Protein tyrosine phosphorylation and regulation of the receptor for platelet-activating factor in rat Kupffer cells

Effect of sodium vanadate

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Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, AGEPC) and sodium vanadate (a phosphotyrosine phosphatase inhibitor) induced a time- and concentration-dependent increase in phosphotyrosine in several proteins and stimulated prostaglandin (PG) E₂ production in cultured rat Kupffer cells. In addition, vanadate induced a decrease in the surface expression of AGEPC receptors and, as a consequence, inhibited AGEPC-stimulated PGE₂ production. The vanadate-induced decrease in the surface expression of AGEPC receptors was time- and concentration-dependent and was partially prevented by genistein, a putative tyrosine kinase inhibitor. Upon removal of vanadate from the culture medium and re-incubation of cells in vanadate-free medium, the surface AGEPC receptors were restored within 7 h after the removal of vanadate. Both AGEPC- and vanadate-stimulated PGE₂ formation was attenuated by genistein. Thus the present investigation demonstrates that both AGEPC and sodium vanadate stimulate tyrosine phosphorylation of cellular proteins, and vanadate induces a decrease in the number of the surface AGEPC receptors. These results suggest that protein tyrosine phosphorylation may play a role, directly or indirectly, in the regulation of surface expression of AGEPC receptors as well as in PGE₂ production in response to vanadate and AGEPC.

INTRODUCTION

Platelet-activating factor ('PAF') is a unique phosphoglyceride which possesses a variety of biological functions in different tissues and cells (see [1–4] for reviews). The chemical structure of this lipid mediator was found to be 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine and is referred to as AGEPC in the present study [5–7]. AGEPC elicits most of its biological effects via specific receptors, which usually are located on the surface of cells and are widely distributed in various tissues and cells [8]. In platelets [9–11], neutrophils [12] and glomerular mesangial cells [13], signal transduction from specific AGEPC receptors to various effector systems involves GTP-binding protein(s). AGEPC stimulates the hydrolysis of polyphosphoinositides, which generates two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol [14–17]. Also, AGEPC stimulates the activity of phospholipase A₂, leading to the release of arachidonic acid from cellular phospholipids through G-protein- [12] and Ca²⁺- [18,19] dependent mechanisms.

Tyrosine kinase activity is associated with receptor-mediated signal transduction of insulin receptors [20], as well as other growth-factor receptors [21,22]. Recent reports have suggested that a tyrosine kinase may be involved in AGEPC-stimulated phosphoinositide metabolism in platelets [23] and arachidonic acid metabolism in the macrophage cell line P388D1 [24]. The tyrosine kinase inhibitor genistein [25] inhibits significantly the stimulatory effect of AGEPC in these two processes [23,24], and AGEPC promotes protein tyrosine phosphorylation in platelets [23], neutrophils [26] and rat liver macrophages [27].

Vanadate is a phosphate analogue which affects enzymic activities involving phosphate-transfer processes such as protein

phosphorylation/dephosphorylation reactions [28] and reactions utilizing ATP [29]. Vanadate has been shown to inhibit phosphotyrosine phosphatase activity [30] and to stimulate tyrosine kinase [31,32], leading to an accumulation of phosphotyrosine in various cellular proteins. Vanadate is believed to modulate receptors for insulin and insulin-like growth factor by stimulating the phosphorylation of tyrosine residues of the respective receptors [33,34]. In addition, vanadate has been shown to stimulate DNA synthesis [35] and inhibit Na⁺/K⁺-ATPase [29].

In the present study, the effect of AGEPC and vanadate on tyrosine phosphorylation in Kupffer cells was investigated, and the possible role of the protein tyrosine phosphorylation on AGEPC-receptor function was explored. It was found that both AGEPC and vanadate induced a time- and concentration-dependent increase in tyrosine phosphorylation of several cellular proteins. Also, evidence is presented indicating that the tyrosine phosphorylation of cellular protein(s) is likely to be involved in the vanadate-induced alteration of surface expression of AGEPC receptors and in AGEPC-receptor-mediated eicosanoid production. A preliminary report of this study has been published [36].

EXPERIMENTAL

Isolation and primary culture of rat Kupffer cells

Rat Kupffer cell suspensions were prepared by centrifugal elutriation [37] and cultured as described previously [38].

