Activation of Extracellular-Signal Regulated Kinase by Platelet-Derived Growth Factor Is Potentiated by Phenylephrine in Primary Cultures of Adult Rat Hepatocytes

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We investigated the effects of the α_1-adrenergic agonist phenylephrine on platelet-derived growth factor (PDGF)-stimulated extracellular signal-regulated kinase (ERK) in primary cultures of adult rat hepatocytes. Hepatocytes were isolated and cultured with PDGF (10 ng/ml) and/or α-adrenergic agonist. Phosphorylated ERK isoforms (ERK1 and ERK2) were detected by Western blotting analysis using anti-phospho mitogen-activated protein kinase (MAPK) antibody. PDGF stimulated phosphorylation of ERK2 (42 kDa MAPK) by 2.0-fold within 3—5 min. The PDGF-induced ERK activation was abolished by AG1296 (10^{-7} M) or LY294002 (10^{-7} M) treatment. MAPK kinase inhibitor, PD98059 (10^{-6} M), completely inhibited the PDGF-induced increase in ERK activity. In addition, PDGF-induced mammalian target of rapamycin activity was completely inhibited by AG1296, LY294002, PD98059, or rapamycin treatment. Phenylephrine alone showed no effects on ERKs, but significantly increased phosphorylation of ERK2 induced by PDGF. Moreover, a synthetic analog of diacylglycerol (DG), phorbol 12-myristate 13 acetate (TPA; 10^{-7} M), potentiated PDGF-induced ERK2 phosphorylation, while ionomycin had no effect (10^{-6} M). The effects of phenylephrine and TPA were antagonized by the phospholipase C (PLC) inhibitor U73122 (10^{-7} M), and the protein kinase C (PKC) inhibitor GF109203X (10^{-7} M), respectively. Accordingly, PDGF-induced DNA synthesis and proliferation in the presence or absence of phenylephrine or TPA were completely inhibited by AG1296, LY294002, PD98059, or rapamycin treatment. These results suggest that activation of PLC/PKC by phenylephrine represent an indirect positive regulatory mechanism for stimulating ERK induced by 10 ng/ml PDGF.

Key words extracellular-signal regulated kinase; platelet-derived growth factor; cross-talk; α_1-adrenergic agonist; hepatocyte

Platelet-derived growth factor (PDGF) is a potent stimulator of the proliferation by connective tissue cells, such as fibroblasts, smooth muscle cells and hepatic stellate cells,[1—4] and may play a role in liver regeneration.[5] PDGF-BB is reported to induce smooth muscle cells proliferation and cell migration more effectively than PDGF-AA or -AB.[1—4] PDGF appears to trigger liver regeneration after partial hepatectomy and chemical-induced acute liver cell necrosis *in vivo*.[6,7] The response of adult rat hepatocytes to PDGF has also been investigated extensively with respect to DNA synthesis and proliferation *in vitro*.[8,9] The receptor for PDGF, similarly to the receptors for epidermal growth factor (EGF), insulin and hepatocyte growth factor (HGF), contains intrinsic tyrosine kinase activity. The mitogenic effects of PDGF are reportedly mediated through the activation of tyrosine kinase-linked receptors.[10] However, little is known about which of the signals originating in the plasma membrane are involved in the induction of hepatocyte DNA synthesis and proliferation by PDGF. We recently reported that PDGF is able to rapidly stimulate hepatocyte DNA synthesis and proliferation during short-term culture.[11] In addition, the hepatocyte DNA synthesis and proliferation produced by PDGF was not inhibited depending on initial plating density. Furthermore, we found that hepatocyte DNA synthesis and proliferation by PDGF were potentiated by both α_1-adrenergic agonists, but not β_2-adrenergic agonists.[11]

More recently, the signal transduction pathways activated in response to PDGF in hepatocytes and other cell types have become more clearly understood.[4,12,13] Using specific inhibitors of signal transducers, we pharmacologically demonstrated that receptor tyrosine kinase, phosphoinositide 3-kinase (PI3K), phospholipase C (PLC) and ribosomal p70 S6 kinase (p70S6K) activities are essential for PDGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.[11]

In addition, extracellular-signal regulated kinase (ERK)1/2, which is the same as mitogen-activated protein kinase (p42 and p44 MAPK), is now known to be activated in response to a large number of mitogenic stimuli, and the enzyme is a key participant in the response to various growth factors and cytokines.[12,14,15] To better understand the PDGF-mediated signaling pathway, we investigated whether activation of ERK isoforms, ERK1 and ERK2, is involved in PDGF-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

