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Protein tyrosine kinase, mitogen-activated protein kinase and protein kinase C are involved in the mitogenic signaling of platelet-activating factor (PAF) in HEC-1A cells

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Abstract

We have recently demonstrated that the phospholipid platelet-activating factor (PAF) mediates an autocrine proliferative loop in the endometrial adenocarcinoma cell line HEC-1A. In the present study we investigated the signaling pathways involved in PAF-mediated increase of proliferation in these cells. In particular, we studied the effect of PAF on protein tyrosine phosphorylation and mitogen-activated protein kinase (MAPK) activity, as well as the effect of protein tyrosine kinase (PTK) and protein kinase C (PKC) inhibition on PAF-induced increase of c-fos gene expression and thymidine incorporation in HEC-1A cells. We found that PAF induced a rapid, time- and dose-dependent increase of tyrosine phosphorylation of a subset of proteins of 42, 44, 78, 99, and 150 kDa molecular weight. We also found that PAF increased tyrosine phosphorylation and activity of p42 MAPK, suggesting the involvement of this important intermediary enzyme in the proliferative effect of PAF. The effect of PAF on c-fos gene expression was not prevented by pre incubation with the PTK inhibitors genistein or methyl-2,5-dihydroxycinnamate, whereas was strongly affected by PKC down regulation after long term incubation with PMA or by PKC inhibition with sangivamycin. We also found that genistein and methyl 2,5-dihydroxycinnamate decreased both basal and PAF-stimulated [^{3}H]thymidine uptake in these cells. Similar results were obtained with PD 098059, a specific inhibitor of MAP kinase cascade. PAF-stimulated [^{3}H]thymidine uptake was also prevented by PKC down regulation after long term exposure to PMA and PKC inhibition with the two inhibitors sangivamycin and *bis*-indolylmaleimide. In conclusion, our results indicate that PAF-induced mitogenesis in HEC-1A cells is mediated by the activation of multiple signaling pathways, involving PTK, MAPK, and PKC activation.

Keywords: Platelet-activating factor; HEC-1A; Endometrial adenocarcinoma; Protein kinase C; Protein tyrosine kinase; Mitogen-activated protein kinase

1. Introduction

Tyrosine phosphorylation of proteins is considered an essential signal in the modulation of cell proliferation [1,2]. Indeed, peptide growth factors induce mitogenesis by stimulating a series of signaling pathways involving intrinsic receptor protein tyrosine kinase (PTK) activation which in turn generates a cascade of events culminating in phosphorylation in tyrosine residues of several cell enzymes. In recent years, evidence is emerging that PTK activation also

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occurs following stimulation with agonists whose response is mediated by G-protein-coupled receptors which stimulate phosphoinositides hydrolysis, mobilization of Ca^{2+} and activation of protein kinase C [3–9]. However, the biological function of these non-receptor protein tyrosine kinases (NRPTK) in proliferating and non-proliferating cells is still largely unclear. So far, a clear linkage between NRPTK and stimulation of proliferation has been demonstrated only for few agents [5–8], while evidence of such a relationship is lacking for other agonists, and the relative importance of PKC-mediated versus NRPTK-mediated pathways in the induction of proliferation is unclear. We have recently demonstrated an unusual mitogenic effect of platelet-activating factor (PAF) in the endometrial adenocarcinoma cell line HEC-1A [10,11]. In particular, the mitogenic effect of PAF appears to be crucial for these cells, since the PAF antagonist L659,989 dramatically blunted cell growth [11]. HEC-1A cells produce large amounts of this phospholipid, which stimulates c-fos gene expression, intracellular calcium increase and thymidine uptake by interaction with specific PAF receptors [10,11]. These receptors, that have recently been cloned [12,13], belong to the family of seven transmembrane domain-G protein coupled receptors. Nonetheless, PAF has been demonstrated to stimulate tyrosine phosphorylation of proteins both in highly differentiated, non-proliferating cells [14–16], and in proliferating cancer cells [17–20]. Moreover, recent data indicate that PAF can stimulate the activity of the serine/threonine mitogen-activated protein kinases (MAPK) $p44^{mapk}$ and $p42^{mapk}$, also named extracellular signal-regulated kinases (ERK1 and ERK2) [20–22], that are considered important intermediary enzymes in converting extracellular signals into intracellular responses [23,24], and are activated by most mitogens. However, while the biological function of such PAF-mediated signaling pathway in non-proliferating cells is emerging [14–16] its possible involvement in mitogenesis in proliferating cells has not been established, and the functional consequence of non-receptor protein tyrosine kinase (NRPTK) activation by PAF in these cells is still unknown. Therefore, HEC-1A cells represent an interesting model to study a possible linkage between NRPTK activation and induction of mitogenesis by PAF.

We designed and performed experiments aimed to investigate: (i) the effect of PAF on tyrosine phosphorylation of proteins in HEC-1A cells; (ii) the effect of PAF on the activity of MAP kinase; (iii) the effect of PTK and MAPK cascade inhibitors on PAF-stimulated thymidine incorporation; (iv) in addition, to study the possible contribution of PKC-mediated pathways in the mitogenic effect of PAF, we evaluated the effect of PKC inhibition on PAF-mediated thymidine uptake and c-fos gene expression.

2. Materials and methods

2.1. Chemicals

Sodium orthovanadate (Na_3VO_4), fatty acid free bovine serum albumin (BSA), gelatine, glycine, trizma base, Temed, APS, Triton X-100, phenylmethylsulfonylfluoride (PMSF), soybean trypsin inhibitor, *o*-phospho-DL-tyrosine, Ponceau S and Commassie R250, EGTA, EDTA, bovine insulin, myelin basic protein (MBP), McCoy's 5A medium, fetal calf serum, penicillin, propidium iodide and streptomycin were from SIGMA (St. Louis, MO). Erbstatin analog (methyl 2,5-dihydroxycinnamate), genistein, daidzein, bisindolylmaleimide-HCl, platelet-activating factor (PAF) and monoclonal anti-p42MAP kinase antibody were from Calbiochem (La Jolla, CA). Sangivamycin was from National Institutes of Health Drug Synthesis and Chemistry Branch (Bethesda, MD). Unconjugated and peroxidase-conjugated monoclonal (PY20) anti-phosphotyrosine antibodies were from ICN (Costa Mesa, CA). Reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were from BIO-RAD Labs (Hercules, CA). Protein molecular weight standards and Tween 20 were from FLUKA Chemie AG (Bucks, Switzerland). Enhanced Chemiluminescence (ECL) Western blotting detection reagents were from Amersham (Buckinghamshire, UK). [*methyl*-^{3}H]thymidine (2 Ci/mmol) was obtained from New England Nuclear (Boston, MA). [γ^{32}P]ATP and [α^{32}P]ATP were from Amersham (Buckinghamshire, UK). bPc-fos(human)1 cDNA probe was obtained from ATTC (Rockville, MD). Organic solvents were purchased from Carlo Erba (Milan, Italy). Polyclonal anti-MAP kinase antibodies αIIcp42 and αIcp44 were a generous gift