[³H]AGEPC binding to cultured rat Kupffer cells

The ligand-binding experiments were performed essentially as

Abbreviations used: AGEPC, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; lysoAGEPC, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; PG, prostaglandin; PMA, phorbol 12-myristate 13-acetate; WEB 2170, 8(*RS*)-6-(2-chlorophenyl)-8,9-dihydro-1-methyl-8-(4-morpholinylcarbonyl)-4*H*,7*H*-cyclopenta[4,5]thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine; WEB 2086, 3-{4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-2-yl}-1-(4-morpholinyl)propan-1-one; L659,989, [*trans*-2-(3-methoxy-5-methylsulphonyl-4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl)]tetrahydrofuran; U-66985, 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphoric acid 6'-trimethylammonium hexyl ester; CV-3988, *rac*-3-(*N*-n-octadecylcarbamoyloxy)-2-methoxypropyl-thiazolioethyl phosphate.

described previously [19,38]. Specific AGEPC binding is defined as the difference between total binding (measured in the absence of 1.0 μM unlabelled AGEPC) and non-specific binding (measured in the presence of 1.0 μM unlabelled AGEPC). Scatchard analysis was performed by a computer-fitting program.

Radioimmunoassay of prostaglandin (PG) E_2

Kupffer cells cultured in regular RPMI 1640 medium were rinsed three times with serum-free Hanks' buffer. The same buffer was added to each plate and the incubation was continued for 15 min, after which the cells were challenged with 10 nM-AGEPC or other compounds for the desired time period. In certain experiments, cells were pretreated with vanadate or genistein in the regular culture medium before stimulation. The amount of PGE_2 released into the incubation medium by cultured Kupffer cells was assayed by a radioimmunoassay as described previously [39].

Western blots of phosphotyrosine

Tyrosine phosphorylation was assayed as follows. Cells cultured in RPMI 1640 medium (4×10^6 cells/35 mm plate) were stimulated with vanadate or AGEPC for different time periods. The reaction was terminated by aspiration of the medium and immediate addition of 50 μl of immunoprecipitation buffer containing 50 mM-Tris/HCl, pH 7.4, 150 mM-NaCl, 0.5 mM-sodium orthovanadate, 5 mM-EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 20 μg of leupeptin/ml, 20 μg phenylmethanesulphonyl fluoride/ml, 100 units of aprotinin/ml and 10 mg of *p*-nitrophenyl phosphate/ml. The precipitation buffer was transferred to an Eppendorf vial containing 50 μl of 2 $\times$ concentrated SDS sample buffer, which contained 0.125 M-Tris/HCl, pH 6.8, 4% SDS, 20% glycerol and 10% mercaptoethanol. The mixture was heated at 95 $^\circ\text{C}$ for 5 min before being subjected to SDS/PAGE. Samples (90 μl) (e.g. containing approx. 50 μg of protein) were applied to 10%-polyacrylamide slab gels and were electrophoresed at constant current (30 mA per gel) as described by Laemmli [40]. Purified protein markers were electrophoresed simultaneously in this system to estimate the molecular masses of the cellular proteins. Proteins on the gel were transferred to a Millipore Immobilon-P transfer membrane (a polyvinylidene difluoride membrane) in cold (4 $^\circ\text{C}$) transfer buffer containing 25 mM-Tris/HCl, pH 8.3, 0.075% SDS, 0.2 M-glycine, 20% methanol and 0.5 mM-sodium vanadate by using a constant current of 200 mA for 4–5 h. The membrane was blocked overnight at 4 $^\circ\text{C}$ in a blocking solution [10 mM-Tris/HCl, pH 7.2, 150 mM-NaCl and 3% BSA (Sigma, fraction V)] and incubated with monospecific anti-phosphotyrosine antibodies (1 $\mu\text{g}/\text{ml}$) in blocking solution at 25 $^\circ\text{C}$ for 2–4 h. The membrane was washed extensively (5 times, 5 min/wash) with rinsing buffer (10 mM-Tris/HCl, pH 7.2, 150 mM-NaCl, 0.05% Tween 20 and 0.05% Nonidet P-40) and immunoblotted by using ^{125}I -Protein A (2–4 h, room temperature). The membrane was washed as described above, dried, and autoradiographed at -80°C using Kodak X-Omat AR film. Some of the autoradiograms were quantified by measuring the integrated density by using a computer imaging system.

Determination of protein

Protein was quantified with the bicinchoninic acid protein assay essentially as described by Smith *et al.* [41]. The reagent kits employed in this assay were provided by Pierce (Rochford, IL, U.S.A.).

Statistical analysis

Data obtained were analysed for statistical significance by Student's *t* test.

