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Evidence that 12-lipoxygenase product 12-hydroxyeicosatetraenoic acid activates p21-activated kinase

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The effect of 12-hydroxyeicosatetraenoic acid (12-HETE), an arachidonic acid metabolite of 12-lipoxygenase, to activate $p21^{Rac/Cdc42}$-activated kinase (PAK1) was studied in a Chinese hamster ovary fibroblast cell line overexpressing the rat vascular type-1a angiotensin II receptor (CHO-AT_{1a}). 12-HETE (0.1 μM) treatment induced a time-dependent activation of PAK1, with a peak effect at 10 min ($335 \pm 16\%$ of control; $n = 3$, $P < 0.001$). The stimulatory effect of 12-HETE on PAK1 activity was dose-dependent, with the maximal activation at 0.01 μM ($350 \pm 15\%$ of control; $n = 3$, $P < 0.001$). A PAK1 fragment encoding the Cdc42/Rac binding domain (amino acid residues 67–150 of hPAK1 termed PBD), was transfected into CHO-AT_{1a} cells. PBD transfection markedly reduced 12-HETE-induced PAK1 activation. Furthermore, transfection of dominant negative Cdc42 and Rac1 inhibited 12-HETE-induced PAK1, strongly suggesting that Cdc42 and Rac1 are the upstream activators of 12-HETE-induced PAK1 activation. Low concentrations (1.5 μM) of LY294002, a highly specific inhibitor of phosphoinositide 3-kinase (PI-3K), abolished 12-HETE-induced PAK1 activation, suggesting that PI-3K activation is upstream of 12-HETE-induced PAK1 activation. Transfection of dominant negative PAK1 blocked 12-HETE-induced PAK1, cJun N-terminal kinase (JNK1) and extracellular-signal-regulated kinase (ERK) activity, while transfection of constitutively active PAK1 stimulated PAK1, JNK1 and ERK activity, suggesting that PAK1 is an upstream activator of 12-HETE-induced JNK1 and ERK activation in these cells. We conclude that 12-HETE can activate Cdc42, Rac1 and PI-3K, which then participate as upstream signalling molecules for PAK1 and JNK1 activation.

Key words: Cdc42, phosphoinositide 3-kinase, Rac.

INTRODUCTION

p21-activated kinases (PAKs) are a novel group of 60–70 kDa serine/threonine kinases [1]. Several distinct members of the PAK family have been identified [2–5]. The C-terminal catalytic domain of PAKs is 60–70% identical to *Saccharomyces cerevisiae* Ste20, an enzyme involved in linking pheromone-activated G protein-coupled receptors to a mitogen-activated protein (MAP) kinase cascade. The N-terminal segment of the PAK family of kinases contains a Cdc42/Rac interactive binding (CRIB) domain and several proline-rich sequences similar to canonical SH3 domain binding regions [6,7]. It has been demonstrated that glutathione S-transferase (GST) fusion proteins containing the SH3 domains of Nck and phospholipase Cγ can bind to PAK3 and PAK1 in an *in vitro* binding assay [6].

PAKs complex specifically with activated (GTP-bound) Cdc42 and Rac proteins, but not Rho proteins, leading to kinase autophosphorylation and activation, while GDP-bound forms of Rac and Cdc42 do not interact with the kinases [1]. The mechanism that underlies the stimulation of kinase activity by these GTP-binding proteins is not fully known. It is likely that the binding of the GTPase to an identified specific region, the CRIB domain, within the N-terminal half of the kinase releases a negative constraint [4]. New evidence shows that PAK proteins contain a basic region, consisting of three contiguous lysine residues (Lys^{66}-Lys^{67}-Lys^{68}) which lie outside of the previously identified CRIB and are required elements for Rac/PAK interaction [8]. It was also recently suggested that Rac functions downstream, instead of upstream, of phosphoinositide 3-kinase (PI-3K) in the insulin signalling cascade leading to activation of PAK65 [9].

Evidence has shown that Cdc42/Rac1 can activate the cJun N-terminal kinases (JNKs) and p38 MAP kinase in certain cell types upon co-transfection [10–13]. The potential candidates for coupling Cdc42/Rac1 to JNK are the PAK family, including PAK1, PAK2 and PAK3. PAK fragments that include the CRIB inhibit epidermal growth factor activation of JNK and interleukin-1 activation of p38. Moreover, constitutively active mutants of PAK1 or PAK3 activate JNK and p38 kinases upon co-transfection. However, the identity of the PAK substrate(s) that couple these kinases to stress-activated protein kinase/JNKs and p38 pathways and the mechanisms involved in this activation have not yet been elucidated [14].

The 12-lipoxygenase (12-LO) enzyme generates active lipids from arachidonic acid. The lipid products of 12-LO, including 12-hydroxyeicosatetraenoic acid (12-HETE), have been shown to lead to growth promoting or inflammatory changes in various types of cells. Our previous studies have demonstrated that 12-LO products can promote cell growth in Chinese hamster ovary fibroblasts stably overexpressing the angiotensin type Ia receptor (CHO-AT_{1a}) [15]. 12-HETE activates extracellular-signal-regulated kinase (ERK) [14] and JNK1 in CHO-AT_{1a} cells when added in the medium [16]. In the present study, we demonstrate for the first time that 12-LO products are able to activate PAK.

Abbreviations used: CHO-AT_{1a} cells, angiotensin II receptor-transfected Chinese hamster ovary cells; JNK, cJun N-terminal kinase; 12-HETE, 12-hydroxyeicosatetraenoic acid; LO, lipoxygenase; PAK, $p21^{Rac/Cdc42}$-activated kinase; PBD, $p21^{Rac/Cdc42}$ binding domain; CRIB, Rac/Cdc42 interactive binding domain; FBS, fetal bovine serum; DTT, dithiothreitol; MLK, mixed lineage kinase; PI-3K, phosphoinositide 3-kinase; ERK, extracellular-signal-regulated kinase; MKK, mitogen-activated protein kinase (MAPK) kinase; MEK, MAPK/ERK kinase.

