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Invited Review for the 2009 Hirosi Kuriyama Award

Regulation of vascular tone and remodeling of the ductus arteriosus

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Received January 6, 2010; Accepted January 10, 2010

Abstract

The ductus arteriosus (DA), a fetal arterial connection between the main pulmonary artery and the descending aorta, normally closes immediately after birth. The DA is a normal and essential fetal structure. However, it becomes abnormal if it remains patent after birth. Closure of the DA occurs in two phases: functional closure of the lumen within the first hours after birth by smooth muscle constriction, and anatomic occlusion of the lumen over the next several days due to extensive neointimal thickening in human DA. There are several events that promote the DA constriction immediately after birth: (a) an increase in arterial oxygen tension, (b) a dramatic decline in circulating prostaglandinE_2 (PGE_2), (c) a decrease in blood pressure within the DA lumen, and (d) a decrease in the number of PGE_2 receptors in the DA wall. Anatomical closure of the DA is associated with the formation of intimal thickening, which are characterized by (a) an area of subendothelial deposition of extracellular matrix, (b) the disassembly of the internal elastic lamina and loss of elastic fiber in the medial layer, and (c) migration into the subendothelial space of undifferentiated medial smooth muscle cells. In addition to the well-known vasodilatory role of PGE_2, our findings uncovered the role of PGE_2 in anatomical closure of the DA. Chronic PGE_2-EP4-cyclic AMP (cAMP)-protein kinase A (PKA) signaling during gestation induces vascular remodeling of the DA to promote hyaluronan-mediated intimal thickening and structural closure of the vascular lumen. A novel target of cAMP, Epac, has an acute promoting effect on smooth muscle cell migration without hyaluronan production and thus intimal thickening in the DA. Both EP4-cAMP downstream targets, Epac and PKA, regulate vascular remodeling in the DA.

Key words: cyclic AMP, exchange protein activated by cyclic AMP (EPAC), protein kinase A, intimal thickening, the ductus arteriosus, vascular remodeling, hyaluronan

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UY was the winner of the Hirosi Kuriyama Award.

Introduction

The ductus arteriosus (DA), a fetal arterial connection between the pulmonary artery and the descending aorta, is indispensable for fetal life. It shunts deoxygenated blood from the main pulmonary artery to the descending aorta. Over half of the blood flow in the descending aorta is diverted to the umbilico-placental circulation (Heymann and Rudolph, 1975), where gaseous exchange takes place. Although patency of the DA is required for fetal survival, the persistence of a patent DA after birth is a major cause of morbidity and mortality, especially in premature infants, leading to severe complications, including pulmonary hypertension, right ventricular dysfunction, postnatal infections and respiratory failure (Hermes-DeSantis and Clyman, 2006). The incidence of DA patency has been estimated to be one in 500 in term newborns and accounts for the majority of all cases of congenital heart diseases in preterm newborns (Mitchell *et al.*, 1971). In preterm babies with birth weights <1,500 g, the incidence of a patent DA exceeds 30% (Van Overmeire *et al.*, 2004). In addition, the presence of a patent DA is more serious in premature infants than in full-term infants since premature infants with a patent DA are more likely to develop problems such as intraventricular hemorrhage, necrotizing enterocolitis, bronchopulmonary dysplasia and congestive heart failure. Therefore, it is important to understand the precise mechanisms of regulation of the DA.

Closure of the human DA is believed to occur in two phases: (1) functional closure of the lumen within the first hours after birth by smooth muscle constriction, and (2) anatomic occlusion of the lumen over the next several days due to extensive neointimal thickening and loss of smooth muscle cells from the inner muscle media (Smith, 1998; Clyman, 2006; Yokoyama *et al.*, 2006b). Although its process is similar in mammalian DA, the time course of two phases is variable among species. There are several events that promote the DA constriction immediately after birth: (a) an increase in arterial oxygen tension, (b) a dramatic decline in circulating prostaglandinE_2 (PGE_2) because of metabolism in the now functioning lungs and elimination of the placental source, (c) a decrease in blood pressure within the DA lumen, and (d) a decrease in the number of PGE_2 receptors in the DA wall (Smith, 1998; Clyman, 2006).

The DA later undergoes permanent closure through structural remodeling and fibrosis. The resulting fibrous band with no lumen persists as the ligamentum arteriosum (Fay and Cooke, 1972). Anatomical closure of the DA is associated with the formation of intimal thickening, which are characterized by (a) an area of subendothelial deposition of extracellular matrix, (b) the disassembly of the internal elastic lamina and loss of elastic fiber in the medial layer, and (c) migration into the subendothelial space of undifferentiated medial smooth muscle cells (Smith, 1998). Some of these changes begin about halfway through gestation and some occur after functional closure of the DA in the neonate (Slomp *et al.*, 1997; Yokoyama *et al.*, 2006b). This cascade of events is thought to orchestrate the subsequent luminal DA reorganization, leading finally to complete obliteration of the DA. In this report, we review the current state of knowledge of the mechanisms of regulating vascular tone and remodeling of the DA.

1. Functional closure of the DA

1-1. Oxygen-induced contraction

During the fetal life, the DA is exposed to an oxygen tension that has been estimated as between 18 to 28 mmHg (Heymann and Rudolph, 1975). After birth, the DA is exposed to arterial blood and arterial oxygen tension rises rapidly after delivery. Rising oxygen tension significantly contracts the DA (Smith, 1998). With the exception of the pulmonary artery, most vascular smooth muscles relax in a low oxygen environment and contract in response to increasing oxygen tension. However, the response of the DA to oxygen is much greater in magnitude, although qualitatively similar to other vessels (Heymann and Rudolph, 1975; Smith and McGrath, 1988).

Several mechanisms have been proposed to explain the contractile effect of raising oxygen tension in the DA. The increase in oxygen tension inhibits ductal smooth muscle voltage-dependent potassium channels (Michelakis *et al.*, 2000; Reeve *et al.*, 2001), such as Kv1.5 and Kv2.1, which results in membrane depolarization, an influx of calcium and DA constriction (Nakanishi *et al.*, 1993; Leonhardt *et al.*, 2003). The inhibition of potassium channels is associated with production of diffusible redox mediator (H_2O_2) by a mitochondrial O_2-sensor, electron transport chain complexes I or III in the DA (Archer *et al.*, 2004). It has been reported that ATP-sensitive potassium channel was inhibited by the raising oxygen tension, resulting in membrane depolarization (Nakanishi *et al.*, 1993). In addition to involvement of potassium channels, recent study reported that depolarization-independent DA contraction is caused by release of calcium from the IP_3-sensitive store in the sarcoplasmic reticulum. Subsequent calcium entry through store-operated channels increases an influx of calcium and DA constriction (Hong *et al.*, 2006). Schematic illustration of the functional closure of the DA is shown in Figure 1.

Calcium entry trough L-type voltage-dependent calcium channels is involved in oxygen-induced DA contraction (Tristani-Firouzi *et al.*, 1996). Our previous study demonstrated that among L-type calcium channels, Cav1.2 was predominant isoform and expression level of Cav1.2 was higher in the rat DA than in the aorta (Yokoyama *et al.*, 2006a). Calcium influx through T-type voltage-dependent calcium channels, especially Cav3.1, also promoted oxygenation-induced DA constriction (Nakanishi *et al.*, 1993; Akaike *et al.*, 2009).