from Dr. M.J. Dunn (Medical College of Wisconsin, Milwaukee, WI). PD 098059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one] was a generous gift of Dr. A. Saltiel (Parke-Davis, Ann Arbor, MI, USA).

2.2. Cells

HEC-1A (obtained from ATCC, Bethesda, MD) is a cell line established in 1968 from explants of endometrial adenocarcinoma. The cells were grown in modified McCoy's 5A (Sigma) supplemented with 10% of fetal calf serum (Gibco, Grand Island, NY, USA), penicillin (10 U/ml), streptomycin (0.1 mg/ml) and 2.2 g/l sodium bicarbonate as suggested by ATCC. Cell cultures were maintained in humidified atmosphere of 5% CO_2 and 95% air at 37°C. Medium was replaced every 2–3 days and the cells were split every 4–5 days using trypsin (0.05%) in Mg^{2+}- and Ca^{2+}-free PBS containing 0.02% EDTA.

2.3. SDS-PAGE and Western blot analysis

After the different treatments, the cells were processed for SDS-electrophoresis as previously described [16]. Briefly, cells (in 6-well plates) were stimulated as indicated and the medium quickly removed. The cells were washed and scraped in PBS containing 1 mM Na_3VO_4, then centrifuged at 400 × *g* at 4°C for 10 min and resuspended in 20 μl lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.25% NP-40, 1 mM Na_3VO_4, 1 mM PMSF). After measurement of proteins, the cell extracts were diluted in 2 × concentrated Laemmli sample buffer (62.5 mM Tris, pH 6.8, containing 10% glycerol, 20% SDS, 2.5% pyronin and 200 mM dithiothreitol), vortexed, incubated at 90°C for 5 min and then loaded onto 7.5% or 10% polyacrylamide-bisacrylamide gels as indicated. After SDS-PAGE, proteins were transferred to nitrocellulose (Sigma, St. Louis, MO), stained with Ponceau to verify equal loading and immunostained with peroxidase conjugated monoclonal anti-phosphotyrosine antibody (PY20). The phosphotyrosine antibody-reacted proteins were revealed by enhanced-chemiluminescence system (ECL, Amersham). Immunoabsorption was obtained by reacting the antibody with 40 mM of *o*-phospho-DL-tyrosine for 1 h at room temperature. Quantification of the bands was made directly on the films by image analysis with a C3077/01 video camera, connected with the video frame grabber M4477 (Hamamatsu Photonics, Japan) [16]. This video frame grabber is a plug-in board used in a Macintosh Ilsi PC (Apple, USA). Acquisition of image was taken by Image quest IQ Base software (Hamamatsu Photonics, Japan). Image processing and analysis was obtained by IMAGE free software, kindly provided by Wayne Rasband (NIMH, Bethesda, MD). For technical reasons, quantifications were performed in ECL low-exposed films.

2.4. Immunoprecipitation of MAP-kinase and MAP-kinase activity

Cells grown on 6-well plates, washed and resuspended in serum-free medium, were stimulated with PAF for the indicated times, then scraped in 1 ml lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.25% NP-40, 1 mM Na_3VO_4, 1 mM PMSF) and centrifuged (12 000 rpm at 4°C for 10 min). The supernatants (200 μg of proteins for each sample) were incubated with 3 μl of the indicated anti-MAPK antibody for 1 h in ice followed by incubation with 20 μl of Protein A-Sepharose (Sigma) for 2 h at 4°C. The immunocomplexes were washed 3 times with 20 mM Tris buffer, resuspended in Laemmli sample buffer, boiled for 5 min and run on 10% SDS-PAGE. Proteins were transferred onto nitrocellulose and MAP-kinase was revealed with polyclonal anti-MAPK antibody (αIIcp42) [25] followed by a peroxidase-conjugated secondary antibody visualized by ECL.

For the MAP-kinase assay the immunoprecipitates obtained as described above, were assayed for the ability to phosphorylate a MAPK specific substrate, myelin basic protein (MBP). Briefly, the extracts were incubated with 30 μl of kinase buffer (10 mM Hepes, pH 7.4, 20 mM $MgCl_2$ 1 mM DTT, 1 mM Na_3VO_4, 25 μM ATP, 0.5 mg/ml MBP, 250 μCi [γ^{32}P] ATP) for 30 min at 30°C. Aliquots of 20 μl were spotted onto phospho-cellulose, washed four times with 1% phosphoric acid and finally with 100% ethanol. The filters were dried and bounded radioactivity determined by liquid scintillation counting.

MAPK activity was also evaluated by in gel kinase assay, which determines the ability of kinases present

in total cell lysates to phosphorylate MBP (0.5 mg/ml) copolymerized in SDS-polyacrylamide gels [26,27]. After the indicated treatments, cells were lysed and sonicated as for immunokinase. After centrifugation, aliquots of the supernatants containing 10 μg of proteins were boiled in 2 × Laemmli's sample buffer, separated on a 10% SDS-polyacrylamide gel containing 0.5 mg/ml MBP. SDS was removed from gel by washing with two changes of 20% 2-propanol in buffer A (50 mM Hepes, pH 7.4, 5 mM 2-βmercaptoethanol) for 1 h at room temperature. The gel was subsequently denaturated with 6 M guanidine HCl in buffer A for 1 h, and then renaturated in buffer A containing 0.04% Tween 20 for 16 h at 4°C. After 1 h preincubation in buffer B (25 mM Hepes, pH 7.4, 10 mM MgCl2, 100 μM Na3VO4, 5 mM 2-βmercaptoethanol, 0.5 mM EGTA), the kinase assay was carried out by incubating the gel at 25°C for 2 h in 5 ml buffer B containing 40 μM ATP and 50 μCi of [γ-^{32}P]ATP. The gel was then extensively washed with 5% trichloroacetic acid containing 1% sodium pyrophosphate, dried under vacuum and subjected to autoradiography. Quantification of the bands was performed as described above.

