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The angiotensin II type 2 (AT_2) receptor: an enigmatic seven transmembrane receptor

Enzo R. Porrello[1, 2], Lea M.D. Delbridge[2], Walter G. Thomas[1]

[1]Molecular Endocrinology Laboratory, Baker Heart Research Institute, Prahran, Victoria 3004, Australia, [2] Department of Physiology, University of Melbourne, Parkville, Victoria 3010, Australia

TABLE OF CONTENTS

1. ABSTRACT

Angiotensin II (AngII) interacts with two receptor subtypes, AT_1 and AT_2, belonging to the seven transmembrane receptor superfamily. Pharmacological investigations initially suggested that AT_2 receptors antagonize AT_1 effects. Data from AT_2 receptor transgenic and knock-out mice have not been entirely consistent with this interpretation. At the cellular level, a clear mechanistic model of AT_2 transduction and signalling has yet to emerge. The AT_2 receptor displays the hallmark motifs and signature residues of a G protein-coupled receptor (GPCR), but fails to demonstrate most of the classic features of GPCR signalling. In recent years, unbiased screens for AT_2-interacting proteins have identified novel partner proteins involved in AT_2 signalling, providing new insight into the mechanisms of AT_2 action. A growing body of evidence suggests that the AT_2 receptor is constitutively active (i.e. signals without AngII). This review critically evaluates controversies surrounding physiological functions and signalling mechanisms of the AT_2 receptor, primarily in a cardiovascular context. Recent advances in the field are highlighted and findings challenging the concept that the AT_2 receptor is a conventional angiotensin receptor are considered.

2. INTRODUCTION

Angiotensin II (AngII) is an important cardiovascular hormone and mediates acute physiological responses including vasoconstriction, adrenal aldosterone release and thirst behaviour. AngII is also important in regulating longer-term responses, including growth and remodelling effects in cardiac, renal and vascular tissue (1).

AngII is known to interact with at least two distinct receptor subtypes, designated AT_1 and AT_2, both of which belong to the superfamily of seven transmembrane receptors. The two receptors share little homology (~34% amino acid sequence identity) (2, 3) and exhibit little resemblance in relation to signalling mechanisms (1). Although both receptors were cloned over 15 years ago (2, 4) and significant advances in the understanding of AT_1 receptor signalling have been made, the AT_2 receptor remains an enigma. Most of the classic actions of AngII are still ascribed to the AT_1 receptor. Transgenic and knock-out approaches in the mouse were anticipated to provide definitive answers to many questions regarding the physiological functions of the AT_2 receptor. However, analyses of these transgenic and knock-out phenotypes have been controversial and have not consistently provided

physiological explanation. At the cellular level, a clear mechanistic model of AT_2 transduction and signalling has yet to emerge. Despite intensive investigation, the AT_2 receptor remains one of the least understood components of the renin-angiotensin system.

This review critically evaluates controversies surrounding the physiological function and signalling mechanisms of the AT_2 receptor, primarily in a cardiovascular context. Recent advances in the identification of novel AT_2 receptor interacting proteins and unconventional ligand-independent signalling are highlighted and findings which challenge the concept that the AT_2 receptor is a conventional angiotensin receptor are considered.

3. AT_2 RECEPTOR PHYSIOLOGY: WHAT DOES THE AT_2 RECEPTOR DO?

Most of the known physiological functions of AngII have been classically ascribed to the AT_1 receptor. With the development of specific compounds which interfered with AngII at its binding sites (5, 6) and the subsequent cloning of the AT_2 receptor (2), the concept of AngII receptor heterogeneity emerged. The most widely studied AT_2 receptor antagonists are PD123177 and PD123319. PD123319 has a high affinity for the AT_2 receptor (K_i ~ 12nM), but a low affinity for the AT_1 receptor (K_i > 100uM) and is approximately 10,000-fold more selective for AT_2 receptors than AT_1 receptors (7). CGP42112 is a highly selective AT_2 receptor agonist at high concentrations (7). PD123319 and CGP42112 have been used in a large number of studies to deduce a physiological role for the AT_2 receptor. With initial reports of AT_2-mediated vasodilator, natriuretic, antigrowth, antiproliferative and proapoptotic effects (1), the notion of the AT_2 receptor antagonizing AT_1 gained momentum. However, data from AT_2 receptor transgenic and knock-out mice are sometimes contradictory and raise questions regarding this classic 'yin-yang' paradigm. In this section, the unique tissue distribution of the AT_2 receptor is reviewed, controversies surrounding the role of the AT_2 receptor in the cardiovascular system are highlighted, and findings from pharmacological and genetic gain- and loss-of-function studies are compared.

3.1. AT_2 receptor expression and tissue distribution

The density of angiotensin receptors is developmentally regulated. The AT_1 receptor is expressed in most adult tissues including the heart, blood vessels, brain and kidney. In contrast, AT_2 receptor expression is largely restricted to embryonic, foetal and neonatal tissues, where it is the dominant subtype (8, 9). Experimental studies indicate that the AT_2 receptor retains a capacity for regulation in the adult – a finding which suggests a potential role for this receptor in human cardiovascular disease. In rodent models AT_2 receptor expression is up-regulated in heart failure (10, 11), and is up- and down-regulated in a temporally-dependent manner post-infarction (12, 13). Surprisingly, little is known about the expression and function of AT_2 receptors in humans. Myocardial AT_2 receptors are up-regulated 3.5-fold in patients with dilated cardiomyopathy, but of particular interest is the finding that 41% of the angiotensin binding sites in the non-failing human heart are of the AT_2 subtype (14). Other studies in humans have also shown that the AT_2 receptor can constitute between 50-70% of angiotensin binding sites in the adult myocardium (15). This situation is in marked contrast to the adult rodent, where it has been suggested that AT_2 receptors are expressed in only about 10% of adult cardiomyocytes (11, 16). Nevertheless, animal studies have shown that AT_1 receptor blockade can increase AT_2 expression levels and circulating AngII levels, creating the potential for increased AngII action at the unblocked AT_2 sites. Targeting AT_2 receptors may therefore be a viable combination therapy in patients treated with AT_1 receptor blockers, which are currently used to treat hypertension and heart failure.

3.2. Developmental regulation: AT_2 involvement in differentiation and apoptosis

The abundant and ubiquitous expression of AT_2 receptors in the foetus indicates a role for this receptor in tissue development and differentiation. It is therefore surprising that few studies have examined the role of AT_2 receptors during early development and tissue differentiation. This is possibly due to the absence of any obvious cardiovascular developmental defects in AT_2 knock-out and transgenic mice. However, AT_2 null mice do have a high incidence of urological abnormalities (17). Polymorphisms in intron 1 of the AT_2 gene (A-1332G) occur with higher frequency in human patients with congenital urinary tract abnormalities (18), suggesting that the AT_2 receptor may play an important role in the development of the urinary tract.

