Arachidonic acid, a principal product of Rac-activated phospholipase A_2, stimulates c-*fos* serum response element via Rho-dependent mechanism

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Abstract Previously, we have reported that phospholipase A_2 (PLA_2) is one of the major downstream targets by which Rac GTPase mediates the activation of c-*fos* serum response element (SRE) in response to agonists such as EGF [FEBS Lett. 407 (1997) 7–12]. Thus, the potential activity of arachidonic acid (AA), a principal product of Rac-activated PLA_2, on c-*fos* SRE stimulation has been suggested. Here, we provide evidence about the biological activity of AA on c-*fos* SRE activation. Further, we observed that co-transfection with expression plasmid of either RhoN19, a dominant negative RhoA mutant, or botulinum C3 transferase which inhibits Rho via ADP ribosylation, selectively repressed AA- or Rac-induced SRE activation, suggesting that Rho activity is critical for the signaling cascade of 'Rac-PLA_2-AA' to c-*fos* SRE. Thus, Rac signaling to the nucleus appears to be, at least partly, mediated by a Rho-linked pathway and this Rac-Rho signaling connection is mediated by AA. In accordance with the role of Rho as a potential mediator of AA signaling to the nucleus, AA induces a rapid translocation of RhoA.

Key words: Arachidonic acid; Rac; Rho; c-*fos* serum response element; Phospholipase A_2

1. Introduction

Arachidonic acid (AA), an important lipid second messenger, is generated via phospholipase A_2 (PLA_2) activation in response to a variety of stimuli, including epidermal growth factor (EGF), inflammatory agents, and cytokines [1–4]. It has been demonstrated that activation of Rac, a member of the Rho family GTPases, leads to AA release, suggesting that PLA_2 is one of the downstream targets of Rac [5–7]. In addition, our recent studies have shown that, in Rat-2 fibroblast cells, AA was released rapidly by EGF or exogenous hydrogen peroxide via Rac-activated PLA_2 and further that the 'Rac-PLA_2-AA cascade' mediates, at least partly, the signaling induced by EGF or hydrogen peroxide [1,2]. Thus, AA, a principal product of PLA_2, is suggested to play a pivotal role in mediating Rac-induced signals in cells.

Recently, several groups have reported on a critical role of AA in the regulation of a variety of cellular activities such as proliferation, differentiation, actin remodeling, superoxide generation, gene expression as well as apoptosis, programmed cell death [5,6,8–11]. Although the exact signaling mechanism of AA mediating those cellular responses remains largely unknown, recent evidence suggests that the metabolism of AA by lipoxygenase (LO) and subsequent production of leukotrienes are likely to be essential, especially in the signaling pathway regulating cellular survival and apoptosis [8]. Also, in actin remodeling in response to EGF, leukotrienes generated by LO are necessary and sufficient for actin stress fiber formation [6]. Similarly, in our recent work on c-*fos* serum response element (SRE) activation induced by RacV12, a constitutively activated mutant of Rac1, AA release via Rac-activated PLA_2 and subsequent metabolism of AA by LO were shown to be essential, thus supporting a potential role of leukotrienes as one of the major downstream mediators of AA signaling [7]. Therefore, the 'Rac-AA-leukotrienes' signaling cascade is likely to be important in regulating several key cellular activities including the c-*fos* SRE stimulation.

It was reported by Peppelenbosch et al. that the addition of exogenous leukotrienes was able to induce actin stress fiber formation [6]. Further, they have demonstrated that leukotriene-dependent stress fiber formation requires Rho proteins, which suggests that the 'Rac-AA-leukotrienes' signaling cascade is one major pathway by which Rac influences Rho-dependent cytoskeletal rearrangements [6]. Therefore, there is an apparent signaling link between Rac and Rho in the actin regulation. However, it is not known whether this 'AA-mediated Rac-Rho signaling link' also exists in other cellular signaling pathways involved in gene regulation, cell survival, or apoptosis.

In the present project, we undertook to understand the downstream part of the 'Rac-AA-leukotrienes signaling cascade' in c-*fos* SRE regulation, and examined whether Rho is necessary for the nuclear signaling cascade of AA to c-*fos* SRE. Our results clearly demonstrate that RhoA is essential for AA-induced c-*fos* SRE stimulation. We propose that AA acts as a common linking molecule between Rac and Rho in both signaling cascades leading to actin remodeling and gene regulation.

