Heart and Vessels
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ORIGINAL ARTICLE

Serum microRNA-30d is a sensitive biomarker for angiotensin II-induced cardiovascular complications in rats

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Abstract

We tested the hypothesis that angiotensin II (Ang II)-induced cardiovascular complications are distinguished from what catecholamine-induced by their serum circulating biomarkers in rats. Infusion of Ang II (1.68 mg/kg/day) significantly increased systolic and diastolic blood pressure assessed at week one or later, accompanied by an increase of heart/body weight ratio. Noradrenaline infusion (5.40 mg/kg/day) produced a similar degree of hypertension, but did not increase heart weight. Ang II-, but not noradrenaline-induced hypertension was associated with a drastic upregulation of serum microRNA-30d (miR-30d) by hundreds of times, accompanied by an increase of miR-30d levels in the atrium but not in the ventricle. Ang II, but not noradrenaline, significantly increased mRNA of brain natriuretic peptide (BNP) in the atrium. Studies using rat neonatal cardiomyocytes in vitro demonstrated that BNP caused an increase of miR-30d when applied for 6 h or longer in the culture medium. In vitro application of Ang II increased the cell size, although BNP and miR-30d were unable to mimic the effect of Ang II. We conclude that serum circulating microRNA-30d is a sensitive biomarker for Ang II-induced cardiovascular complications. It is also postulated that Ang II-induced cardiomyocyte hypertrophy could be independent of miR-30d/BNP signaling pathways.

Keywords Angiotensin II · MicroRNA-30d (miR-30d) · Brain natriuretic peptide (BNP) · Biomarker · Hypertension · Circulation miR

Abbreviations

Ang II	Angiotensin II
miR	MicroRNA
BNP	Brain natriuretic peptide
RAS	Renin-angiotensin system
AF	Atrial fibrillation
RT-PCR	Reverse transcription-polymerase chain reaction
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
HR	Heart rate
ARB	Angiotensin receptor blocker
ACE	Angiotensin converting enzyme

Introduction

Cardiac muscle excitation and contraction depend on multiple neuronal and humoral factors in physiological and pathophysiological conditions of the heart [1–8]. Among them, hypertension and related cardiovascular complications are largely associated with the high levels of catecholamines in blood. Increased concentration of catecholamines may lead to structural and functional derangement in the myocardium, resulting in catecholamine-induced cardiomyopathy [9]. High catecholamine levels cause cellular calcium overload, free radical production, promotion of mitochondrial permeability transition, and cardiomyocyte cell death [10]. On the other hand, various forms of hypertension are associated with inappropriate activation of the renin–angiotensin system (RAS), in which pressure-dependent and pressure-independent patterns of cardiovascular damage have been described. Angiotensin II (Ang II) stimulates noradrenaline release modulated by presynaptic receptors on sympathetic nerves. Also, increased sympathetic nerve activity or increased actions of noradrenaline elevates serum renin activity following activation of RAS [11]. Thus,

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noradrenaline and Ang II play major roles in hypertension, which are themselves believed to be closely interlinked. Although the underlying mechanism of most cases of hypertension are highly complex and likely multifactorial, therapies targeting RAS and catecholaminergic α/β-adrenoceptors are often effective in reducing elevated blood pressure to a certain degree. Therefore, activation of sympathetic activity and RAS should be largely present in the patients with essential hypertension. However, there is no quantitative internal correlation between the degree of sympathetic activity and RAS activity in patients with hypertension. Because of this, an easily accessible biomarker of sympathetic nerve activity and RAS activation in patients with hypertension is needed. Recent studies indicate that circulating biomarkers are associated with elevated blood pressure [12], however, whether they mediate or represent biomarkers of RAS activation is unknown.

MicroRNAs (miRs), a group of highly conserved short noncoding RNAs that post-transcriptionally regulate gene expression through a partial base-pairing mechanism, have emerged as key regulators of biological processes in animals and humans. Large number of studies revealed that the expression of miR was highly altered in various cardiovascular diseases and their alterations are linked with expression of key components of cardiovascular pathophysiology. Thus, miRs are considered as a central part of the development of various cardiac disorders, which subsequently leads to cardiac remodeling and progression to cardiac complications [13]. Circulating miRs have also emerged as potent regulators of cellular signaling. Abnormalities in the expression of these miRs can result in pathological conditions, including hypertension [14, 15]. Regarding cardiovascular effects of miRs, we have previously shown that miR-30d is upregulated in human cardiomyocytes with persistent atrial fibrillation (AF), in response to cellular Ca^{2+}-overload [16]. Although it is known that abnormal Ca^{2+} dynamics may lead to the development of AF as well as hypertension, mechanisms for miR-30d up-regulation in AF cardiomyocytes have not been elucidated. Brain natriuretic peptide (BNP) is a well-known biomarker for congestive heart failure associated with cardiac hypertrophy and hypertension. A previous study suggests that BNP gene could be activated by Ang II in mice [17]. Based on these backgrounds, we hypothesized that RAS-mediated cardiovascular complications and catecholamine-mediated ones could be quantitatively distinguishable by biomarkers which are related to BNP and miR-30d signals.