Pharmacological blockade of the AT_2 receptor with PD123319 from E16 to E21 significantly decreases DNA synthesis in the developing aorta (19). Moreover, AT_2 null mice have reduced levels of the vascular smooth muscle cell differentiation markers calponin and caldesmon at 2 and 4 weeks after birth (20). These data strongly suggest that the AT_2 receptor is involved in vascular smooth muscle differentiation and vasculogenesis.

AT_2 receptor stimulation induces neurite outgrowth and regulates neurofilaments in neural cell lines (21). Pro-apoptotic effects have also been ascribed to the AT_2 receptor, but these studies have predominantly been limited to cell lines of neuronal origin (22, 23). The pro-apoptotic effects of the AT_2 receptor appear to be largely restricted to *in vitro* studies and are not applicable to all cell types. For instance, *in vivo* studies utilising AT_2 knock-out mice have failed to demonstrate an important role for the AT_2 receptor in mediating cardiomyocyte apoptosis (24). AT_2 knock-out mice display an increase in neuronal cell number in certain brain structures associated with learning and memory (25) and have central neurological abnormalities (26, 27). However, it is still unclear whether the increase in cell number in AT_2 knock-out mice is due to increased neuronal proliferation or a suppression of apoptosis. The role of the AT_2 receptor in regulating more subtle aspects of embryogenesis and tissue differentiation has not been explored in detail.

Table 1. Cardiovascular phenotypes associated with AT_2 receptor transgenic over-expression (TG) and knock-out (KO) mice

Animal Model	Progenitor Strain	Mortality	Cardiac Hypertrophy (basal)	Cardiac Hypertrophy (chronic pressure overload)	Interstitial Cardiac Fibrosis	Blood Pressure (basal)	Blood Pressure Response to AngII	References
AT_2-TG	C57BL/6 (alpha-MHC)	No effect on neonatal mortality	↔	↔	↓	↔	↓	24, 44, 53, 54
AT_2-TG	FVB/N (MLC-2v)	No effect on neonatal mortality	↑	↓	↑	↓	?	45, 56
AT_2-KO	C57BL/6	No effect on neonatal mortality	↔	↓	↓	↔	↔	26, 43, 52
AT_2-KO	FVB/N	No effect on neonatal mortality	↔	?	?	↑	↑	27

Effect of genetic manipulation: ↑ increased effect, ↓ decreased effect, ↔ no effect, ? unknown effect.

3.3. Vascular responsiveness: AT_2 dilator and constrictor actions

AngII has long been known to be a potent vasoconstrictor and an important mediator in the genesis of hypertension. AT_1 receptor antagonism has proven to be an efficacious therapy for the treatment of hypertension (28). Numerous lines of evidence support the notion that the AT_2 receptor causes vasodilatation in a number of isolated arteries and exerts depressor effects that oppose the actions of AT_1 *in vivo* (reviewed in (29)). However, the physiological effects of AT_2 receptor signalling in the vasculature are complex and can be disparate (i.e. vasoconstriction vs. vasodilatation) depending on the context.

Studies employing the AT_2 receptor antagonist PD123319 have demonstrated that the AT_2 receptor exerts vasodilator effects in a range of isolated rodent arteries (reviewed in (29)), as well as in human coronary microarteries (30). The AT_2 agonist CGP42112 has also been used to confirm the AT_2 vasodilator effects, which are often only seen in the presence of concomitant AT_1 receptor blockade (31, 32).

In 1995, two independent groups generated AT_2 null mice by targeted gene deletion (26, 27). Under basal conditions, blood pressure was found to be either unchanged (26) or increased (27) in these AT_2 null mice (Refer to Table 1). The pressor responses to AngII also differ between these AT_2 null lines. Ichiki *et al* reported that the pressor response to AngII was greater in AT_2 null mice than their controls, consistent with a depressor effect of the AT_2 receptor. Hein *et al* could not verify this observation in an independent study. The discrepancies between these AT_2 knock-out animals are difficult to reconcile, and have been unsatisfactorily ascribed to genetic differences in the background strains (C57BL/6 vs. FVB/N) and to methodological differences in the AngII administration protocols used. Targeted over-expression of AT_2 receptors in vascular smooth muscle cells of transgenic mice causes vasodilatation, supporting the contention that the AT_2 receptor exerts depressor effects *in vivo* (33).

More recently, reports that the AT_2 receptor mediates vasoconstriction in mesenteric resistance arteries of Spontaneously Hypertensive Rats (SHR) and senescent rats have provided an additional challenge in understanding the role of this receptor in vascular regulation. You *et al* demonstrated that AngII stimulation in the presence of an AT_1 receptor antagonist induced a vasoconstriction in untreated SHR resistance arteries (34). Intriguingly, non-selective antihypertensive treatment for 4 weeks restored the vasodilator function in SHR resistance arteries, which was attributed to an up-regulation of the AT_2 receptor. However, as the ability of PD123319 to reverse the vasodilator effects was not tested in this study, a causative link between the AT_2 up-regulation and restoration of vasodilator function in the SHR was not conclusively demonstrated. Pinaud *et al* recently reported that the AT_2 receptor mediates a different response in the resistance arteries of old and young rats, with the AT_2 receptor inducing vasodilator effects in young rats and vasoconstrictive effects in old rats (35). Interestingly, this study also reported an up-regulation of AT_2 receptor expression in vascular smooth muscle cells with the aging process. Because AT_1 receptor antagonists are most often prescribed in elderly and hypertensive patients, resolving the apparently conflicting roles of the AT_2 receptor in these contexts should be a focus for future studies.

3.4. Cardiac fibrosis: AT_2 proliferative and antiproliferative actions

The AT_1 receptor induces mitogenic effects in many tissues and cell types. In contrast, the AT_2 receptor is often reported to exert an anti-proliferative effect which opposes the AT_1.

Stoll *et al* (1995) were the first to demonstrate the involvement of the AT_2 receptor subtype in the control of cell proliferation. In 1995, they reported that the AT_2 receptor offset the growth promoting effects induced by AT_1 receptor activation in coronary endothelial cells (36). This observation was subsequently confirmed by reports of AT_2-mediated anti-proliferative effects in macro- and micro-vascular endothelial cells, vascular smooth muscle cells, neuronal cells, pheochromacytoma cells, and fibroblasts (reviewed in (11)).

