Essential role of Rac GTPase in hydrogen peroxide-induced activation of c-fos serum response element

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Abstract In the present study, we investigated whether hydrogen peroxide activates *c-fos* serum response element (SRE) in Rat-2 fibroblast cells. By transient transfection analysis, exogenous H_2O_2 stimulated SRE-dependent reporter gene activity in a dose and time-dependent manner. Also, we examined the role of Rac GTPase and phospholipase A_2 (PLA_2) in the H_2O_2-induced SRE activation. Either transfection of a dominant negative Rac mutant, RacN17, plasmid or pretreatment of mepacrine, a potent inhibitor of PLA_2, blocked H_2O_2-induced SRE activation dramatically. Together, these findings suggest a critical role of 'Rac and subsequent activation of phospholipase A_2' in the signaling pathway of H_2O_2 to SRE.

Key words: Hydrogen peroxide; Rac GTPase; *c-fos*; Serum response element; Phospholipase A_2; Arachidonic acid

1. Introduction

The Rac GTPase, a member of Rho family GTPases, regulates the growth factor-induced re-organization of actin filament, known as membrane ruffling [1–3]. Recently, several groups reported that Rac activates the *c-Jun* amino-terminal kinase (JNK) signaling cascade, composed of MEKK (MEK kinase-1), JNKK (JNK kinase) and JNK (*Jun* N-terminal kinase); this pathway is also termed the stress-activated protein kinase (SAPK) pathway [4,5]. The JNK cascade has been shown to be predominantly activated by environmental stress and cytokines to stimulate *c-fos* SRE (serum response element) via TCF (ternary complex factor)/Elk-1-dependent manner [4,5]. The *c-fos* SRE has been shown to be necessary and sufficient for the rapid induction of *c-fos* gene transcription in response to serum stimulation and is a primary nuclear target for many extracellular signals such as growth factors, cytokines, and environmental stress [6,7].

Recently, hydrogen peroxide (H_2O_2) has been shown to induce the expression of several immediate early genes including *c-fos* [8]. However, neither the H_2O_2-responsive element on *c-fos* promoter nor the mediator components of H_2O_2 signaling cascade has not been clearly elucidated. As SRE is an essential cis element responsible for the rapid induction of *c-fos* promoter in response to environmental stress, we determined whether *c-fos* SRE also responds to H_2O_2 which will cause an oxidative stress in the cell. For this study, Rat-2 cells were transiently transfected with a reporter plasmid, pSRE-Luc containing *c-fos* SRE fused to luciferase coding sequences. SRE activation in response to H_2O_2 was monitored by luciferase activities normalized with co-transfected β-galactosidase activity. In the present study, we demonstrate that exogenous H_2O_2 stimulates SRE in a dose- and time-dependent manner in Rat-2 fibroblast cells. Also, we show that Rac and subsequent phospholipase A_2 activation are essential for the H_2O_2 signaling pathway to *c-fos* SRE. In addition, our studies indicate that exogenous H_2O_2 stimulates *c-fos* SRE via Elk-1/TCF (ternary complex factor)-independent mechanism.

2. Materials and methods

2.1. Chemicals and reagents

Phorbol 12-myristate 13-acetate (PMA), H_2O_2, *N*-acetyl cysteine, calphostin C, mepacrine were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS), gentamycin, Dulbecco's modified eagle's medium (DMEM) were from Gibco-BRL (Gaithersburg, MD). All other chemicals were from standard sources and were molecular biology grade or higher.

2.2. Plasmids and DNA manipulations

The reporter genes pSREwt-Luc and pSREmt-Luc were derived from pFos-lcf [9]. They contain positions −53 to +45 of the *c-fos* promoter upstream of the luciferase gene with wild-type SRE or mutant SRE oligonucleotides inserted at the −53 position [9]. pEXV and pEXV-RacN17 plasmids were gifts from Dr. Alan Hall and all the Rac family proteins were expressed as N-terminally 9E10 epitope-tagged derivatives under SV40 promoter [10].

