Oxidative Stress Causes Mineralocorticoid Receptor Activation in Rat Cardiomyocytes
Role of Small GTPase Rac1

Miki Nagase, Nobuhiro Ayuzawa, Wakako Kawarazaki, Kenichi Ishizawa, Kohei Ueda, Shigetaka Yoshida, Toshiro Fujita

Abstract—Overactivation of the mineralocorticoid receptor signaling is implicated in cardiovascular disease, including hypertensive heart disease. Oxidative stress is suggested to augment mineralocorticoid receptor signal transduction, but the precise mechanisms remain unclear. Mineralocorticoid receptor activity is regulated by multiple factors, in addition to plasma ligand levels. We previously identified Rac1 GTPase as a modulator of mineralocorticoid receptor activity. Here we show that oxidative stress induces mineralocorticoid receptor activation in a ligand-independent, Rac1-dependent manner in cardiomyocytes. Oxidant stress was induced in rat cultured cardiomyocytes (H9c2) by L-buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis. BSO depleted intracellular glutathione and concomitantly increased reactive oxygen species (199%; \( P < 0.01 \)). BSO significantly enhanced the corticosterone-induced, mineralocorticoid receptor–dependent luciferase reporter activity (186%; \( P < 0.01 \)) and basal luciferase activity without ligand stimulation. These effects were inhibited by the antioxidant N-acetylcysteine. The ligand independency of BSO action was indicated using a mutant mineralocorticoid receptor that does not bind ligands. With this mutant mineralocorticoid receptor, BSO-evoked mineralocorticoid receptor activation remained intact, whereas ligand-induced mineralocorticoid receptor activation was abolished. We next examined the involvement of Rac1. BSO increased active Rac1 in a redox-dependent fashion, and Rac inhibition suppressed the enhancing effect of BSO. Constitutively active Rac1, indeed, potentiated mineralocorticoid receptor transactivation. Furthermore, mineralocorticoid receptor transactivation by BSO was accompanied by enhanced nuclear accumulation of mineralocorticoid receptor. We conclude that alteration of redox state modulates mineralocorticoid receptor–dependent transcriptional activity via Rac1 in the heart. This redox-sensitive, ligand-independent mineralocorticoid receptor activation may contribute to the processes by which oxidant stress promotes cardiac injury. (Hypertension. 2012;59[part 2]:500-506.)

Key Words: mineralocorticoid receptor ■ cardiomyocytes ■ oxidative stress ■ Rac1 ■ buthionine sulfoximine

Overactivation of the mineralocorticoid receptor (MR), a receptor for corticosteroids, has been implicated in cardiovascular disease. High circulating levels of aldosterone and cortisol were reportedly associated with poor prognosis in patients with heart failure.1 Genetically engineered animals with augmented MR signaling in the heart exhibited cardiac hypertrophy, heart failure, coronary dysfunction, arrhythmia, perivascular inflammation, and fibrosis.2–4 Conversely, cardiomyocyte-specific MR knockout mice were protected against cardiac remodeling and contractile dysfunction after myocardial infarction5 and chronic pressure overload.6 The significance of MR activation in heart failure and hypertensive heart disease was highlighted by large-scale randomized clinical trials.7,8 Although aldosterone excess is an important stimulus to MR, it was revealed that MR can also be activated in normal or even low-aldosterone states.9–11 We recently identified an alternative, ligand-independent pathway of MR activation by Rac1, a Rho family small GTPase, through the analysis of RhoGDI\textsuperscript{-}knockout mice.12 This “Rac1-mediated MR activation” actually played a critical role in the pathogenesis of chronic kidney disease and salt-sensitive hypertension.12,13

Oxidative stress is widely recognized as a major contributor to the cardiac pathologies.14 Several reports suggest that oxidative stress augments MR signal transduction. For example, glucocorticoids activated cardiomyocyte MR during experimental myocardial infarction, a condition with increased oxidants, and aggravated cardiac damage.15 MR-mediated chronotropic, proarrhythmogenic response in cultured cardiomyocytes was augmented by oxidative stress.16 We reported that MR signaling was paradoxically enhanced in the heart and kidney of obese hypertensive rats with excessive salt intake and that antioxidant Tempol suppressed MR activation and concomitantly improved target organ.
damage. However, the precise mechanisms by which oxidant stress induces MR activation have not been clearly elucidated. In the present study, we investigated whether oxidant stress causes MR activation in cultured rat cardiomyocytes and explored the underlying mechanisms, focusing on the involvement of Rac1.

