCs of bradykinin and PA-bradykinin. Possibly therefore, the 40-50% reduction in caveolar abundance that has been reported to occur in rabbit aortic rings following exposure to 2% cyclodextrin (the same concentration that was used in the present study, and that resulted in a reduction of the effect of acetylcholine in rabbit aorta rings) (Darblade *et al.*, 2001) is insufficient to affect B_2 receptor-mediated relaxations, or the reduction in PCAs is less than 40%.

Furthermore, nystatin at a concentration of 20 µg/mL tended to reduce the SNAP-induced relaxations (Figure 9), and a significant reduction occurred at a concentration of 50 µg/mL, thus not allowing us to investigate the effect of higher nystatin concentrations on the bradykinin CRCs.

Importantly however, although caveolar disruption appeared to reduce the relaxant effect mediated by B_2 receptors (for instance because of interference with their interaction with eNOS in this compartment), it did not affect the leftward shift induced by quinaprilat (or the absence thereof in the case of PA-bradykinin), nor did it prevent the quinaprilat-induced relaxation in desensitized preparations. Based on these data, it therefore seems unlikely that ACE inhibition within caveolae underlies its bradykinin-potentiating effects. Apparently therefore, the ACE-B_2 receptor co-localization occurs elsewhere, for instance in coated pits or non-caveolar lipid rafts.

5.5 Conclusion and perspective

Bradykinin potentiation by ACE inhibitors in PCAs is a metabolic process based on the co-localization of ACE and B_2 receptors on the endothelial cell membrane. NEP does not appear to be present in the micro-environment of coronary B_2 receptors, and the ACE inhibitor-induced effect on bradykinin metabolism most likely does not occur in caveolae, i.e. it precedes the coupling of B_2 receptors to eNOS in this compartment. The co-localization of ACE and B_2 receptors mimics the co-localization of ACE and AT_1 receptors (Saris *et al.*, 2002; Schuijt *et al.*, 2002). Thus, ACE is located in a strategic position to allow maximal efficiency of B_2 receptor and AT_1 receptor stimulation.

CHAPTER 6

ACE- versus Chymase-Dependent Angiotensin II Generation in Human Coronary Arteries: A Matter of Efficiency?

Summary

We investigated ACE- and chymase-dependent Ang I-II conversion in human coronary arteries. Human coronary artery rings were mounted in organ baths, and concentration response curves to Ang II, Ang I, and the chymase-specific substrate Pro^{11}-D-Ala^{12}-angiotensin I (PA-Ang I) were constructed. All angiotensins displayed similar efficacy. For a given vasoconstriction, bath (but not interstitial) Ang II during Ang I and PA-Ang I was lower than during Ang II, indicating that interstitial (and not bath) Ang II determines vasoconstriction. PA-Ang I increased interstitial Ang II less efficiently than Ang I. Separate inhibition of ACE (with captopril) and chymase (with C41 or chymostatin) shifted the Ang I concentration response curve ≈5-fold to the right, whereas a 10-fold shift occurred during combined ACE and chymase inhibition. Chymostatin, but not captopril and/or C41, reduced bath Ang II, and abolished PA-Ang I-induced vasoconstriction. Perfused human coronary artery segments, exposed luminally or adventitially to Ang I, released Ang II into the luminal and adventitial fluid, respectively, and this release was blocked by chymostatin. In conclusion, both ACE and chymase contribute to the generation of functionally active Ang II in human coronary arteries. However, as Ang II 'loss' in the organ bath is chymase-dependent, ACE-mediated conversion occurs more efficiently (i.e., closer to AT_1 receptors) than chymase-mediated conversion.

Based on: Tom B., Garrelds I.M., Scalbert E., Stegmann A.P.A., Boomsma F., Saxena P.R. and Danser A.H.J., (2003). ACE- versus chymase-dependent angiotensin II generation in human coronary arteries: A matter of efficiency?, *Arterioscler. Thromb. Vasc. Biol.,* **23: 251-256.**

6.1. Introduction

ACE inhibitors are widely used for the treatment of hypertension and heart failure. Their beneficial effects are believed to be due to blockade of the generation of Ang II from Ang I. In contrast with this concept, during chronic ACE inhibitor therapy, plasma and tissue Ang II levels are unchanged or elevated as compared to the pre-treatment situation (Farquharson *et al.*, 2002; van Kats *et al.*, 2000). This is not a methodological artifact (Farquharson *et al.*, 2002; van Kats *et al.*, 2000). Several explanations may therefore be put forward. First, the ACE inhibitor dose may have been too low to obtain sufficient ACE inhibition. Indeed, high dose ACE inhibition appears to be more effective than low dose (Jorde *et al.*, 2000). Second, the rise in renin and Ang I that occurs when Ang II no longer suppresses renin release may, at least in part, overcome ACE inhibition (Mooser *et al.*, 1990; van Kats *et al.*, 1998). Third, ACE upregulation is known to occur both as a consequence of chronic ACE inhibitor therapy, and during the progression of cardiovascular diseases (Farquharson *et al.*, 2002). Fourth, *in-vitro* studies have shown that there are alternative enzymes capable of converting Ang I into Ang II (Maassen van den Brink *et al.*, 1999; Richard *et al.*, 2001; Urata *et al.*, 1990). The most important of these is the serine protease chymase. Although one *in-vivo* study recently reported that the chymase-specific substrate Pro^{11}-D-Ala^{12}-Ang I (PA-Ang I) induced AT_1 receptor mediated vasoconstriction in human dorsal hand veins (McDonald *et al.*, 2001), *in-vivo* evidence for chymase-dependent Ang II generation from native Ang I could not be obtained in humans (Saris *et al.*, 2000).

In this respect, it is important to realize that the location of ACE and chymase differs greatly. ACE is a membrane-associated enzyme that, due to proteolytic cleavage of its membrane anchor, also occurs in a soluble form in the extracellular fluid. ACE-expressing cells include endothelial cells, vascular smooth muscle cells and cardiomyocytes (Coulet *et al.*, 2001; van Kesteren *et al.*, 1999). In contrast, chymase is located intracellularly, mainly in the cytosol of mast cells that are present in the adventitia (Urata *et al.*, 1993). It has also been demonstrated in endothelial (Urata *et al.*, 1993) and vascular smooth muscle cells (Guo *et al.*, 2001) and in the extracellular matrix (Urata *et al.*, 1993), although not all studies agree on this matter (Yamada *et al.*, 2001). Based on this distribution pattern, it is generally believed that

chymase is predominantly active in the interstitial space (Wei *et al.*, 1999). However, studies investigating interstitial Ang II generation could not (de Lannoy *et al.*, 2001) or only partly (Wei *et al.*, 1999) confirm this concept, either because interstitial fluid contains endogenous inhibitors of chymase (e.g., α_1-antitrypsin) (Kokkonen *et al.*, 1997) or due to species differences (Akasu *et al.*, 1998).

It was the aim of the present study to provide an explanation for the discrepancy between *in-vivo* and *in-vitro* studies with regard to the contribution of chymase to Ang I-to-II conversion in humans. Previous *in-vitro* studies measured either Ang II generation in tissue homogenates (Akasu *et al.*, 1998; Urata *et al.*, 1990), or evaluated Ang II-mediated constriction following the application of Ang I or PA-Ang I to intact human arteries (Richard *et al.*, 2001). Here, using human coronary arteries (HCAs), we measured Ang II release, Ang II-mediated contractile responses, and interstitial Ang II levels following the application of PA-Ang I and native Ang I, with or without inhibitors of ACE and/or chymase. The underlying assumption of our studies was that because of the different location of ACE and chymase, chymase-dependent generation of functionally active Ang II occurs less efficiently, i.e. further away from AT_1 receptors and thus (because of rapid angiotensin metabolism in the interstitial space) requires more Ang I to lead to vasoconstriction. HCAs are particularly useful for this purpose, because they contain ACE, chymase and AT_1 receptors (Maassen van den Brink *et al.*, 1999).

6.2. Methods

Drugs

Ang I, Ang II, captopril, chymostatin, PD123319, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and substance P were purchased from Sigma. C41 (3-[3,4-dimethoxyphenylsulfonyl]-1-3,4-dimethylphenylimidazoline-2,4-dione) was synthesized by dr. M.C. Viaud, Tours, France. Irbesartan was a gift from Bristol-Myers Squibb and PA-Ang I (purity>95%) was a gift from the Institut de Recherches Internationales Servier. Chymostatin and C41 were dissolved in dimethylsulfoxide and irbesartan was dissolved in ethanol. All other compounds were dissolved in distilled water.

Tissue collection and preparation

HCAs were obtained from 17 'heart beating' organ donors (6 men, 11 women; age 18-58 years, mean±S.E.M. 46±3 years) who died of non-cardiac causes (14 cerebrovascular accident, 3 head trauma) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the aortic and pulmonary valves for transplantation purposes. The study was approved by the Ethics Committee of the Erasmus MC. Immediately after circulatory arrest, the hearts were stored in an ice-cooled sterile organ-protecting solution (Maassen van den Brink *et al.*, 1999). After arrival in the laboratory, the HCA (diameter ≈3-4 mm) was removed and stored overnight in a 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. Vessels containing macroscopically visible atherosclerotic lesions were excluded.

Organ bath studies

Vessels were cut into segments of ≈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. 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). The segments, stretched to a stable force of about 15 mN, were exposed to 30 mM KCl twice. Subsequently, segments were exposed to 100 mM KCl to determine the maximal contractile response to 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 or without the AT_1 receptor antagonist irbesartan (1 μM), the AT_2 receptor antagonist PD123319 (1 μM), the chymase inhibitor chymostatin (100 μM), the ACE inhibitor captopril (10 μM) and/or the chymase inhibitor C41 (10 μM). Thereafter, concentration response curves (CRCs) to Ang I, Ang II, PA-Ang I were constructed. Endothelial integrity was verified at the end of each experiment by observing relaxation to 1 nM substance P after pre-contraction with 1 μM $PGF_{2\alpha}$.

To measure Ang II in organ bath fluid, a 50 µL sample was obtained from the organ bath at the time the vasoconstrictor response had reached a plateau. Samples were rapidly mixed with 10 µL angiotensinase inhibitor solution (Maassen van den Brink *et al.*, 1999) and stored at -80°C. Tissue Ang II was measured in a limited number of vessel segments only. Based on previous measurements in vessel segments, showing that vascular Ang II remains stable for at least 1 hour after the addition of Ang I (Schuijt *et al.*, 2002), the segments were removed 1 hour after the addition of 1 µM Ang I or PA-Ang I, washed in fresh Krebs solution, dried on tissue paper, frozen in liquid nitrogen and stored at -80°C.

Perfusion studies

Vessels were cut into segments of 1-2 cm length. Side branches, if present, were tied off with silk sutures. Each segment was mounted horizontally in a double-jacketed 4 mL organ bath and perfused from a 6 mL reservoir, using a roller pump (Ismatec IPS; flow 1 mL/min). Both organ bath and reservoir contained Krebs bicarbonate solution, aerated with 95% O_2 / 5% CO_2, and were maintained at 37°C. The perfusate was collected in the reservoir and reperfused through the vessel, thus creating a closed perfusion circuit (Danser *et al.*, 1995) allowing both adventitial (i.e., into the bath) and luminal (i.e., into the reservoir) drug application. Ang I (1 µM) was added to the bath or the reservoir, under control conditions, in the presence of 10 µM captopril, and in the presence of 10 µM captopril plus 100 µM chymostatin, respectively. Luminal and adventitial fluid samples (50 µL) were obtained as described above immediately before, and 30 and 60 min after the addition of Ang I. Prior to each subsequent Ang I application, bath and reservoir fluid were refreshed. All experiments were performed in the presence of 1 µM irbesartan, to prevent AT_1 receptor-mediated vasoconstriction.

Biochemical measurements

Ang II in organ bath and reservoir fluid was measured with sensitive radioimmunoassays (Maassen van den Brink *et al.*, 1999). Ang II in vessel segments was measured by radioimmunoassay following SepPak extraction and HPLC separation (van Kats *et al.*, 1998).

Data analysis

Data are given as mean±S.E.M. Contractile responses are expressed as a percentage of the maximal contraction to 100 mM KCl (63.9±4.9 mN, n=11). CRCs were analyzed as described earlier (Maassen van den Brink *et al.*, 1999) to obtain pEC_{50} ($-^{10}\log EC_{50}$) values. Statistical analysis was performed by one-way ANOVA, followed by post hoc evaluation (according to Tukey or Dunnett where appropriate). $P<0.05$ was considered significant.

6.3. Results

Organ bath studies

Ang I, Ang II and PA-Ang I displayed similar maximal effects (Figure 1, Table 1). Irbesartan, but not PD123319, prevented the PA-Ang I-induced vasoconstriction (Figure 1), indicating that its contractile effects, like those of Ang I in this model (Maassen van den Brink *et al.*, 1999), are mediated via AT_1 receptors.

Table 1. pEC_{50} and E_{max} of Ang I, Ang II and PA-Ang I in human coronary arteries with or without inhibitors.

	Ang I		**PA-Ang I**		**Ang II**	
Inhibitor	*pEC_{50}*	*E_{max}, % of 100 mM K^+*	*pEC_{50}*	*E_{max}, % of 100 mM K^+*	*pEC_{50}*	*E_{max}, % of 100 mM K^+*
none	7.35±0.13	23±4.4	6.87±0.14#	17±2.7	7.63±0.05	24±6.3
10 µM captopril	7.25±0.13	20±3.9	7.00±0.12	17±2.9		
10 µM C41	7.14±0.12	18±3.7	6.75±0.11	14±2.7	7.55±0.06	17±3.8
10 µM captopril +10 µM C41	6.58±0.05*	21±6.2	6.67±0.07	17±3.7		
100 µM chymostatin	6.90±0.13†	18±4.4	<6†	6.7±1.7†‡		

Data are mean±S.E.M. of 8 experiments. #, $P<0.05$ vs. Ang I and Ang II; *, $P<0.05$ and †, $P<0.01$ vs. none; ‡, contractile response at the highest concentration of Ang I that was tested.

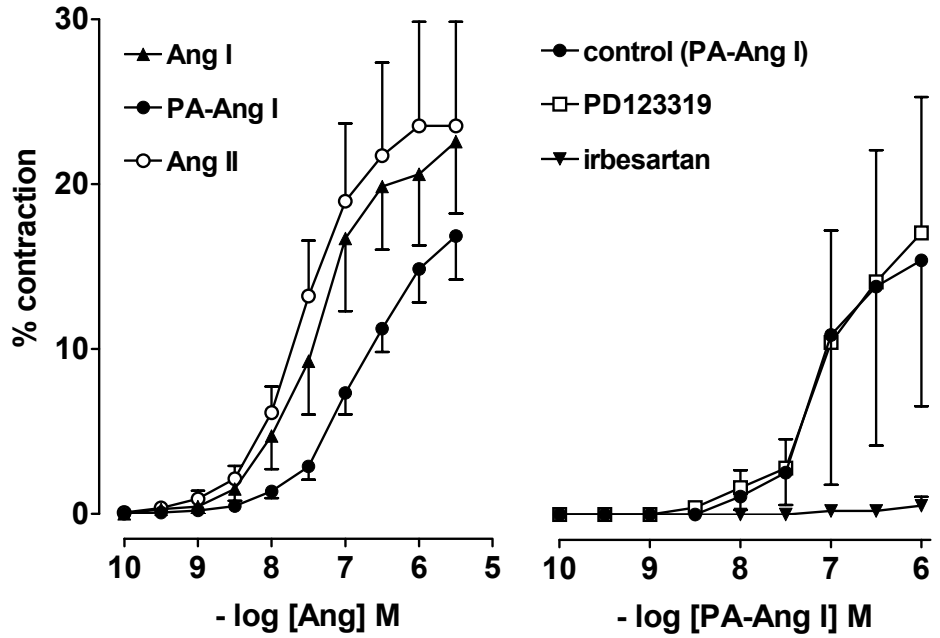


Figure 1. *Left panel: Contractions of human coronary arteries (HCAs) to Ang I, Ang II and PA-Ang I. Right panel: Contractions of HCAs to PA-Ang I in the absence (control) or presence of irbesartan or PD123319. Data (mean±S.E.M., n=8 and n=3 respectively) are expressed as a percentage of the response to 100 mM KCl.*

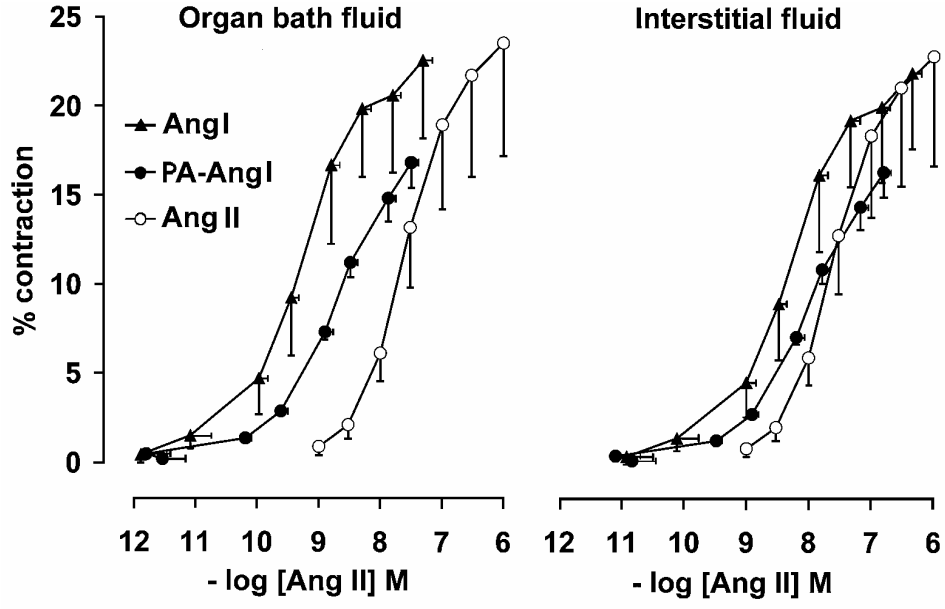


Figure 2. *Contractions of HCAs versus organ bath fluid (left) and interstitial (right) Ang II levels at the time of constriction after the addition of Ang I, Ang II and PA-Ang I. Data (mean±S.E.M., n=8) are expressed as a percentage of the response to 100 mM KCl.*

PA-Ang I was less potent (P<0.05) than Ang I and Ang II. At the moment of constriction, Ang II levels in the organ bath fluid during both Ang I and PA-Ang I amounted to <5% of the Ang I and PA-Ang I levels (Figure 2), and the pEC_{50} values calculated from these Ang II levels (9.20±0.25 and 8.90±0.15 for Ang I and PA-Ang I, respectively; P=NS) were 20-40 times higher (P<0.01) than the pEC_{50} for Ang II. Thus, for a given constriction, the organ bath fluid Ang II level was lower during Ang I and PA-Ang I application than during Ang II application.

Captopril (P=NS), C41 (P=NS) and chymostatin (P<0.05) modestly shifted the Ang I CRC to the right, whereas a ≈5-10-fold rightward shift (P<0.01) occurred during C41 plus captopril (Figure 3, Table 1). Chymostatin, but not captopril and/or C41 abolished the effects of PA-Ang I. C41 did not affect the Ang II CRC (Table 1).

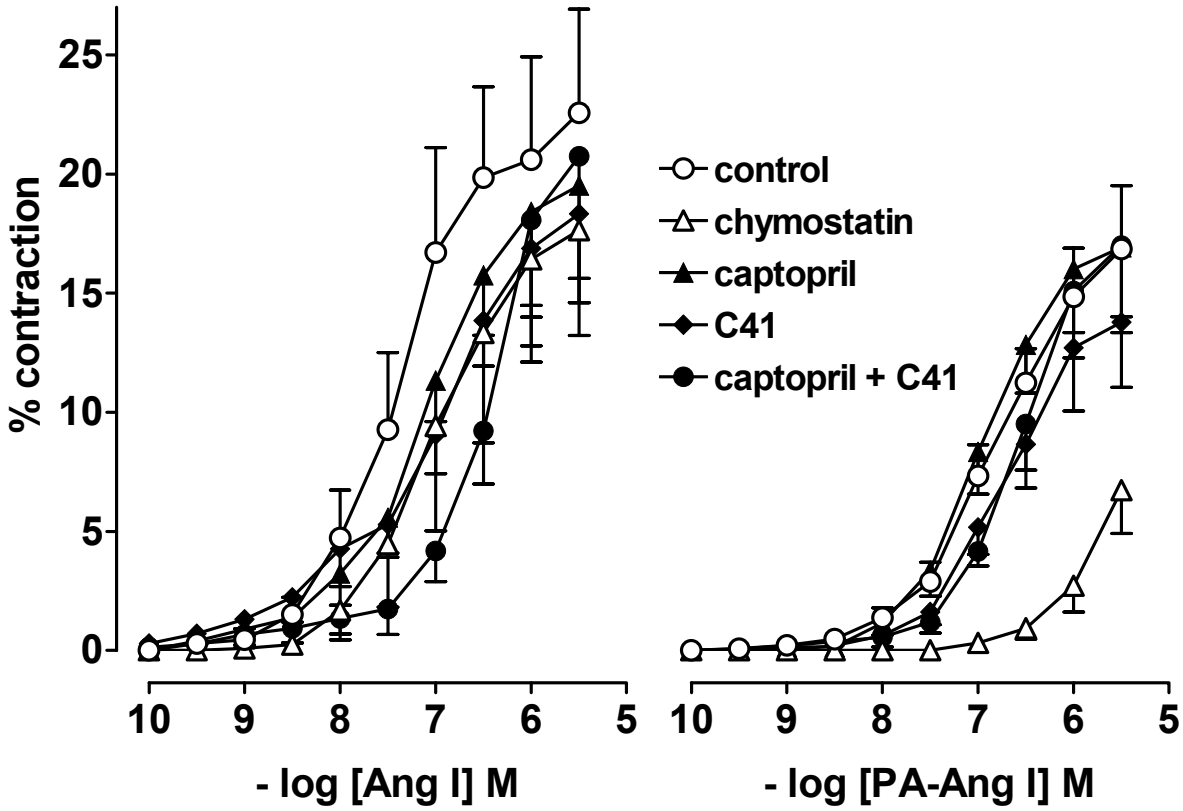


Figure 3. *Contractions of human coronary arteries to Ang I (left) and PA-Ang I (right) in the absence (control) or presence of chymostatin, captopril, C41 or captopril + C41. Data (mean±S.E.M., n=8) are expressed as a percentage of the response to 100 mM KCl.*

Chymostatin reduced the Ang II release into the organ bath during Ang I and PA-Ang I application by >75% ($P<0.05$, Figure 4). Neither captopril, nor C41, nor their combination significantly affected the Ang II levels in the organ bath during Ang I and PA-Ang I application.

