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AT_2 receptors: Functional relevance in cardiovascular disease

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Abbreviations:
ACE, angiotensin converting enzyme
ACE2, angiotensin converting enzyme 2
Ang II, angiotensin II
Ang III, angiotensin III
Ang IV, angiotensin IV
Ang (1–7), angiotensin (1–7)
ATBP50, AT_2R-binding protein of 50 kDa
ATIP-1, AT_2 receptor interacting protein-1
AT_1R, angiotensin II type 1 receptor
AT_2R, angiotensin II type 2 receptor
AT_4R, angiotensin II type 4 receptor
BK, bradykinin
BP, blood pressure
cGMP, cyclic guanine 3′,5′-monophosphate
ECM, extracellular matrix
eNOS, endothelial nitric oxide synthase
ERK-1/2, extracellular-regulated kinases-1,2
IRAP, insulin-regulated aminopeptidase
L-NAME, N^G-nitro-L arginine methyl ester
LVH, left ventricular hypertrophy
MAPK, mitogen-activated protein kinase
MCP-1, monocyte chemoattractant protein-1
MI, myocardial infarction
MMP, matrix metalloproteinase
mRNA, messenger ribonucleic acid
NF-κβ, nuclear transcription factor-κβ
NO, nitric oxide
O_2^-, superoxide
PC12W, rat pheochromocytoma cell line
RAS, renin angiotensin system
ROS, reactive oxygen species
SHR, spontaneously hypertensive rat
TIMP-1, tissue inhibitor of metalloproteinase-1
TNFα, tumour-necrosis factor α
VSMC, vascular smooth muscle cell
WKY, Wistar-Kyoto rat

ABSTRACT

The renin angiotensin system (RAS) is intricately involved in normal cardiovascular homeostasis. Excessive stimulation by the octapeptide angiotensin II contributes to a range of cardiovascular pathologies and diseases via angiotensin type 1 receptor (AT_1R) activation. On the other hand, tElsevier Inc.he angiotensin type 2 receptor (AT_2R) is thought to counter-regulate AT_1R function. In this review, we describe the enhanced expression and function of AT_2R in various cardiovascular disease settings. In addition, we illustrate that the RAS consists of a family of angiotensin peptides that exert cardiovascular effects that are often distinct from those of Ang II. During cardiovascular disease, there is likely to be an increased functional importance of AT_2R, stimulated by Ang II, or even shorter angiotensin peptide fragments, to limit AT_1R-mediated overactivity and cardiovascular pathologies.

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1. Introduction

1.1. Conventional aspects of renin angiotensin system

The major effector peptide of the renin angiotensin system (RAS) is the octapeptide angiotensin II (Ang II), which was originally considered a circulating endocrine hormone (Unger et al., 1996; Csikos et al., 1997), with major effects involving vascular/cardiac contractility and fluid and electrolyte homeostasis. However, identification of components of the RAS in various organs has extended our understanding of the RAS (Zimmerman & Dunham, 1997; Akasu et al., 1998), and it is now well accepted that in addition to systemic effects, local synthesis of Ang II exerts both autocrine and paracrine functions, influencing cellular growth and regional haemodynamics in a tissue-specific manner (Johnston, 1992; Zimmerman & Dunham, 1997; de Gasparo et al., 2000; Paul et al., 2006).

As has been well documented, the 'classical'- or 'renal'-RAS involves the conversion of the hepatic-derived precursor peptide angiotensinogen to angiotensin I (Ang I), which is catalysed by circulating renin released from the kidney. The conversion of Ang I to Ang II is then primarily facilitated by angiotensin converting enzyme (ACE) which is found in endothelial cells, and due to the large surface area, this conversion is prominent in the lungs. Alternatively (but to a much lesser degree), Ang I may be directly converted to Ang (1–7) by tissue endopeptidases (Ferrario et al., 1997). Ang II is susceptible to degradation by aminopeptidases, resulting in fragments such as Ang III and Ang IV, which may in themselves possess biological activity (Ferrario & Iyer, 1998; de Gasparo et al., 2000). (see Fig. 1 and Section 2.4).

1.2. Angiotensin receptors and peptides

Based on the differing affinities of several natural and synthetic ligands, two major subtypes of Ang receptors have been identified and cloned, the Ang subtype 1 receptor (AT_1R) and subtype 2 receptor (AT_2R) (de Gasparo et al., 2000). Both AT_1 and AT_2R possess similar affinity for Ang II (Timmermans et al., 1991; Griendling et al., 1996), however, affinity for entities such as the biphenyl tetrazoles, losartan and candesartan, and compounds such as PD123319 and CGP42112, vary significantly. For example, the AT_1R antagonist, losartan, displays ~10 000-fold greater affinity for the AT_1 than the AT_2R (Timmermans et al., 1991), and candesartan cilexetil, which in vivo is metabolised to candesartan, has 80 times higher affinity for the AT_1R than losartan (Shibouta et al., 1993; Morsing, 1999). On the other hand, the tetrahydroimidazopyridine compounds, PD123177 and PD123319, have been shown to be 3500-fold more selective for the AT_2R than the AT_1R (Timmermans et al., 1991; Timmermans et al., 1993), while CGP42112 exhibits over 1000-fold selectivity for AT_2R (Whitebread et al., 1989).

All of the classical actions of Ang II, including vasoconstriction, effects on fluid and electrolyte homeostasis, and influences on cellular growth and differentiation, have been shown to be due to stimulation of AT_1R located on the plasma membrane of cells. In addition, there has been much recent interest in the cardiovascular effects of other peptides of the RAS, such as Ang 1–7, Ang III and Ang IV, which are formed by various amino- and endopeptidases (see Fig. 1), and are believed to influence cardiovascular function both due to modulation of classical Ang II-mediated actions, and by exerting their own specific biological actions via individual cognate receptors.

For example, the G-protein-coupled receptor encoded by the *Mas* protooncogene was identified as a functional receptor for Ang (1–7) (Santos et al., 2003). A number of cardiovascular effects have been attributed to Ang (1–7) (Santos et al., 2000) acting via MasR (Ferrario et al., 2005b; Santos & Ferreira, 2007) although there is evidence that Ang (1–7) can act independently of Ang (1–7)/MasR (Walters et al., 2005). As will be discussed later, the resurgence of interest in the

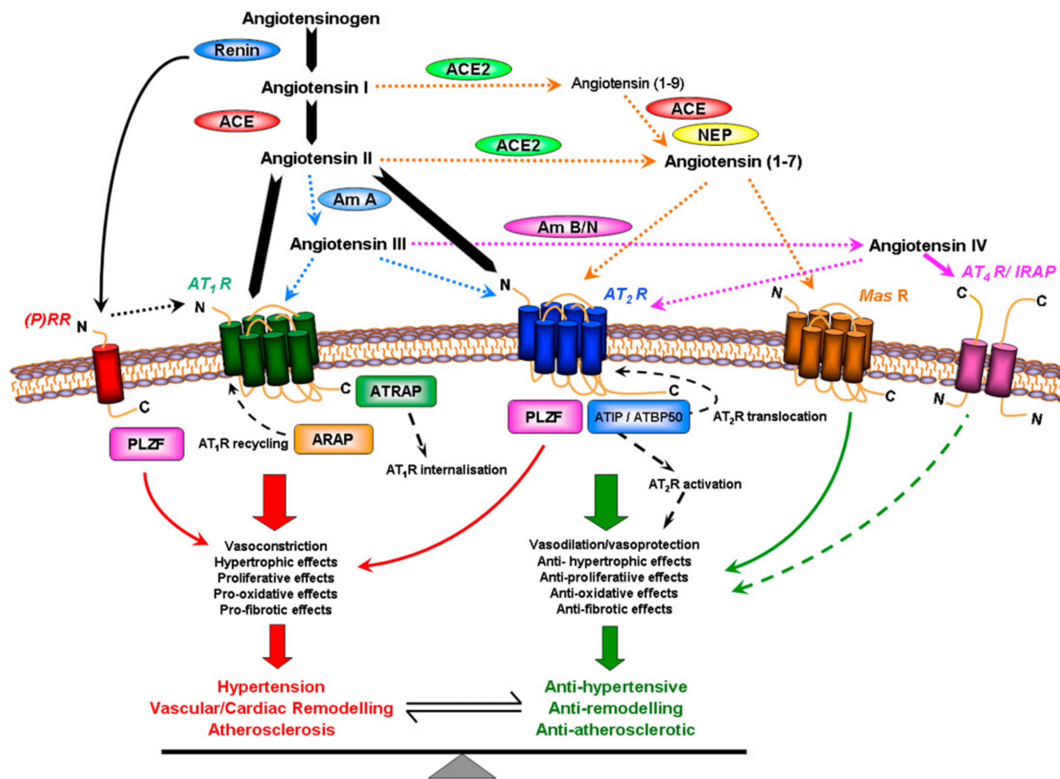

Fig. 1. Summary of the RAS incorporating the Ang peptide family and physiological effects mediated via ATR subtypes. Under the classical RAS schema, Ang II is produced, via renin and ACE, to act with equal affinity on two ATR subtypes, AT_1R and AT_2R (large arrows). However, it is now appreciated that a number of breakdown products of Ang II, namely Ang (1–7), Ang III and Ang IV, exert their own unique effects that are distinct (and often opposite) to those of Ang II. Such effects are often mediated via newly recognized receptors such as MasR for Ang (1–7) and AT_4R (also known as IRAP) for Ang IV, or additionally via AT_2R stimulation. ACE2 is also a new pathway for the formation of Ang (1–7). Newly identified Ang receptor binding proteins associated with different ATR subtypes may also modify ATR activation. Thus, over-stimulation of AT_1R (and (P)RR) by Ang II, which can contribute to a plethora of cardiovascular disease processes, may be counter-regulated by a number of non-AT_1R mechanisms. Most notably, AT_2R stimulation usually causes opposing effects to AT_1R, as indicated. It is also likely that the MasR exerts a similar counter-regulatory role, whereas the evidence is more preliminary and speculative for AT_4R/IRAP. In terms of mediators, Ang II itself stimulates AT_2R whereas the shorter Ang peptides stimulate their cognate receptors and possibly also AT_2R.

effects of Ang (1–7) has been buoyed by the discovery of angiotensin converting enzyme 2 (ACE2). There is also evidence for a binding site which recognizes Ang IV, but which has no affinity for either losartan or PD123319 (Wright et al., 1993), and has been designated the angiotensin II type 4 receptor (AT_4R) (Swanson et al., 1992; Wright et al., 1995). However, this site has recently been purified from bovine adrenal membranes, and identified as the enzyme, insulin-regulated aminopeptidase (IRAP) (Albiston et al., 2001). IRAP has been localised in tissues such as heart, lung, kidney, and brain, and ligand binding results in effects such as enhancement of cognitive function (Wright et al., 1993; Wright et al., 1999) modulation of blood flow (Swanson et al., 1992; Coleman et al., 1998), increased natriuresis (Ardaillou & Chansel, 1997) and inhibition of cardiomyocyte hypertrophy (Baker & Aceto, 1990; Baker et al., 1992).

Thus, at present, there are 4 ATR subtypes (see Fig. 1). However, for the purpose of this review, the following discussion will predominantly focus on the AT_2R and its functional relevance in cardiovascular disease.

1.3. Angiotensin AT_2R

Like the well characterised AT_1R, the AT_2R is a member of the 7 transmembrane spanning receptor family. The AT_2R shares 34% sequence homology to the AT_1R (Guthrie, 1995; Unger et al., 1996), and is expressed abundantly in foetal tissue, but levels decline rapidly after birth. Consequently, it was originally believed that the AT_2R was primarily involved in cellular growth and differentiation (Capponi, 1996; Akishita et al., 1999; Yamada et al., 1999). In adults, the AT_2R has been localised to the heart, kidney, adrenal gland, brain, uterus, pancreas, retina, skin, and both endothelial and vascular smooth muscle cells (VSMCs) of the vasculature (Viswanathan & Saavedra, 1992; Leung et al., 1997; Nora et al., 1998; Allen et al., 1999; Wang et al., 1999; Wheeler-Schilling et al., 1999; Roulston et al., 2003), and importantly, expression is also increased in numerous cardiovascular pathologies (see Section 3 and Table 2).

Signaling mechanisms differ markedly to those associated with AT_1R (Touyz & Berry, 2002). The topic of AT_2R signalling has recently been extensively reviewed (Hannan & Widdop, 2004; Kaschina & Unger, 2003; Steckelings et al., 2005), and so will only be mentioned briefly. One of the most frequently reported signaling pathways for AT_2R stimulation in vasculature has been increased production of cyclic guanine 3′,5′-monophosphate (cGMP), nitric oxide (NO) and bradykinin (BK), as was first described by Siragy and Carey (1996, 1997): see Widdop et al. (2003) for review. In cultured endothelial (Chaki & Inagami, 1993; Caputo et al., 1995; Saito et al., 1996; Pueyo et al., 1998), PC12W (Zhao et al., 2003), and N1E-115 neuroblastoma cells, and in isolated blood vessels from animal (Boulanger et al., 1995; Seyedi et al., 1995; Thorup et al., 1998; Thorup et al., 1999; Hannan et al., 2003), and human (Batenburg et al., 2004) studies, Ang II has been shown to increase NO and/or cGMP levels via AT_2R and/or AT_1R stimulation. Cross talk between AT_2R and AT_1R is also implicated by the recent finding that NO/cGMP activates a cGMP-dependent protein kinase causing decreased RhoA activity and consequently decreased AT_1R-mediated vasoconstriction (Savoia et al., 2006a, 2005). Involvement of BK and NO/cGMP signalling pathways has also been demonstrated following AT_2R stimulation in vivo, in the vasculature of rats (Siragy & Carey, 1996, 1997, 1999; Siragy et al., 2000, 2001; Walters et al., 2005), and mice (Tsutsumi

et al., 1999), and in cardiac growth responses (Liu et al., 1997; Bartunek et al., 1999).

Several signaling pathways involve activation of protein phosphatases, whose function is to dephosphorylate and thus inactivate MAP kinases such as ERK-1 and ERK-2, resulting in inhibition of both AT_1R- and other 'classical' growth factor-mediated pathways involved in cellular growth and differentiation (Stoll et al., 1995; Tsuzuki et al., 1996). Increased PTPase activity following AT_2R stimulation has been demonstrated in PC12W cells (Bottari et al., 1992; Brechler et al., 1994), N1E-115 neuroblastoma cells (Nahmias et al., 1995) and R3T3 fibroblasts (Tsuzuki et al., 1996). In PC12W cells and adult rat ventricular myocytes, induction of the PTPase MKP-1 is G-protein-dependent (Horiuchi et al., 1997; Fischer et al., 1998). However, studies performed in N1E-115 cells demonstrated that AT_2R-mediated activation of PTPase did not involve a G-protein, but rather induced the soluble PTPase, SHP-1 (Bedecs et al., 1997). Thus Ang II stimulation of AT_2R may result in activation of PTPases via both G-protein-dependent and -independent mechanisms. In addition, stimulation of serine/threonine phosphatases, and subsequent inhibition of ERK-1 and -2, appears to be particularly important in neuronal tissue, in which activation of the serine/threonine phosphatase PP2A has been demonstrated to cause opening of a delayed rectifier K^+ channel (Kang et al., 1994), upregulation of AT_2R mRNA, and increased apoptosis (Shenoy et al., 1999).