Methods

Cell Culture

Rat embryonic heart-derived myogenic cell line, H9c2 (American Type Culture Collection CRL-1446), was grown in DMEM supplemented with 10% FBS and antibiotics in a humidified incubator with 5% CO2 at 37°C. Before steroid treatment, FBS was replaced with charcoal-stripped FBS (HyClone).

Glutathione Depletion and Glutathione Assay

L-Buthionine-((S,R))-sulfoximine (BSO), a selective inhibitor of γ-glutamylcysteine synthetase, was used to induce oxidative stress by depleting glutathione (GSH), a potent intracellular thiol-containing antioxidant.18 Cellular glutathione content was measured using a Bioxytech GSH-400 assay kit (Oxis International Inc). Glutathione levels were normalized to protein concentration.

Measurement of Reactive Oxygen Species

Levels of intracellular reactive oxygen species (ROS) were determined using the probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (Molecular Probes), which is oxidized to highly fluorescent 2',7'-dichlorofluorescein in the presence of ROS.19 The fluorescence was quantitated using Accuri C6 flow cytometer (Tomy Digital Biology).

Transfection and Luciferase Assay

Transient transfection with Lipofectamine 2000 (Invitrogen) and luciferase assay was carried out as described previously.12 Plasmids used were pCMX-FLAG-hMR, pMRE-LUC, pEF-BOS-My-hCARac1G12V, pEF-BOS-My-hDNRac1 T17N, and pCMX-FLAG-hMR-GFP.

Rac1 Activation Assay

Rac1 activity was assessed by glutathione S-transferase pull-down assay using PAK1-PBD beads (Millipore).12

Immunoblotting

Immunoblotting was performed as described previously.12 Nuclear fractions were extracted with commercially available kits (BioVision). Whole cell lysates were prepared using SDS buffer (62.5 mmol/L of Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and Complete protease inhibitor mixture).

Mutagenesis

Plasmid-encoding mutant MR, pCMX-FLAG-hMR<sup>T3158C(L979P)</sup>, was created with a QuikChange II site-directed mutagenesis kit (Agilent Technologies). The primer used was 5'-GGGACGCGCAAGCCGCTACTTCCACCGGAAG-3'. This mutation in the ligand-binding domain, causing loss of ligand binding, was found in patients with type 1 pseudohypoaldosteronism.20

Statistical Analysis

Data are expressed as mean±SEM. Statistical significance (P<0.05) was determined by ANOVA followed by Tukey post hoc tests (for multiple comparisons) or unpaired t test (for 2 groups).

Results

BSO Induces Oxidant Stress in Rat Cardiomyocytes

To alter cellular redox state, we used BSO, an inhibitor of glutathione synthesis. Exposure of H9c2 cells to BSO (0.5 mmol/L) for 24 hours severely depleted glutathione (84% decrease; P<0.01; Figure 1A). Concomitantly, BSO increased ROS levels, as assessed by 2',7'-dichlorofluorescein fluorescence (199%; P<0.01; Figure 1B).

Effects of BSO on MR-Dependent Transcriptional Activity

To evaluate whether BSO-evoked oxidant stress modulates the transactivational function of MR, we transfected plasmids encoding MR and mineralocorticoid response element-luciferase into H9c2 cells and performed luciferase reporter assay. Because cardiomyocytes do not possess the enzyme 3β-hydroxysteroid dehydrogenase 2,21 we used corticosterone as an MR ligand. Corticosterone (10<sup>−12</sup> to 10<sup>−6</sup> mol/L) elevated luciferase reporter activity in a concentration-dependent manner (Figure 2A). The response elicited by corticosterone (10<sup>−9</sup> mol/L) was completely blocked by spironolactone (10<sup>−9</sup> mol/L) but was not affected by RU486 (10<sup>−6</sup> mol/L), certificating that this dose of corticosterone transactivates the reporter solely via MR (Figure 2B).