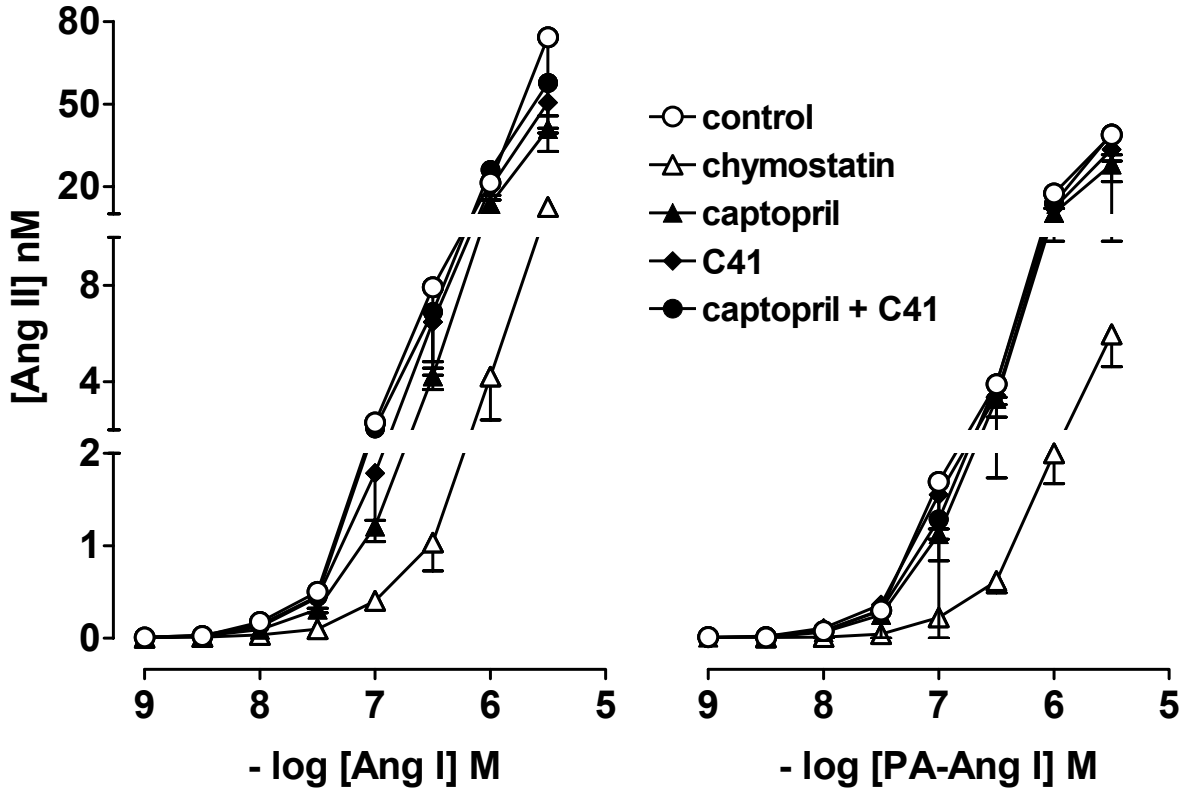


Figure 4. *Ang II levels in organ bath fluid measured at the moment of contraction of human coronary arteries during the concentration response curves to Ang I (left) and PA-Ang I (right) in the absence (control) or presence of chymostatin, captopril, C41 or captopril + C41. Data are mean±S.E.M. of 8 experiments.*

Interstitial Ang II and vasoconstriction

One hour after the application of 1 μM Ang I to the organ bath, the level of Ang II in the arterial segment (wet weight 24±2 mg; n=24) was 18.7±4.3 pmol/g wet weight (n=4). Vascular Ang II was not affected by irbesartan (18.9±6.0 pmol/g; n=4) or

PD123319 (29.4±12.1 pmol/g; n=4). Comparable observations were made following the application of 1 μM PA-Ang I (vascular Ang II respectively 11.4±1.4, 9.9±1.4 and 7.6±2.4 pmol/g; n=4 for each condition), although the vascular Ang II levels during PA-Ang I were 2-4 times ($P<0.01$) lower than during Ang I. The organ bath fluid levels of Ang II in these experiments were not affected by irbesartan or PD123319, and resembled those in Figure 4 (data not shown).

The lack of effect of irbesartan and PD123319 on vascular Ang II indicates that the majority of tissue Ang II is not bound to either cell surface or internalized AT_1 or AT_2 receptors, and thus is located extracellularly (i.e., in interstitial fluid). Since interstitial fluid accounts for ≈15% of tissue weight (Schuijt *et al.*, 2002), it can be calculated that the interstitial Ang II levels during Ang I and PA-Ang I application are 148.7±35.6 and 64.3±5.1 nM (mean of all measurements, including those with AT receptor blockers), respectively. This is 9.4±4.2 and 5.1±1.5 ($P<0.05$ for difference) times higher than the organ bath fluid Ang II levels at the time of vasoconstriction. The interstitial Ang II levels during Ang II application are as high as the Ang II levels in the organ bath (Schuijt *et al.*, 2002). The right panel of Figure 2 illustrates the consequence of this concept. It is clear that, for a given vasoconstriction, the interstitial Ang II levels during Ang I, PA-Ang I, and Ang II are comparable. However, it requires more PA-Ang I than Ang I to reach a certain interstitial Ang II level.

Perfusion studies

Luminal Ang I application resulted in the appearance of Ang II in luminal but not adventitial fluid, whereas adventitial Ang I application resulted in the appearance of Ang II in adventitial, but not luminal fluid (Figure 5). Ang II levels increased over time and were ≈4 times higher in adventitial fluid after adventitial Ang I application than in luminal fluid after luminal Ang I application. Chymostatin plus captopril, but not captopril alone, fully prevented luminal and adventitial Ang II release.

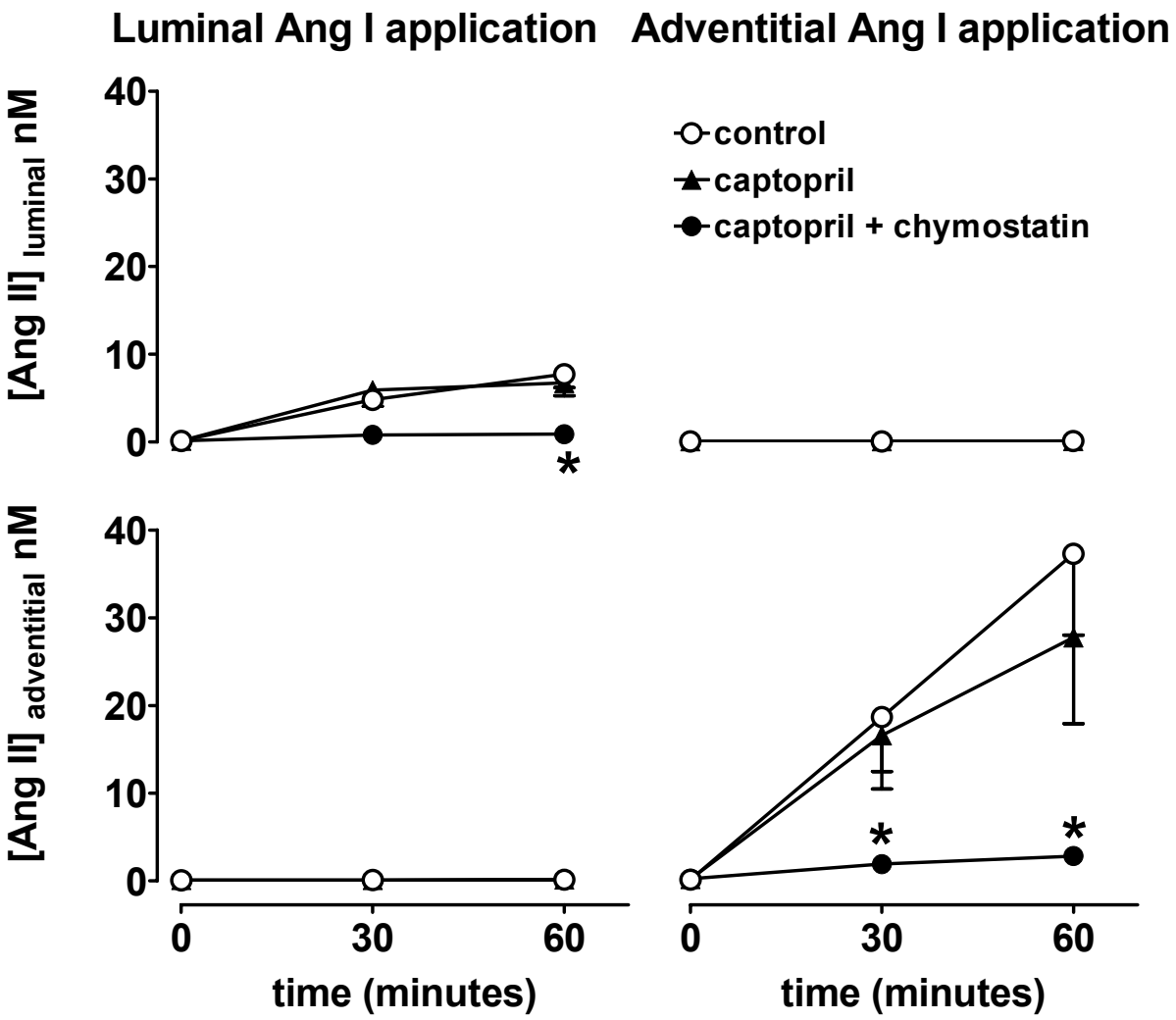


Figure 5. *Luminal and adventitial Ang II levels following the luminal or adventitial application of Ang I (1 μM) under control conditions, in the presence of captopril or in the presence of captopril + chymostatin. In the panels providing the control data only, Ang II was undetectable under all conditions. S.E.M., if not shown, is smaller than the symbol. Data are mean±S.E.M. of 5 experiments (*, P<0.05 vs. control).*

6.4. Discussion

The present study compares, in HCAs mounted in organ baths, the vasoconstrictor efficiency of Ang II generated by ACE and by chymase, using both native Ang I and the chymase-specific substrate PA-Ang I. The results show that Ang I is 5 to10 times more potent than PA-Ang I, and that Ang II release into the organ bath is almost exclusively chymase-dependent. Furthermore, chymase inhibition with chymostatin is sufficient to block the vasoconstrictor effects of PA-Ang I, whereas, in the case of Ang I, combined inhibition of ACE and chymase results in a better blockade of vasoconstriction than separate inhibition of ACE and chymase. Thus, both ACE and chymase contribute to the generation of functionally active Ang II from Ang I in isolated HCAs, but the generation of Ang II by chymase is far less efficient, resulting in loss of Ang II into the organ bath fluid, thereby requiring more Ang I to obtain a certain degree of constriction. These data may offer an explanation for the discrepancy between *in-vitro* and *in-vivo* findings with regard to the enzyme(s) contributing to Ang I-II conversion (Maassen van den Brink *et al.*, 1999; Richard *et al.*, 2001; Saris *et al.*, 2000).

In agreement with previous *in-vitro* (Maassen van den Brink *et al.*, 1999) and *in-vivo* (Saris *et al.*, 2000) studies, Ang I was as potent as Ang II in the present experimental setup. This is remarkable in view of the low Ang II concentrations that were detected in the organ bath fluid during the construction of the Ang I CRC. In fact, for a given constriction the organ bath fluid concentrations during Ang I were approximately 40 times lower than those during Ang II. The explanation for this apparent discrepancy, confirmed in the present study, is that the vascular interstitial Ang II concentrations at equimolar Ang I and Ang II levels are comparable (Schuijt *et al.*, 2002), and thus that vasoconstriction is determined by interstitial rather than organ bath fluid Ang II. The findings on PA-Ang I parallel those on Ang I. Taken together therefore, the levels of Ang II in the organ bath fluid are not representative for the Ang II levels seen by the receptor, and rather represent non-functional Ang II that has been ‘lost’ in the organ bath. Similarly, coronary constriction in the isolated perfused rat heart during exposure to either renin or Ang II did not relate to the Ang II levels in the effluent, suggesting that also in this preparation (representative for resistance vessels, as opposed to the conduit-type artery used in the present study) constriction is determined by tissue (interstitial) Ang II (Müller *et al.*, 1998).

Chymostatin, but none of the other inhibitors used in this study (including the chymase-selective inhibitor C41) prevented the release of Ang II into the organ bath fluid during the application of both Ang I and PA-Ang I. The lack of effect of 10 μM C41, despite its potent inhibitory effects towards chymase in human heart homogenates (IC_{50} 22 nM) (Richard *et al.*, 2001), suggests either that C41 does not have sufficient access to chymase in intact tissue, or that chymostatin, being a non-selective chymase inhibitor, inhibits alternative converting enzymes (e.g., cathepsin G). The latter conclusion would also imply that PA-Ang I is not a chymase-specific substrate, as has already been suggested based on studies in rat arteries (Inoue *et al.*, 1999). In humans however, using aprotinin (an inhibitor of cathepsin G but not chymase), evidence for cathepsin G-mediated Ang I-II conversion could not be obtained (Urata *et al.*, 1990). Thus, the most likely explanation for the limited effect of C41 as compared to chymostatin, in agreement with a recent study in human mammary arteries (Richard *et al.*, 2001), is that, at the concentration we used, its inhibitory capacities are more modest than those of chymostatin. It does have access to chymase, as evidenced by our observation that the Ang I CRC is shifted to the right in the presence of captopril and C41, but not when these inhibitors are given separately. The limited availability of C41 prevented us from constructing Ang I and PA-Ang I CRCs at higher C41 concentrations.

Captopril, at a dose that is sufficient to obtain complete ACE inhibition (Danser *et al.*, 1995; Tom *et al.*, 2001a), did not reduce Ang II release in the organ bath. In contrast, ACE inhibition resulted in complete suppression of Ang II release during incubation of porcine arteries with Ang I and during perfusion of rat hearts with renin or Ang I (Danser *et al.*, 1995 ; Müller *et al.*, 1998; Schuijt *et al.*, 2002). Thus, although ACE-dependent Ang II release does occur *in-vitro*, in human arteries it is apparently modest in comparison with the chymase-dependent Ang II release. In view of the fact that chymase is predominantly located adventitially (Urata *et al.*, 1993), whereas ACE is largely present on endothelial cells (Coulet *et al.*, 2001), we attempted to distinguish endothelial from adventitial Ang II release, by quantifying Ang II in luminal and adventitial fluid obtained from perfused HCAs following the application of Ang I to either the luminal or the adventitial compartment. The data confirm the concept that chymase is located predominantly adventitially, since the chymase-dependent Ang II release following adventitial Ang I application was 4 times

higher than that following luminal Ang II release. Angiotensin diffusion from the adventitial compartment to the luminal compartment or *vice versa* did not occur, in agreement with earlier work suggesting that such exchange probably takes place at the level of the capillaries (Danser *et al.*, 1995; van Kats *et al.*, 2001). Remarkably however, ACE inhibition did not affect luminal Ang II release. This contrasts with *in-vivo* studies measuring venous Ang II levels during arterial Ang I infusion (Saris *et al.*, 2000), as well as with studies in Ang I-perfused porcine arteries (Danser *et al.*, 1995). Since chymase has been demonstrated in endothelial cells (Urata *et al.*, 1993), release of this chymase under *in-vitro* conditions may have resulted in a greater contribution of chymase to endothelial Ang II release *ex-vivo*, thus making it impossible to detect ACE-dependent Ang II release. Along the same lines of reasoning, the contribution of adventitial chymase is probably also larger *ex-vivo*. Explanations for the greater role of chymase *in-vitro* than *in-vivo*, are first that tissue storage and handling may have resulted in chymase release from intracellular storage sites, and second the absence of endogenous chymase inhibitors (e.g., α_1-antitrypsin and secretory leukocyte protease inhibitor) (Kokkonen *et al.*, 1997; Takao *et al.*, 2001) in isolated vessel preparations.

Irrespective of the cause of the major contribution of chymase to Ang II generation in isolated HCAs, it is clear that ACE is located more strategically than chymase, i.e., closer to AT_1 receptors, thereby allowing it to contribute to Ang II generation in a highly efficient manner, with little or no loss of Ang II to the organ bath. The higher interstitial Ang II levels during Ang I application as compared to PA-Ang I application confirm this concept. Similarly efficient ACE-dependent Ang II generation has been demonstrated in porcine femoral arteries and in cultured rat cardiomyocytes (Saris *et al.*, 2002; Schuijt *et al.*, 2002).

The question arises whether chymase does play a role at all *in-vivo*. In view of its location (adventitial, largely intracellular), it may not face the same Ang I levels as ACE. Moreover, endogenous inhibitors in interstitial fluid suppress its activity, thereby counteracting its more efficient cleavage of Ang I as compared to ACE (k_{cat}/K_m 198 vs. 125 μM^{-1} min^{-1}) (Kokkonen *et al.*, 1997; Takao *et al.*, 2001; Urata *et al.*, 1990). Thus, it will probably only generate Ang II when Ang I levels are high, e.g. during ACE inhibition. In support of this concept, all *in-vivo* studies using the

chymase-specific substrate PA-Ang I (Garrison *et al.*, 1997; Inoue *et al.*, 1999; McDonald *et al.*, 2001; Nishimura *et al.*, 1998) show that the concentrations of this substrate required to induce vasoconstriction are 10-100 times higher than the concentrations of Ang I required to reach the same degree of vasoconstriction, despite the fact that the affinities of Ang I and PA-Ang I for chymase are similar (Richard *et al.*, 2001). Furthermore, even when Ang II is generated by adventitial chymase at high Ang I levels, its functional activity may be limited, since it is will be exposed to significant metabolism on its way to AT_1 receptors (de Lannoy *et al.*, 2001; Schuijt *et al.*, 1999).

In conclusion therefore, ACE-dependent Ang II generation results in more efficient AT_1 receptor stimulation than chymase-dependent Ang II generation, and the importance of the latter pathway is overestimated under *in-vitro* conditions.

CHAPTER 7

Superoxide Does Not Mediate the Acute Vasoconstrictor Effects of Angiotensin II: A Study in Human and Porcine Arteries.

Summary

We investigated whether superoxide mediates Ang II-induced vasoconstriction. Human and porcine coronary or femoral arteries (HCAs, PCAs, PFAs) were mounted in organ baths and concentration response curves to Ang II, the NO donor S-nitroso-N-acetylpenicillamine (SNAP) and the NAD(P)H oxidase substrate NADH were constructed in the absence and presence of superoxide inhibiting and activating drugs. Extracellular superoxide was measured using cytochrome c reduction. Ang II constricted both HCAs and PFAs. In HCAs, the NAD(P)H inhibitors diphenyleneiodonium (DPI) and apocynin and the xanthine oxidase (XO) inhibitor allopurinol, but not the superoxide dismutase (SOD) mimetic tempol or the SOD inhibitor diethyldithiocarbamate (DETCA), reduced this constriction. Catalase potentiated Ang II in HCAs, indicating a vasodilator role for H_2O_2. DPI, tempol and SOD did not affect Ang II in PFAs. DPI, apocynin and allopurinol relaxed preconstricted HCAs. Although the relaxant effects of the NO donor SNAP in PCAs was reduced by DETCA, indicating that superoxide-induced constrictions depend on NO inactivation, the apocynin-induced relaxations were NO-independent. Moreover, NADH relaxed all vessels, and this effect was blocked by KCl but not DPI or NO removal. Xanthine plus XO also relaxed HCAs and PCAs. Incubation of human or porcine arteries with Ang II or NADH did not result in detectable increases of extracellular superoxide within 1 hour. In conclusion, acute vasoconstriction by Ang II is not mediated via superoxide generated through NAD(P)H oxidase and/or XO activation. Such activation, if occurring, rather results in the generation of the vasodilator H_2O_2.

Based on: Schuijt M.P., Tom B., de Vries R., Saxena P.R., Sluiter W., van Kats J.P. and Danser A.H.J., (2003). Superoxide does not mediate the acute vasoconstrictor effects of angiotensin II. A study in human and porcine arteries, *J. Hypertension,* **in press.**

7.1. Introduction

Ang II, through activation of NAD(P)H oxidase and/or xanthine oxidase (XO), stimulates superoxide generation in the vascular wall (Berry *et al.*, 2000; Griendling *et al.*, 1994; Mervaala *et al.*, 2001; Touyz *et al.*, 1999). Since superoxide inactivates the endothelium-derived vasodilator NO, it is generally assumed that Ang II-induced vasoconstriction is mediated, at least in part, via superoxide. Ang II infusion studies in rats and mice appear to support this concept (Laursen *et al.*, 1997; Rajagopalan *et al.*, 1996; Wang *et al.*, 2001). However, the Ang II-induced hypertensive effects in such studies were only observed after several days or weeks of Ang II infusion, and may thus reflect the well-known longterm effects of Ang II on vascular remodeling rather than its acute vasoconstrictor effects.

Superoxide generation occurs in all layers of the vascular wall (Griendling *et al.*, 1994; Pagano *et al.*, 1997; Rueckschloss *et al.*, 2002; Touyz *et al.*, 1999). However, since superoxide, unlike NO, is not membrane-permeable, its actions will be restricted to the subcellular compartment where it has been generated. This would favor a role as a signaling molecule rather than as an endothelial NO-inactivating agent. Furthermore, dismutation of superoxide anions by superoxide dismutase (SOD) yields hydrogen peroxide (H_2O_2), a membrane-permeable vasodilator that has been reported to act as an endothelium-derived hyperpolarizing factor (EDHF) (Kobayashi *et al.*, 2002; Matoba *et al.*, 2002). The abundant presence of extracellular SOD (ecSOD) in blood vessels (Stralin *et al.*, 1995) might thus be expected to immediately counteract any vasoconstriction due to a rise in superoxide.

A limited number of studies have investigated the contribution of superoxide to the acute vasoconstrictor effects of Ang II. These studies were performed in isolated rat arteries (which, because of their low ecSOD content, differ from human blood vessels) (Karlsson *et al.*, 1988) and depended on the use of either SOD or its mimetic tempol (Kawazoe *et al.*, 2000; Shastri *et al.*, 2002). Both drugs modestly reduced Ang II-induced vasoconstriction. Their effects were observed at micromolar (i.e., non-physiological) concentrations of Ang II only, suggesting interference with AT_1 receptor desensitization (Hilgers *et al.*, 2002) rather than superoxide inactivation as an explanation of their effects. Unfortunately, the drugs that are currently used to inhibit

NAD(P)H oxidase (diphenyleneiodonium (DPI) and apocynin) lack specificity (Dodd-o *et al.*, 1997; 2000), and thus may also counteract Ang II-induced vasoconstriction independently of their effect on superoxide generation.

It was the aim of the present study to address the importance of superoxide in the acute contractile effects of Ang II in human vessels mounted in organ baths. Because of the non-specificity of most of the drugs that are currently available to interfere with superoxide generation/degradation, and in view of the uncertainty to which degree these drugs reach the (intracellular) compartment where they are supposed to act, we used a wide range of enzyme inhibitors/mimetics, the underlying hypothesis being that contradictory findings could relate to their non-specific effects. For comparison, we also studied the effects of these drugs in combination with NO donors, the NAD(P)H oxidase substrates NADH and NADPH, and contractile agents that do not exert their actions in a NAD(P)H oxidase- (Touyz *et al.*, 2002) and/or superoxide-dependent (Shastri *et al.*, 2002) manner ET-1 and the prostaglandin $F_{2\alpha}$ analogue U46619. Finally, we quantified superoxide generation in parallel with the organ bath studies. Due to the limited availability of human arteries, some issues were addressed in porcine arteries. The angiotensin responsiveness of these vessels largely resembles that of human arteries (Maassen van den Brink *et al.*, 1999; Schuijt *et al.*, 2002; Tom *et al.*, 2003).