In this investigation, our data suggests a causative role for miR-30d in Ang II-mediated hypertension in rats. Employing a rat model of hypertension, the findings point to a pro-hypertensive interplay between Ang II activity and miR-30d-amediated RAS activity. This is particularly important from a translational perspective, implicating miR-30d as a sensitive serum biomarker for Ang II action and the tool for quantitating the RAS activity.

Materials and methods

Animal model and assessment of serum miRNA expression

All experimental protocols met the guidelines stipulated by the Physiological Society of Japan and the Ethical Committee of Oita University for Animal Experiments. Male Wistar rats (Kyudo, Japan, 250–280 g) were housed under standard laboratory conditions (12 h light/dark cycle, 22 ± 2 °C, lights on at 08:00, food and water ad libitum). Under sterile condition, rats were implanted intraperitoneally with 2-weeks osmotic minipumps (Alzet Model 2001; Muromachi Kikai Co., Tokyo, Japan) contained with Ang II or noradrenaline to deliver controlled doses of 1.68 mg/kg/day (Ang II) or 5.40 mg/kg/day (noradrenaline). In separate experiments, acute hypertension models were treated with a single intraperitoneally injection of Ang II (2 mg/kg). After 6 h, blood sample and the heart were collected. Serum samples were obtained after centrifugation at 3000 rpm for 20 min at room temperature, and total RNA was isolated using the mirVana PARIS RNA isolation kit (Ambion, Austin, TX, USA). Expression profiling of miR was performed using an ABI TaqMan microRNA Array kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol.

Blood pressure and heart rate monitoring

Conventional non-invasive blood pressure and heart rate in conscious rats were measured using the tail-cuff method (Model, MK-2000ST, Muromachi) after heating the animal according to manufacturers' directions, and analyzed by a software DCS-2000ST-1 (Muromachi).

Preparation and culture of neonatal cardiomyocytes and transfection

Neonatal cardiomyocytes were prepared from 3- to 5-day-old Wistar rats as described previously [8, 18]. Twenty-four hours after plating, myocytes were incubated for 24 h with Ang II (1 μM), noradrenaline (1 μM) or vehicle (saline) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum for 3–24 h. Cells were transfected with small RNAs (see below) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The following small RNAs were used: pre-miR-30d precursor miR and negative control precursor miR (Ambion).

Quantitative real time-polymerase chain reaction (RT-PCR) for miRNA

All rat tissues and cardiomyocyte samples were subjected to quantitative RT-PCR for miR. Quantitative stem-loop quantitative reverse transcription (RT) was performed with a Taqman miRNA RT kit (Applied Biosystems), and then quantitative PCR for mature miRNAs was performed with Taqman microRNA assays (Applied Biosystems). miR expression levels relative to U6 were calculated from a standard curve.

Gene expression analyses

Total RNA was extracted from neonatal rat cardiomyocytes using TRIzol (Invitrogen, Carlsbad, CA, USA) 24 h after the treatment with agents described above. The single-stranded cDNA was synthesized from 1 μg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Molecular System Inc., Alameda, CA). Real-time PCR was performed on Light Cycler (Roche) using the FastStart DNA Master SYBR Green I (Roche) as a detection reagent. The primers for BNP were designed according to the published gene sequences (GeneBank accession no.NM_031545, forward; 5′–GCTCTTCTT TCCCCAGCTCT–3′ and reverse; 5′–CACTGTGGCAAG TTTGTGCT–3′). Rat Glyceraldehyde–3–phosphate dehydrogenase (GAPDH; GenBank accession no.M17701, forward; 5′–GCCATCAACGACCCCTTCAT–3′ and reverse; 5′–TTCACACCCATCACAAACAT–3′) mRNA was used as an internal control. Data were calculated by $2^{-\Delta\Delta CT}$ and presented as fold change of transcripts for BNP gene in cardiomyocytes and normalized to GAPDH (defined as 1.0-fold).

Immunofluorescence staining

The details of the experiments protocol were performed as described previously [19]. For detection, appropriate secondary antibody conjugated with Alexa Fluor™ 594 (Invitrogen) were used, and the nuclei were counterstained with DAPI ProLong Diamond antifade regent (molecular probes). Images were acquired using a 63× oil objective (Plan-Apochromat 63× [numerical aperture, 1.46] oil immersion objective for differential interference contrast [DIC]; Carl Zeiss). All sections were analyzed using a confocal laser microscopy system and software (LSM710; Carl Zeiss) that was built around an inverted microscope (Axio Observer Z1; Carl Zeiss, Germany). Images were saved in TIFF format and analyzed by ImageJ software (Wayne Rasband, National Institutes of Health, USA).