Coceani *et al.* reported that cytochrome P_{450} was the oxygen sensor and its activation promoted DA contraction through production of endothelin-1 (ET-1) from endothelium and smooth muscle of the DA (Coceani and Kelsey, 1991; Coceani *et al.*, 1992). They demonstrated stimulation of endothelin A receptor (ET_A) is associated with oxygen-induced DA contraction using mice with genetic disruption of ET_A (Coceani *et al.*, 1999). However, the DA was normally closed after birth in ET_A knockout mice. Consequently, ET-1- ET_A signaling plays a role in oxygen-induced contraction, but not in anatomical closure in the DA.

Vitamin A and/or retinoic acid signaling is a candidate for the activator of oxygen sensitivity, because the retinoic acid response element is strongly expressed in the mouse DA (Colbert *et al.*, 1996), and maternally administrated vitamin A accelerated development of the oxygen-sensing mechanism of the rat DA (Wu *et al.*, 2001). Our study demonstrated that

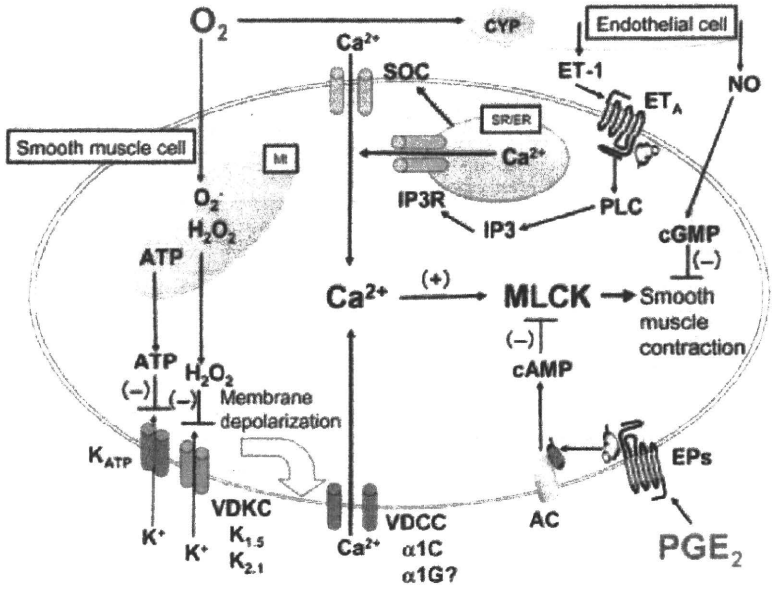

Fig. 1. A schematic model of functional closure of the DA. K^+: potassium ion, Ca^{2+}: calcium ion, O_2: oxygen, O_2^-: superoxide anion, H_2O_2: hydrogen peroxide, ATP: adenosine triphosphate, K_{ATP}: ATP-dependent potassium channel, VDCK: voltage-dependent potassium channel, VDCC: voltage-dependent calcium channel, SOC: store-operated calcium channel, Mt: mitochondria, SR/ER: sarco/endoplasmic reticulum, IP3: inositol triphosphate, IP3R: IP3 receptor, PLC: phopholipase C, MLCK: myosin light chain kinase, CYP: cytochrome P_{450}.

maternally administrated vitamin A increased the expression levels of Cav1.2 and Cav3.1 in the rat DA (Yokoyama *et al.*, 2006a).

1-2. Rapid withdrawal of the vasodilator effect of PGE_2

PGE_2 is produced in the placenta (Smith, 1998) and the DA (Clyman *et al.*, 1978; Coceani *et al.*, 1978) and contributes to the DA patency in utero. Stimulation of PGE_2 receptors activates adenylyl cyclases (Bouayad *et al.*, 2001). The increased intracellular concentrations of cyclic AMP (cAMP) inhibit myosin light chain kinase, resulting in the DA relaxation (Smith, 1998). The dilator effect of PGE_2 on the rabbit DA was mediated by the PGE_2 receptor, EP4 (Smith and McGrath, 1994). After birth, the high fetal circulating concentrations of PGE_2 dramatically decline because the placenta is removed and the lung promotes catabolism of PGE_2 (Smith, 1998). Further, the expressions of PGE_2 receptors were decreased in the DA wall (Smith, 1998; Clyman, 2006).

Both isoforms of the enzyme responsible for synthesizing PGE_2, cyclooxygenase (COX)-1 and COX-2, are expressed in the fetal DA (Takahashi *et al.*, 2000). Since COX-2 expression in the fetal DA significantly increased with advancing gestational age (Trivedi *et al.*, 2006), COX-2 inhibitor-induced DA contraction is weaker in preterm rats on the 19th day of gestation than in near-term on the 21st day (Toyoshima *et al.*, 2006). A COX inhibitor is widely used for the patent DA, however, this may not be a better therapy for premature infants with patent DA.

1-3. Other factors mediating contraction of the DA

It has been reported that nitric oxide (NO) plays a role in vasodilation of the DA. NO is synthesized by endothelial nitric oxide synthase (eNOS) in the luminal endothelium and the vasavasorum endothelium and induces relaxation of the DA through cyclic GMP (cGMP) signaling (Clyman, 2006). The relative importance of the cAMP and cGMP has been studied. Adenylyl cyclase stimulator, forskolin completely reversed the combined contractile effects of elevated oxygen tension, norepinephrin and COX inhibitor, whereas inhibition of cGMP signaling by sodium nitroprusside caused 4% of the effect of forskolin (Smith and McGrath, 1993). This implies that cAMP signaling is more important than cGMP signaling in near-term DA. On the other hand, in premature DA, the combined use of an NO synthase-inhibitor and COX inhibitor produces a much greater degree of the DA contraction than COX inhibitor alone (Seidner *et al.*, 2001).

2. Anatomical closure of the DA

2-1. Histological change during perinatal period

After birth, there is extensive remodeling of the DA wall, which leads to permanent closure of the DA. Intimal thickening, a characteristic developmental remodeling process in the DA, is required for postnatal DA closure (Rabinovitch, 1996; Mason *et al.*, 1999; Yokoyama *et al.*, 2006b). Intimal thickening starts with lifting of the endothelial cells (Gittenberger-de Groot *et al.*, 1985) and accumulations of hyaluronan in the subendothelial region, creating a space that is suitable for migration of smooth muscle cells through the fragmented elastic lamina into the subentothelial region (De Reeder *et al.*, 1988). Figure 2 shows histological change of the rat DA. Intimal thickening is developed in mature rat DA on the 21st day of gestation, while it is lacked in immature DA on the19th day of gestation. Since intimal thickening is poorly developed in human patent DA patients and animal models of patent DA (Gittenberger-de Groot *et al.*, 1980; Gittenberger-de Groot *et al.*, 1985; Tada *et al.*, 1985), this process plays an important role in permanent closure of the DA after birth.

2-2. Molecular mechanisms of regulating intimal thickening

PGE_2 plays a primary role in maintaining the patency of DA, however, previous studies have demonstrated that genetic disruption of the PGE receptor EP4 paradoxically results in fatal patent DA in mice (Nguyen *et al.*, 1997; Segi *et al.*, 1998). In addition, double mutant mice in which COX-1 and COX-2 are disrupted also exhibit patent DA (Loftin *et al.*, 2001). We found that intimal thickening was completely absent in the DA from EP4-disrupted neonatal mice (Yokoyama *et al.*, 2006b). Moreover, a marked reduction in hyaluronan production was found in EP4-disrupted DA, whereas a thick layer of hyaluronan deposit was present in wile-type DA. PGE_2-EP4-cAMP-protein kinase A (PKA) signaling up-regulates hyaluronan synthase type 2 mRNA, which increases hyaluronan production in the DA. Accumulation of hyaluronan then promotes smooth muscle cell migration into the subendothelial layer to form intimal thickening (Yokoyama *et al.*, 2006b). Signals through PGE_2-EP4 have two essential roles in DA development, namely, vascular dilation and intimal thickening.

