Hypertension Research (2011) 34, 701–706

www.nature.com/hr

ORIGINAL ARTICLE

Non-activated APJ suppresses the angiotensin II type 1 receptor, whereas apelin-activated APJ acts conversely

Xiao Sun[1], Shinichiro Iida[2], Ayumu Yoshikawa[3], Rina Senbonmatsu[4], Kazuhito Imanaka[1], Kei Maruyama[3], Shigeyuki Nishimura[2], Tadashi Inagami[5] and Takaaki Senbonmatsu[3]

Apelin and its G-protein-coupled receptor APJ are potent regulators of the cardiovascular system. Recent studies have suggested that apelin–APJ reverses the function of angiotensin II (Ang II)–the Ang II type 1 receptor (AT_1). However, the mechanism remains unclear because of the accumulating evidences that apelin–APJ may contribute to both cardioprotection and pathological progression. In human embryonic kidney 293 cells, we found that coexpression with APJ significantly suppressed the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) induced by Ang II–AT_1, whereas apelin abolished this attenuation through activated APJ independently of its heterodimerization. Pretreatment with the Gi/o-specific inhibitor pertussis toxin (PTX) restituted the ERK1/2 phosphorylation level similar to that found with AT_1 and APJ coexpression without apelin stimulation. In contrast, coexpression of the beta-2-adrenergic receptor or the pharmacologically non-activated Ang II type 2 receptor (AT_2) pretreated with the AT_2-specific antagonist, PD123319, did not affect ERK1/2 phosphorylation through AT_1. Pretreatment with 30 nM of the AT_1 blocker (ARB) TA-606A suppressed 50% of the AT_1-mediated ERK1/2 phosphorylation, whereas 30 nM of TA-606A achieved 75% suppression when the non-activated APJ was coexpressed without ligand or PTX. However, 120 nM of TA-606A failed to reach the target phosphorylation when it was coexpressed with activated APJ with apelin. Based on these results, we demonstrated that non-activated APJ may suppress Ang II–AT_1 signaling, whereas this ligand-independent function was diminished with apelin activation. These results may be relevant to the potential contribution of apelin–APJ to ARB treatment in the clinical realm.
Hypertension Research (2011) **34,** 701–706; doi:10.1038/hr.2011.19; published online 17 March 2011

Keywords: apelin; APJ; angiotensin II type 1 receptor; angiotensin II type 1 receptor blocker

INTRODUCTION

The 380-amino acid, 7-transmembrane domain G-protein-coupled receptor (GPCR) called APJ, which is most closely related to the angiotensin II (Ang II) type 1 receptor (AT_1) but does not bind to Ang II, was identified through the Human Genome Project.[1] In 1998, its endogenous ligand called apelin was identified.[2] Apelin and APJ are highly expressed in cardiomyocytes, endothelial cells and vascular smooth muscle cells (VSMC). Apelin activates APJ, leading to intracellular signaling both locally and in an endocrine fashion.[3,4] Since its discovery, the apelin–APJ system has emerged as an important regulator of cardiovascular homeostasis and may have a critical role in the pathophysiological development of cardiovascular diseases; it has also been regarded as a new therapeutic research and drug design target.[5–11] The functions of apelin–APJ appear to be complex and have yet to be defined by consensus. The accumulating evidences have indicated that the functions of apelin–APJ may be dependent on its target organs and its physiological/pathophysiological state. The experiments performed using cardiomyocytes have shown that apelin–APJ evokes contractility and promotes growth; however, in the vasculature, apelin–APJ acts as a vasodilator as part of its cardioprotective function under physiological but not pathophysiological conditions.[5,8]

Recent studies have revealed that APJ and AT_1 have similar expression patterns in a number of physiological and pathophysiological states, and apelin–APJ contributes to opposing effects against Ang II–AT_1.[10,11] Blood pressure in AT_1 and APJ double-knockout mice was significantly elevated compared with that in AT_1 knockout mice.[11] Chun *et al.* reported that apelin–APJ antagonizes Ang II–AT_1 by increasing nitric oxide (NO) production via intracellular signaling in mouse models of atherosclerosis.[12] However, researchers have not yet concluded that the apelin–APJ system is a counterpart of the Ang II–AT_1 system. In contrast to Chun *et al.*, Hashimoto *et al.* demonstrated that in mouse models of atherosclerosis, apelin–APJ aggravates oxidative stress through increasing the nicotinamide adenine dinucleotide phosphate oxidase subunits, which are major sources of superoxide anion in the cardiovascular system.[13] Similarly,

[1]Department of Cardiovascular Surgery, Saitama Medical Center, Saitama, Japan; [2]Division of Cardiovascular Medicine, Department of Cardiovascular Medicine, Saitama International Medical Center, Saitama, Japan; [3]Department of Pharmacology, Saitama Medical University, Saitama, Japan; [4]Department of Neuroscience, Bates College, Lewiston, ME, USA and [5]Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN, USA
Correspondence: Dr T Senbonmatsu, Department of Pharmacology, Saitama Medical University, 38 Morohongo Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan.
E-mail: senbont@saitama-med.ac.jp
Received 2 September 2010; revised 17 December 2010; accepted 26 December 2010; published online 17 March 2011