The inhibitory effects of the AT_2 receptor on cellular proliferation received particular attention because of the potential clinical ramifications for the treatment of proliferative pathologies including cardiac fibrosis. AngII

is mitogenic in rat cardiac fibroblasts (37), which produce extracellular matrix proteins including collagen. An increase in the extracellular matrix proteins and fibronectin is found in conditions of load-induced cardiac hypertrophy (38). The finding that AT_1-mediated proliferative effects of AngII in cultured neonatal fibroblasts were apparent only when the AT_2 receptor was blocked, suggested that the ability of AngII to induce fibroblast proliferation may critically depend on the activation status of the AT_2 receptor. Furthermore, this finding suggested that the ability of AngII to induce cellular proliferation in a given tissue may depend on the relative AT_1/AT_2 receptor ratio (39). *In vivo*, PD123319 increases cardiac and renal fibrosis, supporting the notion that the AT_2 receptor is anti-fibrotic (10, 40). However, there are also conflicting reports that chronic PD123319 administration reduces collagen content in the thoracic aorta in a model of AngII-induced hypertension in rats (41). Findings from studies of AT_2 knock-out and transgenic mice have been ambiguous in evaluating the physiological role of the AT_2 receptor in fibrosis. While Wu *et al* demonstrated that myocardial perivascular fibrosis is increased in AT_2 null mice (42), Inagami's group showed that the AT_2 receptor was essential for cardiac interstitial fibrosis induced by AngII infusion in a different AT_2 knock-out strain (43) (see Table 1). Again, the inconsistent findings obtained using the different AT_2 knock-out models renders interpretation problematic. With respect to fibrosis these differences may relate to the different fibrotic indices used and/or may reflect underlying differences in AT_2 involvement in matrix deposition at perivascular and interstitial sites.

Even more perplexing are results obtained from AT_2 cardiac-specific transgenic mice. Kurisu *et al* reported that over-expression of the AT_2 receptor in the heart under the control of the cardiac-specific alpha-MHC promoter significantly inhibited AngII-induced increases in perivascular fibrosis (44). Yan *et al* created an AT_2 transgenic mouse in which the AT_2 receptor was over-expressed under the control of the ventricle-specific MLC2v promoter. Intriguingly, these mice display an increase in interstitial collagen (45). The relevance of these studies with respect to the specific role of the AT_2 receptor in fibroblast proliferation is unclear, as the AT_2 receptor was presumably over-expressed exclusively in cardiomyocytes in both models and the effects on fibrosis are likely to be secondary physiological adaptations to the hypertrophic phenotype, which also differs between the two strains (see below).

3.5. Myocardial hypertrophy: AT_2 pro-growth and anti-growth actions

The trophic actions of AngII are well described, including a role in cardiac growth, which is a major predictor of cardiovascular morbidity and mortality. Experimental studies have extensively characterised AngII as an important cardiotrophic factor *in vitro* and *in vivo* (46, 47). AngII directly promotes protein synthesis and cell growth in cultured embryonic chick myocytes (48) and in cultured neonatal rat cardiomyocytes (49), and these effects are mediated by the AT_1 receptor subtype (49). The synthesis rate of both DNA and protein significantly increases in neonatal cardiomyocytes in the presence of the selective AT_2 receptor antagonist PD123319, suggesting that this effect is dependent on the cellular AT_1/AT_2 receptor ratio (39). However, these interpretations are based on *in vitro* findings where endogenous receptor expression is extremely low (50). Experiments involving adenoviral manipulation of AT_2 receptor expression in cultured neonatal cardiomyocytes have demonstrated that this receptor can mediate myocyte hypertrophy independently of AngII (51), suggesting that the AT_2 receptor *per se* is pro-hypertrophic.

In vivo experiments involving AT_2 receptor manipulation have also produced conflicting results. In some contexts AT_2 knockout prevents the induction of hypertrophy (43, 52), whereas over-expression may or may not produce hypertrophy (45, 53). While neither of the AT_2 knock-out mice displayed defects in heart size under basal conditions (26, 27), an antigrowth role for the AT_2 receptor is not supported by findings obtained using the Inagami AT_2 null mice. The hypertrophic response to pressure overload is completely suppressed in these mice, suggesting the AT_2 receptor plays an obligatory role in the hypertrophic process (52). Further supporting this interpretation is the finding that AngII infusion does not lead to cardiac hypertrophy in this model (43). The AT_2 receptor may thus be essential for pressure-overload cardiac hypertrophy, directly challenging the view that this receptor exerts AT_1 antagonistic actions.

Transgenic mice over-expressing the AT_2 receptor under the control of alpha-MHC in cardiomyocytes have similar heart weight to body weight ratios to their wild-type controls at baseline (53, 54) and develop the same degree of hypertrophy (compared to their respective controls) following AngII infusion (24). In contrast, over-expression of the AT_2 receptor under the control of the ventricle-specific MLC2v promoter in mice causes a dilated cardiomyopathy and heart failure at maturity (45). Nakayama and colleagues went on to show that ventricular myocytes from MLC2v-AT_2TG mice have an impaired inotropic response to AngII, which was Ca^{2+}-dependent and was associated with reduced activity of the Na^+/H^+ exchanger (55). On the basis of these results, Yan *et al* concluded that the AT_2 receptor mediates pro-growth effects on the myocardium and noted that the alpha-MHC-AT_2TG mouse model may have been confounded by atrial transgene expression and the subsequent modification of cardiac chronotropic properties (45). However, it should be noted that cardiac hypertrophy was only evident under basal conditions in the line of MLC2v-AT_2TG mice with the highest level of AT_2 receptor expression (18 copies of transgene) (45). MLC2v-AT_2TG mice with a lower level of AT_2 receptor over-expression (9 copies of transgene, 706 fmol/mg protein vs 884 fmol/mg protein) did not display any signs of cardiac hypertrophy or heart failure under basal conditions at 18 weeks of age (45). Furthermore, a recent study by Yan *et al*, whereby MLC2v-AT_2TG mice were subjected to pressure overload by aortic banding, demonstrated that the AT_2 receptor significantly reduced left ventricular myocyte diameter, left ventricular systolic pressure, and collagen compared to aortic banded non-