2.3. 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) FBS and gentamycin as described before [11]. Transient transfection analysis was performed by calcium phosphate/DNA precipitation method [12,13]. To control for variations in both cell numbers and transfection efficiency, all clones were co-transfected with pCMV-bGAL, an eucaryotic expression vector in which *E. coli* β-galactosidase (lacZ) structural gene is under the transcriptional control of the CMV promoter. 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. Luciferase activity was determined as described previously [14]. Transfection experiments were performed in triplicate with two independently isolated sets and the results were averaged.

2.4. [^{3}H]Arachidonic acid release

Rat-2 cells in DMEM medium containing 10% FBS were plated to 1×10^5 cells per well (12-well plate). After 3 h 0.5 μCi/ml of [^{3}H]arachidonic acid (250 μCi; Amersham) was supplemented to each well and incubated for 24 h, then washed at least 8 times with DMEM containing 0.5% FBS. Then, cells were labeled with [^{3}H]arachidonic acid at 0.5 μCi/ml for 48 h in DMEM/0.5% FBS,

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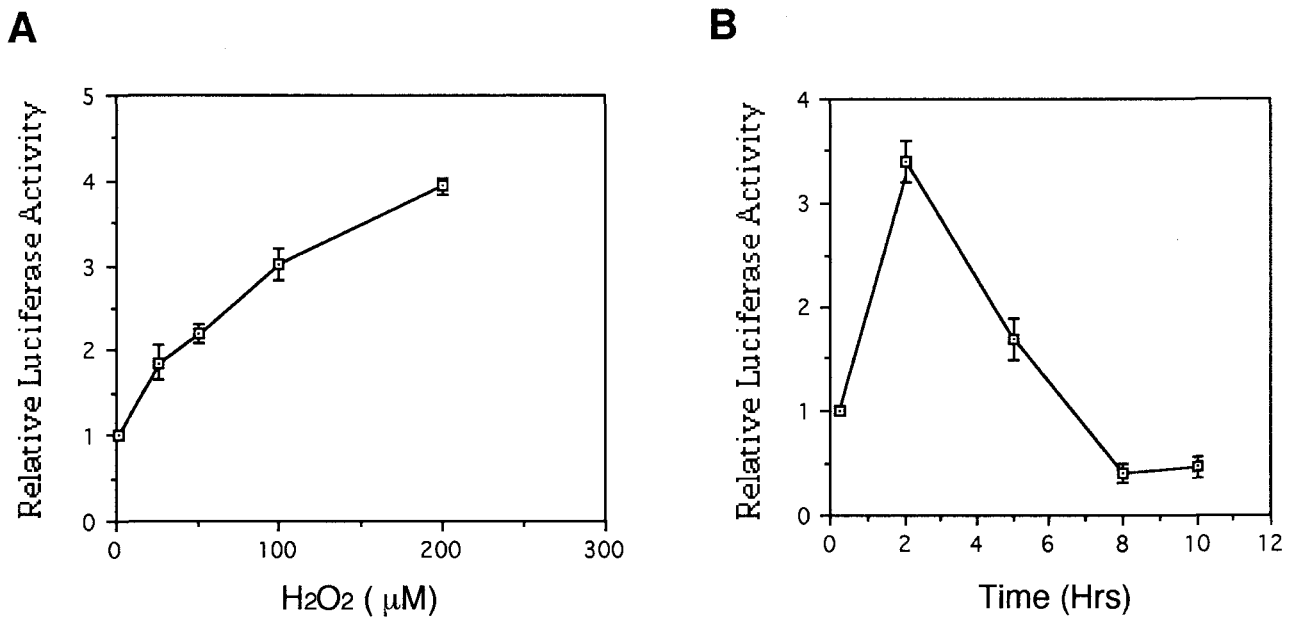

Fig. 1. H_2O_2 activates SRE in a dose- and time-dependent manner. After transient transfections with pSREwt-Luc (3 μg) plasmid, Rat-2 cells were serum-starved in DMEM/0.5% FBS for 36 h before adding H_2O_2. A: Dose response of H_2O_2 on SRE activation. Various amounts of H_2O_2 (0, 25, 50, 100, 200 μM) were treated for 2 h to serum-starved cells. B: Time-dependent response of H_2O_2 (100 μM) on SRE activation. Serum-starved Rat-2 cells were treated with H_2O_2 for the lengths of time indicated. Luciferase and β-galactosidase activity were measured as described in Section 2.