Liu *et al.* reported that apelin promotes VSMC proliferation through the phosphoinositide 3-kinase/Akt signaling pathway.[14] These results indicate that apelin–APJ may have growth-promoting effects similar to those of Ang II–AT_1 in the pathological development of cardiovascular diseases. Taken together, all of these results convincingly demonstrate that apelin–APJ may have dual functions. However, the specific correlation between both GPCRs' functions still remains controversial. In the present study, we investigated the relationship among apelin, APJ, Ang II and AT_1 in an *in vitro* experimental model. APJ alone but not apelin–APJ may be a crucial therapeutic candidate in inhibiting Ang II–AT_1.

METHODS

Reagents

The following reagents and antibodies were purchased: Ang II (Sigma-Aldrich, St Louis, MO, USA); anti-phosphorylated extracellular signal-regulated kinases 1/2 (anti-pERK1/2) antibody; anti-AT_1 antibody; anti-angiotensin II type 2 receptor (anti-AT_2) antibody; anti-APJ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-beta-2-adrenergic receptor (anti-β_2AR) antibody (Abnova, Taipei, Taiwan); anti-extracellular signal-regulated kinases 1/2 (anti-ERK1/2) antibody; anti-pAkt antibody; anti-Akt antibody (Cell Signaling Technology, Boston, MA, USA); pertussis toxin (PTX; Seikagaku, Tokyo, Japan); and the AT_2-specific antagonist, PD123319 (Sigma-Aldrich). The AT_1 blocker (ARB) TA-606A was a gift from the Mitsubishi Tanabe Pharmaceutical company (Osaka, Japan). We also purchased pyroglutamyl (Glp-1) apelin-13 (American Peptide Company, Vista, CA, USA) and used this as apelin.

Vector construction

The construction of the human full-length AT_1, APJ, AT_2 and β_2AR were performed by PCR amplification using the human Multiple Tissue cDNA panel I (Clontech Laboratories, Palo Alto, CA, USA). The construction of the double-cassette vector system was previously described.[15–17] Myc tag was inserted into pcDNA5/FRT/TO (Invitrogen, Carlsbad, CA, USA) to construct pcDNA5/FRT/TO-myc-His. Then, elongation factor 1α (EF1α) promoter, multiple cloning sites and bovine growth hormone site were inserted into pcDNA5/FRT/TO-myc-His. Finally, pcDNA5/FRT/TO-EF1α-V5-His-CMV-myc-His, which contained two expression cassettes, was constructed. Thus, the vectors that expressed AT_1-myc-His; APJ-V5-His; AT_1-myc-His plus APJ-V5-His; AT_1-myc-His plus AT_2-V5-His; and AT_1-myc-His plus β_2AR-V5-His were created.

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C. Transient transfection of HEK293 cells was performed using a Fugene6 reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) as per the manufacturer's protocols. At 30 h after transfection, the cells were serum starved for 6 h before agonist stimulation. For PTX pretreatment, the cells were incubated with 200 ng ml^{-1} of PTX for 3 h. For PD123319 pretreatment, the cells were incubated with 10 μM of PD123319 for 1 h. The cells were pretreated with 15, 30, 60 and 120 nM of TA-606A for 90 min as previously described.[18]

Immunoblotting

The cells were stimulated with Ang II (100 nM); apelin (100 nM) or Ang II plus apelin at 37 °C in serum-free media after preincubation with the indicated reagents. Then, cells were lysed in an ice-cold RIPA buffer (50 mmol l^{-1} Tris-HCl, pH 8.0, 150 mmol l^{-1} NaCl, 5 mmol l^{-1} EDTA, 1% Nonidet P-40, 0.1% SDS and 0.5% deoxycholate) containing 100 mmol l^{-1} phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) at 4 °C and centrifuged at 20 000 *g* for 60 min at 4 °C. The protein concentration of the supernatant was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Then, 4 μg of protein from each sample was subjected to SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and was transferred to a polyvinylidene fluoride membrane. The polyvinylidene fluoride membrane was blotted with a primary antibody as specified in each case. After exposure to the primary antibodies, blots were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The reactive bands were visualized with enhanced chemiluminescence substrates and analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Immunoprecipitation

Cells were stimulated with Ang II (100 nM), Ang II plus 10 nM or 100 nM apelin at 37 °C in serum-free media for 2 min. The whole-cell lysate from the RIPA buffer was incubated with anti-APJ antibody or anti-rabbit immunoglobulin G antibody overnight at 4 °C for immunoprecipitation and incubated with protein G agarose (GE Healthcare, Piscataway, NJ, USA) for an additional 3 h at 4 °C, followed by a brief centrifugation on a bench-top centrifuge. The pellet was washed three times in RIPA buffer and dissolved in the SDS-polyacrylamide gel electrophoresis-loading buffer. Immunoblotting of the heterodimer was performed using an anti-AT_1 antibody.