Materials

1-*O*-[1',2'- ^3H]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (NET-668) (40.5–59.5 Ci/mmol) and ^{125}I -Protein A (NEX-148) (86.6 $\mu\text{Ci}/\mu\text{g}$) were purchased from Du Pont-New England Nuclear (Boston, MA, U.S.A.). Unlabelled AGEPC ($\text{C}_{16:0}$) was obtained from Bachem BioScience (Bubendorf, Switzerland). Anti-phosphotyrosine antibody was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Genistein, leupeptin and aprotinin were purchased from Calbiochem (San Diego, CA, U.S.A.). Sodium orthovanadate, Nonidet P-40, phenylmethanesulphonyl fluoride, deoxycholic acid and *p*-nitrophenyl phosphate were purchased from Sigma (St. Louis, MO, U.S.A.). Radioimmunoassay kits for PGE_2 analyses were obtained from Advanced Magnetics (Cambridge, MA, U.S.A.). Tween 20 was from Bio-Rad (Richmond, CA, U.S.A.).

RESULTS

Protein tyrosine phosphorylation in cultured Kupffer cells

AGEPC-stimulated protein tyrosine phosphorylation. AGEPC, at nanomolar concentrations, stimulated tyrosine phosphorylation of several proteins in Kupffer cells, as assessed by an immunoblotting technique employing a monoclonal antibody to phosphotyrosine. As shown in Figs. 1 and 2, at least five cellular proteins were phosphorylated on tyrosine residues in this cellular system. The predominant phosphorylated protein (pp80) exhibited an approximate molecular mass of 80 kDa. The stimulatory effect of AGEPC (10 nM) was time-dependent (5 s–5 min) with maximal stimulation seen within 2 min [27] and was concentration-dependent (Fig. 1). When the phosphorylation of pp80 was quantified, an EC_{50} value of 0.8 nM was obtained (Fig. 1). LysoGEPC, an inactive metabolite and immediate precursor of AGEPC, at concentrations as high as 100 nM had no effect on tyrosine phosphorylation in this system (Fig. 2). AGEPC-receptor antagonists such as U66985, CV3988 and

Fig. 1. Concentration-dependency of AGEPC-stimulated protein tyrosine phosphorylation in cultured Kupffer cells

Cells (4×10^6 cells/plate) were washed and stimulated with various concentrations of AGEPC (10 pM–10 nM) for 5 min. The autoradiogram was analysed by measuring integrated density with a computer imaging system. The results are expressed in arbitrary units. The inset contains the autoradiogram from which the graphics were derived.

Fig. 2. Effect of AGEPC-receptor antagonists on protein tyrosine phosphorylation stimulated by AGEPC

Cells were washed and incubated with buffer alone (lane 1), 100 nM-lysoGEPC (lane 2), 10 nM-AGEPC (lane 3) or 10 nM-AGEPC in the presence of various AGEPC receptor antagonists (lanes 4–7): 20 μ M-WEB 2170 (lane 4), 20 μ M-WEB 2086 (lane 5), 13.7 μ M-CV3988 (lane 6) or 10 μ M-U66985 (lane 7). The stimulation interval for AGEPC or lysoGEPC was 5 min. The AGEPC-receptor antagonists were added 2–5 min before addition of AGEPC.

WEB 2170 inhibited completely the protein tyrosine phosphorylation stimulated by AGEPC, whereas WEB 2086 showed a partial inhibitory effect (Fig. 2). AGEPC-promoted tyrosine phosphorylation was inhibited by genistein, a specific tyrosine kinase inhibitor [25,42], in the concentration range 25–100 μ M (Fig. 3). Furthermore, the detection of the phosphotyrosine in the cellular proteins was inhibited competitively by 2 mM-phosphotyrosine, but not by the same concentration of phosphoserine or phosphothreonine during immunoblotting, suggesting the tyrosine-specificity of the immunoblotting assay (results not shown).

Vanadate-stimulated protein tyrosine phosphorylation. The vanadate-promoted protein tyrosine phosphorylation was examined by two different protocols: (a) high concentrations (mM) for a short period (minutes) (Fig. 4a) and (b) low concentrations (μ M) for a long term (hours) (Fig. 4b). In Fig. 4(a), cultured Kupffer cells were incubated with 10 mM-vanadate for a brief period of time (1–35 min) in RPMI 1640 medium. Under these conditions, cells were viable as determined by Trypan Blue exclusion, cell morphology and protein measurement after a washing procedure. Vanadate stimulated the accumulation of phosphotyrosine in several cellular proteins in a time-dependent fashion. The molecular masses of these cellular proteins ranged between 30 and 130 kDa. Similarly, when cells were incubated with low concentrations of vanadate (5–30 μ M) for a relatively longer period of time (24 h), cellular phosphotyrosine was increased in a concentration-dependent fashion (Fig. 4b). The tyrosine phosphorylation induced by vanadate was strongly inhibited by pretreatment of cells with genistein (Fig. 4c).

