NADPH oxidase-mediated Rac1 GTP activity is necessary for nongenomic actions of the mineralocorticoid receptor in the CA1 region of the rat hippocampus

Fumiko Kawakami-Mori,1 Tatsuo Shimosawa,1,2 Shengyu Mu,1 Hong Wang,1 Sayoko Ogura,1,3 Yutami Yatomi,2 and Toshiro Fujita1

Departments of 1Nephrology and Endocrinology and 2Clinical Laboratory, University of Tokyo Faculty of Medicine; and 3Division of Laboratory Medicine, Faculty of Medicine, Department of Pathology and Microbiology, Nihon University of Medicine, Tokyo, Japan

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The mineralocorticoid receptor (MR) is a member of the nuclear receptor superfamily mainly known for its actions in controlling sodium transport across epithelial cells, such as the epithelium of the distal portions of the nephrons and the colonic epithelium. The genomic actions of MRs have also been implicated in pathophysiological effects, such as inflammatory, fibrotic, and hypertrophic changes in the peripheral tissues. For example, MR activation has been shown to induce hypertrophy and fibrosis of the cardiomyocytes (17, 43). MR inhibition mitigates myocardial fibrosis and apoptosis by reducing oxidative stress without affecting systolic blood pressure (22). Interestingly, Yanomamo Indians, members of a Brazilian tribe with very low salt intakes and high serum aldosterone levels, are predisposed to neither hypertension nor arteriosclerosis (31). High salt loading in obese, spontaneously hypertensive rats induced the production of reactive oxygen species (ROS), which improved with the administration of an MR blocker (29). Rac1, which is downstream of ROS production, induces actions of MRs independently of ligand binding (41). These findings suggest that organ damage associated with MRs might be independent of ligands but are closely related to ROS production.

The brain is one of the key targets of glucocorticoid actions (25, 39). MR expression is localized to specific regions and is important for cognitive memory, fear memory, and salt preference (3, 37, 40). Corticosterone (CS) is known to bind to both MRs and glucocorticoid receptors (GRs) (35). MRs have a 10-fold higher affinity ($K_d = 0.5–1$ nM) for CS than GRs ($K_d = 2–5$ nM) (6, 35), and under normal conditions, CS binds preferentially to the MR. With increases in the production of CS under conditions of stress, CS binds to low-affinity and membrane-bound MRs in the hippocampal region (33). MR activation can exert rapid nongenomic actions and enhance long-term potentiation induction (1, 13), whereas blockade of MRs, which can pass through the blood-brain barrier (37), attenuates memory consolidation (2, 18). Meanwhile, GRs mediate slow, long-acting effects through transcriptional pathways (genomic actions), and long-term potentiation is conserved even after the levels of CS have returned to baseline, normal levels. GR actions also facilitate long-term depression by inducing sustained elevation of CS production (7, 8). Thus, MRs and GRs can cope with stress by coordinating memory formation through both nongenomic and genomic actions. Furthermore, it has recently been reported that cerebral ischemia induces MR expression and ROS production in the cortex and that MR blockers reduce ROS accumulation (32). MR expression has also been found to govern the fate of neurons in ischemic areas (23). Although a number of studies have shed light on the intracellular signaling associated with the nongenomic actions of MRs in the central nervous system (3, 19, 30), and some reports have shown ROS involvement in MR signaling in the peripheral organs, the relationship between the nongenomic actions of MR and ROS in the brain remains unknown.

The aim of this study was to determine whether acute increases in ROS production might modulate the nongenomic actions of MRs in the hippocampal region and which MR signals might be affected by ROS.
MATERIALS AND METHODS

Slice preparation. Experimental procedures involving live animals were conducted with the approval of the Tokyo University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (6–7 wk of age) were decapitated under diethyl ether anesthesia. The decapitations were performed at a fixed time (10:00–11:00 AM). After the hippocampi were quickly isolated, transverse slices (350 μm thick) were prepared with a tissue slicer (Leica VT1000 S; Leica Microsystems, Barcelona, Spain) in an ice-cold artificial cerebrospinal fluid solution composed of (in mM) NaCl 119, KCl 2.5, NaH₂PO₄ 1.0, NaHCO₃ 26.2, glucose 11, CaCl₂ 2, and MgCl₂ 1.0 in pH 7.2 to 7.3 aerated with a 95% O₂-5% CO₂ mixture. The slices were incubated for at least 60 min in a humidified interface holding chamber at room temperature (22–28°C). After incubation, the slices were submerged in a recording chamber held by a platinum grid attached with nylon string and superfused (2 ml/min) with the artificial cerebrospinal fluid solution, as previously described (28).