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). EGF was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Fetal bovine serum (FBS), gentamicin, and Dulbecco's modified Eagle's medium (DMEM) were from Gibco-BRL (Gaithersburg, MD, USA). Polyclonal antibody to RhoA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were from standard sources and were molecular biology grade or higher.

2.2. Cell culture, transfections and luciferase assay

Rat-2 fibroblast cells were obtained from the American Type Culture Collection (ATCC, CRL 1764). Cells were grown in DMEM supplemented with 2 mM glutamine, 10% (v/v) fetal bovine serum and gentamicin as described before [1,2]. Transient transfection analysis was performed as described previously [1,2]. To control for variations in both cell numbers and transfection efficiency, all clones were

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co-transfected with 1 μg of pCMV-βGAL, an eukaryotic expression vector in which the *Escherichia coli* β-galactosidase (*lacZ*) structural gene is under the transcriptional control of the CMV promoter [12]. Lysates prepared from the harvested cells were assayed for both luciferase activity and β-galactosidase activity, which was used as an internal standard to normalize the luciferase activity directed by the test plasmid [1,2]. Luciferase activity was determined as described previously [1,2]. Transfection experiments were performed in triplicate with two independently isolated sets and the results were averaged.

2.3. Subcellular fractionation of cell lysates and Western blotting

Rat-2 cells were serum-starved for 18 h in serum-free DMEM and appropriate agonists were treated for the indicated times. The medium was removed and the cells were washed twice with ice-cold PBS, scraped, harvested by micro-centrifugation, and resuspended in 0.2 ml of buffer A (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, pH 7.5). The resuspended cells were then sonicated twice for 15 s each or alternatively lysed by 20 passes through a 21.1-gauge needle on ice. Lysates were centrifuged at $10000 \times g$ for 1 h to prepare cytosolic and total particulate fractions. The particulate fractions containing membrane fraction were washed twice and resuspended in 50 μl of buffer A. The supernatant fractions were precipitated with 5 volumes of acetone, incubated on ice for 5 min, centrifuged, and the pellet was resuspended in buffer A. Protein concentrations were determined routinely using the Bradford procedure with Bio-Rad Dye Reagent and bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis was performed on 12% acrylamide gels, and proteins were transferred onto polyvinylidene difluoride membranes for 2 h at 100 V using a Novex wet transfer unit. The membrane was blocked overnight with TBS (PBS containing 0.01% (v/v) Tween 20) with 5% (w/v) non-fat dried milk. Blots were incubated for 2 h with primary antibody (to RhoA) in TBS and then for 1 h with a horseradish peroxidase-conjugated secondary antibody, prior to development using an enhanced chemiluminescence kit (Amersham Corp.). Bands corresponding to RhoA were measured by densitometry and the purified recombinant proteins were used as standards to calibrate the densitometer.

3. Results and discussion

3.1. Exogenous AA stimulates c-fos SRE in a dose- and time-dependent manner

To understand the potential role of AA as a downstream mediator of the Rac signaling cascade leading to the activation of c-*fos* SRE, a well-known primary nuclear target for many extracellular signals such as growth factors, cytokines, and environmental stress [13–15], we first tested whether the exogenous addition of AA, a principal product of Rac-activated PLA_2, induces c-*fos* SRE activation. Following transient transfections with a reporter plasmid, pSRE-Luc [1] containing c-*fos* SRE fused to luciferase coding sequences, Rat-2 cells were serum-starved in DMEM containing 0.5% FBS for 36 h before adding AA exogenously. As shown in Fig. 1, exogenous AA stimulated the c-*fos* SRE-dependent reporter gene activity in a dose- and time-dependent manner. An approximately 5-fold increase in the luciferase activity occurred 2 h after the addition of AA and by 6 h, the c-*fos* SRE luciferase level declined (Fig. 1). Maximal luciferase activity was detected at an AA concentration of 100 μM (Fig. 1). As a control, pO-luciferase [1], a vector without SRE, was transfected and no stimulation of the luciferase activity was observed by AA (data not shown). Together, these results are well in agreement with the suggested role of AA as a major downstream mediator of Rac in the signal transduction cascade leading to c-*fos* SRE stimulation.