On the other hand, catecholamines (*e.g.*, norepinephrine and its analogs) have been shown to be involved in the regulation of liver function (*e.g.*, lipid metabolism, carbohydrate metabolism and cell growth). There are several types of catecholamine receptor; β_1- and β_2-adrenergic receptors stimulate adenylate cyclase, while α_2-adrenergic receptors inhibit its activity.[16] α_1-Adrenergic receptors are related to phospholipase C (PLC) activation and subsequent increases in inositolphosphate turnover and diacylglycerol production, and protein kinase C (PKC) activation.[11,17] There have been few studies regarding the adrenergic regulation of ERK activity induced by growth factors in liver cells. Therefore, we examined whether α_1- and β_2-adrenergic agonists can modulate the PDGF-induced activity of ERK isoforms, as there are indications that hepatocyte DNA synthesis and proliferation are modified by α_1-adrenergic receptor-mediated responses in the presence of PDGF.[11,18]

The results obtained here indicate that PDGF rapidly phosphorylates one of the ERK isoforms, ERK2 (p42 MAPK),

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but not ERK1 (p44 MAPK). Moreover, phenylephrine potentiated the ERK2 phosphorylation induced by PDGF. The physiological significance of cross-talk between the PDGF signaling pathway and α_1-adrenergic receptor-mediated pathway in regulating hepatocyte proliferation is also discussed.

MATERIALS AND METHODS

Materials The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.): AG1296 (*N*-[3-chlorophenyl]-6,7-dimethoxy-4-quinazolinamine); aprotinin; 8-bromo-cAMP; dexamethasone; GF109203X hydrochloride (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide hydrochloride; H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride); ionomycin; metaproterenol hemisulfate; LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one hydrochloride); and phenylephrine hydrochloride. PD98059 (2′-amino-3′-methoxyflavone), platelet-derived growth factor (PDGF)-BB (human recombinant), phenylephrine hydrochloride, U-73122 (1-[6-[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amin]ohexyl]-1*H*-pyrrole-2,5-dione) and UK14304 (5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine) were obtained from Enzo Life Sciences. (Farmingdale, NY, U.S.A.). Rapamycin and 12-*O*-tetradecanoylphorbol-13-acetate (phorbol ester, TPA) were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Williams' medium E and newborn calf serum were purchased from Flow Laboratories (Irvine, Scotland). Collagenase (type II) was obtained from Worthington Biochemical Co. (Freehold, NJ, U.S.A.). Anti-phospho-p42/p44 MAPK monoclonal antibody and anti-p42/p44 MAPK monoclonal antibody, anti-phospho-mammalian target of rapamycin (mTOR) (Ser2481) polyclonal antibody, and anti-mTOR antibody were obtained from Cell Signaling Technology (Danvers, MA, U.S.A.). [Methyl-^{3}H]thymidine (20 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). All other reagents were of analytical grade.

Animals Male Wistar rats (weight 200—220 g) were obtained from Saitama Experimental Animal Co. (Saitama, Japan). Adaptation to a light-, humidity- and temperature-controlled room occurred over a minimum 3-d period prior to the start of experiments. Rats were fed standard diet and given tap water *ad libitum*. Animals used in this study were handled in accordance with the "Guiding Principles for the Care and Use of Laboratory Animals," as approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Guidelines for the Care and Use of Laboratory Animals of Josai University.

Hepatocyte Isolation and Culture Male Wistar rats were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg). Two-step *in-situ* collagenase perfusion was performed in order to facilitate disaggregation of adult rat livers, as described previously.[19] After perfusion, cells were dispersed in Ca^{2+}-free Hanks' solution. Cells were then washed three times by slow centrifugation (120×***g*** for 1 min). Hepatocyte viability was monitored by Trypan blue dye exclusion assay. On average, more than 96% of the cells remained intact.

Isolated hepatocytes were plated onto collagen-coated plastic culture dishes (diameter, 35 mm) at a density of 3.3×10^4 cells/cm^2 in Williams' medium E containing 5% bovine calf serum, 0.1 nM dexamethasone for 3 h at 37 °C in 5% CO_2 in air. The medium was then changed, and cells were cultured in serum- and dexamethasone-free Williams' medium E containing PDGF (10 ng/ml) with or without α_1-, α_2- and β_2-adrenergic agonists and/or specific effectors or inhibitors of signal transducers for the indicated times at 37 °C.

Measurement of Extracellular-Signal Regulated Kinase (ERK) Activity Phosphorylated ERK isoforms (ERK2; p42 MAPK, and ERK1; p44 MAPK) were identified by Western blotting analysis using anti-phospho-ERKs (pERK1 and pERK2) monoclonal antibody as described by Towbin *et al.*[20] Phosphorylated ERK activity was normalized against total ERKs (ERK1 and ERK2). Briefly, cultured hepatocytes were washed once with ice-cold phosphate-buffered saline (pH 7.4) and 0.2 ml of lysis buffer (10 mM Tris–HCl buffer (pH 7.4), 150 mM NaCl, 2 mM ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) was added, after which hepatocytes were harvested. After centrifugation at 16300×***g*** for 30 min at 4 °C, cell lysates were denatured in boiling water for 5 min. Samples of supernatant (30 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide resolving gel, in accordance with the method of Laemmli.[21] After electrophoresis, proteins were transferred to Immobilon-P membranes.