AT_2R stimulation may also activate lipid-signalling pathways. Ang II stimulation of neonatal rat cardiomyocytes (Lokuta et al., 1994), rabbit proximal tubule epithelia (Jacobs & Douglas, 1996), and cultured neurons (Zhu et al., 1998), increased PLA_2 activity and arachadonic acid (AA) release. Long-term stimulation of AT_2R by Ang II has also been shown to increase synthesis of ceramides, which may then activate stress kinases and caspases involved in the induction of apoptosis (Gallinat et al., 1999; Lehtonen et al., 1999).

2. Emerging aspects of renin angiotensin system

2.1. Renin/prorenin

It is now recognized that renin exists in two forms, mature renin which can actively cleave angiotensinogen, and the proenzyme, prorenin. Prorenin lacks enzymatic activity, but is transformed into mature renin following cleavage of the 43-amino acid N-terminal propeptide which covers the enzymatic cleft and prevents angiotensinogen access and subsequent cleavage. Interestingly, although synthesized in a limited number of tissues, it has been suggested that prorenin represents up to 90% of total plasma renin in normal subjects, and in certain physiological and pathological conditions, such as pregnancy and diabetes, can circulate at 100-fold higher concentrations than mature renin (Danser et al., 1998). This excess circulating prorenin cannot be activated in the circulation, which has sparked research into the existence of a renin/prorenin receptor. Indeed it was demonstrated that a major source of renin/prorenin in cardiac tissue is due to sequestration and uptake from the circulation (Danser et al., 1994; Muller et al., 1998) suggesting a functional role for prorenin.

A specific renin/prorenin receptor (P)RR was first identified in cultured human mesangial cells (Nguyen et al., 2002), and has since been found to be expressed at relatively high levels in rat and human heart, brain, placenta and adipocytes, and at lower levels in kidney and liver (Danser & Deinum, 2005; Nguyen, 2006; Achard et al., 2007). The (P)RR consists of 350 amino acids, possesses a single transmembrane domain, and exclusively binds renin/prorenin. Binding of renin/prorenin to (P)RR has been shown to have 2 major consequences: increased catalytic activity of renin/prorenin, and activation of (P)RR-mediated signal transduction cascades. Binding of renin to its receptor increases angiotensinogen conversion to Ang I by five-fold (Nguyen et al., 2002), and prorenin, which is virtually inactive in solution, also displays enzymatic activity following receptor binding. This activation of prorenin is not due to proteolysis of the pro-segment which covers the catalytic site, but rather it has been hypothesized that prorenin undergoes a conformational change when bound to the (P)RR, which unmasks the catalytic site and thus activates the proenzyme without removal of the propeptide (Nguyen et al., 2002). Importantly, increased renin/prorenin activity at the cell surface may result in greater Ang I and Ang II levels in the immediate vicinity of Ang receptors and thus increase efficiency of Ang II binding. In addition, receptor-bound renin/prorenin appears to induce intracellular signaling via activation of the MAP kinases, ERK1/2, which is distinct from Ang II-mediated effects (Nguyen et al., 2002). Thus activation and potentiation of renin/prorenin enzymatic activity, together with specific (P)RR-mediated signaling, could conceivably have striking effects on cardiovascular regulation.

In light of these data, recent studies have investigated the role of renin/prorenin and its receptor in physiological and pathophysiological conditions. In particular, a role for the (P)RR in animal models of diabetes and hypertension has recently been identified. Ichihara et al., used a decoy peptide which corresponds with the handle region in the prorenin pro-segment (handle region peptide = HRP), to competitively prevent non-proteolytic activation of prorenin. This group reported that HRP treatment prevented the development of diabetic nephropathy in streptozotocin (STZ)-induced diabetic rats (Ichihara et al., 2004), and also decreased perivascular fibrosis and left ventricular hypertrophy (LVH) in spontaneously hypertensive stroke prone rats (Ichihara et al., 2006). However, several groups have since demonstrated that HRP is unable to prevent renin/prorenin binding and subsequent Ang I generation in mouse VSMCs (Batenburg et al., 2008), or to inhibit renin/prorenin-induced ERK1/2 phosphorylation in cultured VSMCs (Feldt et al., 2008b), or monocytes (Feldt et al., 2008a), casting doubt on the validity of HRP as a peptide to prevent prorenin activation. Similarly, recent studies have been unable to confirm the beneficial effects of HRP in vivo, and thus saw no improvement in end-organ damage following HRP administration in double-transgenic rats overexpressing renin and angiotensinogen genes (Feldt et al., 2008b), or in renovascular hypertensive 2-kidney, 1-clip (2K1C) rats (Muller et al., 2008).

Nevertheless, over-expression of (P)RR in rats increased systolic blood pressure and heart rate which was shown to gradually worsen with increased aged (Burckle et al., 2006), lending weight to the notion of (P)RR-mediated cardiovascular regulation. Although the exact mechanisms by which these effects occur require further investigation, it appears that they are not simply due to increased synthesis of Ang II and subsequent potentiation of Ang II-mediated effects. Huang et al. (2006), demonstrated that treatment of cultured mesangial cells with human and rat recombinant renin increased levels of the pro-fibrotic cytokine transforming growth factor, TGF-β1, and that this effect was not influenced by an inhibitor of the enzymatic activity of renin (RO 42-5892), an AT_1R antagonist (losartan) or an ACE inhibitor (enalapril), but was significantly inhibited by renin siRNA (Huang et al., 2006). Furthermore, evidence of a direct interaction between (P)RR and the transcription factor, promyelocytic zinc finger protein (PLZF), has recently identified a novel signal transduction cascade involving renin/(P)RR/PLZF, activation of which results in cellular proliferation via upregulation of PI3K-p85α (Schefe et al., 2006). Interestingly, PLZF has also been found to associate with AT_2R in the heart (Senbonmatsu et al., 2003), and such an interaction has been suggested as explanation for the AT_2R-mediated cardiac growth-promoting effects deduced from several AT_2R knockout studies (Senbonmatsu et al., 2000; Ichihara et al., 2001, 2002) (see Section 3.2).

Recently the non-peptide renin inhibitor, aliskiren, was approved for the treatment of hypertension. Animal and clinical studies have revealed striking depressor effects of aliskiren (Jensen et al., 2008). More recently, aliskiren has proven to be beneficial in several pathophysiological settings including hypertension and diabetic

nephropathy (Feldman et al., 2008), cardiac remodelling and fibrosis (Pilz et al., 2005; Whaley-Connell et al., 2008) as well as atherosclerosis (Lu et al., 2008). Moreover, Nussberger et al. (2008) reported that in comparison with anti-hypertensive treatments including AT_1R blockade, a β-blocker and calcium channel antagonist, aliskiren was shown to have equally potent blood pressure lowering effects as well as anti-atherosclerotic effects. The history and efficacy of aliskiren in the treatment of hypertension has recently been reviewed by Jensen et al. 2008).

Therefore, these recent studies support a role for the (P)RR in pathological states and future studies using (P)RR knockout mice may provide more insight into the therapeutic potential of aliskiren and the development of (P)RR inhibitors.

2.2. ACE2

ACE2 is a recently discovered homologue of ACE, with 56% similar homology with the N-terminal domain of ACE (Donoghue et al., 2000; Tipnis et al., 2000; Vickers et al., 2002). In contrast to the wide distribution of ACE, ACE2 expression was initially thought to be restricted to endothelial and VSMCs of the heart and kidney (Donoghue et al., 2000; Tipnis et al., 2000; Crackower et al., 2002) although a more widespread distribution is emerging (see Hamming et al., 2007). Unlike the dipeptidyl carboxypeptidase ACE, ACE2 cleaves a single amino acid from the C-termimal of the peptide substrate. Thus, ACE2 cleaves Ang I to the inactive Ang (1–9), which may then be converted to the vasodilator peptide, Ang (1–7), by ACE. More importantly, ACE2 may also directly metabolise Ang II to form Ang (1–7), and this reaction occurs at a faster rate than the formation of Ang (1–9) from Ang I (Carey & Siragy, 2003; Rice et al., 2004). Thus, ACE2 may counter-regulate ACE activity by simultaneously decreasing Ang II levels and increasing Ang (1–7) formation (Hamming et al., 2007). While ACE2 shares significant sequence homology with ACE, it is not sensitive to ACE inhibitors.

Early investigations regarding ACE2 suggested that ACE2 mRNA was increased in both human and animal models of heart failure (Goulter et al., 2004; Burrell et al., 2005) and decreased in genetically hypertensive rats (Crackower et al., 2002), sparking interest that ACE2 may play an important modulatory role on the RAS in certain cardiovascular pathologies. Furthermore, Ishiyama et al. (2004) have reported that ACE2 mRNA expression was increased by AT_1R inhibition following myocardial infarction (MI), and spironolactone treatment also increased ACE2 in heart failure patients (Keidar et al., 2005). Both AT_1R blockade and ACE inhibition increased cardiac ACE2 expression in Lewis rats, whereas activity of the enzyme was increased by AT_1R inhibition only (Ferrario et al., 2005a). However, in direct contrast, Batlle et al. (2006) found no evidence of increased ACE2 activity in biopsies of human heart failure patients. Such inconsistency in results regarding ACE2 expression and activity may be at least partially due to the methods employed, as only poor correlation between ACE2 mRNA and both protein expression and activity has recently been reported in diabetic mice (Wysocki et al., 2006).

Initial studies regarding ACE2 function performed in ACE2 knockout mice (Crackower et al., 2002), resulted in severe impairment of cardiac contractility, which was normalized in double ACE/ACE2 knockout mice, suggesting a counter-regulatory function of ACE2 on the RAS (Crackower et al., 2002). However, several alternative lines of ACE2 knockout mice have since been generated, which exhibit modestly elevated basal blood pressure and normal cardiac phenotype, despite significantly elevated circulating Ang II levels (Gurley et al., 2006). Furthermore, normal cardiac function has been shown in ACE2 knockout mice (Yamamoto et al., 2006), although ACE2 deletion did result in a greater hypertrophic response to pressure overload compared to wild type mice. Similarly, in normotensive rats, ACE2 over-expression induced by lentiviral administration of ACE2 mRNA, did not affect basal cardiac function, however, transgenic animals displayed significantly blunted cardiac hypertrophic and fibrotic responses to Ang II infusion compared to control animals (Huentelman et al., 2005). In an analogous study performed by the same group, ACE2 over-expression in SHR and WKY rats, reduced blood pressure (BP), decreased left ventricular wall thickness, increased left ventricular end diastolic pressure, and attenuated cardiac perivascular fibrosis in hypertensive animals only (Diez-Freire et al., 2006). These data suggest that cardiac phenotype is not solely dependent on ACE2 expression, but support the notion of a cardioprotective role for ACE2 in situations of cardiac stress.

Interestingly, all strains of ACE2 knockout mice reported to date have increased plasma and tissue levels of Ang II, due to both decreased metabolism of plasma Ang II, and increased tissue synthesis as a result of elevated Ang I, suggesting an important function of ACE2 to regulate Ang II levels (Crackower et al., 2002; Gurley et al., 2006; Yamamoto et al., 2006). In addition, ACE2 is known to undergo proteolytic shedding of its extracellular ectodomain to release a soluble form of ACE2 in plasma that maintains catalytic activity (Lambert et al., 2005; Warner et al., 2005). Moreover, ACE2 acts as a receptor for the severe-acute respiratory syndrome (SARS) coronavirus (Li et al., 2003) where it may serve a protective role (Hamming et al., 2007).

2.3. Ligand-independent effects of AT_2R

Although the AT_2R is a member of the G-protein-coupled receptor (GPCR) superfamily, it is well recognized that AT_2R signal transduction does not always occur via classic G-protein-dependent pathways. Recent studies of GPCR modulation and function, with particular focus on areas such as constitutive activity, formation of homo- and hetero-oligimers, and interaction with receptor-associated proteins have afforded fresh insights into GPCR signalling, and provide information that may assist in resolving previous controversies regarding AT_2R-mediated function.

2.3.1. Constitutive activity

AT_2R may possess constitutive activity, as several investigators have reported that AT_2R expression exerts cellular effects without ligand binding. Over-expression of AT_2R in cultured fibroblasts, CHO cells and VSMCs, caused apoptosis via p38 MAPK and caspase-3 signalling pathways (Miura & Karnik, 2000). Moreover, the degree of apoptosis was not sensitive to either Ang II or PD123319, but showed significant correlation with the level of AT_2R protein expression (Miura & Karnik, 2000). In addition, over-expression of AT_2R in cultured VSMC has also been shown to downregulate $AT_{1a}R$ in a ligand-independent manner (Jin et al., 2002). This effect on $AT_{1a}R$ expression was suggested to be due to potentiated BK/NO signaling, as not only was BK and iNOS protein increased by AT_2R over-expression, but both the B_2R antagonist, HOE 140, and the NO synthase inhibitor, L-NAME, ameliorated the decrease in $AT_{1a}R$ expression (Jin et al., 2002). The same group also reported a similar downregulation of AT_{1a} and TGF-β receptor expression in VSMC from WKY, which was associated with reduced basal and Ang II-induced DNA synthesis (Su et al., 2002). Interestingly, AT_{1a} and TGF-β receptor expression, and both basal and Ang II-stimulated markers of cellular growth, were not altered by AT_2R over-expression in VSMCs from SHR, suggesting a disturbance of gene regulation in this model of genetic hypertension, which was suggested to contribute to the exaggerated growth of VSMCs from SHR (Su et al., 2002).

A recent study by D'Amore et al. (2005) also showed constitutive activity of AT_2R in the context of cardiac growth. In this study, transfection of increasing titres of AT_2R in cultured neonatal cardiomyocytes resulted in cellular hypertrophy, which was not influenced by AT_2R ligand binding, and also did not affect AT_1R-mediated hypertrophic signaling, providing evidence for parallel stimulatory roles of AT_1R and AT_2R in cardiac hypertrophy. Interestingly, Falcon et al. utilised microarray expression analysis to identify

genes whose expression was regulated by AT_2R expression. This group identified ~5224 genes which were regulated independently of AT_2R-ligand binding, with proposed functions on cell migration, protein processing, intracellular signaling and DNA repair (Falcon et al., 2005). Moreover, it was found that AT_2R over-expression inhibited human coronary arterial endothelial cells migration in a ligand-independent manner, albeit in an experimental system in which AT_2R expression was increased to levels much greater than those which occur in either physiological or pathophysiological settings (Falcon et al., 2005).