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The data indicate that small-molecular-mass GTP-binding proteins are involved as upstream activators of PAK, and moreover, that PI-3K is involved in activating PAK in response to 12-HETE. Finally, we demonstrate that PAK is an upstream activator of JNK and ERK in CHO-AT_{1a} cells.

MATERIALS AND METHODS

Reagents

Ham's F-12 medium and fetal bovine serum (FBS) were supplied by Irvine Scientific (Santa Ana, CA, U.S.A.). BSA (fatty acid free), leupeptin and aprotinin were from Sigma. 12-HETE and LY294002 were from Biomol (Plymouth, Meeting, PA, U.S.A.). Wortmannin was from Alexis Corporation. Dr Michael Karin (UC San Diego, CA, U.S.A.) kindly provided the GST–cJun (amino acid residues 1–79) plasmid. JNK1 antibody was from Santa Cruz Biotechnology. [γ-^{32}P]ATP was from New England Nuclear Corp. (Bannockbure, IL, U.S.A.). PAK1 antibodies [2] and the following plasmids: pCVM6M–PBD (residues 67–150); wild-type PAK1; dominant negative PAK1, PAK1K299R; constitutively active PAK1, PAK1T423E; empty vector, pRK5; dominant negative Rac, pRK5–RacT17N; and dominant negative CDC42, pRK5–Cdc42T17N were used.

Cell culture and preparation of cell extracts

CHO-AT_{1a} cells were maintained in 100 mm dishes in Ham's F-12 media with 10% FBS, as described in [15–17]. Cells were growth-arrested by incubation in depletion medium (Ham's F-12 medium containing 1 mg/ml BSA and 20 mM Hepes, pH 7.4, for 72 h prior to use. Cells were treated with 12-HETE in serum-free, BSA-containing medium. After washing twice with cold PBS, the cells were lysed by lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1% Nonidet P40, 2.5% glycerol, and 1 mM sodium orthovanadate, containing the protease inhibitors PMSF, leupeptin and aprotinin). The lysate was centrifuged at 14000 × ***g*** at 4 °C for 10 min. Protein determination of lysate was done by the Bradford method.

Transient transfection in CHO-AT_{1a} cells

Plasmids used were endotoxin-free and prepared by EndoFree plasmid kit (Qiagen) using the standard protocol. The DNA-transfection method used was a cationic liposome-mediated transfection with DOSPER liposomal transfection reagent (Boehringer Mannheim) which followed the manufacturer's instructions. Briefly, the cells were plated the day before the transfection experiment at 3×10^6 cells per 100 mm dish. Next day, cells were washed with Opti-MEM® reduced-serum medium (Gibco BRL) and incubated in 5 ml of Ham's F-12 with 1% FBS. A 45 μg DOSPER/15 μg plasmid mixture was prepared and added to each dish. After 5 h incubation the transfection medium was replaced and 8 ml of fresh depletion medium was added, containing 1% FBS, for overnight incubation. The cells were washed twice with depletion medium, incubated in the same medium for another 32 h, and harvested.

Immunoprecipitation and PAK1 activity assay

For immunoprecipitation, 300 μg of lysate protein was incubated with PAK1 antibody (1:20) [2] in the lysis buffer overnight at 4 °C, followed by incubation with 60 μl of a 50% slurry of Protein A beads for 60 min. After washing 3 times with lysis buffer and twice with kinase buffer, the kinase activity was measured in 60 μl of kinase buffer [50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 0.2 mM dithiothreitol (DTT)] containing 2 μg of myelin basic protein and 20 μM ATP and 5 μCi of [γ-^{32}P]ATP. After incubation for 30 min at 30 °C, the reaction was stopped with 5 × Laemmli sample buffer and resolved on an SDS/12% polyacrylamide gel, followed by autoradiography.

Immunoprecipitation and Jun kinase assay

For immunoprecipitation, 50 μg of lysate protein in diluted WCE buffer (20 mM Hepes, pH 7.7, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 5 μg/ml leupeptin and aprotinin and 0.1 mM PMSF) was used, mixed with 10 μl of JNK-1 antibody, and the mixture was rotated at 4 °C overnight and added to 60 μl of Protein A–Sepharose. After 1 h incubation at 4 °C, the beads were washed 4 times with diluted WCE buffer, the pelleted beads were resuspended in 60 μl of kinase buffer, 20 mM Hepes at pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 0.1 mM sodium orthovanadate and 2 mM DTT, containing 2 μg of GST–cJun (residues 1–79) and 20 μM ATP and 5 μCi of [γ-^{32}P]ATP. After 30 min at 30 °C, the reaction was stopped with 5 × Laemmli sample buffer and resolved on an SDS/12% polyacrylamide gel, followed by autoradiography. According to the manufacturer's data, the antibody JNK-1 is specific for the JNK-1 protein and does not react to any significant degree with JNK-2.

Immunoprecipitation and ERK assay

Immunoprecipitation and kinase assay were also used for ERK1 activity measurement. A 50 μg sample of lysate protein was added in 500 μl of ERK buffer [50 mM Hepes, pH 7.4, 1% Triton X-100, 80 mM β-glycerophosphate, 10 mM EGTA, 2 mM EDTA, 1 mM nitrophenyl phosphate, 10 mM NaF, 10 mM pyrophosphate, 1 mM sodium orthovanadate, 20 μg/ml of leupeptin and aprotinin, and 0.1 mM PMSF) and mixed with 10 μl of ERK1 antibody. The mixture was rotated at 4 °C overnight, then the solution was added to 60 μl of Protein A–Sepharose. After 1 h incubation at 4 °C, the beads were washed 4 times with diluted ERK buffer, the pelleted beads were resuspended in 60 μl of kinase buffer (20 mM Hepes at pH 7.4, 10 mM $MgCl_2$, 5 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 0.1 mM sodium orthovanadate, 2 mM DTT, 2 μg of myelin basic protein and 20 μM ATP and 5 μCi of [γ-^{32}P]ATP). After 30 min at 30 °C, the reaction was stopped with 5 × Laemmli sample buffer and resolved on an SDS/12% polyacrylamide gel, followed by autoradiography. The antibody against ERK1 was from Santa Cruz.