Effect of vanadate on the surface expression of the AGEPC receptor

Concentration- and time-dependency of vanadate effect on specific [^{3} H]AGEPC binding. Kupffer cells in monolayer culture were incubated at 37 $^{\circ}$ C in the absence or presence of various

Fig. 3. Effect of genistein on AGEPC-stimulated protein tyrosine phosphorylation

Cells were pretreated with genistein at the concentrations indicated in regular culture medium for 2 h. The cells were then washed and incubated in Hanks' buffer for another 15 min before stimulation with AGEPC (10 nM). Autoradiograms were analysed by measuring integrated density with a computer system. The results are expressed in arbitrary units. The inset shows the autoradiogram from which the graphics were derived.

concentrations of vanadate (dissolved in regular culture medium) for 1, 2 or 3 h. After incubation, the cells were rinsed with serum-free Hanks' buffer, followed by assessment of [^{3} H]AGEPC binding. As illustrated in Fig. 5, pretreatment with vanadate resulted in a concentration-dependent decrease in the surface expression of specific [^{3} H]AGEPC-binding sites. The EC_{50} value for the effect of vanadate was dependent on the length of the pretreatment period. The EC_{50} for the vanadate-initiated decrease in specific [^{3} H]AGEPC binding was approx. 0.25 mM, 0.65 mM and 2 mM respectively, when the vanadate exposure time was 3 h, 2 h and 1 h. The vanadate-induced decrease in the surface expression of [^{3} H]AGEPC-binding sites was time-dependent (Fig. 5, inset). When vanadate (10 mM) was applied to the cells, specific [^{3} H]AGEPC-binding sites on the Kupffer cells decreased rapidly, e.g. 50 % of the binding sites were lost within 10 min and 80 % were lost within 30 min. Also, the time-course of the specific [^{3} H]AGEPC binding was examined. Specific binding of [^{3} H]AGEPC to cultured Kupffer cells occurred rapidly during the first 20 min and reached a steady state within 30 min, with a maximal binding of about 40 fmol/plate. In contrast, cells pretreated with vanadate (10 mM; 40 min) did not bind significant amounts of [^{3} H]AGEPC for the initial 30 min of incubation; specific [^{3} H]AGEPC binding was inhibited throughout the binding period (90 min) (results not shown). The saturation curve of specific [^{3} H]AGEPC binding indicated that pretreatment with vanadate decreased the specific binding at all concentrations of [^{3} H]AGEPC tested in the range 0.05–2.5 mM (see Fig. 6a). Scatchard analysis revealed that the B_{max} decreased from 95 fmol to 28 fmol, but the slope of the Scatchard plot did not change appreciably ($K_d = 0.41$ nM in the control versus 0.51 nM in the vanadate-treated) when cells were pretreated with 10 mM-vanadate (Fig. 6b), suggesting that the decrease in the specific [^{3} H]AGEPC binding was due primarily to a decrease in the number of specific AGEPC-binding sites and not to an alteration of the binding affinity.

The receptor-regulatory effect of vanadate also was examined at lower concentrations (2.5–30 μ M) for a relatively longer period of time (24 h or 48 h). Similar to the results shown in Fig. 5, vanadate caused a decrease of surface specific [^{3} H]AGEPC

Fig. 4. Vandate-stimulated protein tyrosine phosphorylation in intact Kupffer cells

(a) Time course of vanadate-induced protein tyrosine phosphorylation. Cultured cells were stimulated with 10 mM-sodium orthovanadate in the RPMI 1640 culture medium containing 10% fetal-bovine serum for the time intervals indicated (1–35 min). (b) Concentration-dependency of vanadate-promoted tyrosine phosphorylation. Cells were incubated with various concentrations of vanadate (5–30 μM) in RPMI 1640 culture medium containing 10% fetal-bovine serum for 24 h. (c) Effect of genistein on vanadate-induced tyrosine phosphorylation. Cells were preincubated with (lanes 5, 6) or without (lanes 1–4) genistein (100 μM) for 3 h in culture medium. The cells were washed and stimulated with (lanes 3–6) or without (lanes 1, 2) vanadate for 40 min at 37 °C. The two lanes in the various portions (e.g. lanes 1 and 2, 3 and 4, 5 and 6) are duplicate samples.