Chemicals

Ang II, noradrenaline and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ang II and noradrenaline were dissolved in acidic distilled water, and then diluted to final concentrations as in osmotic mini pump solutions.

Data analysis

Group data are shown as means ± SE. Analysis of variance and Tukey–Kramer procedure or Holm-Sidak method were used for multiple comparisons, and Student's *t* test was used for the comparison of two groups. Differences were considered significant when p values were less than 0.05.

Results

Experimental animal model

We first evaluated long-term effects of Ang II and noradrenaline on blood pressure and heart rate in rats. To assess the impact of both hormones on the cardiovascular system, we carefully selected adequate concentration of each hormone judging from the degree of blood pressure elevation. Osmotic minipumps were used as an alternative to repetitive injections for prolonged delivery of Ang II and noradrenaline in adult rats. After repetition of preliminary experiments by use of various concentrations of Ang II and noradrenaline, we determined the concentration of Ang II (1.68 mg/kg/day) and noradrenaline (5.40 mg/kg/day) to monitor blood pressure, heart rate, plasma BNP, and serum miR-30d up to the observation period for 2 weeks. Figure 1 summarizes the results of actions of Ang II and noradrenaline on systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), and heart/body weight ratios. Importantly, the increase of blood pressures, SBP and DBP were statistically identical between the Ang II group and the noradrenaline group, although heart rates were unchanged in both groups up to 2 weeks. We noted that heart weight was significantly increased by Ang II but not by noradrenaline in spite of the similar increase of blood pressures. Because the impacts of both hormones on blood pressure elevation were identical, we assumed that cardiovascular complications in both groups were comparable.

miRs as possible biomarkers for cardiovascular complication

Our laboratory has previously identified that miR-30d is able to induce the remodeling of the acetylcholine-sensitive potassium channel in the atrial tissue with AF [16]. It is

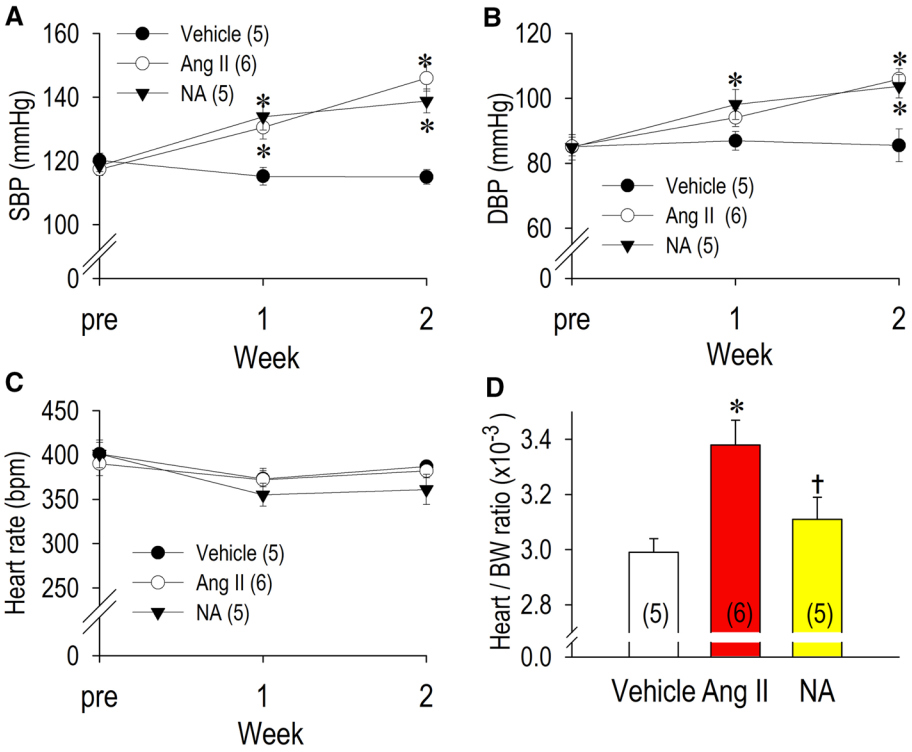

Fig. 1 Construction of hypertension model rats by use of Ang II and noradrenaline infusion. Intraperitoneal implantation of osmotic minipumps releasing Ang II (1.68 mg/kg/day) and noradrenalin (5.40 mg/kg/day) caused changes of systolic blood pressure (SBP) in panel **a**, diastolic blood pressure (DBP) in panel **b**, heart rate (HR) in panel **c**, and heart weight (HW) vs. body weight (BW) ratio (HW/BW) in 2 weeks in panel **d**. Numbers of animals are shown in parentheses. *$p < 0.05$ vs. pre or vehicle