Detection of PAK1 and PAK2 in CHO-AT_{1a} cells by immunoblotting

A sample of protein (20–30 μg) from CHO-AT_{1a} cell lysate was resolved with SDS/7.5% PAGE, transferred on to nitrocellulose, and probed with antisera detecting both PAK1 and PAK2, as described in [2].

Data analysis

The results are expressed as means ± S.E.M. from combined experiments, as noted in each legend. ANOVA with Dunnett's, Tukey–Kramer's multiple comparisons tests, or Student's *t*-tests were used to analyse the data. Autoradiograms of the JNK activity studies were analysed with an automated computerized

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densitometer (SCISCAN 5000; USB, Cleveland, OH, U.S.A.). Measurements were made in the linear range and the values expressed as arbitrary attenuance units or fold over control.

RESULTS

12-LO products activate PAK activity

The time frame for PAK1 activation induced by the 12-LO product, 12-HETE, was first assessed in CHO-AT_{1a} cells serum depleted for 3 days. The data in Figure 1 show that 0.1 μM 12-HETE treatment elicited PAK1 activation as early as 5 min ($140 \pm 11\%$ of control; $n = 3$) and the effect reached a peak at 10 min ($335 \pm 16\%$ of control; $n = 4$, $P < 0.001$). PAK1 activation was sustained to 10 min ($290 \pm 9\%$ of control; $n = 3$, $P < 0.001$) and decreased thereafter. However, 12-HETE still significantly activated PAK1 activity at 60 min ($196 \pm 21\%$ of control, $n = 3$, $P < 0.01$).

The dose-dependent activation of PAK1 by 12-HETE was also studied. Concentrations of 12-HETE as low as 0.1 nM could activate PAK1 activity ($183 \pm 20\%$ of control; $n = 3$, $P < 0.05$). The peak effect for activation was reached at 0.01 μM 12-HETE ($350 \pm 15\%$ of control; $n = 3$, $P < 0.001$). These data indicate that 12-HETE can dose-dependently increase PAK1 activity in CHO-AT_{1a} cells. To elucidate the particular PAK isoforms involved in 12-HETE activation, we first examined whether PAK1 or PAK2 is expressed in CHO-AT_{1a} cells. Figure 2(a) represents a Western blot with an antibody which can detect both PAK1 and PAK2, showing bands at 67 and 62 kDa, indicating that the PAK1 and PAK2 isoforms are expressed in CHO-AT_{1a} cells. A protein lysate from human neutrophils were taken as a control showing the same two bands. Figure 2(b) shows that 12-HETE activates predominantly PAK1. The antibody used was specific to PAK1 in the PAK1 activity assay, or specific to PAK2 in the PAK2 activity assay.

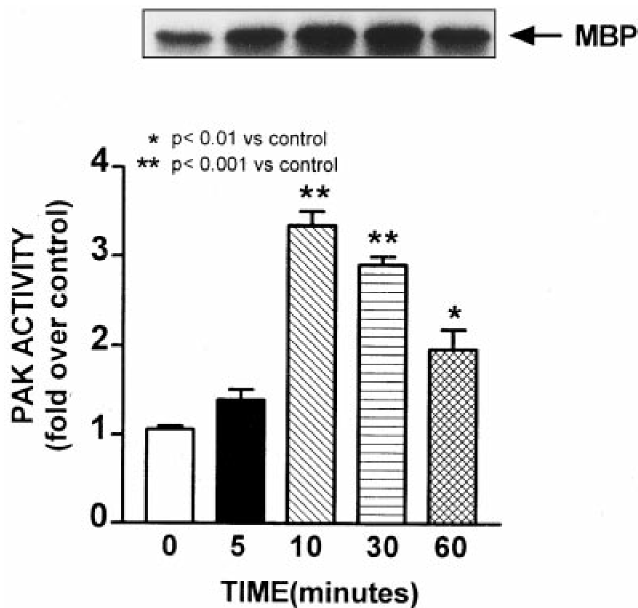

Figure 1 Time course of PAK1 activation by 12-HETE in CHO-AT_{1a} cells

The cells were gently washed and placed in depletion medium for 72 h prior to use. After incubation for 30 min, the cells were treated with 12-HETE (0.1 μM) or with ethanol. The 12-HETE treatment was terminated by washing twice with PBS and adding 300 μl of lysis buffer as described in the Materials and methods section. Top panel shows a representative autoradiogram of phosphorylated MBP bands from a gel. PAK1 activity was measured with MBP as a substrate. Bottom panel shows the densitometric quantification of PAK1 activity stimulated with 0.1 μM 12-HETE or ethanol in the control for the time indicated. Each point is an average (means $\pm$ S.E.M.) from at least 3 separate experiments. Results are expressed as stimulation over control.