7.2. Methods

Drugs

Acetovanillone (apocynin), Ang II, allopurinol, bradykinin, catalase, cytochrome c, diethyldithiocarbamate (DETCA), 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619), DPI, ET-1, 4-hydroxy-TEMPO (tempol), hydroxocobalamin, NADH, NADPH, N^{ω}-nitro-L-arginine methyl ester (L-NAME), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), substance P, SOD from bovine erythrocytes, tiron, xanthine, and XO from buttermilk were from Sigma-Aldrich (Zwijndrecht, The Netherlands). DPI, allopurinol and apocynin were dissolved in dimethylsulfoxide. All other compounds were dissolved in distilled water.

Tissue collection

Human coronary arteries (HCAs) were obtained from 29 heart-beating organ donors (15 men, 14 women; ages 5-64 years, mean±S.E.M. 45±3 years) who died of noncardiac causes (22 cerebrovascular accident, 6 head trauma, 1 hypoxia) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the aortic and pulmonary valves for transplantation purposes. The study was approved by the Ethics Committee of the Erasmus MC. Immediately after circulatory arrest, the hearts were stored in an ice-cooled sterile organ-protecting solution (Maassen van den Brink *et al.*, 1999). Porcine femoral and coronary arteries (PFAs, PCAs) were obtained from twentyfour 2-3 month-old pigs (Yorkshire x Landrace, weight 10-15 kg) that had been used in *in vivo* experiments studying the effects of α_2 or CGRP receptor (ant)agonists under pentobarbital anaesthesia, and from 58 pigs at the local slaughterhouse. The Ethics Committee of the Erasmus MC dealing with the use of animals for scientific experiments approved the protocol for this investigation. Arteries were either removed at the end of the experiment, or after the heart from the slaughterhouse had been brought to the laboratory in cold 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. Vessels were stored overnight in cold, oxygenated Krebs bicarbonate solution. Overnight storage neither affected U46619 (30 nM)-induced constrictor responses (contraction of stored arteries 99±13 % of that of freshly isolated arteries, n=9) nor the subsequent endothelium-dependent relaxations induced by 10 nM substance P (relaxation of stored arteries 106±14 % of that of freshly isolated arteries, n=9). These data, which are in agreement with earlier data on the similar responsiveness of freshly isolated and stored myocardial trabeculae (Tom *et al.*, 2001b), argue against a storage-induced change in redox state affecting contractile or relaxant responses.

Organ bath studies

Vessels were cut into segments of ≈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. Vessel segments were allowed to equilibrate for at least 30 minutes, and the organ bath fluid was refreshed every 15 minutes during this period. Changes in tissue contractile force were recorded with a Harvard isometric

transducer (South Natick). The segments, stretched to a stable force of ≈15 mN (coronary arteries) or ≈50 mN (femoral arteries), were exposed to 30 mM KCl twice. Subsequently, the tissue was exposed to 100 mM KCl to determine the maximal contractile response to KCl. The rings were then allowed to equilibrate in fresh organ bath fluid for 30 minutes. Next, segments were preincubated for 30 minutes with or without DPI (10 μM) (Berry *et al.*, 2000; Touyz *et al.*, 1999), apocynin (1 mM) (Dodd-o *et al.*, 2000; Hamilton *et al.*, 2002), NADH (100 μM), allopurinol (1 mM) (Berry *et al.*, 2000), SOD (6 U/mL), tempol (1 mM) (Shastri *et al.*, 2002), DETCA (100 μM) (Berry *et al.*, 2000), catalase (1000 U/mL) (Hamilton *et al.*, 2001), tiron (10 mM) (Didion *et al.*, 2002b; Griendling *et al.*, 1994), or L-NAME (100 μM) (Hamilton *et al.*, 2002) + hydroxocobalamin (200 μM) (Danser *et al.*, 2000). Thereafter, concentration response curves (CRCs) to Ang II, ET-1, the thromboxane A_2 analog U46619, SNP, SNAP, NADH, NADPH, DPI, apocynin, allopurinol, or H_2O_2 were constructed, either at baseline or following preconstriction with 1 μM $PGF_{2\alpha}$ in HCAs (to 50±5.5% of 100 mM KCl, n=7), 1 μM U46619 or 40 mM KCl in PCAs (to 65±3.0% (n=27) and 69±4.9% (n=8) of 100 mM KCl, respectively) and 10 nmol/mL U46619 in PFAs (to 72±6.9% of 100 mM KCl, n=19). The effect of xanthine (100 μM) + XO (5 mU/mL) was studied in preconstricted HCAs and PCAs. Endothelial integrity was verified at the end of each experiment by observing relaxation to 1 nM substance P or 100 nM bradykinin after preconstriction with U46619 or $PGF_{2\alpha}$.

Superoxide measurement

Extracellular superoxide release was measured in vessel segments as SOD-inhibitable reduction of 10 μM oxidized cytochrome c. Segments were suspended in test tubes containing 1 mL Krebs bicarbonate solution, aerated with 95% O_2/5% CO_2, and maintained at 37°C. After a 15-min equilibration period, oxidized cytochrome c with or without 200 U/mL SOD was added in the presence or absence of 100 μM xanthine + 5 mU/mL XO, 100 (HCA) or 10 (PCA, PFA) nM Ang II, 100 μM NADH, 100 μM NADPH, 10 μM DPI or their combination. After 15 and 60 min, 300 μl samples were removed and measured spectrophotometrically against Krebs bicarbonate solution at 550 - 540 nm. The amount of reduced cytochrome c was calculated using the molecular extinction coefficient 21 mM^{-1} cm^{-1} (Leslie *et al.*, 1987).

Data analysis

Data are given as mean±S.E.M. Contractile responses are expressed as a percentage of the contraction to 100 mM KCl (59±4.0 mN in HCAs, n=19; 88±4.1 mN in PCAs, n=50; 109±8.6mN in PFAs, n=19). Dilator responses are expressed as a percentage of preconstriction. CRCs were analyzed as described (Maassen van den Brink *et al.*, 1999) to obtain pEC_{50} ($-^{10}\log EC_{50}$) values. In the presence of inhibitors, agonists did not always reach the same maximum (E_{max}) at their highest concentration. In such cases we determined the concentration required to obtain 5% (Ang II) or 30% (ET-1) of the KCl-induced contraction, in order to calculate the $pEC_{5\%KCl}$ or $pEC_{30\%KCl}$ value (Maassen van den Brink *et al.*, 1999). Statistical analysis was by paired t-test or one-way ANOVA, followed by post hoc evaluation according to Dunnett. $P<0.05$ was considered significant.

7.3. Results

Ang II, ET-1 and U46619

Ang II concentration-dependently constricted HCAs (pEC_{50} 7.73±0.07; E_{max} 15.7±2.4%, n=15; Figure 1) and PFAs (pEC_{50} 8.84±0.06; E_{max} 51±5.7%, n=12; Figure 2). Ang II-induced constrictions were not studied in PCAs, because these vessels do virtually not respond to Ang II (Maassen van den Brink *et al.*, 1999).

SOD and tempol did not affect the contractile responses to Ang II in HCAs or PFAs. The SOD inhibitor DETCA decreased the Ang II-induced contractions in PFAs by 37±10% ($P<0.05$), but not in HCAs. However, in PFAs DETCA also caused a ≈5-fold rightward shift ($P<0.05$) of the U46619 CRC (pEC_{50} and E_{max} under control conditions respectively 7.92±0.08 and 130±13%, n=4), suggesting that its inhibitory effect towards Ang II is non-specific (Figure 2).

The NAD(P)H oxidase inhibitors DPI and apocynin, as well as the XO inhibitor allopurinol decreased the Ang II-induced E_{max} to 8.7±2.4, 5.5±3.1 and 3.5±1.3%, respectively ($P<0.05$ vs. control for all). DPI did not affect the Ang II CRC in PFAs. Catalase (n=6) potentiated Ang II ≈5-fold ($pEC_{5\%KCl}$ 8.31±0.23 vs. 7.81±0.09; $P<0.05$) in HCAs, and a similar tendency (P=NS) was observed for the radical scavenger tiron ($pEC_{5\%KCl}$ 8.18±0.14, n=6).

The inhibitory effects of DPI and apocynin towards Ang II in HCAs would be in agreement with the concept that superoxide mediates Ang II-induced vasoconstriction. However, the Ang II CRC was unaffected by the NAD(P)H oxidase substrate NADH (Figure 1), despite the rise in vascular superoxide production that has has been reported to occur following the addition of NADH to a vessel segment (Rajagopalan *et al.*, 1996). Moreover, SOD and DETCA were still ineffective in the presence of NADH, and if anything, the inhibitory effects of DPI, apocynin and allopurinol were enhanced rather than decreased in the presence of this substrate.

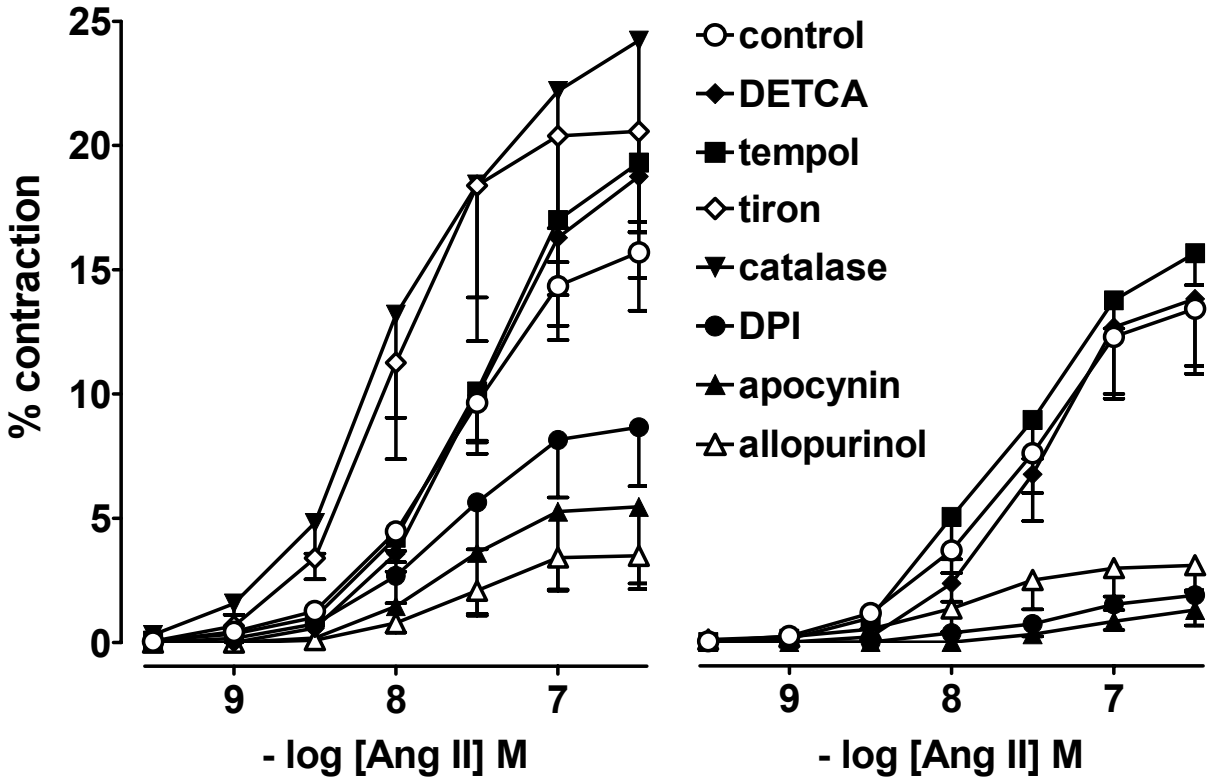


Figure 1. *Contractions of human coronary arteries to Ang II in the absence (left panel) or presence (right panel) of NADH without (control) or with DPI, apocynin, allopurinol, DETCA, tempol, tiron or catalase. Data (mean±S.E.M., n=6-15) are expressed as a percentage of the response to 100 mM KCl.*

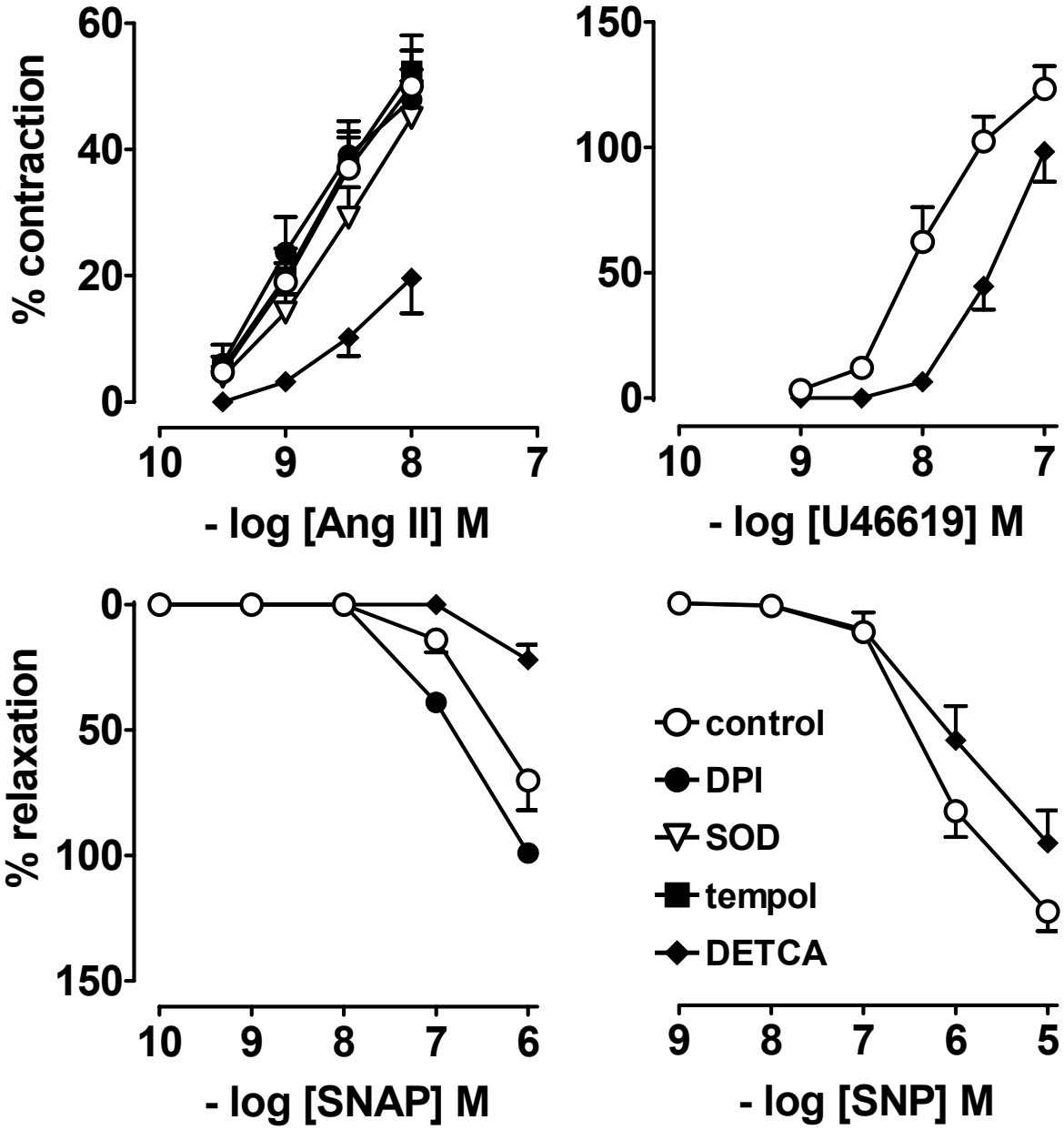


Figure 2. *<u>Top panels</u>: Contractions of porcine femoral arteries to Ang II (left) and U46619 (right) without (control) or with DPI, SOD, tempol or DETCA. Data (mean±S.E.M., n=4-12) are expressed as a percentage of the response to 100 mM KCl. <u>Bottom panels</u>: Relaxations of porcine coronary arteries (left) or porcine femoral arteries (right) to SNAP and SNP without (control) or with DPI or DETCA. Data (mean±S.E.M.; n=6-7) are expressed as a percentage of the preconstriction to U46619.*

ET-1 constricted HCAs in a concentration-dependent manner (pEC_{50} 7.61±0.06, n=7; Figure 3) and its efficacy was 4-5 times as large as that of Ang II (E_{max} 84±4.4%; P<0.01 vs. Ang II). With the exception of tiron, all drugs affecting the Ang II CRC in HCAs similarly affected the ET-1 CRC, although their effects were more modest as compared with their effects towards Ang II.

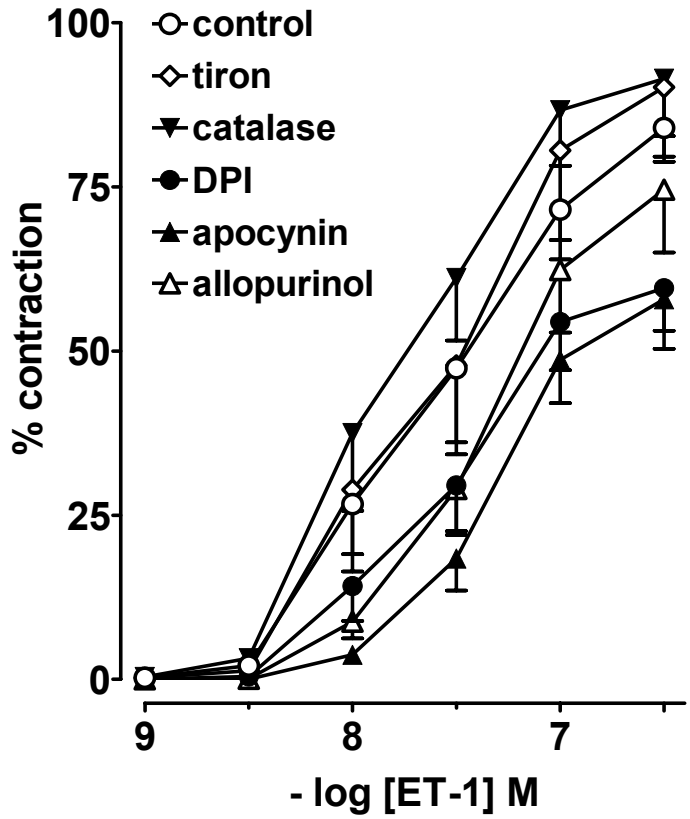


Figure 3. *Contractions of human coronary arteries to ET-1 without (control) or with DPI, apocynin, allopurinol, tiron or catalase. Data (mean±SEM.; n=4-7) are expressed as a percentage of the response to 100 mM KCl.*

This probably relates to the much higher efficacy of ET-1. Catalase (n=4) marginally shifted the ET-1 CRC to the left ($pEC_{30\%KCl}$ 7.96±0.13 vs. 7.74±0.18), and DPI, apocynin and allopurinol (n=7 for all) shifted the ET-1 CRC to the right ($pEC_{30\%KCl}$ 7.52±0.16, 7.31±0.09 and 7.47±0.11, respectively; P<0.05 vs. control for all).

NADH, NADPH, H_2O_2, and xanthine + XO

Following preconstriction with U46619 or $PGF_{2\alpha}$, NADH concentration-dependently relaxed HCAs (pEC_{50} 4.54±0.14, n=7), PCAs (pEC_{50} 4.48±0.17, n=9) and PFAs

(pEC_{50} 4.87±0.20, n=6) by maximally 87±4.9, 74±8.1 and 60±15%, respectively (Figure 4).

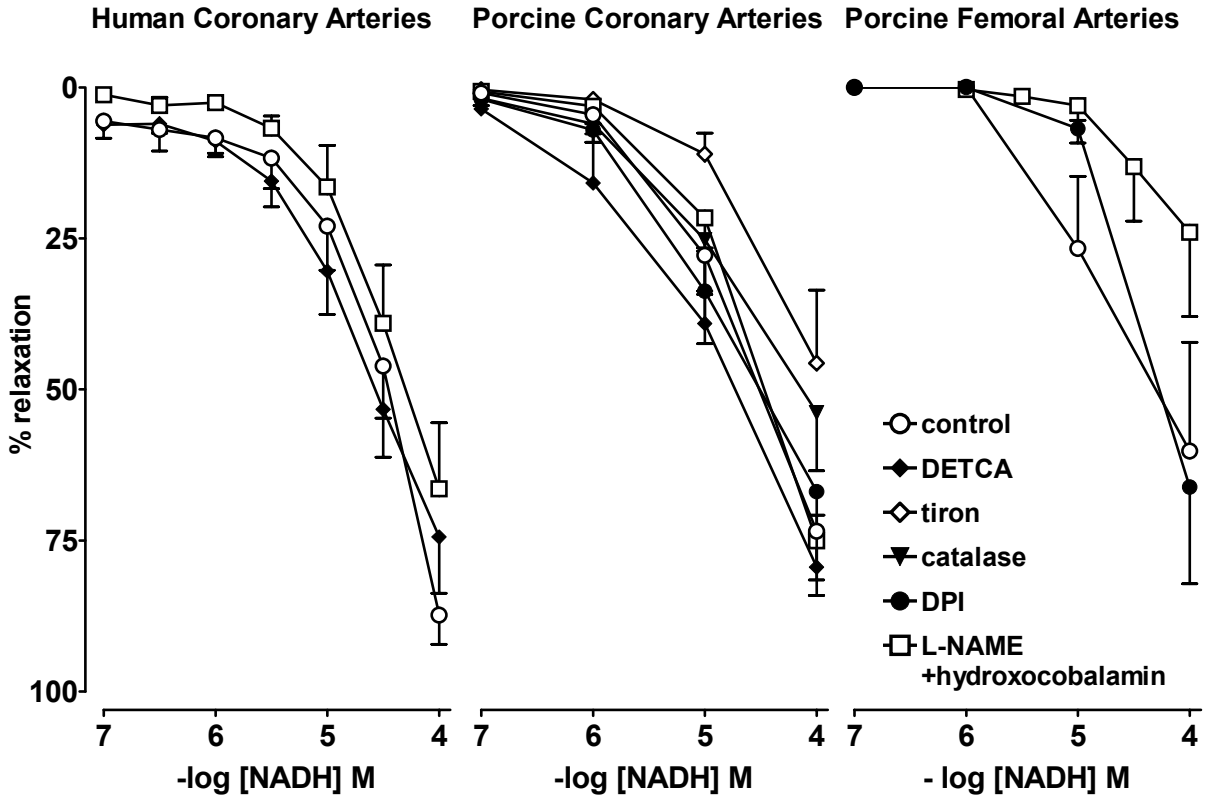


Figure 4. *Relaxation of human coronary, porcine coronary, or porcine femoral arteries to NADH without (control) or with DPI, DETCA, L-NAME + hydroxocobalamin, tiron or catalase. Data (mean±S.E.M.; n=6-9) are expressed as percentage of the preconstriction to U46619 or* $PGF_{2\alpha}$.