For detection of phosphorylated extracellular-regulated protein kinases (pERK1 and pERK2), membranes were immersed in Tris-buffered saline (pH 7.4) containing 1% bovine serum albumin. Membranes were then incubated with antibody (1 μg/ml) against phospho-ERK and/or ERK, and were washed as described previously.[20] Antibody binding was visualized by incubation with a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG) secondary antibody (1 : 3000 dilution)[22]; followed by enhanced chemiluminescence detection (ECL Kit, Amersham, Little Chalfont, U.K.). Densitometry analysis was performed using NIH Image version 1.68 for Macintosh. Data were calculated in arbitrary units and are expressed as means±S.E.M.

Cytosolic protein in hepatocytes was quantified using a modified Lowry procedure with bovine serum albumin as a standard.[23]

Determination of the Mammalian Target of Rapamycin (mTOR) Activity The mTOR protein was identified by immunoprecipitation and subsequent immunoblotting with the corresponding anti-phospho-mTOR antibody according to the protocol of the manufacturer's instructions.[24] In brief, hepatocytes were freshly isolated and seeded at a cell density of 3.3×10^4 cells/cm^2 and cultured in Williams' medium E containing 5% newborn bovine serum. Cultured hepatocytes were washed once with ice-cold phosphate-buffered saline (pH 7.4) and 0.2 ml lysis buffer (10 mM Tris buffer, pH 7.6, 1% Triton X-100, 50 mM NaCl, 5 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), 30 mM sodium pyrophosphate, 0.01 mM sodium orthovanadate, 50 mM NaF 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) was added. Cell lysates were obtained by scraping the cells in lysis buffer followed by sonication for 1 min. Cell lysates were then spun down (15000×***g*** for 10 min at 4 °C),

and the supernatants were denatured in boiling water for 5 min. For immunoblotting analysis using anti-phospho-mTOR (Ser2481) polyclonal antibody, samples of the supernatant (30 μg/lane) were resolved by SDS-PAGE (7.5% polyacrylamide gel), transferred to polyvinylidene difluoride (PVDF) membrane, and immunoblotted with anti-phosphotyrosine antibodies. Blots were developed using enhanced chemiluminescence following incubation with horseradish peroxidase-conjugated secondary antibodies.[22] The supernatant protein concentration was determined using Lowry's assay method.[23]

Quantification was performed by densitometry after development of the membrane with the enhanced chemiluminescence reagent and exposure to Hyperfilm (Kodak, Japan). Densitometric analysis was performed using the NIH image program version 1.6 for Macintosh. Data were calculated in arbitrary units and are expressed as means±S.E.M. The autodiagram is representative of three experiments using different cell preparations.

Measurement of Hepatocyte DNA Synthesis and Proliferation Hepatocyte DNA synthesis was assessed by measuring by [^{3}H]-thymidine incorporation into acid-precipitable materials.[25] After an initial attachment period of 3 h, the hepatocytes were washed twice with serum-free Williams' medium E and cultured in a medium containing PDGF (10 ng/ml) for a further 4 h. The cells were pulsed at 2 h and post-PDGF-stimulation for 2 h with [^{3}H]-thymidine (1.0 μCi/well). Incorporation of [^{3}H]-thymidine into DNA was determined as described previously.[11] Hepatocyte protein content was determined by a modified Lowry procedure,[23] using bovine serum albumin as the standard. Data are expressed as dpm/h/mg cellular protein.

The number of nuclei rather than the number of cells was counted using a modified version of the procedure described previously.[11,16]

Statistical Analysis Group comparisons were performed by analysis of variance (ANOVA) for unpaired data followed by *post-hoc* analysis using Dunnett's multiple comparison tests. Differences showing $p<0.05$ were considered to be statistically significant.

RESULTS

Time Course and Patterns of ERK Isoform Activity Stimulated by PDGF It has been reported that ERK plays an important role in the proliferation of hepatocytes and other cells induced by growth factors and cytokines.[26,27] The typical detectable pattern of phospho-ERK on Western blotting analysis is shown in Fig. 1A. The phosphorylated ERK2 band (pERK2) was induced after only 1 min and peaked (about 2-fold increase) between 3 and 5 min after addition of 10 ng/ml PDGF, a concentration that stimulated hepatocyte proliferation (Fig. 1B), and the band returned to basal levels within 60 min (Fig. 1B). ERK1 was not significantly affected by either medium alone (control) or 10 ng/ml PDGF treatment (Fig. 1C).