also widely accepted that RAS and high blood pressure play important roles in the onset of AF. Because of these pathological backgrounds, we focused on the possible relation between Ang II-related myocardial complication and roles of miR-30d. Accordingly, we examined expression of miR-30d in the ventricle and the atrium using Ang II- and noradrenaline-induced hypertension model rats. Although Ang-II had no actions on miR-30d expression in the atrium and the ventricle when applied for 6 h, long-term administration of Ang II significantly increased expression of miR-30d in the atrium but not in the ventricle when applied for 2 weeks (Fig. 2a, b). On the other hand, noradrenaline had no impact on miR-30d expression in the atrium and the ventricle. In the next, serum concentration of miR-30d was examined in Ang II- and noradrenaline-induced hypertension model rats. Surprisingly, serum miR-30d concentration was elevated more than hundred times when measured 6 h after the onset of Ang II application, and the elevation was continued for 2 weeks (Fig. 2c). Notably, noradrenaline did not change the serum concentration of miR-30d at all, which indicates that Ang II-mediated cardiovascular complications are distinct from what caused by catecholamines in terms of serum circulating miR-30d concentration. It is also implied that Ang II-mediated cardiovascular damages could be quantitatively assessed by serum miR-30d levels. Based on the fact that Ang II upregulates BNP expression in the atrium but not in the ventricle, it is postulated that upregulated serum BNP is derived from the atrial cardiomyocytes but not from the ventricular cardiomyocytes.

To further confirm the result and investigate the cellular background mechanism, we have employed an in vitro experiment by use of rat neonatal cardiomyocytes. Importantly, Ang II increased expression of miR-30d when applied for 3 h or longer in neonatal cardiomyocytes (Fig. 2d). It was also confirmed that noradrenaline had no impact on miR-30d expression. In the same cell milieu, changes of BNP gene were validated by real-time PCR demonstrating that BNP-mRNA was upregulated by Ang II when applied for 6 h or longer (Fig. 2e). This result was somewhat identical to changes in miR-30d, suggesting that miR-30d and BNP could be linked or connected as down-stream signals of Ang II/miR-30d-mediated changes in cardiomyocytes. In contrast, noradrenaline did not exert any effect on BNP expression in the present study.

Interactions between BNP and Ang II

Whole animal studies by use of Ang II-induced hypertension model rats revealed that atrial expression of miR-30d was significantly increased, and in vitro studies of neonatal cardiomyocytes revealed that miR-30d and BNP expression were significantly increased by Ang II. In addition, serum miR-30d was drastically elevated in response to Ang II application in animals. Thus, we hypothesized that BNP and miR-30d are associated as down-stream target signals of Ang II-mediated complications in cardiomyocytes. To confirm the interaction, miR-30d expression in neonatal cardiomyocytes was evaluated in response