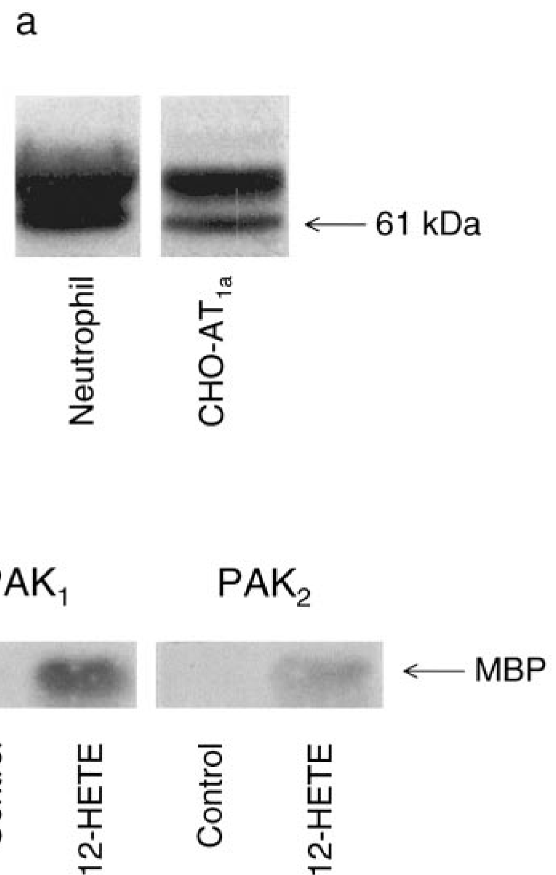

Figure 2 Western blot of PAK1 and PAK2 expression and 12-HETE activates PAK1 and PAK2 activity in CHO-AT1a cells

(**a**) Neutrophil cytosol protein (20 μg; left lane) or 30 μg of CHO-AT_{1a} cell cytosol protein (right lane) was resolved by SDS/PAGE, transferred on to a nitrocellulose membrane, and probed with antisera recognizing PAK1 and PAK2. The upper band is located at 67 kDa, the lower band at 62 kDa. (**b**) CHO-AT_{1a} cells were treated with 0.1 μM 12-HETE or vehicle (ethanol) in the control for 10 min. Detergent-containing lysates were immunoprecipitated with anti-PAK1 or anti-PAK2 antibodies. Phosphorylation reactions were conducted with MBP as substrate. The two left bands show the basal and 12-HETE-stimulated PAK1 activity, the right bands show the basal and 12-HETE-stimulated PAK2 activity. Identical results were seen in 3 experiments.

Small-molecular-weight GTP-binding proteins are the direct activators of PAK

To elucidate the mechanism of PAK1 activation by 12-HETE, a PBD plasmid encoding 84 amino acids (hPAK1, residues 67–150) which includes the CRIB domain was transfected into CHO-AT_{1a} cells. Figure 3 illustrates that PBD transfection almost completely inhibited 12-HETE-induced PAK1 activation ($n = 4$, $P < 0.001$). PBD transfection itself had no effect on basal PAK1 activity. Cells were treated with the transfection reagents only in the non-PBD-transfected group (without empty vector) under the same conditions. Dominant negative Rac1 and Cdc42 GTPase mutants were used to confirm this result. Plasmids

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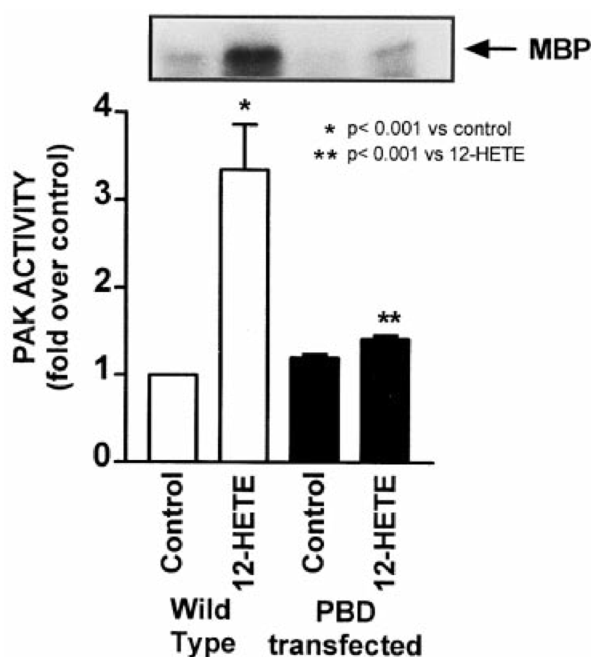

Figure 3 Inhibitory effect of transient transfection of PBD plasmid on 12-HETE-induced PAK1 activation

For the PBD-transfected group, CHO-AT_{1a} cells were transiently transfected with 15 μg of PBD plasmid as described in the Materials and methods section. For the non-PBD transfected group, CHO-AT_{1a} cells were treated with the same transfection reagents as the PBD-transfected group. Cells were then treated with 12-HETE or ethanol for 10 min. The top panel illustrates a representative autoradiogram of phosphorylated MBP bands, the bottom panel illustrates the densitometric quantification. Each point is an average (means ± S.E.M.) of at least 3 separate experiments. Results are expressed as stimulation over control. The PAK1 activity was measured as described in the Materials and methods section.

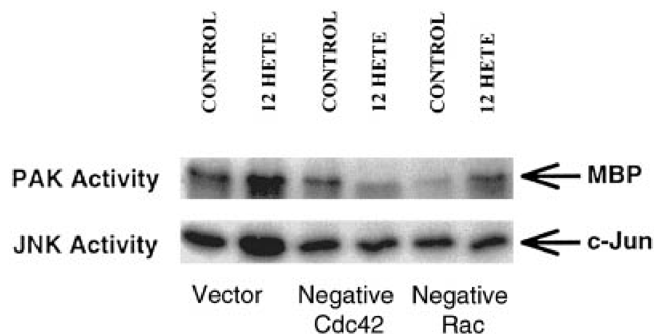

Figure 4 The effects of transfection of Rho GTPase mutants on PAK1 or JNK1 activity in CHO-AT_{1a} cells

CHO-AT_{1a} cells were transiently transfected with various dominant negative Rac1 and Cdc42 GTPases or empty vector as described in the Materials and methods section. Cells then were treated with 12-HETE or ethanol (control) for 10 min. Cell extracts were used for PAK1 and JNK1 activity measurement. Similar results were obtained from two experiments.