Drug treatment and experimental procedure. In the electrophysiological experiment, hippocampal slices were preincubated with blockers for at least 30 min, except for NSC23766, a Rac1 inhibitor, which was applied to the slices 2 h before the electrical stimulation; 1 μM NADPH was applied for 5 min and washed out thereafter, and 100 nM aldosterone (Ald), or 10 nM dexamethasone (Dex) was applied for 20 min.

Western blotting. Hippocampal slices were prepared as described above, and each sample for protein extraction was collected from three or four of the slices. The slices were stimulated with drugs by adding them to artificial cerebrospinal fluid aerated with a 95% O₂-5% CO₂ mixture. After stimulation, the samples were quickly frozen on dry ice, and the CA1 region was dissected. These samples were lysed in ice-cold magnesium-containing lysis buffer composed of 25 mM HEPES, 150 mM NaCl, 1% igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol, 25 mM NaF, and 1 mM NaVO₄ supplemented with a phosphatase inhibitor (Phosstop; Roche, Basel, Switzerland). A 10-μg amount of total protein was separated by 10% SDS-PAGE, and the proteins were analyzed by immunoblotting with anti-mouse monoclonal Rac1 antibody (Genescript, Piscataway, NJ). We used the Rac1 activation assay kit (Millipore, Billerica, MA), which leverages the affinity to the p21-activated kinase-1 protein-binding domain peptide, to measure the total Rac1. To measure the GTP-bound form of Rac1, i.e., the GTP-bound form of Rac1, as previously reported (40, 41). Hippocampal slices were lysed as described above, and 10 μg of total protein was used to measure the total Rac1. To measure the GTP-bound form of Rac1, a 1-mg sample was incubated with 10 μg of p21-activated kinase-1 protein-binding domain beads for 1 h. After being washed adequately with lysis buffer, the beads were boiled for 5 min in Laemmli sample buffer. The lysates were resolved by 15% SDS-PAGE, and the proteins were analyzed by immunoblotting with anti-mouse monoclonal Rac1 antibody.

Statistical analysis. Data are expressed as means ± SE. Differences in values between groups were tested for significance by the t-test or ANOVA and Tukey’s or Scheffé’s post hoc test. A probability level of <0.05 was accepted as representative of statistical significance.

RESULTS

Nongenomic actions of corticosteroids in CA1 synapses are mediated by MR. First, we confirmed that the nongenomic actions of corticosteroids in the CA1 region were mediated by MRs, as previously reported (19). CS rapidly increased the slope of the fEPSPs (124 ± 2.3%, n = 7; Fig. 1, A and B), and this effect was blocked by the MR antagonists Spi (106 ±
4.5%, n = 6; Fig. 1, A and B) and Epl (n = 4.96 ± 6.4%), but not by the GR antagonist RU-486 (118 ± 4.9%, n = 5; Fig. 1, A and B). Furthermore, the effect of 1 nM Ald (121 ± 1.4%, n = 5; Fig. 1C), 1/100 dose of CS, which is thought to be distributed in the hippocampal tissue (44) and binds selectively to the MR at this concentration, was comparable to the effect of CS, whereas 10 nM Dex did not significantly increase the slope of the fEPSPs (104 ± 3.6%, n = 4; Fig. 1C). To confirm that the nongenomic action of MRs is through membranous MR, we used membrane-impermeable CS (BSA-conjugated CS, 500 nM) and observed fEPSPs responses similar to that of CS (Fig. 2A).