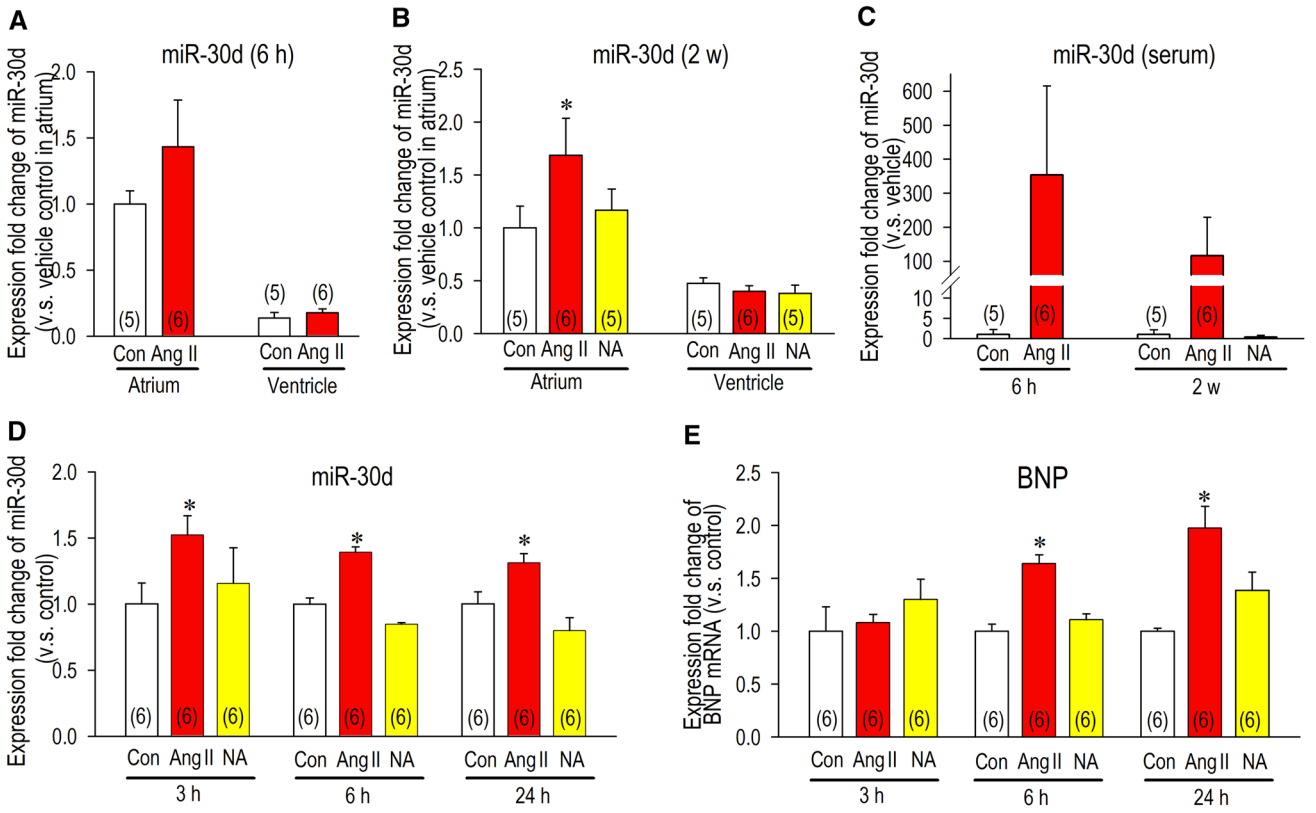

Fig. 2 Expression of miR-30d and BNP in cardiomyocytes and in the serum. **a**, **b** Quantitative RT-PCR performed on miR-30d in cardiomyocytes of hypertension model rats (adult rat) sampled from the atrium and the ventricle after infusion of each hormone for 6 h **a** or 2 weeks **b**. **c** Serum miR-30d derived from hypertension model rats 6 h and 2 weeks after osmotic minipumps implantation. **d**, **e** Expression of miR-30d and BNP in neonatal cardiomyocytes cultured with Ang II **d** or noradrenaline (NA) **e** for 3 h, 6 h and 24 h. *$p < 0.05$ vs. control (con)

to BNP application to the culture medium (Fig. 3). BNP upregulated expression of miR-30d when applied for 6 h or longer, and the upregulation was in a dose-dependent manner, suggesting that Ang II increases expression of miR-30d through BNP-mediated signal pathways in cardiomyocytes.

Are BNP and miR-30d responsible for Ang II-mediated cellular hypertrophy?

To further confirm and differentiate cellular effects of BNP and miR-30d as down-stream signal molecules of Ang II, cell size or cell surface area of cardiomyocytes were assessed with BNP or miR-30 application, and compared

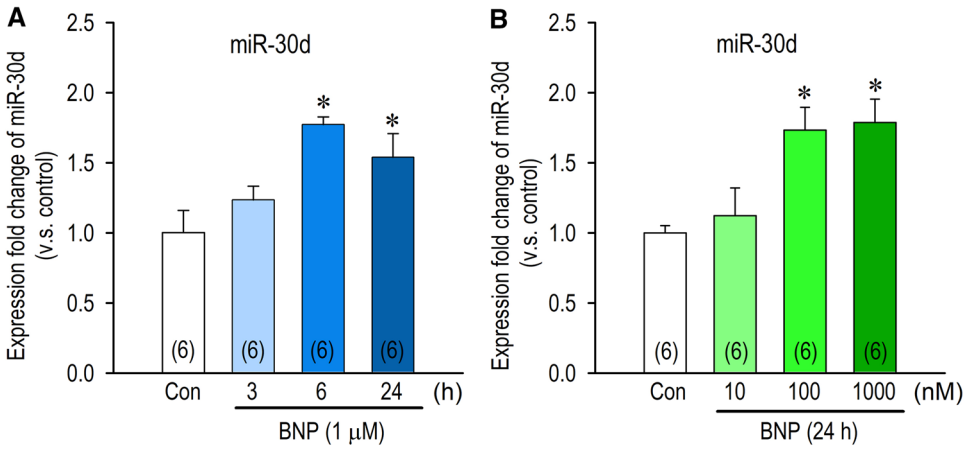

Fig. 3 Time- and concentration-dependent upregulation of miR-30d by BNP in neonatal cardiomyocytes. BNP (1 μM) was applied in the culture medium for 3 h, 6 h and 24 h to assess the time-dependent effect of BNP on miR-30d regulation **a**. BNP of 10 nM, 100 nM and 1,000 nM concentration was applied in the cultured medium for 24 h to assess the concentration-dependent effect of BNP on miR-30d regulation **b**. *$p < 0.05$ vs. control (con)

with those of Ang II in the culture medium for 6 h and 24 h. Ang II increased the cell size when applied for 24 h, which is consistent with the in vivo result in Fig. 1d demonstrating the rise of heart weight as assessed in Ang II-mediated hypertension rats. However, BNP and miR-30d were unable to modify the cell size (Fig. 4b), suggesting that Ang II-mediated signaling elicits diverse outputs dependently and independently of BNP/miR-30d actions on cardiomyocytes.