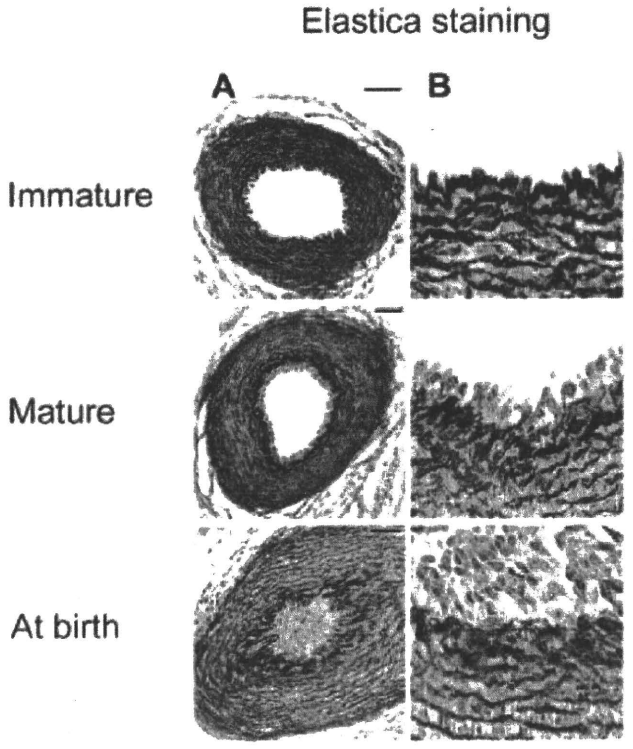

Fig. 2. Developmental changes in intimal thickening in rat DA. (A) Elastica van Gieson staining shows that intimal thickening was poor on the 19th day of gestation (immature), whereas it became apparent on the 21st day of gestation (mature) and 4 hours after birth (at birth). (B) DA intimal thickening are also shown at higher magnification.

A new target of cAMP, *i.e.*, an exchange protein activated by cAMP, has recently been discovered; it is called Epac (de Rooij *et al.*, 1998). Epac has been known to exhibit a distinct cAMP signaling pathway that is independent of PKA (Bos, 2003). Our previous study demonstrated that Epac, which is up-regulated during the perinatal period, had an acute promoting effect on smooth muscle cell migration without hyaluronan production and thus intimal thickening in the DA (Yokoyama *et al.*, 2008). Therefore, both EP4-cAMP downstream targets, Epac and PKA, induced intimal thickening in the DA (Fig. 3).

T-type voltage-dependent calcium channels, especially Cav3.1, promoted oxygenation-induced DA constriction (Akaike *et al.*, 2009). Our study revealed that Cav3.1 was significantly up-regulated in oxygenated rat DA tissue and in the region of intimal thickening of DA and that Cav1.3 promoted smooth muscle cell migration. These results indicate that Cav1.3 promotes oxygenation-induced DA closure through smooth muscle cell migration and vasoconstriction in rats (Akaike *et al.*, 2009). We also found that a novel spliced variant of the alpha1C-subunit was highly expressed in the neointima cushion of the DA (Yokoyama *et al.*, 2006a), although a role of the novel isoform is needed to be studied.

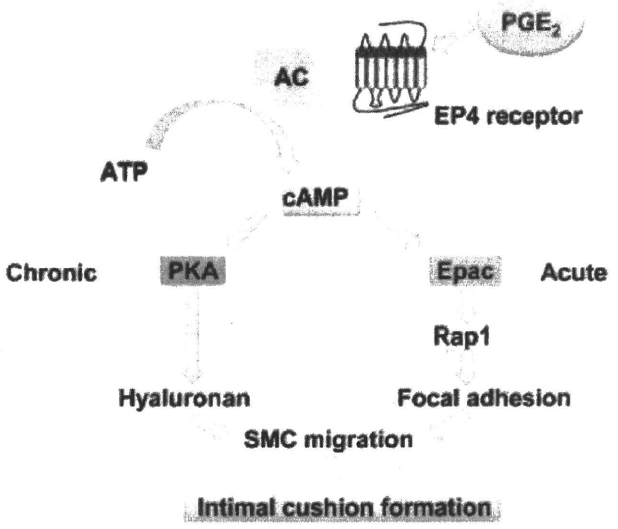

Fig. 3. A schematic model of the diverse cAMP signaling pathway. Both Epac and PKA synergistically promoted intimal cushion formation in the DA, but they work in two distinct ways: Epac-mediated (acute) and PKA-mediated (chronic) promotion. AC: adenylyl cyclase.

2-3. Extracellular matrix in the DA

Vascular cells are defined by the ways in which they regulate their extracellular matrix, and changes in the extracellular matrix, in turn, determine vascular cell phenotype, *i.e.*, the ability to differentiate, proliferate, and migrate (Rabinovitch, 1996). It has been reported that DA smooth muscle cells produce two-fold more fibronectin than aortic smooth muscle cells (Rabinovitch, 1996). Mason *et al.* have demonstrated that preventing fibronectin-dependent intimal thickening would be a feasible manipulation to cause patent DA as a mode of treatment of congenital heart diseases (Mason *et al.*, 1999). Our previous study also demonstrated that maternally administrated viamin A increased fibronectin production and intimal thickening in the rat DA (Yokoyama *et al.*, 2007). Transforming growth factor beta and NO induce extracellular matrix including hyaluronan and fibronectin in DA smooth muscle cell (Rabinovitch, 1996).

Versican, an hyaluronan binding proteoglycan, plays an important role in proliferation and migration of vascular smooth muscle cells (Evanko *et al.*, 1999). Tanacin, a hexameric glycoprotein, also has been known to regulate vascular smooth muscle cell proliferation (Cowan *et al.*, 2000). Therefore, fibronectin and hyaluronan may not be the sole constituents of extracellular matrix in DA intimal thickening.

Closing remarks

The ductal closure occurs in two phases. During first few hours after birth in term newborns, there is acute and functional closure as a result of smooth muscle contraction of the

DA, which is triggered by an increase in oxygen tension and a decline in levels of circulating PGE_2. Importantly, prior to this, anatomical luminal narrowing develops through intimal thickening that occludes the vascular lumen and results in permanent closure after birth. Both PGE_2-EP4-cAMP downstream targets, Epac and PKA, induce intimal thickening in the DA. Chronic activation of EP4 induces hyaluronan-mediated smooth muscle cell migration via PKA, resulting in intimal thickening. Epac has an acute promoting effect on smooth muscle cell migration without hyaluronan production and thus intimal thickening in the DA.

Acknowledgements

This work was supported by grants from the Grant-in-Aid for Scientific Research (KAKENHI) (U.Y.), the Ministry of Health Labor and Welfare (S.M., Y.I.), the Ministry of Education, Culture, Sports, Science and Technology of Japan (S.M., Y.I.), the Yokohama Foundation for Advanced Medical Science (U.Y., S.M.), the Kanae Foundation for the Promotion for Medical Science (U.Y.), the Miyata Cardiology Research Promotion Founds (U.Y., S.M.), the Takeda Science Foundation (U.Y., S.M.), the Sumitomo Foundation (U.Y.), the Japan Heart Foundation Research Grant (U.Y.), the Kowa Life Science Foundation (U.Y.), the Cosmetology Research Foundation (Y.I.), the Uehara Memorial Foundation (U.Y.), the Kitsuen Research Foundation (Y.I.), "High-Tech Research Center" Project for Private Universities: MEXT (S.M.), Waseda University Grant for Special Research Projects (S.M.), The Vehicle Racing Commemorative Foundation (S.M.), and the Japan Space Forum (Y.I.).