washed 4 times with DMEM/0.5% FBS, and finally added with 2 ml of DMEM/0.5% FBS containing H_2O_2 (100 μM) for 2 h. The released [^{3}H]arachidonic acid into the medium was quantitated by scintillation counting of 0.5 ml of medium each time. At the end of experiments, the cells were solubilized with 0.5 ml of ethyl alcohol (EtOH) for the determination of intracellular total incorporation. Counts were corrected for total incorporation.

3. Results

3.1. H_2O_2 stimulates SRE in a dose- and time-dependent manner

The SRE-stimulating activity of exogenous H_2O_2 was analyzed by transient transfection analysis of a reporter plasmid, pSRE-Luc containing *c-fos* SRE fused to luciferase coding sequences. Following transient transfection, Rat-2 cells were serum-starved in DMEM containing 0.5% FBS for 36 h before treating H_2O_2. SRE activation was monitored by luciferase activities normalized with co-transfected β-galactosidase activity. As shown in Fig. 1, exogenous H_2O_2 stimulated the *c-fos* SRE-dependent reporter gene activity in a dose- and time-dependent manner (Fig. 1). A 3.5-fold increase in the luciferase activity occurred 2 h after the addition of exogenous H_2O_2 and by 8 h, the *c-fos* SRE luciferase level declined (Fig. 1B). A 3-fold increase in the luciferase activity was detected at 100 μM concentration of H_2O_2 (Fig. 1A). In a control experiment to demonstrate the specific action of H_2O_2 toward SRE,

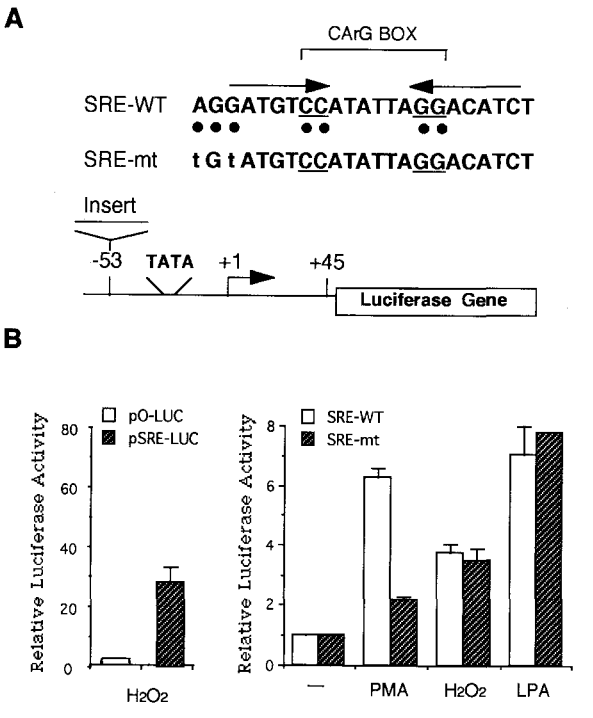

Fig. 2. H_2O_2-dependent SRE activation by TCF-independent pathway. A: Diagram of pSRE-luciferase reporter gene plasmids that were used. The structures of constructs containing wild-type or mutant SRE oligonucleotide sequences (23 mers) inserted to −53 position of the truncated *c-fos* promoter fused to luciferase gene are shown. Also, the methylation interference pattern for SRF ternary complex with TCF is designated as closed circles. The mutant SRE has two point mutations (*AGG* to *TGT*) in the TCF-binding region that abolish the SRF/TCF ternary complex formation. B (left): Transient transfection assay with pO-Luc or pSREwt-Luc. H_2O_2 (100 μM) was treated for 2 h before harvest and luciferase and β-galactosidase activities were measured. The relative activation of pSRE-luciferases to pO-luciferase was calculated. B (right): Transient transfection assay with pSREwt-Luc or pSREmt-Luc (TCF-binding defective SRE). H_2O_2 (100 μM), PMA (10 ng/ml), or LPA (10 μM) was treated for 2 h before harvest. The relative promoter activity was determined as described in Section 2 and histograms of the results of the luciferase are shown. Values were representative of multiple transfections.