Statistical analysis

All data were expressed as a mean ± s.e.m. The data were analyzed using a Student's *t*-test for parametric data or a two-way analysis of variance with Tukey–Kramer multiple comparison tests for comparing multiple groups. A value of $P<0.05$ was considered statistically significant.

RESULTS

Relationship between apelin–APJ and Ang II–AT_1 signaling

To investigate the relationship between apelin–APJ and Ang II–AT_1 signaling, ERK1/2 phosphorylation was evaluated in HEK293 cells transfected with AT_1 alone or dual transfected with AT_1 and APJ. There was no significant difference in the expression level of AT_1 in either of the transfected cell lines. In HEK293 cells dual transfected with AT_1 and APJ, ERK1/2 phosphorylation was significantly reduced compared with that of AT_1 alone on Ang II stimulation ($P<0.05$ at 2, 5 and 10 min; Figure 1a). However, apelin stimulation dramatically increased the ERK1/2 phosphorylation level beyond the level of AT_1 alone ($P<0.05$ at 2 and 5 min; Figure 1a). To further support the idea that non-activated APJ suppresses AT_1, PTX was used to create the pharmacologically non-activated APJ. Interestingly, pretreatment with PTX attenuated the ERK1/2 phosphorylation level by apelin, resulting in the same level of ERK1/2 phosphorylation in AT_1 and APJ that was observed without the ligand (Figure 1a). Although immunoprecipitation of these receptors revealed that AT_1 and APJ formed heterodimers, apelin stimulation never commuted their heterodimerization (Figure 1b).

Specificity of PTX

To determine the relationship between apelin–APJ and Ang II–AT_1, several experiments were performed. Apelin evoked ERK1/2 phosphorylation via an APJ peak at 2 min and returned to the control level at 10 min (Figure 2a). In Figure 1a, the peak level of ERK1/2 phosphorylation of Ang II–AT_1 plus apelin–APJ was beyond that of Ang II–AT_1 because of the level of ERK1/2 phosphorylation of apelin–APJ. AT_1 coexpression did not affect the ERK1/2 phosphorylation induced by apelin–APJ (Figure 2a). The ERK1/2 and Akt phosphorylation via APJ on apelin stimulation were completely inhibited by pretreatment with PTX, whereas the ERK1/2 phosphorylation through Ang II–AT_1 was not attenuated by PTX (Figures 2b-d).

Crosstalk between AT_1 and other GPCRs

To confirm the specificity of APJ for Ang II–AT_1 signaling, the combination of AT_1 and other GPCRs was evaluated. HEK293 cells dual transfected with AT_1 and β_2AR were generated with the same AT_1 expression level. The ERK1/2 phosphorylation level of the dual-transfected HEK293 cells was not significantly different from that of

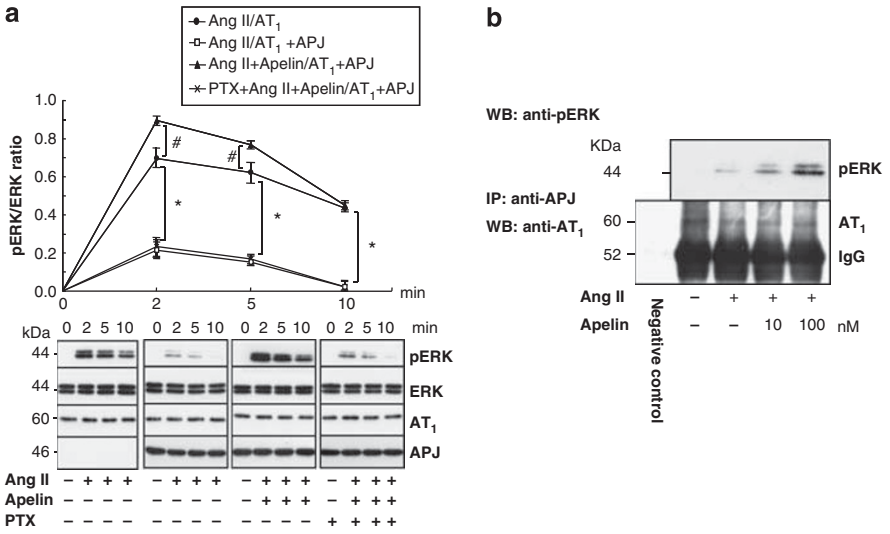

Figure 1 The relationship between apelin–APJ and AT_1 signaling. (**a**) HEK293 cells that expressed AT_1 alone or AT_1 plus APJ with or without PTX pretreatment were stimulated by Ang II or Ang II plus apelin as indicated. pERK1/2, ERK1/2, AT_1 and APJ were detected by western blot. (**b**) HEK293 cells that expressed AT_1 plus APJ were stimulated by Ang II or Ang II plus apelin as indicated. pERK1/2 was detected by western blot. Immunoprecipitation was performed using an anti-APJ antibody, followed by western blot using an anti-AT_1 antibody. Data represent the mean ± s.e.m. from three independent experiments (*Ang II–AT_1+APJ, #Ang II+apelin–AT_1+APJ, $P<0.05$ *vs.* Ang II–AT_1). The molecular weights are indicated in kilodaltons (kDa).