Rac1T17N, Cdc42T17N and empty vector pRK5 were transiently transfected into CHO-AT_{1a} cells. The transfected cells of each construct were divided into two samples, treated with ethanol or with 12-HETE, then PAK1 and JNK1 activity of the lysates was measured as shown in Figure 4. The upper line of Figure 4 shows the effect of Rac and Cdc42 mutant transfection

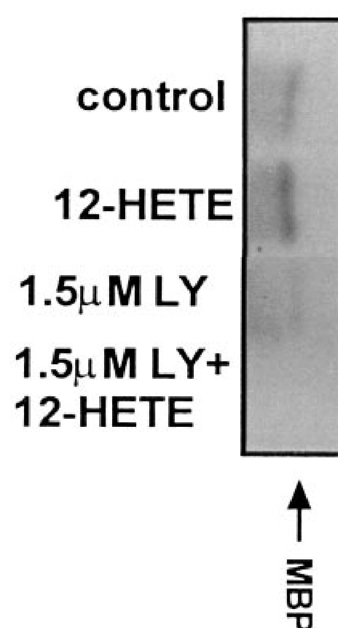

Figure 5 The PI-3-K inhibitor LY294002 inhibits 12-HETE-induced PAK1 activity

Cells were pretreated with different concentrations of LY294002 or DMSO in the control for 30 min, then treated with 12-HETE (0.1 μM) or ethanol in the control for 10 min. PAK1 activity was measured as described in the Materials and methods section. The Figure shows a representative autoradiogram of phosphorylated MBP bands from 3 similar experiments.

on 12-HETE-induced PAK1 activation. 12-HETE stimulated PAK1 activity in the cells expressing the empty vector compared with the PAK1 activity of cells treated with ethanol. The transfection of dominant negative Cdc42 (Cdc42T17N) completely attenuated 12-HETE-induced PAK1 activation. Interestingly, dominant negative Rac1 (Rac1T17N) significantly inhibited the basal PAK1 activity. Although 12-HETE could still increase PAK1 activity, 12-HETE addition could not reverse PAK activity to the basal level seen in empty vector-transfected cells. These results clearly support the role of Cdc42 and Rac1 in 12-HETE-induced activation of PAK1. Dominant negative Cdc42 and Rac1 mutants were also used to further evaluate the role of GTPases in the response of JNK1 activation by PAK. Part of cell extract from the samples for measuring the effect of mutant transfection on PAK1 activity was used for measuring JNK1 activity. The results of these studies are seen on the bottom line of Figure 4. 12-HETE stimulated JNK1 activity in cells expressing empty vector. However, 12-HETE no longer activated JNK1 activity in cells expressing dominantly negative Cdc42 (Cdc-42T17N) and negative Rac1 mutant (Rac1T17N), thus strongly suggesting that the 12-HETE activation pathway on JNK1 is mediated by Cdc42 and/or Rac1.

Evidence that PI-3K is an upstream activator of PAK

To evaluate whether PI-3K lies upstream of PAK1 in the 12-HETE-activated signalling pathway, the selective PI-3K inhibitor LY294002 was used. As shown in Figure 5, LY294002 at a concentration of 1.5 μM completely blocked 12-HETE-induced PAK activation. LY294002 had no detectable effect on basal PI-3K activity. Wortmannin, another inhibitor of PI-3K, at 100 nM also significantly reduced 12-HETE-induced PAK activation (results not shown).

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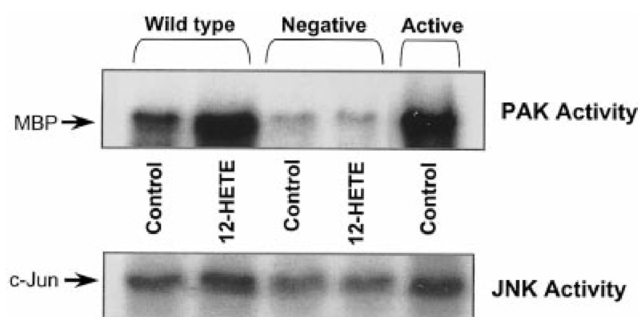

Figure 6 Effect of transient transfection of PAK1 mutants on 12-HETE-induced PAK1 and JNK1 activity

CHO-AT_{1a} cells were transfected with wild-type PAK1, dominant negative PAK1 plasmid, or constitutively active PAK1 as described in the Materials and methods section. The transfected cells were treated with 12-HETE (0.1 μM) or ethanol (control) for 10 min. The same lysate was used for both PAK1 and JNK1 activity measurements. Shown are representative autoradiograms of phosphorylated MBP bands (top panel) and phosphorylated GST–cJun (residues 1–79) (bottom panel). Results are representative of 3 similar experiments.

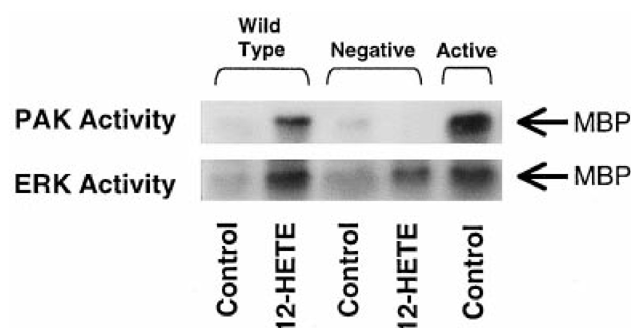

Figure 8 Effect of transient transfection of PAK1 mutants on 12-HETE-induced PAK1 and ERK1/2 activity

CHO-AT_{1a} cells were transfected with wild-type PAK1, dominant negative PAK1 or constitutively active PAK1 respectively, as described in the Materials and methods section. The transfected cells were treated with 12-HETE (0.1 μM) or ethanol in the control for 10 min. The same lysate was used for both PAK1 and ERK1/2 activity measurements. The top panel shows PAK1 activity and the lower panel shows the ERK1/2 activity representative of results from 2 similar experiments. Both kinase assays used MBP as substrate.