Fig. 5. Concentration- and time-dependency of vanadate-induced decrease in specific $[^3\text{H}]\text{AGEPC}$ binding

Cells were incubated with vanadate at several concentrations (1 μM –10 mM) for 1 h (●), 2 h (○) or 3 h (▲), after which the cells were washed and specific $[^3\text{H}]\text{AGEPC}$ binding was assessed. The results are presented as percentages of the amount of specific $[^3\text{H}]\text{AGEPC}$ binding in the cells which received no vanadate treatment. Each data point and bar represent the mean $\pm$ s.d. of three separate experiments. Inset: time course of vanadate-induced decrease in specific $[^3\text{H}]\text{AGEPC}$ binding. Cells were preincubated with vanadate (10 mM) for various time points followed by a wash procedure and incubation with $[^3\text{H}]\text{AGEPC}$ (0.83 $\pm$ 0.09 nM). The results are presented as percentages of the amount of specific $[^3\text{H}]\text{AGEPC}$ binding observed before vanadate treatment (64.5 $\pm$ 14.8 fmol). Each data point represents the mean $\pm$ s.d. of two separate experiments.

Fig. 6. Effect of vanadate on the specific $[^3\text{H}]\text{AGEPC}$ binding

Cells were incubated without (○) or with (●) vanadate (10 mM) at 37 °C for 40 min, followed by an extensive wash procedure. The cells were then incubated with various concentrations of $[^3\text{H}]\text{AGEPC}$ (0.11–2.49 nM) in the absence or presence of excess unlabelled AGEPC. At the end of the incubation interval, the cells were washed and the specific $[^3\text{H}]\text{AGEPC}$ binding was assayed. (a) Saturation curve; (b) Scatchard plot.

Fig. 7. Effect of low concentrations of vanadate (2.5–30 μM) on AGEPC binding

Cells were incubated with various concentrations of vanadate (2.5–30 μM) in culture medium containing 10% fetal-bovine serum at 37 °C for 24 h (●) or 48 h (○), followed by a washing procedure with serum-free Hanks' buffer and [^3H]AGEPC binding assessment. The results are presented as percentages of the amount of specific [^3H]AGEPC binding in cells which received no vanadate. Each data point and bar represents the mean value $\pm$ S.D. of 2 (48 h) or 3 (24 h) separate experiments.

Fig. 8. Recovery of AGEPC receptor after incubation with vanadate

Cells were incubated with 15 μM -vanadate in serum-containing medium for 24 h. At the end of incubation, cells were either washed (○) three times with vanadate-free medium and incubated in fresh vanadate-free medium or further incubated (●) in the vanadate-containing medium at 37 °C for various time intervals as indicated in the Figure. At each time point, the cells were rinsed and the [^3H]AGEPC binding was assessed. The results are presented as percentages of the amount of [^3H]AGEPC bound to the cells which received no vanadate pretreatment. Each data point and bar represent the mean $\pm$ S.D. of 2–3 separate experiments.

binding in a concentration-dependent manner, with an EC_{50} of 15 μM after a 24 h incubation period (Fig. 7). The lost specific AGEPC-binding sites were restored upon removal of extracellular vanadate by washing the cells with vanadate-free buffer. As shown in Fig. 8, the specific [^3H]AGEPC-binding sites returned to the control level within 7 h after removal of vanadate.

Temperature-dependency of the effect of vanadate on AGEPC receptor binding. The vanadate-induced decrease in specific AGEPC receptors was temperature-dependent. Low temperature (4 °C) attenuated markedly the loss of the AGEPC receptors induced by vanadate (results not shown).

Effect of genistein on the vanadate-induced decrease in the surface expression of the AGEPC receptor. Since genistein

Table 1. Effect of genistein on vanadate-induced loss of the surface expression of the specific [^3H]AGEPC-binding sites

Cells were incubated with genistein at various concentrations (10–100 μM) or with dimethyl sulphoxide (DMSO) in culture medium for 3 h, followed by incubation with vanadate (10 mM) for 20 min. At the end of incubation interval, the cells were rinsed twice with Hanks' buffer containing 0.1% BSA and then incubated with [^3H]AGEPC (0.89 ± 0.08 nM) for 60 min at 25 °C in the presence or absence of excess unlabelled AGEPC. Specific [^3H]AGEPC binding was assessed as described in the Experimental section. The results are expressed as percentages of the amount of specific [^3H]AGEPC binding (42.1 ± 3.9 fmol) in cells pretreated with genistein but without vanadate treatment. Genistein pretreatment had no effect on [^3H]AGEPC binding. Each value represents the mean $\pm$ S.D. of three separate experiments: * $P \leq 0.01$ versus DMSO; ** $P < 0.05$ versus *; *** $P < 0.01$ versus *.