In contrast, NADPH marginally affected U46619-preconstricted PCAs (relaxation <20%, n=7; data not shown). DPI and DETCA did not affect the NADH CRCs. Tiron, catalase, and L-NAME + hydroxocobalamin tended to reduce the NADH effects in PCAs, but significance ($P<0.05$) was reached with tiron only. Following preconstriction with 40 mM KCl, NADH was unable to cause relaxation (n=8, data not shown).

H_2O_2 relaxed U46619-preconstricted PFAs (pEC_{50} 3.41±0.02, n=6) by maximally 124±25% (data not shown).

Xanthine + XO relaxed preconstricted HCAs and PCAs by 22±7.5% (n=4) and 57±11% (n=9). Relaxations in PCAs tended to be reduced by catalase (to 38±8.9%, n=9) and DETCA (to 34±16%, n=9), but the differences were not significant.

SNAP and SNP

The NO-donors SNAP and SNP dose-dependently relaxed preconstricted PCAs (pEC_{50} 6.63±0.06, n=6) and PFAs (pEC_{50} 6.31±0.10, n=7) by maximally 70±13 and 123±8%, respectively (Figure 2). DPI shifted the SNAP CRC in PCAs ≈5-fold to the left (pEC_{50} 6.87±0.05, n=6, $P<0.05$ vs. control), suggesting that superoxide inactivates exogenous NO. In agreement with this concept, SOD inhibition with DETCA largely prevented the SNAP-induced vasodilation in PCAs (maximal relaxation 22±6%, n=6, $P<0.01$ vs. control) and tended to decrease the SNP-induced vasodilation in PFAs (maximal relaxation 96±13%, n=7, P=0.06 vs. control). When repeated in the presence of NADH, the findings with SNP (n=7) and SNAP (n= 6) with or without DPI and DETCA were unaltered (data not shown).

Inhibitors/mimetics of superoxide generating and degrading enzymes

DPI, apocynin and allopurinol dose-dependently relaxed preconstricted HCAs (Figure 5). Relaxation amounted to 17±6.4, 107±4.1 and 89±7.1% at the highest concentration tested (i.e., the concentration used in the experiments with Ang II and ET-1). With the exception of the DPI-induced relaxation, all findings in HCAs were mimicked in PCAs. DPI constricted PFAs to the same degree as PCAs (n=5; data not shown). The apocynin-induced relaxations in PCAs were not affected by L-NAME + hydroxocobalamin (Figure 5). In PCAs, tiron (n=4) and catalase (n=4) had no effect on baseline constriction, wherease DETCA, tiron, and catalase did not affect U46619-induced constrictions (70±5.1, 80±4.6 and 72±8.3 vs. 75±4.2% of 100 mM KCl, n=8 for each), thereby indirectly supporting the idea that these drugs did not exert relaxant/constrictor effects.

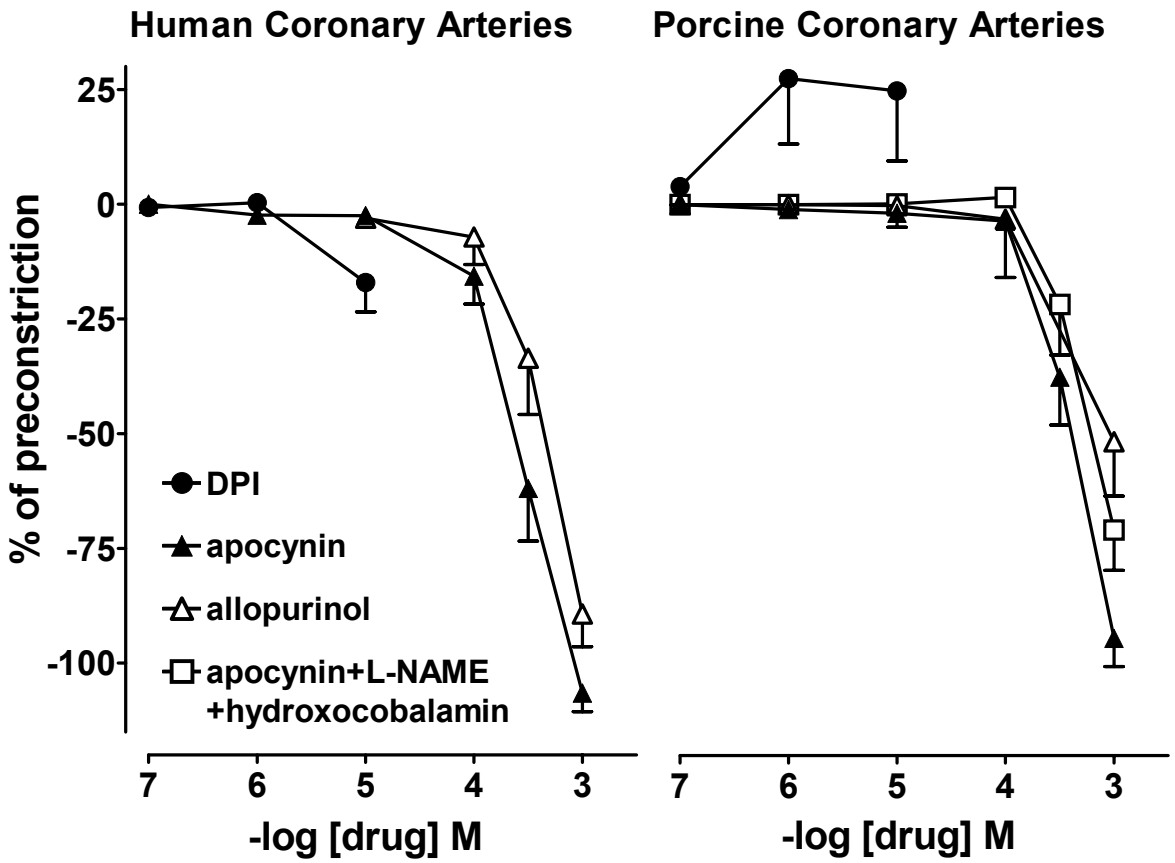


Figure 5. *Effects DPI, apocynin (without or with L-NAME + hydroxocobalamin), and allopurinol in preconstricted human coronary arteries (left panel) or porcine coronary arteries (right panel). Data (mean±S.E.M.; n=3-8) are expressed as a percentage of the preconstriction to U46619.*

Superoxide measurement

Incubation of vessel segments with xanthine + XO resulted in an increase in cytochrome c reduction at 15 min (Table). SOD blocked this increase, thereby confirming that it was due to the generation of superoxide. Results at 60 min did not differ from those at 15 min, most likely due to XO deactivation during turnover (Hodges *et al.*, 2000). No increases in cytochrome c reduction were observed during incubation of segments with Ang II (at concentrations that induced maximal contractile effects) for 15 or 60 minutes. NADH and NADPH both increased

cytochrome c reduction, but these increases were not blocked by SOD, indicating that they were not due to superoxide generation. DPI reduced the NADPH-induced increases in cytochrome c reduction, but, in view of the absence of an effect of SOD, this decrease could also not be attributed to a reduction in superoxide generation. Apocynin (in the presence of NADH or NADPH), DETCA and tiron at the concentration used in the organ bath set-up increased cytochrome c reduction in the absence of a vessel segment (n=5-7, data not shown) in a non-SOD-inhibitable manner, thus impairing the use of these drugs in the present assay. Results with tempol were identical to those with SOD (n=4 for vehicle, xanthine + XO, Ang II and NADH in HCAs; data not shown).

7.4. Discussion

This study does not support the concept that the acute vasoconstrictor effects of Ang II in healthy human and porcine arteries are mediated via superoxide generated through NAD(P)H oxidase and/or XO activation. The data rather suggest that such activation results in vasodilation, most likely in an H_2O_2-dependent manner.

SOD and its mimetic tempol did not affect the Ang II CRCs in human and porcine arteries. This contrasts with previous findings in rat aortic rings, where these agents modestly reduced the contractions obtained at micromolar Ang II concentration (Kawazoe *et al.*, 2000; Shastri *et al.*, 2002). It could be argued that their lack of effect in the present study is due to the fact that human and porcine vessels possess much higher endogenous ecSOD levels than rat vessels (Karlsson *et al.*, 1988), thus masking any vasoconstrictor effect of superoxide. Such constrictor effects should then become apparent during SOD inhibition with DETCA. However, DETCA was ineffective in HCAs and reduced (rather than increased) Ang II constrictions in PFAs. The latter may have been due to a non-specific action of DETCA, because it was also observed in relationship with U46619-induced constrictions. The absence of DETCA-induced Ang II potentiation is not due to its inability to block SOD in the present experimental setup, since DETCA did diminish the vasodilator effects of NO donors in porcine arteries. Thus, in agreement with a previous study in mouse aortic rings (Didion *et al.*, 2002b), an increase in superoxide is capable of causing vasoconstriction through NO inactivation. Conversely, a decrease in superoxide, as obtained with the NAD(P)H oxidase inhibitor DPI, enhanced the vasodilation by NO donors.

Table: Cytochrome c reduction.

			nmol/mL		pmol/mg wet weight							
vessel	incubation time (min)	SOD	vehicle	X+ XO	vehicle	Ang II	DPI	DPI + Ang II	NADH	NADPH	DPI + NADH	DPI + NADPH
HCA	15	-	0.81±0.19	2.85±0.35†	20±7.1	14±6.1	5.0±8.6	12±7.9	65±9.0†	80±2.5†	66±9.2†	46±5.0‡
	15	+	0.97±0.24	1.33±0.42*	24±7.8	20±9.5	3.9±15	7.7±12	68±7.2†	77±7.5†	66±3.6†	64±4.8
	60	-	0.88±0.17	2.34±0.30†	25±6.4	18±5.8	12±7.9	17±8.4	127±21†	99±6.8†	114±17†	60±4.1
	60	+	0.95±0.24	1.06±0.36*	23±8.3	23±8.9	7.5±16	12±11	121±17†	106±11†	128±22†	98±24
PCA	15	-	0.32±0.12	2.45±0.44†	24±3.0	22±2.3	28±6.4	25±6.7	71±9.1†	49±8.4†	58±11†	32±4.1‡
	15	+	0.37±0.16	0.74±0.68*	26±4.8	27±5.6	20±5.1	16±2.3	60±7.7†	45±5.3†	64±11†	26±2.0‡
	60	-	0.46±0.10	2.03±0.27†	31±2.5	25±3.2	30±7.5	27±5.1	109±10†	60±9.1†	92±16†	38±5.3‡
	60	+	0.47±0.15	0.69±0.53*	31±4.6	28±5.3	19±2.7	18±2.1‡	91±10†	52±5.9†	86±12†	33±2.3‡
PFA	15	-	0.06±0.08	1.49±0.41†	8.6±16	-7.4±14	-	-	-	-	-	-
	15	+	0.01±0.02	-0.2±0.09*†	1.7±4.5	-8.3±23	-	-	-	-	-	-
	60	-	0.16±0.10	1.12±0.29†	26±19	25±14	-	-	-	-	-	-
	60	+	0.11±0.08	-0.13±0.09*†	20±14	21±22	-	-	-	-	-	-

Values are mean±S.E.M. in human coronary arteries (HCA, n=7-10, except n=3 for NADPH and DPI+NADPH), porcine coronary arteries (PCA, n=5-19) and porcine femoral arteries (PFA, n=5). SOD, superoxide dismutase; X, xanthine; XO, xanthine oxidase; Ang II, angiotensin II; DPI, diphenylene iodonium; * $P<0.05$ vs. without SOD, † $P<0.05$ vs. vehicle, ‡ $P<0.05$ vs. without DPI.

In agreement with the absence of DETCA-induced Ang II potentiation, DPI did also not affect the Ang II CRC in PFAs. The contrasting data between NO donors and Ang II in porcine arteries may relate to 1) the high (pharmacological) NO concentrations that occur following the addition of SNAP or SNP, and 2) the site of superoxide-NO interaction. Under physiological conditions, the NO concentrations are ~3 orders of magnitude below the levels of SOD (Koppenol, 1998), and thus superoxide inactivation will be due to SOD rather than its reaction with NO. DETCA will reverse this situation, particularly following the addition of exogenous NO donors (or endothelial NO-dependent vasorelaxants such as acetylcholine (de Saram *et al.*, 2002; Paravicini *et al.*, 2002)). NO-superoxide interaction may occur anywhere between the NO generation site (i.e., NOS in endothelial cells) and the NO action site (i.e., guanylyl cyclase in vascular smooth muscle cells). Particularly if superoxide is of adventitial origin (Rey *et al.*, 2002), the most likely site of interaction is the extracellular space. Using the cytochrome c reduction, we were however unable to demonstrate an increase in extracellular superoxide over a period of 1 hour following Ang II application. This implies that the previously described Ang II-induced increases in superoxide generation (Berry *et al.*, 2000; Griendling *et al.*, 1994; Laursen *et al.*, 1997; Wang *et al.*, 2001) occur intracellularly and result in limited or no superoxide release to the interstitium. Yet, DETCA (a cell-permeable SOD inhibitor), tempol (a cell-permeable SOD mimetic) nor DPI affected the Ang II-induced vasoconstriction in PFAs. This suggests that the Ang II-induced increases in superoxide occur in an intracellular compartment that does not allow interaction with NO. The superoxide that inactivates NO following the addition of NO donors or acetylcholine apparently resides in a compartment (e.g., the extracellular space) that does allow such interaction, and may thus be of different origin.

In apparent contrast with the above line of reasoning, DPI markedly reduced the effects of Ang II in HCAs, and even larger reductions of the Ang II-induced vasoconstriction were observed in the presence of the NAD(P)H oxidase inhibitor apocynin and the XO inhibitor allopurinol. These data are however difficult to reconcile with the lack of effect of both tempol and DETCA on Ang II-induced vasoconstrictions in human vessels. They also disagree with the limited contribution of XO to vascular superoxide generation (Berry *et al.*, 2000). Consequently, functional (non-superoxide-related) antagonism might underlie these findings. Indeed,

all 3 inhibitors of superoxide generation concentration-dependently relaxed preconstricted HCAs. The relaxant effect of apocynin was unaffected by NOS inhibition and NO scavenging, thereby arguing against the proposal that it is due to a reduction in superoxide generation (and, as a consequence, a rise in NO) (Hamilton *et al.*, 2002). The nonspecific actions of DPI include the blockade of many flavoprotein-dependent enzymes other than NAD(P)H oxidase (e.g., NOS), inhibition of K^+ and Ca^{2+} channels (Weir *et al.*, 1994), and activation of guanylate cyclase (Dodd-o *et al.*, 1997). Thus, it is not surprising that DPI relaxed HCAs, and modestly constricted porcine arteries. Non-specific actions of allopurinol are supported by its cardioprotective properties in rabbits, a species which virtually lacks XO (Godin *et al.*, 1987).

The possibility that functional antagonism, unrelated to the inhibition of superoxide generation, underlies the effects of DPI, apocynin and allopurinol towards Ang II, is further supported by our finding that the inhibitory effects of these drugs were mimicked in relationship with the NAD(P)H oxidase (Touyz *et al.*, 2002)- and/or superoxide (Shastri *et al.*, 2002)-independent vasoconstrictor ET-1.

In further agreement with the concept of functional antagonism, catalase and tiron potentiated Ang II. This suggests that reactive oxygen species cause vasodilation rather than vasoconstriction. Catalase (which does not enter the cell) also tended to potentiate ET-1, and a similar catalase-induced potentiation of norepinephrine has been reported (Kobayashi *et al.*, 2002). Since intracellularly generated H_2O_2, the substrate of catalase, has been proposed to act as an EDHF, the most likely explanation of these findings is that endogenous H_2O_2 (a membrane-permeable factor derived from superoxide, either through spontaneous dismutation or generated by SOD) counteracts vasoconstriction following its release into the extracellular space. Accordingly, exogenous H_2O_2 was found to relax PFAs.

H_2O_2-induced relaxations are believed to depend on activation of large conductance Ca^{2+}-sensitive K^+ channels (BK_{Ca} channels) (Barlow *et al.*, 1998) and/or stimulation of eNOS-dependent NO release (Thomas *et al.*, 2002). AT_1 receptor mediated, H_2O_2-induced NO release has recently been demonstrated in endothelial cells following NAD(P)H oxidase stimulation by Ang II (Cai *et al.*, 2002). Importantly, preliminary findings in the latter study revealed that membranes of

vascular smooth muscle and endothelial cells produce H_2O_2 rather than superoxide when stimulated with NADH. This finding, which the authors contributed to electrostatical hindrance of superoxide release (thereby favoring its spontaneous dismutation to H_2O_2), is in full agreement with our observation of NADH-induced relaxation in human and porcine vessels and the inability of DETCA to block this relaxation. It also explains why no increases in superoxide release were observed following the addition of either NADH or NADPH to human or porcine vessels. It should be noted that, although our data support the likelihood that extracellularly applied NADH reacts with NAD(P)H oxidase, the membrane orientation of vascular NAD(P)H oxidase is still a matter of debate (Griendling *et al.*, 2000). In agreement with the proposal that the classical inside-out orientation is not applicable in non-phagocytic cells (Satriano *et al.*, 1993), several authors have shown superoxide generation following the addition of exogenous NAD(P)H to isolated arteries (Didion *et al.*, 2002a; Pagano *et al.*, 1997).

The NADH-induced relaxations in the present study were unaffected by L-NAME + hydroxocobalamin, and blocked by KCl, thereby favouring a direct relaxant effect of H_2O_2 through BK_{Ca} channels (i.e., in an EDHF-like manner) rather than an NO-dependent effect. The lack of effect of catalase towards NADH is more difficult to explain, but may relate to its inability to fully block excessive H_2O_2 generation following the addition of NADH. The relaxations observed following the addition of xanthine and XO to the organ bath might be explained in the same way, i.e., a predominance of H_2O_2 generation, in particular because Pomposiello et al. (Pomposiello *et al.*, 1999) reported earlier that both 30 mM KCl and catalase prevent xanthine + XO-induced relaxations in PCAs.

In conclusion, our data, when taken together, do not support the idea that acute Ang II-induced vasoconstriction depends on superoxide release and subsequent NO inactivation. This does not exclude the possibility that superoxide, following its intracellular generation, contributes as a signaling messenger to the longterm growth and remodeling effects of Ang II (Liu *et al.*, 2003). In addition, superoxide dismutation, particularly in endothelial cells (Cai *et al.*, 2002), results in the generation of the membrane-permeable vasodilator H_2O_2. Dismutation of NAD(P)H oxidase-derived superoxide, whether spontaneous or mediated via SOD, not only explains why NADH causes vasodilation, but may also underlie the vasodilator effects

that have been observed previously through activation of endothelial AT_1 receptors (Boulanger *et al.*, 1995).

CHAPTER 8
Thesis Summary and General Discussion

8.1. Summary in English

Introduction and aim (Chapter 1)

ACE inhibitors are widely used for the treatment of hypertension and heart failure. Their beneficial effects are usually attributed to blockade of Ang I-to-II conversion. However, since ACE also degrades the vasodilator bradykinin, bradykinin accumulation may also underlie ACE inhibitor-induced effects. In addition, recent studies suggest that ACE inhibitors potentiate bradykinin independent of their effects on bradykinin hydrolysis, through induction of ACE-B_2 receptor 'crosstalk'. Such crosstalk might also be enhanced by the ACE-inhibiting peptide Ang-(1-7), a metabolite of both Ang I and Ang II. Simultaneously, this peptide may exert effects via as yet unidentified 'Ang-(1-7) receptors'.

It was the aim of this thesis to study bradykinin potentiation by ACE inhibitors and Ang-(1-7). Bradykinin potentiation by inhibitors of neutral endopeptidase (NEP), a second enzyme contributing to bradykinin catabolism, was also addressed. In addition, we verified whether bradykinin-induced vasodilation and Ang II-induced vasoconstriction are mediated via NO and superoxide, respectively. Finally, we investigated whether enzymes other than ACE contribute to Ang I-II conversion. Studies were performed in human and porcine coronary arteries, as well as in porcine atrial trabeculae.

Bradykinin-related aspects (Chapters 2-5)

ACE inhibitors as well as Ang-(1-7) enhanced the bradykinin-induced relaxations of porcine coronary arteries. These drugs not only shifted the bradykinin concentration-response curve to the left, but also induced relaxant effects in vessels that, following repeated exposure to bradykinin, no longer responded to bradykinin (desensitized preparations). The effect of Ang-(1-7) was half that of an ACE inhibitor, due to the fact that Ang-(1-7) blocks only one of the two active centers of ACE. Other, non-ACE-related effects of Ang-(1-7) were not observed. Remarkably, despite the many

reports on ACE inhibitor-induced bradykinin potentiation independent of hydrolysis, we were unable to show potentiation when using ACE-resistant bradykinin analogues. Yet, ACE inhibitors did not affect the half-life of bradykinin added to porcine coronary arteries in organ baths. We therefore propose that ACE and B_2 receptors co-localize in the endothelial membrane, allowing ACE to determine the bradykinin concentration in the immediate vicinity of the B_2 receptor. According to this concept, ACE inhibition causes a rise in the bradykinin concentrations that are seen by the B_2 receptor, allowing relaxation to occur even in desensitized preparations. NEP-B_2 receptor co-localization most likely does not occur, since NEP inhibitors did not potentiate bradykinin in porcine coronary arteries. Sterol-binding agents, capable of disrupting caveolae, did not prevent the ACE inhibitor-induced potentiation of bradykinin, suggesting that ACE-B_2 receptor co-localization occurs outside of caveolae.

Bradykinin exerted a modest, short-lasting negative inotropic effect in porcine atrial trabeculae. This effect, as well as the vasorelaxant effect of bradykinin in porcine coronary arteries, was only partially blocked by an inhibitor of NOS, L-NAME. Since more complete inhibition was obtained with the NO scavenger hydroxocobalamin, and in view of the fact that desensitization occurred more rapidly in the presence of L-NAME, our data raise the possibility that bradykinin-induced effects depend on NO from storage sites (e.g., S-nitrosothiols) in addition to *de-novo* synthesized NO.

Angiotensin-related aspects (Chapters 6-7)

Ang I and II equipotently constricted human coronary arteries. The chymase-specific substrate Pro^{11}-D-Ala^{12}-Ang I was less potent than the endogenous angiotensins. Separate ACE or chymase inhibition modestly shifted the Ang I concentration-response curve to the right, whereas a much larger rightward shift was observed when both enzymes were inhibited. Remarkably, despite the similar potency of Ang I and II, the Ang II levels in the organ bath during the Ang I experiments were <5% of the Ang I levels. This suggests that tissue Ang II rather than organ bath fluid Ang II determines vasoconstriction. In support of this concept, the vascular interstitial Ang II levels were found to be equal for a given constriction induced by either Ang I, Ang II, or Pro^{11}-D-Ala^{12}-Ang I. Yet to reach a certain interstitial Ang II level >2 times more

Pro[11]-D-Ala[12]-Ang I than Ang I was required. Moreover, only chymase inhibition diminished the Ang II levels in the organ bath reached in the presence of Pro[11]-D-Ala[12]-Ang I or Ang I, suggesting that Ang II generated by chymase is 'lost' in the organ bath. Apparently therefore, ACE-mediated Ang I-II conversion occurs more efficiently (i.e., closer to AT_1 receptors, with little loss into the organ bath) than chymase-mediated conversion.