BSO significantly increased the MR-driven luciferase activity evoked by corticosterone (10<sup>−9</sup> mol/L; 36-fold versus 20-fold; P<0.01; Figure 2C, right). In addition, BSO en-
The transcriptional activation of the mineralocorticoid receptor (MR) was also observed when we stimulated MR with 10^{-2}E), showing that the enhancing effect of BSO was not abolished by RU486, which did not suppress the BSO-induced elevation of corticosterone-stimulated luciferase activity (Figure 2B). The enhancing effects of BSO were abrogated by an antioxidant N-acetylcysteine (Figure 2D). On the other hand, RU486 did not suppress the BSO-induced elevation of corticosterone-stimulated luciferase activity (Figure 2E), showing that the enhancing effect of BSO was not mediated via glucocorticoid receptor. BSO-induced MR activation was also observed when we stimulated MR with 10^{-9}mol/L of aldosterone (Ald; 10^{-9}mol/L), whereas overexpression of CARac1 significantly increased the basal and corticosterone-stimulated MR transcriptional activity in H9c2 cells (Figure 4B). These findings indicate that Rac1 GTPase plays an indispensable role in oxidative stress-induced MR activation.

**Luciferase Assay With Mutant MR**

To determine the ligand dependency of BSO- and CARac1-induced MR activation, we performed luciferase reporter assay using a mutant MRL979P (Ald979P), which does not bind ligands (Figure 4). With this mutant MRL979P, corticosterone failed to increase luciferase activity. On the other hand, MR activation in response to BSO or CARac1 remained intact both in the presence and absence of ligands. These findings support the ligand independence of MR transactivation by Rac1.

**BSO Facilitates Nuclear Accumulation of MR**

To assess nuclear translocation of MR, we administered BSO 4 hours posttransfection of MR-green fluorescent protein (GFP) and analyzed MR-GFP in the nuclear fraction 48 hours after transfection. BSO increased the amount of nuclear MR-GFP in the basal condition without corticosterone (Figure 5A). On stimulation with corticosterone (10^{-9}mol/L; 1 hour), MR-GFP was translocated into the nucleus. Again, BSO facilitated nuclear accumulation of MR-GFP. BSO-evoked increment of nuclear MR-GFP was prevented by EHT1864 (Figure 5B). MR-GFP in whole cell lysates was shown to validate equivalent transfection. These data indicate...
that BSO-induced MR transactivation may be attributed, at least in part, to altered nuclear-cytoplasmic shuttling of MR.

**Discussion**

We reported previously that MR activity is modulated by Rac1 in a ligand-independent fashion and that overactivation of the Rac1-MR pathway contributes to the pathogenesis of chronic kidney disease\textsuperscript{12} and salt-sensitive hypertension.\textsuperscript{13} The present study extrapolates these studies and demonstrates, using cultured rat cardiomyocytes, that BSO-evoked oxidative stress activates Rac1, which, in turn, causes MR activation, as evidenced by enhanced MR-driven transcriptional activity and nuclear accumulation of MR. Two Rac inhibitors, NSC23766\textsuperscript{12} and EHT1864,\textsuperscript{22} as well as DNRac1, effectively abrogated the enhancing effects of BSO. Thus, our data indicate for the first time that the “Rac1-MR pathway” exists in the heart as well and participates in the pathophysiology of ROS-induced cardiac dysfunction.