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Hypertension
Journal of the American Heart Association

Cardiac-Specific Activation of Angiotensin II Type 1 Receptor Associated Protein Completely Suppresses Cardiac Hypertrophy in Chronic Angiotensin II Infused Mice

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Hypertension 2010;55;1157-1164; originally published online Mar 15, 2010;
DOI: 10.1161/HYPERTENSIONAHA.109.147207
Hypertension is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0194-911X. Online ISSN: 1524-4563

Cardiac-Specific Activation of Angiotensin II Type 1 Receptor–Associated Protein Completely Suppresses Cardiac Hypertrophy in Chronic Angiotensin II–Infused Mice

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Abstract—We cloned a novel molecule interacting with angiotensin II type 1 receptor, which we named ATRAP (for angiotensin II type 1 receptor–associated protein). Previous in vitro studies showed that ATRAP significantly promotes constitutive internalization of the angiotensin II type 1 receptor and further attenuates angiotensin II–mediated hypertrophic responses in cardiomyocytes. The present study was designed to investigate the putative functional role of ATRAP in cardiac hypertrophy by angiotensin II infusion in vivo. We first examined the effect of angiotensin II infusion on endogenous ATRAP expression in the heart of C57BL/6J wild-type mice. The angiotensin II treatment promoted cardiac hypertrophy, concomitant with a significant decrease in cardiac ATRAP expression, but without significant change in cardiac angiotensin II type 1 receptor expression. We hypothesized that a downregulation of the cardiac ATRAP to angiotensin II type 1 receptor ratio is involved in the pathogenesis of cardiac hypertrophy. To examine this hypothesis, we next generated transgenic mice expressing ATRAP specifically in cardiomyocytes under control of the α-myosin heavy chain promoter. In cardiac-specific ATRAP transgenic mice, the development of cardiac hypertrophy, activation of p38 mitogen-activated protein kinase, and expression of hypertrophy-related genes in the context of angiotensin II treatment were completely suppressed, in spite of there being no significant difference in blood pressure on radiotelemetry between the transgenic mice and littermate control mice. These results demonstrate that cardiomyocyte-specific overexpression of ATRAP in vivo abolishes the cardiac hypertrophy provoked by chronic angiotensin II infusion, thereby suggesting ATRAP to be a novel therapeutic target in cardiac hypertrophy. (***Hypertension.*** **2010;55:1157-1164.)**

Key Words: basic science ■ receptors ■ gene expression/regulation ■ hypertrophy/remodeling ■ angiotensin receptors

Evidence suggests that the activation of angiotensin II (Ang II) type 1 receptor (AT_1R) through the tissue renin-angiotensin system may play an important role in the development of cardiac hypertrophy. The carboxyl-terminal portion of AT_1R is involved in the control of AT_1R internalization independent of G protein coupling, and it plays an important role in linking receptor-mediated signal transduction to the specific biological response to Ang II.[1,2]

We previously cloned a novel AT_1R-associated protein (ATRAP) that specifically interacts with the carboxyl-terminal domain of AT_1R.[3–6] We showed that ATRAP is broadly expressed in many tissues, as is AT_1R, and suppresses Ang II–mediated pathological responses in cardiomyocytes and vascular smooth muscle cells by promoting the constitutive internalization of AT_1R.[7–9] However, the function of ATRAP in cardiac hypertrophy in vivo still remains to be demonstrated. Thus, the present study was carried out to investigate whether there is a role for ATRAP in the cardiac hypertrophy induced by chronic Ang II treatment in vivo. We first examined an effect of chronic Ang II infusion on endogenous cardiac expression of ATRAP in C57BL/6J wild-type (Wt) mice. Next, we examined whether cardiac ATRAP attenuates the pathological hypertrophic response provoked by chronic Ang II infusion using transgenic (Tg) mice with cardiomyocyte-specific overexpression of ATRAP.

Materials and Methods

This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All of the animal studies were reviewed and approved by the animal studies committee of Yokohama City University.

Received November 4, 2009; first decision November 23, 2009; revision accepted February 11, 2010.

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Hypertension **is available at http://hyper.ahajournals.org** **DOI: 10.1161/HYPERTENSIONAHA.109.147207**

Animals and Treatment

Male Wt mice were purchased from Charles River Laboratories. Tg mice expressing the ATRAP specifically in cardiomyocytes were generated on a C57BL/6J background using standard techniques. Littermates genotyped as Wt were used as the littermate control (LC) mice in this study. Mice aged 8 to 12 weeks were used in the present study.

Ang II (200 ng/kg per minute) or vehicle was continuously infused into mice subcutaneously via an osmotic minipump (model 1003D, 2001, 2001D, 2002; ALZET) for 0, 15, 30, and 60 minutes and 3 and 14 days. Olmesartan (RNH6270) was provided by Sankyo Pharmaceuticals. It was dissolved in drinking water for oral administration and given to Wt mice for 2 weeks. The olmesartan dosage (10 mg/kg per day) was determined from previous reports.[10]

Blood Pressure Measurements by Tail-Cuff Method and Echocardiography

Systolic blood pressure (BP; SBP) and heart rate were measured indirectly by the tail-cuff method (BP-monitor MK-2000; Muromachi Kikai Co), as described.[7,11] Under anesthesia with an intraperitoneal injection of Avertin, transthoracic echocardiography was performed with an echo cardiographic system equipped with a 12.0-MHz phase-array transducer (Aplio SSA-700A; Toshiba), as described previously.[12,13] Left ventricular (LV) diameter, wall thickness, and the ejection fraction were measured using M-mode tracings and averaged for 3 cycles.

BP Measurements by Radiotelemetry

Direct BP measurement was performed by a radiotelemetric method in which a BP transducer (PA-C10, Data Sciences International) was inserted into the left carotid artery. Ten days after transplantation, each mouse was housed individually in a standard cage on a receiver under a 12-hour light-dark cycle. Direct BP was recorded every minute by radiotelemetry, as described previously.[14]

Western Blot Analysis of ATRAP and AT_1R

The characterization and specificity of the antimouse ATRAP antibody was described previously.[7–9] The anti-AT_1R antibody (sc-1173, lot E2508) was purchased from Santa Cruz Biotechnology, Inc.[7] To examine the specificity of the antibody, an AT_1R-selective blocking peptide (sc-1173p) was used. Western blot showed a single protein band of ≈42 kDa, which was abolished by an AT_1R-selective blocking peptide (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). Western blot analysis was performed as described previously.[7,8] Briefly, tissue extracts were used for electrophoresis, and membranes (Millipore) were incubated with an anti-ATRAP antibody or an anti-AT_1R antibody and subjected to enhanced chemiluminescence (Amersham Biosciences). The images were analyzed quantitatively using FUJI LAS3000 Image Analyzer (FUJI Film) for determination of the ATRAP and AT_1R protein levels. To measure the cardiac expression ratio of ATRAP/AT_1R, each ATRAP protein level was divided by the corresponding AT_1R protein level obtained by reprobing and, thus, derived from the same extract.

Histological Analysis

After 2 weeks of vehicle or Ang II infusion, both the LC and Tg mice hearts were cleared by perfusion with PBS at 70 mm Hg through the coronary arteries and then fixed by perfusion with 4% paraformaldehyde. Tissue sections were stained with hematoxylin/eosin and immunohistochemical antibody (antidystrophin monoclonal antibody, Novocastra) for cell size measurement, because this antibody binds to myocardial cellular membranes. Cross-sectional area of cardiomyocytes in the LV free wall was measured digitally using Image-Pro Plus software, as described previously.[15]

Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted from the LV with ISOGEN (Nippon Gene), and cDNA was synthesized using the SuperScript III First-Strand System (Invitrogen). Real-time quantitative RT-PCR was performed by incubating the reverse transcription product with TaqMan PCR Master Mix and a designed TaqMan probe (Applied Biosystems).[11] RNA quantity was expressed relative to the 18S rRNA control.