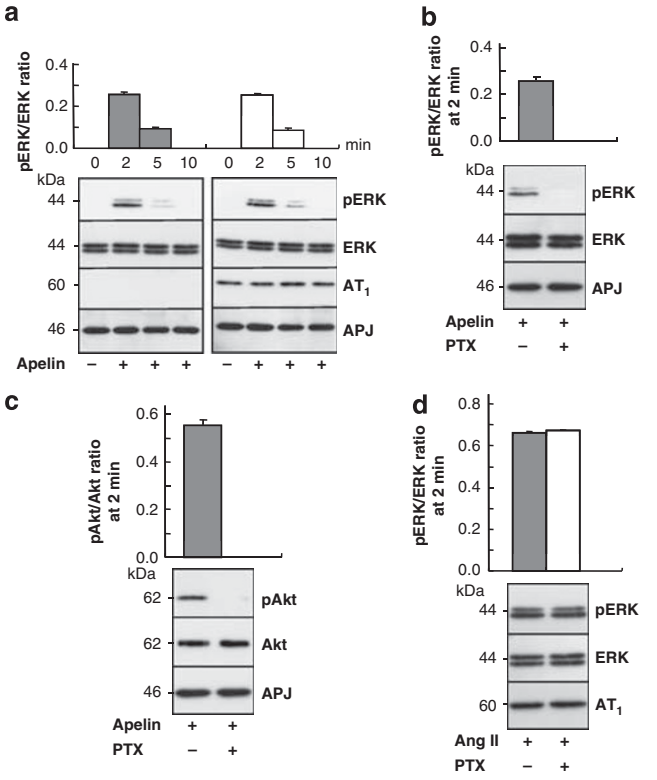

Figure 2 Specificity of PTX. (**a**) HEK293 cells that expressed APJ alone or AT_1 plus APJ were stimulated by apelin as indicated. (**b, c**) HEK293 cells that expressed APJ upon apelin stimulation for 2 min in the absence or presence of PTX pretreatment. (**d**) HEK293 cells that expressed AT_1 upon Ang II stimulation for 2 min with or without PTX pretreatment. pERK1/2, ERK1/2, pAkt, Akt, AT_1 and APJ were detected by western blot. Data represent the mean ± s.e.m. from three independent experiments.

AT_1 alone on Ang II stimulation (Figure 3a). Next, we evaluated the relationship between AT_1 and AT_2. Because AT_1 and AT_2 share the same ligand, the AT_2-specific antagonist, PD123319, was utilized to make the pharmacologically non-activated AT_2. The coexpression of non-activated AT_2 impacted the ERK1/2 phosphorylation level by the action of Ang II–AT_1 (Figure 3b).

Relationship among apelin, APJ and ARB

To reveal whether APJ may affect AT_1 blocking by ARB, TA-606A was used, and the ERK1/2 phosphorylation level at 2 min after Ang II stimulation was evaluated at various concentrations of TA-606A pretreatment (Figure 4). In HEK293 cells transfected with AT_1 alone, pretreatment with 30 nM of TA-606A significantly decreased the ERK1/2 phosphorylation level (50% decrease, $P<0.05$). Meanwhile, in the HEK293 cells dual transfected with AT_1 and non-activated APJ without ligand or PTX, the ERK1/2 phosphorylation level was decreased by 60–61% of that of AT_1 alone without TA-606A, and pretreatment with 30 nM TA-606A decreased the ERK1/2 phosphorylation level by 73–75%. In contrast, in the cells dual transfected with AT_1 and apelin-activated APJ, pretreatment with 120 nM TA-606A only suppressed the ERK1/2 phosphorylation level by 34% compared with AT_1 alone without TA-606A (Figure 4).

DISCUSSION

The present study demonstrated that non-activated APJ specifically suppressed Ang II–AT_1 signaling. Apelin stimulation eliminated this ligand-independent function of APJ, and the Gi/o-specific inhibitor PTX elicited the pharmacologically non-activated APJ upon apelin stimulation out of relation to its heterodimerization.

Relationship between AT_1 and APJ

Non-activated APJ significantly suppressed Ang II–AT_1 function, whereas apelin-activated APJ attenuated this suppression. Although Chun *et al.* never mentioned the relevance between the non-activating and the activating APJ, they demonstrated that AT_1 and APJ form receptor heterodimers in the transfected HEK293