2.3.2. Receptor dimerisation

It is well established that GPCRs are susceptible to receptor dimerisation, and that such an interaction between receptors may affect both receptor activation and signaling, as has been well described for AT_1R (Oro et al., 2007). Like AT_1R which are reported to form heterodimers (AbdAlla et al., 2000, 2001b), both hetero- and homo-oligomers of AT_2Rs have been reported. AT_2R were first shown to directly bind to AT_1R in PC12W cells and fetal fibroblasts, and subsequent stimulation of cells with Ang II resulted in decreased expression of the of AT_1R-associated G-proteins, $G\alpha_{i/o}$ and $G\alpha_{q/11}$, suggesting that such an interaction between receptor subtypes may contribute to AT_2R-mediated antagonism of AT_1R (AbdAlla et al., 2001a). Furthermore, this effect on AT_1R-mediated signaling was independent of AT_2R stimulation, as PD123319 had no influence on $G\alpha_{i/o}$ and $G\alpha_{q/11}$ activation (AbdAlla et al., 2001a). In the same study, AT_2–AT_1R heterodimers were decreased in myometrial biopsies from pregnant compared to non-pregnant women, and expression levels paralleled levels of AT_1R-mediated signaling, demonstrating a functional relevance of heterodimerisation. However, a study in which cultured cardiomyocytes were transfected with AT_1R and AT_2R failed to detect any influence of AT_2R over-expression on AT_1R signaling pathways (D'Amore et al., 2005), suggesting that further confirmation of AT_1/AT_2R heterodimerisation is required.

AT_2Rs have also recently been shown to form heterodimers with BK receptors (B_2R) (Abadir et al., 2006). Similarly to that seen by AT_1–AT_2R dimerisation in PC12W cells, Abadir et al. found that the rate of formation of AT_2–B_2R heterodimers was influenced by the level of expression of both receptors, and was not dependent on ligand binding. Furthermore, conditions which maximized AT_2–B_2R dimer expression also resulted in maximal NO and cGMP production (Abadir et al., 2006), demonstrating that heterodimers are indeed functional.

In addition, homooligomerisation due to disulfide bonding between AT_2R was shown to occur in transfected CHO cells, and was localised to the plasma membrane (Miura et al., 2005). In this cell line, such an interaction between AT_2R resulted in apoptosis, as was indicated by increased caspase3-like activity. Interestingly, apoptosis was unaffected by treatment with either Ang II or PD123319, but was prevented by inhibition of disulfide bonding by dithiothreitol, suggesting that not only was AT_2R-mediated apoptosis ligand-independent, but that homodimerisation of the AT_2R was essential to the observed effect (Miura et al., 2005).

2.3.3. Angiotensin receptor binding proteins

GPCRs are now thought to interact with a range of other accessory proteins (see Bockaert et al., 2003). Recent studies have identified and sequenced 2 distinct AT_1R-associated binding proteins, ARAP1 and ATRAP, which either promote recycling of the AT_1R to the plasma membrane (ARAP1), or induce receptor internalization (ATRAP) (Daviet et al., 1999; Guo et al., 2003; Lopez-Ilasaca et al., 2003). Renal-specific over-expression of ARAP1 in mice resulted in hypertension, decreased urine output, and renal hypertrophy (Guo et al., 2006), and as these effects were abrogated by AT_1R inhibition, indicate a functional role for ARAP1 in potentiation of AT_1R signaling. Conversely, ATRAP has been found to be co-localised with AT_1R in renal tubules (Tsurumi et al., 2006), and in vitro studies have demonstrated that over-expression of this protein decreases AT_1R-mediated signaling and cellular proliferation, thus suggesting that ATRAP may act as a negative regulator of AT_1R signaling (Cui et al., 2000; Lopez-Ilasaca et al., 2003). This field has recently been reviewed (Mogi et al., 2007).

Similar proteins that modulate AT_2R expression at the cell membrane have also been identified. ATIP1 (AT_2-interacting protein 1) is the product of the human Mitochondrial Tumour Suppressor gene, MTUSI, and, in contrast to AT_2R, is widely expressed throughout the body suggesting both AT_2R-dependent and -independent functions (Nouet et al., 2004). Alternatively, this mismatch in ATIP/AT_2R expression could represent an important mechanism by which ATIP modulates AT_2R function in situations of pathological re-expression of AT_2R. Binding of endogenous ATIP1 to the C-terminal tail of the AT_2R inhibited mitogenic pathway signalling, an effect which was potentiated by ligand activation, but was also present in the absence of such stimuli (Nouet et al., 2004). An identical protein, designated ATBP50 (AT_2R-binding protein of 50 kDa), has also been identified in mice. Binding of the Golgi membrane-associated ATBP50 to AT_2R was shown to promote AT_2R cell-surface expression, and this effect was prevented by downregulation of ATBP50 by use of siRNA (Wruck et al., 2005). In mouse neuroblastoma N1E-115 cells, stimulation of AT_2R inhibited EGF-induced ERK1/2 activation and cellular proliferation, and interestingly, not only was this effect blocked by PD123319, but it was also significantly inhibited by ATBP50 siRNA, indicating a functional modulatory interaction between ATBP50 and AT_2R-mediated anti-mitogenic signalling (Wruck et al., 2005).

By contrast, AT_2R also exert growth-promoting effects when coexpressed with the transcription factor PLZF (Senbonmatsu et al., 2003). PLZF is highly expressed in cardiac tissue, and was found to colocalise with AT_2R in the cell membrane upon Ang II stimulation. Within 30 minutes of Ang II administration, AT_2R and PLZF were shown to translocate to the internal compartment, and nuclear PLZF induced $p70^{S6}$ kinase activation (essential for protein synthesis), via increased expression of the phosphatidylinositol-3 kinase $p85\alpha$ subunit (Senbonmatsu et al., 2003).

Taken collectively, these recent data on interacting proteins demonstrate that AT_2R-mediated growth effects may vary dramatically depending on the presence and type of AT_2R-binding proteins, and highlights the importance of future determination of AT_2R-modulatory factors, in addition to AT_2R expression, in the elucidation of AT_2R function in a given tissue (e.g., see Section 3.2 for differential cardiac modulation).

2.4. Angiotensin peptide fragments

Another emerging concept of the RAS is the unique roles of shorter Ang II peptide fragments, such as Ang (1–7), Ang III and Ang IV. These peptides were initially thought to be inactive breakdown products of Ang II, however, they are now recognized as active components of the RAS, often with their own unique biological profile.

2.4.1. Ang (1–7)

There has been a resurgence of interest in the actions of the N-terminal heptapeptide Ang (1–7) since the discovery of ACE2 and the realization that this peptide can be efficiently produced by this additional pathway. As already mentioned, Ang (1–7) can be formed directly from Ang I via neutral endopeptidase. Alternatively, ACE2 is a carboxypeptidase that cleaves the C-terminal amino acid from either Ang I or Ang II to form Ang (1–9) or Ang (1–7), respectively. Ang (1–7) evokes a range of acute central and peripheral effects, the most prominent being vasodilatation, inhibition of VSMC proliferation, vasopressin release and fluid and electrolyte homeostasis (Santos et al., 2000). Interestingly, the mechanism of action of Ang (1–7) is not always straightforward since it can mediate multiple effects via a variety of ATRs including AT_1R, AT_2R or an Ang (1–7)-sensitive site that is recognized by the analogue A-779 (for review, see Santos et al.,

2000). More recently, it was postulated that Ang (1–7) is the endogenous ligand for the MasR, mainly on the basis of cardiovascular actions of Ang (1–7) being abolished in MasR-deficient mice (Santos et al., 2003; Pinheiro et al., 2004). Other studies have suggested that the MasR can heterodimerise with AT_1R to inhibit the effects of Ang II (Kostenis et al., 2005). In addition, extracellular matrix (ECM) remodeling in the heart leading to collagen accumulation and impaired heart function was seen in MasR-deficient mice (Santos et al., 2006). In the last few years, a number of important chronic effects of Ang (1–7) have been identified as a result of exogenous infusion of the peptide. In particular, Ang (1–7) exerted anti-growth and anti-fibrotic effects in rat models of MI, neointimal formation and fibrosis (Strawn et al., 1999; Loot et al., 2002; Benter et al., 2006; Grobe et al., 2006, 2007). The ATR subtype responsible for these cardiovascular protective effects was examined in only one of the afore-mentioned studies, in which it was only partially mediated by the MasR (Grobe et al., 2007), and AT_2R involvement was not examined. By contrast, we found that Ang (1–7) caused vasodepressor effects, during AT_1R blockade, utilizing BK and NO pathways, that were abolished by AT_2R blockade but not Ang (1–7) receptor blockade (Walters et al., 2005). Analogous findings of specific AT_2R- but not MasR-sensitivity to Ang (1–7) have recently been reported (Lara Lda et al., 2006). The likely physiological relevance of these findings is underscored by the fact that the cleavage of Ang (1–7) by ACE2 from Ang II provides a double effect, i.e. the shunting towards Ang (1–7) promotes the formation of a counter-regulatory peptide while reducing the levels, and thus action, of pro-excitatory Ang II (Hamming et al., 2007). Updated biochemical and functional aspects of Ang (1–7) have recently been reviewed (Ferrario et al., 2005b; Ferrario, 2006; Reudelhuber, 2006; Santos & Ferreira, 2007).

2.4.2. Ang III

The Ang (2–8) fragment, Ang III, is readily cleaved from Ang II via aminopeptidase A. Ang III is usually considered a less potent analogue of Ang II that exerts similar AT_1R-mediated effects to the parent octapeptide (de Gasparo et al., 2000). This lack of potency of Ang III is not usually the case in the central nervous system where it is proposed that Ang III is the main mediator of centrally-mediated pressor effects of Ang II, following conversion from the latter (Reaux et al., 2001; Wright et al., 2003). However, this view has recently been challenged (Kokje et al., 2007). Interestingly, large bolus doses of Ang III were reported to exert a biphasic effect on BP in anaesthetized rats consisting of an initial pressor followed by depressor effect; the latter component being AT_2R-mediated (Scheuer & Perrone, 1993). More recently, an AT_2R-mediated vasodepressor effect of Ang III was unmasked during AT_1R blockade in conscious SHR and involved NO and BK signaling pathways (Walters et al., 2003), in an identical manner to Ang (1–7) (Walters et al., 2005). Similarly, Padia et al.showed that Ang III evoked natriuretic effects in conscious rats via AT_2R stimulation, and that inhibition of aminopeptidase N (and thus prevention of the conversion of Ang III to Ang IV), potentiated sodium excretion (Padia et al., 2006, 2007). Furthermore, Ang III-mediated natriuresis was shown to involve the NO/BK cascade which this group has championed as being a hallmark of AT_2R activation (Siragy & Carey, 1996, 1997, 1999; Padia et al., 2006).

2.4.3. Ang IV

Ang IV is formed by the cleavage of Ang III by aminopeptidase B or N. The role of Ang IV in cardiovascular pathophysiology, like other Ang peptide fragments, is emerging as a possible mediator in cardiovascular disease. Following identification of the AT_4R as an aminopeptidase (Albiston et al., 2001), it has been proposed that AT_4R ligands act by inhibiting IRAP catalytic activity, thereby reducing IRAP cleavage of substrates such as lys-bradykinin and vasopressin and prolonging their biological activity (Lew et al., 2003). The precise mechanisms of IRAP modulation by Ang IV is yet to be elucidated, however, the recent identification of a site of Ang IV interaction distinct from the active site of IRAP, suggests that Ang IV may utilise an allosteric mechanism to modulate IRAP activity (Caron et al., 2003). Currently, available data suggests that Ang IV exerts central effects on learning and memory (Chai et al., 2004). AT_4R/IRAP is also upregulated in a rabbit balloon vascular injury model suggesting a possible role in vascular repair or remodeling (Moeller et al., 1999). On the other hand, Esteban et al. (2005) recently demonstrated that Ang IV activated NFκB and subsequently increased pro-inflammatory mediator expression in cultured VSMCs, via AT_4R and independently of AT_1R or AT_2R. The latter two studies suggest Ang IV may play a role in diseases such as atherosclerosis or neointimal hyperplasia. However, Ang IV also induced vasodilatation, (Kramar et al., 1997; Patel et al., 1998; Chen et al., 2000) and has been shown to increase eNOS activation and subsequent NO release (Patel et al., 1998; Hill-Kapturczak et al., 1999) which represents a protective effect in the vasculature. In this context, we have recently shown that chronic treatment with Ang IV improved endothelial dysfunction in ApoE-deficient mice, and this vasoprotective effect most likely resulted from increased NO bioavailability (Vinh et al., 2008a,b). Clearly further research is required to elucidate the role of Ang IV in cardiovascular pathology.

2.5. Endogenous AT_2R ligands?

Given the contextual actions of the various Ang peptide fragments, it is likely that there will be a greater scrutiny of the relative levels of various peptides in the future. For example, it was recently reported that hypercholesterolemia, evoked by a fat-enriched diet in LDL-deficient mice, stimulated production of many angiotensin peptide fragments, with the greatest increases seen in Ang II and Ang IV plasma concentrations (Ang (1–7) was not analysed) (Daugherty et al., 2004). Whether or not all of these peptide fragments have modulatory roles in the progression of atherosclerosis or other cardiovascular diseases remains to be seen. It is well known that plasma Ang II levels are elevated following AT_1R blockade. However, in many instances, AT_1R antagonists as well as ACE inhibitors both elevate plasma Ang (1–7) (Campbell et al., 1991; Campbell et al., 1994), and a similar finding has been made for plasma Ang IV levels in hypertensive patients (Shibasaki et al., 1999). Of course, these changes do not necessarily reflect changes in tissue levels which are notoriously difficult to measure. Nevertheless, it is well established that there is differential regulation of Ang II and related peptides in plasma versus tissue (Campbell et al., 1991, 1994, 1995; Campbell, 1996). Clearly, the plasma and tissue profiles of various Ang peptides are likely to be an important consideration for the full understanding of cardiovascular pathophysiology. New mas spectroscopy techniques (Jankowski et al., 2005) for detection and quantification of plasma and tissue Ang peptides simultaneously should help address this issue.