Determination of Mitogen-Activated Protein Kinase Activity

Western blot analysis was performed for phosphorylated p38, extracellular signal-regulated protein kinase 1/2 (ERK), and c-Jun N-terminal kinase (JNK) using antiphospho-p38 antibody (V1211, Promega), antiphospho-ERK antibody (4370, Cell Signaling Technology), and antiphospho-JNK antibody (4668, Cell Signaling Technology), which recognize only activated p38, ERK1/2, and JNK, respectively, as described previously.[8] To detect total p38, ERK, and JNK, the anti-p38 mitogen-activated protein kinase (MAPK) antibody (sc-728, Santa Cruz Biotechnology), anti-ERK antibody (4695, Cell Signaling Technology), and anti-JNK antibody (sc-571, Santa Cruz Biotechnology), were used.

Statistical Analysis

For the statistical analysis of differences among groups, unpaired Student t test or ANOVA followed by Scheffe F test was used. All of the quantitative data are expressed as mean±SE. Values of $P<0.05$ were considered statistically significant.

Results

Effects of Ang II Infusion on Cardiac Hypertrophy in Wt Mice

In the first experiment, age-matched Wt mice were divided into 3 groups: (1) a vehicle-infused group; (2) an Ang II (200 ng/kg per minute)–infused group without ARB treatment; and (3) an Ang II (200 ng/kg per minute)–infused group with ARB treatment. Ang II infusion significantly increased diastolic intraventricular septum and diastolic LV posterior wall thickness, as estimated by echocardiography and heart weight (HW)/body weight (BW) ratio, and these hypertrophic responses to Ang II treatment were completely prevented by angiotensin receptor blocker (ARB) treatment (Table 1). Ang II infusion also increased cardiac hypertrophy-related gene

Table 1. BP, BW, Heart Rate, Tissue Weight, and Echocardiographic Measurements 14 Days After Ang II Infusion in Wt Mice

Variable	Vehicle	Ang II	Ang II+ARB
SBP, mm Hg	112±6	120±5	110±7
BW, g	27.0±0.6	27.0±0.4	26.9±0.4
HR, bpm	685±19	693±32	715±22
HW/BW, mg/g	4.05±0.05	4.58±0.13*	3.94±0.06
KW/BW, mg/g	5.59±0.07	5.80±0.10	5.85±0.18
Echocardiography			
IVSd, mm	0.60±0.01	0.75±0.03*	0.58±0.02
LVPWd, mm	0.58±0.01	0.75±0.05*	0.60±0.02
LVEDD, mm	3.96±0.07	3.78±0.12	3.95±0.12
LVESD, mm	2.70±0.06	2.43±0.12	2.81±0.12
EF, %	68.4±0.9	73.3±3.1	65.6±2.9

HR indicates heart rate; IVSd, intraventricular septum, diastolic; LVPWd, left ventricular posterior wall, diastolic; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; EF, ejection fraction. All of the values are mean±SE (n=6 to 8).

*$P<0.05$ vs vehicle group.

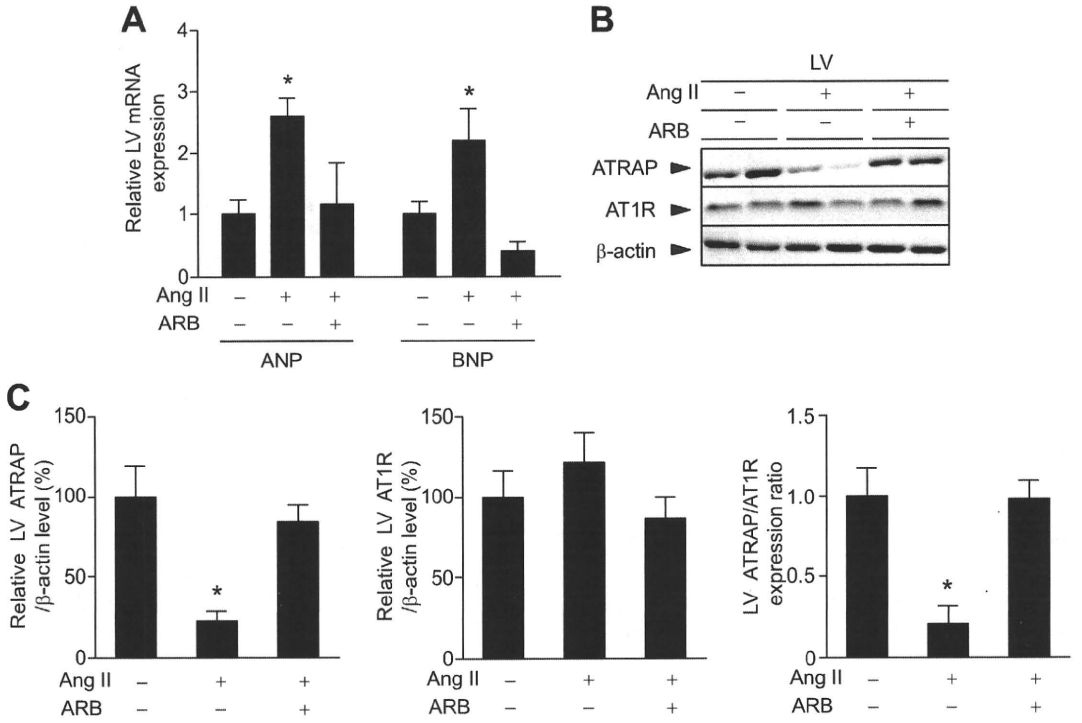

Figure 1. Expression of cardiac hypertrophy-related mRNAs and cardiac ATRAP and AT_1R proteins by Ang II infusion into Wt mice. A, Effects of Ang II infusion and the AT_1R antagonist olmesartan (ARB) on cardiac atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) mRNA expression in Wt mice. Values are calculated relative to those achieved with extracts in the vehicle-infused group (Ang II and ARB) and expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs vehicle. B, Representative Western blot analysis of the effects of Ang II infusion and the AT_1R antagonist olmesartan (ARB) on cardiac ATRAP and AT_1R protein expression in Wt mice. C, Quantitative analysis of the effects of Ang II infusion and the AT_1R antagonist olmesartan (ARB) on cardiac ATRAP and AT_1R protein expression in Wt mice. Values are calculated relative to those achieved with extracts in the vehicle-infused group (Ang II and ARB) and expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs vehicle.

expression in Wt mice (Figure 1A). LV mRNA levels of atrial natriuretic peptide (ANP) and brain natriuretic peptide were increased 2.2- and 2.6-fold by Ang II infusion, respectively, and the mRNA upregulation of these peptides was abolished by ARB treatment.