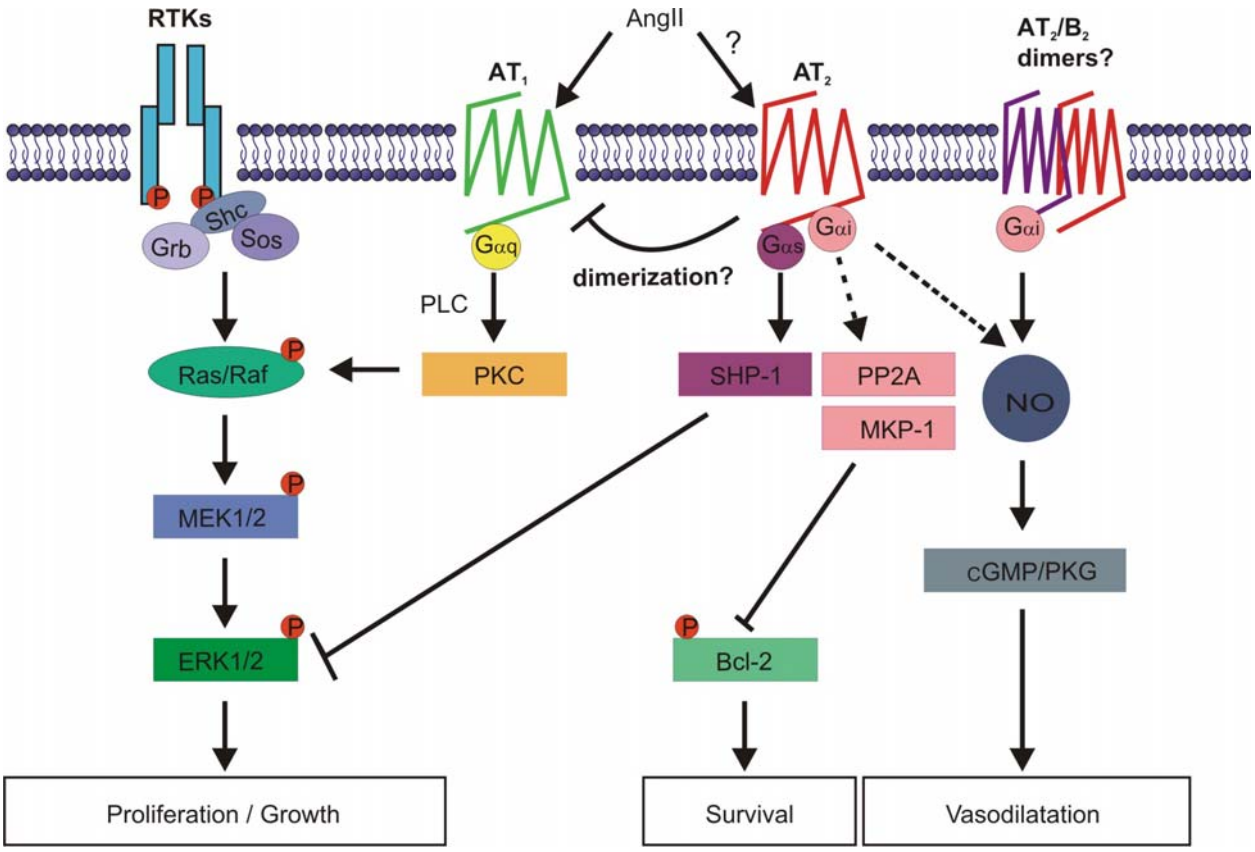

Figure 1. Putative signalling pathways involved in AT_2-mediated anti-proliferative/anti-growth, apoptotic, and vasodilator responses. ERK1/2 plays an important role in cellular proliferation. AT_2-dependent activation of the protein phosphatases SHP-1, PP2A and MKP-1 leads to dephosphorylation of ERK1/2, trans-inactivation of RTKs, and inhibition of cell proliferation. Trans-inactivation of RTKs by the AT_2 receptor also involves inhibition of auto-phosphorylation of the RTK, an early step required for receptor activation, and may involve heterodimerization of the AT_2 receptor with RTKs such as the ErbB3 receptor for EGF (not shown in Figure 1, discussed in Section 5.1). As well as dephosphorylating ERK1/2, activation of MKP-1 can also result in dephosphorylation of the pro-survival protein Bcl-2, which induces apoptosis. While the AT_2 receptor classically couples to $G_{alpha\text{-}i}$, studies have shown that AT_2-mediated activation of SHP-1 may depend on $G_{alpha\text{-}s}$. AT_2 coupling to $G_{alpha\text{-}i}$ is linked with activation of cGMP second messenger signalling. However, AT_2-dependent production of NO-cGMP may involve a functional heterodimerization with the bradykinin B_2 receptor. AT_2 coupling to NO-cGMP signalling causes vasodilatation. The AT_2 receptor antagonizes AT_1-induced signalling, exerting anti-proliferative and vasodilator effects, and this may be mediated by a direct physical interaction (i.e. heterodimerization) between the two receptor subtypes.

transgenic controls (56). This study utilized MLC2v-AT_2TG mice expressing a low level of AT_2 receptor (9 copies) at a young age (4-5 weeks) when cardiac hypertrophy is not evident under basal conditions (45); unfortunately, non-banded AT_2 transgenic mice were not used as controls in this study (56). It is also unclear whether the line of MLC2v-AT_2TG mice with a higher level of AT_2 expression, which display signs of overt heart failure at 18 weeks of age, respond similarly to pressure overload.

At present, a consensus with regard to the physiological role of the AT_2 receptor remains elusive. While it can be concluded that the localization, expression levels and physiological context all appear to be critical determinants of AT_2 receptor function *in vivo,* further work is required to characterize the functions of this enigmatic receptor.

4. AT_2 RECEPTOR SIGNALLING: THE ONGOING SEARCH FOR G PROTEIN-COUPLED SIGNALS

Given the variety and variability of functions which have been attributed to the AT_2 receptor, it is not surprising that the AT_2 signalling pathway (s) have been difficult to elucidate. Since the molecular cloning of both angiotensin receptors in the early '90s, AT_2 receptor signalling has been the subject of great controversy. Despite fifteen years and over 2100 publications since the cloning of the AT_2 receptor, resolution regarding the signalling mechanisms involved has not yet been achieved. Although the AT_2 receptor displays all of the classic motifs and signature residues of a G protein-coupled receptor (GPCR), it fails to demonstrate most of the classic features of GPCR activation and signalling (see Figure 1).

4.1. G Protein-coupling

Before the AT_2 receptor was cloned and identified as a member of the GPCR superfamily, it was generally thought not to be G protein-coupled (57). This view was based on studies which failed to demonstrate AT_2 receptor-induced modulation of cytosolic Ca^{2+} or cyclic AMP, which argued against coupling to G_s and G_i-dependent signalling pathways (1). Furthermore, stimulation of the AT_2 receptor did not result in an increase in the binding of $(^{35}S)GTP_{gamma}S$, and agonist binding did not induce receptor internalization - both classic features of GPCRs (57, 58). Subsequent studies in which the cloned rat AT_2 receptor was stably over-expressed in human embryonic kidney 293 (HEK293) cells verified many of the early findings and failed to show any effect of AngII stimulation on cAMP levels, cGMP levels, arachidonic acid release, or phosphotyrosine phosphatase activity (59).