3.2. Rho GTPase activity is required for AA-induced SRE activation

It has been reported that AA metabolites are involved in the activation of Rho by Rac in the regulation of actin remodeling in Swiss 3T3 cells [5,6]. To obtain further insight into the mechanism by which AA activates c-*fos* SRE in Rat-2 fibroblasts, we examined whether AA-induced SRE activation is also dependent on functional Rho. For this analysis, botulinum C3 transferase expression was used. Expression of C3 transferase has been shown to specifically inhibit Rho via ADP-ribosylation at Asn-41 [15]. Cells were co-transfected with EF (vector) or EF-C3 (C3 transferase) together with SRE-luciferase reporter gene to determine whether C3 transferase co-transfection blocks AA-induced SRE activation. As shown in Fig. 2A, we observed that EF-C3 blocked dramatically AA-induced SRE activation (~80% reduction of luciferase activity) whereas EGF (50 ng/ml)-induced SRE activation was only slightly affected (Fig. 2A). LPA (10 μM)-induced SRE activation was also blocked completely by C3 transferase, which is in agreement with a previous report showing that LPA activates c-*fos* SRE principally via a

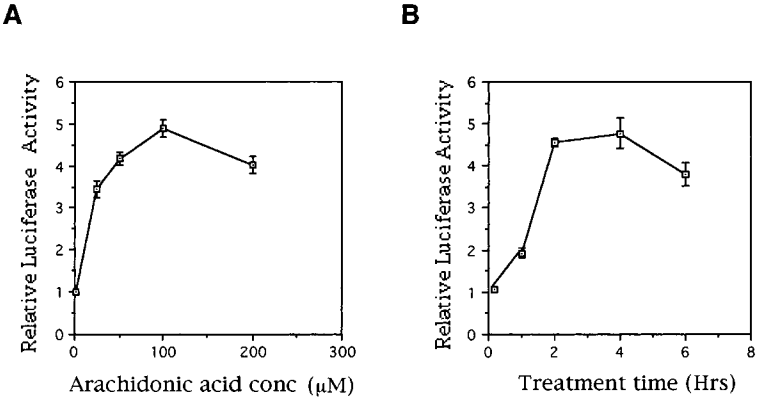

Fig. 1. Dose- and time-dependent c-*fos* SRE stimulation by AA. After transient transfections with pSRE-Luc (3 μg), Rat-2 cells were serum-starved in 0.5% FBS/DMEM for 36 h before treatment with AA. A: Dose response of AA on SRE activation. Various amounts of AA (0, 25, 50, 100, 200 μM) were added for 2 h to serum-starved transfected cells. B: Time-dependent response of AA (100 μM) on SRE activation. Serum-starved Rat-2 cells were treated with AA for the lengths of time indicated. Luciferase and β-galactosidase activities were measured as described in Section 2.

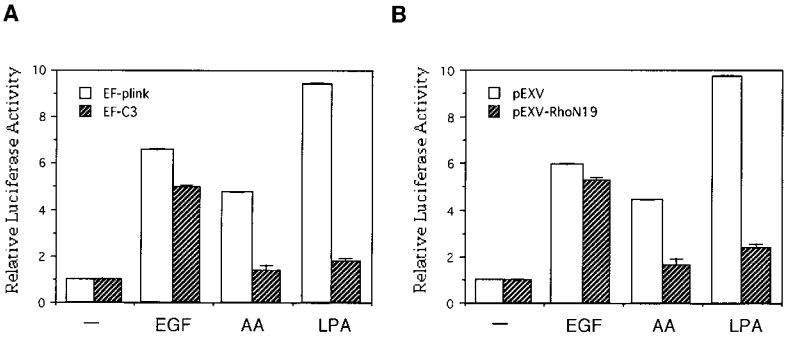

Fig. 2. Inhibition of AA-induced SRE activation by RhoN19 or C3-transferase. A reporter gene plasmid, pSRE-Luc (3 μg) was transiently co-transfected with 1 μg of pEFC3, a C3-transferase expression plasmid (A) or 5 μg of pEXV-RhoN19, a dominant negative RhoA expression plasmid (B). The total amount of DNA was kept at 20 μg with calf thymus carrier DNA. Transfected cells were serum-deprived in 0.5% FBS/DMEM for 36 h before treatment with AA (100 μM), EGF (50 ng/ml), or LPA (10 μM). After 2 h of treatment, luciferase and β-galactosidase activities were measured. Values are representative of triplicate transfections.