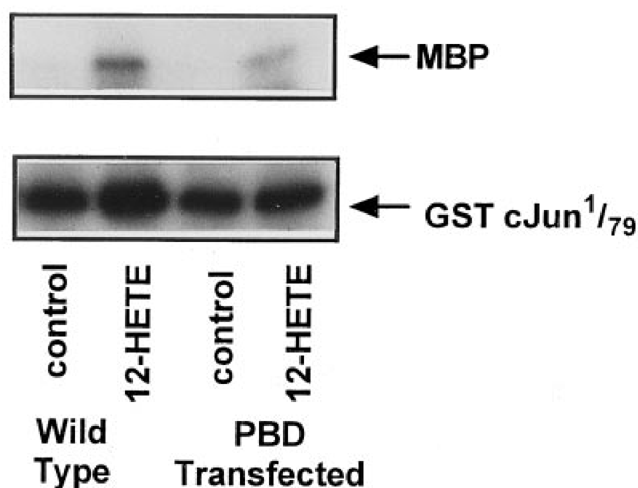

Figure 7 Effect of PAK1-PBD on 12-HETE-induced PAK1 and JNK1 activity

The transfection procedure was performed as described in the Materials and methods section. Cells were treated with 12-HETE (0.1 μM) or ethanol (control) for 10 min. The same lysate was used for both PAK and JNK activity measurements. Autoradiograms of phosphorylated MBP bands (top panel) and phosphorylated GST–cJun (residues 1–79) (bottom panel) are representative of results from 3 similar experiments.

PAK lies upstream of JNK in the 12-HETE-induced pathway

To further elucidate whether PAK1 is an upstream activator of JNK1 in the 12-HETE-induced pathway, PAK1 wild-type (PAK1wt), dominant negative PAK1 (PAK1K229R) [12] and constitutively active PAK1 (PAK1T423E) were transiently transfected into CHO-AT_{1a} cells. After stimulation with 12-HETE or ethanol, the cells were lysed to measure PAK1 and JNK1 activity. As shown in top panel of Figure 6, 12-HETE treatment stimulated PAK1 activity in wild-type PAK1 transfected cells. In contrast, 12-HETE-induced PAK1 activity was clearly inhibited in dominant negative PAK1 transfected cells. PAK1 activity was increased in constitutively active PAK1 transfected cells in the absence of 12-HETE treatment. The same lysates from these transfection experiments were collected to analyse the effect of transfection of these PAK1 mutants on JNK1 activity. These results are shown in the lower panel of Figure 6. 12-HETE treatment stimulated JNK1 activity in wild-type PAK1 transfected cells compared with cells treated with vehicle alone. In addition, 12-HETE-induced JNK1 activity was inhibited in dominant negative PAK1 transfected cells. However, JNK1 activity was markedly increased in dominant active PAK1 transfected cells. Transient transfection of the PBD plasmid was also used to evaluate the role of GTPase and PAK kinase activity in JNK1 activation by 12-HETE. Figure 7 shows that PBD transfection almost completely blocked both 12-HETE-induced PAK1 activation and 12-HETE-induced JNK1 activation. The activities of both kinases could be stimulated by 12-HETE treatment in non-PBD-transfected CHO-AT_{1a} cells, which were treated only with the transfection reagents, thus indicating that transfection agents alone were not responsible for the inhibition or the 12-HETE response.

PAK lies upstream of ERK activation in 12-HETE-induced pathway

To dissect the relationship between PAK1 and ERK1/2, transient transfection of the PAK1 mutants into CHO-AT_{1a} cells was again utilized. The effect of PAK1 mutants on PAK1 activation is shown in the upper panel, and the effect on ERK1/2 activation is shown in lower panel, in Figure 8. The results clearly indicate that 12-HETE stimulates PAK1 and ERK1/2 activation in wild-type PAK1-transfected cells; however, the 12-HETE stimulation of ERK1/2 was partially blocked by dominant negative PAK1. Interestingly, constitutively active PAK itself could activate ERK1/2 activity, indicating that PAK1 can activate ERK1/2 in CHO-AT_{1a} cells.

DISCUSSION

In this report we describe for the first time that an arachidonic acid metabolite of 12-LO, 12-S-HETE, can activate PAK1 activity in CHO fibroblasts. The 12-HETE-induced PAK1 stimulation was time-dependent, with activation seen as early as 5 min and a peak effect at 10 min. PAK1 activation was also sustained until 30 min and decreased thereafter. However, 12-HETE still significantly activated PAK1 activity at 60 min (Figure 1). The stimulatory effect of 12-HETE on PAK1 activity was also dose-

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dependent, with concentrations of 12-HETE as low as 0.1 nM exhibiting PAK1 activation. The maximal effect for activation was reached at 0.01 μM 12-HETE. Interestingly, the precursor of 12-HETE, arachidonic acid, does not stimulate PAK activity [18], indicating that 12-HETE stimulation of PAK1 activity is a specific response that is not due to non-specific hydrophobic- or detergent-like properties of this lipid. Current studies are underway to investigate whether 12-HETE acts by binding to a specific receptor.