Using a wide range of inhibitors and mimetics of superoxide generating/degrading enzymes, we were unable to support the idea that superoxide mediates the acute vasoconstrictor effects of Ang II in human and porcine arteries. In fact, activation of various superoxide-generating pathways resulted in vasodilation rather than vasoconstriction, possibly due to (spontaneous) dismutation of superoxide into the vasodilator H_2O_2. This does not mean that superoxide is unable to cause vasoconstriction, since inhibition of superoxide dismutation reduced the relaxant effect of NO, thereby confirming that superoxide causes vasoconstriction though NO inactivation.

8.2. Samenvatting in het Nederlands

Introductie en doel (Hoofdstuk 1)

ACE remmers worden veel gebruikt bij de behandeling van hypertensie en hartfalen. Hun gunstige effecten worden doorgaans toegeschreven aan de blokkade van de omzetting van Ang I in Ang II. Echter, aangezien ACE ook de vaatverwijdende stof bradykinine afbreekt, zou de accumulatie van bradykinine ook een rol kunnen spelen bij de effecten van ACE remmers. Tevens suggereren recente studies dat ACE remmers bradykinine potentiëren, onafhankelijk van hun effect op de hydrolyse van bradykinine, namelijk via de inductie van ACE-bradykinine type 2 (B_2) receptor "crosstalk". Dergelijke crosstalk zou ook versterkt worden door het ACE-remmende peptide Ang-(1-7), een metaboliet van zowel Ang I als Ang II. Tegelijkertijd zou dit peptide kunnen werken via tot nog toe onbekende "Ang-(1-7) receptoren".

Het doel van dit proefschrift is om de bradykinine potentiëring door ACE remmers en Ang-(1-7) te bestuderen. Tevens wordt de bradykinine potentiëring door remmers van neutraal endopeptidase (NEP), een ander enzym dat bijdraagt aan

bradykinine katabolisme, besproken. Vervolgens wordt geverifieerd of de bradykinine-geïnduceerde vasodilatatie en de Ang II-geïnduceerde vasoconstrictie gemedieerd worden door respectievelijk NO en superoxide. Tenslotte wordt onderzocht of andere enzymen dan ACE een rol spelen in de Ang I-II conversie. De experimenten worden uitgevoerd met humane en varkenskransslagaders, alsmede met atriale trabekels van het varken.

Aspecten gerelateerd aan bradykinine (Hoofdstukken 2-5)

Zowel ACE remmers als Ang-(1-7) versterkten de bradykinine-geïnduceerde relaxatie van kransslagaders van het varken. Door de voorgaande stoffen trad niet alleen een linksverschuiving van de concentratie-effect curves op, maar werd ook een relaxerend effect geïnduceerd in bloedvaten die, na herhaalde blootstelling aan bradykinine, niet langer reageerden op bradykinine (gedesensitiseerde preparaten). Vergeleken met een ACE remmer was het effect van Ang-(1-7) de helft, veroorzaakt door het feit dat Ang-(1-7) slechts één van de twee actieve centra van ACE blokkeert. Andere, niet-ACE gerelateerde, effecten van Ang-(1-7) werden niet geobserveerd. Ondanks de vele studies die ACE remmer-geïnduceerde bradykinine potentiëring onafhankelijk van hydrolyse gerapporteerd hebben, is het opmerkelijk dat wij niet in staat waren om potentiëring aan te tonen gebruik makend van ACE-resistente bradykinine analogen. Aan de andere kant beïnvloedden ACE remmers niet de halfwaarde tijd van bradykinine, toegevoegd aan kransslagaders van varkens in een orgaanbad.

Daarom stellen wij voor dat ACE en B_2 receptoren gecolokaliseerd zijn in het endotheliale membraan, ervoor zorgend dat ACE de bradykinine concentratie bepaalt in de onmiddelijke aanwezigheid van de B_2 receptor. Volgens dit concept veroorzaakt ACE inhibitie een stijging van de bradykinine concentratie welke bemerkt wordt door de B_2 receptor, waardoor relaxaties zelfs in gedesensitiseerde preparaten voorkomen. Er is hoogstwaarschijnlijk geen sprake van NEP-B_2 colokalisatie, aangezien NEP remmers bradykinine niet potentieerden in kransslagaders van het varken. Sterol-bindende middelen, die caveolae kunnen vernietigen, voorkwamen de ACE remmer geïnduceerde bradykinine potentiëring niet, wat suggereert dat ACE-B_2 receptor colokalisatie voorkomt buiten de caveolae.

Bradykinine veroorzaakte een bescheiden, kortdurend negatief inotroop effect op atriale trabekels van het varken. Dit effect, maar ook het vaatverwijdende effect van bradykinine in kransslagaders van het varken, werd slechts gedeeltelijk geblokkeerd door L-NAME, een NOS remmer. Aangezien een completere inhibitie werd gezien met de NO scavenger hydroxycobolamine en gezien het feit dat desensitisatie veel sneller optrad in de aanwezigheid van L-NAME, kan geconcludeerd worden dat de bradykinine-geïnduceerde effecten afhankelijk zijn van NO in opslagplaatsen (bv. In de vorm van S-nitrosothiolen) en van de-novo gesynthetiseerd NO.

Aspecten gerelateerd aan angiotensine (Hoofdstukken 6-7)

Ang I and II contraheerden humane kransslagaders op een equipotente wijze. Het chymase-specifieke substraat Pro^{11}-D-Ala^{12}-Ang I was minder potent dan de endogene angiotensines. Separate ACE of chymase remming veroorzaakte een bescheiden rechtsverschuiving van de Ang I concentratie-effect curve, terwijl een meer uitgesproken rechtsverschuiving werd geobserveerd na remming van beide enzymen tegelijkertijd. Het is opmerkelijk dat, ondanks de gelijke potenties van Ang I en II, de Ang II concentraties <5% van de Ang I concentraties waren gedurende de Ang I experimenten. Dit suggereert dat voornamelijk Ang II in het weefsel, en niet in de orgaan bad vloeistof, de vasoconstrictie bepaalt. Dit concept wordt bevestigd door de bevinding dat de Ang II concentraties in het vasculaire interstitium gelijk bleven bij constricties geïnduceerd door Ang I, Ang II of Pro^{11}-D-Ala^{12}-Ang I. Echter om een bepaalde concentratie van Ang II in het interstitium te bereiken was een 2 maal hogere concentratie Pro^{11}-D-Ala^{12}-Ang I dan Ang I benodigd. Tevens werden de Ang II concentraties in de orgaan baden, bereikt in de aanwezigheid van Pro^{11}-D-Ala^{12}-Ang I of Ang I, alleen geëlimineerd door chymase inhibitie, wat suggereert dat Ang II, gegenereerd door chymase, "verdwijnt" in het orgaan bad. Het lijkt er dus op dat de ACE gemedieerde conversie van Ang I naar Ang II efficiënter (of: dichterbij de AT_1 receptoren, met minder verlies in het orgaan bad) verloopt dan de chymase gemedieerde conversie.

Gebruik makend van verscheidene remmers en mimetica van superoxide aanmakende/afbrekende enzymen, waren wij niet in staat om het idee te onderbouwen dat superoxide de acute vasoconstrictieve effecten van Ang II medieert in slagaderen van de mens en het varken. De activatie van superoxide-genererende pathways

resulteerde zelfs in vasodilatatie in plaats van vasoconstrictie, waarschijnlijk veroorzaakt door (spontane) dismutatie van superoxide naar de vaatverwijder H_2O_2. Dit betekent niet dat superoxide niet in staat is vasoconstrictie te bewerkstelligen, aangezien de inhibitie van superoxide dismutatie het relaxerende effect van NO verminderde, wat bevestigt dat superoxide vasoconstrictie veroorzaakt door NO inactivatie.

8.3. General discussion

Despite the many studies on ACE inhibitor-induced bradykinin potentiation independent of hydrolysis, we were unable to confirm this finding in intact porcine coronary arteries. Various explanations for this discrepancy may be put forward. First, in many studies bradykinin analogues (e.g., [Hyp^3-$Tyr(Me)^8$]-bradykinin) were used that are not ACE-resistant (Danser *et al.*, 2000; Dendorfer *et al.*, 2001a; Gobeil *et al.*, 2002). Second, most studies that investigated ACE-B_2 receptor crosstalk made use of transfected cells that overexpress both ACE and B_2 receptors (Deddish *et al.*, 1998; Marcic *et al.*, 1999; Minshall *et al.*, 1997b). Such high expression levels could lead to interactions that normally do not occur. For instance, co-expression of both NEP and B_2 receptors in CHO cells resulted in NEP-B_2 receptor crosstalk (Deddish *et al.*, 2002), whereas in isolated porcine coronary arteries, despite the presence of NEP in porcine vessels (Miyamoto *et al.*, 2002), NEP inhibition did not affect bradykinin-induced relaxations. Furthermore, expression levels strongly affect ligand-induced sequestration of B_2 receptors in transfected cells, so that B_2 receptor desensitization and/or resensitization will be different in transfected cells (Faussner *et al.*, 2003).

If ACE inhibitor-induced bradykinin potentiation is a matter of metabolism, why does ACE inhibition neither increase the tissue bradykinin levels in patients (Campbell *et al.*, 1999) nor alter the half-life of bradykinin in organ bath fluid in the presence of coronary arteries? The most likely explanation is that ACE co-localizes with B_2 receptors in a compartment in which degradation dramatically impairs bradykinin availability. Application of an ACE inhibitor under such conditions will immediately increase the bradykinin concentrations in the micro-environment of the B_2 receptors (Figure 1), but not at the tissue level. In support of this concept, the existence of a tissue compartment with highly effective bradykinin degradation has been demonstrated in the isolated rat heart during bradykinin distribution studies

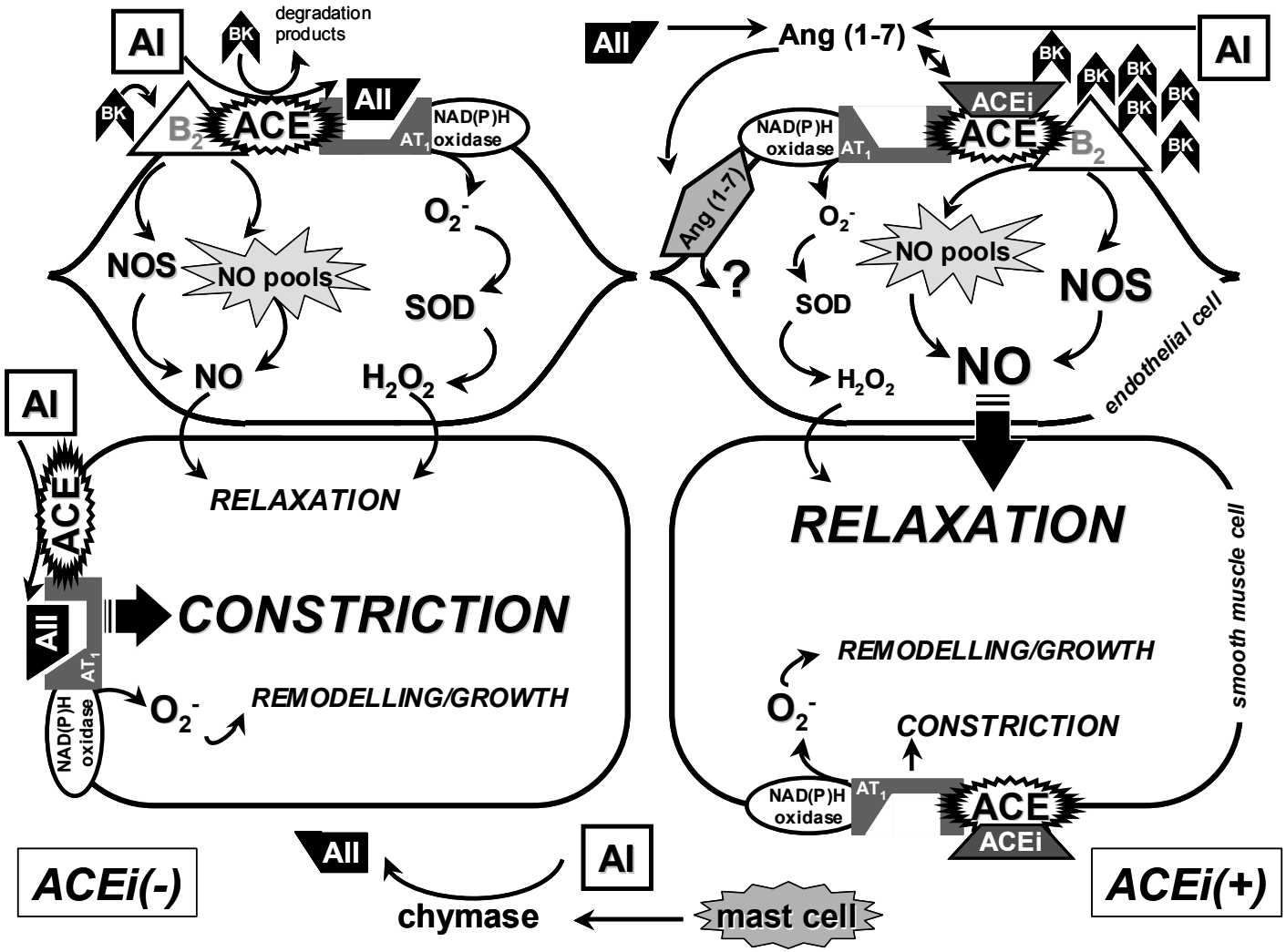


Figure 1: *Unifying scheme*

(Dendorfer *et al.*, 1997b). A potential candidate for the ACE and B_2 receptor-containing compartment are caveolae, since both components have been demonstrated in these structures (Benzing *et al.*, 1999; Haasemann *et al.*, 1998). However, disruption of caveolae with cholesterol-depleting agents did not affect bradykinin potentiation by ACE inhibitors. Thus, co-localization must occur elsewhere, for instance in coated pits or non-caveolar lipid rafts.

The high local efficacy of ACE is not restricted to the inactivation of bradykinin: in the organ bath setup, ACE also effectively activated Ang I locally, resulting in vascular (interstitial) Ang II levels that were up to 27-fold higher than the Ang II levels in the organ bath (Schuijt *et al.*, 2002; Tom *et al.*, 2003). Since interstitial rather than circulating Ang II was found to determine vasoconstriction, these data suggest that ACE and AT_1 receptors, like ACE and B_2 receptors, co-localize in the same compartment (Figure 1). Further evidence for the close anatomical localization of ACE, B_2 receptors and AT_1 receptors comes from a recent observation on AT_1-B_2 receptor heterodimerization, resulting in enhanced G-protein activation and altered receptor sequestration (AbdAlla *et al.*, 2000). Chymase did not co-localize with AT_1 receptors, and thus Ang II generation by this enzyme is less efficient, i.e., chymase needs to generate more Ang II to exert a certain effect, because chymase-derived Ang II is exposed to significant metabolism on its way to AT_1 receptors. This conclusion implies that the high (tissue) Ang II levels that are sometimes observed during ACE inhibition, if due to chymase, do not necessarily result in AT_1 receptor activation.

ACE also degrades Ang-(1-7), a metabolite of both Ang I and Ang II. Ang-(1-7) inhibits the ACE C-domain, and is cleaved by the ACE N-domain. Since bradykinin is cleaved by both ACE domains, potentiation will be maximal at ACE inhibitor concentrations that block both domains, and half-maximal in the presence of Ang-(1-7). All Ang-(1-7)-induced effects described in this thesis were of metabolic origin (i.e., related to ACE inhibition): Ang-(1-7) did not potentiate ACE-resistant bradykinin analogues, and no direct effects of Ang-(1-7) could be detected. The latter finding indicates that the recently identified Ang-(1-7) receptor (Mas protooncogene) is absent in human and porcine coronary arteries.

Finally, AT_1-B_2 receptor co-localization may result in interaction(s) at the post-receptor level. Bradykinin-induced relaxations, as well as its negative inotropic effects, were largely due to *de-novo* synthesized NO and NO from storage sites. AT_1 receptor activation in endothelial cells also results in NO release (Boulanger *et al.*, 1995). NAD(P)H-derived H_2O_2 has been reported to be the mediator of this latter release (Cai *et al.*, 2002). At the same time, H_2O_2 might act as 'endothelium-derived hyperpolarizing factor' (Matoba *et al.*, 2002), i.e., it may mediate bradykinin-induced relaxations in the absence of NO.

Ang II, through activation of NAD(P)H oxidase and/or xanthine oxidase, stimulates superoxide generation in the vascular wall. Although superoxide induces vasoconstriction through NO inactivation, the acute Ang II-induced constrictor effects in human and porcine arteries could not be attributed to superoxide. This does not exclude the possibility that superoxide, following its intracellular generation, contributes to the long-term growth and remodelling effects of Ang II. Indeed, Liu et al. (Liu *et al.*, 2003) recently provided evidence that Ang II-enhanced NAD(P)H oxidase plays a role in the induction of ICAM-1 expression, leukocyte infiltration, and vascular hypertrophy, independently of changes in blood pressure.

CHAPTER 9
Appendix

9.1. Acknowledgement (Dankwoord)

I would like to express my gratitude to the people below who helped me to put this thesis together and shared a wonderful time with me in Holland.

I would like to start with Prof. Dr. Saxena who has opened a new world for me by giving me the opportunity to come to Holland and join his group. Pramod, thank you for giving me this chance, but also for welcoming me in your house and giving me faith and full support. You sat with me behind your computer, taught me how to write an article, how to think and present. I also learned from you that I do not have to know everything, and when this happens simply to say "I do not know".

I would like to thank Prof. Dr. F.C. Tulunay for giving me the chance to come and work with Prof. Dr. Saxena.

Also, I want to express my thanks to Prof. Dr. A.H.J. Danser. Jan, I appreciate your support very much. You not only guided me, but also taught me how to be efficient, to perform research, to write, to think and to develop a personal point of view. Your enthusiasm has always been a challenge for me. Without your deadlines, I would have never been so productive. I also very much enjoyed the scientific and non-scientific discussions we had during the congresses. You were not only a 'boss' but also nice company. I sincerely hope whatever the future brings we will stay in touch.

My thanks also goes to Prof. Dr. D.J.G.M. Duncker, Prof. Dr. P.A.B.M. Smits and Dr. J.W. de Jong for critically reading my thesis, Prof. Dr. Y.S. Bakhle for coming all the way from London and to Prof. Dr. M.A.D.H. Schalekamp and Prof. Dr. J.M.J. Lamers for being part of my promotion committee.

My thankfulness also goes to Jan Heiligers who has done most of the work during my first 1,5 years and provided me with the fresh tissues during the 4 years. Thanks Jan and I hope to enjoy more of your tasteful dinners. I appreciate deeply the technical assistance of René de Vries, Ingrid Garrelds, Jeanette van Gool and René de

Bruijn and the outstanding cooperation with the members of the Rotterdam Heart Valve Bank for providing the human hearts for my experiments.

My appreciation also goes to Magda Busscher-Lauw, Ria Manten and Birgitte Breemerkamp for the administrative work, Corné Tak and Richard van Veghel for helping me with my computer problems.

And of course, the Danser group "Wendy, Martin, Jasper". Thanks a lot for your input and the fun during the congresses. I really had a good time.

Also, my other (ex-)colleagues "Antoinette, Carlos, Sue, Erik, Mechteld, Roeland, Remon, Kapil, Vijay, Suneet, Pankaj, Regien, Uday, Inge, Wenxia, Saurabh, Aloys, Mark and Andor" for the nice time especially during coffee breaks.

I would like to mention some people who helped me to build a new life in Holland. I would like to start with the family Kula (Ergun, Şükriye, Funda, Filiz and Erkan). Since the day I came to The Netherlands you have been a second family for me (Hepinize bana gösterdiğiniz yakın ilgiden dolayı çok teşekkür ederim). My friends "Gül-Togan, Beste(paranymph)-Jerome, Yasemin(paranymph)-Serkan, Pelin-Mustafa, Güçlü, Ruth-Marco, Serap-Mehtap, Zeyno-Bald, Yakut, Guy-Danielle, Mark-Ingrid, Arjan, Hale-Abraham" thanks a lot for the unforgettable days and nights in Holland.

Furthermore, I would like to thank my parents in-law, Nell and Isse. You have accepted me right away and even call me 'daughter'. You have always been supportive, thoughtful and helpful.

It is very hard for me to put into words my gratitude to my parents. You have given me all the love, concern, faith, support and motivation. You worked very hard throughout your lives, faced all the difficulties to provide me and my sisters comfortable, peaceful lives. Indeed, you did succeed... (Anneciğim ve babacığım, size olan minnet duygularımı kelimelerle ifade etmek çok zor. Siz bana sevgi, ilgi, güven, destek ve motivasyon verdiniz. Bütün hayatınız boyunca bana ve kardeşlerime rahat ve huzurlu bir hayat sağlamak için her türlü güçlükle mücadele ettiniz ve bunu başardınız da. Size çok çok teşekkür ederim). I would also like to thank my sisters Berna-Berrak and their husbands Faruk-Elvan and the kids Göktuğ-Bora.

Lastly, I would like to thank Peter for being tremendous support to me with his love, faith and help. My love, I am so happy to be with you and I already look forward to living together in our new house.

9.2. About the author

Beril Tom was born in Kırıkkale, Turkey on 10 January 1972. After attending "T.E.D. Ankara College" in Ankara, Turkey for secondary schooling (1983-1990), she studied medicine at the Ankara University. In 1996, she obtained her medical degree. Thereafter, she started to work as assistant-in-training in Ankara University Department of Pharmacology and Clinical Pharmacology in 1997. During this period, she had the opportunity to work in the Department of Pharmacology Erasmus University Medical Center, Rotterdam, The Netherlands, under the supervision of Prof. Dr. Saxena on migraine research (1999). In 2000, she joined the PhD programme in the same department under the supervision of Prof. Dr. A.H.J. Danser on renin-angiotensin system.