Effects of Ang II Infusion on Cardiac ATRAP and AT_1R Expression in Wt Mice

We also examined the effects of Ang II infusion on endogenous ATRAP and AT_1R protein expression in the hearts of Wt mice. With respect to the regulation of cardiac AT_1R

Table 2. BP, BW, Heart Rate, Tissue Weight, and Echocardiographic Measurements 14 Days After Ang II Infusion in LC, Tg46, and Tg52 Mice

	LC		Tg46		Tg52	
Variable	Vehicle	Ang II	Vehicle	Ang II	Vehicle	Ang II
BW, g	23.5±0.4	23.8±0.9	24.5±0.9	23.7±0.6	24.1±1.0	24.7±0.6
HW/BW, mg/g	4.15±0.11	4.79±0.12*	4.19±0.21	4.24±0.13	4.17±0.15	3.97±0.13
KW/BW, mg/g	5.88±0.27	5.85±0.23	5.40±0.23	5.89±0.26	5.75±0.17	5.69±0.06
Echocardiography						
IVSd, mm	0.56±0.02	0.75±0.03*	0.61±0.02	0.63±0.03	0.64±0.05	0.67±0.02
LVPWd, mm	0.59±0.02	0.74±0.07*	0.64±0.03	0.61±0.03	0.63±0.05	0.63±0.02
LVEDD, mm	4.05±0.12	4.0±0.15	3.85±0.15	3.86±0.13	4.18±0.60	4.25±0.11
LVESD, mm	2.64±0.09	2.65±0.21	2.42±0.17	2.56±0.12	2.95±0.10	2.99±0.11
EF, %	68.4±0.9	67.5±1.6	71.3±2.9	70.7±1.8	65.8±1.9	63.8±1.7

IVSd indicates intraventricular septum, diastolic; LVPWd, left ventricular posterior wall, diastolic; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; EF, ejection fraction. All of the values are mean±SE (n=6 to 8).

*$P<0.05$ vs vehicle-infused LC mice.

expression by Ang II infusion, previous studies reported increased, decreased, or unaltered cardiac AT_1R levels after Ang II infusion, probably because of differences in the dose and duration of the Ang II infusion.[16–18] In the present study, treatment with Ang II did not affect cardiac AT_1R protein or mRNA levels (Figure 1B and 1C and data not shown). On the other hand, Ang II infusion significantly decreased the cardiac ATRAP protein level, thereby resulting in downregulation of the cardiac ATRAP/AT_1R ratio (Figure 1B and 1C). However, ARB treatment by olmesartan (10 mg/kg per day) recovered the cardiac expression ratio of ATRAP to AT_1R so as to be comparable with the vehicle-infused group.

Effects of Ang II Infusion on Cardiac Hypertrophy in Cardiac-Specific ATRAP Tg Mice

Because chronic Ang II treatment significantly decreased the endogenous ATRAP expression in the heart concomitant with the development of cardiac hypertrophy in Wt mice, we hypothesized that an increase in cardiac ATRAP expression might suppress it in vivo. Thus, to validate the antihypertrophic properties of ATRAP in vivo, we generated Tg mice with cardiac-specific overexpression of ATRAP by the use of mouse ATRAP cDNA linked to the α-major histocompatibility complex promoter.[19] Quantitative analysis of ATRAP expression at the protein level revealed the highest and a moderate expression level of ATRAP in lines 52 and 46 (Tg52 and Tg46), respectively, among 10 obtained lines of Tg mice, and these 2 lines of Tg mice are characterized in Figure S2.

Age-matched LC and 2 independent lines of Tg mice (Tg46 and Tg52) were divided into 6 groups: (1) vehicle-infused LC mice; (2) Ang II (200 ng/kg per minute)–infused LC mice; (3) vehicle-infused Tg46 mice; (4) Ang II (200 ng/kg per minute)–infused Tg46 mice; (5) vehicle-infused Tg52 mice; and (6) Ang II (200 ng/kg per minute)–infused Tg52 mice. Although Ang II infusion significantly increased the diastolic intraventricular septum and diastolic LV posterior wall thickness, as estimated by echocardiography and HW/BW ratio in LC mice, these cardiac hypertrophic responses to Ang II infusion were completely suppressed in both Tg52 mice and Tg46 mice (Table 2). Thus, Tg52 mice were further characterized in comparison with LC mice.

The results of SBP measurement by the tail-cuff method did not result in significant Ang II–mediated BP responses in the LC or Tg mice (Table S1). Thus, to examine diurnal BP profiles and strictly compare the effects of Ang II infusion on BP, direct BP measurement by radiotelemetric devices was performed in LC and Tg mice. In LC mice, Ang II infusion for 2 weeks tended to increase SBP in the light period (118.2±4.0 versus 132.7±3.7 mm Hg; $P=0.075$) and significantly increased SBP in the dark period (129.3±2.7 versus 144.7±4.1 mm Hg; $P<0.05$; Figure 2A). Similarly, in Tg52 mice, Ang II infusion significantly increased SBP in both the light (115.2±2.8 versus 130.5±5.0 mm Hg; $P<0.05$) and dark (128.7±2.0 versus 143.3±4.6 mm Hg; $P<0.05$) periods. Although radiotelemetric SBP of the vehicle-infused Tg52 mice was significantly lower than that of the vehicle-infused LC mice at 12:00 AM (107.2±2.7 versus 116.2±4.5 mm Hg; $P<0.05$) and 3:00 PM (112.0±2.0 versus 123.7±3.5 mm Hg; $P<0.05$; Figure 2B), SBP of the Ang II–infused Tg52 mice was comparable to that of the Ang II–infused LC mice throughout the light-dark cycle (Figure 2C). Regarding other parameters obtained by radiotelemetry, the mean BP and heart rate of Tg52 mice were comparable to those of LC mice with or without Ang II infusion (Figure S3).

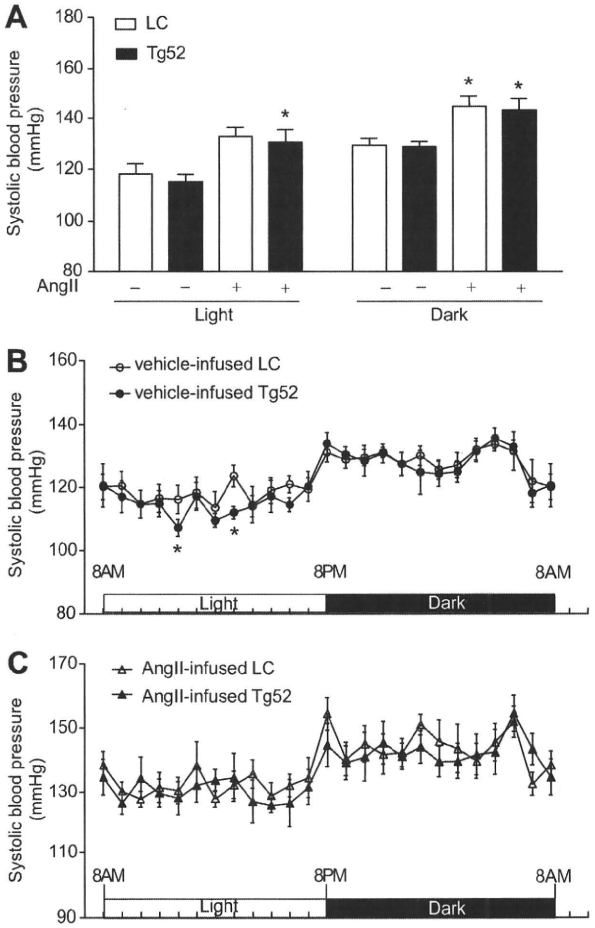

Figure 2. Diurnal BP profiles and effects of Ang II infusion analyzed by the radiotelemetric method in LC mice and cardiac-specific ATRAP transgenic (Tg52) mice. A, Effects of Ang II infusion on SBP measured by the radiotelemetric method in LC and Tg52 mice in the light and dark periods. Values are expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs vehicle. B, Diurnal SBP profile of LC and Tg52 mice infused with vehicle. Values are expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs LC mice. C, Diurnal BP profile of LC and Tg52 mice infused with Ang II. Values are expressed as the mean±SE (n=6 in each group).