2.5. Northern blot analysis of mRNA

For Northern analysis of c-fos mRNA expression, cells were treated with PAF in the presence of the indicated PTK or PKC antagonists for 30 min. Total cellular RNA was prepared by the hot-phenol method and quantificated by spectrofluorimetric analysis at 260 nm. 20 μg of RNA were fractionated in a 1% agarose-3% formaldehyde gel, transferred onto nylon membrane (Nytran, Schleicher and Schuell) and baked at 80°C for 2 h [11]. The membranes were pre-hybridized for 1 h and then hybridized overnight at 65°C with Church and Gilbert solution (pH 8) containing 10 mg/ml BSA, 7% sodium dodecyl sulfate (SDS), 0.25 M sodium phosphate buffer, 1 mM EDTA and 0.2 mg/ml hot-denatured sonicated herring sperm DNA. The probe used for RNA hybridization was bPc-fos(human)1 cDNA. To correct possible differences in RNA loading, the same nylon membranes were concomitantly or subsequently hybridized to rat cyclophyllin (coding region) cDNA probe. The probes were labelled with deoxycytidine 5′-[α^{32}P]triphosphate by a random priming kit (Boheringer Mannheim Italy, Milan, Italy), chromatographed (Nu-Clean D25 Disposable Spun Columns, IBI, New Haven, CT, USA) and hot-denatured before use. The membrane was washed four times with a solution containing 1% SDS, 0.2 M Church and Gilbert buffer and 0.1 mM EDTA (pH 8) at 65°C for 10 min. The nylon membrane was then submitted to autoradiography using Kodak X-Omat AR film and Kodak X-Omatic regular intensifying screens at −80C. Quantification of the bands was performed as described above.

2.6. Mitogenic assay ([^{3}H]thymidine incorporation and cell counting by cytofluorimeter)

[^{3}H]thymidine incorporation was assessed as described previously [11]. Briefly, HEC-1A cells were plated into 24-well dishes, grown until 80% confluence in complete medium, maintained for 24 h in serum-free medium and the indicated concentrations of stimuli or vehicles (10 μl) added in triplicate for 24 h. Cells were then pulsed with 1 μCi/well of [^{3}H]thymidine for 4 h, washed twice with ice-cold 5% TCA and solubilized with 0.25 N NaOH in SDS (0.1%) at 37 C for 20 min. Aliquots were transferred to plastic vials and counted by liquid scintillation.

For flow cytometry analysis, cells were incubated or not with PAF (1 μM) for the indicated times, washed twice with cold phosphate buffer saline (PBS) without calcium and magnesium and 1 ml of fluorochrome solution (Propidium Iodine [PI] 0.05 mg/ml, dissolved in 0.1% sodium citrate and 0.1% Triton X-100) added. Dishes were kept for 60–90 min in the dark at 4°C, then cells were dislodged by gentle scraping and repeated pipetting and transferred in test tubes for cytofluorimetric analysis. Complete removal of cells from substrate was checked by phase contrast microscopy. Cell counting was performed by FACScan flow cytometer (Becton Dickinson, Mountain View, CA) analysis. We used a suspension of fluorescent beads as volume standard to determinate the absolute cell number. The concentration of the beads in the standard suspension was ascertained by a hemocytometer and resulted 755 beads/μl. 20 μl of the fluorescent particles suspension were added to each sample before flow cytometer analysis. The machine was set to count a constant number of events. The green fluorescence of the beads was

detected in the 515–555 nm wavelength band and DNA fluorescence due to PI staining in the 563–607 nm wavelength band on linear scale. Cells with a normal DNA content were considered as viable. Number of either beads or viable cells was calculated with the computer HP 9000 (model 340, Palo Alto, CA) interfaced to the flow cytometer. The standard beads were gated out from linear DNA histograms. The cell number in the test tube was calculated by multiplication of the counted number of nuclei by the ratio of the added to counted number of the standard beads. Results are expressed as number of viable cells/dish.

2.7. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical comparisons were performed by paired Student's *t*-test.

3. Results

3.1. Effect of PAF on protein tyrosine phosphorylation and MAPK activation in HEC-1A cells

Time-course of PAF-induced tyrosine phosphorylation of proteins in HEC-1A cells is shown in Fig. 1A. The effect of PAF was present after 30 s incubation, reached a maximum at 2 min and slightly declined after 5 min (Fig. 1A). PAF induced an increase of tyrosine phosphorylation in several protein bands. Among these, bands in the molecular weight ranges of 42, 44, 78, 99, and 150 kDa were dose-dependently modulated by PAF (Fig. 1B). The specificity of the immunoreactions with the anti-phosphotyrosine antibody to phosphotyrosine residues was verified by previous immunoabsorption of the antibody with *o*-phospho-DL-tyrosine [16], which completely abolished the immunoreactivity in the different protein bands (not shown). The prominent effect of PAF on tyrosine phosphorylation of a 42-kDa, as well as, albeit smaller, on a 44-kDa protein band (Fig. 1B) prompted us to investigate the possible immunological identity of such bands with mitogen-activated protein kinases (MAPK), by using monoclonal and polyclonal antibodies against these proteins. We first studied the effect of PAF on tyrosine phosphorylation of MAPK by immunoprecipitation/anti-phosphotyrosine Western blot analysis. In these experiments, the polyclonal antibody αIIcp42 was used to immunoprecipitate MAP kinase. Such antibody has been shown to recognize both 42 and 44 kDa isoforms of MAPK [25]. As shown in Fig. 2A, PAF induced a sustained increase of tyrosine phosphorylation in a protein band of approx. 42–45 kDa, which was already evident at early time points. Such