NADPH oxidase activation has additive effects on the nongenomic actions of MRs. ROS have been reported to induce synaptic enhancement in the hippocampus (21). To determine whether this ROS action might influence the nongenomic actions of MRs, we simultaneously applied NADPH (1 μM) and CS (20–100 nM) and compared their effect with the effect of CS alone. NADPH alone increased the slope of the fEPSPs (138 ± 12.0%, n = 6; Fig. 3, A and B), CS increased fEPSPs dose dependently (Fig. 3C), and coapplication of NADPH and CS increased the fEPSP slope markedly (1 μM NADPH + 100 nM CS 163 ± 10.0%, n = 6) compared with CS application alone (Fig. 3, B and C) at each concentration. Furthermore, we examined the effect of another ROS mediator, EGF, on the nongenomic action of MR (Fig. 4); 10 ng/ml EGF also enhanced the nongenomic action of MR. Therefore, it was indicated that ROS production enhanced nongenomic MR action.

To clarify the additive effect of NADPH oxidase activity on MR signaling, we examined the effect of Apo (100 μM; Fig. 5, A, B, and E), a blocker of the NADPH oxidase. Surprisingly, Apo blocked the effect of both CS (102 ± 4.0%, n = 5; Fig. 5A) and Ald (107 ± 6.4%, n = 5; Fig. 5E). These findings...

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**Fig. 2.** Membranous MR induced not only fEPSP increase but also Rac1 activity. A: effect of 500 nM CS-BSA on field EPSPs. Membrane-impermeable CS quickly increased fEPSPs. B: Rac1 activation by 500 nM CS-BSA. Rac1 was also activated by CS-BSA.

**Fig. 3.** NADPH and CS additively enhanced fEPSPs. A: representative fEPSP traces after exposure to 1 μM NADPH and 100 nM CS + NADPH are shown. B: exposure to NADPH for 5 min increased the slope of the fEPSPs. Simultaneous application of NADPH + 20–100 nM CS had an additive effect in increasing the slope of the fEPSPs.

**Fig. 4.** Another ROS mediator, EGF, increased fEPSPs. EGF (20 ng/ml) also quickly increased fEPSP slopes, and application both EGF and CS further increased fEPSPs.

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These findings provide evidence for the nongenomic actions of MRs in the hippocampus and highlight the role of NADPH oxidase in mediating these effects.
indicate that the nongenomic actions of MR require ROS via NADPH oxidase.

MR and NADPH oxidase have an additive effect on ERK1/2 phosphorylation. The increase in presynaptic glutamate release in response to the nongenomic actions of the MR has been reported to be mediated by ERK1/2 phosphorylation (19). We investigated whether ERK1/2 is also phosphorylated by MR activity and ROS and whether MR and ROS might have an additive effect on ERK1/2 phosphorylation. Simultaneous application of CS and NADPH significantly increased the level of phosphorylation of ERK1/2 (1.88 ± 0.33, n = 7, P < 0.05) compared with application of CS (1.29 ± 0.13, n = 7) or NADPH (1.44 ± 0.17, n = 7) alone (Fig. 6A). We investigated whether the ERK1/2 phosphorylation by CS and ROS could be suppressed by Spi, RU-486, Apo, and the ROS scavenger Temp. ERK1/2 phosphorylation induced by CS was blocked by Spi, Apo, and Temp but not by RU-486 (Fig. 6C; CS, 1.32 ± 0.06; RU-486, 1.29 ± 0.04; Spi, 1.04 ± 0.04; Apo, 0.92 ± 0.92; Temp, 0.81 ± 0.04). Furthermore, Apo and Temp, but neither RU-486 nor Spi, blocked the effect of NADPH (Fig. 6E; NADPH, 1.53 ± 0.24; Spi, 1.64 ± 0.23; Apo, 1.37 ± 0.12; Temp, 1.24 ± 0.27; n = 7–9). These results indicate that the MR and NADPH oxidase actions share a common signaling pathway and that NADPH activation is required for the nongenomic actions of the MR.