Despite the initial identification of structural features and motifs in the AT_2 receptor, qualifying it as a member of the GPCR superfamily, it took several years before the first report demonstrating direct AT_2 coupling to the G proteins $G_{ialpha2}$ and $G_{ialpha3}$ (60). Only a few studies have subsequently demonstrated AT_2 receptor coupling to Gi and have directly linked downstream signals to activation of this class of G protein. Hayashida *et al* reported that the intracellular third loop (ICL3) of the AT_2 receptor is an important determinant for its coupling to $G_{alpha-i}$ (61). ICL3 was subsequently shown to be involved in AT_2-induced apoptotic responses in the neuronal lineage PC12W cells (62) and has been implicated in AT_2-mediated inhibition of IP_3 generation in *Xenopus* oocytes (63). More recent studies have implicated the involvement of Gi in AT_2 receptor-dependent increases in nitric oxide synthase expression (64) and in AT_2-mediated inhibition of proximal tubule Na^+-ATPase by the angiotensin peptide fragment Ang1-7 (65). However, a definitive demonstration that Gi coupling is *necessary* for AT_2 function has not yet been possible, although such an experiment might be feasible using Gi knock-out cell lines (66). It also remains unclear whether ICL3 is important for coupling of the full-length AT_2 receptor to Gi. The initial identification of an interaction between Gi and ICL3 of the AT_2 receptor reported by Hayashida *et al* employed a synthetic ICL3 peptide fragment (61). Whether mutations in ICL3 are sufficient to uncouple the full-length AT_2 receptor from Gi has not yet been determined.

Notably, several groups have reported that AT_2-mediated activation of the intracellular protein tyrosine phosphatase SH2 domain-containing phosphatase 1 (SHP-1) is pertussis toxin-insensitive, which does not support an involvement of $G_{alpha-i}$ (67, 68). Feng *et al* also demonstrated that the AT_2 receptor-mediated activation of SHP-1 is associated with a $G_{beta-gamma}$-independent constitutive association of the receptor with $G_{alpha-s}$ (68). The limited availability of data and discrepant findings in relation to establishing G protein-coupling with AT_2 receptor signalling presents a major research challenge and constrains the development of a full understanding of AT_2 signalling mechanisms.

4.2. NO-cGMP

Nitric oxide (NO) increases the catalytic activity of soluble guanylyl cyclases, which in turn generates cGMP (69). Initial studies investigating cGMP involvement in AT_2 signalling were inconclusive. While a number of *in vitro* findings suggested that AngII reduced absolute and/or basal cGMP levels via the AT_2 receptor in neuronal cells (70-73), other contradictory evidence did not support AT_2 coupling to cGMP (58, 74-76). In 1996, Siragy and Carey published a landmark study, which demonstrated that renal AT_2 activation *in vivo* increased cGMP generation (77). The discrepancies between the initial *in vitro* studies in neuronal cell lines and the later *in vivo* link made between AT_2 activation and increased cGMP production in the kidney have not been resolved. However, the findings reported by Siragy and Carey represented an important milestone in the field, with subsequent studies predominantly focussing on AT_2-mediated NO-cGMP signalling in cardiovascular tissues *in vivo*.

Further evidence for an involvement of NO-cGMP in AT_2-mediated cardiovascular effects emerged from studies by Liu *et al*, demonstrating that the beneficial therapeutic effects of AT_1 receptor blockade involved kinin stimulation and cGMP production (78). Gohlke *et al* also showed that cGMP levels were increased in the rat aorta following AT_2 receptor stimulation (79). Subsequent studies in gene targeted mice confirmed these initial observations. Siragy *et al* noted that AT_2 knock-out mice had low basal levels of cGMP in renal interstitial fluid compared to wild type (80). Correspondingly, AT_2 transgenic mice have elevated levels of cGMP in the aorta (33). A modest reduction in eNOS expression has also been reported in the myocardium of AT_2 knock-out mice (81). However, it is unclear whether this slight reduction in eNOS expression in the heart of AT_2 knock-out mice is localized to cardiomyocytes or the coronary vasculature.

In this context, studies of AT_2 transgenic and knock-out mice implicating bradykinin in the AT_2-mediated increases in cGMP production are of particular interest. Tsutsumi *et al* elegantly showed that the AT_2 receptor stimulates bradykinin production in vascular smooth muscle cells and AT_2-mediated increases in cGMP could be blocked with the bradykinin receptor antagonist icatibant (33). The authors subsequently concluded that the AT_2 receptor stimulates the production of bradykinin, which in turn promotes the production of NO/cGMP in a paracrine manner. Importantly, AT_2-mediated vasodilatation of human coronary microarteries also appears to be mediated by bradykinin and NO (30). The complexity of the proposed mechanism for AT_2-dependent NO/cGMP production, and in particular the involvement of paracrine signalling mechanisms, might explain some of the inconsistencies in the initial pharmacological studies, which were predominantly restricted to cell lines of neuronal origin.

More recently, clues to the mechanism of AT_2-mediated increases in NO have emerged. AT_2 receptor activation by AngII induces phosphorylation of eNOS via a PKA-mediated signalling pathway (suggestive of Gs-

cAMP signalling), which leads to sustained activation of eNOS in the thoracic aorta of mice with abdominal aortic banding (82). Interestingly, bradykinin can induce PKA-dependent phosphorylation of eNOS and the data from the study by Yayama and colleagues are consistent with the model of AT_2-mediated stimulation of bradykinin release and downstream activation of NO/cGMP signalling. Furthermore, recent evidence for a functional heterodimerization of AT_2 and bradykinin (B_2) receptors is provided by confocal fluorescence resonance energy transfer studies (FRET) in PC12W cells, which suggest that the AT_2 and B_2 receptors physically associate with each other (83). While the possibility that AT_2-dependent increases in NO production are mediated by a functional heterodimerization with bradykinin B_2 receptors is intriguing, more detailed studies employing receptor mutagenesis in heterologous expression systems are required to elucidate the structural determinants and specificity of the AT_2-B_2 interaction.

4.3. Activation of phosphatases and dephosphorylation of MAPKs

AT_2 receptor coupling to the activation of protein phosphatases was one of the first identified signals generated by AT_2 receptor stimulation. Since the first report of an involvement of a vanadate-sensitive tyrosine phosphatase in AT_2 signalling (84), AT_2-mediated activation of phosphatases has emerged as a key mechanism accounting for the anti-growth and apoptotic effects of the AT_2 receptor (22, 85).

Mitogen-activated protein kinase (MAPK) signalling plays a key role in cellular proliferation. A number of studies have demonstrated that extracellular signal-regulated kinases 1 and 2 (ERK1/2) are desphosphorylated following activation of the AT_2 receptor (22, 67, 86-88). This finding has been corroborated by studies in AT_2 knock-out mice, which display elevated levels of ERK1/2 at baseline and in response to serum (86). AT_2-mediated dephosphorylation of ERK1/2 appears to involve three phosphatases: SHP-1, mitogen-activated protein kinase phosphatase 1 (MKP-1), and protein phosphatase 2A (PP2A) (22, 67, 88).