Rho-dependent pathway [15]. This result indicates that the signaling pathway of AA to SRE is dependent upon functional Rho.

Similarly, by co-transfection experiments with pEXV-RhoN19 encoding a dominant negative RhoAN19, we observed a similar mode of inhibition of the AA-induced SRE activation. For this test, cells were transiently co-transfected with 5 μg of pEXV (control vector) or pEXV-RhoN19 along with pSRE-Luc (3 μg). As shown in Fig. 2B, AA-induced SRE activation is significantly inhibited by co-transfection with pEXV-RhoN19 (approximately 75–80% reduction of luciferase activity), not with pEXV (vector), again suggesting that Rho activity is critical for the c-*fos* SRE stimulation by AA. Together, these results suggest that AA-induced c-*fos* SRE activation requires Rho activity in Rat-2 fibroblast cells.

3.3. *RacV12-induced SRE activation requires Rho*

To obtain evidence regarding the role of Rho in Rac-induced signaling to c-*fos* SRE, we tested whether Rac-induced c-*fos* SRE activation also requires Rho. pEXV-RacV12 encoding a constitutively active GTPase-deficient allele of Rac1, termed RacV12, was transiently co-transfected with a reporter plasmid, pSRE-Luc. As shown in Table 1, RacV12-induced stimulation of SRE luciferase activity was significantly diminished in a dose-dependent manner (up to ~60% reduction of luciferase activity) by co-transfection with EF-C3, but not with EF. Similarly, by co-transfection with pEXV-RhoN19, significant inhibition of the AA-induced SRE activation was observed in a dose-dependent manner (Table 1, bottom). These results indicate that the Rac signaling cascade leading to c-*fos* SRE activation is largely mediated via a Rho-dependent pathway although we cannot rule out the existence of alternative signaling routes which will stimulate c-*fos* SRE independently of Rho. These results are well in agreement with our proposed model for 'PLA_2-AA' as one of the major downstream elements of Rac in the nuclear signaling pathway.

3.4. *AA induces a translocation of RhoA GTPase protein*

The essential role of Rho in the AA signaling cascade was further examined by determining whether exogenous AA stimulates the translocation of Rho protein. To do this, serum-starved Rat-2 cells were stimulated with 100 μM of AA for 10 min. Then, the cells were lysed, and the particulate fractions were analyzed for RhoA by Western blotting. As shown in Fig. 3A, the level of RhoA protein localized in the particulate membrane fraction is induced to increase by AA (~1.8-fold increase as shown in lane 2). Thus, this result indicates stimulation of RhoA during the AA signaling cascade in Rat-2 fibroblast cells. As shown in Fig. 3A, lanes 3–6, the AA-induced translocation of RhoA was not affected by mepacrine (a specific PLA_2 inhibitor) or indomethacin (a cyclooxygenase

Table 1
Inhibition of RacV12-induced SRE activation by co-transfection with pEF-C3 or pEXV-RhoN19

	+Co-transfected DNA	Amounts of co-transfected DNA (μg)			
		0	0.5	0.75	1.0
pEXV	+pEF	1	1.1±0.1	0.9±0.2	0.9±0.2
pEXV-RacV12	+pEF	7.2±0.2	7.1±0.2	7.0±0.4	6.8±0.3
pEXV	+pEF-C3	1	0.9±0.2	1.0±0.1	0.8±0.2
pEXV-RacV12	+pEF-C3	7.2±0.2	5.5±0.2	3.9±0.3	2.7±0.2
	+Co-transfected DNA	Amounts of co-transfected DNA (μg)			
		0	1.0	2.0	5.0
pEXV	+pEXV	1	1.0±0.1	0.9±0.2	0.8±0.3
pEXV-RacV12	+pEXV	7.2±0.2	7.0±0.2	6.9±0.7	6.6±0.5
pEXV	+pEXV-RhoN19	1	0.8±0.2	1.1±0.4	0.8±0.3
pEXV-RacV12	+pEXV-RhoN19	7.2±0.2	5.8±0.4	4.2±0.5	3.0±0.4

A reporter gene plasmid, pSRE-Luc (3 μg) and 5 μg of pEXV or pEXV-RacV12 were transiently co-transfected with indicated amounts of pEF or pEF-C3 (top) or indicated amounts of pEXV or pEXV-RhoN19 (bottom). The total amount of DNA was kept at 20 μg with calf thymus carrier DNA. Transfected cells were serum-deprived in 0.5% FBS/DMEM for 36 h before assays for luciferase and β-galactosidase activities. The results are means ± S.D. obtained with three samples.