Rac1 activation is additively induced by MR and NADPH oxidase activation. We next investigated what signal underlies the need for NADPH oxidase-induced ROS for the nongenomic actions of MR. It is known that certain subfamilies of NADPH oxidase, namely NOX1 and NOX2, are regulated by small GTPase Rac1 (26, 27). It was recently reported that prolonged exposure to Ald also activates Rac1 and enhances ROS generation, probably by genomic actions, in vascular smooth muscle cells (15) and that Rac1 GTP enhances MR translocation to the nuclei of the renal podocytes via the p21-activated kinase pathway (34, 40). We postulated that Rac1 activation is the key to the additive effect of ROS and

![Fig. 5. Involvements of NADPH oxidase and Rac1 in nongenomic action of MR:A: pretreatment with 100 μM apocynin (Apo) blocked the nongenomic actions of the MR. B: Apo also blocked the increase of the slope of the fEPSPs induced by 1 μM NADPH. C: summary of actions of CS, Ald, and NADPH in the presence of Apo. JEPSPs (%) increased after drug application. Effect of 50 μM NSC23766 (Rac inhibitor) on fEPSP amplitude following exposure to 100 nM CS (D) or 1 μM NADPH (E) F: summary of actions of CS, Ald, and NADPH in the presence of NSC23766.

![Fig. 6. A: ERK1/2 phosphorylation ratio after application of CS, NADPH, and NADPH + CS. B: simultaneous stimulation with CS + NADPH significantly increased Rac1 GTP activity. C and D: actions of CS were blocked by Spi, Apo, and Temp (4-hydroxy TEMPO), but not by RU-486. E and F: NADPH actions were blocked only by Apo and Temp. *P < 0.05, C and D, significant difference is shown vs. CS application or vs. NADPH application.](http://ajpendo.physiology.org/)
MR signaling. We measured the changes in Rac1 GTP activity in the CA1 region of the hippocampus immediately after the application of CS and NADPH. As shown in Fig. 6B, application of CS or NADPH immediately increased the Rac1 GTP activities (CS: 1.29 ± 0.13, n = 8; NADPH: 1.44 ± 0.17, n = 8), and when both were applied concomitantly, an even greater degree of Rac1 GTP activation was observed (1.81 ± 0.33, n = 8, P < 0.05). Rac1 GTP activation by CS was also reconfirmed using membrane-impermeable CS (BSA-CS; Fig. 2B). We next investigated the effect of each blocker on the Rac1 GTP activation induced by CS and/or NADPH. Rac1 activation induced by CS was blocked by Spi, Apo, and Temp but not by RU-486 (CS, 1.27 ± 0.07; RU-486, 1.26 ± 0.06; Spi, 1.03 ± 0.07; Apo, 0.79 ± 0.06; Temp, 0.65 ± 0.08). Activation induced by NADPH was blocked by Apo and Temp but not by Spi. These results are comparable to ERK1/2 phosphorylation, confirming that the nongenomic actions of MRs require NADPH oxidase activity, and NADPH oxidase activation is at least in part via membranous MRs.

Rac1 activity is indispensable for synaptic enhancement mediated by the nongenomic actions of MR and NADPH oxidase activation. To determine whether Rac1 activation is required for synaptic enhancement induced by MR activity and ROS, we investigated the effect of a Rac1 inhibitor (NSC23766; NSC) that blocks the interaction between Rac1 and guanine nucleotide exchange factor (GEF), such as Tiam-1 (10), and might also block the synaptic enhancement. Since preincubation with NSC for 2 h blocked the synaptic potentiation induced by both CS (1.01 ± 0.08, n = 6; Fig. 5C) and NADPH (0.94 ± 0.11, n = 5; Fig. 5D), the results indicate that Rac1 activation is also necessary for nongenomic MR signaling.

**Fig. 7.** A: pretreatment with NSC23766 blocked ERK1/2 phosphorylation induced by CS and NADPH. MEK inhibitor (100 μM U-0126) decreased the ERK1/2 phosphorylation ratio (B) but did not block Rac1 GTP activation by CS (C).

**DISCUSSION**

The nongenomic action of MR is known to occur in various organs other than the brain, such as vascular smooth muscle cells, endothelial cells, and heart. It acts quickly and in a GRE-independent fashion when CS and/or Ald levels are elevated, and it is supposed to be responsible for quick reaction under stress (24). As previously noted, the genomic action of MR is associated with ROS (15), but little is known about the precise relationship of between nongenomic action of MR and ROS (42).

The results of this study demonstrate for the first time that NADPH oxidase activity is indispensable for the nongenomic actions of the MR, which is likely induced by membranous MR, and that it exerts an additive effect on the nongenomic actions of the MR in the hippocampus. We further demonstrate that Rac1 activation mediates both MR activation and ERK1/2 phosphorylation (Fig. 8).