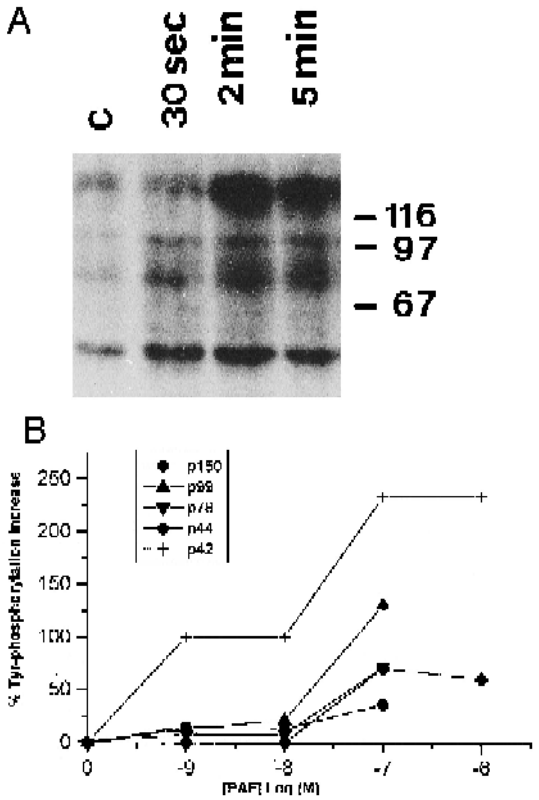

Fig. 1. Panel A: Time-course of PAF-induced increase of tyrosine phosphorylation in HEC-1A cells. Cells were incubated with 0.1 μM PAF for the indicated times. Cell lysates were run onto 7.5% polyacrylamide-bisacrylamide gels followed by transfer to nitrocellulose. Tyrosine phosphorylated proteins were revealed using the monoclonal antibody PY20-HRP followed by the ECL detection system. Molecular weight markers ($\times 10^3$) are indicated to the right of the blot. Representative of two similar experiments. Panel B: Cumulative results of at least three different experiments showing the concentration-dependence of PAF (5-min stimulation)-induced increase of tyrosine phosphorylation in the different protein bands in HEC-1A cells. To obtain two separate bands at 44 and 42 kDa, cell proteins were run onto 10% polyacrylamide-bisacrylamide gels. Quantification of the bands was performed as described in Section 2.

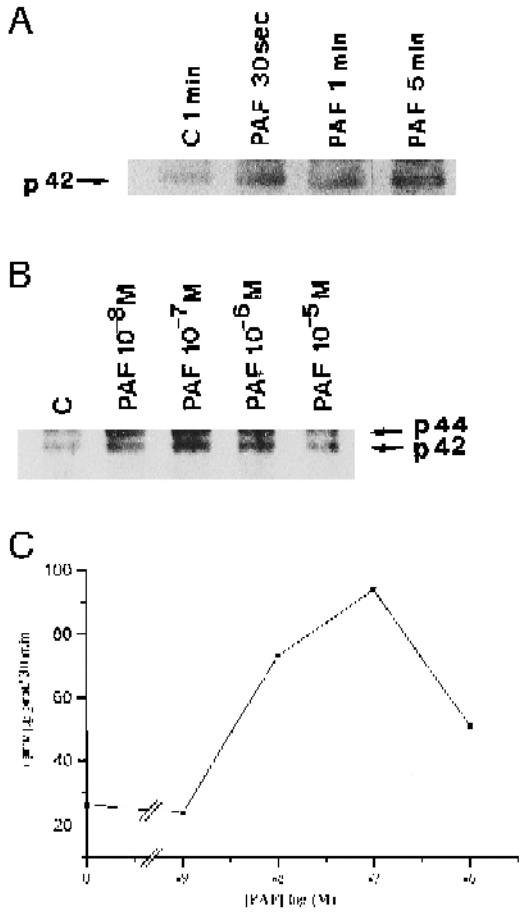

Fig. 2. Time-course (Panel A) and dose-dependence (Panel B) of PAF-induced increase of tyrosine phosphorylation of MAPK in HEC-1A cells. After stimulation with PAF, cell proteins were immunoprecipitated with αIIcp42 antibody. After SDS-PAGE onto 10% polyacrylamide-bisacrylamide gel, and transfer to nitro-cellulose, tyrosine phosphorylated proteins were revealed using the monoclonal antibody PY20-HRP followed by the ECL detection system. The arrows indicate p42 (panel A) and p42 and p44 (panel B) protein bands. Representative of two similar experiments. (Panel C) Effect of increasing concentrations of PAF on MAPK kinase activity, evaluated by immunokinase assay in the presence of the MAPK substrate MBP and [^{32}P]γ-ATP. Quantification of phosphorylated MBP was made by liquid scintillation. Results are mean of duplicate of a representative out of three similar experiments.

increase was dose-dependent (Fig. 2B) with a maximal activation at 0.1 μM concentration. In the latter experiments, PAF-mediated increase of tyrosine phosphorylation was evident in two protein bands respectively of 42 and 44 kDa (Fig. 2B), indicating an effect of the phospholipid on both MAPK isoforms. We next evaluated MAP kinase activity in PAF-stimulated HEC-1A cells by immunokinase assay in the presence of MBP and [γ^{32}P]-ATP. When the p42 isoform of MAPK was immunoprecipitated by a monoclonal antibody (anti-MAPK ERK2, Calbiochem), we found that PAF dose-dependently stimulated a three to four fold increase of MAPK activity with a maximum at 0.1 M concentration (Fig. 2C). When MAPK was immunoprecipitated with the polyclonal αIIcp42 antibody, similar results were obtained (data not shown).

