Rac1-Mediated Activation of Mineralocorticoid Receptor in Pressure Overload–Induced Cardiac Injury

Nobuhiro Ayuzawa, Miki Nagase, Kohei Ueda, Mitsuhiro Nishimoto, Wakako Kawarazaki, Takeshi Marumo, Atsu Aiba, Takayuki Sakurai, Takayuki Shindo, Toshiro Fujita

Abstract—There is increasing evidence for a crucial role of aberrant mineralocorticoid receptor (MR) activation in heart failure, with clinical studies showing beneficial effects of MR blockade. However, the mechanisms of MR activation in heart failure remain unclear. In this study, we observed that the small GTPase Rac1 contributes to myocardial MR activation, whereas Rac1-MR pathway activation leads to cardiac dysfunction. Mouse hearts subjected to chronic pressure overload induced by transverse aortic constriction showed Rac1 activation and increased nuclear accumulation of MR and expression of MR target genes, suggesting MR activation. Pharmacological inhibition of Rac1 and heterozygous deletion of Rac1 in cardiomyocytes suppressed Rac1-induced MR signaling and reduced NADPH oxidase 4 gene induction and reactive oxygen species overproduction, which attenuated transverse aortic constriction–induced cardiac hypertrophy and dysfunction. Consistently, treatment with the selective MR antagonist eplerenone blocked transverse aortic constriction–induced MR signaling and NADPH oxidase 4 gene upregulation, which improved cardiac hypertrophy and dysfunction. These findings suggest that Rac1-MR pathway activation in the myocardium is involved in development of heart failure induced by pressure load via recruitment of the responsible isoform of NADPH oxidase. Thus, the cardiac Rac1-MR–NADPH oxidase 4 pathway may be a therapeutic target for treatment of the pressure-overloaded heart. (Hypertension. 2016;67:99-106. DOI: 10.1161/HYPERTENSIONAHA.115.06054.) • Online Data Supplement

Key Words: aldosterone ■ heart failure ■ hypertension ■ NADPH oxidase ■ Rac1

Heart failure can often result from chronic neurohormonal and mechanical stress and remains a leading cause of death worldwide. The mineralocorticoid receptor (MR), a steroid receptor for corticosteroids, plays a pathophysiological role in the development of heart failure. High circulating levels of MR ligands were reportedly associated with poor prognosis in patients with heart failure, whereas recent clinical trials of MR ligands were reportedly associated with poor prognosis in patients with heart failure, whereas recent clinical trials of MR ligands showed beneficial effects of MR blockade on morbidity and mortality. 

Although the aldosterone–MR system was conventionally associated with renal sodium absorption in the distal tubules, experimental studies have revealed a direct effect of aldosterone on the heart and a pathological role of MR in the myocardium. Aldosterone excess or MR activation in the myocardium leads to unfavorable responses, including hypertrophy, fibrogenic signaling, and arrhythmogenic changes. Furthermore, MR ablation in cardiomyocytes was shown to attenuate cardiac dysfunction in a mouse model of myocardial infarction and chronic pressure overload.

The mechanism for MR activation in cardiac injury remains unclear. Although ligand excess is an important stimulus, aberrant MR activation can occur with a normal or even low aldosterone status in some pathological conditions, leading to end-organ damage. Indeed, Dahl salt-sensitive rats fed a high-salt diet develop cardiac injury with enhanced MR signaling, despite low levels of circulating aldosterone, whereas eplerenone dramatically retards progression of cardiac remodeling. Recently, several studies have suggested that MR activity is also affected by factors other than its ligands. We previously identified Rac1, a rho family small GTPase, as a novel modulator of MR activity and demonstrated the pathological role of Rac1-mediated MR activation in the kidney of proteinuric disease and salt-sensitive hypertension. Further, overexpression of constitutively active mutant Rac1 in rat cardiomyocytes promoted nuclear accumulation of MR and increased MR-dependent transcriptional activity, regardless of the ligand, implicating a potential contribution of Rac1-MR signaling in cardiac diseases.

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From the Division of Clinical Epigenetics, Research Center for Advanced Science and Technology (N.A., K.U., M. Nishimoto, W.K., T.M., T.F.), and Laboratory of Animal Resources, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine (A.A.), The University of Tokyo, Tokyo, Japan; Department of Anatomy and Life Structure, Faculty of Medicine, Juntendo University, Tokyo, Japan (M. Nagase); CREST, Japan Science and Technology Agency, Tokyo, Japan (T.M., T.F.); and Department of Cardiovascular Research, Institute of Pathogenesis and Disease Prevention, Shinshu University Graduate School of Medicine, Matsumoto, Japan (T. Sakurai, T. Shindo).

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Correspondence to Toshiro Fujita, Division of Clinical Epigenetics, Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan. E-mail Toshiro.FUJITA@rcast.u-tokyo.ac.jp

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In the present study, we found that Rac1 mediates MR activation in the myocardium, which leads to cardiac dysfunction by recruitment of the responsible isoform of NADPH oxidase, NOX4, in pressure overload–induced heart failure.

Methods

Methods are available in the online-only Data Supplement.