AT_2 receptor gain-of-function studies have provided a somewhat more controversial view with respect to coupling to phosphatase signalling. Nakajima *et al* used *in vivo* gene transfer of the AT_2 receptor in balloon-injured carotid arteries to demonstrate that the AT_2 receptor attenuated neointimal formation and inhibited AngII-induced MAPK activity in a PD123319-sensitive manner (19). Similarly, studies in vascular-targeted AT_2 transgenic mice provided convincing evidence that AT_2 receptor activation is linked with an up-regulation of SHP-1, as early as 1 min after stimulation with AngII (89). In contrast, adenovirus-mediated over-expression and stimulation of the human AT_2 receptor in porcine cardiac fibroblasts did not modulate proliferation and actually inhibited protein tyrosine phosphatases (90). D'Amore *et al* recently showed that adenovirus-mediated over-expression of AT_2 receptors in neonatal cardiomyocytes increased growth in a constitutive and ERK1/2-independent manner (51).

Conflicting reports with respect to the role of phosphatase signalling in AT_2-mediated pro-apoptotic effects also exist. While AT_2-induced apoptosis in PC12W cells is AngII-dependent and mediated by dephosphorylation of Bcl-2 by MKP-1 (22), Miura and Karnik found that apoptosis is a constitutive function of the AT_2 receptor (i.e. does not require AngII) and involves activation of p38 MAPK (91). These contradictions only serve to further highlight the context-specific nature of the AT_2 receptor and may point to a particular sensitivity of this GPCR to over-expression.

5. NOVEL AT_2 INTERACTING PROTEINS: SCREENING FOR NEW AT_2 TARGETS

The unconventional signalling pathways and enigmatic nature of the AT_2 receptor have prompted the search for new AT_2 targets. In recent years, a number of studies have employed yeast two-hybrid screening to identify novel proteins that interact with the C-terminus of the AT_2 receptor. These interacting proteins, which include ErbB3, ATIP/ATBP50, and PLZF, appear to be important for AT_2 receptor signalling, trafficking and function (see Figure 2).

5.1. ErbB3

The AT_2 receptor negatively cross-talks with receptor tyrosine kinases (RTKs) such as fibroblast growth factor (FGF), epidermal growth factor (EGF) and insulin receptors (85, 92, 93). Trans-inactivation of RTKs by the AT_2 receptor involves rapid activation of tyrosine phosphatases and inhibition of auto-phosphorylation of the RTK (85, 93), an early step required for receptor activation. However, AT_2-mediated trans-inactivation of the insulin receptor in Chinese hamster ovary cells does not involve protein dephosphorylation or the G proteins G_i/G_o (85), suggesting that other mechanisms are also involved. One intriguing possibility is that AT_2-mediated trans-inactivation of RTKs involves a direct physical interaction between the two receptors. Using a yeast two-hybrid protein interaction assay with the C-terminus of the AT_2 receptor as bait, Knowle *et al* identified the ErbB3 EGF receptor as an AT_2 interacting partner (94). In subsequent studies using mutated and chimeric AT_2 receptors, Knowle *et al* showed that replacing ICL3 of the AT_2 receptor with that of AT_1 abolishes the interaction between AT_2 and ErbB3 (94, 95). ICL3 plays an important role in AT_2-mediated inhibitory effects on cell proliferation (ie. activation of apoptosis) and appears to be an important determinant of AT_2 coupling to G_i/G_o (see Section 4.1), as well as SHP-1 activation (68). Therefore, ICL3 appears to be an integral regulator of AT_2 signalling and function, and the involvement of AT_2 interactions with ErbB3 in ICL3-dependent AT_2 signalling warrants further investigation. Furthermore, the possibility that the AT_2 receptor also physically interacts with other RTKs and that this represents a general model for AT_2-mediated trans-inactivation of RTKs is enticing and should be an area of future study.

5.2. ATIP/ATBP50

Recently, two independent groups identified ATIP/ATBP50 as a novel AT_2 interacting protein through yeast 2-hybrid screening of the C-terminus of the AT_2