Effects of Specific Inhibitors of Signal Transducers on PDGF-Stimulated ERK Isoform Activity In order to characterize the involvement of ERK in the mitogenic pathway induced by PDGF, we investigated the effects of the PDGF receptor tyrosine kinase inhibitor AG1296 (10^{-7} M[28]), the phospholipase C (PLC) inhibitor U-73122 (10^{-6} M[29]), the PI3 kinase inhibitor LY294002 (10^{-7} M[30]), the MEK inhibitor PD98059 (10^{-6} M[27]), the mammalian target of rapamycin (mTOR) inhibitor rapamycin (10 ng/ml[31]) and the PKC inhibitor GF109203X (10^{-7} M[32]) on PDGF-induced ERK activity. As shown in Fig. 2, the phosphorylation of ERK2 induced by PDGF (10 ng/ml) at 3 min was almost completely blocked by AG1296, U-73122, LY294002 and PD98059. However, rapamycin and GF109203X did not affect the PDGF-induced ERK2 activity. In addition, phosphorylation of ERK1 in the presence of 10 ng/ml PDGF was not affected by these inhibitors.

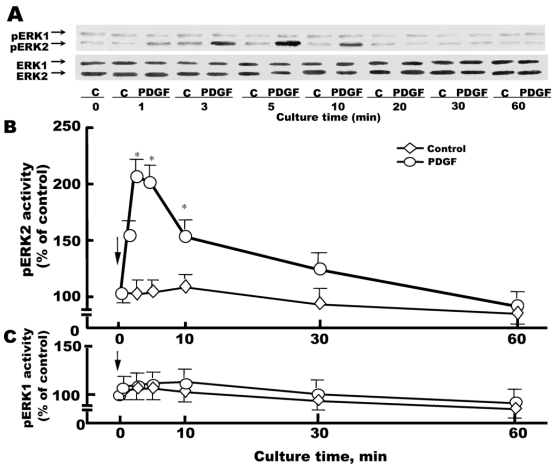

Fig. 1. Time Course and Patterns of ERK Isoform Activity Induced by PDGF

Isolated hepatocytes cultured for 3 h were washed and incubated in either the absence (control; C: medium alone) or presence of 10 ng/ml PDGF for the indicated times at 37 °C. Phosphorylated ERK isoforms (pERK1/pERK2) were identified using anti-phospho ERK antibody, as described in Materials and Methods. Intensity of the M_r 44-kDa and 42-kDa bands corresponding to phosphorylated ERK1 and ERK2 (pERK1 and pERK2) was normalized against total ERK (ERK1/ERK2). (A) Typical Western blotting band, phosphorylated ERK1/ERK2 (pERK1/pERK2), total ERK1/ERK2 (ERK1/ERK2); (B) time course of phosho-ERK2 activity (pERK2); (C) time course of phosho-ERK1 activity (pERK1). Results are expressed as a percentage of the respective control values (mean±S.E.M. of three experiments). $*p<0.05$ *vs.* respective controls.

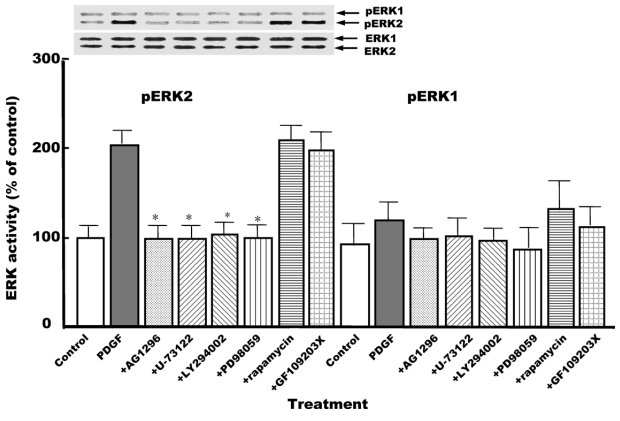

Fig. 2. Effects of Specific Inhibitors of Signal Transducers on ERK Isoform Activity Induction by PDGF

Hepatocytes were stimulated with PDGF (10 ng/ml) in the presence of specific inhibitors of signal transducers, such as AG1296 (10^{-7} M), U-73122 (10^{-6} M), LY294002 (10^{-7} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml) and GF109203X (10^{-7} M). Phosphorylated ERK isoforms (pERK1 and pERK2) were determined after incubation for 3 min with the test agents. Phosphorylated ERK isoforms were identified using anti-phospho-ERK1/2 (p42/p44 MAPK) antibody as described in Materials and Methods. Results are expressed as a percentage of the respective control values (mean±S.E.M. of three experiments). $*p<0.05$ *vs.* respective controls (PDGF alone).

