Arachidonic acid induces the activation of the stress-activated protein kinase, membrane ruffling and H_2O_2 production via a small GTPase Rac1

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Abstract Arachidonic acid (AA) is generated via Rac-mediated phospholipase A2 (PLA2) activation in response to growth factors and cytokines and is implicated in cell growth and gene expression. In this study, we show that AA activates the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in a time- and dose-dependent manner. Indomethacin and nordihydroguaiaretic acid, potent inhibitors of cyclooxygenase and lipoxygenase, respectively, did not exert inhibitory effects on AA-induced SAPK/JNK activation, thereby indicating that AA itself could activate SAPK/JNK. As Rac mediates SAPK/JNK activation in response to a variety of stressful stimuli, we examined whether the activation of SAPK/JNK by AA is mediated by Rac1. We observed that AA-induced SAPK/JNK activation was significantly inhibited in Rat2-Rac1N17 dominant-negative mutant cells. Furthermore, treatment of AA induced membrane ruffling and production of hydrogen peroxide, which could be prevented by Rac1N17. These results suggest that AA acts as an upstream signal molecule of Rac, whose activation leads to SAPK/JNK activation, membrane ruffling and hydrogen peroxide production.

Key words: Arachidonic acid; Rac1; Stress-activated protein kinase/c-Jun N-terminal kinase; Membrane ruffling; Hydrogen peroxide

1. Introduction

Arachidonic acid (AA), an important lipid messenger, is generated via phospholipase A2 (PLA2) activation in response to a variety of stimuli including growth factors and inflammatory cytokines [1–3]. AA and its metabolites are implicated in the regulation of several biological processes such as inflammation, cell growth and differentiation, and apoptosis [3–6]. Although the exact signaling mechanism of AA mediating these cellular responses remains largely unknown, recent evidence suggests that a small GTPase Rac mediates epidermal growth factor (EGF)-induced AA release [1,2,7]. In Rat2 fibroblast cells, AA is also released by exogenous hydrogen peroxide through Rac-mediated activation of PLA2 [8]. Therefore, it was suggested that PLA2 is one of the downstream targets of Rac and that 'Rac-PLA2-AA cascade' mediates, at least partly, the signaling induced by EGF or hydrogen peroxide. Rac is a member of the Rho GTPase family, which consists of Rho, Rac and Cdc42 [9]. Rho family proteins were originally found to play key roles in growth factor-induced actin reorganization. Rho controls stress fiber formation, whereas Rac and Cdc42 govern the formation of membrane ruffling and filopodia, respectively [9–11]. Recently it was also reported that Rac and Rho are implicated in gene expression. For example, Rac GTPase mediates the activation of c-*fos* serum responsive element (SRE) in response to agonists such as EGF [1]. PLA2 and its principal product, AA, also play a critical role in EGF-induced c-*fos* SRE activation [2].

AA can be metabolized to leukotrienes and prostaglandins by lipoxygenase (LOX) and cyclooxygenase (COX), respectively. These AA metabolites are involved in the regulation of EGF-induced actin remodeling. Especially, leukotrienes are necessary and sufficient for the formation of actin stress fiber [12]. Leukotrienes were also known to play an important role in the signaling pathway regulating cellular survival and apoptosis [6]. Thus, AA is likely to act as an intracellular second messenger in the EGF-mediated signal pathway. However, AA can be released from cells in response to stimuli and therefore could act as an intercellular signal molecule in a paracrine or autocrine manner [3,13].

AA is a potent activator of several protein kinases. It activates mitogen-activated protein kinase (MAPK) in vascular smooth muscle cells via protein kinase C (PKC) and Ras [14,15]. In addition, AA has also been demonstrated to activate stress-activated protein kinase (SAPK)/c-Jun N-terminal protein kinase (JNK) [16–18]. SAPK/JNK can be activated by a variety of stressful conditions such as UV irradiation, hyperosmolarity, heat shock, and proinflammatory cytokines [19,20] and has been implicated in cell proliferation and stress-induced programmed cell death (apoptosis) [19,21,22]. Recently, it was demonstrated that stress-induced SAPK/JNK activation is mediated by Rac GTPase [19,23], thereby indicating that Rac is upstream of SAPK/JNK. However, the signal pathway by which AA activates SAPK/JNK is not clearly defined.

In this study, we examined whether the activation of SAPK/JNK by AA is mediated by Rac1. Our results showed that Rac1 is critical for AA-induced SAPK/JNK activation. We further showed that AA induced membrane ruffling and H_2O_2 production in a Rac1-dependent manner. Therefore,

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we propose that AA could act as an upstream signal molecule of Rac1.