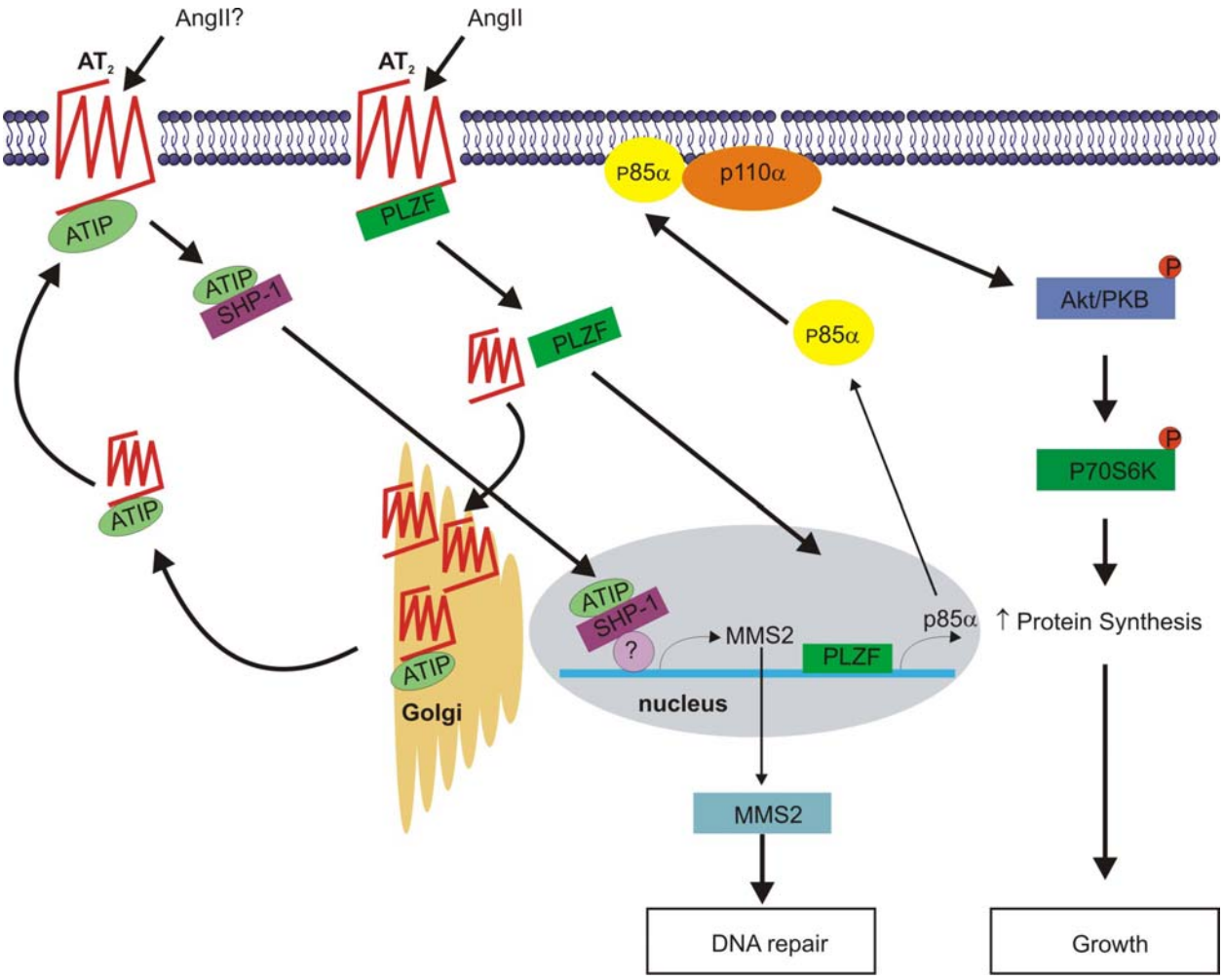

Figure 2. Putative AT_2 receptor signalling pathways mediated by novel AT_2 interacting proteins. ATIP interacts with the C-terminus of the AT_2 receptor and is involved in transporting the AT_2 receptor from the Golgi to the plasma membrane. AT_2 receptor stimulation at the plasma membrane induces translocation of ATIP to the nucleus and promotes the formation of an ATIP/SHIP-1 complex, which leads to transcriptional activation of the DNA repair enzyme MMS2. The AT_2 receptor also interacts with the transcription factor PLZF. Following AngII stimulation, PLZF is activated and translocated from the cytosol to the plasma membrane where it drives AT_2 and PLZF to internalize. Whereas AT_2 receptors accumulate in the perinuclear region, PLZF emerges in the nucleus where it increases transcription of the PI3K regulatory subunit p85 alpha. The downstream activation of $P70^{S6}$ kinase (P70S6K) leads to an increase in protein synthesis and cell growth.

receptor (96, 97). Nouet *et al* were the first to report the interaction of a novel protein termed ATIP1 (AT_2 interacting protein) with the C-terminal tail of the AT_2 receptor (96). ATIP1 belongs to a family of at least four members (ATIP1-4) that all possess the same domain required for interaction with the AT_2 receptor. Ectopic expression of ATIP in Chinese hamster ovary cells leads to a significant inhibition of insulin, basic fibroblast growth factor and epidermal growth factor-induced ERK1/2 activation and DNA synthesis, in a similar manner to AT_2 receptor activation alone (96). Interestingly, the ATIP-mediated repression of ERK activity requires AT_2 receptor expression, but not extracellular activation by AngII (96). More recently, Li *et al* showed that the AT_2 receptor induced neural differentiation by increasing expression of methane methylsulfonate-sensitive 2 (MMS2), which plays an important role in the ubiquitin proteasome system and DNA repair (98). siRNA-mediated knock-down of ATIP significantly inhibits AngII-dependent increases in MMS2 mRNA and protein levels. Furthermore, AT_2 receptor stimulation induces translocation of ATIP to the nucleus and promotes the formation of an ATIP/SHP-1 complex, which could provide a mechanism for the AT_2-mediated transcriptional activation of MMS2 and downstream induction of neural differentiation (98). MMS2 expression is increased following permanent cerebral artery occlusion in wild-type, but not AT_2 knock-out mice, consistent with a protective role for the AT_2 receptor in brain injury (99).

ATIP appears to play an important role in the trafficking of the AT_2 receptor to the plasma membrane. siRNA-mediated knock-down of the AT_2 receptor binding

protein of 50kDa (ATBP50, which is identical to ATIP), reduces cell surface expression of the AT_2 receptor and suppresses its anti-proliferative effect (97). However, it is still unclear whether ATIP/ATBP50 is involved in the transport of other GPCRs from the Golgi to the plasma membrane and this protein may prove to be an important generic regulator of GPCR cell surface expression. Studies of ATIP knock-out mice are required to identify physiological roles for this protein, which likely has a function extending beyond a specific involvement in AT_2 receptor trafficking and signalling.

5.3. PLZF

Yeast 2-hybrid studies with the AT_2 receptor C-terminal tail as bait have also revealed an interaction with a transcription factor, promyelocytic zinc finger protein (PLZF), which is highly expressed in the heart (100). Following AngII stimulation, PLZF is activated, translocated from the cytosol to the plasma membrane and then drives AT_2 and PLZF to internalize. Whereas AT_2 accumulates in the perinuclear region, PLZF localizes to the nucleus where it binds to a consensus sequence for the PI3K p85 alpha regulatory subunit gene, increases p85 alpha transcription and enhances $p70^{S6}$ kinase activity (which is essential for protein synthesis) (100). Furthermore, AT_2 interaction with G_i appears to be involved in the interaction with PLZF and translocation of PLZF to the nucleus (100).

In prior studies, Senbonmatsu *et al* also showed that AT_2 knock-out mice fail to undergo a hypertrophic response to pressure overload following aortic banding (52). This phenotype was seen in association with a failure of AT_2 null hearts to up-regulate $p70^{S6}$ kinase following pressure overload (52). Following the identification of PLZF as an important AT_2 interacting protein, Senbonmatsu *et al* went on to show that AT_2 null hearts fail to up-regulate p85 alpha expression in response to AngII infusion, even though PLZF levels are not different under basal conditions between wild type and AT_2 null hearts (100). These data strongly suggest that the AT_2 receptor is important for the induction of cardiac hypertrophy.

Understanding how the AT_2 receptor suppresses cellular growth in some contexts by an apparent antagonism of insulin signalling, yet appears to be capable of enhancing one of the major components of the insulin signalling pathway (p85 alpha) in the heart to promote cellular growth is a major challenge. More detailed studies in primary cardiomyocyte cultures are required to fully characterize the upstream signalling pathways that lead to PLZF translocation following AT_2 receptor activation. The finding that AT_2 accumulates in the perinuclear region following dissociation from PLZF is also particularly interesting, given that prior studies indicated that AT_2 does not normally internalize (59, 101). Furthermore, validation of PLZF involvement in AT_2 receptor signalling and function in independent studies and resolution of the conflicting data from different AT_2 knock-out mouse models must be resolved (see Section 3.5 and Table 1) if coupling to PLZF is to emerge as an important piece in the AT_2 puzzle.