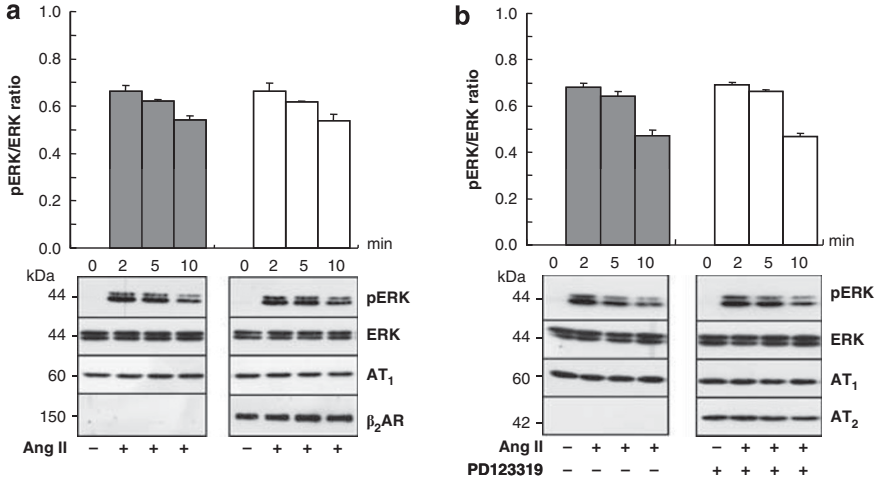

Figure 3 Crosstalk between AT_1 and other GPCRs. (**a**) HEK293 cells that expressed AT_1 alone or AT_1 plus β_2AR were stimulated by Ang II as indicated. (**b**) AT_1-expressing HEK293 cells and PD123319-pretreated AT_1- and AT_2-coexpressing HEK293 cells were stimulated by Ang II as indicated. pERK1/2, ERK1/2, AT_1, AT_2 and β_2AR were probed by western blot. Data represent the mean ± s.e.m. from three independent experiments.

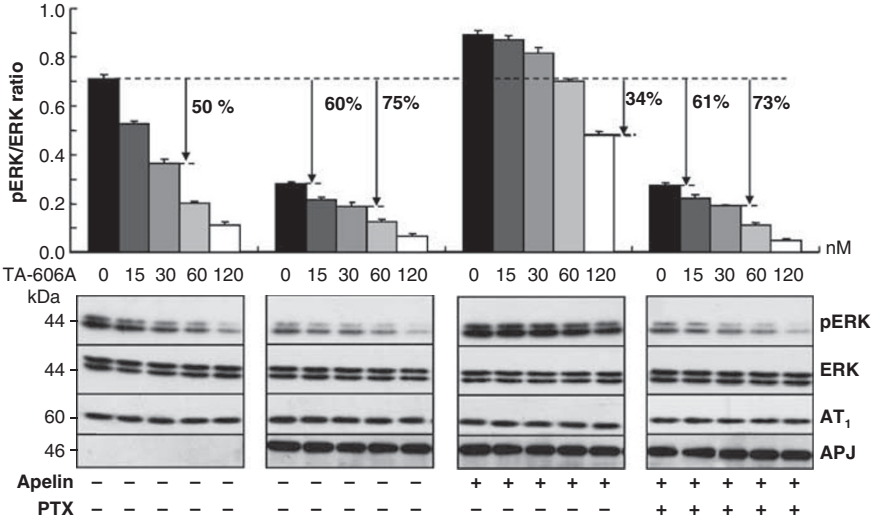

Figure 4 The relationship among apelin, APJ and ARB. HEK293 cells that expressed AT_1 alone or AT_1 plus APJ were pretreated with a designed concentration of TA-606A with or without a combination of PTX pretreatment as indicated. Next, cells were simulated by Ang II or Ang II plus apelin for 2 min. pERK1/2, ERK1/2, AT_1 and APJ were detected by western blot. Data represent the mean ± s.e.m. from three independent experiments (the decreased percentage of the ERK1/2 phosphorylation level with Ang II–AT_1 without TA-606A is indicated).

cells.[12] Most GPCRs, traditionally considered to function as monomers, actually create homodimers and heterodimers with other GPCRs. These GPCRs' oligomerization may exist in native tissues and may have important consequences to receptor functions.[19] Interestingly, apelin stimulation never altered the heterodimerization of APJ and AT_1, despite changing their function; however, we also demonstrated that APJ and AT_1 formed receptor heterodimers in the transfected HEK293 cells by immunoprecipitation. Accumulating evidences of the heterodimerization of GPCRs show that heterodimerization may change their receptor functions. Levoye *et al*. reported that the orphan GPCR, GPR50 receptor, specifically inhibits melatonin receptor type 1A function through heterodimerization independently of its ligand.[20] Occasionally agonists stabilize and/or increase the level of dimers, but they can have the opposite effect.[21,22] Gines *et al*. reported that dopamine D_1 and adenosine A_1 receptors constitutively form heterodimers in the Ltk-fibroblast cells, and the dopamine D_1 receptor agonist causes a decrease in the heterodimer level, leading to a cyclic adenosine monophosphate increase.[23] However, we need to distinguish the relationship between AT_1 and APJ from the heterodimerization of other GPCRs. Apelin stimulation may change AT_1 function through APJ without its conversion to heterodimers. Furthermore, the Gi/o inhibitor PTX was used to make a functional non-activated APJ.[24–26] PTX-induced, pharmacologically non-activated APJ could also suppress the Ang II–AT_1 signaling, as was the case with the physiologically non-activated APJ. Although the detailed mechanisms of this relationship have been studied, this study