These results of direct BP measurement by the radiotelemetric method confirmed no significant BP difference between the LC and Tg52 mice after Ang II infusion. With respect to histological analysis, Ang II infusion significantly increased the cross-sectional area of LC mice (251.5±6.7 versus 302.0±10.4 μm^2; $P<0.01$) but not Tg52 mice (267.7±11.2 versus 277.3±11.5 μm^2; Figure 3A). There were no significant increases in interstitial fibrosis in either the LC mice or Tg52 mice on Masson staining at this stage (data not shown). These results indicate that the cardiac hypertrophy effects induced by Ang II infusion were com-

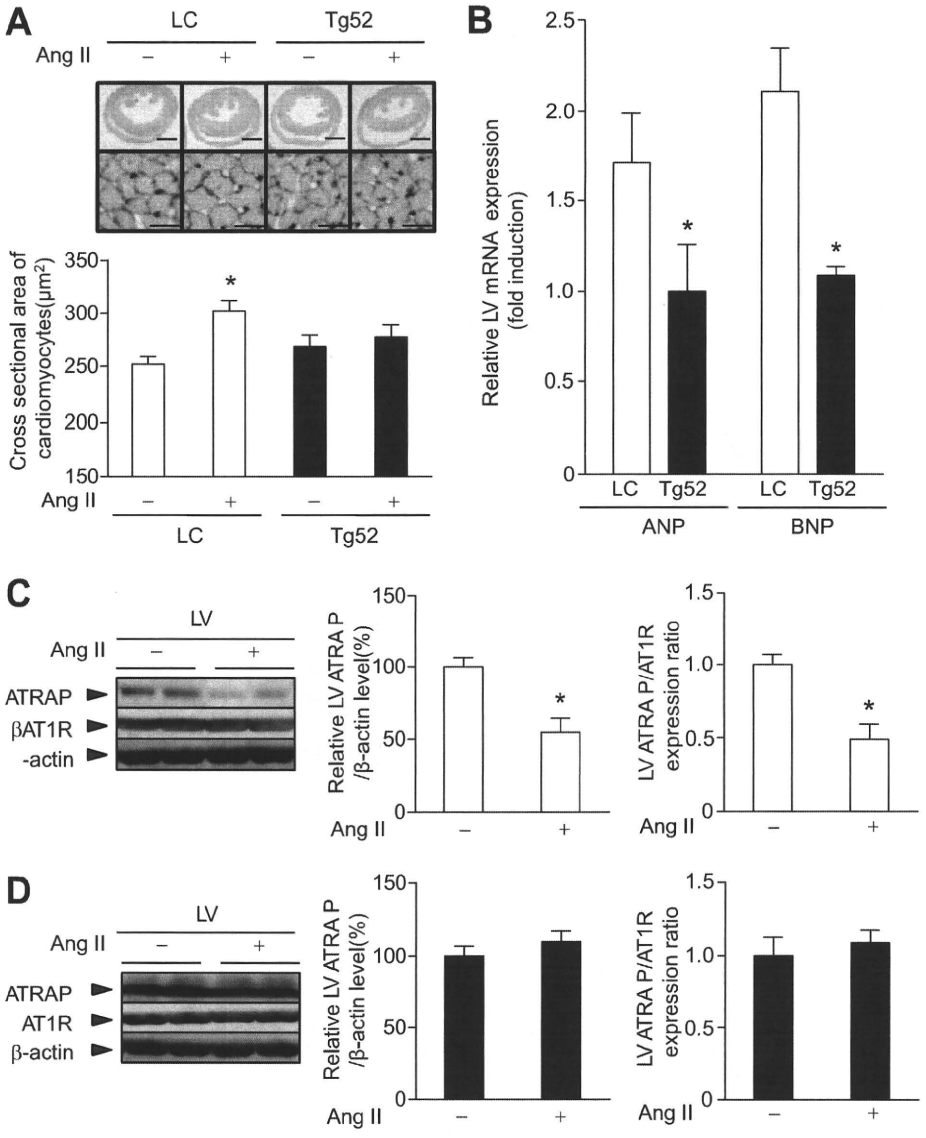

Figure 3. Cardiac hypertrophic responses by Ang II infusion into cardiac-specific ATRAP transgenic (Tg52) mice and LC mice. A, Effects of Ang II infusion on cardiac hypertrophy in Tg mice and LC mice. Representative images of hematoxylin and eosin–stained section of the hearts of all 4 groups examined (top, bar: 1 mm), representative images of antidystrophin monoclonal antibody–stained section of the LV for cell size measurement in all 4 groups (middle, magnification: ×400; bar: 20 μm), and quantitative analysis of a cardiomyocyte cross-sectional area of the LV (bottom). *$P<0.01$ vs vehicle. B, Effects of Ang II infusion on cardiac atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) mRNA expression in LC and Tg52 mice. Values are calculated as the fold induction of those achieved with extracts in the vehicle-infused group and expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs LC. C, Representative Western blot and quantitative analysis of the effects of Ang II infusion on LV ATRAP and AT_1R protein expression in LC mice. Values are calculated relative to those achieved with extracts in the vehicle-infused group (Ang II) and expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs vehicle. D, Representative Western blot and quantitative analysis of the effects of Ang II infusion on LV ATRAP and AT_1R protein expression in Tg52 mice. Values are calculated relative to those achieved with extracts in the vehicle-infused group (Ang II) and expressed as the mean±SE (n=6 in each group). *$P<0.05$ vs vehicle.

pletely suppressed in Tg52 mice with cardiac-specific overexpression of ATRAP.

Effects of Ang II Infusion on Hypertrophy-Related Gene Expression in Cardiac-Specific ATRAP Tg Mice

We examined whether cardiac-specific overexpression of ATRAP attenuates the cardiac hypertrophy-related gene expression induced by chronic Ang II infusion. Although Ang II infusion in LC mice increased the LV mRNA expression levels of atrial natriuretic peptide and brain natriuretic peptide by 1.7- and 2.1-fold induction, the upregulation of these mRNAs in response to Ang II infusion was completely suppressed in Tg52 mice (Figure 3B). With respect to the inhibitory effect of Ang II treatment on the cardiac ATRAP/AT_1R ratio, although Ang II infusion significantly decreased the cardiac ATRAP/AT_1R ratio through suppression of cardiac ATRAP expression in LC mice (Figure 3C), Ang II treatment did not affect the ratio at all in Tg52 mice (Figure 3D).