6. SIGNALLING WITHOUT ANGIOTENSIN II: IS THE AT_2 RECEPTOR CONSTITUTIVELY ACTIVE?

As alluded to above, several lines of evidence suggest that the AT_2 receptor is constitutively active (i.e. functions in the absence of its ligand). Unlike the AT_1 receptor, which is in a constrained conformation and is activated when bound to AngII, the AT_2 receptor displays ligand pharmacology that is consistent with it being in a 'relaxed', constitutively active conformation (102). While side-chain modifications of AngII are detrimental to AT_1 receptor binding affinity, the same modifications are well tolerated by the AT_2 receptor (102). The AT_2 receptor induces apoptosis in the absence of AngII stimulation, and this effect is not modulated by PD123319 (91). Similarly, in neonatal cardiomyocytes, adenoviral-mediated AT_2 receptor expression induces myocyte growth, and this effect is not modulated by AngII, PD123319 or CGP42112 (51). Gene expression profiling studies of human coronary artery endothelial cells have shown that subsequent to lentiviral delivery of the AT_2 receptor a large number (5224) of genes are regulated in an AT_2 receptor ligand-independent manner (103). In contrast, much fewer genes (1235) were differentially expressed in response to the AT_2 receptor-specific ligand CGP42112. This finding suggests that expression of the AT_2 receptor *per se* may be a major determinant of function and that many cellular effects of AT_2 expression are not contingent on ligand interaction with this receptor.

The detailed mechanisms underpinning constitutive activation of the AT_2 receptor are not well understood. Homo-oligomerization of AT_2 receptors, which is mediated by disulfide bonding between Cys^{35} in one AT_2 receptor and Cys^{290} in its dimerization partner, appears to be important for the constitutive induction of apoptosis in Chinese hamster ovary cells (104). However, the mechanisms which drive homo-oligomerization and constitutive activity of AT_2 receptors remain poorly defined. Furthermore, whether receptor dimerization and/or constitutive activity are simply a nuance of receptor over-expression needs to be determined. Nevertheless, mounting *in vitro* evidence for a constitutively active AT_2 receptor must prompt a reconsideration of the assumptions about AT_2 receptor pharmacology and physiology, which are largely based on pharmacological studies employing selective AT_2 receptor agonists and antagonists. The possibility that up-regulation of a constitutively active GPCR under pathological conditions allows the renin-angiotensin system to escape regulatory control from its ligand is tantalizing, and may open up new strategies for the treatment of hypertension and cardiac hypertrophy.

7. AT_2 RECEPTOR DIMERIZATION: FACT OR FANTASY?

GPCRs were traditionally thought to act as monomers, but recent evidence suggests that GPCRs may form dimers and higher-order oligomers as part of their normal trafficking and transduction function (105). Angiotensin receptor dimerization has received particular attention because of the potential for developing new

cardiovascular therapeutics. In a landmark study, AbdAlla *et al* reported that intracellular factor XIIIA transglutaminase crosslinks AT_1 receptor homodimers on monocytes at the onset of atherosclerosis (106). AbdAlla *et al* also demonstrated that AT_1 receptors heterodimerize with the bradykinin B_2 receptor in patients with preeclampsia and contribute to AngII hypersensitivity (107). In 2001, the same group detected AT_1 and AT_2 receptor heterodimers on cells ectopically expressing AngII receptors, as well as in foetal fibroblasts and in myometrial biopsies (108). The AT_2 receptor was found to antagonize AT_1 signalling by direct association (108). Collectively, these studies by Quitterer's group were the first to provide evidence for AngII receptor dimerization *in vitro* and *in vivo,* and highlighted the potential clinical significance of GPCR dimers.

However, before AT_2/AT_1 receptor heterodimerization is accepted as an important regulatory aspect of AngII signalling and function, the initial important observation that AT_1 and AT_2 receptors heterodimerize awaits confirmation. The use of more sophisticated approaches such as bioluminescence resonance energy transfer (BRET), which depends on energy transfer between bioluminescent donor and fluorescent acceptor proteins to identify intermolecular interactions, have the potential to resolve this issue in the future. Finally, further studies are required to determine whether AT_2 heterodimers are regulated by AngII and how dimerization affects the pharmacology, signalling and internalization of the AT_1 receptor.

8. PERSPECTIVE

The AT_2 receptor has been an enigma since it was cloned in the early 90's and remains possibly the most controversial element of the renin-angiotensin system. As highlighted in a review by Steckelings and colleagues, perhaps one reason for the difficulty in understanding the role of the AT_2 receptor is that it does not appear to be a conventionally 'active' receptor which evokes a specific response (11). The AT_2 receptor is unconventional and its actions do not appear to involve most of the classic GPCR signalling pathways, and in many instances do not even require binding of the ligand AngII. While pharmacological studies have been informative, the data are not entirely consistent with genetic gain and loss of function studies, suggesting that the AT_2 receptor may indeed have AngII-independent actions.

To resolve the outstanding questions relating to AT_2 function, new studies focussing on the relevance of AT_2 ligand-independent pathways *in vivo* are required. These studies should reveal novel aspects of AT_2 receptor biology, previously unappreciated, which may explain the anomalies observed between different experimental models. More sophisticated studies employing unbiased approaches to identify AT_2 targets (e.g. microarrays, siRNA libraries, yeast 2-hybrid) will likely identify new intermediates in AT_2 signalling and will bring insight to understanding the AT_2 receptor enigma.

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Abbreviations: AngII: angiotensin II, GPCR: G protein-coupled receptor, SHR: Spontaneously Hypertensive Rat, alpha-MHC: alpha myosin heavy chain, MLC2v: myosin light chain 2v, HEK293: human embryonic kidney 293, cAMP: cyclic adenosine monophosphate, cGMP: cyclic guanosine monophosphate, ICL3: intracellular third loop, SHP-1: SH2 domain-containing phosphatase 1, NO: nitric oxide, eNOS: endothelial nitric oxide synthase, MAPK: mitogen-activated protein kinase, ERK1/2: extracellular signal-regulated kinases 1 and 2, MKP-1: mitogen-activated protein kinase phosphatase 1, PP2A: protein phosphatase 2A, RTK: receptor tyrosine kinase, FGF: fibroblast growth factor, EGF: epidermal growth factor, ATIP: AT_2-interacting protein, MMS2: methane methylsulfonate-sensitive 2, PLZF: promyelocytic zinc finger protein.

Key Words: Angiotensin II type 2 Receptor, AT2 Receptor, Angiotensin II, G Protein-Coupled Receptor, GPCR, Signalling, Constitutive Activity, Review

Send correspondence to: Enzo R. Porrello, Department of Physiology, The University of Melbourne, Parkville, Victoria 3010, Australia, Tel: 61-3 8344 5821, Fax: 61-3 8344 5897, E-mail: e.porrello@pgrad.unimelb.edu.au

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