2. Materials and methods

2.1. Chemicals and reagents

AA was obtained from BioMol (Plymouth Meeting, PA, USA) and Sigma Chemical Co. (St. Louis, MO, USA). Indomethacin and nordihydroguaiaretic acid (NDGA) were from Sigma Chemical Co. EGF was purchased Boehringer Mannheim GmbH (Mannheim, Germany). Fetal bovine serum (FBS), glutamine, and Dulbecco's modified Eagle's medium (DMEM) were from Gibco-BRL (Gaithersburg, MD, USA).

2.2. Cell culture and transfection

Rat2 fibroblast cells were obtained from the American Type Culture Collection (ATCC, CRL 1764) and grown in DMEM supplemented with 2 mM glutamine and 10% (v/v) FBS. Rat2 stable clones expressing a dominant-negative Rac1N17 protein were prepared as described previously [1,2]. pEXV-myc and pEXV-myc-RacN17 plasmids (gifts from Dr. Alan Hall) were expressed as N-terminally 9E10 epitope-tagged derivatives under SV40 promoter [10,11]. Rat2 cells were stably cotransfected with pEXV-RacN17 or control vector plasmid, pEXV, along with the NeoR gene. In the presence of G418 (500 μg/ml), several stable colonies were picked and expanded.

2.3. SAPK/JNK activity assay and Western blotting

SAPK/JNK activity was determined as described by Brenner et al. [22]. Rat2 cells were extracted with buffer A (1% Triton X-100, 25 mM HEPES, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 10 nM okadaic acid, 0.1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and the cell extracts were incubated with anti-SAPK antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h and then protein A-agarose for an additional 1 h. After centrifugation, the precipitates were incubated in a mixture of 20 mM HEPES (pH 7.4), 2 mM sodium orthovanadate, 2 mM DDT, 100 mM $MgCl_2$, [^{32}P]ATP, and 4 μl GST-c-Jun at 30°C for 30 min. To stop the reaction, 4×Laemmli sample buffer was added to the precipitates and heated at 100°C for 5 min. After centrifugation, the supernatant was analyzed by SDS-PAGE and autradiography.

For Western analysis, Rat2 cells were washed in cold phosphate-buffered saline (PBS) and lysed in SDS sample buffer by heating at 100°C for 10 min. The protein content was quantified by using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins (50 μg) were separated by SDS-PAGE. The resulting gels were transferred to nitrocellulose papers and the membranes were blocked with 10% bovine serum albumin in PBS-T (PBS plus 0.1% Tween) for 1 h at room temperature. The membranes were then incubated with primary antibody for 1–2 h at room temperature. After washing in PBS-T three times, the blot was incubated with peroxidase-conjugated secondary antibody and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp.).

2.4. F-actin staining

Rat2 cells were grown on round coverslips in multiwell culture plates and exposed to EGF (100 ng/ml) and AA for indicated times. The cells were fixed with 3.7% (w/v) formaldehyde in PBS for 30 min on ice. The fixed cells were then permeabilized by incubating in 0.2% Triton X-100 in PBS for 15 min on ice. The cells were then stained with 0.165 M NBD-phallacidin for 30 min at room temperature. The stained cells were washed three times in PBS for 15 min and mounted on slide glasses with Gelvitol which was prepared by mixing 100 ml of 23% polyvinyl alcohol in PBS with 50 ml glycerol. Then the cells were observed under a fluorescence microscope.

2.5. Measurement of intracellular H_2O_2

The amount of intracellular H_2O_2 was measured as described by Ohba et al. [24]. Briefly, cells were grown on coverslips for 2 days and serum-starved for another 2 days. The cells were stabilized in serum-free medium without phenol red for at least 30 min and stimulated with AA for various times. For the last 10 min of stimulation, 5 μM H2DCFDA was added to monitor intracellular H_2O_2. Then, the cells were immediately observed under a laser scanning confocal microscope (Carl Zeiss LSM 410). The samples were excited by a longpass 515 nm filter. Thirty cells were randomly selected from three independent experiments and dichlorofluorescein fluorescence intensities of treated cells were compared with those of unstimulated cells.