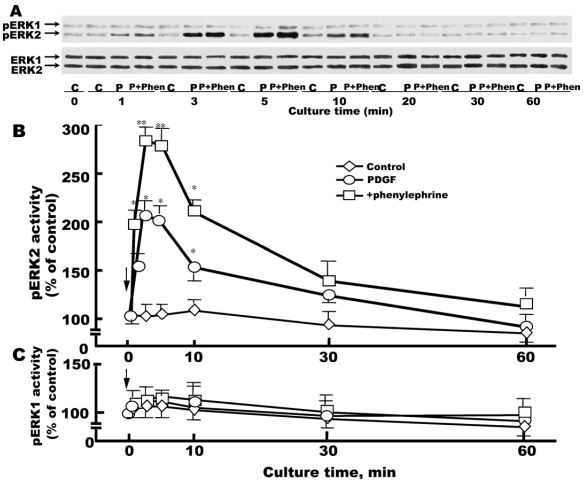

Fig. 3. Effects of Phenylephrine on Time Course and Patterns of ERK Isoform Activity Induced by PDGF

Hepatocytes were stimulated with PDGF (10 ng/ml) in the absence or presence of phenylephrine (10^{-6} M). Phosphorylated ERK ((A) typical Western blotting band; (B) pERK2; and (C) pERK1) were identified using anti-phospho-ERK1/2 (p42/44 MAPK) antibody and/or anti-ERK, as described in Materials and Methods. Results are expressed as a percentage of the respective control values (mean±S.E.M. of three experiments). $*p<0.05$; $**p<0.01$ *vs.* respective controls. C: control; P: PDGF; Phen: phenylephrine.

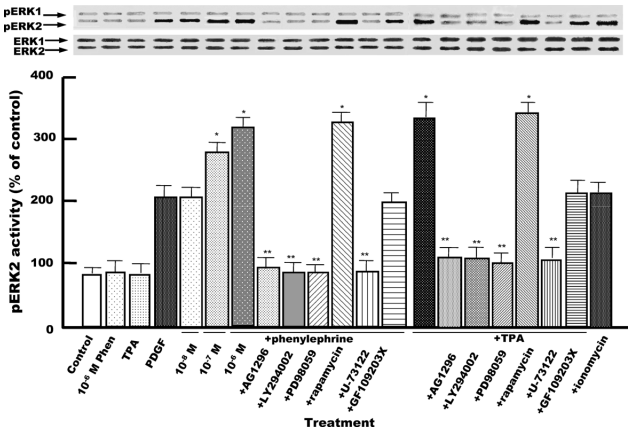

Fig. 4. Effects of Specific Inhibitors of Signal Transducers on Phenylephrine and/or TPA-Induced ERK Isoform Activity in the Presence of PDGF

Hepatocytes were stimulated with PDGF (10 ng/ml) plus phenylephrine (10^{-8}—10^{-6} M), and/or TPA (10^{-7} M) in the presence of specific inhibitors of signal transducers, such as AG1296 (10^{-7} M), LY294002 (10^{-7} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml), U-73122 (10^{-6} M), GF109203X (10^{-7} M) and ionomycin (10^{-7} M). Phosphorylated ERK isoforms (pERK1 and pERK2) were determined after incubation for 3 min with test agents. Phosphorylated ERK isoforms (pERK1 and pERK 2) were identified using anti-phospho-ERK1/2 (p42/44 MAPK) antibody, as described in Materials and Methods. Results are expressed as a percentage of the respective control values (mean±S.E.M. of three experiments). $*p<0.05$; $**p<0.01$ *vs.* respective controls (PDGF alone).

Activation of ERK2 Produced by PDGF Is Potentiated by α_1-Adrenergic Agonist Phenylephrine The potentiating effects of α_1-adrenergic agonists on the induction of ERK pathway by 10 ng/ml PDGF were investigated using the α_1-adrenergic agonist phenylephrine. PDGF in the presence of phenylephrine (10^{-6} M) caused a more rapid and significant increase in ERK2 activity than PDGF alone. ERK2 activity peaked at 3 min (about 3.0-fold increase from baseline) after addition and rapidly decreased to basal levels within 60 min (Figs. 3A, B). In contrast, phenylephrine alone did not significantly stimulate ERK2 activity. Phenylephrine (10^{-6} M) in the presence of 10 ng/ml PDGF did not significantly stimulate ERK1 phosphorylation (Fig. 3C). The present findings indicate that phenylephrine potentiates PDGF-induced ERK2 activity.