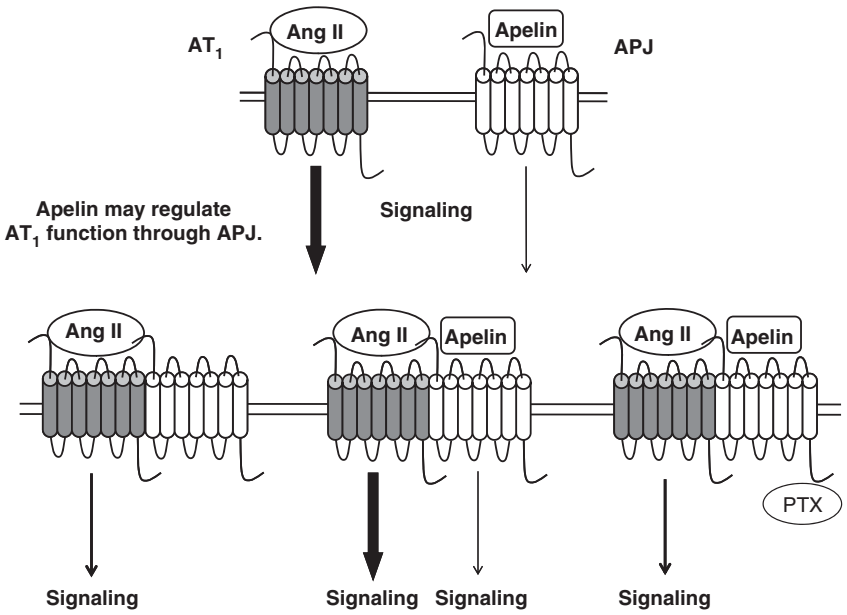

Figure 5 The relationship between APJ and AT_1. Both receptors form the heterodimers. Non-activated APJ suppresses AT_1 signaling, whereas apelin diminishes this suppression. PTX reverses apelin function.

is the first to demonstrate that the crosstalk between APJ and AT_1 was determined by whether or not they received apelin stimulation independently of their heterodimerization. These findings possibly suggest that non-activated APJ, rather than apelin–APJ signaling, had a critical role in inhibiting Ang II–AT_1. Various accumulating evidences clearly imply that apelin–APJ signaling has the dual functions of cardioprotection and pathologic progression. This study's results suggested that even if the apelin–APJ signaling elicits NO production via activation of endothelial NO synthase leading to cardioprotection, apelin-activated APJ loses Ang II–AT_1 suppression. Taken together, these evidences indicated that apelin–APJ may have dual functions through the regulation of Ang II–AT_1 (Figure 5). When NO production via apelin–APJ signaling is not enough to neutralize the non-activating APJ function that suppresses Ang II–AT_1, apelin–APJ signaling may demonstrate its pathological progressive effects. Alternatively, it may behave positively, as is the case with its protective character when there is sufficient NO production.

AT_1 was shown to have interactions with several GPCRs in a functional manner.[27–30] Barki-Harrington *et al.* reported that interactions exist between AT_1 and β_2AR, which are not affected by ligand stimulation, and a single receptor antagonist effectively blocks downstream signaling and trafficking of both receptors simultaneously.[28] In our study, the coexpression of β_2AR did not affect Ang II–AT_1 signaling. AT_2 works as an AT_1 antagonist.[29,30] However, the pharmacologically non-activated AT_2 did not affect the Ang II–AT_1 signaling either. Miura *et al.* also demonstrated that the AT_1 signaling may be mainly blocked by the AT_2 signaling through their negative crosstalk in phospholipase-β_3 phosphorylation rather than by heterodimerization of both receptors on the cell surface.[31] Collectively, the ligand-independent function of APJ in suppressing AT_1 signaling may be specific.

Role of apelin–APJ in inhibiting Ang II–AT_1 by ARB

ARBs, the effective AT_1 uncompetitive antagonists, have been proven to be safe and well tolerated for chronic use and work as a critical component of therapy for hypertension, atherosclerosis and heart diseases.[32–34] ARBs also prolong life in humans.[35,36] In the present study, the relationship among apelin, APJ and Ang II–AT_1 suggested that the non-activated APJ may work as an endogenous AT_1 antagonist, whereas apelin stimulation attenuated this suppression. Therefore, endogenously enhanced ARB efficiency through the non-activated APJ became a substantial pathway for consideration. The enhancing effect of the non-activated APJ on ARBs immediately converted to an abating effect by apelin stimulation. Consequently, our findings may signify that APJ alone but not apelin–APJ signaling may contribute to inhibit Ang II–AT_1 by ARB. Taken together, considering the antagonizing Ang II–AT_1 effect of APJ with/without apelin, the expression level of APJ and apelin in the damaged cardiovascular cells, such as the endothelial cells and VSMC, may have a potential contribution to ARB treatment in the clinical setting.