As is clearly evident from the preceding discussion, there are an increasing number of reports that Ang peptides other than Ang II can cause a range of cardiovascular effects via non-AT_1R (see Table 1). However, there are often mismatches when comparing between functional and binding studies. For example, Ang (1–7) has relatively low binding affinity for AT_2R relative to Ang II and Ang III (Rowe et al., 1995; Bouley et al., 1998), and a low affinity for AT_1R (Rowe et al., 1995; Santos et al., 2003) and yet many of its reported physiological effects occur via interaction with these sites (see Santos et al., 2000). Indeed, we have reported that Ang (1–7) can lower BP via functional stimulation of AT_2R (Walters et al., 2005). Moreover there are other functional and molecular data to support this claim that Ang (1–7) can stimulate AT_2R (Jaiswal et al., 1993; Muthalif et al., 1998; Hansen et al., 2000; Heitsch et al., 2001; De Souza et al., 2004; Castro et al., 2005; Lara Lda et al., 2006) often despite low potency (Hansen et al., 2000). Much less data are available pertaining to Ang IV in this respect. This hexapeptide has a much lower binding affinity than Ang II for AT_2R (Bouley et al., 1998; Hansen et al., 2000), unlike AT_4R; nevertheless

Table 1
Endogenous Ang peptides and synthetic ligands and their relative affinity for various ATR subtypes

	Peptide structure	AT_1R	AT_2R	MasR	AT_4R
Endogenous ligands					
Ang II (1–8)	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	+++	+++	+	
Ang II (1–7)	Asp-Arg-Val-Tyr-Ile-His-Pro	+	++	+++	
Ang III (2–8)	Arg-Val-Tyr-Ile-His-Pro-Phe	++	+++		
Ang IV (3–8)	Val-Tyr-Ile-His-Pro-Phe	+	+		+++
Synthetic ligands					
Sartan compounds	–	(+++)			
PD123319	–		(+++)		
CGP42112	Nicotinoyl-Tyr-Lys(Z-Arg)-His-Pro-Ile		+++		
Compound 21	–		+++		
AVE 0991	–		+	+++	
A-779	Asp-Arg-Val-Tyr-Ile-His-D-Ala			(+++)	
Divalinal-Ang IV	Valψ(CH_2-NH_2)-Tyr-Valψ(CH_2-NH_2)-His-Pro-Phe				(+++)

+ indicates relative affinity for receptor based on binding and functional data (+++) indicates compounds that are antagonists.

there are functional data indicating that Ang IV exerts vascular effects via AT_2R (Loufrani et al., 1999; Faure et al., 2006). Indeed, our own recent data has demonstrated that chronic Ang IV treatment resulted in vasoprotective effects on endothelial function via both AT_4R and AT_2R stimulation (Vinh et al., 2008a,b), consistent with findings in a stroke model (Faure et al., 2006).

On the other hand, there are substantial binding data suggesting that Ang III has 5–10 times higher affinity for AT_2R over AT_1R (Dudley et al., 1990; Timmermans et al., 1991; Rosenstrom et al., 2004), and indeed possesses higher affinity than Ang II itself at AT_2R (Mukoyama et al., 1995; Bouley et al., 1998; Hansen et al., 2000). These binding data fit with the relatively few functional studies that have examined ATR subtype effects of Ang III, since both in vivo (Walters et al., 2003; Padia et al., 2006, 2007, 2008) and cellular signaling (Lorenzo et al., 2002) studies have implicated an AT_2R-selective effect of this peptide.

Intriguingly, Ang (1–7) and Ang III both exerted AT_2R-mediated effects (Walters et al., 2003, 2005; Padia et al., 2006) under conditions in which Ang II itself was ineffective (Gohlke et al., 1998), which raises the distinct possibility that these smaller Ang peptide fragments are endogenous AT_2R ligands. Likewise, the effects of chronic Ang (1–7) or Ang IV infusions are strikingly different to those of Ang II itself (Grobe et al., 2006, 2007; Vinh et al., 2008a,b). Therefore, at the very least, we should consider that Ang peptides other than Ang II have a major role in the cardiovascular system as endogenous non-AT_1R ligands stimulating multiple ATR subtypes (see Table 1).

Of particular note, AT_2R does not desensitize since concentration–response curves to AT_2R-mediated vasorelaxation are highly reproducible, unlike AT_1R-mediated contractile effects (Widdop et al., 2002). The reproducible nature of AT_2R function is consistent with a lack of AT_2R internalization previously reported (Mukoyama et al., 1995; Hein et al., 1997) and hence lack of desensitization. Cellular trafficking of both AT_1 and AT_2R expressed in human embryonic kidney 293 cells indicated that AT_2R cell-surface binding was not altered after prolonged exposure to Ang II (Mukoyama et al., 1995), and fluorescently labeled AT_2R were also not internalized after agonist exposure (Hein et al., 1997). By contrast, AT_1R were rapidly internalized (Hein et al., 1997; Thomas, 1999), which is consistent with functional data (Widdop et al., 2002). Thus it is possible that, in the event of raised circulating or tissue levels of Ang peptides, such fragments may maintain efficacy at least at the AT_2R. Indeed, the ability of failing human hearts to produce Ang (1–7) via ACE2 was directly correlated with AT_2R, but not AT_1R, density (Zisman et al., 2003), further supporting the concept of endogenous AT_2R ligands modulating the effects of Ang II.

2.6. Novel ATR ligands

The Ang II derived peptide CGP42112 has long been the gold standard for determining functional and selective AT_2R activity (Whitebread et al., 1989; de Gasparo et al., 2000). Recently, non-peptide selective AT_2R agonists have been developed. Initially, a non-peptide agonist for both AT_1R and AT_2R was identified (Wan et al., 2004a) and this was soon followed by the first selective non-peptide AT_2R agonist, Compound 21, which was active in 2 in vivo bioassays where it enhanced alkaline secretion from rat intestine and lowered BP in anaesthetized SHR (Wan et al., 2004b). In addition, a number of other peptide-based AT_2R mimetics have been made by the same research group following extensive structure–activity relationships using Ang II or analogues (Johannesson et al., 2004; Georgsson et al., 2005; Rosenstrom et al., 2005; Georgsson et al., 2006). More recently, new peptide-based ligands have been designed that may become lead compounds for future drug development (Georgsson et al., 2007).

AVE0991 is a non-peptide compound that was first described as a Ang (1–7) mimetic, as it competed for Ang (1–7) binding in bovine aortic endothelial cells, and increased NO release in a similar manner to Ang (1–7) (Wiemer et al., 2002). The functional antidiuretic and vasodilator effects of AVE0991 are absent in MasR-deficient mice (Pinheiro et al., 2004; Lemos et al., 2005), although in some instances either MasR blockade (with A-779) or AT_2R blockade (with PD123319) can markedly attenuate the effects of AVE0991 (Wiemer et al., 2002; Pinheiro et al., 2004). More recently, chronic AVE0991 administration was reported to attenuate heart failure induced by MI (Ferreira et al., 2007) although the ATR subtype mediating this effect was not investigated.

Ang IV mimetics also exist. Modifications to the valine in position 1 of Ang IV lead to the formation of an analogue, norleucine-Ang IV (Nle^1-Ang IV), which exhibits similar agonistic properties but 100 fold higher affinity for IRAP compared with Ang IV (Sardinia et al., 1994). In addition, Leu-Val-Val-hemorphin-7 (LVV-hemorphin-7), which was first identified as a ligand based on its ability to displace ^{125}I-Ang IV (Lee et al., 2003) also mimics the biological effects of Ang IV such as enhanced memory and learning retention. In contrast, divalinal-Ang IV, is used as an antagonist to inhibit Ang IV mediated effects (Wright et al., 1995).

3. Role of AT_2R in cardiovascular pathological states

Having discussed the physiological and pharmacological effects mediated by AT_2R, it is appropriate to discuss the specific role of AT_2R in cardiovascular pathologies, given that this ATR subtype is usually upregulated in a range of settings (refer to Table 2).

3.1. Hypertension

Essential or primary hypertension refers to the condition of elevated arterial BP without known cause, and although usually asymptomatic in its earlier stages, has been shown to be closely correlated with the occurrence of future cardiovascular disorders such as left ventricular hypertrophy (LVH), cardiac failure, arteriosclerosis, and stroke (Unger et al., 1996; Weber, 1997; Simon et al., 1998). Considering the well documented anti-hypertensive effect of AT_1R antagonists, and that AT_2R oppose AT_1R-mediated actions in many situations, it is tempting to speculate that AT_2R stimulation may contribute to BP regulation (Widdop et al., 2003).

Initially, in vivo studies deduced a vasodilator function of the AT_2R since Ang II evoked either an augmented vasodepressor effect in the presence of AT_1R blockade or an enhanced vasoconstriction during AT_2R blockade (Scheuer & Perrone, 1993; Munzenmaier & Greene, 1996). However, similar studies performed in SHR were unable to demonstrate corresponding Ang II-mediated vasodilatation during AT_1R blockade (Gohlke et al., 1998). Such divergence in responses in

Table 2
Status of AT_2R expression and function in different cardiovascular pathologies

Disease/setting	AT_2R expression (direction of change; localization)	Function	References
Hypertension (vessels)	↑ aorta SHR, 2K1C, banding (↑ young, ↓ adult- mesenteric SHR)	Anti-hypertrophic (vasoconstriction)	14, 24, 28, 34, 39, 41, 42
Normotension (vessels)	Present VSMC, EC	Vasodilatation	
LVH	↑'s and ↓'s reported	Hypertrophic/anti-hypertrophic	18, 26, 33, 35
Heart failure	Mainly ↑ infarcted heart (fibrotic regions)	Anti-growth	2, 7, 12, 16, 22, 35, 38
Cardiac fibrosis	Mainly ↑	Anti-fibrotic	6, 35, 38
Stroke	↑ infarcted brain	Neuroprotective	19, 43
Renal disease	Mainly ↑	Renoprotective Pronatriuretic	4, 8
Diabetes: Type 1	↑'s & ↓'s kidney, ↑ heart, ↑ vasculature	Renoprotective	1, 5, 10, 11, 17, 20, 29, 30, 37
Type 2	↑ kidney (tubular)	Pronatriuretic	
Atherosclerosis	↑ plaque and vessel wall	Vasoprotective/anti-growth	15, 36, 44
Neointimal formation	↑ neointima	Vasoprotective/anti-growth	21, 23, 32, 40
Females	Mainly ↑ vasculature, kidney	Vasoprotective	3, 9, 23, 27, 31
Aging	↑ heart, aortic and mesenteric arteries	(Vasoconstriction-mesentery)	13, 25, 39

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these experiments may be reconciled by the subtle hypotensive effect of AT_2R stimulation, which may have been masked by the concomitant dominant AT_1R-mediated pressor action of Ang II infusion. In order to avoid such confounding influences of AT_1R stimulation on potential AT_2R vasodilator function, investigators have assessed the effect of selective AT_2R agonists and antagonists during AT_1R blockade (see Fig. 2). Using this approach, selective stimulation of AT_2R by CGP42112 lowered BP, provided that there was a background of AT_1R blockade in conscious rats (Barber et al., 1999; Carey et al., 2001). Furthermore, this BP-lowering response to AT_2R stimulation was shown to be associated with increased blood flow in renal, mesenteric and hindquarter circulations indicating widespread vasodilatation (Li & Widdop, 2004), which highlights the fact that modest AT_2R-mediated vasorelaxation observed in isolated blood vessels can translate into significant in vivo haemodynamic effects. A similar acute BP-lowering effect of AT_2R stimulation was also deduced in a recent study in which the anti-hypertensive action of losartan was potentiated in rats following transient peripheral over-expression of AT_2R. This hypotensive action was blocked by PD123319, and persisted over the same time frame as enhanced AT_2R expression (reduced towards basal levels 7 days after viral transduction), lending weight to the importance of relative AT_1/AT_2R expression to AT_2R-mediated functional effects (Li et al., 2006).

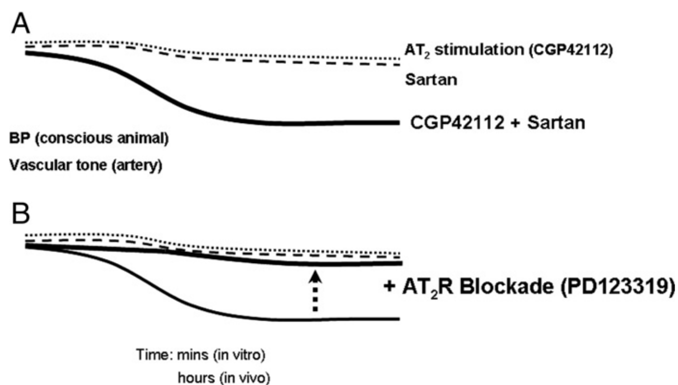

Fig. 2. The in vitro and in vivo vasodilator effects of AT_2R stimulation are often difficult to detect because of the overriding effects of AT_1R-mediated vasoconstriction. This state can be dramatically changed by performing AT_2R stimulation against a background of low-dose AT_1R antagonist (sartan), even using sartan doses that are sub-threshold for BP-lowering (**A**). Under these circumstances, AT_2R-mediated vasodilatation can be unmasked and subsequently abolished by concomitant AT_2R blockade using PD123319 (**B**).

These acute in vivo findings are consistent with numerous reports of AT_2R-mediated vasorelaxation in a wide variety of locations, including mesenteric, renal, coronary, cerebral and uterine vascular beds, which has been shown to be via BK/NO/cGMP signaling pathways. AT_2R-mediated vasorelaxation has also been indirectly implicated in conduit vessels such as the aorta since aortic banding markedly increased aortic AT_2R expression as well as activating the eNOS/cGMP axis (Hiyoshi et al., 2004; Yayama et al., 2004, 2006). Consequently, Ang II-mediated contraction via AT_1R stimulation was reduced in this vessel Yayama, 2004 #1686; Hiyoshi, 2004 #1685}. Recent evidence further suggests that such recruitment of NO/cGMP mechanisms activates a cGMP-dependent protein kinase (cGKI) resulting in downregulation of RhoA activity, which is known to involved in AT_1R-mediated vasoconstriction (Savoia et al., 2006a). Interestingly, AT_1R blockade was shown to increase AT_2R expression (2–3-fold) and NO production, and to suppress NAD(P)H-driven superoxide generation, in arteries of hypertensive SHRSP, but not normotensive WKY rats (Savoia et al., 2006a). This potentiated NO signaling decreased RhoA/Rho kinase activation, reduced MLC phosphorylation, and subsequently unmasked AT_2R-mediated vasorelaxation to Ang II, providing evidence for mechanisms of AT_1/AT_2R crosstalk at a signaling level (Savoia et al., 2005). For further detailed analysis of AT_2R-mediated relaxation/vasodilatation, see (Hannan & Widdop, 2004; Henrion et al., 2001; Widdop et al., 2008).

The vast majority of in vitro vascular studies on AT_2R have used arteries obtained from normotensive animals; interestingly, there is little evidence of AT_2R-mediated vasorelaxation in arteries taken from untreated hypertensive animals (Matrougui et al., 1999; Matrougui et al., 2000; Cosentino et al., 2005) which contrasts the afore-

mentioned in vivo data (Barber et al., 1999; Li & Widdop, 2004; Walters et al., 2005). Moreover, in some cases, Ang II in fact evoked contraction of mesenteric arteries obtained from SHR that involved both AT_1R and AT_2R (Touyz et al., 1999).

Pressure overload induced by aortic banding in rats (Yayama et al., 2004) and mice (Hiyoshi et al., 2004), or 2K1C hypertension (Hiyoshi et al., 2005), has been shown to upregulate vascular AT_2R expression and thus attenuate the AT_1R-mediated contractile response to Ang II. Increased AT_2R expression in the context of pressure overload was suggested to be due to stimulation of AT_1R by increased circulating Ang II, as AT_1R inhibition prevented the upregulation of AT_2R expression due to aortic banding (Yayama et al., 2004). On the other hand, vascular AT_2R was reduced in mesenteric arteries, but increased in aortae, in spontaneously hypertensive rats compared to normotensive controls (Touyz et al., 1999; Widdop et al., 2008). Thus, site-specific changes in vascular AT_2R expression associated with hypertension, and the need to block tonic AT_1R activity, may account for some of the discrepancies between in vitro and in vivo hypertensive studies.