Animals and Experimental Design

All animal procedures were approved by the Animal Care Committee of The University of Tokyo. Chronic pressure overload induced by transverse aortic constriction (TAC) surgery was performed as previously described19 using a 27-gauge needle. In the drug treatment study, 9-week-old male C57BL/6J mice (Tokyo Laboratory Animals Sciences, Tokyo, Japan) were infused with NSC23766 (Tocris Biosciences, Bristol, UK); at a dose of 15 mg/kg/d via an osmotic minipump (Alzet, Palo Alto, CA) starting from 7 days before TAC surgery or fed eplerenone (Pfizer, New York, NY; 1.25 g/kg, in chow) from 3 days after TAC surgery. NSC23766 is a selective small-molecule Rac1 inhibitor, which inhibits the activation of Rac1 by hindering its binding to the Rac-specific guanine nucleotide exchange factor without altering the access of the related RhoA or Cdc42 to their specific guanine nucleotide exchange factors.20,21 A similar dose of NSC23766 used in our previous report reduced Rac1 activity in the kidney without apparent evidence of organ toxicity in mice.15 Cardiomyocyte-specific Rac1-deficient mice were generated by crossing Rac1-floxed mice22 on C57BL/6J background with transgenic mice expressing Cre recombinase under control of α-myosin heavy chain promoter (Myh6-cre)23,24 on a C57BL/6J background. In the Rac1-floxed mice, theloxP site is inserted to delete the entire exon 1 of the Rac1 gene as previously described.22 Transgenic Myh6-cre mice were kindly provided by Dr Sano (Keio University School of Medicine, Department of Cardiology). On mating these 2 lines, we were unable to obtain live offspring with the Rac1−/− genotype, indicating the embryonic lethality of the Rac1−/− mice.19,20 Myh6-cre−/+ mice were bred as previously described19 using a 27-gauge needle. In the early period (2 weeks after treatment), suggesting MR activation in the myocardium, which leads to cardiac hypertrophy and dysfunction in the myocardium (Figure 2F).

Results

Pharmacological Inhibition of Rac1 Abolished MR Activation in the Pressure-Overloaded Heart and Subsequent Cardiac Hypertrophy and Dysfunction

To clarify the role of Rac1-evoked MR activation in cardiac injury, we performed an in vivo study using a mouse model of TAC. In our experiment, chronic pressure overload induced by TAC resulted in cardiac hypertrophy at 2 weeks of treatment, which was followed by gradually developing cardiac dysfunction 7 weeks later. Two weeks after surgery, the expression of activated form of Rac1 protein was significantly increased in the pressure-overloaded heart, without any change in the total amount of Rac1 protein, indicating Rac1 activation (Figure S1A). As reported previously,25 MR protein was mainly located in the nuclear fraction rather than in cytosol in the heart at steady state (Figure S1B). Nuclear accumulation of MR started to increase in the pressure-overloaded heart during the early period (2 weeks after treatment), suggesting MR activation (Figure S1C).

The Rac1 activation induced by TAC persisted during the 7-week treatment (Figure 1A), which was accompanied by further increased accumulation of nuclear MR (Figure 1B). Consistent with MR activation, gene expression of plasminogen activator inhibitor-1 (Serpinel) and the serine (or cysteine) peptidase inhibitor, clade A, member 3 N (Serpina3n), which are downstream targets of MR in cardiomyocytes, was upregulated in pressure-overloaded hearts (Figure 1C), despite slightly, but not significantly, elevated circulating aldosterone and unchanged corticosterone levels (Table S1).

To clarify the relationship between Rac1 activity and MR signaling, we evaluated the effects of pharmacological intervention with the Rac1 inhibitor NSC23766 or the MR blocker eplerenone on the TAC-induced changes. Treatment with NSC23766 inhibited TAC-induced activation of Rac1 (Figure 1A) and was associated with a significant reduction in MR signaling (Figure 1B and 1C), without altering the levels of ligands (Table S1). This inhibition of MR signaling by the Rac1 inhibitor is consistent with the result of treatment with eplerenone, a selective MR antagonist (Figure 1B and 1C). These findings suggest that cardiac Rac1 stimulated by pressure overload induces MR activation in a ligand-independent manner, as previously reported in renal diseases.14–17

Next, we examined the impact of pharmacological intervention of the Rac1-MR pathway on TAC-induced cardiac injury. A 7-week TAC treatment markedly increased the size of cardiomyocytes and heart weight and reduced ejection fraction in the left ventricle (Figure 2A–2E; Table S1), with increased gene expression of atrial natriuretic peptide (Nppa) and β-myosin heavy chain (Myh7), which are markers of pathological hypertrophy in the myocardium (Figure 2F). Both the TAC-induced cardiac hypertrophy and dysfunction were significantly attenuated by treatment with NSC23766 or eplerenone (Figure 2A–2F; Table S1). Overall, these findings suggest that Rac1-mediated MR activation is involved in the pathogenesis of cardiac hypertrophy and dysfunction in the pressure-overloaded heart.

Rac1-MR Pathway Was Associated With Nox4-Related Reactive Oxygen Species Production in the Pressure-Overloaded Heart

To further investigate the underlying mechanism linking the Rac1–MR pathway and heart failure, we assessed reactive oxygen species (ROS) generation and its major source of NADPH oxidase activity because of their reported association with Rac126,27 and MR9,11,28 in cardiac injury. We found that pressure overload markedly increased the amount of 4-hydroxy-2-nonenal, a cytotoxic lipid peroxidation product, and NADPH-dependent O2·− production. These were suppressed by either NSC23766 or eplerenone (Figure 3A and 3B).
Nox2 and Nox4, the major isoforms in the myocardium,\textsuperscript{29,30} play different roles in cardiac injury. Recent experiments demonstrated that Nox4,\textsuperscript{31,32} but not Nox2,\textsuperscript{33} is the isoform responsible for pressure overload–induced cardiac injury. Consistently, in the current study, gene expression of Nox4,\textsuperscript{31,32} but not Nox2,\textsuperscript{33} is the isoform responsible for pressure overload–induced cardiac injury. To confirm that myocardial Rac1 serves as a modulator of aberrant MR activation in the pressure-overloaded heart, we generated cardiomyocyte-specific heterozygous Rac1-deficient mice by crossing Rac1-\textit{floxed} mice\textsuperscript{22} with Myh6-\textit{cre} \textit{flox/+}; Rac1\textsuperscript{CM+/−} transgenic mice.\textsuperscript{23,24} Rac1\textsuperscript{CM+/−}; Myh6-\textit{cre} \textit{flox/+} (termed Rac1\textsuperscript{CM+/−}) showed cardiomyocyte-specific expression of Cre recombinase (Figure S2A–S2C) and a 34\% reduction in gene expression of C\textit{yb} (Nox2) gene expression (Figure 3C).