9.3. Publications

Full papers:

Tom B., de Vries P., Heiligers J.P.C., Willems E.W., Scalbert E., Delagrange P. and Saxena P.R. (2001). The lack of effect of the pineal hormone melatonin in an animal model predictive of antimigraine activity. *Cephalalgia, 21(6), 656-663.*

Tom B., de Vries R., Saxena P.R., Danser A.H.J. (2001). Negative inotropic effect of bradykinin in porcine isolated atrial trabeculae: Role of nitric oxide. *J. Hypertension, 19(7), 1289-1293.*

Tom B., de Vries R., Saxena P.R., Danser A.H.J. (2001). Bradykinin potentiation by angiotensin-(1-7) and ACE inhibitors correlates with ACE C- and N- domain blockade. *Hypertension, 38(1), 95-99.*

Tom B., de Vries P., Heiligers J.P.C., Willems E.W., Kapoor K., John G.W. and Saxena P.R. (2002). Effects of donitriptan on carotid haemodynamics and cardiac output distribution in anaesthetised pigs. *Cephalalgia, 22(1), 37-47.*

Tom B., Dendorfer A., de Vries R., Saxena P.R., Danser A.H.J., (2002). Bradykinin potentiation by ACE inhibitors: A matter of metabolism. *Br. J. Pharmacol., 137(2), 276-284.*

Tom B., Dendorfer A., Danser A.H.J., (2003). Bradykinin, angiotensin-(1-7), and ACE inhibitors: How do they interact? *Int. J. Biochem. Cell Biol., 1413:1-10.*

Tom B., Scalbert E., Stegmann A.P.A., Boomsma F., Saxena P.R., Danser A.H.J. (2003). ACE- versus chymase-dependent angiotensin II generation in human coronary arteries: A matter of efficiency? *Arterioscler. Thromb. Vasc. Biol., 23(2):251-256.*

Tom B., Batenburg W.W., van Gool J.M.G., de Vries R., Danser A.H.J. (2003). ACE C-domain inhibition is sufficient to block angiotensin II generation by tissue ACE. *Submitted (Hypertension).*

Danser A.H.J., Tom B., de Vries R., Saxena P.R. (2000). L-NAME-resistant bradykinin-induced relaxation in porcine coronary arteries is NO-dependent. Effect of ACE inhibition. *Br. J. Pharmacol., 131, 195-202.*

Opgaard O.S., Tom B., de Vries R., Edvinson L., Saxena P.R. (2000). Modulation of contractile force by endothelin receptors in porcine myocardial trabeculae. *Pharmacology & Toxicology, 87(4), 185-192.*

Opgaard O.S., Tom B., de Vries R, Saxena PR, Edvinsson L (2000). Inotropic effects of endothelin compared to noradrenaline in porcine myocardial trabeculae. *J. Cardiovasc. Pharmacol., 36 (5 Suppl. 1), S172-S175.*

Schuijt M.P., Tom B., de Vries R., Saxena P.R., Sluiter W., van Kats J.P., Danser A.H.J. (2003). Superoxide does not mediate the acute vasoconstrictor effects of angiotensin II. A study in human and porcine arteries. *J. Hypertension, in press.*

Opgaard O.S., de Vries R., Tom B., Edvinson L., Saxena P.R. (1999). Positive inotropy of calcitonin gene-related peptide and amylin on porcine isolated myocardium. *Eur. J. Pharmacol., 385(2-3), 147-154.*

Opgaard O.S., Knutsson M., de Vries R., Tom B., Saxena P.R., Edvinsson L. (2001). Vasoactive intestinal peptide has a direct positive inotropic effect on isolated human myocardial trabeculae. *Clin. Sci. (Lond), 101(6), 637-43.*

Willems, E.W., Heiligers, J.P.C., de Vries, P., Tom B., Kapoor K., Villalón, C.M. & Saxena, P.R. (2001). A61603-induced vasoconstriction in the porcine carotid vasculature: involvement of a non-adrenergic mechanism. *Eur. J. Pharmacol. 417(3), 195-201.*

Willems E.W., Heiligers J.P.C., de Vries P., Kapoor K., Tom B., Villalón C.M. & Saxena P.R. (2001). α_1-Adrenoceptor subtypes mediating vasoconstriction in the carotid circulation of anaesthetised pigs; possible avenues for antimigraine drug development. *Cephalalgia, 21(2), 110-119.*

Abstracts:

Tom B., Derici K., Demirel Yılmaz E. (1998). Guanylate cyclase inhibition by methylene blue depressed cardiac functions. *Naunyn-Schmiedeberg's Archives of Pharmacology (Suppl. 1), 358(1):1824.*

Tom B., de Vries R., Saxena P.R., Danser A.H.J. (2000). Biphasic inotropic effect of bradykinin in isolated atrial trabeculae. Role of ACE and NO. *J. Hypertension (Suppl. 4), 18: S112.*

Tom B., Danser A.H.J. (2000). Angiotensin (Ang) II synthesized insitu by ACE and chymase in human coronary arteries: Comparison of its vasoconstrictor effectiveness with that of circulating Ang II. *Circulation (Suppl. S), 102 (18):1191.*

Tom B., de Vries R., Saxena P.R., Danser A.H.J. (2001). Bradykinin potentiation by ACE inhibitors: Inhibition of metabolism, protein kinase C (PKC) or phosphatases? *Hypertension 38(3):P56.*

Tom B., de Vries P., Heiligers J.P.C., Willems E.W., Kapoor K., John G.W., Saxena P.R. (2001). Systemic and carotid haemodynamic effects of the novel 5-$HT_{1B/1D}$ receptor agonist donitriptan in anaesthetised pigs. *Eur. Neuropsycopharmacology (Suppl. 3), 11:S340.*

Tom B., Schuijt MP, Saxena P.R., Danser A.H.J. (2001). Angiotensin II-induced vasoconstriction in human coronary arteries: Importance of superoxide. *Circulation (Suppl. S), 104(17):877.*

Tom B., de Vries R., Saxena P.R., Danser A.H.J. (2002). ACE-mediated angiotensin I-II conversion in human coronary arteries occurs more efficiently than chymase-mediated conversion. *J. Hypertension (Suppl. 4), 20:S12, O045.*

Tom B., Schuijt M.P., de Vries R., Saxena P.R., Danser A.H.J. (2002). Does NAD(P)H oxidase-dependent superoxide production mediate angiotensin (Ang) II-induced vasoconstriction? *Hypertension 40(4):PC07.*

Tom B., Schuijt MP, Saxena P.R., Danser A.H.J. (2003). No role for oxygen-derived free radicals in angiotensin II-mediated vasoconstriction of human coronary arteries. *Br. J. Pharmacol. (Suppl. S), 138:33P.*

Ergün H., Tom B., Ülgen N., Derici K., Tulunay F.C. (1998). Double-blind study with dipyrone versus diclofenac sodium in acute renal colic pain in Turkish patients. *The Pain Clinics, 239:270.*

Ergün H, Tom B., Mihoglu H., Tulunay F.C. (1998). Quality of life survey in Turkish migraine and tension type headache patients. *Functional Neurology, (13)2:152.*

Akın D., Tom B., Onaran O.H., Gürdal H. (1998). Contribution of spontaneous beta-adrenoceptor activity in forskolin stimulated adenylyl cyclase activity in rat heart membranes. *Naunyn-Schmiedeberg's Archives of Pharmacology (Suppl. 2), 358(1):686.*

Derici M.K., Tom B., Samsar U., Yılmaz E.D. (1998). The effects of L-NAME on cardiac functions. *Naunyn-Schmiedeberg's Archives of Pharmacology (Suppl. 1), 358(1):318.*

Schuijt M.P., Tom B., de Vries R., Saxena P.R., Danser A.H.J. (2002). Opposing role of superoxide in angiotensin II-induced vasoconstriction in human and porcine vessels. *J. Hypertension (Suppl. 4), 20:S108, P0424.*

Schuijt M.P., Tom B., de Vries R, Saxena PR, Danser A.H.J. (2002). Does superoxide generation mediate angiotensin II-induced vasoconstriction: A matter of compartmentalization. *Hypertension 40(3):61.*

Schuijt M.P., Tom B., de Vries R., Sluiter W., Saxena P.R., Danser A.H.J. (2003). Superoxide: A direct or an indirect vasoconstrictor? *Br. J. Pharmacol. (Suppl. S), 138:228P.*

Danser A.H.J., de Vries R., Tom B., Saxena P.R. (2000). Potentiation of bradykinin by angiotensin-(1-7), quinaprilat and captopril correlates directly with inhibition of the ACE C- and N-terminal domains. *Hypertension 36(4):699-699.*

Danser A.H.J., de Vries R., Tom B., Saxena P.R., Dendorfer A. (2002). Bradykinin potentiation by quinaprilat and angiotensin-(1-7): An effect beyond blocking hydrolysis. *J. Hypertension (Suppl. 4), 20:S286, P1210.*

Ergün H., Özdomaniç I.F., Açar H.I., Tom B., Mihoglu H., Tulunay F.C. (1998). The relationship between verbal score, visual analog scale and percentile score in Turkish patients. *The Pain Clinics, 169:119.*

Willems E.W., de Vries P., Heiligers J.P.C., Tom B., Villalón C.M. & Saxena P.R. (2000). Carotid vascular effects mediated by alpha(1)-adrenoceptors in anaesthetised pigs: Possible implications for migraine therapy. *Br. J. Pharmacol. (Suppl. S), 129:54P.*

9.4. List of abbreviations

ACE	angiotensin-converting enzyme
Ang	angiotensin
PA-Ang I	Pro^{11}-D-Ala^{12}-angiotensin I
B_1	bradykinin type 1
B_2	bradykinin type 2
NO	nitric oxide
NOS	nitric oxide synthase
eNOS	endothelial nitric oxide synthase
NEP	neutral endopeptidase
PEP	prolyl endopeptidase
PCP	prolyl carboxypeptidase
AT_1	angiotensin II type 1
AT_2	angiotensin II type 2
CHO	Chinese hamster ovary
PKC	protein kinase C
ANP	atrial natriuretic peptide
SHR	spontaneously hypertensive rats
BK	bradykinin
PCA	porcine coronary artery
HCA	human coronary artery
PFA	porcine femoral artery
BK_{Ca}	large-conductance voltage and Ca^{2+}-activated K^+-channel
SK_{Ca}	small-conductance Ca^{2+}-activated K^+-channel
CRC	concentration response curve
H_2O_2	hydrogen peroxide
SOD	superoxide dismutase
ecSOD	extracellular SOD
EDHF	endothelium-derived hyperpolarizing factor
DPI	diphenyleneiodonium
XO	xantine oxidase
ET-1	endothelin 1
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
U46619	9,11-dideoxy-11α,9α- epoxymethano-prostaglandin $F_{2\alpha}$
L-NAME	N^{ω}-nitro-L-arginine methyl ester HCl

9.5. References

Abbas, A., Gorelik, G., Carbini, L.A. & Scicli, A.G. (1997). Angiotensin-(1-7) induces bradykinin-mediated hypotensive responses in anesthetized rats. *Hypertension*, **30,** 217-221.

AbdAlla, S., Lother, H. & Quitterer, U. (2000). AT_1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. *Nature*, **407,** 94-98.

Akasu, M., Urata, H., Kinoshita, A., Sasaguri, M., Ideishi, M. & Arakawa, K. (1998). Differences in tissue angiotensin II-forming pathways by species and organs in vitro. *Hypertension*, **32,** 514-520.

Anning, P.B., Grocott-Mason, R.M., Lewis, M.J. & Shah, A.M. (1995). Enhancement of left ventricular relaxation in the isolated heart by an angiotensin-converting enzyme inhibitor. *Circulation*, **92,** 2660-2665.

Arnal, J.F., Clamens, S., Pechet, C., Negre-Salvayre, A., Allera, C., Girolami, J.P., Salvayre, R. & Bayard, F. (1996). Ethinylestradiol does not enhance the expression of nitric oxide synthase in bovine endothelial cells but increases the release of bioactive nitric oxide by inhibiting superoxide anion production. *Proc. Natl. Acad. Sci. U.S.A.*, **93,** 4108-4113.

Auch-Schwelk, W., Bossaller, C., Claus, M., Graf, K., Gräfe, M. & Fleck, E. (1993). ACE inhibitors are endothelium dependent vasodilators of coronary arteries during submaximal stimulation with bradykinin. *Cardiovasc. Res.*, **27,** 312-317.

Bachvarov, D.R., Houle, S., Bachvarova, M., Bouthillier, J., Adam, A. & Marceau, F. (2001). Bradykinin B_2 receptor endocytosis, recycling, and down-regulation assessed using green fluorescent protein conjugates. *J. Pharmacol. Exp. Ther.*, **297,** 19-26.

Barlow, R.S. & White, R.E. (1998). Hydrogen peroxide relaxes porcine coronary arteries by stimulating BK_{Ca} channel activity. *Am. J. Physiol.*, **275,** H1283-H1289.

Baumgarten, C.R., Linz, W., Kunkel, G., Schölkens, B.A. & Wiemer, G. (1993). Ramiprilat increases bradykinin outflow from isolated hearts of rat. *Br. J. Pharmacol.*, **108,** 293-295.

Benter, I.F., Ferrario, C.M., Morris, M. & Diz, D.I. (1995). Antihypertensive actions of angiotensin-(1-7) in spontaneously hypertensive rats. *Am. J. Physiol.*, **269,** H313-H319.

Benzing, T., Fleming, I., Blaukat, A., Müller-Esterl, W. & Busse, R. (1999). Angiotensin-converting enzyme inhibitor ramiprilat interferes with the sequestration of the B_2 kinin receptor within the plasma membrane of native endothelial cells. *Circulation*, **99,** 2034-2040.

Berry, C., Hamilton, C.A., Brosnan, M.J., Magill, F.G., Berg, G.A., McMurray, J.J. & Dominiczak, A.F. (2000). Investigation into the sources of superoxide in human blood vessels : Angiotensin II increases superoxide production in human internal mammary arteries. *Circulation*, **101,** 2206-2212.

Bjornstad-Ostensen, A., Holte, H.R. & Berg, T. (1997). Amplification of kinin-induced hypotension by nitric oxide synthesis in spontaneously hypertensive rats. *Hypertension*, **29,** 53-57.

Blais, C., Drapeau, G., Raymond, P., Lamontagne, D., Gervais, N., Venneman, I. & Adam, A. (1997). Contribution of angiotensin-converting enzyme to the cardiac metabolism of bradykinin: an interspecies study. *Am. J. Physiol.*, **273,** H2263-H2271.

Blais, C., Jr., Fortin, D., Rouleau, J.L., Molinaro, G. & Adam, A. (2000). Protective effect of omapatrilat, a vasopeptidase inhibitor, on the metabolism of bradykinin in normal and failing human hearts. *J. Pharmacol. Exp. Ther.*, **295,** 621-626.

Blaukat, A., AbdAlla, S., Lohse, M.J. & Müller-Esterl, W. (1996). Ligand-induced phosphorylation/dephosphorylation of the endogenous bradykinin B_2 receptor from human fibroblasts. *J. Biol. Chem.*, **271,** 32366-32374.

Bolotina, V.M., Najibi, S., Palacino, J.J., Pagano, P.J. & Cohen, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, **368,** 850-853.

Bönner, G., Preis, S., Schunk, U., Toussaint, C. & Kaufmann, W. (1990). Hemodynamic effects of bradykinin on systemic and pulmonary circulation in healthy and hypertensive humans. *J. Cardiovasc. Pharmacol.*, **15,** S46-S56.

Boulanger, C.M., Caputo, L. & Levy, B.I. (1995). Endothelial AT_1-mediated release of nitric oxide decreases angiotensin II contractions in rat carotid artery. *Hypertension*, **26,** 752-757.

Boulanger, C.M., Heymes, C., Benessiano, J., Geske, R.S., Levy, B.I. & Vanhoutte, P.M. (1998). Neuronal nitric oxide synthase is expressed in rat vascular smooth muscle cells: activation by angiotensin II in hypertension. *Circ. Res.*, **83,** 1271-1278.

Brosnihan, K.B., Li, P. & Ferrario, C.M. (1996). Angiotensin-(1-7) dilates canine coronary arteries through kinins and nitric oxide. *Hypertension*, **27,** 523-528.

Cai, H., Li, Z., Dikalov, S., Holland, S.M., Hwang, J., Jo, H., Dudley, S.C., Jr. & Harrison, D.G. (2002). NAD(P)H oxidase-derived hydrogen peroxide mediates endothelial nitric oxide production in response to angiotensin II. *J. Biol. Chem.*, **277,** 48311-48317.

Campbell, D.J., Duncan, A.M. & Kladis, A. (1999). Angiotensin-converting enzyme inhibition modifies angiotensin but not kinin peptide levels in human atrial tissue. *Hypertension*, **34,** 171-175.

Campbell, D.J., Kladis, A. & Duncan, A.M. (1993a). Bradykinin peptides in kidney, blood, and other tissues of the rat. *Hypertension*, **21,** 155-165.

Campbell, D.J., Kladis, A. & Duncan, A.M. (1993b). Nephrectomy, converting enzyme inhibition, and angiotensin peptides. *Hypertension*, **22,** 513-522.

Chappell, M.C., Pirro, N.T., Sykes, A. & Ferrario, C.M. (1998). Metabolism of angiotensin-(1-7) by angiotensin-converting enzyme. *Hypertension*, **31,** 362-367.

Cheng, C.P., Onishi, K., Ohte, N., Suzuki, M. & Little, W.C. (1998). Functional effects of endogenous bradykinin in congestive heart failure. *J. Am. Coll. Cardiol.*, **31,** 1679-1686.

Clerk, A., Gillespie-Brown, J., Fuller, S.J. & Sugden, P.H. (1996). Stimulation of phosphatidylinositol hydrolysis, protein kinase C translocation, and mitogen-activated protein kinase activity by bradykinin in rat ventricular myocytes: dissociation from the hypertrophic response. *Biochem. J.*, **317,** 109-118.

Cohen, R.A., Plane, F., Najibi, S., Huk, I., Malinski, T. & Garland, C.J. (1997). Nitric oxide is the mediator of both endothelium-dependent relaxation and hyperpolarization of the rabbit carotid artery. *Proc. Natl. Acad. Sci. U.S.A.*, **94,** 4193-4198.

Colombari, E., Davisson, R.L., Shaffer, R.A., Talman, W.T. & Lewis, S.J. (1998). Hemodynamic effects of L-glutamate in NTS of conscious rats: a possible role of vascular nitrosyl factors. *Am. J. Physiol.*, **274,** H1066-H1074.

Coulet, F., Gonzalez, W., Boixel, C., Meilhac, O., Pueyo, M.E. & Michel, J.B. (2001). Endothelium-independent conversion of angiotensin I by vascular smooth muscle cells. *Cell. Tissue Res.*, **303,** 227-234.

Cyr, M., Lepage, Y., Blais, C., Gervais, N., Cugno, M., Rouleau, J.L. & Adam, A. (2001). Bradykinin and des-Arg$^{(9)}$-bradykinin metabolic pathways and kinetics of activation of human plasma. *Am. J. Physiol.*, **281,** H275-H283.

Danser, A.H., Chowdury, S., de Lannoy, L.M., van der Giessen, W.J., Saxena, P.R. & Schalekamp, M.A. (1995). Conversion and degradation of [^{125}I] labelled angiotensin I in isolated perfused porcine coronary and carotid arteries. *Cardiovasc. Res.*, **29,** 789-795.

Danser, A.H.J., de Vries, R., Schoemaker, R.G. & Saxena, P.R. (1998). Bradykinin-induced release of nitric oxide by the isolated perfused rat heart: importance of preformed pools of nitric oxide-containing factors. *J. Hypertens.*, **16,** 239-244.

Danser, A.H.J., Tom, B., de Vries, R. & Saxena, P.R. (2000). L-NAME resistant bradykinin-induced relaxation in porcine coronary arteris is NO-dependent: effect of ACE inhibition. *Br. J. Pharmacol.*, **131,** 195-202.

Darblade, B., Caillaud, D., Poirot, M., Fouque, M., Thiers, J.C., Rami, J., Bayard, F. & Arnal, J.F. (2001). Alteration of plasmalemmal caveolae mimics endothelial dysfunction observed in atheromatous rabbit aorta. *Cardiovasc. Res.*, **50,** 566-576.

Davie, A.P. & McMurray, J.J. (1999). Effect of angiotensin-(1-7) and bradykinin in patients with heart failure treated with an ACE inhibitor. *Hypertension*, **34,** 457-460.

Davisson, R.L., Bates, J.N., Johnson, A.K. & Lewis, S.J. (1996). 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*, **28,** 354-360.

de Lannoy, L.M., Schuijt, M.P., Saxena, P.R., Schalekamp, M.A. & Danser, A.H. (2001). Angiotensin converting enzyme is the main contributor to angiotensin I-II conversion in the interstitium of the isolated perfused rat heart. *J. Hypertens.*, **19,** 959-965.

de Lean, A., Munson, P.J. & Rodbard, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.*, **235,** E97-E102.

de Saram, K., McNeill, K.L., Khokher, S., Ritter, J.M. & Chowienczyk, P.J. (2002). Divergent effects of vitamin C on relaxations of rabbit aortic rings to acetylcholine and NO-donors. *Br. J. Pharmacol.*, **135,** 1044-1050.

de Vries, P., Willems, E.W., Heiligers, J.P.C., Villalón, C.M. & Saxena, P.R. (1999). Investigation of the role of 5-HT_{1B} and 5-HT_{1D} receptors in the sumatriptan-induced constriction of porcine carotid arteriovenous anastomoses. *Br. J. Pharmacol.*, **127,** 405-412.

Deddish, P.A., Marcic, B., Jackman, H.L., Wang, H.Z., Skidgel, R.A. & Erdös, E.G. (1998). N-domain-specific substrate and C-domain inhibitors of angiotensin-converting enzyme: angiotensin-(1-7) and keto-ACE. *Hypertension*, **31,** 912-917.

Deddish, P.A., Marcic, B.M., Tan, F., Jackman, H.L., Chen, Z. & Erdös, E.G. (2002). Neprilysin inhibitors potentiate effects of bradykinin on B_2 receptor. *Hypertension*, **39,** 619-623.

Dendorfer, A., Reissmann, S., Wolfrum, S., Raasch, W. & Dominiak, P. (2001a). Potentiation of kinin analogues by ramiprilat is exclusively related to their degradation. *Hypertension*, **38,** 142-146.

Dendorfer, A., Vordermark, D. & Dominiak, P. (1997a). Degradation of bradykinin by bovine tracheal epithelium and isolated epithelial cells. *Br. J. Pharmacol.*, **120,** 121-129.

Dendorfer, A., Wolfrum, S., Schafer, U., Stewart, J.M., Inamura, N. & Dominiak, P. (2000). Potentiation of the vascular response to kinins by inhibition of myocardial kininases. *Hypertension*, **35,** 32-37.

Dendorfer, A., Wolfrum, S., Wagemann, M., Qadri, F. & Dominiak, P. (2001b). Pathways of bradykinin degradation in blood and plasma of normotensive and hypertensive rats. *Am. J. Physiol.*, **280,** H2182-H2188.

Dendorfer, A., Wolfrum, S., Wellhoner, P., Korsman, K. & Dominiak, P. (1997b). Intravascular and interstitial degradation of bradykinin in isolated perfused rat heart. *Br. J. Pharmacol.*, **122,** 1179-1187.

Didion, S.P. & Faraci, F.M. (2002a). Effects of NADH and NADPH on superoxide levels and cerebral vascular tone. *Am. J. Physiol.*, **282,** H688-H695.

Didion, S.P., Ryan, M.J., Baumbach, G.L., Sigmund, C.D. & Faraci, F.M. (2002b). Superoxide contributes to vascular dysfunction in mice that express human renin and angiotensinogen. *Am. J. Physiol.*, **283,** H1569-H1576.