In summary, the present study has demonstrated that APJ specifically suppresses Ang II–AT_1 signaling, whereas apelin stimulation inhibits this ligand-independent function. These results suggest that the crosstalk between APJ and AT_1 may be distinguishable with or without apelin stimulation. Moreover, we showed that the similarities between apelin–APJ and Ang II–AT_1 were also reflected in ARB function, and may contribute to ARB treatment in the clinical setting.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr Makoto Endo for valuable suggestions and critical reading of the paper. This study was supported in part by the Takeda Science Foundation, the Maruki Memorial Foundation, research grant HL58205 from the NIH (to TI) and the Saitama Medical University grant (to TS).

1 O'Dowd BF, Heiber M, Chan A, Heng HH, Tsui LC, Kennedy JL, Shi X, Petronis A, George SR, Nguyen T. A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene* 1993; **136**: 355–360.

2 Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, Kawamata Y, Fukusumi S, Hinuma S, Kitada C, Kurokawa T, Onda H, Fujino M. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem Biophys Res Commun* 1998; **251**: 471–476.
3 Hosoya M, Kawamata Y, Fukusumi S, Fujii R, Habata Y, Hinuma S, Kitada C, Honda S, Kurokawa T, Onda H, Nishimura O, Fujino M. Molecular and functional characteristics of APJ. Tissue distribution of mRNA and interaction with the endogenous ligand apelin. *J Biol Chem* 2000; **275**: 21061–21067.
4 Kleinz MJ, Skepper JN, Davenport AP. Immunocytochemical localisation of the apelin receptor APJ, to human cardiomyocytes, vascular smooth muscle and endothelial cells. *Regul Pept* 2005; **126**: 233–240.
5 Kleinz MJ, Davenport AP. Emerging roles of apelin in biology and medicine. *Pharmacol Ther* 2005; **107**: 198–211.
6 Lee DK, George SR, O'Dowd BF. Unravelling the roles of the apelin system: prospective therapeutic applications in heart failure and obesity. *Trends Pharmacol Sci* 2006; **27**: 190–194.
7 Kidoya H, Naito H, Takakura N. Apelin induces enlarged and non-leaky blood vessels for functional recovery from ischemia. *Blood* 2010; **115**: 3166–3174.
8 Farkasfalvi K, Stagg MA, Coppen SR, Siedlecka U, Lee J, Soppa GK, Marczin N, Szokodi I, Yacoub MH, Terracciano CM. Direct effects of apelin on cardiomyocyte contractility and electrophysiology. *Biochem Biophys Res Commun* 2007; **357**: 889–895.
9 Japp AG, Cruden NL, Barnes G, van Gemeren N, Mathews J, Adamson J, Johnston NR, Denvir MA, Megson IL, Flapan AD, Newestern bloty DE. Acute cardiovascular effects of apelin in humans: potential role in patients with chronic heart failure. *Circulation* 2010; **121**: 1818–1827.
10 Iwanaga Y, Kihara Y, Takenaka H, Kita T. Down-regulation of cardiac apelin system in hypertrophied and failing hearts: possible role of angiotensin II-angiotensin type 1 receptor system. *J Mol Cell Cardiol* 2006; **41**: 798–806.
11 Ishida J, Hashimoto T, Hashimoto Y, Nishiwaki S, Iguchi T, Harada S, Sugaya T, Matsuzaki H, Yamamoto R, Shiota N, Okunishi H, Kihara M, Umemura S, Sugiyama F, Yagami K, Kasuya Y, Mochizuki N, Fukamizu A. Regulatory roles for APJ a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure *in vivo*. *J Biol Chem* 2004; **279**: 26274–26279.
12 Chun HJ, Ali ZA, Kojima Y, Kundu RK, Sheikh AY, Agrawal R, Zheng L, Leeper NJ, Pearl NE, Patterson AJ, Anderson JP, Tsao PS, Lenardo MJ, Ashley EA, Quertermous T. Apelin signaling antagonizes Ang II effects in mouse models of atherosclerosis. *J Clin Invest* 2008; **118**: 3343–3354.
13 Hashimoto T, Kihara M, Imai N, Yoshida S, Shimoyamada H, Yasuzaki H, Ishida J, Toya Y, Kiuchi Y, Hirawa N, Tamura K, Yazawa T, Kitamura H, Fukamizu A, Umemura S. Requirement of apelin-apelin receptor system for oxidative stress-linked atherosclerosis. *Am J Pathol* 2007; **171**: 1705–1712.
14 Liu C, Su T, Li F, Li L, Qin X, Pan W, Feng F, Chen F, Liao D, Chen L. PI3K/Akt signaling transduction pathway is involved in rat vascular smooth muscle cell proliferation induced by apelin-13. *Acta Biochim Biophys Sin* 2010; **42**: 396–402.