**Heterozygous Deletion of Rac1 in Cardiomyocytes Confirmed the Role of Rac1-MR Pathway in Pressure Overload–Induced Cardiac Injury**

To confirm that myocardial Rac1 serves as a modulator of aberrant MR activation in the pressure-overloaded heart, we generated cardiomyocyte-specific heterozygous Rac1-deficient mice by crossing Rac1-\textit{floxed} mice\textsuperscript{22} with Myh6-\textit{cre} \textit{flox/+}; Myh6-\textit{cre} \textit{flox/+} (termed Rac1\textsuperscript{CM+/−}) showed cardiomyocyte-specific expression of Cre recombinase (Figure S2A–S2C) and a 34\% reduction in gene expression of C\textit{yb} (Nox2) gene expression (Figure 3C).

**Figure 1.** The Rac1 inhibitor NSC23766 (NSC) and the mineralocorticoid receptor (MR) blocker eplerenone (EPL) both suppressed MR signaling in the pressure-overloaded heart. Mice were subjected to 7 weeks of Sham or transverse aortic constriction (TAC) with or without treatment with NSC or EPL. A, Immunoblot of active and total Rac1 protein in the left ventricle (LV). Actin was used as the loading control. Bar graph shows the results of densitometric analysis (n=6 per group). B, MR abundance in the nuclear fraction of LV. cAMP response element–binding protein (CREB) was used as the loading control of the nuclear fraction (n=6–9 per group). C, Expression of MR target genes, Serpine1 and Serpina3n, in the LV assessed by quantitative reverse transcriptase polymerase chain reaction (RT-PCR; n=6–10 per group). Values are presented as means±SEM fold change relative to Sham group. *\textit{P}<0.05 and **\textit{P}<0.01 vs Sham; ##\textit{P}<0.01 vs TAC.

**Figure 2.** Protective effects of pharmacological Rac1 inhibition or mineralocorticoid receptor (MR) blockade on pressure overload–induced cardiac hypertrophy and dysfunction. Mice were subjected to 7 weeks of Sham or transverse aortic constriction (TAC) with or without treatment with NSC23766 (NSC) or eplerenone (EPL). A, Representative photomicrographs of Masson’s trichrome-stained left ventricle (LV) sections. Scale bars, 50 \textmu m. B, Cross-sectional areas (CSA) of cardiomyocytes in the LV (n=6–10 per group). C, Representative M-mode LV echocardiogram. D, LV ejection fraction (EF; n=5–10 per group). E, Heart weight/body weight ratio (HW/BW; n=6–10 per group). F, Quantitative analysis of \textit{Nppa} and Myh7 gene expression in the LV (n=6–10 per group). Values are presented as fold change relative to Sham group. Data are expressed as means±SEM. *\textit{P}<0.05 and **\textit{P}<0.01 vs Sham; ***\textit{P}<0.01 vs TAC. AW indicates LV anterior wall; LVID, LV internal diameter; and PW, LV posterior wall.
Rac1 in the left ventricle compared with Rac1\(^{WT}\); Myh6-cre\(^{−/−}\) (Rac1\(^{WT}\)) mice (Figure S2D). Isolated cardiomyocytes from Rac1\(^{CM+/−}\) mice showed markedly reduced expression of Rac1 protein compared with those from Rac1\(^{WT}\) mice (Figure S2E).

Next, we applied Sham or TAC to Rac1\(^{CM+/−}\) and Rac1\(^{WT}\) mice. Seven weeks after surgery, the amount of active Rac1 and MR signaling in the left ventricle in Rac1\(^{WT}\) mice, whereas these abnormalities were significantly attenuated in Rac1\(^{CM+/−}\) mice (Figure 4A–4C). These results support the hypothesis that myocyte-Rac1 is involved in the aberrant activation of MR in the pressure-overloaded heart. Furthermore, TAC-induced NADPH-dependent O\(_2^−\) production and Nox4 gene expression were suppressed in Rac1\(^{CM+/−}\) mice compared with Rac1\(^{WT}\) mice (Figure 4C and 4D).

With the suppression of Rac1-MR signaling and the resulting reduction in Nox4-related ROS production, the hearts of Rac1\(^{CM+/−}\) mice were protected against TAC-induced hypertrophy and dysfunction. After 7 weeks of TAC, either the increase in heart weight and size of cardiomyocytes or the induction of Nppa and Myh7 genes were ameliorated in Rac1\(^{CM+/−}\) mice (Figure 5C and 5D; Tables S2 and S3) following NSC23766 and eplerenone treatment (Figure 4A–4C). These findings suggest a pathological role of Rac1-mediated MR activation in the failing heart.

Although several studies have revealed a pathological role of myocardial MR in heart failure, the mechanism of MR activation in the failing heart remains unclear. In tubular epithelial cells of the classical aldosterone-sensitive distal nephron, where 11\(^β\)-HSD2 protects MR from cortisol, with improvement of ejection fraction (Figure 5A and 5B; Table S3). Finally, the results of hemodynamic analysis in the left ventricle clearly showed a protective effect of cardiomyocyte-specific Rac1 deletion on TAC-induced cardiac dysfunction (Figure 5E).