MAPK activity was also evaluated by measuring MBP kinase activity of HEC-1A cell proteins in polyacrylamide gels copolimerized with MBP. As shown in Fig. 3A, at least three proteins with the ability to phosphorylate MBP were demonstrated in HEC-1A cells. Among these proteins, PAF treatment (5 min incubation) caused an increase of MBP kinase activity of a 42–44 (indicated in the figure by an arrow) and a 80–100 kDa proteins (Fig. 3A). Such increase was inhibited by the tyrosine kinase inhibitors genistein (Fig. 3A) and erbstatin (not shown), and by the MAPK cascade inhibitor PD098059 (Fig. 3A), which has been indicated as a specific inhibitor of MAPK kinase (MEK) activity [28–30]. The inhibitory effect of PD098059 was already maximum at the concentration of 1 μM (Fig. 3B). However, in our hands, the compound also inhibited basal as well as stimulated activity of other bands (Fig. 3A) irrespectively of the concentration used (not shown).

3.2. Effect of tyrosine kinase inhibition on basal and PAF-stimulated c-fos gene expression

In a previous report, we showed that mRNA expression for the early oncogene c-fos in HEC-1A cells was enhanced by treatment with PAF [11]. In order to define the role of tyrosine kinase activation on PAF-stimulated c-fos gene expression, we utilized the two tyrosine kinase inhibitors genistein [31] and the erbstatin analog methyl-2,5-dihydroxycinnamate [32] as pharmacological tools. Results of these experiments are repoted in Figs. 4 and 5. The upper panels report the hybridizing bands using a cDNA probe for c-fos (upper blot) and for the housekeeping gene cyclophyllin (lower blot). In the lower panels the percentage increase of c-fos expression with the dif-

ferent treatments compared to control is reported. These values represent percentage increase of the ratios between the optical densities of c-fos and cyclophillin mRNA bands. Pre-treatment of the cells with 10 and 30 μM concentrations of genistein (30 min pre incubation) did not affect basal nor inhibited PAF (0.1 μM)-stimulated c-fos gene expression (Fig. 4). This result was consistently obtained in at least two different experiments and was confirmed also using 100 μM genistein (not shown). Similarly, PAF-stimulated c-fos mRNA expression was not inhibited by pretreatment with the structurally unrelated tyrosine kinase inhibitor methyl 2,5-dihydroxycin-

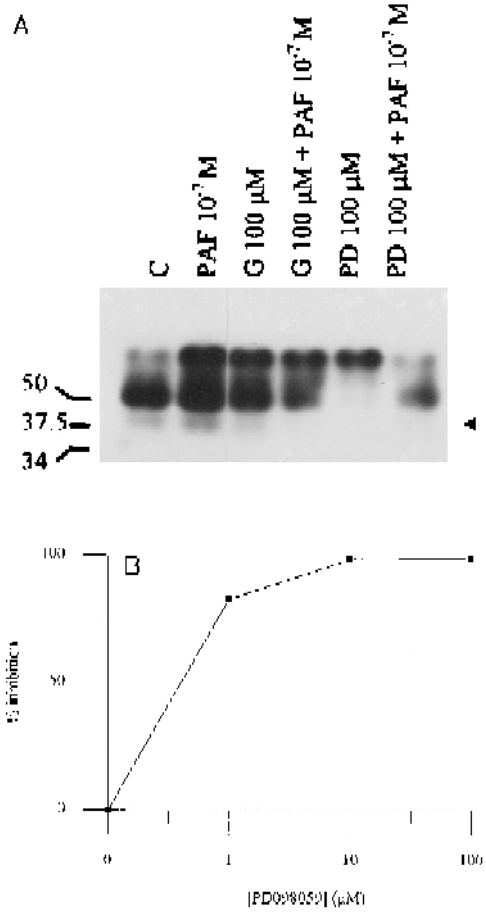

Fig. 3. Panel A: effect of PAF (5 min incubation) in the presence or absence of genistein (G) or PD 098059 (PD) on MBP-phosphorylating activity of HEC-1A cells. Cell lysates (10 μg/lane) were resolved on a 10% SDS polyacrylamide gel containing MBP (0.5 mg/ml) for kinase renaturation assay. The arrow indicates the protein band at 42–44 kDa. Molecular weight markers ($\times 10^3$) are indicated to the left of the blot. Panel B: dose-response curve of the inhibitory effect of PD 098059 on PAF-stimulated MBP kinase activity in the 42–44 kDa protein band in HEC-1A cells. Quantification of the band was performed by image analysis as described in Section 2.

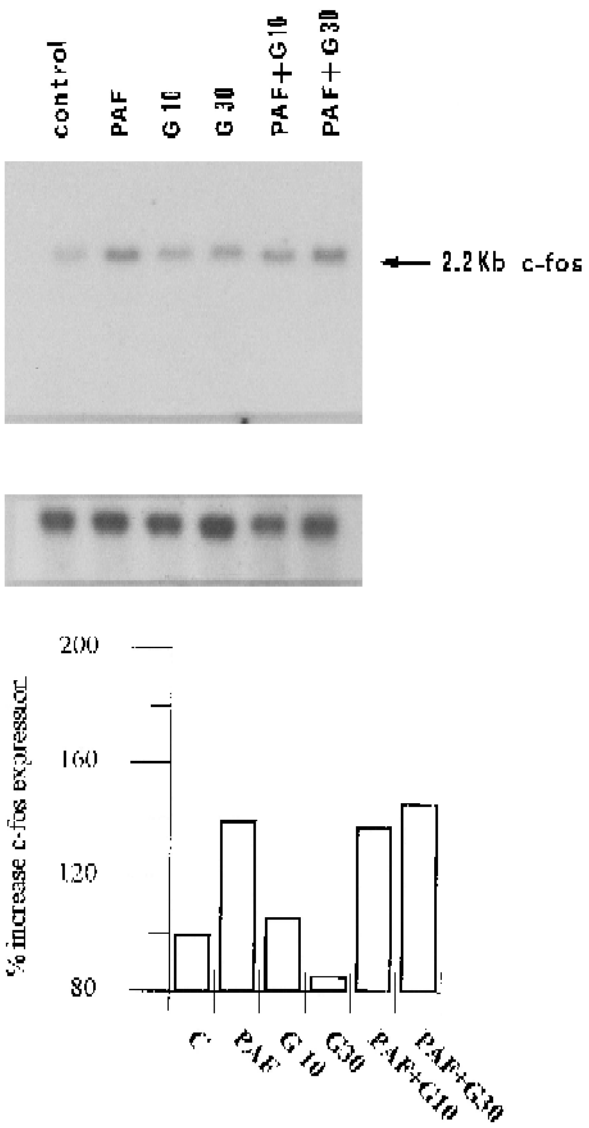

Fig. 4. Effect of genistein (G, 10 and 30 μM, 30 min preincubation) on PAF-stimulated c-fos gene expression in HEC-1A cells. Cells were incubated with PAF (0.1 μM) for 30 min. In the upper panel hybridization of HEC-1A RNA with a cDNA probe for c-fos (upper blot) and with rat-cyclophyllin cDNA probe (lower blot) is shown. In the lower panel the percentage increase of c-fos gene expression with the different treatments calculated by densitometric analysis of the bands is reported. Representative of at least two similar experiments.