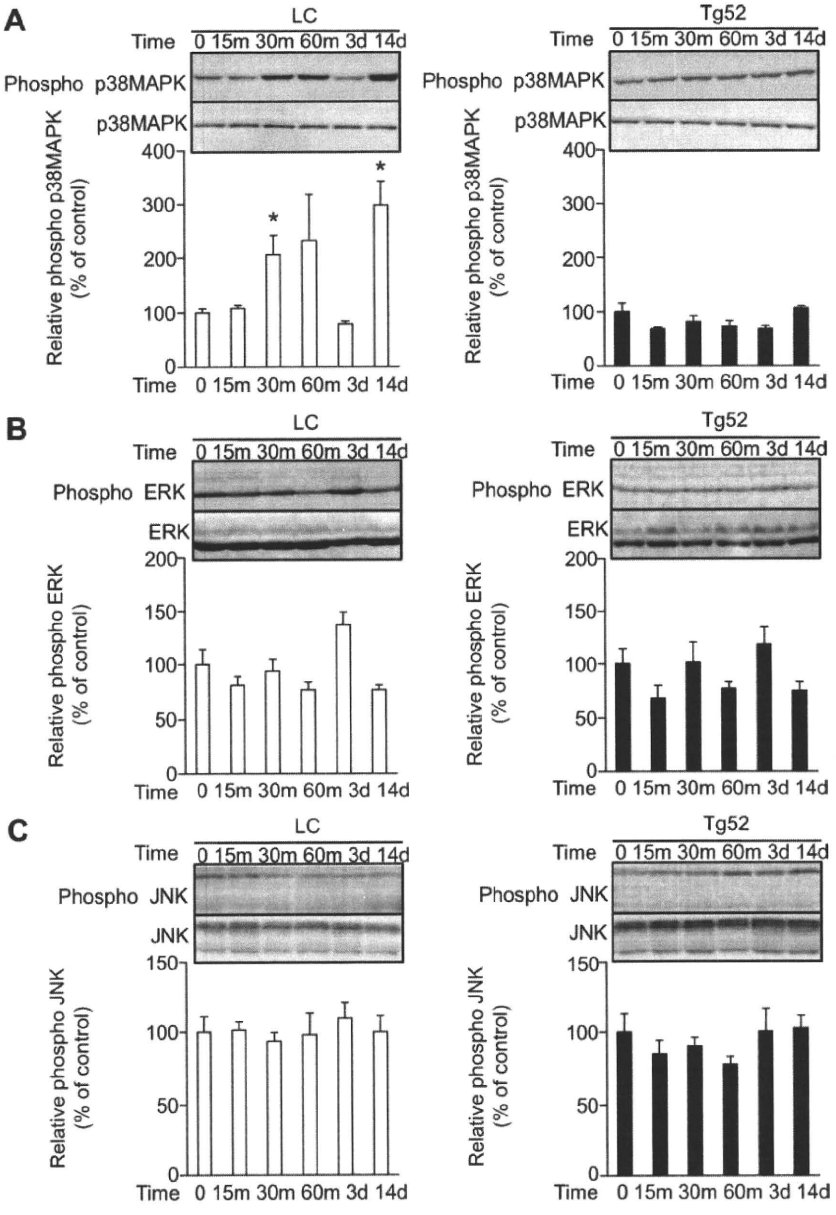

Figure 4. Effects of Ang II Infusion on MAPK activation in cardiac-specific ATRAP transgenic (Tg52) mice. Representative Western blots (top) and quantitative analysis (bottom) of the effects of Ang II infusion for 0, 15, 30, and 60 minutes and 3 and 14 days on phosphorylated and total MAPK (A, p38; B, ERK; and C, JNK) in LC mice (left, LC) and Tg52 mice (right). Values are calculated relative to those achieved with extracts in the mice at baseline (time 0) and expressed as the mean±SE (n=6 in each group). m indicates minutes; d, days. *$P<0.05$ vs baseline.

Effects of Ang II Infusion on MAPK Activation in Cardiac-Specific ATRAP Tg Mice

As the downstream effector of the AT_1R signaling pathway, MAPK plays an important role in the development of cardiac hypertrophy.[20–22] Thus, ultimately we examined the time course of LV MAPK activation in LC and Tg52 mice during Ang II treatment. LV p38 was significantly activated after 30 minutes and 14 days of Ang II infusion in LC mice (Figure 4A). However, LV p38 was not at all activated in Tg52 mice during Ang II treatment. On the other hand, LV JNK and ERK were not significantly activated by Ang II infusion in either LC or Tg52 mice (Figure 4B and 4C).

Discussion

This is the first report to our knowledge of a novel inhibitory function of cardiac ATRAP on cardiac hypertrophy in vivo. Activation of AT_1R signaling through the tissue renin-angiotensin system provokes sequential activation of signaling pathways, which leads to cardiac hypertrophy,[23–26] and chronic elevation of circulating Ang II causes sustained hypertension and associated cardiac hypertrophy.[27] The carboxyl-terminal portion of AT_1R is important for receptor internalization and activation of downstream signaling pathways.[28,29] ATRAP was cloned as a specific interacting molecule with the carboxyl-terminal domain of the AT_1 receptor by a yeast 2 hybrid screening system.[3] The results of in vitro studies showed that ATRAP suppresses Ang II–induced hypertrophic and proliferative responses by promoting a constitutive internalization of AT_1R and decreasing the p38 activity and transforming growth factor β production in cardiomyocytes and vascular smooth muscle cells, respectively, thereby suggesting that ATRAP is an endogenous inhibitor of AT_1R signaling.[8,9]

In the present study, we first showed that chronic infusion of Ang II significantly decreased the cardiac expression ratio

of ATRAP/AT_1R with the development of cardiac hypertrophy, for which the effect was completely inhibited by an AT_1R antagonist. We observed recently that treatment with an AT_1R antagonist recovered a constitutive decrease in the ratio of cardiac expression of ATRAP/AT_1R in spontaneously hypertensive rats, which was accompanied by a decrease in cardiac p38 activity and a suppression of cardiac hypertrophy.[30] Previous studies have shown that increases in cardiac p38 activity through the activation of AT_1R signaling are profoundly involved in cardiac hypertrophy and the damage incurred in genetic and experimental hypertension models, including spontaneously hypertensive rats and Ang II infusion.[31,32]

Because we had hypothesized that cardiac-specific upregulation of the ATRAP/AT_1R ratio suppresses the cardiac hypertrophy induced by Ang II infusion, we produced the cardiomyocyte-specific ATRAP Tg mice, with a constitutively high expression of cardiac ATRAP and a resultant upregulation of the cardiac ATRAP/AT_1R ratio, to examine this hypothesis. We obtained in vivo evidence that ATRAP attenuates the cardiac hypertrophy by chronic Ang II infusion concomitant with a suppression of cardiac p38 activation. Because the physiological parameters, such as BW, heart rate, radiotelemetric BP, cardiac functions, and HW/BW ratio, are comparable at baseline in LC and Tg mice, the effect of cardiac-specific overexpression of ATRAP is likely to be exerted only in the context of a hypertensive challenge.

The present study shows the enhancement of cardiomyocyte-specific ATRAP expression in vivo protects against the hypertrophic responses in mice induced by an Ang II–induced BP increase. Nevertheless, a limitation of the present study is that the results do not allow us to dissociate the direct effect of ATRAP enhancement on cardiac hypertrophy from the influence of pressure overload. In fact, several previous studies have indicated that cardiac hypertrophy can occur even in the absence of cardiac AT_1R,[33,34] that high doses of Ang II–released specifically in the heart do not result in cardiac hypertrophy,[35] and that if the kidneys do not express AT_1R, infusion of a very high dose of Ang II does not result in cardiac hypertrophy.[36] Therefore, the role of ATRAP in the regulation of cardiac hypertrophy in vivo needs to be further investigated in other models of hypertension and/or pressure or volume overload.

Perspectives

Because the results obtained in this study are essentially derived from Tg mice, it is important to exercise caution in interpreting the finding to be relevant to the pathophysiology of human cardiac hypertrophy. Nevertheless, the findings of the present study provide important information for the further investigation of the in vivo functional roles of ATRAP in the pathogenesis of cardiac hypertrophy and suggest the potential benefit of an ATRAP activation strategy. Additional studies to elucidate the molecular mechanisms of the antihypertrophic properties of cardiac ATRAP may enable a clinical application of ATRAP in the near future, such as the use of activating ligands for more efficient inhibition of AT_1R signaling in combination with inhibitors of the renin-angiotensin system.

Sources of Funding

This study was supported by grants from the Japan Society for the Promotion of Science; Yokohama Foundation for Advancement of Medical Science; Takeda Science Foundation; Salt Science Research Foundation (No. 0911); Kidney Foundation, Japan (JKFB09-25); Mitsubishi Pharma Research Foundation; and the Strategic Research Project of Yokohama City University. S.M. was supported by the High-Tech Research Center Project for Private Universities with a matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology.

Disclosures

None.

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