Vasoconstrictor effect of aldosterone via angiotensin II type 1 (AT1) receptor: possible role of AT1 receptor dimerization

Masahiro Yamada, Motoi Kushibiki, Tomohiro Osanai, Hirofumi Tomita, and Ken Okumura*

Department of Cardiology, Hirosaki University Graduate School of Medicine, 5 Zaifu-Chō, Hirosaki 036-8562, Japan

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Aims
We recently demonstrated that aldosterone induces a non-genomic vasoconstrictor effect on rat coronary arterioles and that this effect was blocked by angiotensin II type 1 receptor (AT1) blockers. Intracellular transglutaminase enhances AT1 signaling by cross-linking AT1 homodimers. The purpose of this study was to confirm the AT1-dependency of the vasoconstrictor effect of aldosterone using AT1a knockout (AT1aKO) mice and to investigate the role of intracellular transglutaminase and AT1 dimerization in this effect.

Methods and results
The mesenteric arterioles (60–160 μm) were isolated from C57BL/6J (wild-type, WT) and AT1aKO mice, and the internal diameter was measured by video microscopy. Aldosterone (10^{-12} to 10^{-6} M), but not hydrocortisone, produced a dose-dependent vasoconstriction in WT mice; the maximal diameter change was \(-8.6 \pm 0.3\%\) from the baseline (\(P < 0.001\)). This vasoconstrictor effect was unaffected by the mineralocorticoid receptor antagonist spironolactone or eplerenone, the AT2 antagonist PD123319, the glucocorticoid receptor antagonist RU486, or endothelium denudation. Aldosterone's vasoconstrictor effect was negligible in AT1aKO mice. The AT1 blockers valsartan or candesartan suppressed aldosterone-induced vasoconstriction in WT mice. The transglutaminase inhibitors cystamine and monodansyl cadaverine also suppressed the vasoconstrictor effect of aldosterone, without affecting the vasoconstrictor effect of angiotensin II in WT mice. AT1 dimer protein levels were increased in WT mesenteric arterioles treated with 10^{-7} M aldosterone, and the transglutaminase inhibitor and AT1 blocker blocked this aldosterone-induced formation of AT1 dimer. Treatment with 10^{-7} M aldosterone for 10 min increased the transglutaminase activity by 2.5 \(\pm\) 0.2-fold in cultured vascular smooth muscle cells and by 1.2 \(\pm\) 0.1-fold in the mesenteric arterioles. These increases were abolished by transglutaminase inhibitors.

Conclusion
Aldosterone produces a non-genomic, endothelium-independent vasoconstrictor effect by enhancing intracellular transglutaminase activity and presumably inducing AT1 dimer formation in mesenteric arterioles.

1. Introduction
In addition to stimulation of NaCl transport in the renal distal tubules and collecting ducts, aldosterone promotes cardiac hypertrophy, fibrosis, and vascular inflammation.1–3 Aldosterone exerts these classical effects through binding to the mineralocorticoid receptor (MR), which is translocated into the nucleus where it initiates the transcription of target genes. In contrast to these genomic actions of aldosterone, rapid non-genomic effects of aldosterone have also been demonstrated. These effects occur within seconds or in a few minutes and are not blocked by either spironolactone or inhibitors of transcription and protein synthesis, suggesting that they are independent of the MR.4 It was recently reported that aldosterone shows rapid vasomotor effects in rabbit afferent and efferent arterioles, canine coronary arteries, and rat mesenteric arteries.5–7 A rapid action of aldosterone has also been reported in humans.8–10 Although these rapid non-genomic effects of aldosterone have been said to be mediated via a putative membrane receptor, the precise mechanism underlying these effects remains unclear.

It was reported that aldosterone increases the number of angiotensin II type 1 (AT1) receptors and their responsiveness11,12; moreover, aldosterone-induced cardiac fibrosis is abolished by an AT1 receptor blocker.13 These findings raise the possibility that aldosterone interacts with AT1 receptor, either directly or indirectly. We recently demonstrated that aldosterone induces a rapidly occurring vasoconstrictor...
effect in isolated rat coronary arterioles, and this effect is inhibited by AT₁ receptor blockers but not by spirono-
lactone. The AT₁ receptor is usually activated by angio-
tensin II, but is also activated by mechanical stimuli, such as stretch, independently of angiotensin II, in cardiomyo-
cytes. A recent study demonstrated that dimerization of AT₁ receptors cross-linked by intracellular transglutaminase enhances signalling downstream of these receptors in human monocytes. In the present study, using mesenteric arter-
ioles isolated from wild-type (WT) and AT₁a knockout (AT₁aKO) mice, we investigated whether AT₁ receptors par-
ticipate in the vasoconstrictor effect of aldosterone, and whether receptor dimerization is involved.