**Discussion**

In this study, we observed that chronic pressure overload activated Rac1, and in turn, Rac1 activation increased nuclear accumulation of MR protein and the expression of MR target genes in mice hearts. In addition to systemic MR blockade, both pharmacological Rac1 inhibition and heterozygous deletion of Rac1 in cardiomyocytes abolished TAC-induced MR activation, resulting in attenuated cardiac hypertrophy and dysfunction, along with reduced TAC-induced ROS overproduction and expression of Nox4 gene. These findings suggest a pathological role of Rac1-mediated MR activation in the failing heart.

**Figure 3.** Effect of an Rac1 inhibitor or mineralocorticoid receptor (MR) blocker on transverse aortic constriction (TAC)–induced reactive oxygen species overproduction and gene induction of NADPH oxidase 4 (Nox4). Mice were subjected to 7 weeks of Sham or TAC with or without treatment with NSC23766 (NSC) or eplerenone (EPL). A, Immunoblot of 4-hydroxy-2-nonenal (4-HNE) in the LV (n=6 per group). B, NADPH-dependent O\(_2^−\) production measured by lucigenin chemiluminescent assay in the LV (n=6–10 per group). C, Quantitative analysis of Nox4 and Cybb (Nox2) gene expression in the LV (n=6 per group).

**Figure 4.** Cardiomyocyte-specific heterozygous deletion of Rac1 reduced transverse aortic constriction (TAC)–induced mineralocorticoid receptor (MR) signaling, gene induction of NADPH oxidase 4 (Nox4), and reactive oxygen species overproduction. Rac1\(^{WT}\) and Rac1\(^{CM+/−}\) mice were subjected to 7 weeks of Sham or TAC. A, Rac1 activity in the LV (n=4–7 per group). B, MR abundance in the nuclear fraction of the LV (n=4–7 per group). C, Expression of Serpina3n and Nox4 genes in the LV (n=4–7 per group). D, NADPH-dependent O\(_2^−\) production in the LV was measured by lucigenin chemiluminescent assay in additional experiments (n=6–12 per group). Data are expressed as mean±SEM fold change relative to Rac1\(^{WT}\)-Sham group. *P<0.05 and **P<0.01 vs Sham; †P<0.05, ‡P<0.01, and §N.S. vs TAC.
MR is located mainly in the cytoplasm and is translocated to the nucleus on aldosterone binding. On the other hand, in nonepithelial tissues like the myocardium, where the expression of 11β-HSD2 is low or undetectable, the MR is supposedly saturated with abundantly circulating cortisol. Hermández-Díaz et al reported that myocardial MR was mainly localized in the nucleus, regardless of circulating corticosteroid levels. However, it is unclear whether the nuclear accumulation of MR in the heart changes in pathological conditions.

In the present study, we found that nuclear accumulation of MR was further promoted in the pressure-overloaded heart, indicating aberrant MR activation. Moreover, the Rac1 inhibitor NSC23766 and heterozygous deletion of Rac1 in cardiomyocytes suppressed this response without affecting the level of circulating ligands, suggesting that activated Rac1 in the myocardium mediates MR activation in the failing heart in a ligand-independent fashion. These findings are compatible with our previous observation in cultured cardiomyocytes that overexpression of active Rac1 induced MR activation in a ligand-independent manner in cells cultured with serum-depleted medium or when the cellular MR gene was replaced with a ligand-resistant mutant. Given that the ligand binding of MR in cardiomyocytes is already saturated at steady state by the absence of 11β-HSD2, Rac1 serves as an enhancer of MR activation via increased nuclear MR translocation in the pressure-overloaded heart, in a similar manner to the essential role for Rac1 in the nuclear localization of β-catenin, a transcription factor involved in canonical Wnt signaling.

Aberrant Rac1-mediated MR activation in the myocardium was supported by changes in the expression of various target genes of MR in the myocardium. Fejes-Tóth et al identified various genes, including Serpine1, as potential targets of the aldosterone–MR system in a cardiomyocyte cell line stably expressing MR. In addition, Latouche et al recently indicated that Serpine1 and Serpina3n genes were MR targets in the myocardium using mice with cardiomyocyte-specific overexpression of MR or glucocorticoid receptor. In the present study, gene expression of Serpine1 and Serpina3n was upregulated in parallel with Rac1 activation and the subsequent nuclear accumulation of MR in the pressure-overloaded heart, both of which were suppressed by either systemic or local inhibition of Rac1. The TAC-induced expression of these genes was also abolished by MR antagonists, along with the blockade of nuclear MR accumulation. Furthermore, all of these interventions targeted at the Rac1-MR pathway ameliorated cardiac dysfunction, which indicates the involvement of Rac1-mediated MR activation in the development of TAC-induced heart failure.