Indeed, vascular AT_2R expression is generally increased by chronic AT_1R blockade (Savoia et al., 2005; You et al., 2005; Savoia et al., 2006a,b), and such treatment can unmask AT_2R-mediated vasorelaxation in previously unresponsive aortic vessels from SHR (Cosentino et al., 2005). In this context, we have recently reported that the AT_2R vascular phenotype is dependent on basal arterial BP and level of AT_2R expression, at least in mesenteric arteries (You et al., 2005). Ang II (in the presence of AT_1R blockade) evoked AT_2R-mediated vasorelaxation in mesenteric arteries from WKY rats whereas analogous experiments using mesenteric arteries from SHR resulted in Ang II causing AT_2R-mediated contraction. At the same time, AT_2R expression, determined by Western blot analysis, was reduced in mesenteric arteries from SHR compared with WKY rats. Strikingly, when basal BP of SHR was normalized to WKY-like levels with either an ACE inhibitor or an AT_1R antagonist, the AT_2R-mediated response was converted from contraction to relaxation. Mesenteric AT_2R expression was increased with chronic anti-hypertensive treatment, in line with AT_2R-mediated vasorelaxation (You et al., 2005). Moreover, this upregulation of AT_2R expression appeared to be related to the level of basal BP since treatment of SHR with the non-specific anti-hypertensive, hydralazine, also increased AT_2R expression and converted AT_2R-mediated vasoconstriction to Ang II in mesenteric vessels into vasorelaxation. Vascular AT_2R localization was also performed using fluorescently-labelled Ang II in the presence of AT_1R blockade. These studies confirmed the upregulation of AT_2R following anti-hypertensive treatments and identified that AT_2R were re-expressed at the level of the endothelium (You et al., 2005). Conversely, it was recently reported that C-reactive protein caused systolic hypertension that was directly related to downregulation of vascular AT_2R (Vongpatanasin et al., 2007).

AT_2R function has also been indirectly examined by determining the effect on BP of AT_2R blockade during AT_1R inhibition. In relatively short-term studies performed in rats with either renovascular hypertension or which had been sodium-depleted, the hypotensive effect of AT_1R blockade was reversed by simultaneous administration of PD123319, suggesting a vasodilator role of the AT_2R (Gigante et al., 1998; Siragy & Carey, 1999; Siragy et al., 2000). However, in analogous experiments, Duke et al. (2005a) reported that AT_2R contributed to the BP-lowering and mesenteric vasodilator effect of candesartan in normotensive, but not hypertensive (SHR, 2K1C) rats.

There are a limited number of clinical studies investigating vascular AT_2R function in vivo, and these studies have not always reported AT_2R-mediated vasodilator effects, which most likely reflect the different patient populations studied. In healthy male volunteers, intravascular administration of PD123319 had no effect on systemic vascular resistance or arterial stiffness, providing no evidence for acute AT_2R-mediated haemodynamic effects (Phoon & Howes, 2001; Brillante et al., 2005). However, in other studies by the same investigators, treatment of adult male subjects (Gilles et al., 2004) or elderly women (Phoon & Howes, 2002) with an AT_1R antagonist, uncovered acute haemodynamic effects due to AT_2R, deduced from short-term infusions of PD123319, thus supporting a role for AT_2R during AT_1R blockade, albeit with some variation that likely reflects the different patient populations (Phoon & Howes, 2002; Gilles et al., 2004).

On the other hand, investigations into *chronic* AT_2-mediated regulation of BP have yielded less conclusive results. In AT_2R knockout mice (Hein et al., 1995; Ichiki et al., 1995; Gross et al., 2000a), and rats administered AT_2R antisense to 'knockdown' receptor expression (Wang et al., 2004), both an increase in basal BP and/ or potentiation of the pressor response to exogenous Ang II have been attributed to the absence of AT_2R modulation of vascular tone. In mice with targeted over-expression of vascular (Tsutsumi et al., 1999) or cardiac (Masaki et al., 1998) AT_2R, there was no change in basal BP, although acute Ang II infusion decreased BP. Stimulation of AT_2R by chronic Ang II infusion during AT_1R blockade failed to decrease BP (Cao et al., 1999; Diep et al., 1999), and similarly, chronic AT_2R inhibition either had no (Liu et al., 1997; Tea et al., 2000) or minimal (Varagic et al., 2001) influence on the anti-hypertensive effect of chronic AT_1R blockade. Thus in terms of chronic haemodynamic control, and in contrast to the well documented acute vasodilator/relaxation effects of AT_2R stimulation, AT_2R activation appears to exert only subtle influences on long-term BP regulation. Further studies using chronic AT_2R agonist treatment are clearly warranted to address this issue.

Compared to the extensively investigated role of AT_2R in vascular reactivity, longer term effects on vascular remodeling in hypertension have been less well studied. Vascular remodelling associated with hypertension occurs at all levels of the vascular tree, and increased local expression of RAS components and subsequent enhanced local Ang II production (Shiota et al., 1992; Jandeleit-Dahm et al., 1997; Wang et al., 2003), implies an important role of the RAS in this process. Furthermore, other factors involved in cellular growth and ECM production which are known to be stimulated by Ang II, such as growth factors (Parker et al., 1998; Su et al., 2002), factors involved in the inflammatory response (Chou et al., 1998) and reactive oxygen species (ROS) (Virdis et al., 2002; Keidar et al., 2004; Touyz et al., 2003), are also upregulated in the hypertensive vasculature. The present overview is limited to discussion of vascular remodeling directly related to hypertension, as reports of AT_2R-mediated vascular effects in the specific contexts of atherosclerosis and diabetes (Savoia et al., 2006b) will be discussed in following sections.

Early direct evidence for a component of the vascular anti-hypertrophic effect of sartan-treatment being due to AT_2R stimulation was provided by Tea et al. (2000), who showed that simultaneous AT_2R inhibition returned aortic mass, smooth muscle cell number and DNA synthesis back to control levels in SHR. We have similarly shown that chronic PD123319 treatment completely reversed sartan-mediated aortic remodeling in both adult and senescent SHR (unpublished data), similarly to aged normotensive rats (Jones et al., 2004). Importantly, this AT_2R-mediated vascular anti-hypertrophic effect was not simply a consequence of sartan-induced BP-lowering, as simultaneous PD123319 administration had no further influence on BP in these studies (Tea et al., 2000; Jones et al., 2004). Similarly, in Ang II-induced hypertension, simultaneous blockade of AT_1 and AT_2R by Sar-Ile resulted in greater media:lumen ratio than that due to AT_1R blockade alone in both aortic and mesenteric vessels, suggesting an AT_2R-mediated anti-hypertrophic effect (Brassard et al., 2005). Regulation of collagen synthesis also appears to be altered in hypertension, since AT_1R-mediated Ang II stimulation of collagen production was potentiated in SHR and occurred via both p38 and ERK1/2 signalling pathways, whereas collagen production was reduced, and dependent on only ERK1/2 activation in WKY (Touyz et al., 2001). In addition, Su et al. (2002) showed that over-expression of AT_2R in

VSMCs from WKY rats decreased AT_1R and TGF-β receptor expression, but that this response was absent in VSMCs of SHR, suggesting a deficiency in inhibitory mechanisms in this model of genetic hypertension.

Furthermore, both basal media:lumen ratio, and vascular hypertrophy due to pressure overload was significantly elevated in aortic, femoral (Brede et al., 2001), and coronary (Akishita et al., 2000b; Wu et al., 2002) vessels of AT_2R knockout mice compared to wild type controls, indicating an AT_2R-mediated protective effect on vascular hypertrophy. Moreover, consistent with results from pharmacological AT_1/AT_2R inhibition in rats, the anti-hypertrophic action of AT_1R inhibition was reduced in coronary vessels from AT_2R knockout mice, supporting a role for AT_2R stimulation in the beneficial remodeling effects of AT_1R antagonists (Wu et al., 2002).

Taken collectively, these experimental results suggest that, although an acute vasodilator role of AT_2R is well documented, chronic BP regulation seems to be only minimally influenced by AT_2R stimulation. However, AT_2R have consistently been shown to be important in the regulation of vascular structure, which may indirectly influence BP maintenance in the longer term. Indeed, restoration of AT_2R-mediated vasorelaxation in mesenteric arteries from SHR was correlated with anti-hypertensive efficacy (You et al., 2005).

3.2. LVH

LVH is considered a major predictor of cardiovascular morbidity and mortality (Levy et al., 1990), and results in changes in the structural organization of the heart, which necessarily influences cardiac function. When the relative proportions of cell types within the heart remain unchanged, hypertrophy is termed adaptive or physiological, however, by far the most prevalent form of cardiac hypertrophy involves disproportionate changes within the cardiac tissue, which ultimately decrease cardiac function, and results in LVH and subsequent progression to heart failure (Swynghedauw, 1999; Zhu et al., 2003). Since the cloning of the AT_2R over a decade ago, and despite considerable investigation into the role of AT_2R in cardiac hypertrophy, the exact function of AT_2R in cardiac tissue still remains somewhat controversial (Booz, 2004; Reudelhuber, 2005).

Importantly, the relatively low expression of the AT_2R compared with that of the AT_1R, is upregulated in certain conditions, such that increased AT_2R expression has been reported in patients with heart failure, ischemic heart disease, and dilated cardiomyopathy (Regitz-Zagrosek et al., 1995; Asano et al., 1997; Haywood et al., 1997; Tsutsumi et al., 1998; Wharton et al., 1998). Moreover, a correlation between AT_2R density and severity of heart failure has been reported (Rogg et al., 1996), raising the obvious question of whether or not such increased AT_2R expression represents a causative or a reactive consequence of LVH.

In animal models, AT_2R expression is also increased by cardiac hypertrophy and heart failure, either in terms of absolute numbers, or relative to AT_1R expression (Suzuki et al., 1993; Lopez et al., 1994; Nio et al., 1995; Ohkubo et al., 1997; Bartunek et al., 1999; Busche et al., 2000). In vitro studies have demonstrated increased AT_2R expression due to stabilization of mRNA following cardiomyocyte stretch, providing a potential mechanism for observed enhancement of AT_2R density during situations of cardiac overload (Kijima et al., 1996).

In accordance with the notion that the AT_2R opposes AT_1R mediated effects, Liu et al. (1997) demonstrated in a rat model of heart failure that the anti-hypertrophic effects of AT_1R blockade on LV volume and cardiomyocyte size were reversed by concomitant AT_2R inhibition. Consistent with a protective function of AT_2R, AT_2R-deficient mice showed a greater hypertrophic response to MI than that of wild type controls (Brede et al., 2003; Oishi et al., 2003), and also attenuated response to AT_1R blockade (Xu et al., 2002). In more recent studies in which AT_2R were over-expressed via a lentiviral vector gene delivery system in cardiomyocytes of 5 day old SHR (Metcalfe et al., 2004), or Sprague-Dawley rats (Falcon et al., 2004), AT_2R over-expression reduced cardiac hypertrophy at 21 weeks of age compared to control SHR, and attenuated Ang II-induced cardiac hypertrophy and fibrosis, respectively. Similarly, in isolated perfused adult hypertrophied hearts (Bartunek et al., 1999), Ang II-induced production of early response genes were enhanced by AT_2R inhibition, providing evidence for AT_2R-mediated regulation of cellular growth at the level of gene expression.

On the other hand, a lack of effect of AT_2R on indices of cellular growth has also been shown in pharmacological studies. We and others have deduced no influence of AT_2Rs on cardiac hypertrophy from 'PD-reversal' studies, in which simultaneous PD123319 administration did not modify the cardiac anti-hypertrophic effect of AT_1R blockade in either aged WKY (Jones et al., 2004) or SHR (Varagic et al., 2001) rats. Similarly, the cardiac growth-inhibitory effect of valsartan, was of similar magnitude in aortic-banded AT_2R knockout mice to that of animals expressing AT_2R (Wu et al., 2002). Ang II infusion also resulted in a comparable increase in cardiac hypertrophy in mice with cardiac specific over-expression of AT_2R, to that of wild type mice (Sugino et al., 2001; Kurisu et al., 2003).

In direct contrast, several in vivo studies have suggested a growth-stimulatory role of AT_2R. Mice in which AT_2R were over-expressed using the ventricular-specific myosin light-chain promoter, exhibited dilated cardiomyopathy which was associated with increases in both myocyte cross sectional area and fibrosis (Yan et al., 2003). Similarly, in AT_2R knockout mice, the cardiac hypertrophic response to aortic banding, Ang II infusion or MI was absent in AT_2R-deficient animals (Senbonmatsu et al., 2000; Ichihara et al., 2001; Ichihara et al., 2002), indicating a requisite presence of AT_2R for cardiomyocyte hypertrophy. Interestingly, D'Amore et al. (2005) recently showed that AT_2R evoked constitutive growth in cultured neonatal cardiomyocytes. The research groups who reported a growth-promoting function of AT_2R also found evidence for increased signalling via $p70^{S6}$ kinase, which is a well documented hypertrophic mechanism (Senbonmatsu et al., 2000; Yan et al., 2003). In a subsequent study (Senbonmatsu et al., 2003), AT_2R-mediated cellular hypertrophy involving $p70^{S6}$ kinase was dependent on PLZF activation as previously discussed (Section 2.3.3). Nevertheless, the cardiac hypertrophic effects of AT_2R are controversial, with either no effect or hypertrophy being attributed to cardiac AT_2R in different AT_2R knockout strains subjected to various cardiac loads (Akishita et al., 2000b; Senbonmatsu et al., 2000; Widdop et al., 2003). Thus, it is possible that these discrepancies may relate to differential PLZF expression and/ or activity in the 2 AT_2R knockout strains. In this context, as previously mentioned, a novel signal transduction pathway involving renin/(P)RR/PLZF has been described, that displayed both excitatory and inhibitory actions of PLZF (Schefe et al., 2006).

Interestingly, a study performed by Lako-Futo et al. may go some way towards explaining such equivocal AT_2R-mediated effects on cardiac hypertrophy. In acute studies performed in rats, blockade of the AT_2R with PD123319 during 62 h Ang II infusion, resulted in enhanced expression of genes associated with the promotion of cardiac hypertrophy (c-fos, endothelin-1, and IGF-1), but also increased expression of anti-hypertrophic factors (ANF and BNP), and decreased growth factors (VEGF and FGF-1) (Lako-Futo et al., 2003). These results suggest that in addition to opposing AT_1R-mediated effects, AT_2R stimulation may produce growth-stimulatory actions which partially offset AT_2R-mediated growth-inhibitory effects. Thus, it is conceivable that even slight alterations in conditions may shift the balance of AT_2R-mediated actions from a situation in which pro- and anti-hypertrophic effects remain in equilibrium, to that in which either growth-promoting or growth inhibiting- pathways are favoured.