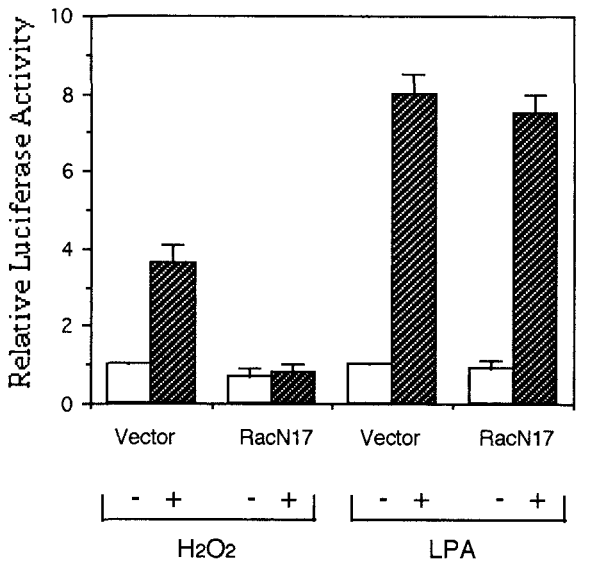

Fig. 3. Rac activity is essential for the H_2O_2-induced SRE activation. A reporter gene plasmid, pSREwt-Luc (3 μg) plasmid was transiently co-transfected with 5 μg of pEXV (vector) or pEXV-RacN17 (RacIN17). Total amounts of DNA was kept at 20 μg with calf thymus carrier DNA. Transfected cells were serum-deprived in DMEM/0.5% FBS for 36 h before harvest. H_2O_2 (100 μM) or LPA (10 μM) was added 2 h prior to cell harvest, and luciferase and β-galactosidase activities were measured. Values were representative of multiple transfections.

Rat-2 cells were transiently transfected with pO-Luc (vector only without SRE insert). No stimulation of luciferase activity, however, was observed in response to H_2O_2 addition, suggesting that the H_2O_2 signal to SRE activation is specific (Fig. 2B).

3.2. H_2O_2 activates SRE in TCF/Elk-1-independent pathway

As *c-fos* SRE activation involves both TCF/Elk-1-dependent and TCF/Elk-1-independent signaling pathways [6,7,9], we examined which signaling pathway is responsible for H_2O_2-induced SRE activation. To do this, Rat-2 cells were transiently transfected with a mutant SRE-luciferase plasmid defective in TCF binding (pSREmt-Luc; Fig. 2A) and measured the luciferase activity after H_2O_2 treatment. The oligonucleotide in pSREmt-Luc plasmid contains two point mutations (*AGA* to *TGT*) which abolish TCF binding [9]. H_2O_2 activated both SRE mutant and SRE wild-type luciferase to the same extent (≈3.5 fold), indicating that H_2O_2 activates *c-fos* SRE in TCF/Elk-1-independent signaling pathway in Rat-2 fibroblasts (Fig. 2B). In contrast, PMA (phorbol-12-myristate-13-acetate; 10 ng/ml) did not activate mutant SRE-dependent luciferase activity. LPA (lysophosphatidic acid; 10 mM), which had been shown to activate SRE by Elk-1-independent pathway, stimulated both SRE mutant- and SRE wild-type-dependent luciferase activity to the same level (Fig. 2B).