It has been established that PAKs are regulated by three different mechanisms: (i) by the binding of GTP-bound Rac or Cdc42 to an N-terminal GTPase-binding domain, (ii) in the case of PAK2, by the caspase-mediated proteolytic removal of the regulatory N-terminus [19], and (iii) direct activation of PAK activity by three classes of lipids such as sphingolipid, phosphatidylinositol and phosphatidic acids [18]. Since 12-HETE is defined as a type of fatty acid with four double bonds, it was of interest to further define whether the 12-HETE action is mediated through small-molecular-weight GTP-binding proteins to PAK1 or by directly interacting with PAK1 similar to sphingosine or phosphatidic acid. The GTPase-binding domain was used to elucidate the role of Rac and Cdc42 in the process of PAK1 activation. PBD is a segment of 84 amino acid residues (hPAK1, residues 67–150) comprising the CRIB domain, which is a region of approx. 16 amino acid residues corresponding to amino acid residues 74–89 of rat $p65^{PAK}\alpha$ [20]. When overexpressed in cells, PBD would compete for the binding site on GTP-bound active Cdc42/Rac1 with PAK1 molecules and thereby block PAK1 activation. The transfection of the PBD almost completely blocked the activation of PAK1 activity by 12-HETE (Figure 3). To further confirm this result, transfection of dominant negative Cdc42d and Rac1 GTPase mutants was performed and PAK1 activity measured. The results in Figure 4 indicated that 12-HETE was able to activate PAK1 activity in cells expressing empty vectors. However, 12-HETE did not activate PAK1 activity in cells expressing negative Cdc42, suggesting that Cdc42 GTPases are the direct upstream activators for 12-HETE-induced PAK1 stimulation. Interestingly, negative Rac mutant significantly inhibited the basal PAK1 activity, while 12-HETE was able to increase PAK1 activity somewhat, but could not reverse it to the basal level in empty-vector-transfected cells. These results suggest that the regulation of PAK1 activation by Rac1 is somewhat different from that by Cdc42. New evidence from other laboratories suggests that Cdc42 and Rac have different mechanisms for activation of PAK. It appears that there are three domains on GTPases of the Rho family including Rac1. These three domains are the residues 26–45 [21], the insert region 124–135 [21], and an intact polybasic domain consisting of six contiguous basic amino acids (residues 183–188) at its C-terminus [8]. Cdc42 shares highly homology with Rac in the Ras-like effector region, residues 26–45 [21], while the insert region is not implicated in PAK activation [21]. New data show that mutation of six basic residues (183–188) to neutral amino acids in C-terminal polybasic domains of Rac 1 dramatically decreased the ability of Rac1 to bind PAK1, and almost completely abolished its ability to stimulate PAK1 activity [8]. This C-terminal polybasic domain is present in several members of the Ras superfamily of small GTP-binding proteins including Rac1 K-Ras4B, Rap1A and Rap1B [8]. These data clearly indicate that Cdc42 and Rac1 share a highly homologous N-terminal Ras-like effector region in regard to PAK activation. However, Rac1 possesses its own unique regions to bind and activate PAK1. Therefore this Rac unique region might account for the further inhibition of basal PAK activity by negative Rac1, while Cdc42 did not. To date, there have been no studies evaluating the possible stimulatory action of 12-HETE on Rac1. However, there is evidence showing that many biologically active lipids are regulators of the Rac–GDP-dissociation-inhibitor complex [22]. Additional studies will be needed to test this hypothesis.

To investigate the role of PI-3K in 12-HETE-introduced PAK1 activation, two distinct pharmacological inhibitors were used. Wortmannin decreased 12-HETE-induced PAK1 activation; but, since Wortmannin can also inhibit myosin light-chain kinase and other kinases, we also used LY294002, a more specific PI-3K inhibitor. At a concentration of 1.5 μM, LY294002 totally suppressed 12-HETE-induced PAK1 activation (Figure 5), clearly indicating that PI-3K is an upstream activator of PAK1. PI-3K products have been shown to play a role in the activation of Rac [23,24], indicating that PI-3K lies upstream of Rac1. However, there is also a report showing that PI-3K is the effector protein of Cdc42/Rac1 [25]. The present study did not evaluate whether PI-3K is upstream of Cdc42 or Rac1, or whether there are parallel, but separate, pathways linking the GTP-binding proteins and PI-3K to PAK1 activation. Further experiments will be needed to elucidate this mechanism.

To explore the role of PAK1 in 12-HETE-induced JNK1 activation in CHO-AT_{1a} cells, dominant negative PAK1 was first used to interrupt the PAK1 activation pathway by 12-HETE. Transient transfection of dominant negative PAK1 blocked both 12-HETE-induced PAK1 and JNK1 activities. In contrast, PAK1 or JNK1 activity was markedly activated in cells overexpressing constitutively active PAK1 (Figure 6). Since we concluded that at least Cdc42 is a direct activator of PAK1, it is reasonable to suggest that JNK1 activity should be blocked if the pathway from Cdc42/Rac to PAK1 was inhibited using PBD. The results demonstrated that when PBD is expressed in the CHO-AT_{1a} cells both 12-HETE induced PAK1 and JNK1 were blocked (Figure 7). The transfection of negative Cdc42 or Rac1 also blocked JNK1 activity (Figure 4). These results strongly suggest that PAK1 is an upstream activator of JNK1 in response to 12-HETE, and the activation pathway by 12-HETE should be 12-HETE $\rightarrow$ Cdc42 $\rightarrow$ PAK $\rightarrow$ JNK. The results also suggest the partial role of Rac in this response. The activation pathway Cdc42/Rac $\rightarrow$ PAK $\rightarrow$ JNK by other agonists has been demonstrated by others [26,27]. However, a new family of mixed lineage kinases (MLK) has been identified [28]. Notably, two members of this family, MLK2 and MLK3, possess an SH3 domain in their N-terminal region, and both have a functional CRIB domain which mediates GTP-dependent association with Rac1 and Cdc42 [29,30]. MLK3 has been demonstrated to activate mitogen-activated protein kinase (MAPK) kinase 4 (MKK4) and MKK7 *in vitro*, both are specific activators of JNK [30].