2. Methods

This investigation conforms with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All experimental procedures were carried out in accordance with the Guidelines for Animal Experiments of Hirosaki University Graduate School of Medicine.

2.1. Animals

Male C57BL/6J WT mice and AT₁aKO mice aged 15–25 weeks were used. AT₁aKO mice were kindly provided by Tanabe Seiyaku Co., Ltd (Osaka, Japan) and have been characterized previously. All mice were kept in a specific pathogen-free facility under controlled temperature (23 ± 1 °C) and humidity (60%), with a 12-hour light and dark cycle, in the Institute for Animal Experiments at our institution. Animals were given free access to standard labora-
tory mouse chow and tap water.

2.2. Preparation for experiments

All experiments were carried out according to the methods established by Duling et al. and Kuo et al. WT and AT₁aKO mice were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the mesenteric arteries were removed and immediately placed in 4°C physiological salt solution (PSS) containing (in mmol/L) NaCl 145.0, KCl 4.7, CaCl₂ 2.0, MgSO₄ 1.17, NaHPO₄ 1.2, glucose 5.0, pyruvate 2.0, ethylenediaminetetraacetic acid 0.02, and 3-morpholinopropanesulfonic acid 3.0. The mesenteric arter-
ioles (60–160 μm in internal diameter) were dissected from the sur-
rrounding intestinal tissue under cold (5°C) PSS containing albumin at pH 6.9. Each isolated microvessel was then transferred for cannula-
tion into a Lucite vessel chamber containing albumin-PSS (pH 7.4) and room temperature at ambient temperature. Both ends of each vessel were cannulated with micropipettes filled with albumin-PSS. Cannulated vessels were then securely tied to micro-
piptettes using 11-0 ophthalmic sutures (Alcon).

2.3. Drugs

Drugs and chemicals were purchased from SIGMA-ALDRICH Japan K.K. (Tokyo, Japan) except where specifically stated. Valsartan and benazepril were gifts from Novartis Pharma K.K. (Tokyo, Japan). Candesartan (CV-11974) was a gift from Takeda Chemical Industries, Ltd (Osaka, Japan). Eplerenone was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

2.4. Instrumentation

After cannulation, vessels were transferred to the stage of an inverted microscope (IX70, Olympus) coupled to a CCD camera (C5330, Olympus) and video micrometer (Microcirculation Research Institute, Texas A&M University Health Science Center). The micropipettes were connected to an independent pressure reservoir system, and intraluminal pressures were measured through the side arms of the two reservoir lines by low-volume-displacement strain-gauge transducers. The isolated vessels were pressurized without flow by setting both reservoirs at the same hydrostatic level. The internal diameters of each vessel were measured throughout the experiment by video microscopic techniques, as described previously.

2.5. Experimental protocols in isolated vessel studies

Each cannulated vessel was bathed in albumin-PSS and equilibrated with room air, and the temperature was maintained at 36–37°C. Vessels were maintained at their in situ length, and allowed to develop basal tone at 60 cmH₂O of intraluminal pressure. After a stabilization period of 60 min, experiments were performed. All chemicals were administered to the bath. First, increasing doses of aldosterone (10⁻¹³ to 10⁻⁶ M) were administered, and the arteri-
olar diameter was measured within a few minutes of adding aldos-
teron. To investigate the effects of the selective AT₁ receptor blockers valsartan (10⁻⁶ and 10⁻⁵ M) and candesartan (10⁻⁷ M), an AT₂ receptor antagonist PD123319 (10⁻⁶ M), the MR antagonists spi-
onolactone (10⁻⁶ M) and eplerenone (10⁻⁵ M), the glucocorticoid receptor (GR) antagonist RU486 (10⁻⁵ M), the angiotensin-
converting enzyme (ACE) inhibitor benazepril (10⁻⁵ M), and the transglutaminase inhibitors cystamine (5×10⁻⁴ M) and monodansyl cadaverine (10⁻⁴ M) on the aldosterone-mediated effects on mesen-
terio arteriole diameter, the effects of aldosterone on mesenteric arteriolar diameter were observed before and after treatments with these inhibitors. All drugs were added to the vessel bath 30 min before the administration of aldosterone, and were present throughout the study. To determine any additional effect of aldosterone on angiotensin II-induced vascular responses, the effect of angiotensin II alone and the combined effects of angiotensin II and aldosterone were examined in the same vessels. To investigate whether another steroid hormone could exert a vasocon-
strictor effect similar to that of aldosterone, the effects of incre-
mental doses of hydrocortisone (10⁻¹³ to 10⁻⁶ M) on arteriolar diameter were examined. At the end of each experiment, the vaso-
constrictor response to 40 mM KCl was estimated to confirm biologi-
cal reactivity. Vessels with constrictor responses <30% of the baseline diameter were excluded from the study.