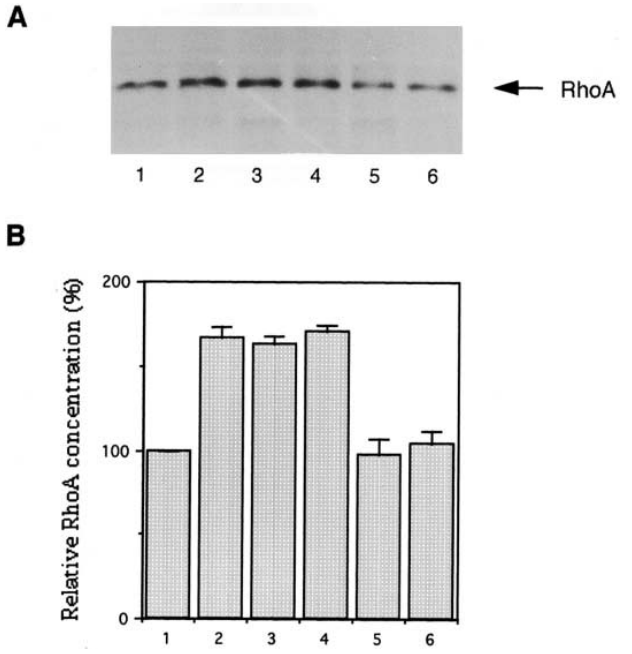

Fig. 3. AA stimulates translocation of RhoA in a LO-dependent manner. A: Cells were stimulated with buffer (lane 1), AA (100 μM, lane 2), AA+mepacrine, 1 μM (lane 3), AA+indomethacin, 10 μM (lane 4), AA+NDGA, 5 μM (lane 5), or AA+KN-62, 0.75 μM (lane 6) for 10 min, lysed, and the membrane fraction was prepared as described in Section 2. The inhibitors were pretreated for 20 min before the addition of AA. The increasing level of RhoA (~25 K_d) was detected by Western blotting of equal amounts of membrane fraction proteins. B: Data are expressed as a relative percentage of the basal value (buffer treatment; 100%) and are the average ± standard error of three experiments.

inhibitor) pretreatment, but was severely inhibited by NDGA (a LO inhibitor) or KN-62 (a CaM kinase II inhibitor) pretreatment. In Fig. 3B, these data are expressed as relative percentages of the basal value obtained with buffer treatment. This result points to a critical role of LO and also possibly CaM kinase II in the AA-induced signaling to RhoA activation.

In the present study, we have shown for the first time that AA, a principal product of Rac-activated PLA_2, activates c-*fos* SRE via a Rho-dependent signaling pathway in Rat-2 fibroblast cells, thus demonstrating a signaling link between Rac and Rho. Our proposed mode of the 'Rac-AA-Rho' signaling cascade to c-*fos* SRE activation seems very similar to that of the actin remodeling pathway suggested by Peppelenbosch et al. [5,6]. In the actin remodeling system, Rac was shown to activate Rho GTPase via AA metabolism to leukotriene production, an event necessary for growth factor-dependent actin stress fiber formation in fibroblast cells [5,6]. Similarly, we previously observed that AA release via Rac-activated PLA_2 and subsequent LO metabolites is critical in mediating Rac signaling to c-*fos* SRE [7]. Thus, AA release and subsequent metabolism by LO appear to be commonly involved in the Rac-mediated signaling pathways to either actin remodeling or gene regulation. Consistent with this idea, we observed that the addition of leukotriene $C_4/D_4/E_4$, metabolic products of 5-LO, induces a significant activation of SRE luciferase activity (data not shown). Further studies aimed at understanding the signaling link between AA and Rho activation in the control of Rac-induced c-*fos* SRE activation will lead to additional insights into the regulation of the Rac-Rho signaling link.

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