Discussion

In this study, we have investigated whether serum miR-30d represents cardiovascular and myocardial complications caused by Ang II in rats. Our main findings were as follows: (1) long-term application of Ang II significantly and drastically increasesd serum miR-30d by hundreds times, (2) long-term application of noradrenaline was without effect on serum miR-30d, (3) long-term application of Ang II upregulated expression of BNP and miR-30d in the atrium, (4) BNP increased miR-30d expression in cardiomyocytes in vitro, and (5) Ang II-mediated signals through BNP/miR-30d were not responsible for cellular hypertrophy. These results suggest that serum circulation miR-30d is a sensitive biomarker that quantitatively represents actions of Ang II. Because cardiovascular complications caused by RAS system is a detrimental cardiac problem due to the reason that it is associated with all kinds of heart diseases, a quantitative assessment of RAS activity by serum miR-30d could be beneficial for the decision of therapeutic direction and appropriate selection of drugs for patients with cardiovascular diseases.

RAS is a complex system that regulates systemic functions through body electrolytes and blood volume control, exerts proliferative action at the heart and vessels [20, 21]. The significant relation between cardiovascular diseases and RAS system is further supported by the fact that angiotensin-converting enzyme (ACE) inhibitors and angiotensin-receptor blockers (ARB) have beneficial effects in patients with hypertension, myocardial infarction, and heart failure [20, 21]. Thus, it is well-acknowledged that RAS and its key mediator Ang II play important roles on signaling pathways involved in the development of multiple cardiac complications. The hypertrophic effects of Ang II on the heart are associated with its vasoconstrictor and hypertensive properties. However, it is currently known that Ang II is a powerful hypertrophic agent independently of its blood pressure effects. Actually, in vitro observation in this study shows that Ang II elevated myocardial cell size independently of cell surface hydrostatic pressure (Fig. 4), suggesting that Ang II activates different hypertrophic signaling pathways in cardiac myocytes [22, 23]. Similarly, the elevation of myocardial Ang II was also observed in hypertrophied heart in animal experiments after overload pressure as well as in patients from heart failure, which suggests that hypertrophy have resulted from local RAS activation [23, 24]. Because of these results and accumulation of knowledges on RAS as well as excessive sympathetic nerve function in cardiovascular diseases, ARB/ACE inhibitors and β-blockers are widely recognized as the first choice drugs for patients with primary hypertension and/or heart failure. It is also accepted that β-blockers and ARB/ACE inhibitors are complementary, and can be started together as soon as the diagnosis

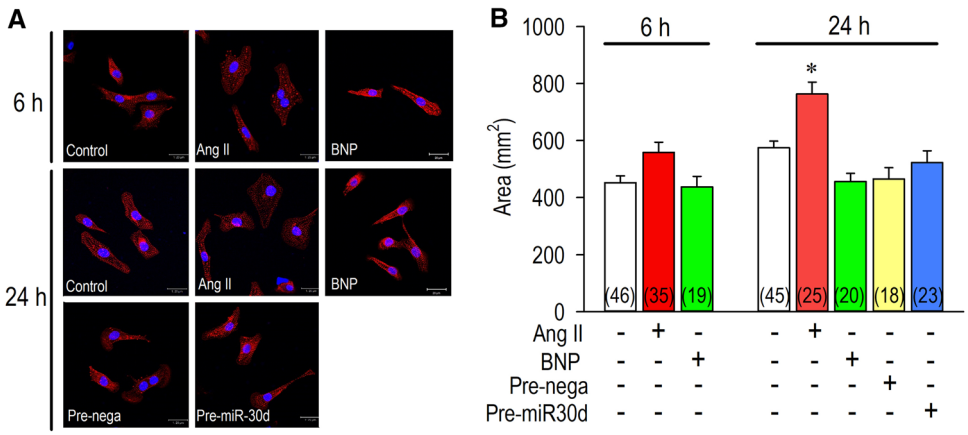

Fig. 4 Assessment of cellular hypertrophy caused by Ang II, BNP and miR-30d. **a** Representative immunostaining of cardiomyocytes for α-actinin and the nucleus marker protein (DAPI) incorporation with Ang II (1 μM), BNP (1 μM) for 6 h (upper), and pre-miR-30d (30 nM) in comparison with control (vehicle) or Pre-nega control miRNA (30 nM) for 24 h (middle and lower) incubation **a** (scale bars represent 20 μm). Assessment of cell surface areas of randomly selected cardiomyocytes cultured with Ang II (1 μM), BNP (1 μM), Pre-nega miRNA (30 nM), and Pre-miR-30d (30 nM). Numbers of cells are indicated in parentheses. $^{*}p < 0.05$ vs. control (con) or white bars