Treatment	Specific [^3H]AGEPC binding (% of control)
DMSO	100 $\pm$ 9.2
DMSO, then vanadate (10 mM, 20 min)	41.0 $\pm$ 6.0*
Genistein (10 μM), then vanadate	48.5 $\pm$ 2.5
Genistein (25 μM), then vanadate	52.7 $\pm$ 4.4**
Genistein (50 μM), then vanadate	61.9 $\pm$ 5.3***
Genistein (100 μM), then vanadate	61.6 $\pm$ 5.9***

inhibited protein tyrosine phosphorylation in Kupffer cells in response to vanadate, it was expected that genistein would be able to attenuate the vanadate-induced decrease in specific [^3H]AGEPC binding if the protein tyrosine phosphorylation was considered as one of the mechanisms involved in the receptor regulatory effect of vanadate. As shown in Table 1, vanadate (10 mM) decreased significantly the specific [^3H]AGEPC binding (59% decrease) within 20 min. Genistein attenuated the inhibitory effect of vanadate in a dose-dependent manner, with a significant effect seen between 25 μM and 100 μM .

Effect of vanadate on AGEPC-receptor-mediated PGE_2 production in Kupffer cells

Effect of vanadate on AGEPC-stimulated PGE_2 production. Cultured cells were pretreated with vanadate for 1 h and washed thoroughly. The cells were then incubated in vanadate-free Hanks' buffer at 37 °C for another 15–20 min, followed by stimulation with various concentrations of AGEPC. At the end of the incubation period, the Hanks' buffer was removed and immediately frozen for subsequent prostaglandin measurement. As illustrated in Fig. 9, vanadate at relatively low concentrations (i.e. between 10 μM and 0.5 mM), did not inhibit the effect of AGEPC, but rather enhanced slightly the subsequent AGEPC-stimulated PGE_2 production as observed in rat liver cell line C-9. As shown in Fig. 5, vanadate in this concentration range (60 min incubation) did not appreciably alter specific AGEPC binding. However, at higher concentrations (i.e. 0.5–10 mM) vanadate caused a concentration-dependent inhibition of AGEPC-stimulated PGE_2 production, with an EC_{50} value of approx. 1.8 mM (Fig. 9). This value was close to the EC_{50} value (2.0 mM) for the inhibitory effect of vanadate on AGEPC receptor binding (Fig. 5).

Effect of genistein on AGEPC- and vanadate-stimulated PGE_2 production. Since vanadate inhibits phosphotyrosine phosphatase

Fig. 9. Effect of vanadate on AGEPC-stimulated PGE₂ production

Cells were incubated with various concentrations of vanadate at 37 °C for 40 min, followed by a washing procedure and incubation with 10 nM-AGEPC. Results are presented as percentages of the net increase in the PGE₂ production [4236 ± 4506 ± 141 (AGEPC-stimulated) - 270 ± 130 (basal production) pg/plate] in the control cells which received no vanadate during the preincubation period and cells stimulated with AGEPC. Each data point represents the mean ± S.D. of two separate experiments.

Fig. 10. Concentration-dependency of AGEPC- and vanadate-stimulated PGE₂ production: effect of genistein

Cells were incubated with (●) or without (○) genistein (30 μM) for 3 h and then washed extensively with Hanks' buffer. The cells were incubated for another 15 min in the same buffer and challenged with various concentrations of vanadate (0.1–10 mM, 30 min; a) or AGEPC (0.1 nM–0.1 μM, 15 min; b).

and possibly stimulates tyrosine kinase [30–32], it was decided to ascertain whether vanadate stimulates PGE₂ production, and whether such an effect of vanadate might be inhibited by genistein in cultured Kupffer cells. Similar to experiments examining tyrosine phosphorylation and ligand binding, PGE₂ production was measured in two experimental protocols: a short term with high concentrations of vanadate and a long period of time with low concentrations of vanadate. Cells were washed with serum-free Hanks' buffer and incubated in the same buffer at 37 °C for

a brief period of time (20 min), followed by incubation with various compounds for another 15 min. As shown in Fig. 10(a), vanadate at concentrations in the range 0.1–10 mM stimulated PGE₂ production, and this effect was inhibited significantly by pretreatment of the cells with genistein (30 μM). When cells were incubated in RPMI 1640 medium (with or without 10% fetal-bovine serum) in the absence or presence of various concentrations of vanadate (5–30 μM) for 24 h, it was found that a large amount of PGE₂ accumulated in medium from the control cells (9.54 ± 1.46 ng/ml; *n* = 3) and that the presence of vanadate (5 μM) did not increase significantly PGE₂ synthesis (10.33 ± 1.51 ng/ml; *n* = 3). No PGE₂ was detected in the medium which had not been incubated with Kupffer cells. In addition, as expected, AGEPC (10 nM) stimulated PGE₂ production in Kupffer cells. Genistein (30 μM) significantly decreased the stimulatory effect of AGEPC by nearly 80% (Fig. 10b).