With regard to the NADPH oxidases involved in the development of heart failure, the 2 major isoforms of NADPH oxidase in cardiomyocytes, Nox2 and Nox4, have markedly different properties in terms of regulation and subcellular localization. Nox2 activation requires stimulus-induced membrane translocation of cytosolic regulatory subunits, including Rac1. Using cardiomyocyte-specific Rac1-deficient mice, several studies have reported a pathological role of Rac1 participating in Nox2 activation in angiotensin II–induced cardiac injuries. By contrast, Nox4

Figure 5. Cardiomyocyte-specific heterozygous deletion of Rac1 prevents pressure overload–induced cardiac hypertrophy and dysfunction. Rac1WT and Rac1CM+/− mice were subjected to 7 weeks of Sham or transverse aortic constriction (TAC). A, Representative M-mode LV echocardiogram. B, LV ejection fraction (EF; n=3–7 per group). A similar result was obtained in an additional experiment (Table S3). C, Heart weight/body weight ratio (HW/BW) and cross-sectional areas (CSA) of cardiomyocytes (n=4–7 per group). D, Quantitative analysis of Nppa and Myh7 gene expression in the LV (n=4–7 per group). Values are presented as fold change relative to Rac1WT-Sham group. E, Hemodynamic analysis of maximum left ventricular pressure (dP/dtmax) and LV end-diastolic pressure (LVEDP) in an additional experiment (n=6–8 per group). Data are expressed as means±SEM. **P<0.01 vs Rac1WT-Sham; #P<0.05 and ##P<0.01 vs Rac1WT-TAC. AW indicates LV anterior wall; LVID, LV internal diameter; and PW, LV posterior wall.
is distributed on the membranes of mitochondria and in the nuclei of cardiomyocytes, and constitutively generates O$_2^-$ to locally regulate ROS generation. It should be noted that Nox2-deletion failed to attenuate TAC-induced cardiac injury, although it showed a protective effect on angiotensin II–induced hypertrophy and remodeling. By contrast, cardiomyocyte-specific deletion of Nox4 attenuated pressure overload–induced cardiac hypertrophy and dysfunction by reducing local ROS overgeneration in mitochondria and nuclei. Consistent with the results of several studies demonstrating the association of Nox4 in MR-related cardiac injuries, our study showed that Nox4 gene induction by pressure overload was suppressed by all 3 interventions targeting the Rac1-MR pathway, in association with improvement in cardiac dysfunction. Taken together, these findings suggest that Rac1 serves as an activator of MR, a nuclear transcriptional factor, which is distinct from the classical role of Rac1 in Nox2 activation, and that Rac1-MR pathway activation plays a critical role in increased Nox4 gene transcription and the resulting cardiac dysfunction in the pressure-overloaded heart.

It should be noted that both myocyte MR and nonmyocyte MR may play a role in the development of heart failure. Indeed, genetic ablation of myeloid MR attenuated cardiac injury induced by TAC, L-NAME/angiotensin II, and DOCA/salt. In addition, recent studies suggest a role of endothelial MR in leukocyte adhesion in human coronary artery disease and endothelial proliferation and angiogenesis in a TAC model. Moreover, endothelial cell-specific MR-deficient mice are protected against DOCA/salt-induced cardiac injury, suggesting the possible involvement of endothelial MR in cardiac injury. However, the role of endothelial Rac1-MR pathway in heart failure remains unknown. Given the lack of aldosterone rise in plasma by the Rac1 inhibitor in contrast to the MR antagonist, cardiac Rac1 was activated in response to TAC but adrenal Rac1 might not be affected by TAC. Moreover, several investigators demonstrated the different response of Rac1 to certain stimuli in the individual tissues: the opposite response of Rac1 activation by mechanical stretch in endothelial cells and vascular smooth muscle cells. Further studies are required to clarify whether the nonmyocyte Rac1-MR pathway is involved in the development of TAC-induced heart failure.

In summary, we found that TAC-induced Rac1 activation induced aberrant MR activation in the myocardium, which leads to cardiac dysfunction via recruitment of the responsible NADPH oxidase, NOX4, in the pressure-overloaded heart. Our results suggest that the Rac1-MR-NOX4 pathway may be an alternative therapeutic target for the treatment of heart failure.

**Perspectives**

MR activation plays a crucial role in the pathogenesis of cardiovascular diseases, and MR blockade is now a standard strategy in the treatment of heart failure. In the present study and others, the beneficial effects of Rac1 inhibition were comparable to MR blockade in both rodent models of cardiac disease and chronic kidney disease, regardless of the levels of plasma aldosterone. Thus, Rac1 inhibition may be an alternative target to MR blockade for the treatment of heart failure and chronic kidney disease. However, given the diverse biological effects of Rac1, including activation of NOX2, a NADPH oxidase, Rac1 inhibitors may be superior to MR blockers in models of heart failure other than TAC. Thus, a suitable dose of Rac1 inhibitor could protect from heart failure by inhibition of both MR activity and NOX2 ROS production, with a reduced incidence of hyperkalemia. Further studies are required to evaluate the roles of MR-independent Rac1 signaling in various models of heart failure.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is New?**

- Rac1 mediates aberrant mineralocorticoid receptor (MR) activation in the pressure-overloaded heart.
- Rac1–MR pathway activation causes cardiac injury via recruitment of NADPH oxidase 4–related reactive oxygen species overproduction.

**What Is Relevant?**

- The findings provide novel insight into the mechanism of MR activation in heart failure and suggest Rac1 as a potential therapeutic target in hypertensive cardiac diseases.