3. Results and discussion

3.1. AA activates the SAPK/JNK

Previously, others have reported that AA activates SAPK/JNK in vascular smooth muscle cells [16] and through NADPH oxidase in rabbit proximal tubular epithelial cells [17]. In this study, we examined whether AA could induce the activation of SAPK/JNK in Rat2 fibroblast cells. SAPK/JNK activity was measured in cell lysates by an immune complex kinase assay using GST-c-Jun fusion protein as a substrate. As shown in Fig. 1A, exposure of cells to 50 μM AA activated SAPK/JNK in a time-dependent manner. The activation of SAPK/JNK was evident at 5 min and peaked at 10 min after exposure to AA. At 30 min, SAPK/JNK activity returned to the basal level (data not shown). SAPK/JNK activation by AA was also dose-dependent in that GST-c-Jun phosphorylation was increased at 5 μM AA and maximal at 50–100 μM (Fig. 1B). SAPK/JNK protein level was not changed by treatment of AA (Fig. 1, lower panels).

AA can be oxidized through the COX, LOX, and/or cytochrome P450 pathways to yield eicosanoids. To test whether AA metabolism through one of the above pathways is required for its activation of SAPK/JNK, Rat2 cells were treated with the COX inhibitor indomethacin (10 μM) or the LOX inhibitor NDGA (10 μM) for 20 min, followed by stimulation with 50 μM AA. Neither indomethacin nor NDGA exerted inhibitory effects on AA-induced activation

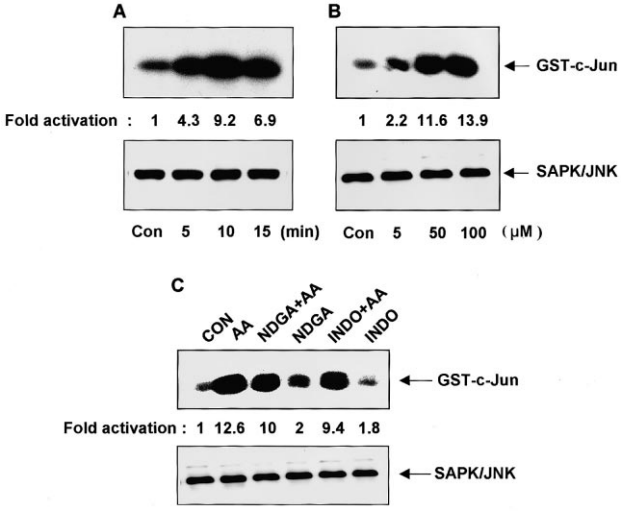

Fig. 1. Time- (A) and dose- (B) dependent activation of SAPK/JNK by AA. A: Growth-arrested Rat2 fibroblast cells were exposed to 50 μM AA for the indicated times. Cell lysates were analyzed either for SAPK/JNK immune complex kinase assay (upper panel) or for Western blotting using anti-JNK1 antibody (lower panel). B: Rat2 cells were treated with various concentrations (5–100 μM) of AA for 10 min and analyzed for SAPK/JNK activity. C: AA activates SAPK/JNK independent of its metabolism via the cyclooxygenase or the lipoxygenase pathways. Growth-arrested Rat2 cells were incubated in the presence and absence of 10 μM NDGA or 10 μM indomethacin (INDO) for 20 min and treated with 50 μM AA for 10 min and the SAPK/JNK activity was measured. The results are representative of those seen in three independent experiments.