2.6. Endothelial denudation

To assess the role of the endothelium in the effect of aldosterone, the endothelium was mechanically denuded by passing air through the lumen. The absence of a dilator response to acetylcholine (10⁻⁶ mol/L) after pre-constriction with 40 mM KCl was used to confirm the adequacy of denudation. Full dilation in response to sodium nitroprusside (10⁻⁶ mol/L) was used to confirm that the vascular smooth muscle cells (VSMCs) remained functionally intact after denudation. The diameter changes in endothelium-intact and endothelial-denuded arterioles in response to acetylcholine were 84.2 ± 2.2 and 7.5 ± 6.3%, respectively, relative to the full dilator response to sodium nitroprusside (10⁻⁶ mol/L). All other experiments of contraction studies were performed using endothelium-intact arterioles.

2.7. Protein extraction

Isolated mesenteric arteriole networks from WT mice were washed in cool phosphate-buffered saline (PBS), and equilibrated in albumin-PSS for 30 min at 37°C, followed by treatment with or without aldosterone (10⁻⁷ M) for 10 min. Some preparations treated with aldosterone were pre-treated with 5×10⁻⁴ M cysta-
mone or 10⁻⁴ M valsartan for 30 min. Then, the mesenteric arter-
ioles were moved to HGNT buffer (20 mM HEPES, 150 mM NaCl, 10% glycerin, 20 mM Na₂HPO₄, 0.1% Triton X-100, pH 7.4), including...
the Complete protease inhibitor cocktail (Roche), and sonicated on ice. Protein concentrations in the buffer were determined by the Bradford’s method.

### 2.8. Western blot analysis

Equal amounts of protein from mesenteric arterioles were applied to SDS-polyacrylamide gels (5–20% gradient gels). Protein was transferred to PVDF membranes (Bio-Rad Laboratories). After blocking for 1 h, membranes were incubated with anti-AT_{1} antibody at 4°C overnight, followed by 1 h incubation with the secondary antibody. Immunoreactive bands were detected using the ECL Plus detection system (Amersham Pharmacia Biotech).

### 2.9. In situ transglutaminase activity

To assess the effect of aldosterone on intracellular transglutaminase activity, the experiment was carried out on the mouse VSMC line (MOVAS) (American Type Culture Collection, #CL-2797) and the mesenteric arteriole networks isolated from WT mice. MOVAS cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium) at 37°C under 5% CO_{2}, and subcultured every 5–7 days with trypsin–EDTA. The media in all cultures were renewed every 2–3 days. Then, the cells were incubated with serum-free DMEM for 12 h prior to experiments. The mesenteric arteriole networks isolated from WT mice were minced and washed in cold PBS. Then, minced mesenteric arterioles were equilibrated in albumin–PSS at 37°C. MOVAS cells and minced mesenteric arterioles were labelled with 2 mM 5-(biontinamido)pentylamine (Pierce), a biotinylated polyamine, for 60 min at 37°C under 5% CO_{2}, and then treated with 10^{-7} M aldosterone for 10 min with or without pre-treatment with transglutaminase inhibitors for 30 min. To test the calcium dependency of transglutaminase activation, in another set of experiments, MOVAS cells were incubated with 10^{-5} M BAPTA-AM for 30 min, followed by 2 mM biotinylated polyamine for 60 min, and then treated with 10^{-7} M aldosterone without extracellular calcium for 10 min. The cells and minced mesenteric arterioles were harvested and sonicated on ice, and protein concentrations were determined. Intracellular transglutaminase activity was quantified by measuring the presence of incorporated 5-(biontinamido)pentylamine into proteins by a microplate assay using horseradish peroxidase-conjugated streptavidin and the TMB colour-developing kit (Pierce), as described by Zhang et al.\(^{27}\) Absorbance values measured at 450 nm were calculated as a percentage of basal absorbance (that is, with no drug additions) within a given group of samples.