In the current study, we clearly demonstrated the ROS-mediated activation of Rac1 in cardiomyocytes: BSO increased the active form of Rac1, which was blocked by antioxidant N-acetylcysteine. Hydrogen peroxide also activated Rac1. Rac1 was reported to be activated by various stimuli, including mechanical stretch, inflammatory cytokines, growth factors, high glucose, angiotensin II, and aldosterone. In the case of ROS, most studies considered ROS as a Rac1 effector, because RacGTPase is a component of NADPH oxidase, and its activation is suggested to stimulate NADPH oxidase activity and ROS generation.\textsuperscript{23} However, a few in vitro studies directly indicated ROS as a Rac1 activator,\textsuperscript{24,25} similar to our observations. In addition, Wang et al\textsuperscript{26} indirectly showed that vascular endothelial growth factor–evoked mitochondrial ROS activated Rac1 and induced cell migration in endothelial cells. Mitochondrial delivery of vitamin E or overexpression of mitochondrial catalase blocked Rac1 activation and suppressed vascular endothelial growth factor–stimulated endothelial cell migration, which was reversed by CARac1. Furthermore, Rac inhibition was shown to be effective against ischemia/reperfusion injury in vivo without scavenging ROS,\textsuperscript{27} which may be explained by ROS-mediated Rac1 regulation. Altogether, ROS activates Rac1, which, in turn, produces ROS via NADPH oxidase activation, leading to the formation of a feed-forward loop between Rac1 and ROS.

Although oxidative stress is suggested to enhance MR activity in cardiomyocytes,\textsuperscript{9,15,16} the precise mechanisms had long been a mystery. The present study demonstrated, for the first time, that Rac1 mediates the BSO-induced, redox-dependent MR activation in cardiomyocytes using a luciferase reporter system. Then how would ROS/Rac1 activate MR? Transcriptional activity of MR, a nuclear receptor, can be regulated at multiple steps, such as the amount and modification (eg, phosphorylation, acetylation, and SUMOylation) of MR, nuclear translocation, epigenetic modification, recruitment of coregulators, and cross-talk with other transcription factors. Among these potential mechanisms, ROS/Rac1 may activate MR by affecting nuclear translocation of MR based on our data showing the increased nuclear shuttling of MR by BSO and Rac1. Supporting this, oxidative stress was shown to alter nucleo-cytoplasmic shuttling of transcription factor pancreatic duodenal homeobox 1 via c-Jun NH\textsubscript{2}-terminal kinase.\textsuperscript{28} In the case of the glucocorticoid receptor, redox state modulated the glucocorticoid-inducible gene expression,\textsuperscript{29} and oxidative stress impaired both ligand-dependent and -independent nuclear translocation of the glucocorticoid receptor.\textsuperscript{30} Similar to oxidative stress, Rac1 was shown recently to regulate nuclear translocation of several transcription factors, such as β-catenin, in the canonical Wnt signaling\textsuperscript{31} and STAT5 in cytokine signaling.\textsuperscript{32} Taken together, the redox-sensitive modulation of nuclear MR translocation may constitute an important mechanism for ROS-induced activation of MR signal transduction.

ROS may otherwise activate MR by altering the recruitment of coregulators, as suggested for coactivator Ubc9\textsuperscript{33} and NADH-dependent corepressor.\textsuperscript{34} Alternatively, ROS/Rac1-induced phosphorylation may have some role. Future studies are necessary to elucidate the precise mechanisms of MR activation by Rac1.

It should be noted that Rac inhibitor EHT1864 not only blocked BSO-evoked enhancement but also profoundly suppressed corticosterone activation of MR (Figure 3B). Because
DNRac1 did not attenuate the corticosterone-mediated luciferase activity. EHT1864-mediated inhibition of MR activity may not be attributable to Rac1 inactivation but to specific actions of EHT1864. Rosenblatt et al. reported recently that EHT1864 inhibited estrogen receptor-α transcriptional activity and suppressed estrogen-induced cell proliferation in breast cancer cells. They suggested that downregulation of estrogen receptor-α expression by EHT1864 may be the underlying mechanism. Further studies are necessary to elucidate the detailed mechanisms of action of EHT1864 on MR signaling.