namate (250 μM, 30 min preincubation, Fig. 5). Taken together these data, obtained with concentrations and incubation times of methyl 2,5-dihydroxycinnamate and genistein previously shown by our group to inhibit PTK activity [16,33] seem to exclude the involvement of tyrosine kinase in PAF-mediated c-fos gene expression.

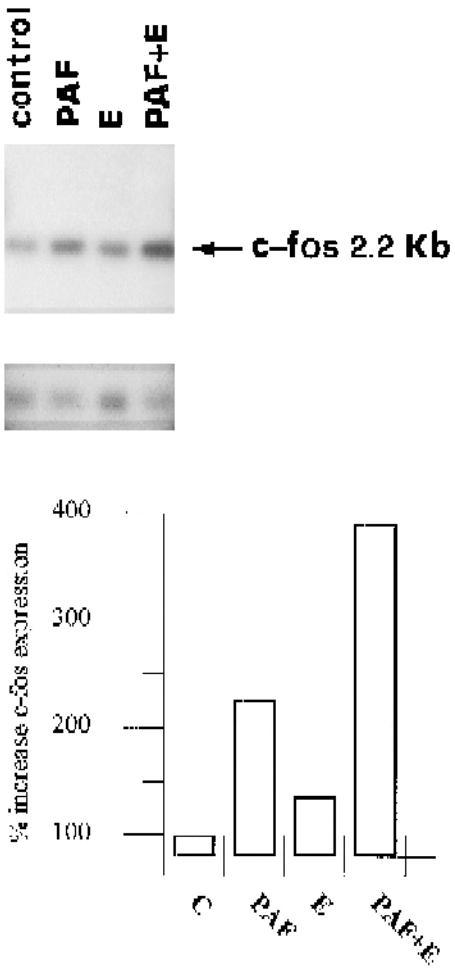

Fig. 5. Effect of the erbstatin analog methyl 2,5-dihydroxycinnamate (E, 250 μM, 30 min preincubation) on PAF-stimulated c-fos gene expression in HEC-1A cells. Cells were incubated with PAF (0.1 μM) for 30 min. In the upper panel hybridization of HEC-1A RNA with a cDNA probe for c-fos (upper blot) and with rat-cyclophyllin cDNA probe (lower blot) is shown. In the lower panel the percentage increase of c-fos gene expression with the different treatments calculated by ratio of densitometric analysis of the c-fos and cyclophyllin bands is reported. Representative of two similar experiments.

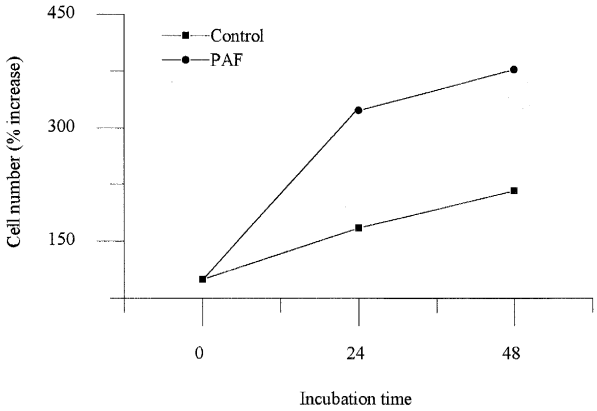

Fig. 6. Growth rate curve of HEC-1A cells in the presence or absence (control) of PAF (1 μM). Cell number was determined by flow cytometer as described in Section 2. Data represent mean values of two different experiments performed in triplicate.

3.3. Effect of tyrosine kinase inhibition on PAF-stimulated thymidine uptake

We then evaluated the effect of genistein and methyl 2,5-dihydroxycinnamate on [^{3}H]thymidine uptake stimulated by PAF. In preliminary experiments (not shown) we demonstrated that the effect of PAF on thymidine uptake was already maximum at 24 h and persisted for 48–72 h, thus 24 h stimulation was chosen for these experiments. The growth rate curve of HEC-1A cells with/without PAF confirmed that the effect of PAF on cell growth was present at such incubation time (Fig. 6). As shown in Fig. 7A, 30 and 100 μM concentrations of genistein inhibited PAF (3 μM)-stimulated [^{3}H]thymidine uptake. Conversely, basal and PAF-stimulated [^{3}H]thymidine uptake were unaffected by daidzein, a genistein analog found to be 100-fold less active than genistein (results not shown). We next investigated the effect of methyl-2,5-dihydroxycinnamate. As shown in Fig. 7B, this compound inhibited basal and PAF (3 μM)-stimulated DNA synthesis starting at the concentration of 10 μM. Hence, these results suggest an involvement of PTKs in PAF-mediated DNA synthesis. We next studied the involvement of MAPK activation in the effect of PAF, by using PD098059. We found that this compound slightly inhibited PAF-stimulated thymidine uptake at the concentration of 10 μM, and completely reversed the effect of PAF at 100 μM