DISCUSSION

Tyrosine-specific protein kinases are involved in the signal-transduction mechanisms of a number of growth factors, e.g. epidermal growth factor [21,22] and platelet-derived growth factor [42,43], and of insulin [20]. Receptors for the growth factors and insulin possess single-pass transmembrane segments and have intrinsic tyrosine kinase catalytic activity. These features distinguish this family of receptors from other receptor families, which for instance have seven transmembrane domains, are linked to various response elements via GTP-binding protein(s), and have no apparent catalytic activity, e.g. catecholamine receptors [44]. Similar to the catecholamine receptor, a recent study demonstrated that AGEPC receptor molecule has seven transmembrane segments and belongs to the superfamily of receptors which interact with GTP-binding proteins [45].

Recent studies have indicated that tyrosine kinase activation is involved in signal transduction stimulation by AGEPC in platelets (phosphoinositide turnover) [23] and in macrophages (arachidonic acid metabolism) [24]. AGEPC was found to stimulate protein tyrosine phosphorylation in rabbit platelets [23] and in human neutrophils [26]. Although the identity of the tyrosine-phosphorylated proteins which were increased in response to AGEPC is unknown, it has been proposed that tyrosine phosphorylation may regulate the activities of both phospholipase C and phospholipase A₂.

The present study was designed to examine whether AGEPC and vanadate, an inhibitor of phosphotyrosine phosphatase, stimulate protein tyrosine phosphorylation in primary cultured rat Kupffer cells, and to explore the possible relationship between the protein tyrosine phosphorylation and vanadate-induced down-regulation of surface AGEPC receptors. Our studies demonstrate that AGEPC stimulates tyrosine phosphorylation of several cellular proteins with estimated molecular masses of 130, 115, 80, 60 and 50 kDa. The effect of AGEPC on protein tyrosine phosphorylation is time- and concentration-dependent, and is receptor-mediated. The identity of the tyrosine-phosphorylated proteins in Kupffer cells in response to AGEPC is unknown at present. Some of the tyrosine-phosphorylated proteins observed in Kupffer cells in response to AGEPC have higher molecular masses (i.e. 80 kDa, 115 kDa, 130 kDa) than those observed in rabbit platelets (50 kDa and 60 kDa) [23], and hence represent different substrates of the tyrosine kinase. It remains to be elucidated whether these tyrosine kinase substrates have any functional relationship with the AGEPC receptor, components of the signal transduction pathways for AGEPC, or related effector systems [such as the phospholipase(s) responsible for arachidonic acid and phosphoinositide metabolism]. In addition, it is noteworthy that receptors for certain growth

factors such as platelet-derived growth factor, upon stimulation by its agonist, promote tyrosine phosphorylation of several cellular proteins, including phosphoinositide 3-kinase (85 kDa), phospholipase C- γ (145 kDa) and GTPase-activating protein (125 kDa), and are physically associated with these signalling components [46].

Vanadate stimulated tyrosine phosphorylation of numerous cellular proteins in intact Kupffer cells in a time- and concentration-dependent manner (Figs. 4a and 4b). Genistein (30 μ M), a specific tyrosine kinase inhibitor, inhibited the stimulatory effect of vanadate (Fig. 4c). Vanadate has been found not only to inhibit phosphotyrosine phosphatase, thereby increasing the accumulation of phosphotyrosine [30], but to stimulate the activity of tyrosine kinase as well [31,32]. Of particular significance is the fact that vanadate causes protein tyrosine phosphorylation of insulin receptors, leading to the down-regulation of the insulin receptor [33].