### 2.10. Assay for angiotensin II

Isolated mesenteric arteriole networks from WT mice were incubated in albumin–PSS at 37°C for 30 min, and aliquots were sampled to measure the basal release of angiotensin II. After treatment with aldosterone at 10^{-7} M for 10 min, the albumin–PSS was sampled again to measure the angiotensin II level. The maximal diameter changes induced by aldosterone, the vasoconstrictor effect, we compared the effects of aldosterone between WT and AT_{1a}KO mice. In contrast to WT mice, only a negligible vasoconstrictor effect was observed in the mesenteric arteriolar network of AT_{1a}KO mice (n = 7) (Figure 3C), despite the fact that the vasoconstrictor response to 40 mM KCl was similar between WT and AT_{1a}KO mice (n = 7) (Figure 3D).

### 3. Results

#### 3.1. Effect of aldosterone on the mesenteric arteriolar diameter and the role of AT_{1} receptor in it

Figure 1A shows the time course of aldosterone-induced vasoconstriction in the mesenteric arterioles (n = 10). Aldosterone (10^{-6} M) produced a rapid vasoconstrictor effect. Notable vasoconstriction occurred at 10 s, and the response reached the maximum within a few minutes in almost every experiment. Figure 1B shows the dose dependency of the vasoconstrictor effect of aldosterone in WT mice. Aldosterone at doses >10^{-12} M caused a significant and dose-dependent vasoconstrictor effect with a maximum diameter change of −8.6 ± 0.3% from the baseline at a dose of 10^{-6} M (n = 54, P < 0.001). A representative example showing the dose-dependent, vasoconstrictor effect of aldosterone is shown in Figure 1C.

Pre-treatment with spironolactone or eplerenone did not affect these aldosterone-induced vasoconstrictor effects, and the maximal diameter changes induced by aldosterone before and after treatment with spironolactone were −8.6 ± 1.0 and −8.3 ± 1.0%, respectively (n = 7), while those before and after treatment with eplerenone were −9.5 ± 0.5 and −9.1 ± 0.7%, respectively (n = 7) (Figure 2A and B).

When vasoconstrictor responses were compared between endothelium-intact and endothelial-denuded arterioles (n = 6), there was no significant difference between them (Figure 2C).

Figure 2D shows the effect of hydrocortisone on the mesenteric arteriolar diameter: only a negligible vasoconstrictor response was noted. The GR antagonist RU486 did not influence the vasoconstrictor effect of aldosterone (diameter change, −8.8 ± 0.5 vs. −8.4 ± 0.7% at 10^{-6} M aldosterone, P = NS) (n = 6) (Figure 2D).

We recently showed that the vasoconstrictor effect of aldosterone in rat coronary arterioles is mediated via AT_{1} receptors.\(^{14}\) As shown in Figure 3A and B, either valsartan or candesartan abolished the vasoconstrictor effect of aldosterone. The maximal diameter changes induced by aldosterone before and after treatment with valsartan at 10^{-6} and 10^{-4} M were −8.8 ± 0.5 and −3.5 ± 0.7%, respectively, for 10^{-6} M (n = 6), and −9.2 ± 0.9 and −2.2 ± 0.4%, respectively, for 10^{-4} M (n = 8), whereas those before and after candesartan treatment were −8.9 ± 0.6 and −3.4 ± 1.1%, respectively (n = 7) (all P < 0.05). To further confirm the participation of AT_{1} receptors in aldosterone’s vasoconstrictor effect, we compared the effects of aldosterone between WT and AT_{1a}KO mice. In contrast to WT mice, only a negligible vasoconstrictor effect was observed in the mesenteric arterioles of AT_{1a}KO mice (n = 7) (Figure 3C), despite the fact that the vasoconstrictor response to 40 mM KCl was similar between WT (diameter change, −41.5 ± 2.0) and AT_{1a}KO mice (−42.1 ± 4.6) (P = NS). The AT_{2} Receptor blocker PD123319 did not influence aldosterone-induced vasoconstriction (diameter change, −9.6 ± 0.6 vs. −9.0 ± 0.7% at 10^{-6} M aldosterone, P = NS) (n = 7) (Figure 3D).
3.2. Mechanism for the vasoconstrictor effect of aldosterone