Effects of Specific Inhibitors of Signal Transducers on α_1-Adrenergic Agonist- and/or TPA-Induced ERK Isoform Activity in the Presence of PDGF In order to investigate the potentiating mechanism for the α_1-adrenergic receptor/protein kinase C (PKC) pathway in induction of ERK2 activity by PDGF, we examined the effects of phenylephrine (indirect PKC activator[33]), 12-*O*-tetradecanoylphorbol-13-acetate, (phorbol ester, TPA; direct PKC activator[18]) and ionomycin (ionophore of the intracellular Ca^{2+} [34]) on the phosphorylation of ERK isoforms induced by 10 ng/ml PDGF. Both phenylephrine (10^{-6} M)- and TPA (10^{-7} M)-induced potentiation of ERK2 phosphorylation in the presence of PDGF were almost completely blocked by AG1296, U-73122, LY294002 and PD98059 (Fig. 4). However, they were not blocked by rapamycin. In contrast, pretreatment of hepatocytes with the PKC inhibitor GF109203X (10^{-7} M) blocked the potentiative effects of phenylephrine and TPA on the phosphorylation of ERK2 induced by PDGF. Moreover, ionomycin (10^{-8}—10^{-6} M), a Ca^{2+}-transporting ionophore that raises intracellular levels of Ca^{2+} in the cytosol from across biological membranes, did not potentiate ERK2 phosphorylation by PDGF. Phenylephrine (10^{-6} M) or TPA (10^{-7} M) alone had no significant effect on the phosphorylation of ERK1 and ERK2 (Fig. 4).

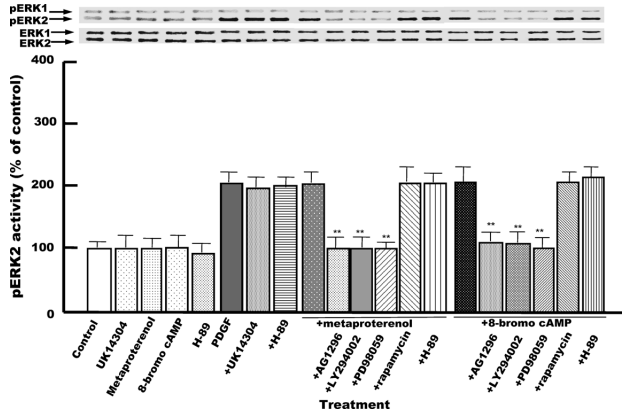

Fig. 5. Effects of Specific Inhibitors of Signal Transducers on Metaproterenol and/or 8-Bromo cAMP-Induced ERK Isoform Activity in the Presence of PDGF

Hepatocytes were stimulated with PDGF (10 ng/ml) plus UK14304 (10^{-6} M), metaproterenol (10^{-6} M) and/or 8-bromo cAMP (10^{-7} M) in the presence of specific signal transducer inhibitors such as AG1296 (10^{-7} M), LY294002 (10^{-7} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml) and H-89 (10^{-7} M). Phosphorylated ERK isoforms (pERK1 and pERK2) were determined after incubation for 3 min with the test agents. Phosphorylated ERK isoforms (pERK1 and pERK2) were identified using anti-phospho-ERK1/2 (p42/44 MAPK) antibody, as described in Materials and Methods. Results are expressed as a percentage of the respective control values (mean±S.E.M. of three experiments). $**p<0.01$ *vs.* respective controls (PDGF alone).

Effects of Specific Inhibitors of Signal Transducers on UK14304, Metaproterenol and/or 8-Bromo cAMP-Induced ERK Isoform Activity in the Presence of PDGF The effects of α_2- and β_2-adrenergic agonists on ERK activity induction by 10 ng/ml PDGF were investigated using the α_2-adrenergic agonist UK14304 or the β_2-adrenergic agonist metaproterenol. When hepatocytes were stimulated with PDGF in the presence of UK14304 (10^{-6} M; indirect AC inhibitor[35]), metaproterenol (10^{-6} M; indirect PKA stimula-

tor[36]) and/or 8-bromo cAMP (10^{-7} M; direct PKA stimulator[37]), significant potentiation of ERK2 activity was not observed when compared with PDGF (10 ng/ml) alone (Fig. 5). PDGF-induced ERK2 phosphorylation in the presence of metaproterenol and 8-bromo-cAMP was also blocked by AG1296, LY294002 and PD98059, but was not blocked by rapamycin. In addition, pretreatment of hepatocytes with the PKA inhibitor H-89 (10^{-7} M[38]) did not block the phosphorylation of ERK2 in the presence of PDGF. UK14304 (10^{-6} M), metaproterenol (10^{-6} M), 8-bromo cAMP (10^{-7} M) and H-89 (10^{-7} M) alone had no significant effect on the phosphorylation of ERK1 and ERK2 (Fig. 5).