of heart failure is made [25]. In the context of therapeutic decision for individual patient on prescription of the appropriate drugs, quantitative assessment of sympathetic nerve actions and RAS system on the pathophysiology is needed. For this purpose, evaluation of serum circulating miR-30d could be a potential benefit as an objective marker for Ang II-mediated diseases particularly for patients with chronic heart failure.

miRs have first been reported as biomarkers for neoplasm in 2008 [26], and ever since after the establishment as a useful tool, their potential use as biomarkers has been demonstrated in the literature for numerous diseases. Among them, miR-30d has been recognized as a valuable diagnostic biomarker for non-invasive screening of cervical cancer [27], nervous system lymphoma [28], prostate cancer [29], non-small cell lung cancer [30], and many other neoplasmic diseases. Of note, recent studies identified that miR-30d is also acting as a biomarker for various cardiovascular diseases or pathophysiological conditions of the heart, including myocardial fibrosis [31], heart failure [32], and myocardial infarction [33]. Of note, the highest expression of miR-30d was found in the left ventricle across human tissues, followed by the lung, the right ventricle, the psoas, the submandibular gland, the red blood cell and others, although they did not evaluate the atria [34]. Their data also suggests a link between serum levels of miR-30d and the heart as a dominant source of miR-30d expression [34]. Because pathological cardiovascular conditions are often associated with the actions of Ang II or RAS activity, our observation demonstrating an intense link between miR-30d and Ang II-mediated cardiovascular complications is largely consistent with these cardiovascular pathophysiology. According to an online database for prediction of functional microRNA targets, miRDB [35], 62 genes including prostaglandin F2 receptor inhibitor (Ptgfrn), phospholipase C, eta 1 (Plch1), and calpain 7 (Capn7) (Table 1). In addition, miRDB also predicts several genes associated with cardiovascular regulations which are expected to interact with miR-30d, including adrenoceptor α2a (Adra2), adrenoceptor α1d (Adra1d), and Ca^{2+}-calmodulin-dependent protein kinase IIδ (Camk2d). By combining miR target prediction data with experimental biological data such as in this study, more specific and reliable biomarkers could be obtained to clarify and quantify the activity of Ang II and RAS in individual subjects.

Although we have successfully demonstrated that Ang II increases expression of miR-30d in the cardiomyocytes and the serum, signal pathway linking Ang II and miR-30d, and their interaction signals with BNP are largely unknown. Ang II was able to upregulated miR-30d as early as 3 h after application (Fig. 2d), however, upregulation of BNP by Ang needed 6 h or longer (Fig. 2e). Taken together with the result shown in Fig. 3, it is postulated that Ang II upregulates miR-30d dependently and independently of BNP actions. Further investigation is obviously needed to clarify the role of these steps.

Various limitations of our study should be addressed, including: (1) This was an experimental study by use of rodent, and the results may not be directly extrapolated to human subjects, (2) signal molecule(s) linking BNP and expression of miR-30d were not identified, (3) pharmacological actions of ARB or RAS inhibition on the results of Ang II in in vivo and in vitro studies were not assessed, (4) we could not demonstrate the concentration-dependent relation between Ang II and miR-30d in the serum, (5) organs of miR-30d production caused by Ang-II application were not identified, and (6) possible actions of miR-30d (Pre-miR-30d) to stimulate BNP expression was not confirmed in in vivo and in vitro, although bioinformatic analysis revealed that there is no potentials binding motif of miR-30d onto BNP-mRNA (Table 1). These questions should be addressed thoroughly in future studies.

In conclusion, we have successfully demonstrated that serum miR-30d represents cardiovascular and myocardial complications caused by Ang II in rats. Our main findings may be helpful for the beneficial decision of therapeutic direction and appropriate selection of drugs based on quantitative assessment of Ang II activity for patients with cardiovascular diseases.