We first examined the possibility that aldosterone produces a vasoconstrictor effect by increasing the level of endogenous angiotensin II in the mesenteric arterioles. As shown in Figure 4A, angiotensin II (\(10^{-2}\) M) caused a significant and dose-dependent vasoconstrictor effect, like aldosterone, on the mesenteric arterioles of WT mice (\(P < 0.05\)), and co-administration of angiotensin II and aldosterone resulted in an additional effect to that induced by angiotensin II alone (\(P < 0.05\), \(n = 8\)). Benazepril showed no influence on the vasoconstrictor effect of aldosterone, and the maximal diameter changes before and after treatment with benazepril were \(-7.0 \pm 0.7\) and \(-7.1 \pm 0.6\)%, respectively (\(n = 7\)) (Figure 4B). When the effect of aldosterone on the release of angiotensin II from the mesenteric arteriole network was examined, no significant increase in angiotensin II concentration was observed after treatment with aldosterone (Figure 4C).

We then examined the role of intracellular transglutaminase activation in the effect of aldosterone. As shown in Figure 5A, both cystamine and monodansyl cadaverine inhibited the vasoconstrictor effect of aldosterone in WT mice. The maximal diameter changes induced by aldosterone before and after treatment with cystamine were \(-7.5 \pm 0.6\) and \(-0.4 \pm 0.4\)%, respectively (\(P < 0.05\), \(n = 7\)), whereas those before and after monodansyl cadaverine treatment were \(-9.2 \pm 1.3\) and \(-2.9 \pm 1.1\)%, respectively (\(P < 0.05\), \(n = 6\)). Neither cystamine nor monodansyl cadaverine affected the vasoconstrictor effect of angiotensin II (Figure 5B).

3.3. AT\(_1\) receptor dimer formation induced by aldosterone

Figure 6A illustrates the representative bands for AT\(_1\) protein in the presence or absence of aldosterone in the mesenteric arterioles (western blot analysis). There was a single immunoreactive band for AT\(_1\) protein at 50–60 kDa in the absence of aldosterone. Higher molecular weight immunoreactive bands for AT\(_1\) at 100–120 kDa emerged at 10 min after treatment with aldosterone. Pre-treatment with cystamine and valsartan inhibited the emergence of these high-molecular-weight immunoreactive bands for AT\(_1\) in response to aldosterone.

3.4. Effect of aldosterone on \textit{in situ} transglutaminase activity

As shown in Figure 6B and C, aldosterone increased the levels of pentynamine-incorporated protein, a measure of \textit{in situ} transglutaminase activity, by \(2.5 \pm 0.2\)-fold in MOVAS cells (\(P < 0.05\)) and by \(1.2 \pm 0.1\)-fold in the
mesenteric arterioles ($P < 0.05$) compared with a control experiment without aldosterone ($P < 0.05$ vs. control). Pretreatment with the transglutaminase inhibitors cystamine or monodansyl cadaverine inhibited this increase ($P < 0.05$). In calcium-free conditions, aldosterone did not influence the transglutaminase activity.

4. Discussion

Wehling et al.\textsuperscript{22} first showed unequivocal rapid effects of aldosterone, which were neither mimicked by cortisol nor blocked by spironolactone, and postulated that these non-genomic effects were mediated by a plasma membrane receptor distinct from the classical MR. In the present study, aldosterone was found to cause vasoconstriction in the resistance arterioles within a few minutes. This effect of aldosterone was not mimicked by hydrocortisone, and was unaffected by MR and GR antagonists, strongly suggesting that the vasoconstrictor effect of aldosterone is non-genomic and independent of classical MR. However, the mechanism underlying this non-genomic vasoconstrictor effect of aldosterone remains unclear.