Effect of Specific Inhibitors of Growth-Related Signal Transducers and on TPA-Induced Mammalian Target of Rapamycin (mTOR) Activity in the Presence of PDGF To obtain further support for the cross-talk between PDGF receptor mediated-ERK2-induced mTOR activation (mTOR phosphorylation), and α_1-adrenergic receptor activities, we examined the effects of specific inhibitors of growth-related signal transducers on PDGF-induced mTOR phosphorylation by α_1-receptor agonist and TPA. Figure 6 shows that PDGF (10 ng/ml) caused an increase in the mTOR phosphorylation of a 289-kDa protein that peaked 5—10 min after addition, at about 2.2-fold (compared with control). When PDGF (10 ng/ml) was added in combination with AG1296 (10^{-7} M), LY294002 (10^{-6} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml), U-73122 (10^{-7} M), and GF109203X (10^{-7} M), respectively, AG1296, LY294002, PD98059, rapamycin, and U-73122 completely abolished the PDGF-induced increase in mTOR phosphorylation (Ser 2481). In contrast, PDGF-induced mTOR activation was not abolished by GF109203X (10^{-7} M) treatment. In addition, both α_1-adrenegic receptor agonist phenylephrine (10^{-6} M) and direct PKC activator TPA (10^{-7} M) increased in PDGF-induced the mTOR phosphorylation at about 4.5-fold (compared with control). The TPA (10^{-7} M)-induced potentiation of mTOR phosphorylation in the presence of PDGF was almost completely blocked by AG1296, U-73122, LY294002, PD98059 and rapamycin (Fig. 6). However, pretreatment of hepatocytes with the PKC inhibitor GF109203X (10^{-7} M) blocked the potentiative effects of TPA on the phosphorylation of mTOR induced by PDGF. Phenylephrine (10^{-6} M) or TPA (10^{-7} M) alone had no significant effect on the phosphorylation of mTOR (Fig. 6).

Effects of Specific Inhibitors of Growth-Related Signal-Transducers on Hepatocyte DNA Synthesis and Proliferation Induced by PDGF In order to confirm the notion that 10 ng/ml PDGF induces hepatocyte DNA synthesis and proliferation through PDGF receptor, ERK or mTOR phosphorylation, we investigated whether or not AG1296, LY294002, PD98059, and rapamycin are able to inhibit PDGF-induced hepatocyte DNA synthesis and proliferation in the presence or absence of phenylephrine or TPA. As shown in Fig. 7, AG1296 (10^{-7} M), LY294002 (10^{-6} M), PD98059 (10^{-6} M), rapamycin (10 ng/ml) and U-73122 (10^{-7} M) almost completely blocked PDGF-induced hepatocyte DNA synthesis and proliferation with or without phenylephrine (10^{-6} M) or TPA (10^{-7} M). In addition, the potentiating effects of phenylephrine (10^{-6} M) or TPA (10^{-7} M) on PDGF-induced hepatocyte DNA synthesis and proliferation was blocked by GF109203X (10^{-7} M) treatment. Phenylephrine (10^{-6} M) or TPA (10^{-7} M) alone had not any effect on PDGF-induced DNA synthesis and proliferation (Fig. 7).

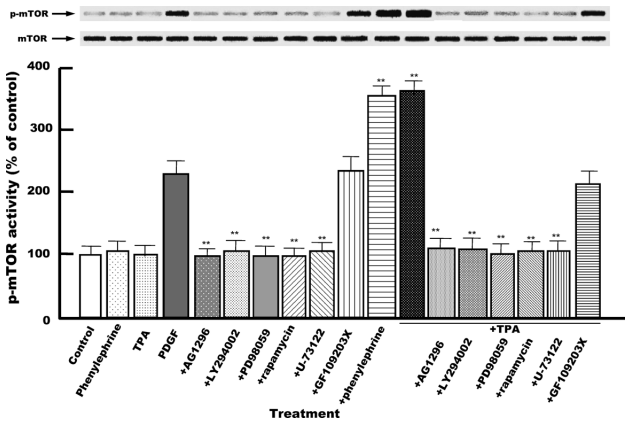

Fig. 6. Effect of Specific Inhibitors of Growth-Related Signal Transducers on PDGF-Induced Mammalian Target of Rapamycin (mTOR) Activity

Freshly isolated hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. After a medium change the hepatocytes were cultured with PDGF, with or without the indicated specific inhibitors of signal transducers, for 10 min. Phosphorylated mTOR (p-mTOR; P-p289 kDa) and mTOR protein (p289 kDa) were determined by Western blotting analysis as described in Materials and Methods. Typical Western bolt images are indicated on the top of the figure. The concentrations used were as follows: PDGF, 10 ng/ml; phenylephrine, 10^{-6} M; TPA, 10^{-7} M; AG1296, 10^{-7} M; LY294002, 10^{-7} M; PD98059, 10^{-6} M; rapamycin, 10 ng/ml; U-73122, 10^{-6} M and GF109203X, 10^{-7} M. The results are expressed as the mean±S.E.M. of three different experiments. $**p<0.01$ compared with respective PDGF-treated controls (PDGF alone).