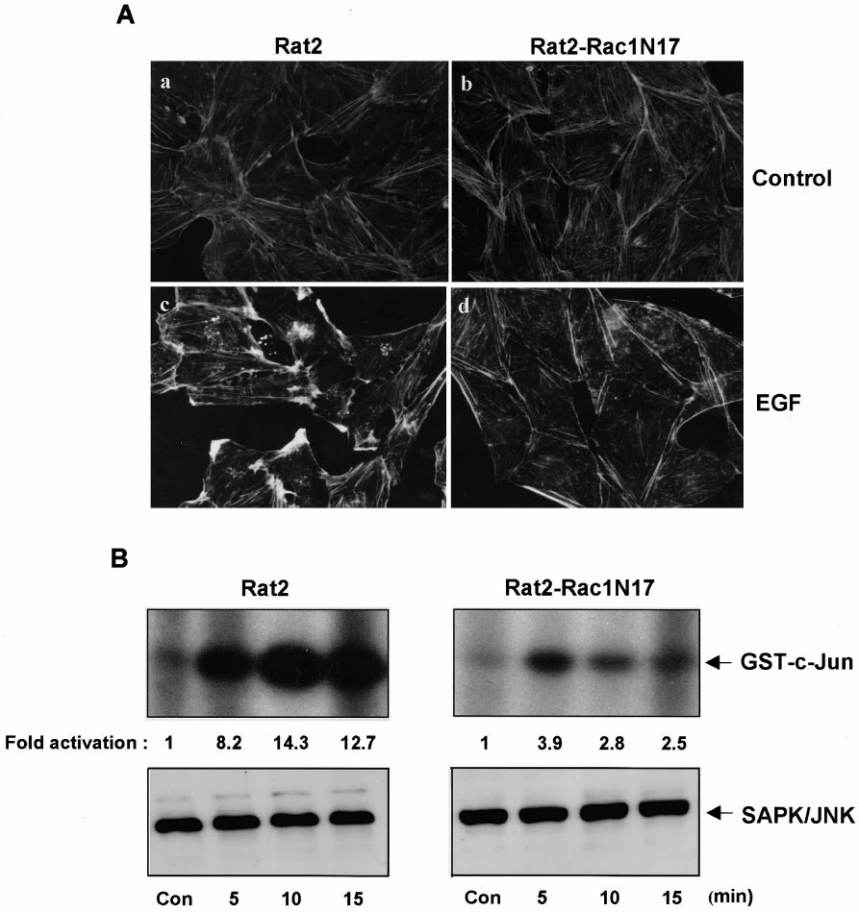

Fig. 2. A: Characterization of Rat2-Rac1N17 mutant cells. Rat2-Rac1N17 mutant cells were prepared as described in Section 2. Serum-starved Rat2 or Rat2-Rac1N17 cells were stimulated with EGF (100 μg/ml) for 10 min, fixed, and stained with NBD-phallacidin before fluorescence microscopic analysis. B: AA activates SAPK/JNK through a small GTPase Rac1. Growth-arrested Rat2 or Rat2-Rac1N17 cells were treated with 50 μM AA for the indicated times and the SAPK/JNK activity was measured by an immune complex kinase assay. The results are representative of those seen in three independent experiments.

of SAPK/JNK (Fig. 1C). Thus, consistent with others' results [16–18], we demonstrated that AA activates SAPK/JNK in a dose- and time-dependent manner in Rat2 fibroblast cells and that AA-induced SAPK/JNK activation does not require subsequent eicosanoid metabolism.

3.2. AA-induced SAPK/JNK activation is mediated by Rac1

A number of unrelated stressful stimuli are known to activate SAPK/JNK via Rac1 [19,20]. These facts led us to examine whether Rac1 plays a role in the activation of SAPK/JNK by AA. To do this, we prepared a dominant-negative Rac1 mutant cell line (Rac1N17) as described by Kim et al. [1]. As shown in Fig. 2A, upon stimulation of cells with EGF, Rac1 mutant cells (Rat2-Rac1N17) did not show any changes in actin filaments, whereas normal cells exhibited extensive membrane ruffling within 10 min, followed by stress fiber formation. Using the Rat2-Rac1N17 mutant cell line, we examined whether SAPK/JNK activation by AA is Rac1-dependent. As shown in Fig. 2B, AA-induced SAPK/JNK activation was significantly prevented in Rac1N17 mutant cells. These results suggest that AA-induced SAPK/JNK activation is mediated by a small GTP-binding protein Rac1.

3.3. AA-induced membrane ruffling is Rac1-dependent

Previous reports showed that Rac1 activation in response to a number of growth factors such as EGF leads to membrane ruffling [9–11]. Therefore, we examined whether AA acts through Rac1 to stimulate membrane ruffling. As shown in Fig. 3, treatment of AA generated membrane ruffling, similar to that observed in EGF-treated cells. And the AA-inducible membrane ruffling was also prominently prevented in dominant-negative Rac1N17 mutant cells (Fig. 3). While Rac1N17 blocked AA-induced membrane ruffling, it did not block AA-induced alteration in actin cytoskeletal structure. Taken together, these results suggest that AA induces the activation of SAPK/JNK and membrane ruffling via activation of a small GTPase Rac1.