3.3. Rac is essential for the H_2O_2-induced signaling pathway to SRE activation

Because Rac, a member of Rho family GTPases, has been reported to be involved in the activation of SRE in response to environmental stress [4,5], we postulated that H_2O_2 may induce *c-fos* SRE activation via Rac activation. To determine whether H_2O_2-induced SRE activation is dependent on Rac activity in Rat-2 cells, we used an expression vector encoding a dominant negative Rac1 mutant (RacN17). As shown in Fig. 3, co-transfection of RacN17 (pEXV-RacN17) led to almost complete inhibition of SRE activation by H_2O_2. On the other hand, LPA (lysophosphatidic acid)-induced SRE activa-

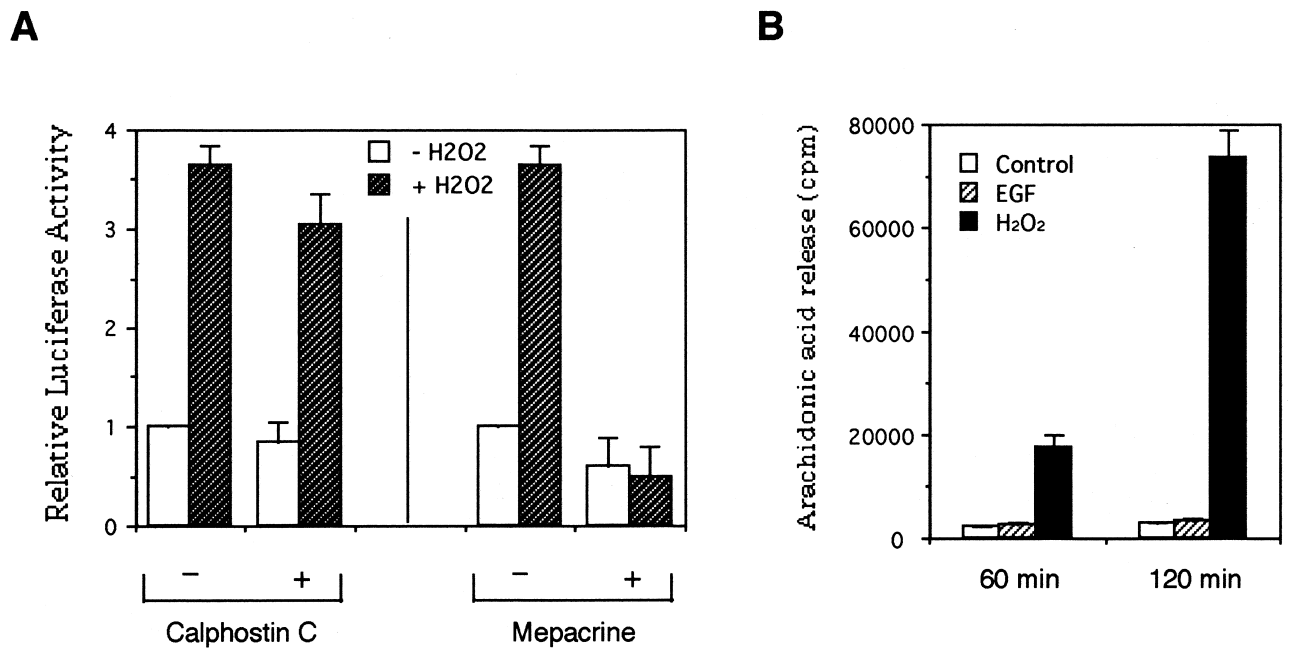

Fig. 4. H_2O_2 leads to the activation of PLA_2 and subsequent release of AA. A: PLA_2 activation is essential for the H_2O_2-mediated signaling pathway to SRE. Subconfluent Rat-2 cells were transiently transfected with 3 μg of pSREwt-Luc plasmid and then serum-starved for 36 h in DMEM/0.5% FBS. Either calphostin C (PKC inhibitor; 50 nM) or mepacrine (PLA_2 inhibitor; 50 nM) was pretreated 30 min before addition of H_2O_2 (100 μM) or control buffer (−H_2O_2). At 2 h later, luciferase activities were measured and normalized with co-transfected β-galactosidase activities. B: H_2O_2 induces the release of arachidonic acid. The release of [^{3}H]arachidonic acid from the pre-labeled cells stimulated with control buffer, H_2O_2 (100 μM), or EGF (50 ng/ml) was measured at two different time points (60, 120 min) as described in Section 2.

tion was not affected by RacN17, indicating that the action of RacN17 is specific to H_2O_2 (Fig. 3). This result suggests an essential role of Rac GTPase in the signaling pathway of H_2O_2 to SRE.