In this context, it is interesting to note that the most consistent reports of a cardioprotective effect of AT_2R in vivo, have been determined from experiments performed in models of MI-induced

heart failure. The vast majority of investigators have deduced AT_2R-mediated improvement of LV function and remodelling from multiple studies performed in mouse models of either AT_2R-deletion (Brede et al., 2003; Oishi et al., 2003) or over-expression (Yang et al., 2002; Bove et al., 2005). In fact, only 1 study has deduced a negative role for AT_2R stimulation in this context (Ichihara et al., 2002), and these discordant results may be due to the alternative strain of knockout mouse used by Ichihara et al., as has been previously discussed (see Widdop et al., 2003). AT_2R-mediated cardioprotection has been shown to involve NO signalling, as NO synthase inhibition prevented the beneficial effects of AT_2R over-expression on LV function (Bove et al., 2004). BK has also been implicated in cardiac AT_2R-mediated effects, as pharmacological inhibition of B_2R in rats (Liu et al., 1997), or B_2R deletion in mice (Isbell et al., 2007), was shown to prevent the anti-fibrotic influence of AT_2R stimulation. In addition, AT_2R stimulation has been shown to be involved in the beneficial effects of sartans post-MI, as AT_1R inhibition was found to be less effective during AT_2R blockade (Liu et al., 1997) or AT_2R-deletion (Xu et al., 2002), and potentiated by AT_2R-over-expression (Voros et al., 2006).

Interestingly, genetic influences in humans have also been reported which add another degree of complexity. The AT_2R gene polymorphism -1332G/A (also known as 1675G/A) has also been linked with LVH. In hypertensive subjects, LVH has been associated with either the A allele (Schmieder et al., 2001) or the G allele (Alfakih et al., 2004) in 2 separate studies, with the method of LVH determination (echocardiography versus magnetic resonance imaging) suggested as explanation for these discrepancies (Alfakih et al., 2004). Premature coronary artery disease has also been associated with the G allele (Alfakih et al., 2005). However, the interpretation of these findings is somewhat unclear as the same group has reported that the G allele represents either decreased AT_2R function (Nishimura et al., 1999; Alfakih et al., 2004; Alfakih et al., 2005) or increased AT_2R function (Warnecke et al., 2005; Strauss & Hall, 2006).

Clearly, neither animal nor human studies to date have been able to delineate a consistent influence of AT_2R stimulation on LVH. Current equivocal results may be partly explained by the range of animal models, indices measured, drugs, doses and treatment times employed by investigators, but most likely reflect the complex and context-specific nature of AT_2R-mediated cardiac growth effects, probably also involving ligand-independent effects. Given the correlation between LVH and the poor long-term prognosis of patients exhibiting such modification of cardiac structure (Levy et al., 1990; Rihal et al., 1994; Senni et al., 1998), this area of AT_2R function undoubtedly requires further research.

3.3. Cardiac fibrosis

In contrast to the range of equivocal results regarding the influence of AT_2R on LVH, the majority of experimental data relating to cardiac fibrosis report anti-fibrotic effects of AT_2R stimulation. Indeed, increased AT_2R expression in the heart has been specifically localised to cardiac fibroblasts, suggesting a link between upregulation of AT_2R expression and fibrosis present in hypertrophied and failing hearts (Brink et al., 1996; Tsutsumi et al., 1998; Wharton et al., 1998). Such an association between AT_2R expression and cardiac disease states, and previous suggestions of AT_2R-mediated anti-growth effects, has fuelled interest in the AT_2R as a potential target for cardiac anti-fibrotic therapies.

It is well accepted that Ang II is an important mediator of ECM homeostasis, with numerous reports of either AT_1R blockade or ACE inhibitor treatment resulting in decreased cardiac fibrosis in both humans and animal models. Importantly, in both physiological (e.g. heart valve leaflets) and pathophysiological (e.g. MI) sites of repair, AT_2R are expressed at high levels, along with other RAS components such as angiotensinogen, ACE, and AT_1R (Weber et al., 1997). Moreover, important factors involved in Ang II-mediated connective tissue formation, including TGF-β_1 and its receptors, and type I and III collagens, are also expressed in AT_2R-rich fibroblasts and myofibroblasts (Katwa et al., 1997; Lijnen et al., 2003).

Although 2 separate groups have reported a stimulatory effect of AT_2R on cardiac collagen accumulation (Senbonmatsu et al., 2000; Ichihara et al., 2001; Ichihara et al., 2002; Yan et al., 2003), the bulk of data from numerous in vivo studies clearly support an inhibitory effect of AT_2R on cardiac fibrosis. In 'PD123319 reversal' experiments, AT_2R inhibition reversed the anti-fibrotic effects of AT_1R blockade both in SHRs (Varagic et al., 2001), and in aged normotensive WKY (Jones et al., 2004) and aged SHR (unpublished data). Similarly, the anti-fibrotic effect of AT_1R inhibition was found to be attenuated in AT_2R-deficient mice, since the sartan-mediated reduction in cardiac fibrosis following aortic banding or Ang II infusion, was of smaller magnitude in AT_2R knockout mice compared to wild type littermates (Wu et al., 2002; Xu et al., 2002). Other investigators have also deduced an inhibitory effect of the AT_2R on cardiac fibrosis, as the promotion of interstitial fibrosis by either aortic banding or L-NAME was augmented in AT_2R knockout mice (Akishita et al., 2000b; Wu et al., 2002; Gross et al., 2004) and cardiac specific over-expression of AT_2R diminished the amount of both perivascular and interstitial fibrosis induced by Ang II infusion (Kurisu et al., 2003).

However, the exact mechanisms by which AT_2R may modulate collagen deposition are not completely understood. The accumulation of collagen within the heart is a dynamic process, with total collagen content representing the net effect of both collagen synthesis and degradation. In terms of collagen production, a stimulatory role for AT_1R in terms of collagen synthesis is well established, however, there are contrasting reports of the contribution of AT_2R to this process. In rat cardiac fibroblasts, collagen production was reduced by inhibition of either AT_1 or AT_2R (Brilla et al., 1994; Brilla et al., 1995), and chronic blockade of both AT_1R and AT_2R with Sar^1Ile^8Ang II in rats also decreased collagen deposition, suggesting a stimulatory role of AT_2R in collagen synthesis (Brassard et al., 2005). In addition, Mifune et al. (2000) also reported that AT_2R stimulation by CGP42112 induced collagen synthesis in VSMCs and mesangial cells, but that CGP42112 treatment had the opposite effect in cultured fibroblasts, indicating heterogeneous AT_2R-mediated responses in different cell types. In contrast, several other investigators have reported a lack of effect of AT_2R on collagen secretion, since Ang II stimulation of collagen production in cultured rat and porcine cardiac fibroblasts, and rat mesenteric VSMCs was inhibited by AT_1R blockade, but unaffected by AT_2R inhibition (Lijnen et al., 2000; Touyz et al., 2001; Warnecke et al., 2001). On the other hand, the influence of AT_2R on collagen degradation has not been extensively investigated. In one of the earliest studies to investigate AT_2R-specific effects on collagen metabolism, the decrease in collagenase activity due to Ang II stimulation of cultured cardiac fibroblasts was abolished by AT_2R blockade but not influenced by AT_1R inhibition (Brilla et al., 1994). In contrast, Min et al. (2004) demonstrated a diminution of collagenase activity by AT_2R blockade which was associated with increased tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) expression, and selective AT_2R stimulation with CGP42112 increased matrix metalloproteinase-1 (MMP-1) production in human monocytes (Kim et al., 2005).

3.4. Stroke and neuroprotection

On the basis of several large-scale clinical trials, it is now accepted that chronic inhibition of the RAS can provide neuroprotection, with reduced occurrence of stroke in high-risk populations (Dahlof et al., 2002; Arnold et al., 2003; Chapman et al., 2004; Schrader et al., 2005). Neuroprotection has also been demonstrated by candesartan cilexetil in an acute setting (Schrader et al., 2003). Moreover, drugs that target the renin–angiotensin system offer benefits that extend beyond the control of BP (Arnold et al., 2003; Chapman et al., 2004).

In accordance with human clinical trials, animal studies have also concluded that inhibition of the RAS protects against neuronal injury following stroke, often in a BP-independent manner (Ito et al., 2002; Groth et al., 2003). Similarly, AT_1R knock out mice, exhibited smaller infarct size following temporary middle cerebral artery occlusion (Walther et al., 2002). Underlying mechanisms may involve the inhibition of AT_1R-mediated cerebrovascular pathological growth and inflammation, as well as improved cerebrovascular compliance and autoregulation (Nishimura et al., 2000; Saavedra et al., 2006). In addition, Sugawara et al. (2005) recently demonstrated that a non-hypotensive dose of an AT_1 receptor antagonist reduced superoxide production and improved neurological outcome following global ischemia (Sugawara et al., 2005).

Not surprisingly considering the contribution of AT_2R to the effects of AT_1R inhibition in other settings, attention has recently turned to the potential role of the AT_2R in neuroprotection, particularly since AT_2R is reported to be upregulated in stroke (Zhu et al., 2000; Li et al., 2005b; Lu et al., 2005), specifically in the ischemic area of the brain (Mogi et al., 2006). Moreover, AT_2R-deficient mice display a larger ischemic area and a more severe neurological deficit following stroke when compared to wild type (Iwai et al., 2004), implicating a neuroprotective role for the AT_2R. In this context, the neuroprotective effect of valsartan was reduced in AT_2R knockout animals (Iwai et al., 2004; Mogi et al., 2006), and either peripheral (Lu et al., 2005) or central (Li et al., 2005b) administration of an AT_2R antagonist reversed the neuroprotection evoked by AT_1R blockade, suggesting opposing roles for AT_1R and AT_2R during stroke.

The potential mechanisms of AT_2R-mediated neuroprotection in reducing infarct volume following stroke are currently under investigation. AT_2R-mediated apoptosis (Zhu et al., 2000; Grammatopoulos et al., 2002) is one possibility. Indeed, AT_2R is capable of inducing apoptosis in PC12W cells through the induction of ceramides and the activation of MKP-1 signalling (Dai et al., 1999). Furthermore, AT_2R knockout mice have higher neuronal numbers compared to controls, suggesting that AT_2R deficit antagonises angiotensin II-induced apoptosis (Von Bohlen und Halbach et al., 2001). Another possible AT_2R-mediated neuroprotective influence involves neuronal regeneration. Earlier studies (Laflamme et al., 1996) demonstrated that in vitro stimulation of AT_2R was associated with intense neurite outgrowth; an effect that was negated by PD123319. Reinecke et al. (2003) demonstrated that angiotensin II can act similarly to NGF, promoting remyelination, neurite outgrowth and functional recovery following sciatic nerve crush in adult rats via AT_2R (Reinecke et al., 2003). In this model of nerve injury, co-administration of losartan and Ang II accelerated regeneration whereas PD123319 alone or in combination with Ang II, prevented recovery. Similar findings were reported using an optic nerve crush model, whereby stimulation of AT_2R on retinal ganglion cells significantly increased neurite outgrowth (Lucius et al., 1998). Stroth et al. (1998) identified that the AT_2R is involved in the regulation of cytoskeleton proteins essential for neurite extension (Stroth et al., 1998). They examined the expression of several microtubule components in PC12W cells and found Ang II and NGF differentially regulate microtubule proteins and, in the case of Ang II, the upregulation of dendritic proteins was mediated via AT_2R. Likewise, it was found that selective activation of AT_2R increased the expression of beta III-tubulin, and the microtubule-associated proteins, tau and MAP2, resulting in increased elongation of neuritis (Cote et al., 1999). In a recent series of elegant studies from Unger's laboratories, it was found that central administration of PD 123319 abolished the neuroprotective effects of central AT_1 receptor blockade in conscious rats, both in terms of infarct size and neurological outcome following a transient unilateral medial cerebral artery occlusion (Li et al., 2005b). These authors indicated that AT_2R was upregulated during stroke and supported neuronal survival and neurite outgrowth in vulnerable peri-ischemic brain areas, as has recently been reviewed (Thone-Reineke et al., 2006).

At first glance, it seems contradictory that AT_2R would be involved in both apoptosis and neuronal regeneration, however, during early stages, neuronal injury will initiate a series of events that are identical for apoptosis and regeneration (Lucius et al., 1998). AT_2R-mediated activation of the MKP-1 pathway brings the cell to a point where it may enter the repair process, or programmed cell death, depending on the energy state of the cell. Thus AT_2R contribute to the cellular mechanisms allowing adaptation to ischemic insult, by stimulating both tissue repair and programmed cell death (Lucius et al., 1999). The apoptotic process can be neuroprotective as it serves to conserve energy by reducing the occurrence of inflammatory reactions, allowing more energy to be directed to the recovery of viable cells. Thus, AT_2R may play a role in determining the fate of damaged neurons. Indeed, unmasking the apoptotic and regenerative effects of the AT_2R by AT_1R blockade could enhance tissue repair following injury and account for the reduction in infarct volume observed in animals treated with AT_1R antagonists (Lucius et al., 1998). In very recent studies, Mogi et al. (2006) have proposed that AT_2R stimulation increases the expression of a neuroprotective factor, methyl methanesulfonate sensitive 2, which is involved in neuronal differentiation (Mogi et al., 2006).

While most research has focused on the neuronal effects of the AT_2R in stroke, the vasodilator role of AT_2R cannot be ignored as a possible contributor to any AT_2R neuroprotective effect, by maintaining perfusion of penumbral regions following stroke. In accordance with many studies describing AT_2R-mediated relaxation of peripheral arteries (see Section 3.1), Ang II caused AT_1R-mediated vasoconstriction in cerebral arteries both in vitro and in vivo; an effect of Ang II that was converted to AT_2R-mediated vasodilatation in the presence of an AT_1R antagonist (Vincent et al., 2005). The fact that a sartan itself leads to an upregulation of AT_2R in cerebral vessels (Zhou et al., 2006) is consistent with a vascular component to the neuroprotective effect of AT_2R stimulation. Thus, the neuroprotective role of the AT_2R appears to be complex, and is likely to involve a delicate interplay with AT_1R on apoptotic, neuronal regenerative and vascular components in the central nervous system (Saavedra et al., 2006). Curiously, the direct effect of AT_2R stimulation in the setting of stroke has not been reported.

3.5. Renal disease

AT_2R are also developmentally regulated in the kidney, with AT_2R expression higher than that of AT_1R expression in the foetal kidney (Ciuffo et al., 1993; Shanmugam et al., 1995; Ozono et al., 1997). However, the relative proportion of these receptors reverses within days of birth, such that AT_1R expression is higher than AT_2R in the adult. AT_2R are detected at relatively low levels in adult kidney, although reports of the extent and location of AT_2R varies considerably depending on techniques used. Generally, AT_2R mRNA and protein have been found to be distributed throughout tubular and vascular segments of the renal cortex and medulla, however, the demonstration of glomerular AT_2R is more variable (Ozono et al., 1997; Miyata et al., 1999; Cao et al., 2000; Ruiz-Ortega et al., 2003). Discrepancies between studies may involve species differences since renal AT_2R are more easily detected in mice than rats (Armando et al., 2002; Baiardi et al., 2005). AT_2R are detected in human kidneys, primarily in associated vasculature (Zhuo et al., 1996; Matsubara et al., 1998), but also in tubular and glomerular tissue of patients with glomerular lesions (Mifune et al., 2001).