We recently demonstrated that aldosterone induces a rapidly occurring vasoconstrictor effect in isolated rat coronary arterioles, and that this effect is not inhibited by spironolactone, but by AT\textsubscript{1} receptor blockers.\textsuperscript{14} Aldosterone potentiates the rennin–angiotensin system by some mechanism(s). It was shown that aldosterone increases local generation of angiotensin II by up-regulation of ACE mRNA expression and its activity.\textsuperscript{23,24} This raises the possibility that the present vasoconstrictor effect of aldosterone might be attributed to the generation of endogenous angiotensin II. However, the effect of aldosterone occurred too rapidly to be caused by \textit{de novo} synthesis of angiotensin II, and the transient stimulation with aldosterone did not increase angiotensin II release from mesenteric arteries. In addition, aldosterone-induced vasoconstriction was unaffected by the ACE inhibitor benazepril. These findings all suggest that locally generated endogenous angiotensin II via ACE activation is not involved in the present vasoconstrictor effect of aldosterone. Although a recent study showed the existence of a non-ACE-dependent angiotensin II-forming system,\textsuperscript{25} the involvement of this non-ACE-dependent system in the action of aldosterone was not established. Furthermore, we demonstrated that angiotensin II caused vasoconstriction, and that the addition of aldosterone resulted in a greater degree of vasoconstriction, indicating that the signalling pathway mediating aldosterone-induced vasoconstriction is not necessarily the same as that involving angiotensin II. It should be pointed out that aldosterone-induced vasoconstriction was small compared with that induced by angiotensin II.

Figure 2  (A and B) Effects of spironolactone and eplerenone on aldosterone-induced changes in mesenteric arteriolar diameter in wild-type mice. Neither spironolactone nor eplerenone influenced aldosterone's effect ($P = \text{NS}, \text{two-way ANOVA}$). (C) Effect of aldosterone on the mesenteric arteriolar diameter in endothelium-denuded arterioles. There was no difference in the vasoconstrictor effects between endothelium-intact and endothelium-denuded vessels ($P = \text{NS}, \text{two-way ANOVA}$). (D) Effects of hydrocortisone on mesenteric arteriolar diameter in wild-type mice, and the effect of the GR antagonist RU486 on the aldosterone-induced vasoconstrictor effect in wild-type mice. RU486 showed no influence on aldosterone's effect ($P = \text{NS}, \text{two-way ANOVA}$).
80% of classical receptor signalling occurs over 2 orders of ligand magnitude, and therefore the aldosterone’s vasoconstrictor effect was atypical. The mechanism of AT1 signalling induced by aldosterone seems not to be mediated via a classical ligand-receptor binding.

Various in vivo/vitro data have shown that aldosterone interacts with angiotensin II signalling via a non-genomic mechanism(s). Chai et al.26 showed that aldosterone potentiated the vasoconstrictor effect of angiotensin II in the coronary arteries, and that this effect was not blocked by spironolactone. Min et al.27 also reported that the synergistic effect of aldosterone and angiotensin II on the mitogenic response in VSMCs (within 15 min) was inhibited by the AT1 receptor blocker olmesartan, but not by spironolactone.27 Therefore, we further investigated the role of AT1 receptors in the vasoconstrictor effect of aldosterone.

The present results clearly show that the blockade of AT1 receptor activation by valsartan or candesartan suppresses the vasoconstrictor effect of aldosterone. Furthermore, as shown by the experiments in AT1aKO mice, a genetic deletion of the AT1a receptor blocked aldosterone-induced vasoconstriction. It should be pointed out that there are two pharmacologically indistinguishable subtypes of angiotensin II receptor, AT1 and AT2 receptors, and AT1 receptors can be further subdivided into AT1a and AT1b subtypes in mice. In this study, we examined the effect of aldosterone only in AT1a knockout mice, but not in AT1b or AT2 knockout mice. It was reported that systemic blood pressure was comparable in AT1b receptor knockout mice and WT mice.28

Regarding the role of AT2 receptor in the present vasoconstrictor effect of aldosterone, the AT2 receptor blocker PD123319 had no influence on this effect of aldosterone. Taken together, these findings suggest that AT1a receptors are key mediators in the signal transduction pathway of aldosterone, and their activation is essential for aldosterone-induced, non-genomic vasoconstriction.