3.4. *Phospholipase A_2 is essential for H_2O_2-induced SRE activation*

As it was reported that the activation of Rac GTPase induces the arachidonic acid (AA) release via phospholipase A_2 (PLA_2) activation, we examined whether Rac-activated phospholipase A_2 is essential for the H_2O_2-induced SRE activation [15–17]. As shown in Fig. 4A, the inhibition of PLA_2, by a 30 min preincubation with mepacrine (50 nM), abolished completely the SRE activation by H_2O_2. On the other hand, the inhibition of protein kinase C (PKC) by calphostin C (50 nM) did not show any inhibition (Fig. 4A). These results suggest that the activity of phospholipase A_2, not protein kinase C, is required for the H_2O_2-induced SRE activation in Rat-2 cells. Next, we determined whether H_2O_2 activates arachidonic acid (AA) release via Rac-PLA_2. To do this, we measured the release of [^{3}H]arachidonic acid from the pre-labeled cells stimulated with H_2O_2 (100 μM) at two different time points (60, 120 min). As shown in Fig. 4B, H_2O_2 stimulated the release of [^{3}H]arachidonic acid dramatically (>10-fold over control) at both time points. The H_2O_2-induced release of [^{3}H]arachidonic acid was completely blocked by pretreatment of mepacrine (50 nM; data not shown). To determine whether H_2O_2-induced SRE activation is due to the oxidative free radicals triggered by H_2O_2 treatment, *N*-acetyl cysteine (NAC), a known free radical scavenger, was treated. NAC (5 mM) treatment inhibited the H_2O_2-induced SRE activation by 80%. These results suggest that H_2O_2-induced oxidative free radicals is responsible for the SRE activation (data not shown).

4. Discussion

As a first step towards understanding the biological mechanism of oxidative stress in the cell, we undertook the analysis of signal transduction pathway of H_2O_2. To do this, we determined whether H_2O_2 leads to the activation of SRE which has been reported to be stimulated by environmental stress [4–6]. As shown in Fig. 1, exogenous H_2O_2 stimulated SRE in a dose- and time-dependent manner, suggesting that SRE is one of the nuclear target sequences of H_2O_2 in Rat-2 fibroblast cells. In an attempt to understand the role of Rac in the H_2O_2-induced signaling pathway to SRE, a dominant negative mutant, RacN17 was transiently transfected to Rat-2 fibroblast cells. As shown in Fig. 3, RacN17 specifically inhibited the H_2O_2-induced SRE activation, suggesting a critical role of Rac GTPase in the signal transduction pathway of H_2O_2 to SRE. Although Rac activation and subsequent arachidonic acid metabolism appear to be essential for H_2O_2 signaling pathway to SRE activation, the downstream kinase cascade that mediates this effect is yet to be identified. Recently, Rac has been reported to activate the *c-Jun* amino-terminal kinase (JNK) signaling cascade in response to cytokines and environmental stress, thereby activating TCF (ternary complex factor) protein, Elk-1 to activate SRE [4–6]. However, it is unlikely that this MAP kinase cascade mediates the H_2O_2-induced SRE activation, as H_2O_2 activates SRE in TCF/Elk-1-independent signaling pathway in Rat-2 fibroblasts. In consistent with our observation, Rho family GTPases including Rac have been shown to play a role in the signaling route to SRE activation through TCF/Elk-1-independent pathway [18]. Thus, Rac may mediate both TCF/Elk-1-dependent and independent signaling pathway to SRE activation depending on the type of agonist or stress.

Our results suggest that Rac and subsequent activation of phospholipase A_2 are essential for the H_2O_2-induced SRE activation. In support of our results, recent report suggests that the activation of Rac GTPase induces the release of arachidonic acid (AA) in response to epidermal growth factor (EGF) via PLA_2 activation [15–17], suggesting a critical role of Rac GTPase in the release of arachidonic acid. In the present study, we demonstrate for the first time that exogenous H_2O_2 activates SRE-reporter gene activity specifically in Rat-2 cells and also we propose an essential role of Rac GTPase in the signaling pathway of H_2O_2 to SRE.

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