3.4. AA-induced H_2O_2 increment is Rac-dependent

Rac has been suggested to play a critical role in EGF-induced reactive oxygen species (ROS) production through the activation of NADPH oxidase complex in phagocytic and non-phagocytic cells [9,25]. In NIH 3T3 cells, transient expression of constitutively activated forms of the small GTP-binding proteins Ras or Rac1 leads to a significant increase in intracellular ROS [25]. Thus, we examined using a laser scanning confocal microscope whether AA activates H_2O_2 production in a Rac1-dependent manner. Our results showed that AA increased the release of H_2O_2 2.5-fold in Rat2 fibroblast cells compared to the control level. And the AA-induced

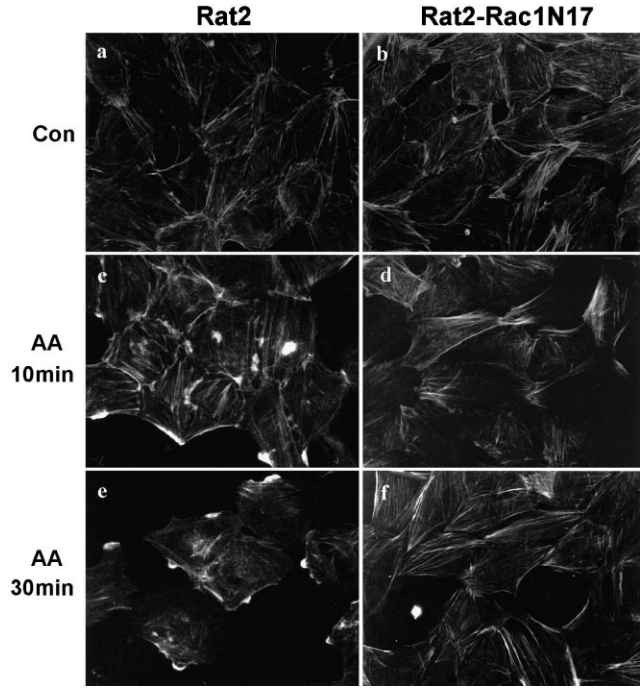

Fig. 3. AA-induced membrane ruffling is Rac1-dependent. Serum-starved Rat2 or Rat2-Rac1N17 cells were stimulated with AA (100 μM) for the indicated times, fixed and stained with NBD-phallacidin and observed under a fluorescence microscope.

H_2O_2 increase was inhibited in Rac1N17 dominant-negative mutant cells (Fig. 4).

Our results clearly demonstrate that AA induces SAPK/JNK activation, membrane ruffling, and hydrogen peroxide production, and that the activities of AA are mediated by Rac1. Indeed, AA is known to activate Rac1. AA and its LOX metabolites possess an ability to activate several GTP-binding proteins such as Ras and Rac through inhibiting GTPase-activating proteins [26]. In phagocytes, AA activates NADPH oxidase, which may be mediated by Rac1 [17]. Thus, AA could act as an upstream signal molecule of Rac.

Recently it was suggested that Rho family GTPases may be linked to the action mechanism of AA. Gong et al. [27] have demonstrated that AA induces Ca^{2+} sensitization of force in α-toxin-permeabilized smooth muscle accompanied by an increase in myosin light chain phosphorylation. It was also reported that Y37632, a Rho kinase inhibitor, inhibits AA-induced Ca^{2+} sensitization and reduces myosin light chain phosphorylation, thereby indicating that Rho as well as Rac may act as downstream signal molecules in the signal transduction of AA [28].

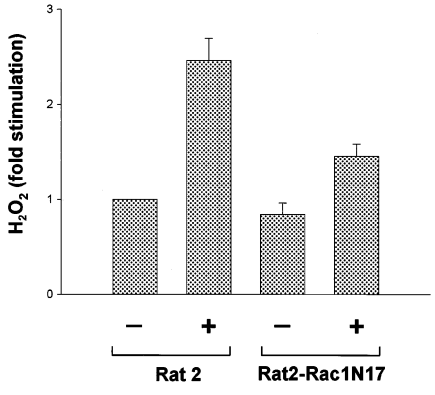

Fig. 4. AA-induced H_2O_2 increment is Rac1-dependent. Serum-starved Rat2 or Rat2-Rac1N17 cells were stimulated with AA for 5 min and stained as described in Section 2. AA-induced H_2O_2 production was determined using a laser scanning confocal microscope. Values shown are the mean ± S.D. of three separate experiments. $P < 0.05$.

It is known that AA is produced through Rac-mediated PLA2 activation in response to growth factors such as EGF and the 'Rac-PLA2-AA cascade' mediates, at least partly, the growth factor-induced gene expression and actin reorganization [1,2,7]. Thus, PLA2 has been suggested to be one of the downstream targets of Rac. In addition, H_2O_2 stimulates external signal-responsive PLA2 in vascular smooth muscle and cultured endothelial cells and produces AA [29,30]. However, we showed that AA could produce hydrogen peroxide in a Rac1-dependent manner (Fig. 4). Taking these results together, we suggest that, in addition to acting downstream of Rac, AA may activate Rac and form a positive feedback loop.

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