The members of the small GTPase Rho family, Rac1, RhoA, and Cdc42, function as critical modulators of actin cytoskeleton remodeling and regulate diverse cellular processes, including adhesion, migration, cell cycle progression, and gene expression. Recent studies have underscored the critical roles of the Rho family in cardiac pathophysiology. Cdc42 functions as an antihypertrophic molecular switch in the heart, by antagonizing nuclear factor of activated T-cells activity via the c-Jun NH2-terminal kinase. RhoA is also cardioprotective against ischemia/reperfusion injury via protein kinase D. On the other hand, growing evidence indicates that Rac1 promotes cardiac injury. Rac1 is an essential mediator of cardiomyocyte hypertrophy and dilated cardiomyopathy. Inactivation of Rac1 by cardiomyocyte-specific Rac1 knockout, Rac specific inhibitors, and adenoviral gene transfer of DNRac1 is beneficial against ischemia/reperfusion, angiotensin II infusion, and diabetic cardiac injury.

Figure 4. Effects of mutant mineralocorticoid receptor (MR) on corticosterone- (Cort), L-buthionine sulfoximine- (BSO), and CARac1-induced MR transactivation. We performed luciferase reporter assay using a mutant MR<sup>3979P</sup> that does not bind ligands. A. Effects of intact MR (left) and mutant MR<sup>3979P</sup> (right) on Cort- and BSO-induced MR activation. Inset shows the result of mutant MR. n = 6 each group. **P < 0.01 vs control (Cont). B. Effects of intact MR (left) and mutant MR<sup>3979P</sup> (right) on Cort- and CARac1-induced MR activation. Inset shows the result of mutant MR. Vec indicates Vector; Rac1, CARac1. n = 6 each group. *P < 0.01 vs Vec.

Figure 5. Effects of L-buthionine sulfoximine (BSO) and Rac inhibition on nuclear accumulation of mineralocorticoid receptor (MR)-green fluorescent protein (GFP). A, Effects of BSO (0.5 mmol/L). H9c2 cells were transfected with pCMX-hMR-GFP, treated with BSO, and stimulated with or without corticosterone (Cort; 10<sup>-9</sup> mol/L, 1 hour) at 48 hours posttransfection. n = 4 each group. *P < 0.05, **P < 0.01. B, Effects of EHT (20 μmol/L) on BSO-evoked potentiation of nuclear translocation in the presence of Cort (10<sup>-9</sup> mol/L, 1 hour). EHT was administered 2 hours before Cort stimulation. n = 4 each group. *P < 0.01 vs control (Cont). EHT indicates EHT1864.
Rac1-induced MR activation may contribute to these pathogenic conditions.

**Perspectives**

We demonstrated that Rac1 induces MR activation in rat cultured cardiomyocytes, contributing to ROS-mediated MR activation and cardiac damage. ROS/Rac1-induced MR transcriptional activation may be attributed, at least in part, to the enhanced nuclear accumulation of MR. We are currently identifying the endogenous target genes downstream of BSO-Rac1-MR cascade and investigating how this pathway culminates in cardiac injury. It should be noted, however, that the present article deals solely with cell-based studies and used a cultured cardiomyocyte cell line derived from an embryonic rat heart. Mature myocytes might behave differently. Furthermore, in the in vivo heart, the initial damage may be a vascular insult modulated by MR with secondary damage to the myocytes. The role of Rac1 in vascular tissues, where 11β-hydroxysteroid dehydrogenase 2 is present and the ligand is aldosterone, might be quite different. Altogether, how Rac1 may be involved in the whole organism may differ from the results obtained in the present study. It is essential to carry out animal experiments to get in vivo evidence of the ROS-Rac1-MR pathway in cardiac pathology, including hypertensive heart disease, and ultimately to identify clinical conditions in which this pathway is critically involved.

**Sources of Funding**

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Takeda Science Foundation, and Japan Foundation for Applied Enzymology.

**Disclosures**

None.

**References**


Oxidative Stress Causes Mineralocorticoid Receptor Activation in Rat Cardiomyocytes:
Role of Small GTPase Rac1
Miki Nagase, Nobuhiro Ayuzawa, Wakako Kawarazaki, Kenichi Ishizawa, Kohei Ueda,
Shigetaka Yoshida and Toshiro Fujita

Hypertension. 2012;59:500-506; originally published online January 9, 2012;
doi: 10.1161/HYPERTENSIONAHA.111.185520
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/59/2/500

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial
Office. Once the online version of the published article for which permission is being requested is located,
click Request Permissions in the middle column of the Web page under Services. Further information about
this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/