**Summary**

We provide novel evidence that Rac1 causes MR activation in the pressure-overloaded heart and that Rac1–MR activation induces cardiac injury via induction of NADPH oxidase 4. These findings indicate an important role of the Rac1–MR pathway in the pressure-overloaded heart and suggest that Rac1 may be a therapeutic target in hypertensive cardiac diseases.
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ONLINE SUPPLEMENT

Rac1-Mediated Activation of Mineralocorticoid Receptor in Pressure Overload-Induced Cardiac Injury

Nobuhiro Ayuzawa¹, Miki Nagase², Kohei Ueda¹, Mitsuhiro Nishimoto¹, Wakako Kawarazaki¹, Takeshi Marumo¹,³, Atsu Aiba⁴, Takayuki Sakurai⁵, Takayuki Shindo⁵, Toshiro Fujita¹,³

¹Division of Clinical Epigenetics, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan
²Department of Anatomy and Life Structure, Faculty of Medicine, Juntendo University, Tokyo, Japan
³CREST, Japan Science and Technology Agency, Tokyo, Japan
⁴Laboratory of Animal Resources, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
⁵Department of Cardiovascular Research, Institute of Pathogenesis and Disease Prevention, Shinshu University Graduate School of Medicine, Matsumoto, Japan

Short title: Rac1 activates cardiac mineralocorticoid receptors

Address correspondence to: Toshiro Fujita
Division of Clinical Epigenetics, Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan
Tel: +81-3-5452-5070; Fax: +81-3-5452-5057; E-mail: Toshiro.FUJITA@rcast.u-tokyo.ac.jp
Methods

**Immunoblot analysis and Rac1 activation assay**
We performed immunoblot analysis as previously described. Nuclear and cytosol extracts were prepared with commercially available kits (BioVision, Milpitas, CA). The primary antibodies used included antibodies for Rac1 (Millipore, Temecula, CA), actin (Sigma-Aldrich, St. Louis, MO), MR (clone 1D5; a gift from Prof. Gomez-Sanchez, University of Mississippi), cyclic AMP response element-binding protein (Millipore, Temecula, CA), 4-hydroxy-2-nonenal (JaICA, Shizuoka, Japan), Cre recombinase (Novagen, Madison, WI), and glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz, Santa Cruz, CA). Activity of Rac1 was assessed by the glutathione S-transferase (GST) pull-down assay as previously described.

**Quantitative RT-PCR**
Gene expression was quantitatively analyzed by real-time reverse-transcription polymerase chain reaction (RT-PCR), as described previously. Rac1, Serpine1, Nox4, and Cybb gene expression was assessed with specific TaqMan gene expression primer-probes (Applied Biosystems, Foster City, CA). Gene expression of cre, Nppa, Myh7, and Serpina3n was quantified by the SYBR green probe method, using the specific primers described in Table S4.

**Histomorphometric analysis**
We fixed hearts in 4% paraformaldehyde solution and embedded them in paraffin. Cross-sections (3 μm) at the papillary muscle level were stained with Masson’s trichrome. For morphometric analysis, sections were digitalized at high magnification (×100–400), and the myocyte cross-sectional area (CSA) and fibrosis fraction were determined by computerized pixel counting using Image J software (National Institutes of Health). Only nucleated cells from transversely cut muscle fibers, around 100 cells per section, were evaluated in CSA analysis. Interstitial and perivascular fibrosis areas were calculated as a percentage of the total myocardial area and vessel area, respectively.

**Immunohistochemistry**
Immunostaining was performed as previously described, with some modification. Briefly, paraffin-embedded sections (4 μm thick) were incubated with rabbit anti-Cre recombinase antibody (Novagen, Madison, WI), followed by biotinylated secondary antibody. Immunoreactivity was detected using a VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA) and a metal-enhanced DAB kit (Pierce, Rockford, IL). Finally, the sections were counterstained with methyl-green.
**Echocardiography**

Trans-thoracic echocardiographic studies were performed with a 12.0 MHz phased-array ultrasound system (Aplio, Toshiba, Tokyo, Japan), as described previously. Mice were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (2.5 mg/kg i.p.) to achieve the appropriate heart rate (per min) control under light anesthesia for echocardiography evaluation.

**Hemodynamic measurement**

For hemodynamic measurement, mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the right carotid artery was cannulated with a 1.2-Fr pressure catheter (FTS-1211B-0018, Scisense, Ontario, Canada), which was then advanced into the left ventricle. Pressure signals were recorded and analyzed using LabScribe2 software (iWorx Systems, Dover, NH) with a sampling rate of 500 Hz. Serial data at a heart rate of approximately 300 beats per min were used in analysis to minimize data deviation.

**Lucigenin chemiluminescent assays**

NADPH-dependent O$_2^-$ production was measured using bis-N-methylacridinium nitrate (lucigenin) chemiluminescence in a microplate luminometer (LB 9507, Berthold Technologies, Bad Wildbad, Germany), as previously described. Briefly, approximately 25 mg of left ventricle tissue was homogenized in 1 mL of Hank’s balanced salt solution (Life Technologies, Carlsbad, CA) containing 10 mmol/L HEPES-NaOH (pH 7.4), and incubated for 20 min at 37°C. Lucigenin (10 μmol/L) and NADPH (100 μmol/L) were then added into the sample, just before luminescence measurement. The O$_2^-$ level was measured as relative light units by chemiluminescence, and standardized by tissue weight.