In terms of models of heightened RAS activation, there have been variable effects reported for renal AT_2R expression. Sodium depletion upregulated renal AT_2R (Ozono et al., 1997); AT_2R was downregulated only in the ischaemic kidney from 2K1C rats (Wang et al., 1999) whereas Ang II infusion per se did not alter renal AT_2R expression (Wang et al., 1999). AT_1R was also downregulated in the former 2 models, while adult Ren-2 gene transgenic rats, TGR(mRen-2)27

exhibited increased AT_1R but not AT_2R (Zhuo et al., 1999). However, the functional consequences of such changes in ATR expression were not examined.

On the other hand, in models of overt renal damage, such as induced by renal ablation, subtotal nephrectomy, or kidney damage caused by protein overload, AT_2R were invariably upregulated (Bautista et al., 2001; Cao et al., 2002; Ruiz-Ortega et al., 2003; Hashimoto et al., 2004; Tejera et al., 2004; Vazquez et al., 2005). Ex vivo analysis indicated that increased AT_2R was associated with enhanced renal vasodilatation in perfused kidneys (Bautista et al., 2001) and increased tubular apoptosis following persistent proteinuria (Tejera et al., 2004). However, Cao et al. (2002) reported that blockade of AT_2R with PD123319 actually conferred renoprotection, as evidenced by a reduction in albuminuria, as did AT_1R blockade. These results suggested that both AT_1R and AT_2R were renoprotective in the subtotal nephrectomy model (Cao et al., 2002), although recent data using the same model disputes those findings. Vazquez et al. (2005) found that there was a time-dependent increase in AT_2R expression over 30 days after renal ablation. Moreover, PD123319 exacerbated renal ischaemia and damage as well as causing a marked increase in telemetered BP (Vazquez et al., 2005). Thus, on balance, AT_2R appear to be renoprotective, in keeping with other studies in which mice treated with PD123319, or in mice with AT_2R deleted, exhibit marked increases in renal fibrosis (Ma et al., 1998; Morrissey & Klahr, 1999). Similarly, vascular over-expression of AT_2R ameliorated glomerular injury in the mouse remnant kidney model since there were reductions in albuminuria and glomerular expression of platelet-derived growth factor and transforming growth factor (Hashimoto et al., 2004).

In addition to the well described AT_2R renal vasodilator function, AT_2R are thought to exert pronatriuretic effects. Seminal studies by Siragy and Carey (1996, 1997, 1999) (Jin et al., 2001; Jin et al., 2004) demonstrated that increased renal interstitial levels of cGMP, NO and BK were dependent on AT_2R activation, and led to natriuresis. Moreover, Carey and colleagues have recently postulated that Ang III may be the endogenous AT_2R ligand in the kidney since, during AT_1R blockade, renal interstitial infusion of Ang III, but not Ang II, caused natriuresis. This effect of Ang III was blocked by PD123319, indicative of an AT_2R mechanism (Padia et al., 2006), which is consistent with Ang III-mediated depressor effects being AT_2R-mediated (Walters et al., 2003). Moreover, stimulation of dopamine D_1-like receptors with fenoldopam caused natriuresis due to recruitment and activation of AT_2R in renal proximal tubules (Salomone et al., 2007). The pressure–natriuresis relationship was also impaired in AT_2R knockout mice which had elevated basal renal resistance and altered arachidonic acid metabolism (Gross et al., 2000b). More recent pharmacological studies have directly addressed the role of AT_2R in natriuresis. In obese Zucker rats, CGP42112 increased urinary and sodium excretion, in line with increased expression of tubular AT_2R, whereas this effect was absent in lean rats. Moreover, PD123319 reversed the natriuretic and diuretic effects of candesartan, suggesting an involvement of AT_2R in the effect of the latter compound (Hakam & Hussain, 2005), consistent with earlier studies (Siragy & Carey, 1999; Siragy et al., 1999). Thus, AT_2R mediates natriuresis directly, or indirectly after AT_1R blockade (Hakam & Hussain, 2005; Padia et al., 2006), thereby opposing the well known antinatriuretic effects of AT_1R activation. Mechanisms underlying the natriuretic effects of AT_2R may involve altered proximal tubule bicarbonate reabsorption involving phosholipase A and arachidonic acid (Haithcock et al., 1999), or by direct inhibition by AT_2R of the proximal tubule sodium pump via NO/cGMP signalling as has recently been postulated (Hakam & Hussain, 2006a,b). Furthermore, renal AT_2R have recently been shown to inhibit renin biosynthesis and subsequent local Ang II production, thus providing an additional mechanism for modulation of AT_1R-mediated effects (Siragy et al., 2007).

The regional haemodynamic effect of Ang II within the rabbit kidney itself has also been investigated by measuring cortical and medullary blood flows. As expected, we found that AT_2R counteracted AT_1R-mediated vasoconstriction in the kidney cortex. By contrast, Ang II evoked AT_1R-mediated medullary vasodilatation that was opposed by activation of AT_2R (Duke et al., 2003). Moreover, in 2K1C hypertensive rats, the AT_2R antagonist increased basal medullary flow, suggestive of AT_2R-mediated medullary vasoconstriction (Duke et al., 2005b). Thus, the medullary circulation behaves somewhat differently to other vascular beds in response to Ang II. Likewise, endogenous Ang II acting via both AT_1R and AT_2R would appear to enhance renal neurovascular function in rabbit kidneys (Rajapakse et al., 2005, 2006).

3.6. Diabetes

Diabetes is one of the major causes of renal failure and blindness and is an important risk factor for the development of cardiovascular disease, where its presence is also associated with a worse prognosis (Gilbert et al., 2003). Although hyperglycaemia is a major determinant in the development of long-term complications of diabetes there are also glucose-independent mechanisms that contribute to the pathogenesis of organ injury in this disease. Activation of local RAS seems to be involved in the progression of kidney damage, with results of large clinical trials confirming the beneficial effects of ACE inhibitors and AT_1R antagonists (Brenner et al., 2001; Lewis et al., 2001; Parving et al., 2001), however as yet the role of the AT_2R in the pathogenesis of diabetic nephropathy remains to be elucidated.

There are relatively few studies that have examined ATR distribution in this disease. Of these, the majority of studies have focussed on the kidney as diabetic nephropathy is one of the major complications associated with both type 1 and type 2 diabetes. Using the STZ-model of type 1 diabetes, both reductions (Kalinyak et al., 1993; Cheng et al., 1994; Brown et al., 1997; Bonnet et al., 2002) and increases (Brown et al., 1997; Wehbi et al., 2001) in AT_1R have been reported. On the other hand, AT_2R expression has been found to be markedly increased in brush-border and basolateral membrane of the kidney (Hakam et al., 2006), and either not detected in the glomeruli of either diabetic or control rats (Hakam et al., 2006) or found to be significantly decreased (Wehbi et al., 2001). In a long-term study of type 1 diabetes, both AT_1R and AT_2R gene and protein levels were reduced in kidneys of diabetic SHR (both glomeruli and tubulo-interstitial cells) compared with both non-diabetic SHR and a normotensive model in which only the AT_2R showed a tendency towards a reduced expression (Bonnet et al., 2002). Less information is available regarding type 2 diabetes. Using the obese hyperinsulinaemic Zucker rat as a model of type 2 diabetes, AT_2R density was also found to be increased in the brush-border and basolateral membrane of the kidney (Hakam & Hussain, 2005) which is consistent with findings from kidney biopsies of patients with type 2 diabetes in which the AT_2R levels were also found to be increased in tubular cells with a concomitant reduction of AT_1R expression (Mezzano et al., 2003).

Thus it is difficult to reach consensus on overall receptor changes in the 2 types of diabetes due to differences in methodologies and duration and severity of diabetes. Unlike experimental renal failure, which is fast-onset and exhibits a clear upregulation of AT_2R, diabetes-induced changes in renal ATR expression reflect the continuum of the diabetic progression. Indeed, it is highly likely that the relative expression of AT_1 and AT_2R in heterogeneous tissue compartments in the kidney is important in determining the effects of Ang II in progressive diabetic nephropathy.

In this context, in STZ-induced diabetic rats, there are 50- and 80-fold increases in AT_2R in brush-border and basolateral membranes. Diabetes elevated urinary flow and urinary sodium excretion which was blocked by the selective AT_2R antagonist, PD123319 in diabetic but not control rats (Hakam et al., 2006). A similar effect of the AT_2R was observed in the type 2 diabetic model, the obese Zucker rat, with the AT_2R agonist, CGP42112, promoting an increase in sodium and

urine excretion in obese but not lean Zucker rats (Hakam & Hussain, 2005). On the basis of the effects of PD123319, there was a basal effect of AT_2R to promote natriuresis in type 1 diabetes (Hakam et al., 2006) but not in type 2 diabetes (Hakam & Hussain, 2005) which most likely reflects the profoundly greater upregulation of tubular AT_2R in the STZ-model compared with the type 2 model. The increase in natriuresis and diuresis produced by administration of an AT_1R antagonist in the obese Zucker rat was abolished by an AT_2R antagonist (Hakam & Hussain, 2005). The AT_2R has since been shown to inhibit the Na^+-K^+-ATPase via an NO/cGMP/protein kinase G pathway (Hakam & Hussain, 2006a,b), thus providing a possible mechanism responsible for AT_2R-mediated natriuresis and diuresis in this model.

In addition, in the diabetic kidney, there is significant upregulation of various angiogenic cytokines, such as VEGF, that is mediated via AT_1R and may thus contribute to the progression of renal injury (Pupilli et al., 1999; Tamarat et al., 2002; Rizkalla et al., 2005). A link between AT_2R and VEGF expression, albeit in a non-diabetic context has also been made (Rizkalla et al., 2003; Sarlos et al., 2003). In the STZ-induced model of diabetes, there is increased gene and protein expression of VEGF and its receptor (VEGF-R2) in the kidney. Chronic AT_1R or AT_2R blockade attenuated expression of cytokines and their receptors (Rizkalla et al., 2005). It should also be noted that there was increased apoptosis involving tubular and interstitial cells of the cortex and medulla of the kidney in STZ-treated rats (Kumar et al., 2004). Losartan and PD123319 inhibited apoptosis in the kidney to similar extents and there was no additive effect using both antagonists. Therefore it is possible that AT_1R and AT_2R signalling pathways can both lead to apoptosis and induction of angiogenic cytokines in the diabetic kidney.

There is evidence that Ang II and VEGF and their cognate receptors participate in retinal angiogenesis and ischaemic retinopathies such as diabetic retinopathy (see Wilkinson-Berka, 2004). The AT_2R has been identified in several ocular tissues including the retina and has been found to predominate in the developing retina (Sarlos et al., 2003) consistent with the view that the AT_2R influences cell growth and differentiation in organ development. There was an increase in AT_2R expression and AT_2R blockade decreased retinal angiogenesis as well as the levels of VEGF, its receptor VEGF-R2 and angiopoietin-2 similarly to that seen in the diabetic kidney (Sarlos et al., 2003). Zhang et al. (2004) showed that blockade of the AT2R in STZ-treated rats decreased VEGF expression, thereby suggesting that VEGF expression is modulated by both AT_1 and AT_2 receptors and implicating these receptor subtypes in retinal diseases such as diabetic retinopathy. Thus blockade of the AT_2R as well as the AT_1R may confer end-organ protection in various retinal diseases.

Limited information is available regarding AT_2R function in other non-renal tissues from diabetic animals or in the clinical setting. Increased expression of AT_2R in normal (Sechi et al., 1994) and cardiomyopathic (Li et al., 2005a) hearts from diabetic rats have been reported. AT_2R-mediated relaxation was also enhanced in aortic tissue obtained from STZ-treated rats compared with controls, and was associated with increased AT_2R density, but not AT_1R, in this vessel (Arun et al., 2004). Recently, it was shown that 1-year treatment of diabetic hypertensive patients with valsartan, but not atenolol, caused an upregulation of AT_2R in gluteal resistance arteries that was associated with improved AT_2R-mediated vasorelaxation in these arteries (Savoia et al., 2007). Furthermore, in line with earlier in vivo vascular AT_2R function (see Section 3.1), Howes' group has recently examined small-artery vascular stiffness and in fact found that there was increased functional expression of vascular AT_2R in patients with insulin resistance (Brillante et al., 2008).

3.7. Atherosclerosis and neointimal formation

Ang II is recognized as a potent mediator in the pathogenesis of atherosclerosis (Daugherty et al., 2000), with AT_1-receptor activation by Ang II promoting vascular inflammation, cellular proliferation and oxidative stress which are considered key stages in the initiation and progression of the disease (Dzau, 2001; Daugherty & Cassis, 2004). The role of Ang II in atherosclerosis is evident in a number of studies using transgenic animal models and human tissues, that demonstrate an increase in AT_1-receptor density in hypercholesterolemia (Nickenig & Bohm, 1997; Warnholtz et al., 1999), as well as increased expression of ACE in atherosclerotic lesions (Diet et al., 1996; Fukuhara et al., 2000). Furthermore, when the AT_1R gene was deleted from 2 widely used models of atherosclerosis, i.e. apolipoprotein E (ApoE)-deficient mice or LDL-receptor-deficient mice, both types of double knockouts exhibited markedly reduced atherosclerotic lesions (Wassmann et al., 2004; Daugherty et al., 2004), suggesting a primary role for AT_1R in the pathogenesis of atherosclerosis. Indeed, recent studies using pharmacological intervention of the RAS have demonstrated beneficial effects in the progression of atherosclerosis and associated cardiovascular outcomes (see (Dzau, 2001; Daugherty & Cassis, 2004).

In the same study that used AT_1R/LDL-receptor double knockouts, AT_2R/LDL-receptor double knockout mice were also reported as a secondary aim. The authors reported that these mice do not exhibit an increase in atherosclerosis (Daugherty et al., 2004), however, no data was presented to support such a statement. By contrast, the AT_2R gene was deleted from ApoE-deficient mice independently by 2 groups (Iwai et al., 2005; Sales et al., 2005), with both studies concluding that AT_2R do in fact exert an anti-atherosclerotic effect.

Iwai et al. (2005) demonstrated that AT2R/ApoE-double knockout (DKO) mice fed a high cholesterol diet display exaggerated atherosclerotic lesion development, together with increased NADPH oxidase activity and superoxide production, when compared with ApoE knockout mice. It is well known that Ang II can induce expression of NADPH oxidase and subsequently increase superoxide production via activation of the AT_1-receptor (Griendling et al., 1994). These results suggest inhibitory modulation of oxidative stress via the AT_2R which is consistent with in vitro data using cultured endothelial cells in which AT_2R blockade with PD123319 enhanced superoxide formation (Sohn et al., 2000). These findings also have further implications in terms of the progression of atherosclerosis, as decreased oxidative stress (typically superoxide anion production) increases the bioavailability of the potent anti-atherosclerotic vasodilator NO (Cai & Harrison, 2000). In addition, the anti-atherosclerotic effect of the AT_1R antagonist, valsartan was markedly reduced in AT_2R/ApoE-DKO compared with ApoE-single knockout mice (Iwai et al., 2005), suggesting that AT_2R contribute to the beneficial effect of AT_1R blockade and are counter-regulatory to AT_1R in the setting of atherosclerosis.