Table 1 62 predicted target genes for rat-miR-30d in MicroRNA target prediction database

Gene symbol	GeneBank accession no	Gene description
Pip4k2a	NM_053926	Phosphatidylinositol–5–phosphate 4–kinase type 2 alpha
Tnrc6a	NM_001107549	Trinucleotide repeat containing 6a
Twf1	NM_001008521	Twinfilin actin-binding protein 1
Celsr3	NM_031320	Cadherin, EGF LAG seven-pass G-type receptor 3
Cyp24a1	NM_201635	Cytochrome P450, family 24, subfamily a, polypeptide 1
Rarg	NM_001135249	Retinoic acid receptor, gamma
Pde7a	NM_031080	Phosphodiesterase 7A
Eed	NM_001106278	Embryonic ectoderm development
Cpsf6	NM_001106785	Cleavage and polyadenylation specific factor 6
RGD1359108	NM_001007702	Similar to RIKEN cDNA 3110043O21
Klhl20	NM_001107192	Kelch-like family member 20
Stim2	NM_001105750	Stromal interaction molecule 2
Spast	NM_001108702	Spastin
Ptgfrn	NM_019243	Prostaglandin F2 receptor inhibitor
Gabrb1	NM_012956	Gamma-aminobutyric acid type A receptor beta 1 subunit
Ddah1	NM_022297	Dimethylarginine dimethylaminohydrolase 1
Rgs8	NM_019344	Regulator of G-protein signaling 8
Runx2	NM_001278484	Runt-related transcription factor 2
Polr3g	NM_001109468	RNA polymerase III subunit G
Rfx6	NM_001106388	Regulatory factor X, 6
Gli2	NM_001107169	GLI family zinc finger 2
Ptpn13	NM_001100789	Protein tyrosine phosphatase, non-receptor type 13
Ythdf3	NM_001108546	YTH N(6)-methyladenosine RNA binding protein 3
Stox2	NM_001134863	Storkhead box 2
Ppp1r18	NM_001126287	Protein phosphatase 1, regulatory subunit 18
Afap1l2	NM_001305184	Actin filament associated protein 1-like 2
Galnt3	NM_001015032	Polypeptide *N*-acetylgalactosaminyltransferase 3
Polr3e	NM_001108503	RNA polymerase III subunit E
Mier3	NM_001168000	MIER family member 3
Stk39	NM_019362	Serine threonine kinase 39
Capn7	NM_001030037	Calpain 7
Lhx8	NM_001012219	LIM homeobox 8
Sox9	NM_080403	SRY box 9
Rimbp2	NM_001100488	RIMS binding protein 2
Plch1	NM_001191707	Phospholipase C, eta 1
Sgcb	NM_001191068	Sarcoglycan, beta
Scn2a	NM_012647	Sodium voltage-gated channel alpha subunit 2
Ano4	NM_001106778	Anoctamin 4
Scn3a	NM_013119	Sodium voltage-gated channel alpha subunit 3
Mfsd6	NM_001106911	Major facilitator superfamily domain containing 6
Ccnjl	NM_001037773	Cyclin J-like
Yaf2	NM_001134871	YY1 associated factor 2
Pcdh17	NM_001107279	Protocadherin 17
Extl2	NM_001100704	Exostosin-like glycosyltransferase 2
Eml4	NM_001108008	EMAP like 4
Rasd1	NM_001270954	Ras related dexamethasone induced 1
Gatm	NM_031031	Glycine amidinotransferase
Ankhd1	NM_001204053	Ankyrin repeat and KH domain containing 1
Prdm1	NM_001107639	PR/SET domain 1
Bnip3l	NM_080888	BCL2 interacting protein 3 like
Ppp3r1	NM_017309	Protein phosphatase 3, regulatory subunit B, alpha

Table 1 (continued)

Gene symbol	GeneBank accession no	Gene description
Klf10	NM_031135	Kruppel-like factor 10
Slc12a6	NM_001109630	Solute carrier family 12, member 6
Ccne2	NM_001108656	Cyclin E2
Cep41	NM_001025770	Centrosomal protein 41
Elavl2	NM_001302217	ELAV like RNA binding protein 2
Tmem181	NM_001130939	Transmembrane protein 181
Ankrd17	NM_001105999	Ankyrin repeat domain 17
Ccnt2	NM_001107171	Cyclin T2
Foxg1	NM_012560	Forkhead box G1
Snapin	NM_001170576	SNAP-associated protein
Desi2	NM_001013873	Desumoylating isopeptidase 2

Possible gene targets of miR-30d was predicted by an algorithm in miRDB (http://mirdb.org/miRDB) with prediction score of 95 or higher. According to miRbase22.1 (miRDB) analysis, 683 predicted target genes were picked up based on target score of 50 or greater, and 62 predicted target genes were picked up based on target score of 95 or higher

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Author contributions All authors gave their informed consent prior to their inclusion in the study. MM conceived, planned and conducted the experiments. MM and KO took the lead in writing the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The experiments conducted in this work were approved by approved in advance by the Ethics Review Committee for Animal Experimentation of Oita University School of Medicine, and in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

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