**Isolation of cardiomyocytes**

Isolation of cardiomyocytes from hearts of adult Rac1$^{CM+/−}$ and Rac1$^{WT}$ mice was performed using the protocol reported by O’Connel et al., with some modification. Briefly, mice were anesthetized with isoflurane (3%) and heparinized (8000 IU/kg). The heart was quickly excised from the opened chest cavity, and extraneous tissues were removed. The ascending aorta was then cannulated with 24-gauge needle and tied with 7-0 silk thread. The heart was perfused at 37°C with perfusion buffer (120.4 mmol/L NaCl, 14.7 mmol/L KCl, 0.6 mmol/L KH$_2$PO$_4$, 0.6 mmol/L Na$_2$HPO$_4$, 1.2 mmol/L MgSO$_4$-7H$_2$O, 10 mmol/L HEPES-NaOH pH7.2, 4.6 mmol/L NaHCO$_3$, 3.75 g/L Taurine, 1 g/L butanedione monoxime, 1 g/L glucose) for 4 min, then switched to perfusion buffer supplemented with 2.4 mg/mL collagenase II (CLS2, Worthington Biochemical, Lake Wood, NJ) to digest the extracellular matrix. After 3 min, CaCl$_2$ was added to the perfusate at a final concentration of 40 μmol/L, and continued
digestion for 8 min. The heart was then cut from the cannula, teased into small pieces, and then gently pipetted several times with 2-mm opening pipette tip until dissociation into cell suspension in perfusion buffer supplemented with 12.5 μmol/L CaCl₂ and 10% fetal bovine serum. The cell suspension was filtered with nylon mesh (400 μm pore size), centrifuged for 3 min at 20 g, and resuspended in perfusion buffer supplemented with 12.5 μmol/L CaCl₂ and 10% fetal bovine serum. Gradual calcium reintroduction was then performed at room temperature. The cell suspension was centrifuged for 3 min at 20 g, resuspended in perfusion buffer supplemented with 10% fetal bovine serum and increasing concentration of CaCl₂, and incubated for 2 min. This three-step process was repeatedly performed with stepwise-increases in CaCl₂ concentration of 100, 400, 900, and 1200 μmol/L. Finally, we obtained purified cardiomyocytes, with approximately 70–80% rod-shaped.
References


Table S1. Characteristics and echocardiographic parameters of mice that received Sham or TAC surgery with or without pharmacological interventions.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham</th>
<th>TAC</th>
<th>TAC/NSC</th>
<th>TAC/EPL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Base line</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>22.6 ± 0.3</td>
<td>22.8 ± 0.2</td>
<td>22.8 ± 0.2</td>
<td>22.7 ± 0.2</td>
</tr>
<tr>
<td><strong>7 week</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>27.7 ± 0.5</td>
<td>27.5 ± 0.4</td>
<td>27.5 ± 0.3</td>
<td>26.4 ± 0.9</td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>122 ± 4</td>
<td>262 ± 9*</td>
<td>214 ± 7*‡</td>
<td>176 ± 10*‡</td>
</tr>
<tr>
<td>PAC (pg/mL)</td>
<td>314 ± 67</td>
<td>413 ± 53</td>
<td>390 ± 96</td>
<td>1833 ± 114*‡</td>
</tr>
<tr>
<td>Corticosterone (ng/mL)</td>
<td>117 ± 27</td>
<td>177 ± 25</td>
<td>117 ± 33</td>
<td>195 ± 26</td>
</tr>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart Rate (/min)</td>
<td>259 ± 11</td>
<td>264 ± 6</td>
<td>276 ± 17</td>
<td>290 ± 16</td>
</tr>
<tr>
<td>LVAWd (mm)</td>
<td>0.64 ± 0.01</td>
<td>1.06 ± 0.04*</td>
<td>0.96 ± 0.03*</td>
<td>0.98 ± 0.03*</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.60 ± 0.03</td>
<td>1.10 ± 0.03*</td>
<td>1.06 ± 0.04*</td>
<td>0.95 ± 0.03*†</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>4.31 ± 0.10</td>
<td>5.15 ± 0.12*</td>
<td>4.75 ± 0.10</td>
<td>4.29 ± 0.14‡</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.87 ± 0.05</td>
<td>3.94 ± 0.12*</td>
<td>3.26 ± 0.09‡</td>
<td>2.91 ± 0.15‡</td>
</tr>
<tr>
<td>FS (%)</td>
<td>33.2 ± 1.3</td>
<td>23.7 ± 0.6*</td>
<td>31.5 ± 0.9‡</td>
<td>32.3 ± 1.6‡</td>
</tr>
</tbody>
</table>

PAC, plasma aldosterone concentration; LVAWd, left ventricular anterior wall dimension in diastole; LVPWd, left ventricular posterior wall dimension in diastole; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; FS, fractional shortening. *P<0.01 vs. Sham; †P<0.05 and ‡P<0.01 vs. TAC.
Table S2. Characteristics and echocardiographic parameters of Rac1\(^{WT}\) and Rac1\(^{CM+/-}\) mice that received Sham or TAC surgery.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rac1(^{WT})</td>
<td>Rac1(^{CM+/-})</td>
</tr>
<tr>
<td>Base line</td>
<td>n=7</td>
<td>n=5</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>34.4 ± 1.3</td>
<td>33.3 ± 1.7</td>
</tr>
<tr>
<td>7 week</td>
<td>n=7</td>
<td>n=5</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>40.8 ± 2.0</td>
<td>40.0 ± 1.6</td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>167 ± 9</td>
<td>146 ± 5</td>
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<tr>
<td>Echocardiography</td>
<td>n=7</td>
<td>n=5</td>
</tr>
<tr>
<td>Heart Rate (/min)</td>
<td>341 ± 21</td>
<td>393 ± 19</td>
</tr>
<tr>
<td>LVAWd (mm)</td>
<td>0.68 ± 0.05</td>
<td>0.71 ± 0.01</td>
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<tr>
<td>LVPWd (mm)</td>
<td>0.73 ± 0.04</td>
<td>0.76 ± 0.06</td>
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<tr>
<td>LVIDd (mm)</td>
<td>4.51 ± 0.17</td>
<td>4.27 ± 0.16</td>
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<tr>
<td>LVIDs (mm)</td>
<td>2.87 ± 0.14</td>
<td>2.73 ± 0.10</td>
</tr>
<tr>
<td>FS (%)</td>
<td>36.6 ± 1.2</td>
<td>36.0 ± 0.8</td>
</tr>
</tbody>
</table>