Using a separately derived AT_2R/ApoE-DKO, Sales et al. (2005) also published data investigating the effect of AT_2R on atherosclerotic disease progression. Although this study did not show increases in lesion size in AT_2R/ApoE-DKO mice, they demonstrated striking differences in plaque composition, cellular proliferation and apoptosis over time. Typically, there is a reduction in macrophage, smooth muscle, lipid and collagen content of plaques with disease progression together with increased apoptosis. However, AT_2R deletion ameliorated this process suggesting that AT_2R influences the cellular composition of the atherosclerotic lesion. Importantly, in ApoE-deficient mice, AT_2R were co-localised with intimal smooth muscle cells and macrophages, and were upregulated following a high cholesterol diet. The authors noted a temporal association of AT_2R expression and reduced smooth muscle cell content during lesion development, emphasizing an anti-inflammatory and antiproliferative role for AT_2R (Sales et al., 2005). Increased AT_2R expression in atherosclerotic plaques in the same ApoE-deficient mouse model was recently confirmed (Johansson et al., 2005; Vinh et al., 2008b), and increased AT_2R and ACE2 immunoreactivity was found in atherosclerotic plaques in rabbits, often co-localised with macrophages (Zulli et al., 2006). Interestingly, Dandapat et al. (2008) have recently reported that over-expression of the AT_2R in LDL-receptor knockout

mice, abrogated atherosclerotic lesion development, and was associated with a reduction in oxidative stress and other factors involved in the pro-inflammatory cascade. Furthermore, using the same mouse model, Hu et al. (2008) have additionally provided evidence of AT_2R-mediated regulation of collagen deposition in atherosclerotic plaques which involved reduced MMP expression, decreased macrophage deposition and increased superoxide dismutase. Indeed these studies strongly support previous studies reporting anti-oxidative and anti-inflammatory effects mediated via the AT_2R, both of which are vital processes that contribute to atherosclerotic lesion formation.

Consistent with the anti-atherosclerotic role of AT_2R deduced from knockout studies, Daugherty et al. (2001) had previously demonstrated that Ang II-induced atherosclerosis and aneurysm formation in male ApoE-KO mice was significantly potentiated with AT_2R blockade. However, Johansson et al. (2005) were unable to reproduce this potentiation of lesion formation in the same model but using female mice. The authors suggested gender differences may have accounted for the disparity, as it has been demonstrated that male hormones may protect against Ang II-mediated lesion development (Henriques et al., 2004). Alternatively, the ability of oestrogen to increase AT_2R expression may play a role (Kintscher & Unger, 2005), which illustrates important gender differences that are emerging with respect to AT_2R expression and function (see Section 3.9). On close inspection of the same study, Johansson et al. (2005) reported that the incidence of abdominal aortic aneurysm increased from 11% with Ang II treatment alone, to 50% when treated with both Ang II and PD123319 in female $ApoE^{-/-}$ mice on a standard diet. These results are consistent with the protective effect of the AT_2R on aneurysm formation (Daugherty et al., 2001), perhaps indicating different mechanisms involved with atherosclerosis and aneurysm formation.

Recent studies from our own laboratory have revealed striking differences between the effects of chronic administration of either Ang II or Ang IV in ApoE-deficient mice fed a high fat diet. As expected, Ang II increased superoxide production and lesion formation in ApoE-deficient mice and worsened aortic endothelial function in wild type mice. By contrast, Ang IV caused vasoprotection in ApoE-deficient mice since there was a marked improvement in endothelial-mediated vasorelaxation that was accompanied by increased eNOS expression and reduced superoxide activity. Moreover, this action of Ang IV to increase NO biovailability was mediated by both AT_2R- and AT_4R-dependent mechanisms (Vinh et al., 2008a,b).

The anti-growth effects of AT_2R have also been reported for neointimal formation following vascular injury. The RAS has been implicated in neointimal formation mainly via the activation of the AT_1R (Kim et al., 1995; Viswanathan et al., 1992). Nakajima et al. (1995) first reported AT_2R involvement in neointimal formation in rats by transfecting an AT_2R expression vector into the balloon-injured rat carotid artery and observed that over-expression of AT_2R attenuated neointimal formation. Similarly, cuff-induced inflammation and subsequent neointimal formation in the femoral artery was substantially enhanced in AT_2R knockout mice compared with wild type (Akishita et al., 2000a; Wu et al., 2001), consistent with an increased AT_2R expression in neointima of wild type mice (Suzuki et al., 2002). It has also been demonstrated that AT_2R knockout mice show decreased apoptosis in the neointima which is consistent with the increased neointimal formation observed following cuff placement (Suzuki et al., 2002). Furthermore, the protective effects of AT_1R blockade using valsartan were decreased in AT_2R knockout mice, suggesting involvement of AT_2R stimulation in the protection associated with AT_1R blockade (Wu et al., 2001). More recently, the effects of a sartan (valsartan) have been compared with an ACE inhibitor (benazepril) on vascular remodeling following carotid artery balloon injury. Interestingly, both RAS inhibitors caused anti-remodelling of balloon-injured rat carotid arteries, but by different molecular mechanisms since only valsartan caused increased AT_2R expression together with increased cGMP production (Barker et al., 2006).

Thus, with a few minor exceptions (Daugherty et al., 2004; Johansson et al., 2005), AT_2R exerts vasoprotective effects and substantial anti-remodelling of atherosclerotic lesions and neointimal formation.

3.8. Aging

Aging is associated with specific cardiovascular changes including cardiac hypertrophy and fibrosis, vascular stiffening and endothelial dysfunction. These cardiovascular adaptations are seen in both elderly normotensive and hypertensive subjects (Lakatta & Levy, 2003), indicating that modifications are more than a mere consequence of confounding cardiovascular complications such as arterial hypertension, decreased aortic compliance and atherosclerosis, which are particularly prevalent in the elderly population. Circulating levels of Ang II are reduced by aging whereas angiotensinogen, ACE, and AT_1R are upregulated in both the heart and vasculature (Heymes et al., 1994, 1998; Wang et al., 2003). AT_1R blockade and ACE inhibition have been shown to be effective in preventing or regressing age-associated effects on cardiovascular remodelling and function in humans and animals (Burrell & Johnston, 1997; Basso et al., 2005).

Importantly, although cardiac AT_2R expression is relatively low in the adult rat heart (Busche et al., 2000), expression may be upregulated in certain disease states which are particularly common in the elderly, such cardiac fibrosis (Tsutsumi et al., 1998), hypertrophy (Lopez et al., 1994), and heart failure (Ohkubo et al., 1997). Surprisingly, only study has investigated the influence of senescence on AT_2R expression in the heart. Heymes et al. (1998) found augmented AT_2R expression in both the left and right ventricles of aged rats, as well as in freshly isolated cardiomyocytes from aged rats. Moreover, there was a correlation between AT_2R expression and fibrosis in the cardiac tissue. Considering the demonstrated involvement of AT_2R stimulation to the cardioprotective effects of AT_1R inhibition in adults, it is tempting to speculate that the AT_2R may also play an important role in the regulation of cardiovascular aging. Indeed, we have shown that AT_2R contribute to the effects of AT_1R inhibition in 20 month old WKY rats, since beneficial actions of candesartan cilexetil administration on both aortic remodeling and cardiac fibrosis were completely abolished by simultaneous PD123319 treatment (Jones et al., 2004).

In terms of vascular AT_2R, the vasoconstrictor response of human isolated coronary microarteries to Ang II was potentiated by PD123319, as seen in in vitro animal studies (Zwart et al., 1998; Hannan et al., 2003), and the magnitude of this effect was positively correlated with the age of the donor (Batenburg et al., 2004). This finding suggests increased functional effects of vascular AT_2R with aging, at least in coronary vasculature. More recently, Pinaud et al. (2007) found increased AT_2R expression in mesenteric arteries that was predominantly localised to the medial smooth muscle layer since confocal imaging of fluorescent Ang II in the presence of candesartan revealed a decrease of AT_2R in the endothelium. Strikingly, in these vessels, the AT_2R phenotype was that of superoxide-mediated contraction, as it was blocked by antioxidant treatment (Pinaud et al., 2007). The effect of aging on aortic AT_2R localisation has also recently been determined. Like in mesenteric arteries, there was a marked increase in AT_2R immunofluorescence, predominantly in the media while AT_2R expression appeared to be preserved in the endothelium (Widdop et al., 2008).

3.9. Gender

Gender differences exist in AT_2R expression in the vasculature and kidney. AT_2R expression in the kidney was generally higher in female, normotensive mice and rats compared with male counterparts; these changes were abolished by ovariectomy and oestrogen upregulated AT_2R in various tissue compartments of the kidney in mice (Armando

et al., 2002) and rats (Baiardi et al., 2005). This gender effect was particularly evident in the renal vasculature of the rat since AT_2R were only detected in arcuate arteries and veins from female rats (Baiardi et al., 2005). An increased ratio of AT_2R/AT_1R was also found in kidneys and vasculature of female SHR compared to male SHR. This effect was due to reciprocal changes in AT_1R (decreased) and AT_2R (increased) in the kidneys whereas AT_1R, but not AT_2R, was downregulated in aortic and mesenteric vessels of female SHR, in an oestrogen-sensitive manner (Silva-Antonialli et al., 2004).

Gender-specific modulation of cardiovascular disease, and the treatment thereof, represents an important area for future research (Regitz-Zagrosek, 2006). Indeed, losartan reduced BP more effectively within 24 h in female SHR than male SHR (Silva-Antonialli et al., 2000). Moreover, the same group has recently reported that losartan increased vascular AT_2R expression after either 24 hour- or 15 day-treatment in female, but not male, SHR, which was associated with the restoration of endothelial function (de P Rodrigues et al., 2006). In addition, (Okumura et al., 2005) reported that the upregulation of vascular AT_2R, but not AT_1R, following cuff injury was greater in female mice, and was associated with a greater reduction in neointimal formation as well as in indices of inflammation and oxidative stress in females versus males. Moreover, valsartan caused greater vessel anti-remodelling in wild type females whereas this sex difference was less marked in AT_2R knockouts (Okumura et al., 2005). Recently, we have made the remarkable finding that chronic low-dose Ang II infusion lowered BP in conscious normotensive female, but not male, rats. This effect occurred via AT_2R-dependent mechanisms and involved enhanced renal AT_2R expression (Sampson et al., 2008).

3.10. AT_2R clinical context

There is much less clinical data pertaining to the effects of AT_2R. Sartans have similar clinical benefit to ACE inhibitors in the majority of cardiovascular diseases. Indeed, large-scale clinical trials have been unable to unequivocally discriminate between these 2 classes of drugs, emphasising the prime importance of disrupting AT_1R function. However, this does not necessarily rule out subtle effects of AT_2R contributing to the beneficial effects of AT_1R antagonists in much the same way that BK may contribute to the effects of ACE inhibition.

One area of uncertainty lies in the use of sartans in high cardiovascular risk patients. The VALUE trial reported a relative increase in first time MI (fatal and non-fatal) in patients on valsartan compared with amlodipine, although there were early differences in BP-reduction between treatment arms which may have contributed to differences in MI rates (Julius et al., 2004). Based on the results of this trial, Verma & Strauss (2004) initiated a debate that is still ongoing, as to the potential harm of sartans for MI in high-risk patients (Verma & Strauss, 2004) (for detailed review, see Strauss & Hall, 2006; Tsuyuki & McDonald, 2006). As explanation for potential differences between sartans and ACE inhibitors, it was argued that the ability of AT_1R antagonists to indirectly facilitate AT_2R stimulation may contribute to the risk of MI in high-risk patients (Strauss & Hall, 2006). On this point, they provided pre-clinical evidence on the potentially deleterious effects of AT_2R stimulation from limited experimental studies (Strauss & Hall, 2006), although a much larger body of evidence, as outlined in this review, supporting a cardioprotective role for AT_2R was overlooked. Indeed, results from the recently completed ONTARGET trial, which evaluated the effects of telmisartan either alone, in combination with ramipril, or ramipril alone, in patients with vascular disease or high-risk diabetes, saw no difference in the incidence of the primary endpoints of cardiovascular death, MI, stroke or heart failure in any treatment arm (Yusuf et al., 2008).

In the meantime, the AT_2R gene polymorphism -1332G/A has also been linked with cardiovascular pathologies such as LVH and premature coronary artery disease, although the interpretation of these data is inconsistent, since either beneficial or deleterious effects of AT_2R have been reported (see Section 3.2). Clinical studies determining forearm vasodilatation or arterial stiffness in small groups of healthy subjects or patients on sartans or with insulin resistance have provided evidence of functional vascular AT_2R, but with some variation likely reflecting the heterogeneous patient populations examined (see Section 3.1). Finally, as outlined, there is a growing body of literature from ex vivo human data which implicates a cardio- and vaso-protective effect of AT_2R. For example there is a marked upregulation of AT_2R that is thought to exert anti-fibrotic effects in human heart failure (see Section 3.3); and vasorelaxation has been directly demonstrated in human isolated coronary (Batenburg et al., 2004) and gluteal (Savoia et al., 2006b) vasculatures.

3.11. Limitations and future directions

There are some limitations in the AT_2R research just discussed that still confound interpretations and hamper future progress. In particular, there is a dearth of selective AT_2R agonists and antagonists, with CGP42112 and PD123319, respectively, being the gold standards for comparison. While these compounds are excellent tools, studies using other compounds are needed since AT_2R knockouts bred on different background strain have most likely contributed to some of the controversial findings regarding AT_2R function. In the future, data using non-peptide drugs such as Compound 21, under both acute and chronic conditions, are likely to contribute to the understanding of AT_2R function and its potential therapeutic role. Indeed, to date there are no data reporting chronic effects of direct pharmacological stimulation of AT_2R to determine definitively any BP and remodelling effects while avoiding potentially confounding issues such as the use of genetically modified animal models, stimulation of AT_2R by Ang II during AT_1R inhibition, or inference of AT_2R function during AT_2R blockade.

4. Conclusions

Over the past decade, a variety of effects caused by stimulation of AT_2R in the cardiovascular system have been reported. In this review, we have highlighted that many such AT_2R effects, and AT_2R itself, are upregulated in a range of cardiovascular disease states. Generally, AT_2R mediates acute haemodynamic effects but do not always translate into marked changes in basal BP inferred from AT_2R knockout and transgenic studies although further studies with AT_2R agonists are required. On the other hand, AT_2R exert substantial anti-growth and anti-remodelling effects that indirectly contributes 'beyond BP control' under chronic conditions. The experimental evidence overwhelmingly indicates that AT_2R makes a major contribution to the beneficial structural and remodelling effects of 'sartans' although this involvement is difficult to gauge in the clinical context. Often, a pharmacological effect of AT_2R is only unmasked during AT_1R blockade suggesting crosstalk between ATR which may take the form of either functional, physical or signalling interactions between AT_2R and AT_1R. Clearly, the level of AT_2R expression, relative to AT_1R, in different pathological states, together with the relative mixture of angiotensin peptide fragments in the local milieu and the prevailing crosstalk, will determine end-organ response. Thus, there is still much to learn about the AT_2R context.

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