The nongenomic actions of CS mediated via the MR in the hippocampal region, affecting both pre- and postsynaptic responses, were recently clarified (5, 30). Synaptic vesicle release is enhanced by L-type Ca channels and is mediated by Src and ERK1/2 phosphorylation (4, 19). Postsynaptic K channel (I_{A} current) suppression and lateral diffusion and insertion of the postsynaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor (8, 20, 30) are also enhanced. These actions might contribute to enhanced induction of long-term potentiation by CS under conditions of stress.
Since we evaluated the nongenomic actions of the MR by measuring fEPSPs, Rac1 GTP activity, and ERK1/2 phosphorylation ratio in slices of the CA1 region of the hippocampus, we could not separate the presynaptic effects from the postsynaptic effects. Paired-pulse ratio (PPR) usually reflects presynaptic release probability, but a postsynaptic IA current would also change the PPR, and the PPR cannot indicate the release probability precisely. It has already been shown that ERK1/2 phosphorylation acts both pre- and postsynaptically (8, 30). Furthermore, Rac1 GTP activation mediates PSD95 phosphorylation, which recruits surface AMPA receptors (20). Thus, the actions of the MR affect both pre- and postsynaptic processes through Rac1 and ERK1/2 activation.

There is another possible mechanism by which ROS mediates the nongenomic actions of the MR. ROS may change the intracellular amounts of CS by increasing the activity of $11\beta$-HSD1 (9). $11\beta$-HSDs are enzymes that metabolize glucocorticoids and consist of two isozymes, $11\beta$-HSD1 and -2. Expression of $11\beta$-HSD2 is confined to certain areas of the brain, including the paraventricular nucleus and nucleus of the solitary tract (11, 45), where they contribute to salt preference (12, 40). In the hippocampus, $11\beta$-HSD1 is dominantly expressed (37). $11\beta$-HSD1 has both dehydrogenase and hydroxylase activity that is activated by NADP$^+$. Therefore, it is possible that local CS concentration (internal CS) might be increased by ROS via $11\beta$-HSD1 activation. However, in the present study, synaptic enhancement by NADPH was not blocked by Spi, and Apo preincubation, which may decrease the internal CS concentration through inhibition of $11\beta$-HSD1, changed neither the synaptic response nor the ERK1/2 phosphorylation ratio (Fig. 9). Furthermore, Apo completely blocked the synaptic enhancement induced by additional CS application. These data indicate that the NADPH pathway is downstream of the MR and that alteration of $11\beta$-HSD1 activation cannot explain the acute, additive effect of ROS and CS in our preparation.

We used CS as the main MR agonist in our preparation because relatively low levels of Ald cross the blood-brain barrier in vivo (6, 44) and CS is the main modulator of MR activity in the brain. In this study, both application of CS at 100 nM and Ald at 1 nM, induced synaptic potentiation, consistent with previous reports (35). Furthermore, we evaluated ERK1/2 phosphorylation of the hippocampus induced by intraperitoneal CS injection in vivo (Fig. 10). This suggests that physiological concentrations of CSs can affect synaptic intensity and cognitive functions.

Our study clarified that MR activation induces Rac1 GTP activity (nongenomically, at least in part via membranous MR; Fig. 2), and this pathway is mediated by NADPH oxidase. Additionally, we demonstrated that simultaneous exposure to CS and NADPH enhances synaptic response. Considering that NADPH activity induces glutamate toxicity (14), CS can also enhance glutamate toxicity via the NADPH pathway. Therefore, it is possible that excessive psychological stress and oxidative stress may cause glutamate toxicity in an additive manner. In conclusion, the present study has revealed that nongenomic action of MR in the brain regulates ROS and Rac1.

![Fig. 9](image-url) Changes in ERK1/2 phosphorylation ratio when hippocampal slices were preincubated with Spi or Apo. We rearranged the sample gel although they were run on the same gel. There were no significant differences between groups. There were no significant changes of normalized fEPSPs after application of Spi or Apo.

![Fig. 10](image-url) Hippocampal ERK1/2 phosphorylation was evaluated in vivo. Ten minutes after intraperitoneal injection of 10 μg/kg CS, rats were immediately killed and hippocampi were removed. PBS-DMSO was used as the vehicle.