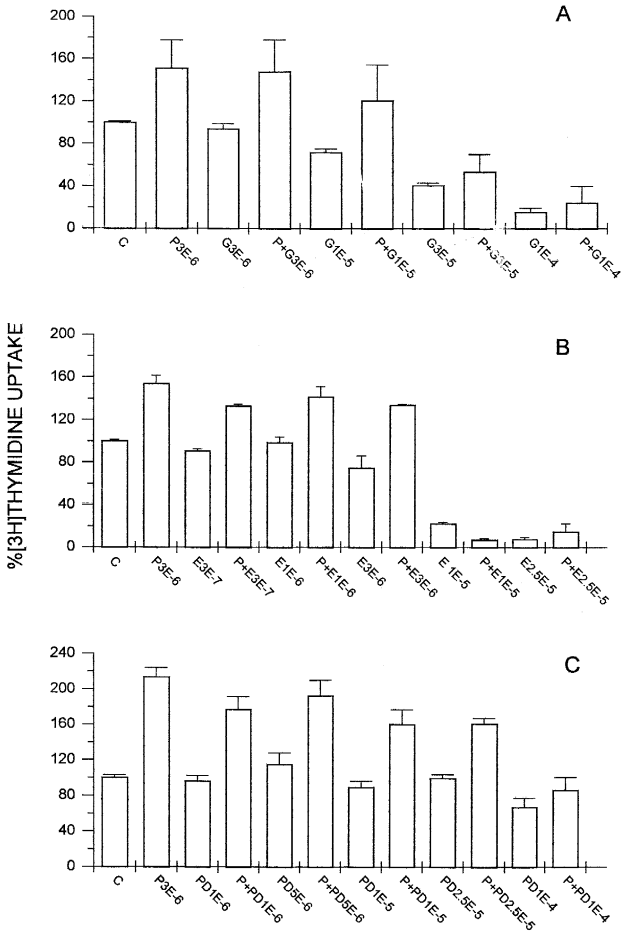

Fig. 7. Effect of increasing concentrations of genistein (G, 30 min preincubation, panel A), the erbstatin analog methyl-2,5-dihydroxycinnamate (E, 30 min preincubation, panel B), and PD 098059 (PD, 30 min preincubation, panel C) on basal (C) and PAF (P) -stimulated [^{3}H]thymidine uptake in HEC-1A cells. Results shown are the mean ± S.E.M. of 5 (panel A) and 3 (panels B and C) experiments, performed in triplicate.

(Fig. 7C), suggesting an involvement of MAPK activation in the mitogenic effect of PAF in HEC-1A cells.

3.4. Effect of PKC inhibition on PAF-induced mitogenesis in HEC-1A cells

We next investigated the possible involvement of PKC activation in PAF-induced c-fos gene expression. In order to obtain down regulation of PMA sensitive PKC isoenzymes, cells were treated for 24 h with the phorbol ester PMA (0.1 μM) before addition of PAF (0.1 μM, 30 min incubation). As shown in Fig. 8 upper panel, c-fos gene expression was strongly inhibited in PKC-depleted cells either in basal conditions or after PAF stimulation. In Fig. 8, lower panel, the percentage increase of c-fos expression after the different treatments, obtained by den-

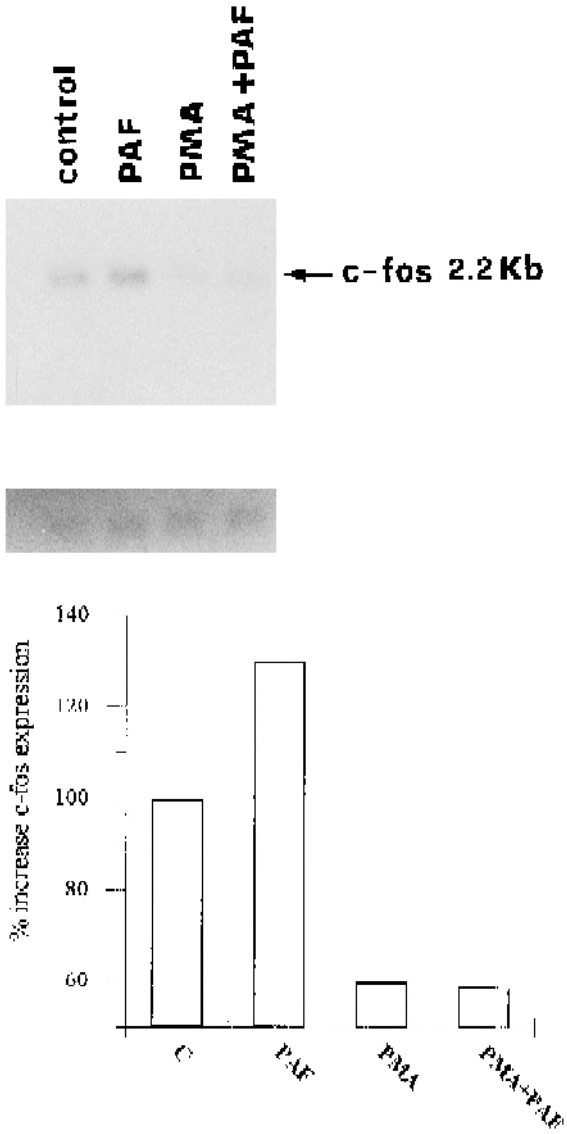

Fig. 8. Effect of PKC depletion after 24 h incubation with 0.1 μM of the phorbol ester PMA on PAF (0.1 μM, 30 min incubation)-stimulated c-fos gene expression in HEC-1A cells. In the upper panel hybridization of HEC-1A RNA with a cDNA probe for c-fos (upper blot) and with rat-cyclophyllin cDNA probe (lower blot) is shown. In the lower panel the percentage increase of c-fos gene expression with the different treatments calculated by ratio of densitometric analysis of the c-fos and cyclphyllin bands is reported.

Table 1
Effect of PKC inhibition on PAF-stimulated [^{3}H]thymidine uptake in HEC-1A cells

Control	B	S	PMA 24h	PAF	PAF + B	PAF + S	PAF + PMA 24h	PMA 5 min	PAF + PMA 5 min
100 ± 2.2 $n = 16$	99.3 ± 8.5 $n = 8$	76.1 ± 0.1 $n = 5$	90.5 ± 6.1 $n = 3$	162.4 ± 3.9 * $n = 10$	104.6 ± 10 $n = 6$	71.6 ± 0.1 $n = 3$	123.5 ± 4.7 $n = 3$	155.6 ± 4.7 * $n = 6$	172 ± 4.9 * $n = 3$

PKC was inhibited with the two different inhibitors sangivamycin (S) and bisindolylmaleimide (B) or depleted after long-term exposure (24 h) to the phorbol ester PMA (0.1 μM). PKC stimulation was obtained with short-term (5 min) exposure to PMA (0.1 μM). Values represent mean ± S.E.M. of percentage thymidine incorporation in the indicated number of observations.
* $P < 0.001$ versus control.

sitometric analysis as described above, is reported. Similar results were obtained when cells were pretreated with the PKC inhibitor sangivamycin (results not shown).