Dodd-o, J.M. & Pearse, D.B. (2000). Effect of the NADPH oxidase inhibitor apocynin on ischemia-reperfusion lung injury. *Am. J. Physiol.*, **279,** H303-H312.

Dodd-o, J.M., Zheng, G., Silverman, H.S., Lakatta, E.G. & Ziegelstein, R.C. (1997). Endothelium-independent relaxation of aortic rings by the nitric oxide synthase inhibitor diphenyleneiodonium. *Br. J. Pharmacol.*, **120,** 857-864.

Du, X.Y., Schoemaker, R.G., X462P, Bos, E. & Saxena, P.R. (1993). Effects of histamine on porcine isolated myocardium: differentiation from effects on human tissue. *J. Cardiovasc. Pharmacol.*, **22,** 468-473.

Duka, I., Kintsurashvili, E., Gavras, I., Johns, C., Bresnahan, M. & Gavras, H. (2001). Vasoactive potential of the B_1 bradykinin receptor in normotension and hypertension. *Circ. Res.*, **88,** 275-281.

Duncan, A.M., Kladis, A., Jennings, G.L., Dart, A.M., Esler, M. & Campbell, D.J. (2000). Kinins in humans. *Am. J. Physiol.*, **278,** R897-R904.

d'Uscio, L.V., Quaschning, T., Burnett, J.C. & Lüscher, T.F. (2001). Vasopeptidase inhibition prevents endothelial dysfunction of resistance arteries in salt-sensitive hypertension in comparison with single ACE inhibition. *Hypertension*, **37,** 28-33.

Dussaule, J.C., Stefanski, A., Bea, M.L., Ronco, P. & Ardaillou, R. (1993). Characterization of neutral endopeptidase in vascular smooth muscle cells of rabbit renal cortex. *Am. J. Physiol.*, **264,** F45-F52.

Edwards, G., Dora, K.A., Gardener, M.J., Garland, C.J. & Weston, A.H. (1998). K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature*, **396,** 269-272.

Emanueli, C., Maestri, R., Corradi, D., Marchione, R., Minasi, A., Tozzi, M.G., Salis, M.B., Straino, S., Capogrossi, M.C., Olivetti, G. & Madeddu, P. (1999). Dilated and failing cardiomyopathy in bradykinin B_2 receptor knockout mice. *Circulation*, **100,** 2359-2365.

Erdös, E.G. (2002). Kinins, the long march - A personal view. *Cardiovasc. Res.*, **54,** 485-491.

Farquharson, C.A. & Struthers, A.D. (2002). 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.*, **39,** 767-775.

Faussner, A., Bauer, A., Kalatskaya, I., Jochum, M. & Fritz, H. (2003). Regulation of cardiovascular signaling by kinins and products of similar converting enzyme systems: Expression levels strongly affect ligand-induced sequestration of B_2 bradykinin receptors in transfected cells. *Am. J. Physiol.*, **284,** H1892-H1898.

Fernandes, L., Fortes, Z.B., Nigro, D., Tostes, R.C., Santos, R.A. & Catelli de Carvalho, M.H. (2001). Potentiation of bradykinin by angiotensin-(1-7) on arterioles of spontaneously hypertensive rats studied in vivo. *Hypertension*, **37,** 703-709.

Ferrario, C.M., Chappell, M.C., Tallant, E.A., Brosnihan, K.B. & Diz, D.I. (1997). Counterregulatory actions of angiotensin-(1-7). *Hypertension*, **30,** 535-541.

Ferreira, A.J., Santos, R.A. & Almeida, A.P. (2001). Angiotensin-(1-7): cardioprotective effect in myocardial ischemia/reperfusion. *Hypertension*, **38,** 665-668.

Fisslthaler, B., Popp, R., Kiss, L., Potente, M., Harder, D.R., Fleming, I. & Busse, R. (1999). Cytochrome P_{450} 2C is an EDHF synthase in coronary arteries. *Nature*, **401,** 493-497.

Gardiner, S.M., Compton, A.M., Kemp, P.A. & Bennett, T. (1990). Regional and cardiac haemodynamic responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 in conscious rats: effects of NG-nitro-L-arginine methyl ester. *Br. J. Pharmacol.*, **101,** 632-639.

Garrison, E.A., Champion, H.C. & Kadowitz, P.J. (1997). [Pro^{11},D-Ala^{12}]angiotensin I has rapid onset vasoconstrictor activity in the cat. *Am. J. Physiol.*, **273,** E1059-E1064.

Garthwaite, J., Southam, E., Boulton, C.L., Nielsen, E.B., Schmidt, K. & Mayer, B. (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol. Pharmacol.*, **48,** 184-188.

Gobeil, F., Halle, S., Blais, P.A. & Regoli, D. (2002). Studies on the angiotensin-converting enzyme and the kinin B_2 receptor in the rabbit jugular vein: modulation of contractile response to bradykinin. *Can. J. Physiol. Pharmacol.*, **80,** 153-163.

Godin, D.V. & Bhimji, S. (1987). Effects of allopurinol on myocardial ischemic injury induced by coronary artery ligation and reperfusion. *Biochem. Pharmacol.*, **36,** 2101-2107.

Gohlke, P., Kuwer, I., Schnell, A., Amann, K., Mall, G. & Unger, T. (1997). Blockade of bradykinin B_2 receptors prevents the increase in capillary density induced by chronic angiotensin-converting enzyme inhibitor treatment in stroke-prone spontaneously hypertensive rats. *Hypertension*, **29,** 478-482.

Gohlke, P., Linz, W., Scholkens, B.A., Kuwer, I., Bartenbach, S., Schnell, A. & Unger, T. (1994). Angiotensin-converting enzyme inhibition improves cardiac function. Role of bradykinin. *Hypertension*, **23,** 411-418.

Golser, R., Gorren, A.C., Leber, A., Andrew, P., Habisch, H.J., Werner, E.R., Schmidt, K., Venema, R.C. & Mayer, B. (2000). Interaction of endothelial and neuronal nitric-oxide synthases with the bradykinin B_2 receptor. Binding of an inhibitory peptide to the oxygenase domain blocks uncoupled NADPH oxidation. *J. Biol. Chem.*, **275,** 5291-5296.

Gonzalez, W., Soleilhac, J.M., Fournie-Zaluski, M.C., Roques, B.P. & Michel, J.B. (1998). Characterization of neutral endopeptidase in vascular cells, modulation of vasoactive peptide levels. *Eur. J. Pharmacol.*, **345,** 323-331.

Gorelik, G., Carbini, L.A. & Scicli, A.G. (1998). Angiotensin-(1-7) induces bradykinin-mediated relaxation in porcine coronary artery. *J. Pharmacol. Exp. Ther.*, **286,** 403-410.

Graf, K., Gräfe, M., Bossaller, C., Niehus, J., Schulz, K.D., Auch-Schwelk, W. & Fleck, E. (1993). Degradation of bradykinin by neutral endopeptidase (EC 3.4.24.11) in cultured human endothelial cells. *Eur. J. Clin. Chem. Clin. Biochem.*, **31,** 267-272.

Griendling, K.K., Minieri, C.A., Ollerenshaw, J.D. & Alexander, R.W. (1994). Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ. Res.*, **74,** 1141-1148.

Griendling, K.K., Sorescu, D. & Ushio-Fukai, M. (2000). NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ. Res.*, **86,** 494-501.

Guo, C., Ju, H., Leung, D., Massaeli, H., Shi, M. & Rabinovitch, M. (2001). A novel vascular smooth muscle chymase is upregulated in hypertensive rats. *J. Clin. Invest.*, **107,** 703-715.

Haasemann, M., Cartaud, J., Müller-Esterl, W. & Dunia, I. (1998). Agonist-induced redistribution of bradykinin B_2 receptor in caveolae. *J. Cell. Sci.*, **111,** 917-928.

Hamilton, C.A., Brosnan, M.J., Al-Benna, S., Berg, G. & Dominiczak, A.F. (2002). NAD(P)H oxidase inhibition improves endothelial function in rat and human blood vessels. *Hypertension*, **40,** 755-762.

Hamilton, C.A., McPhaden, A.R., Berg, G., Pathi, V. & Dominiczak, A.F. (2001). Is hydrogen peroxide an EDHF in human radial arteries? *Am. J. Physiol.*, **280,** H2451-H2455.

Hecker, M., Pörsti, I., Bara, A.T. & Busse, R. (1994). Potentiation by ACE inhibitors of the dilator response to bradykinin in the coronary microcirculation: interaction at the receptor level. *Br. J. Pharmacol.*, **111,** 238-244.

Hilgers, K.F. & Stumpf, C. (2002). Angiotensin II, the endothelium and superoxide anions. *J. Hypertens.*, **20,** 1271-1273.

Hirooka, Y., Imaizumi, T., Masaki, H., Ando, S., Harada, S., Momohara, M. & Takeshita, A. (1992). Captopril improves impaired endothelium-dependent vasodilation in hypertensive patients. *Hypertension*, **20,** 175-180.

Hodges, G.R., Young, M.J., Paul, T. & Ingold, K.U. (2000). How should xanthine oxidase-generated superoxide yields be measured? *Free Radic. Biol. Med.*, **29,** 434-441.

Holubarsch, C., Hasenfuss, G., Schmidt-Schweda, S., Knorr, A., Pieske, B., Ruf, T., Fasol, R. & Just, H. (1993). Angiotensin I and II exert inotropic effects in atrial but not in ventricular human myocardium. An in vitro study under physiological experimental conditions. *Circulation*, **88,** 1228-1237.

Hornig, B., Kohler, C. & Drexler, H. (1997). Role of bradykinin in mediating vascular effects of angiotensin-converting enzyme inhibitors in humans. *Circulation*, **95,** 1115-1118.

Ignarro, L.J. (1990). Nitric oxide. A novel signal transduction mechanism for transcellular communication. *Hypertension*, **16,** 477-483.

Ignjatovic, T., Tan, F., Brovkovych, V., Skidgel, R.A. & Erdös, E.G. (2002). Novel mode of action of angiotensin I-converting enzyme inhibitors: direct activation of bradykinin B_1 receptor. *J. Biol. Chem.*, **277,** 16847-16852.

Inoue, K., Nishimura, H., Kubota, J. & Kawamura, K. (1999). Alternative angiotensin II formation in rat arteries occurs only at very high concentrations of angiotensin I. *Hypertension*, **34,** 525-530.

Ishigai, Y., Mori, T., Ikeda, T., Fukuzawa, A. & Shibano, T. (1997). Role of bradykinin-NO pathway in prevention of cardiac hypertrophy by ACE inhibitor in rat cardiomyocytes. *Am. J. Physiol.*, **273,** H2659-H2663.

Ishihata, A. & Endoh, M. (1995). Species-related differences in inotropic effects of angiotensin II in mammalian ventricular muscle: receptors, subtypes and phosphoinositide hydrolysis. *Br. J. Pharmacol.*, **114,** 447-453.

Iyer, S.N., Averill, D.B., Chappell, M.C., Yamada, K., Allred, A.J. & Ferrario, C.M. (2000). Contribution of angiotensin-(1-7) to blood pressure regulation in salt-depleted hypertensive rats. *Hypertension*, **36,** 417-422.

Iyer, S.N., Chappell, M.C., Averill, D.B., Diz, D.I. & Ferrario, C.M. (1998a). Vasodepressor actions of angiotensin-(1-7) unmasked during combined treatment with lisinopril and losartan. *Hypertension*, **31,** 699-705.

Iyer, S.N., Ferrario, C.M. & Chappell, M.C. (1998b). Angiotensin-(1-7) contributes to the antihypertensive effects of blockade of the renin-angiotensin system. *Hypertension*, **31,** 356-361.

Jaspard, E., Wei, L. & Alhenc-Gelas, F. (1993). Differences in the properties and enzymatic specificities of the two active sites of angiotensin I-converting enzyme (kininase II). Studies with bradykinin and other natural peptides. *J. Biol. Chem.*, **268,** 9496-9503.

Joly, G.A., Ayres, M., Chelly, F. & Kilbourn, R.G. (1994). Effects of NG-methyl-L-arginine, NG-nitro-L-arginine, and aminoguanidine on constitutive and inducible nitric oxide synthase in rat aorta. *Biochem. Biophys. Res. Commun.*, **199,** 147-154.

Jorde, U.P., Ennezat, P.V., Lisker, J., Suryadevara, V., Infeld, J., Cukon, S., Hammer, A., Sonnenblick, E.H. & Le Jemtel, T.H. (2000). Maximally recommended doses of angiotensin-converting enzyme (ACE) inhibitors do not completely prevent ACE-mediated formation of angiotensin II in chronic heart failure. *Circulation*, **101,** 844-846.

Ju, H., Venema, V.J., Marrero, M.B. & Venema, R.C. (1998). Inhibitory interactions of the bradykinin B_2 receptor with endothelial nitric-oxide synthase. *J. Biol. Chem.*, **273,** 24025-24029.

Karlsson, K. & Marklund, S.L. (1988). Extracellular superoxide dismutase in the vascular system of mammals. *Biochem. J.*, **255,** 223-228.

Kasel, A.M., Faussner, A., Pfeifer, A., Muller, U., Werdan, K. & Roscher, A.A. (1996). B_2 bradykinin receptors in cultured neonatal rat cardiomyocytes mediate a negative chronotropic and negative inotropic response. *Diabetes*, **45,** S44-S50.

Kawazoe, T., Kosaka, H., Yoneyama, H. & Hata, Y. (2000). Acute production of vascular superoxide by angiotensin II but not by catecholamines. *J. Hypertens.*, **18,** 179-185.

Kentsch, M. & Otter, W. (1999). Novel neurohormonal modulators in cardiovascular disorders. The therapeutic potential of endopeptidase inhibitors. *Drugs R.D.*, **1,** 331-338.

Kitakaze, M., Minamino, T., Node, K., Komamura, K., Shinozaki, Y., Mori, H., Kosaka, H., Inoue, M., Hori, M. & Kamada, T. (1995). Beneficial effects of inhibition of angiotensin-converting enzyme on ischemic myocardium during coronary hypoperfusion in dogs. *Circulation*, **92,** 950-961.

Kobayashi, T. & Kamata, K. (2002). Modulation by hydrogen peroxide of noradrenaline-induced contraction in aorta from streptozotocin-induced diabetic rat. *Eur. J. Pharmacol.*, **441,** 83-89.

Kojda, G. & Kottenberg, K. (1999). Regulation of basal myocardial function by NO. *Cardiovasc. Res.*, **41,** 514-523.

Kokkonen, J.O., Kuoppala, A., Saarinen, J., Lindstedt, K.A. & Kovanen, P.T. (1999). Kallidin- and bradykinin-degrading pathways in human heart: degradation of kallidin

by aminopeptidase M-like activity and bradykinin by neutral endopeptidase. *Circulation*, **99,** 1984-1990.

Kokkonen, J.O., Lindstedt, K.A., Kuoppala, A. & Kovanen, P.T. (2000). Kinin-degrading pathways in the human heart. *Trends Cardiovasc. Med.*, **10,** 42-45.

Kokkonen, J.O., Saarinen, J. & Kovanen, P.T. (1997). Regulation of local angiotensin II formation in the human heart in the presence of interstitial fluid. Inhibition of chymase by protease inhibitors of interstitial fluid and of angiotensin-converting enzyme by Ang-(1-9) formed by heart carboxypeptidase A-like activity. *Circulation*, **95,** 1455-1463.

Koppenol, W.H. (1998). The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radic. Biol. Med.*, **25,** 385-391.

Krassoi, I., Pataricza, J., Torday, L.L., Kun, A. & Papp, J.G. (2000). Improvement by phosphoramidon of damaged endothelial function in porcine coronary artery. *Ann. Thorac. Surg.*, **70,** 878-882.

Kuga, T., Mohri, M., Egashira, K., Hirakawa, Y., Tagawa, T., Shimokawa, H. & Takeshita, A. (1997). Bradykinin-induced vasodilation of human coronary arteries in vivo: role of nitric oxide and angiotensin-converting enzyme. *J. Am. Coll. Cardiol.*, **30,** 108-112.

Laursen, J.B., Rajagopalan, S., Galis, Z., Tarpey, M., Freeman, B.A. & Harrison, D.G. (1997). Role of superoxide in angiotensin II-induced but not catecholamine- induced hypertension. *Circulation*, **95,** 588-593.

Lawrence, A.C., Evin, G., Kladis, A. & Campbell, D.J. (1990). An alternative strategy for the radioimmunoassay of angiotensin peptides using amino-terminal-directed antisera: measurement of eight angiotensin peptides in human plasma. *J. Hypertens.*, **8,** 715-724.

Lee, K.M., Toscas, K. & Villereal, M.L. (1993). Inhibition of bradykinin- and thapsigargin-induced Ca^{2+} entry by tyrosine kinase inhibitors. *J. Biol. Chem.*, **268,** 9945-9948.

Leslie, R.G. & Allen, R. (1987). Evaluation and improvements of a rapid microassay for measuring superoxide anion production by phagocytes. 2. Biochemical aspects. *J. Immunol. Methods*, **103,** 261-266.

Li, C.G. & Rand, M.J. (1999). Effects of hydroxocobalamin and carboxy-PTIO on nitrergic transmission in porcine anococcygeus and retractor penis muscles. *Br. J. Pharmacol.*, **127,** 172-176.

Liu, J., Yang, F., Yang, X.P., Jankowski, M. & Pagano, P.J. (2003). NAD(P)H oxidase mediates angiotensin II-induced vascular macrophage infiltration and medial hypertrophy. *Arterioscler. Thromb. Vasc. Biol.*, **23,** 776-782.

Llorens-Cortes, C., Huang, H., Vicart, P., Gasc, J.M., Paulin, D. & Corvol, P. (1992). Identification and characterization of neutral endopeptidase in endothelial cells from venous or arterial origins. *J. Biol. Chem.*, **267,** 14012-14018.

Loot, A.E., Roks, A.J., Henning, R.H., Tio, R.A., Suurmeijer, A.J., Boomsma, F. & van Gilst, W.H. (2002). Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. *Circulation*, **105,** 1548-1550.

Maassen van den Brink, A., de Vries, R., Saxena, P.R., Schalekamp, M.A.D.H. & Danser, A.H.J. (1999). Vasoconstriction by in situ formed angiotensin II: role of ACE and chymase. *Cardiovasc. Res.*, **44,** 407-415.

Mahon, J.M., Carr, R.D., Nicol, A.K. & Henderson, I.W. (1994). Angiotensin(1-7) is an antagonist at the type 1 angiotensin II receptor. *J. Hypertens.*, **12,** 1377-1381.

Marceau, F., Hess, J.F. & Bachvarov, D.R. (1998). The B_1 receptors for kinins. *Pharmacol. Rev.*, **50,** 357-386.

Marcic, B., Deddish, P.A., Jackman, H.L. & Erdös, E.G. (1999). Enhancement of bradykinin and resensitization of its B_2 receptor. *Hypertension*, **33,** 835-843.

Marcic, B., Deddish, P.A., Skidgel, R.A., Erdös, E.G., Minshall, R.D. & Tan, F. (2000a). Replacement of the transmembrane anchor in angiotensin I-converting enzyme (ACE) with a glycosylphosphatidylinositol tail affects activation of the B_2 bradykinin receptor by ACE inhibitors. *J. Biol. Chem.*, **275,** 16110-16118.

Marcic, B.M. & Erdös, E.G. (2000b). Protein kinase C and phosphatase inhibitors block the ability of angiotensin I-converting enzyme inhibitors to resensitize the receptor to bradykinin without altering the primary effects of bradykinin. *J. Pharmacol.Exp. Ther.*, **294,** 605-612.

Marin-Castano, M.E., Schanstra, J.P., Neau, E., Praddaude, F., Pecher, C., Ader, J.L., Girolami, J.P. & Bascands, J.L. (2002). Induction of functional bradykinin B_1-receptors in normotensive rats and mice under chronic angiotensin-converting enzyme inhibitor treatment. *Circulation*, **105,** 627-632.

Matoba, T., Shimokawa, H., Kubota, H., Morikawa, K., Fujiki, T., Kunihiro, I., Mukai, Y., Hirakawa, Y. & Takeshita, A. (2002). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. *Biochem. Biophys. Res. Commun.*, **290,** 909-913.

McAllister, B.S., Leeb-Lundberg, F. & Olson, M.S. (1993). Bradykinin inhibition of EGF- and PDGF-induced DNA synthesis in human fibroblasts. *Am. J. Physiol.*, **265,** C477-C484.

McClean, D.R., Ikram, H., Garlick, A.H., Richards, A.M., Nicholls, M.G. & Crozier, I.G. (2000). The clinical, cardiac, renal, arterial and neurohormonal effects of omapatrilat, a vasopeptidase inhibitor, in patients with chronic heart failure. *J. Am. Coll. Cardiol.*, **36,** 479-486.

McDonald, J.E., Padmanabhan, N., Petrie, M.C., Hillier, C., Connell, J.M. & McMurray, J.J. (2001). Vasoconstrictor effect of the angiotensin-converting enzyme-resistant, chymase-specific substrate [Pro^{11}-D-Ala^{12}] angiotensin I in human dorsal hand veins: in vivo demonstration of non-ace production of angiotensin II in humans. *Circulation*, **104,** 1805-1808.

McDonald, K.M., Mock, J., D'Aloia, A., Parrish, T., Hauer, K., Francis, G., Stillman, A. & Cohn, J.N. (1995). Bradykinin antagonism inhibits the antigrowth effect of converting enzyme inhibition in the dog myocardium after discrete transmural myocardial necrosis. *Circulation*, **91,** 2043-2048.

Mervaala, E.M., Cheng, Z.J., Tikkanen, I., Lapatto, R., Nurminen, K., Vapaatalo, H., Muller, D.N., Fiebeler, A., Ganten, U., Ganten, D. & Luft, F.C. (2001). Endothelial dysfunction and xanthine oxidoreductase activity in rats with human renin and angiotensinogen genes. *Hypertension*, **37,** 414-418.

Michaud, A., Williams, T.A., Chauvet, M.T. & Corvol, P. (1997). Substrate dependence of angiotensin I-converting enzyme inhibition: captopril displays a partial selectivity for inhibition of N-acetyl-seryl-aspartyl-lysyl-proline hydrolysis compared with that of angiotensin I. *Mol. Pharmacol.*, **51,** 1070-1076.

Miki, T., Miura, T., Ura, N., Ogawa, T., Suzuki, K., Shimamoto, K. & Iimura, O. (1996). Captopril potentiates the myocardial infarct size-limiting effect of ischemic preconditioning through bradykinin B_2 receptor activation. *J. Am. Coll. Cardiol.*, **28,** 1616-1622.

Minshall, R.D., Erdös, E.G. & Vogel, S.M. (1997a). Angiotensin I-converting enzyme inhibitors potentiate bradykinin's inotropic effects independently of blocking its inactivation. *Am. J. Cardiol.*, **80,** 132A-136A.

Minshall, R.D., Nakamura, F., Becker, R.P. & Rabito, S.F. (1995). Characterization of bradykinin B_2 receptors in adult myocardium and neonatal rat cardiomyocytes. *Circ. Res.*, **76,** 773-780.