15 Yoshida T, Mishina M. Neuron-specific gene manipulations to transparent zebrafish embryos. *Methods Cell Sci* 2003; **25**: 15–23.
16 Kawai M, Maruyama H, Bessho K, Yamamoto H, Miyazaki J, Yamamoto T. Simple strategy for bone regeneration with a *BMP*-2/7 gene expression cassette vector. *Biochem Biophys Res Commun* 2009; **390**: 1012–1027.
17 Li Z, Zhang H, Lundin L, Thullberg M, Liu Y, Wang Y, Claesson-Welsh L, Stromblad S. p21-activated kinase 4 phosphorylation of integrin beta5 Ser-759 and Ser-762 regulates cell migration. *J Biol Chem* 2010; **285**: 23699–23710.
18 Hashimoto Y, Harada Y, Narita H, Naito K, Murata S. The pharmacologic profile of 606A, a novel angiotensin II receptor antagonist. *J Cardiovasc Pharmacol* 1997; **29**: 284–290.
19 Prinster SC, Hague C, Hall RA. Heterodimerization of g protein-coupled receptors: specificity and functional significance. *Pharmacol Rev* 2005; **57**: 289–298.
20 Levoye A, Dam J, Ayoub MA, Guillaume JL, Couturier C, Delagrange P, Jockers R. The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *EMBO J* 2006; **25**: 3012–3023.
21 Terrillon S, Bouvier M. Roles of G-protein-coupled receptor dimerization. *EMBO Rep* 2004; **5**: 30–34.
22 Rios CD, Jordan BA, Gomes I, Devi LA. G-protein-coupled receptor dimerization: modulation of receptor function. *Pharmacol Ther* 2001; **92**: 71–87.
23 Gines S, Hillion J, Torvinen M, Le Crom S, Casado V, Canela EI, Rondin S, Lew JY, Watson S, Zoli M, Agnati LF, Verniera P, Lluis C, Ferre S, Fuxe K, Franco R. Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. *Proc Natl Acad Sci USA* 2000; **97**: 8606–8611.
24 Masri B, Lahlou H, Mazarguil H, Knibiehler B, Audigier Y. Apelin (65-77) activates extracellular signal-regulated kinases via a PTX-sensitive G protein. *Biochem Biophys Res Commun* 2002; **290**: 539–545.
25 Masri B, Morin N, Pedebernade L, Knibiehler B, Audigier Y. The apelin receptor is coupled to Gi1 or Gi2 protein and is differentially desensitized by apelin fragments. *J Biol Chem* 2006; **281**: 18317–18326.
26 Hamada J, Kimura J, Ishida J, Kohda T, Morishita S, Ichihara S, Fukamizu A. Evaluation of novel cyclic analogues of apelin. *Int J Mol Med* 2008; **22**: 547–552.
27 AbdAlla S, Lother H, el Massiery A, Quitterer U. Increased AT(1) receptor heterodimers in preeclampsia mediate enhanced angiotensin II responsiveness. *Nat Med* 2001; **7**: 1003–1009.
28 Barki-Harrington L, Luttrell LM, Rockman HA. Dual inhibition of beta-adrenergic and angiotensin II receptors by a single antagonist: a functional role for receptor-receptor interaction *in vivo*. *Circulation* 2003; **108**: 1611–1618.
29 AbdAlla S, Lother H, Abdel-tawab AM, Quitterer U. The angiotensin II AT2 receptor is an AT1 receptor antagonist. *J Biol Chem* 2001; **276**: 39721–39726.
30 Horiuchi M, Hayashida W, Akishita M, Tamura K, Daviet L, Lehtonen JY, Dzau VJ. Stimulation of different subtypes of angiotensin II receptors AT1 and AT2 receptors, regulates STAT activation by negative crosstalk. *Circ Res* 1999; **84**: 876–882.
31 Miura S, Matsuo Y, Kiya Y, Karnik SS, Saku K. Molecular mechanisms of the antagonistic action between AT1 and AT2 receptors. *Biochem Biophys Res Commun* 2010; **391**: 85–90.
32 Eberhardt RT, Kevak RM, Kang PM, Frishman WH. Angiotensin II receptor blockade: an innovative approach to cardiovascular pharmacotherapy. *J Clin Pharmacol* 1993; **33**: 1023–1038.
33 Israili ZH. Clinical pharmacokinetics of angiotensin II (AT1) receptor blockers in hypertension. *J Hum Hypertens* 2000; **14** (Suppl 1): S73–S86.
34 Wassmann S, Czech T, van Eickels M, Fleming I, Böhm M, Nickenig G. Inhibition of diet-induced atherosclerosis and endothelial dysfunction in apolipoprotein E/angiotensin II type 1A receptor double-knockout mice. *Circulation* 2004; **110**: 3062–3067.
35 Benigni A, Corna D, Zoja C, Sonzogni A, Latini R, Salio M, Conti S, Rottoli D, Longaretti L, Cassis P, Morigi M, Coffman TM, Remuzzi G. Disruption of the Ang II type 1 receptor promotes longevity in mice. *J Clin Invest* 2009; **119**: 524–530.
36 Cassis P, Conti S, Remuzzi G, Benigni A. Angiotensin receptors as determinants of life span. *Pflugers Arch* 2010; **459**: 325–332.