The inhibitory effect of PKC inhibition on PAF-mediated c-fos gene expression prompted us to study the possible involvement of PKC on PAF-stimulated thymidine uptake. As shown in Table 1, PKC inhibition with the two inhibitors sangivamycin and bisindolylmaleimide blunted PAF-mediated [^{3}H]thymidine incorporation. Similar results were obtained in PKC-depleted cells after long-term stimulation with 0.1 μM PMA (Table 1). The involvement of PKC in PAF-mediated thymidine uptake was further suggested by the absence of an additive effect of PAF on PMA (0.1 μM, 5 min stimulation)-induced mitogenesis (Table 1).

4. Discussion

In the present paper we report evidence that PAF-induced mitogenesis in HEC-1A cells is mediated by a network of signaling pathways involving NRPTK, MAPK and PKC activation. Our data demonstrate that PAF stimulates a rapid (1–2 min) phosphorylation in tyrosine residues of several cellular proteins in HEC-1A cells. In addition, we report that tyrosine kinase inhibition with the two structurally and mechanicistically unrelated PTK inhibitors genistein and methyl-2,5-dihydroxycinnamate blunted PAF-induced thymidine incorporation. These results suggest the involvement of NRPTK activation in the mitogenic effect of PAF. Recent evidence indicates that the two PTK inhibitors used in the present study may have additional effects beside PTK inhibition. In particular, genistein has been shown to inhibit type II topoisomerases [34], S6 kinase [35] and to revert certain insulin actions independently of PTK inhibition [36]. Similarly, the cytotoxic effect of methyl-2,5-dihydroxycinnamate has been shown to be independent of PTK inhibition [37]. In this light, the possibility that non-specific effects of the two compounds might contribute to inhibit basal and stimulated mitogenesis in HEC-1A cells cannot be excluded. On the other hand, both the erbstatin analog and genistein did not prevent PAF-induced increase of c-fos gene expression whereas this effect was abolished by PKC down regulation and pharmacological inhibition. This finding, while suggesting the involvement of PKC activation in c-fos gene expression, excludes that the two compounds have non-specific effects on PKC activity and suggests that activation of PKC by PAF in HEC-1A cells is independent of PTK activity.

Among the different proteins showing increased tyrosine phosphorylation in response to PAF, two proteins respectively in the 42 and 44 kDa molecular weight range were immunologically identified by immunoprecipitation studies as p42 and p44 MAPKs. Immunokinase experiments performed either by using a monoclonal antibody directed against the p42 isoform and a polyclonal antibody that recognize both p42 and p44 isoforms, demonstrated that the increased tyrosine phosphorylation stimulated by PAF in the two proteins was associated with an increase of their activity. These results were also confirmed by evaluation of phosphorylating activity of HEC-1A cell proteins of MBP copolimerized with polyacrylamide ('in-gel' kinase assays), which revealed the presence of several MBP phosphorylating proteins including one at 42–44 kDa. In addition, pharmacological inhibition of MAPK cascade activation by PD

098059 [28–30] blunted the stimulatory effect of PAF on MAPK activity and DNA synthesis. Taken together, these findings strongly suggest, for the first time, that MAPKs are involved in a biological effect of PAF in proliferating cells. However, it must be mentioned that in our hands, PD 098059 inhibited PAF-stimulated MBP phosphorylation in other protein bands besides the 42–44 kDa and stimulated MBP phosphorylation by a protein band of about 80–100 kDa. In different cells, this compound has been shown to enhance basal activity of c-raf as well as its activation by growth factors [30], and to induce a persistent activation of the ras pathway [38]. It is possible that MAPK activation by PAF in HEC-1A cells is involved in activation of other kinases able to phosphorylate MBP by the phospholipid. The finding that PD 098059 inhibits PAF-stimulated thymidine uptake at higher concentrations than those required to inhibit MAPK activation, further indicates that multiple signaling pathways are involved in growth of HEC-1A cells. Similarly, in cells transformed towards a tumorigenic phenotype PD 098059 was found to inhibit cell growth only at very high concentrations [29].

Finally, we report evidence of the involvement of PKC-mediated pathways in the mitogenic effect of PAF. Indeed, we show here that PKC down regulation after long-time exposure to PMA as well as its inhibition with sangivamycin were able to revert the stimulatory effect of PAF. Moreover, two different PKC inhibitors and long term exposure to PMA antagonized PAF-stimulated thymidine incorporation. Although long-term treatment with PMA does not down regulate all PKC isoenzymes and PKC inhibitors may have other effects beside PKC inhibition, taken together, our results indicate that mitogenic signaling by PAF also requires signaling pathways leading to PKC activation. This hypothesis is substantiated by the finding that short-term stimulation of PKC with PMA was able to induce mitogenesis in these cells, and that no additive effect was observed when cells were concomitantly stimulated with the two agents.

In conclusion, our results suggest that multiple signaling pathways are involved in PAF-mediated mitogenesis in HEC-1A cells. In particular, we show evidence that PKC activation is involved in PAF-mediated c-fos gene expression. In addition, PAF induces an increase of tyrosine phosphorylation of proteins which is involved in the activation of MAPK. The inhibitory effect of PTK, MAPK cascade and PKC inhibition on PAF-stimulated thymidine incorporation suggests that these proliferative signals may support PAF-induced mitogenesis in this cell line. As previously reported in different cell systems [5,8], cross-talks among NRPTK and PKC may also occur further contributing in enhancing mitogenic signaling. In a different tumor cell line, PAF stimulation of c-fos gene expression was found to be blunted by both PKC and PTK inhibition [19], suggesting cross-talks between the two pathways in these cells.

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