Minshall, R.D., Nedumgottil, S.J., Igic, R., Erdös, E.G. & Rabito, S.F. (2000). Potentiation of the effects of bradykinin on its receptor in the isolated guinea pig ileum. *Peptides*, **21,** 1257-1264.

Minshall, R.D., Tan, F., Nakamura, F., Rabito, S.F., Becker, R.P., Marcic, B. & Erdös, E.G. (1997b). Potentiation of the actions of bradykinin by angiotensin I-converting enzyme inhibitors. The role of expressed human bradykinin B_2 receptors and angiotensin I-converting enzyme in CHO cells. *Circ. Res.*, **81,** 848-856.

Minshall, R.D., Vogel, S.M. & Rabito, S.F. (1997c). Are the inotropic and antiarrhythmic effects of bradykinin due to increases in coronary flow? *Am. J. Cardiol.*, **80,** 148A-152A.

Minshall, R.D., Yelamanchi, V.P., Djokovic, A., Miletich, D.J., Erdös, E.G., Rabito, S.F. & Vogel, S.M. (1994). Importance of sympathetic innervation in the positive inotropic effects of bradykinin and ramiprilat. *Circ. Res.*, **74,** 441-447.

Miyamoto, A., Murata, S. & Nishio, A. (2002). Role of ACE and NEP in bradykinin-induced relaxation and contraction response of isolated porcine basilar artery. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **365,** 365-370.

Mombouli, J.V., Ballard, K.D. & Vanhoutte, P.M. (2002). Kininase-independent potentiation of endothelium-dependent relaxations to kinins by converting enzyme inhibitor perindoprilat. *Acta Pharmacol. Sin.*, **23,** 203-207.

Mombouli, J.V., Illiano, S., Nagao, T., Scott-Burden, T. & Vanhoutte, P.M. (1992). Potentiation of endothelium-dependent relaxations to bradykinin by angiotensin I converting enzyme inhibitors in canine coronary artery involves both endothelium-derived relaxing and hyperpolarizing factors. *Circ. Res.*, **71,** 137-144.

Mombouli, J.V. & Vanhoutte, P.M. (1997). Endothelium-derived hyperpolarizing factor(s): updating the unknown. *Trends Pharmacol. Sci.*, **18,** 252-256.

Moore, P.K., Babbedge, R.C., Wallace, P., Gaffen, Z.A. & Hart, S.L. (1993). 7-Nitro indazole, an inhibitor of nitric oxide synthase, exhibits anti- nociceptive activity in the mouse without increasing blood pressure. *Br. J. Pharmacol.*, **108,** 296-297.

Mooser, V., Nussberger, J., Juillerat, L., Burnier, M., Waeber, B., Bidiville, J., Pauly, N. & Brunner, H.R. (1990). Reactive hyperreninemia is a major determinant of plasma angiotensin II during ACE inhibition. *J. Cardiovasc. Pharmacol.*, **15,** 276-282.

Müller, D.N., Fischli, W., Clozel, J.P., Hilgers, K.F., Bohlender, J., Menard, J., Busjahn, A., Ganten, D. & Luft, F.C. (1998). Local angiotensin II generation in the rat heart: role of renin uptake. *Circ. Res.*, **82,** 13-20.

Munch, P.A. & Longhurst, J.C. (1991). Bradykinin increases myocardial contractility: relation to the Gregg phenomenon. *Am. J. Physiol.*, **260,** R1095-R1103.

Myers, P.R., Minor, R.L., Jr., Guerra, R., Jr., Bates, J.N. & Harrison, D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. *Nature*, **345,** 161-163.

Neufeld, E.B., Cooney, A.M., Pitha, J., Dawidowicz, E.A., Dwyer, N.K., Pentchev, P.G. & Blanchette-Mackie, E.J. (1996). Intracellular trafficking of cholesterol monitored with a cyclodextrin. *J. Biol. Chem.*, **271,** 21604-21613.

Nishimura, H., Buikema, H., Baltatu, O., Ganten, D. & Urata, H. (1998). Functional evidence for alternative Ang II-forming pathways in hamster cardiovascular system. *Am. J. Physiol.*, **275,** H1307-H1312.

Nolly, H., Carbini, L.A., Scicli, G., Carretero, O.A. & Scicli, A.G. (1994). A local kallikrein-kinin system is present in rat hearts. *Hypertension*, **23,** 919-923.

Nolly, H.L., Lama, M.C., Carretero, O.A. & Scicli, A.G. (1992). The kallikrein-kinin system in blood vessels. *Agents Actions (Suppl)*, **38,** 1-9.

Ogiwara, T., Murdoch, G., Chik, C.L. & Ho, A.K. (1995). Tyrosine kinase inhibitors enhance cGMP production in rat pinealocytes. *Biochem. Biophys. Res. Commun.*, **207,** 994-1002.

Pagano, P.J., Clark, J.K., Cifuentes-Pagano, M.E., Clark, S.M., Callis, G.M. & Quinn, M.T. (1997). Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. *Proc. Natl. Acad. Sci. U.S.A.*, **94,** 14483-14488.

Palmer, R.M., Rees, D.D., Ashton, D.S. & Moncada, S. (1988). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153,** 1251-1256.

Paravicini, T.M., Gulluyan, L.M., Dusting, G.J. & Drummond, G.R. (2002). Increased NADPH oxidase activity, gp91phox expression, and endothelium-dependent vasorelaxation during neointima formation in rabbits. *Circ. Res.*, **91,** 54-61.

Perich, R.B., Jackson, B. & Johnston, C.I. (1994). Structural constraints of inhibitors for binding at two active sites on somatic angiotensin converting enzyme. *Eur. J. Pharmacol.*, **266,** 201-211.

Pomposiello, S., Rhaleb, N.E., Alva, M. & Carretero, O.A. (1999). Reactive oxygen species: role in the relaxation induced by bradykinin or arachidonic acid via EDHF in isolated porcine coronary arteries. *J. Cardiovasc. Pharmacol.*, **34,** 567-574.

Pörsti, I., Bara, A.T., Busse, R. & Hecker, M. (1996). Corrigendum. *Br. J. Pharmacol.*, **117,** 231.

Pörsti, I., Bara, A.T., Busse, R. & Hecker, M. (1994). Release of nitric oxide by angiotensin-(1-7) from porcine coronary endothelium: implications for a novel angiotensin receptor. *Br. J. Pharmacol.*, **111,** 652-654.

Quignard, J.F., Feletou, M., Thollon, C., Vilaine, J.P., Duhault, J. & Vanhoutte, P.M. (1999). Potassium ions and endothelium-derived hyperpolarizing factor in guinea-pig carotid and porcine coronary arteries. *Br. J. Pharmacol.*, **127,** 27-34.

Raidoo, D.M., Ramsaroop, R., Naidoo, S., Müller-Esterl, W. & Bhoola, K.D. (1997). Kinin receptors in human vascular tissue: their role in atheromatous disease. *Immunopharmacology*, **36,** 153-160.

Rajagopalan, S., Kurz, S., Munzel, T., Tarpey, M., Freeman, B.A., Griendling, K.K. & Harrison, D.G. (1996). Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J. Clin. Invest.*, **97,** 1916-1923.

Raut, R., Rouleau, J.L., Blais, C., Jr., Gosselin, H., Molinaro, G., Sirois, M.G., Lepage, Y., Crine, P. & Adam, A. (1999). Bradykinin metabolism in the postinfarcted rat heart: role of ACE and neutral endopeptidase 24.11. *Am. J. Physiol.*, **276,** H1769-H1779.

Rees, D.D., Palmer, R.M., Schulz, R., Hodson, H.F. & Moncada, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br. J. Pharmacol.*, **101,** 746-752.

Reissmann, S., Schwuchow, C., Seyfarth, L., Pineda De Castro, L.F., Liebmann, C., Paegelow, I., Werner, H. & Stewart, J.M. (1996). Highly selective bradykinin agonists and antagonists with replacement of proline residues by N-methyl-D- and L-phenylalanine. *J. Med. Chem.*, **39,** 929-936.

Ren, Y., Garvin, J.L. & Carretero, O.A. (2002). Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension*, **39,** 799-802.

Rey, F.E., Li, X.C., Carretero, O.A., Garvin, J.L. & Pagano, P.J. (2002). Perivascular superoxide anion contributes to impairment of endothelium-dependent relaxation: role of $gp91_{phox}$. *Circulation*, **106,** 2497-2502.

Rhaleb, N.E., Drapeau, G., Dion, S., Jukic, D., Rouissi, N. & Regoli, D. (1990). Structure-activity studies on bradykinin and related peptides: agonists. *Br. J. Pharmacol.*, **99,** 445-448.

Richard, V., Hurel-Merle, S., Scalbert, E., Ferry, G., Lallemand, F., Bessou, J.P. & Thuillez, C. (2001). Functional evidence for a role of vascular chymase in the production of angiotensin II in isolated human arteries. *Circulation*, **104,** 750-752.

Roks, A.J.M., van Geel, P.P., Pinto, Y.M., Buikema, H., Henning, R.H., de Zeeuw, D. & van Gilst, W.H. (1999). Angiotensin-(1-7) is a modulator of the human renin-angiotensin system. *Hypertension*, **34,** 296-301.

Rothberg, K.G., Heuser, J.E., Donzell, W.C., Ying, Y.S., Glenney, J.R. & Anderson, R.G. (1992). Caveolin, a protein component of caveolae membrane coats. *Cell*, **68,** 673-682.

Rouleau, J.L., Pfeffer, M.A., Stewart, D.J., Isaac, D., Sestier, F., Kerut, E.K., Porter, C.B., Proulx, G., Qian, C. & Block, A.J. (2000). Comparison of vasopeptidase inhibitor, omapatrilat, and lisinopril on exercise tolerance and morbidity in patients with heart failure: IMPRESS randomised trial. *Lancet*, **356,** 615-620.

Rueckschloss, U., Quinn, M.T., Holtz, J. & Morawietz, H. (2002). Dose-dependent regulation of NAD(P)H oxidase expression by angiotensin II in human endothelial cells: protective effect of angiotensin II type 1 receptor blockade in patients with coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.*, **22,** 1845-1851.

Rump, L.C., Berlit, T., Schwertfeger, E., Beyersdorf, F., Schollmeyer, P. & Bohmann, C. (1997). Angiotensin converting enzyme inhibition unmasks the sympathofacilitatory effect of bradykinin in human right atrium. *J.Hypertens.*, **15,** 1263-1270.

Saris, J.J., van den Eijnden, M.M., Lamers, J.M., Saxena, P.R., Schalekamp, M.A.D.H. & Danser, A.H.J. (2002). Prorenin-induced myocyte proliferation: no role for intracellular angiotensin II. *Hypertension*, **39,** 573-577.

Saris, J.J., van Dijk, M.A., Kroon, I., Schalekamp, M.A. & Danser, A.H. (2000). Functional importance of angiotensin-converting enzyme-dependent in situ angiotensin II generation in the human forearm. *Hypertension*, **35,** 764-768.

Satriano, J.A., Shuldiner, M., Hora, K., Xing, Y., Shan, Z. & Schlondorff, D. (1993). Oxygen radicals as second messengers for expression of the monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, CSF-1, in response to tumor necrosis factor-alpha and immunoglobulin G. Evidence for involvement of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase. *J. Clin. Invest.*, **92,** 1564-1571.

Schnitzer, J.E., Oh, P., Pinney, E. & Allard, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell Biol.*, **127,** 1217-1232.

Schoemaker, R.G., Du, X.Y., Bax, W.A. & Saxena, P.R. (1992). 5-Hydroxytryptamine increases contractile force in porcine right atrium but not in left ventricle. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **346,** 486-489.

Schuijt, M.P., Basdew, M., van Veghel, R., de Vries, R., Saxena, P.R., Schoemaker, R.G. & Danser, A.H.J. (2001). AT_2 receptor-mediated vasodilation in the heart: effect of myocardial infarction. *Am. J. Physiol.*, **281,** H2590-H2596.

Schuijt, M.P., de Vries, R., Saxena, P.R., Schalekamp, M.A.D.H. & Danser, A.H.J. (2002). Vasoconstriction is determined by intertitial rather than circulating angiotensin II. *Br. J. Pharmacol.*, **135,** 275-283.

Schuijt, M.P., van Kats, J.P., de Zeeuw, S., Duncker, D.J., Verdouw, P.D., Schalekamp, M.A.D.H. & Danser, A.H.J. (1999). Cardiac interstitial fluid levels of angiotensin I and II in the pig. *J. Hypertens.*, **17,** 1885-1891.

Seyedi, N., Win, T., Lander, H.M. & Levi, R. (1997). Bradykinin B_2-receptor activation augments norepinephrine exocytosis from cardiac sympathetic nerve endings. Mediation by autocrine/paracrine mechanisms. *Circ. Res.*, **81,** 774-784.

Shastri, S., Gopalakrishnan, V., Poduri, R. & Di Wang, H. (2002). Tempol selectively attenuates angiotensin II evoked vasoconstrictor responses in spontaneously hypertensive rats. *J. Hypertens.*, **20,** 1381-1391.

Soleilhac, J.M., Lucas, E., Beaumont, A., Turcaud, S., Michel, J.B., Ficheux, D., Fournie-Zaluski, M.C. & Roques, B.P. (1992). A 94-kDa protein, identified as neutral endopeptidase-24.11, can inactivate atrial natriuretic peptide in the vascular endothelium. *Mol. Pharmacol.*, **41,** 609-614.

Stralin, P., Karlsson, K., Johansson, B.O. & Marklund, S.L. (1995). The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. *Arterioscler. Thromb. Vasc. Biol.*, **15,** 2032-2036.

Su, J.B., Houel, R., Heloire, F., Barbe, F., Beverelli, F., Sambin, L., Castaigne, A., Berdeaux, A., Crozatier, B. & Hittinger, L. (2000). Stimulation of bradykinin B_1 receptors induces vasodilation in conductance and resistance coronary vessels in conscious dogs: comparison with B_2 receptor stimulation. *Circulation*, **101,** 1848-1853.

Sung, C.P., Arleth, A.J., Shikano, K. & Berkowitz, B.A. (1988). Characterization and function of bradykinin receptors in vascular endothelial cells. *J. Pharmacol. Exp. Ther.*, **247,** 8-13.

Takao, K., Takai, S., Ishihara, T., Mita, S. & Miyazaki, M. (2001). Isolation of chymase complexed with physiological inhibitor similar to secretory leukocyte protease inhibitor (SLPI) from hamster cheek pouch tissues. *Biochim. Biophys. Acta.*, **1545,** 146-152.

Tallant, E.A., Lu, X., Weiss, R.B., Chappell, M.C. & Ferrario, C.M. (1997). Bovine aortic endothelial cells contain an angiotensin-(1-7) receptor. *Hypertension*, **29,** 388-393.

Taylor-McCabe, K.J., Ersahin, C. & Simmons, W.H. (2001). Bradykinin metabolism in the isolated perfused rabbit heart. *J. Hypertens.*, **19,** 1295-1299.

Thomas, S.R., Chen, K. & Keaney, J.F., Jr. (2002). Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. *J. Biol. Chem.*, **277,** 6017-6024.

Tio, R.A., Tobe, T.J., Bel, K.J., de Langen, C.D., van Gilst, W.H. & Wesseling, H. (1991). Beneficial effects of bradykinin on porcine ischemic myocardium. *Basic Res. Cardiol.*, **86,** 107-116.

Tom, B., de Vries, R., Saxena, P.R. & Danser, A.H.J. (2001a). Bradykinin potentiation by angiotensin-(1-7) and ACE inhibitors correlates with ACE C- and N-domain blockade. *Hypertension*, **38,** 95-99.

Tom, B., de Vries, R., Saxena, P.R. & Danser, A.H.J. (2001b). Negative inotropic effect of bradykinin in porcine isolated atrial trabeculae: role of nitric oxide. *J. Hypertens.*, **19,** 1289-1293.

Tom, B., Dendorfer, A., de Vries, R., Saxena, P.R. & Danser, A.H.J. (2002). Bradykinin potentiation by ACE inhibitors: a matter of metabolism. *Br. J. Pharmacol.*, **137,** 276-284.

Tom, B., Garrelds, I.M., Scalbert, E., Stegmann, A.P., Boomsma, F., Saxena, P.R. & Danser, A.H. (2003). ACE-versus chymase-dependent angiotensin II generation in human coronary arteries: a matter of efficiency? *Arterioscler. Thromb. Vasc. Biol.*, **23,** 251-256.

Touyz, R.M., G., Y., Chen, X. & Schiffrin, E.L. (2002). Redox-mediated MAP kinase signaling by angiotensin II and endothelin-1 are differentially regulated in smooth muscle cells from human resistance arteries. *J. Hypertens.*, **20,** S8 (Abstract).

Touyz, R.M. & Schiffrin, E.L. (1999). Ang II-stimulated superoxide production is mediated via phospholipase D in human vascular smooth muscle cells. *Hypertension*, **34,** 976-982.

Ueda, S., Masumori-Maemoto, S., Ashino, K., Nagahara, T., Gotoh, E., Umemura, S. & Ishii, M. (2000). Angiotensin-(1-7) attenuates vasoconstriction evoked by angiotensin II but not by noradrenaline in man. *Hypertension*, **35,** 998-1001.

Ueda, S., Masumori-Maemoto, S., Wada, A., Ishii, M., Brosnihan, K.B. & Umemura, S. (2001). Angiotensin(1-7) potentiates bradykinin-induced vasodilatation in man. *J. Hypertens.*, **19,** 2001-2009.

Urata, H., Boehm, K.D., Philip, A., Kinoshita, A., Gabrovsek, J., Bumpus, F.M. & Husain, A. (1993). Cellular localization and regional distribution of an angiotensin II-forming chymase in the heart. *J. Clin. Invest.*, **91,** 1269-1281.

Urata, H., Kinoshita, A., Misono, K.S., Bumpus, F.M. & Husain, A. (1990). Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *J. Biol. Chem.*, **265,** 22348-22357.

van Kats, J.P., Danser, A.H.J., van Meegen, J.R., Sassen, L.M., Verdouw, P.D. & Schalekamp, M.A.D.H. (1998). Angiotensin production by the heart: a quantitative study in pigs with the use of radiolabeled angiotensin infusions. *Circulation*, **98,** 73-81.

van Kats, J.P., Duncker, D.J., Haitsma, D.B., Schuijt, M.P., Niebuur, R., Stubenitsky, R., Boomsma, F., Schalekamp, M.A.D.H., Verdouw, P.D. & Danser, A.H.J. (2000). 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*, **102,** 1556-1563.

van Kats, J.P., Schalekamp, M.A.D.H., Verdouw, P.D., Duncker, D.J. & Danser, A.H.J. (2001). Intrarenal angiotensin II: Interstitial and cellular levels and site of production. *Kidney Int.*, **60,** 2311-2317.

van Kesteren, C.A.M., Saris, J.J., Dekkers, D.H.W., Lamers, J.M.J., Saxena, P.R., Schalekamp, M.A.D.H. & Danser, A.H.J. (1999). Cultured neonatal rat cardiac myocytes and fibroblasts do not synthesize renin or angiotensinogen: evidence for stretch-induced cardiomyocyte hypertrophy independent of angiotensin II. *Cardiovasc. Res.*, **43,** 148-156.

Vedernikov, Y.P., Mordvintcev, P.I., Malenkova, I.V. & Vanin, A.F. (1992). Similarity between the vasorelaxing activity of dinitrosyl iron cysteine complexes and endothelium-derived relaxing factor. *Eur. J. Pharmacol.*, **211,** 313-317.

Venturini, C.M., Palmer, R.M. & Moncada, S. (1993). Vascular smooth muscle contains a depletable store of a vasodilator which is light-activated and restored by donors of nitric oxide. *J. Pharmacol. Exp. Ther.*, **266,** 1497-1500.

Vickers, C., Hales, P., Kaushik, V., Dick, L., Gavin, J., Tang, J., Godbout, K., Parsons, T., Baronas, E., Hsieh, F., Acton, S., Patane, M., Nichols, A. & Tummino, P. (2002). Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J. Biol. Chem.*, **277,** 14838-14843.

Vila-Petroff, M.G., Younes, A., Egan, J., Lakatta, E.G. & Sollott, S.J. (1999). Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. *Circ. Res.*, **84,** 1020-1031.

Wang, H.D., Xu, S., Johns, D.G., Du, Y., Quinn, M.T., Cayatte, A.J. & Cohen, R.A. (2001). Role of NADPH oxidase in the vascular hypertrophic and oxidative stress response to angiotensin II in mice. *Circ. Res.*, **88,** 947-953.

Wang, L., Sadoun, E., Stephens, R.E. & Ward, P.E. (1994). Metabolism of substance P and neurokinin A by human vascular endothelium and smooth muscle. *Peptides*, **15,** 497-503.

Wei, C.C., Meng, Q.C., Palmer, R., Hageman, G.R., Durand, J., Bradley, W.E., Farrell, D.M., Hankes, G.H., Oparil, S. & Dell'Italia, L.J. (1999). Evidence for angiotensin-converting enzyme- and chymase-mediated angiotensin II formation in the interstitial fluid space of the dog heart in vivo. *Circulation*, **99,** 2583-2589.

Weir, E.K., Wyatt, C.N., Reeve, H.L., Huang, J., Archer, S.L. & Peers, C. (1994). Diphenyleneiodonium inhibits both potassium and calcium currents in isolated pulmonary artery smooth muscle cells. *J. Appl. Physiol.*, **76,** 2611-2615.

Widdop, R.E., Sampey, D.B. & Jarrott, B. (1999). Cardiovascular effects of angiotensin-(1-7) in conscious spontaneously hypertensive rats. *Hypertension*, **34,** 964-968.

Willems, E.W., Heiligers, J.P.C., De Vries, P., Tom, B., Kapoor, K., Villalon, C.M. & Saxena, P.R. (2001). A61603-induced vasoconstriction in porcine carotid vasculature: involvement of a non-adrenergic mechanism. *Eur. J. Pharmacol.*, **417,** 195-201.

Willems, E.W., Trion, M., De Vries, P., Heiligers, J.P., Villalon, C.M. & Saxena, P.R. (1999). Pharmacological evidence that $alpha_1$-and $alpha_2$-adrenoceptors mediate vasoconstriction of carotid arteriovenous anastomoses in anaesthetized pigs. *Br. J. Pharmacol.*, **127,** 1263-1271.

Yamada, K., Iyer, S.N., Chappell, M.C., Ganten, D. & Ferrario, C.M. (1998). Converting enzyme determines plasma clearance of angiotensin-(1-7). *Hypertension*, **32,** 496-502.

Yamada, M., Ueda, M., Naruko, T., Tanabe, S., Han, Y.S., Ikura, Y., Ogami, M., Takai, S. & Miyazaki, M. (2001). Mast cell chymase expression and mast cell phenotypes in human rejected kidneys. *Kidney Int.*, **59,** 1374-1381.

Zeiher, A.M., Drexler, H., Saurbier, B. & Just, H. (1993). Endothelium-mediated coronary blood flow modulation in humans. Effects of age, atherosclerosis, hypercholesterolemia, and hypertension. *J. Clin. Invest.*, **92,** 652-662.