Abbreviations as in Table S1. \(^*P<0.05\) and \(^\dagger P<0.05\) vs. Rac1\(^{WT}\)-Sham; \(^\ddagger P<0.05\) and \(^\S P<0.01\) vs. Rac1\(^{WT}\)-TAC.
Table S3. Characteristics, echocardiographic parameters, and hemodynamic analysis of Rac1\textsuperscript{WT} and Rac1\textsuperscript{CM+/−} mice that received Sham or TAC surgery in an additional trial.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rac1\textsuperscript{WT}</td>
<td>Rac1\textsuperscript{CM+/−}</td>
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<tr>
<td>Base line</td>
<td>n=10</td>
<td>n=14</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>25.8 ± 0.9</td>
<td>25.8 ± 0.7</td>
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<tr>
<td>7 week</td>
<td>n=10</td>
<td>n=14</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>29.3 ± 1.1</td>
<td>29.0 ± 0.6</td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>129 ± 7</td>
<td>131 ± 5</td>
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<tr>
<td>HW/BW (mg/g)</td>
<td>4.4 ± 0.1</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>Echocardiography</td>
<td>n=10</td>
<td>n=7</td>
</tr>
<tr>
<td>Heart Rate (/min)</td>
<td>308 ± 17</td>
<td>314 ± 10</td>
</tr>
<tr>
<td>LVAWd (mm)</td>
<td>0.56 ± 0.01</td>
<td>0.56 ± 0.02</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.56 ± 0.02</td>
<td>0.55 ± 0.01</td>
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<tr>
<td>LVIDd (mm)</td>
<td>4.46 ± 0.14</td>
<td>4.41 ± 0.11</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.38 ± 0.13</td>
<td>2.31 ± 0.15</td>
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<tr>
<td>FS (%)</td>
<td>47.0 ± 1.6</td>
<td>47.8 ± 2.6</td>
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<tr>
<td>EF (%)</td>
<td>83.5 ± 1.4</td>
<td>83.9 ± 2.0</td>
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<tr>
<td>Hemodynamics</td>
<td>n=8</td>
<td>n=7</td>
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<tr>
<td>Heart Rate (/min)</td>
<td>334 ± 19</td>
<td>352 ± 28</td>
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<tr>
<td>LVP\textsubscript{max} (mmHg)</td>
<td>92.7 ± 4.3</td>
<td>92.4 ± 3.5</td>
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<tr>
<td>dP/dt\textsubscript{min} (mmHg/sec)</td>
<td>−4502 ± 354</td>
<td>−4638 ± 413</td>
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<tr>
<td>Tau (Glantz, msec)</td>
<td>18.6 ± 1.5</td>
<td>16.9 ± 0.8</td>
</tr>
</tbody>
</table>

dP/dt\textsubscript{max}, peak rate of pressure rise; dP/dt\textsubscript{min}, peak rate of pressure decline; Tau, relaxation time constant calculated using the Glantz method. Other abbreviations as in Table S1.

*P<0.05 and †P<0.05 vs. Rac1\textsuperscript{WT}-Sham; ‡P<0.01 vs. Rac1\textsuperscript{WT}-TAC.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Pair (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cre</td>
<td>Forward: GCGTTCGAACGCACTGATTTC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TACACCAGAGACGGAAATCCA</td>
</tr>
<tr>
<td>Nppa</td>
<td>Forward: TTCGGGGGTAGGATTGACAGGA</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGTGACACACCACAAGGGCTTA</td>
</tr>
<tr>
<td>Myh7</td>
<td>Forward: TACAGGCTGCGGCTACCTCTCTA</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCCAGCCCTCCCTTCTCAGACTTC</td>
</tr>
<tr>
<td>Serpina3n</td>
<td>Forward: AGCTGGCTGTTTCAGCTCTGTA</td>
</tr>
<tr>
<td></td>
<td>Reverse: CATCCATTCCAACGTGCCATCTG</td>
</tr>
</tbody>
</table>
Figure S1. Activation of Rac1 and MR in the pressure-overloaded heart. A, Immunoblot of GTP-bound, active Rac1, and total Rac1 in the left ventricle (LV) of mice after 2 weeks of Sham or TAC surgery. Actin was used as the loading control. B, MR abundance in the nuclear and cytosolic fraction of LV of mice at steady state. cAMP response element binding protein (CREB) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as the loading control of nuclear and cytosolic fraction, respectively. C, Immunoblot of nuclear and cytosolic MR protein in LV of mice after 2 weeks of Sham or TAC.
Figure S2. Cardiomyocyte-specific heterozygous deletion of Rac1 in mice. A, Gene expression of cre in the left ventricle (LV), liver, kidney, and brain, assessed by quantitative RT-PCR. Cre recombinase mRNA was detected only in LV of Rac1WT and Rac1CM+/- mice (n=5–7 per group). Data are expressed as the fold change relative to LV of Rac1WT mice. B, Immunoblot of Cre recombinase in the nuclear fraction of the LV, liver, kidney, and brain. Cre recombinase was detected only in the nuclear fraction of the LV in Rac1WT (WT) and Rac1CM+/- (+/-) mice. C, Representative immunohistochemical staining for Cre recombinase in the LV of Rac1CM+/- mice. Cre recombinase was expressed in nuclei of cardiomyocytes (arrows). Scale bars, 25 μm. D, Gene expression of Rac1 in the LV, assessed by quantitative RT-PCR. Rac1 mRNA expression in the LV was significantly reduced in Rac1CM+/- mice compared with Rac1WT mice (n=5–7 per group). Data are expressed as the fold change relative to Rac1WT mice. E, Immunoblot of Rac1 protein expression in isolated cardiomyocytes. Data are expressed as mean ± SEM. **P<0.01.