Our previous studies have shown that 12-HETE is a potent growth-promoting agent and stimulator of ERK1/2 activity in CHO-AT_{1a} cells [15]. The link between cell growth and PAK activation has not been clarified. However, there are some clues supporting a role of PAK1 in cellular growth. New data demonstrates that PAK1 phosphorylates MAP/ERK kinase 1 (MEK1) on serine-298, a site important for binding of Raf-1 to MEK1 *in vivo* [30]. In the present study, we demonstrate that PAK1 is an upstream mediator in 12-HETE-induced ERK1/2 activation by transfection of PAK1 mutants. This conclusion is based on data showing that a negative PAK1 mutant suppresses 12-HETE-induced ERK1/2 activity, while constitutive active PAK1 mutant stimulates ERK1/2 activity without the presence of 12-HETE (Figure 8). These results suggest that PAK1 is an upstream activator of ERK1/2 in the 12-HETE-induced action pathway.

In summary, this study provides evidence for the first time that PAK1 is an upstream mediator in 12-HETE-induced JNK1 and

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ERK1/2 activation. These results also provide evidence for the role of small GTP-binding proteins in PAK1 and JNK1 activation in a 12-HETE-induced signal transduction pathway. Therefore, 12-LO products should be considered as activators of the PAK pathway.

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REFERENCES

1 Manser, E., Leung, T., Salihuddin, H., Zhao, Z.-S. and Lim, L. (1994) Nature (London) **367**, 40–46
2 Knaus, U. G., Morris, S., Dong, H.-J., Chernoff, J. and Bokoch, G. M. (1995) Science **269**, 221–223
3 Manser, E., Chong, C., Zhao, Z.-S., Leung, T., Michael, G., Hall, C. and Lim, L. (1995) J. Biol. Chem. **270**, 25070–25078
4 Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J. and Cerione, R. A. (1995) J. Biol. Chem. **270**, 22731–22737
5 Martin, G. A., Bollag, G., McCormick, F. and Abo, A. (1995) EMBO J. **14**, 1970–1978
6 Galisteo, M. L., Chernoff, J., Su, Y.-C., Skolnik, E. Y. and Schlessinger, J. (1996) J. Biol. Chem. **271**, 20997–21000
7 Bokoch, G. M., Wang, Y., Bohl, B. P., Sells, M. A., Quilliam, L. A. and Knaus, U. G. (1996) J. Biol. Chem. **271**, 25746–25749
8 Knaus, U. G., Wang, Y., Reilly, A. M., Warnock, D. and Jackson, J. H. (1998) J. Biol. Chem. **273**, 21512–21518
9 Tsakiridis, T., Taha, C., Grinstein, S. and Klip, A. (1996) J. Biol. Chem. **271**, 19664–19667
10 Coso, O. A., Chiariello, M., Yu, J.-C., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J. S. (1995) Cell **81**, 1137–1146
11 Minden, A., Lin, A., Claret, F.-X., Abo, A. and Karin, M. (1995) Cell **81**, 1147–1157
12 Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J. and Bokoch, G. M. (1995) J. Biol. Chem. **270**, 23934–23936
13 Bagrodia, S., Derijard, B., Davis, R. J. and Cerione, R. A. (1995) J. Biol. Chem. **270**, 27995–27998
14 Teramoto, H., Coso, O. A., Miyata, H., Igishi, T., Miki, T. and Gutkind, T. S. (1996) J. Biol. Chem. **271**, 27225–27228
15 Wen, Y., Nadler, J. L., Gonzales, N., Scott, S., Clauser, E. and Natarajan, R. (1996) Am. J. Physiol. **271**, C1212–C1220
16 Wen, Y., Scott, S., Liu, Y., Gonzales, N. and Nadler, J. L. (1997) Circ. Res. **81**, 651–655
17 Teutsch, B., Bihoreau, C., Monnot, C., Bernstein, K. E., Murphy, J., Alexander, R. W., Corvol, P. and Clauser, E. (1992) Biochem. Biophys. Res. Commun. **18**, 1381–1388
18 Bokoch, G. M., Reilly, A. M., Daniels, R. H., King, C. C., Olivera, A., Spiegel, S. and Knaus, U. G. (1998) J. Biol. Chem. **273**, 8137–8144
19 Rudel, T. and Bokoch, G. M. (1997) Science **276**, 1571–1574
20 Burbelo, P. D., Drechsel, D. and Hall, A. (1995) J. Biol. Chem. **270**, 29071–29074
21 Freeman, J. L., Abo, A. and Lambeth, J. D. (1996) J. Biol. Chem. **271**, 19794–19801
22 Welch, H., Eguinoa, A., Stephens, L. R. and Hawkins, P. T. (1998) J. Biol. Chem. **273**, 11248–11256
23 Nimnual, A. S., Yatsula, B. A. and Bar-Sagi, D. (1998) Science **279**, 560–563
24 Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J. and Parise, L. V. (1997) Nature (London) **390**, 632–636
25 Coso, O. A., Chiariello, M., Yu, J., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J. S. (1995) Cell **81**, 1137–1146
26 Minden, A., Lin, A., Claret, F., Abo, A. and Karin, M. (1995) Cell **81**, 1147–1157
27 Merritt, S. E., Mata, M., Nihalani, D., Zhu, C., Hu, X. and Holzman, L. B. (1999) J. Biol. Chem. **274**, 10195–10202
28 Nagata, K. I., Puls, A., Futter, C., Schaefer, A. P., Nakata, T., Hirokawa, N. and Hall, A. (1998) EMBO J. **17**, 149–158
29 Teramoto, H., Coso, O. A., Miyata, H., Igishi, T., Miki, T. and Gutkind, J. S. (1996) J. Biol. Chem. **271**, 277225–27228
30 Frost, J. A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P. E. and Cobb, M. H. (1997) EMBO J. **16**, 6426–6438

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