An important finding in the present study was that vanadate down-regulated the surface expression of the AGEPC receptors in rat Kupffer cells. Pretreatment of cells with vanadate caused a time- and concentration-dependent decrease in specific [^{3} H]AGEPC binding, with a $t_{1/2}$ of 10 min at 10 mM-vanadate and an EC_{50} of 0.65 mM when the incubation time was 2 h (Fig. 5). The EC_{50} was much smaller, e.g. 15 μ M, when the vanadate pretreatment time was 24 h or 48 h (Fig. 7). The decrease in specific AGEPC binding was due primarily to a loss of specific AGEPC binding sites rather than to an alteration of the binding affinity for AGEPC, as assessed by Scatchard analysis of the binding data (Fig. 6). Compared with the time course of the effect of vanadate on the insulin receptor (e.g. the inhibition is not seen until 6 h after addition of vanadate) [34,47], the vanadate-induced down-regulation of AGEPC receptors was rapid, with the half-maximal effect seen within 10–20 min. Similarly to the present study, vanadate (10 mM) induced a rapid decrease in the surface asialo-orosomucoid receptor in rat liver parenchymal cells, reaching a half-maximal effect within 2.5–5 min [48]. Pretreatment with vanadate followed by an extensive washing also attenuated the AGEPC-stimulated cellular responses, i.e. PGE₂ formation. The EC_{50} values for the inhibitory effect of vanadate on PGE₂ production and the regulation of the AGEPC receptor were comparable, suggesting that the vanadate-induced inhibition of PGE₂ production stimulated by AGEPC was a consequence of a loss of surface AGEPC receptors.

The molecular mechanism for the vanadate-induced loss of surface AGEPC receptors is not known. Numerous lines of evidence from several different systems (cell-free preparations and intact cells) indicate that vanadate treatment inhibits phosphotyrosine phosphatase, whereas it has little effect on phosphoserine or phosphothreonine phosphatases [30,49–51]. Since vanadate causes an increase in tyrosine phosphorylation of numerous proteins (more than nine cellular proteins) in the intact Kupffer cells, tyrosine phosphorylation of the AGEPC receptor protein or other cellular proteins which may have a regulatory effect on the receptor could be proposed as a possible molecular regulatory mechanism. The findings that genistein inhibited the vanadate-stimulated tyrosine phosphorylation (Fig. 4c) and attenuated significantly the vanadate-induced loss of surface AGEPC receptors (Table 1) suggested that these two events may be related. However, it should be pointed out that, although we believe that vanadate induces AGEPC-receptor down-regulation through a mechanism(s) involving tyrosine phosphorylation, either directly or indirectly, the possibility remains that vanadate alters AGEPC-receptor metabolism through effects independent of phosphorylation reactions.

Vanadate at concentrations between 0.1 and 10 mM for a brief

period of time (30 min) stimulated PGE₂ production in the cultured Kupffer cell. Similar to the AGEPC-stimulated PGE₂ production, the vanadate-elicited response was attenuated in cells pretreated with genistein. This result supports the hypothesis that both AGEPC- and vanadate-stimulated PGE₂ production may involve activation of tyrosine-specific protein kinase(s). Supporting this hypothesis was the observation that vanadate, at a concentration of 11 μ M, amplified the eicosanoid production of rat liver cells (C-9 cell line) stimulated with AGEPC [52]. It should be noted that this stimulatory effect of vanadate observed in the present study differs from its inhibitory effect on AGEPC-stimulated PGE₂ production, which may be due to the decreased expression of AGEPC receptor during the vanadate preincubation. The regulatory effect of tyrosine kinase on AGEPC-stimulated phosphoinositide turnover in platelets [23] and on lipopolysaccharide-primed AGEPC-stimulated arachidonic acid metabolism in macrophage P388D1 cells [24] has been observed. In addition to tyrosine kinase, other protein kinases such as protein kinase C also may play a role in AGEPC-stimulated arachidonic acid metabolism [53,54]. Recent studies in our laboratory have indicated that AGEPC-stimulated PGE₂ production in cultured Kupffer cells was inhibited significantly by two protein kinase C inhibitors, staurosporine (which interacts with the catalytic domain of protein kinase C) and calphostin C (which interacts with the regulatory domain of protein kinase C), and in the cells down-regulated for protein kinase C [27]. These results suggest that AGEPC-stimulated arachidonic acid metabolism in various cell-types can be regulated by several protein kinases, including tyrosine kinase and protein kinase C [27,39,53,54]. Activation of these protein kinase(s) is likely to be an important regulatory component in AGEPC-elicited cellular responses.

In summary, the present investigation demonstrates that both AGEPC and vanadate stimulate tyrosine phosphorylation of several cellular proteins in Kupffer cells. The stimulated protein tyrosine phosphorylation appears to be related to the vanadate-induced decrease in surface expression of AGEPC receptors. Furthermore, eicosanoid production in response to AGEPC and vanadate may involve activation of a tyrosine kinase in Kupffer cells. The identities of the tyrosine-phosphorylated proteins induced by AGEPC require investigation.

This work was supported by National Institutes of Health Grant AM-33538.

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Received 30 December 1991/28 May 1992; accepted 16 June 1992