AT1 receptors are activated not only by angiotensin II, but also by other mechanisms such as mechanical stress or receptor dimerization.15,16 Growing evidence suggests that receptor homo- and heterodimerization specifically modify the signalling downstream of individual receptor subtypes. AT1 receptors form heterodimers with other receptors, such as bradykinin B2 receptors,29 angiotensin II type 2 receptors,30 β2-adrenergic receptors,31 and dopamine D1 receptors.32 For example, heterodimerization of the AT1 receptor with bradykinin B2 receptor specifically enhances angiotensin II-stimulated signalling in pre-eclampsia.33 Recently, AbdAlla et al.16 demonstrated that the intracellular factor XIIIa transglutaminase cross-links with agonist-induced AT1 receptor homodimers, and that these cross-linked dimers display enhanced AT1 signalling and desensitization. Therefore, we investigated the involvement of AT1 receptor dimerization in the aldosterone-induced non-genomic vasoconstriction. The results showed that
treatment of mesenteric arterioles with aldosterone leads to the generation of AT1 dimers. We further showed that pre-treatment with the transglutaminase inhibitor cystamine not only blocked the dimerization of AT1 receptors, but blunted the vasoconstrictor effect of aldosterone, while showing no influence toward the vasoconstrictor effect of angiotensin II. Moreover, an in situ transglutaminase activity assay showed that aldosterone increased intracellular transglutaminase activity. These findings suggest that the activation of AT1 receptors by aldosterone is dependent on the dimerization of these receptors via the activation of intracellular transglutaminase, and is clearly distinct from the activation of AT1 receptors induced by angiotensin II binding. Transglutaminase is a calcium-dependent enzyme that catalyzes the posttranslational modification of proteins by transamidation of specific polypeptide-bound glutamine residues. Wehling et al. reported that aldosterone non-genomically increases the intracellular calcium level in VSMCs, which is consistent with our finding of aldosterone-induced, calcium-dependent transglutaminase activation.

Valsartan and candesartan were recently described as inverse agonists of AT1 receptors, blocking AT1 signalling by inducing a conformational change to an inactive structure. In this study, valsartan not only blocked aldosterone-induced vasoconstriction, but also the formation of AT1 dimers. This indicates that the inverse agonist-induced conformational change in AT1 receptors may contribute to the inhibition of the formation of AT1 dimers.

5. Implications

In addition to the regulation of salt and water balance, aldosterone has been shown to exert some deleterious
effects, including cardiovascular remodelling, endothelial dysfunction, and renal injury. With the use of MR antagonists, the randomized aldactone evaluation study (RALES) and eplerenone neurohormonal efficacy and survival study (EPHESUS) established the beneficial effects of chronic blockade of aldosterone on morbidity and mortality in patients with chronic heart failure. Recently, we reported that aldosterone caused a significant, mild degree of vasoconstriction in rat coronary arterioles, which may result in the exacerbation of myocardial ischaemia and ventricular function. The present study clearly demonstrates that this vasoconstrictor effect of aldosterone is dependent on AT1 receptors, and that their activation may be mediated by receptor dimerization. Importantly, this

Figure 6  (A) Immunoblot detection of AT1 receptor monomer and dimer in mesenteric arterioles. Aldosterone increased the levels of dimer protein, whereas the transglutaminase inhibitor cystamine and the AT1 receptor blocker valsartan inhibited dimer formation induced by aldosterone. (B and C) Quantitative analysis of transglutaminase-catalyzed incorporation of 5-(biotinamido)pentylamine into protein as a measure of in situ activity in VSMCs line (B) (n = 6–8) and mouse mesenteric arterioles (C) (n = 8–10). Aldosterone induced an increase in intracellular transglutaminase activity. Pre-treatment with the transglutaminase inhibitor cystamine (Cys) or monodansyl cadaverine (Mono) inhibited this increase. In a calcium-free condition (Ca free), aldosterone did not influence transglutaminase activity. *P < 0.05.
effect of aldosterone was not blocked by MR antagonist, but by AT1 receptor blockers. AT1 receptor blockers are among the most beneficial drugs that improve the prognosis of patients with chronic heart failure. An inhibitory effect of AT1 receptor blockers on aldosterone-induced vasoconstriction may partly underlie some of the benefits of these drugs.

6. Limitation

Despite this novel activation mechanism of AT1 receptors as the initial signalling event in the vasoconstriction of resistance arterioles induced by aldosterone, the upstream signalling pathway leading to transglutaminase activation and dimerization of AT1 receptors still remains unclear. Furthermore, we did not identify which type of transglutaminase is involved, and we did not show which out of homodimers and heterodimers including the AT1 receptor is induced by aldosterone. Further biochemical investigations are needed. The vessels were pressurized from the luminal side without flow, and each drug was administered to the bath side. This seems to be different from the physiological condition in which drugs affect both inner and outer sides.

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