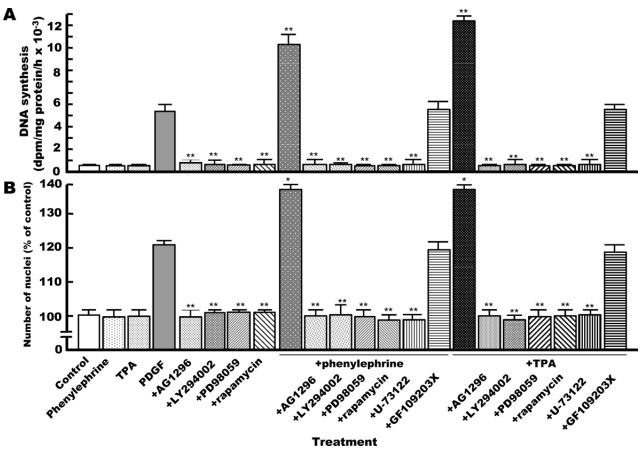

Fig. 7. Effects of Specific Inhibitors of Growth-Related Signal Transducers on Hepatocyte DNA Synthesis and Proliferation Induced by PDGF in the Presence or Absence of Phenylephrine or TPA

Hepatocytes were plated at a density of 3.3×10^4 cells/cm^2 and cultured as described in the legend to Fig. 1. Specific signal-transducer inhibitors were added without or with 10 ng/ml PDGF immediately after the medium change, and the cells were cultured for a further 4 h. The concentrations were as follows: phenylephrine, 10^{-6} M; TPA, 10^{-7} M; AG1296, 10^{-7} M; LY294002, 10^{-6} M; PD98059, 10^{-6} M; and rapamycin, 10 ng/ml; U-73122, 10^{-6} M; GF109203X, 10^{-7} M. The rate of hepatocyte DNA synthesis is expressed as dpm/mg protein/h (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei compared with the control culture (B). The results are expressed as means±S.E.M. of three independent experiments. $*p<0.05$, $**p<0.01$ compared with the respective control.

DISCUSSION

The proliferative pathway by which PDGF activates the receptor tyrosine kinase/ERK cascade (ERK pathway) has been described in many normal cells and transformed cells.[4,5,13—15] However, there have been few studies regarding the PDGF-induced activation of ERK isoforms and cross-

talk with adrenergic signal transduction pathways in primary cultures of adult rat hepatocytes. Therefore, we investigated the possible roles of ERK isoform activity and other signal transducers induced by mitogenic dose of PDGF in the absence or presence of adrenergic receptor agonists.

As shown in Figs. 1 and 2, we demonstrated that PDGF rapidly stimulates ERK2 activity, but not ERK1 activity. Cárcamo-Orive *et al.*, show that continued expression of cyclin-D1 in the G_0/G_1 phase is dependent on ERK2 expression by PDGF, and that the ablation of ERK1 influences neither the proliferation capacity of human mesenchymal stem cells, nor the expression pattern of cyclin-D1. In agreement with a recent report, our data are suggested that ERK2 play a key role in hepatocyte proliferation.[39,40] The ERK2 activity was blocked by the MEK inhibitor PD98059. Moreover, hepatocyte ERK2 activity induced by PDGF was almost completely blocked by specific inhibitors of growth-related signal transducers, such as AG1296, U-73122 and LY294002, but not rapamycin or GF109203X (Fig. 2). These results suggest that signal transducers such as receptor tyrosine kinase of PDGF, PLC, ERK2, PI3K and mTOR play an essential role in the mitogenic activity induced by PDGF under these experimental conditions (Fig. 6). The present results are consistent with reports that ERK2 acts upstream of mTOR/p70S6K and downstream of receptor tyrosine kinase, PLC and PI3K in primary cultures of adult rat hepatocytes and cancer cell lines.[11,14,41] In addition, GF109203X-sensitive PKC is not involved in the PDGF signaling pathway.[42]

There have been very few studies on the adrenergic agonist-induced regulation of ERK activity. Thus, we examined whether α_1-, α_2- and β_2-adrenergic agonists modulate the PDGF-induced changes in the activity of each ERK isoform. Phenylephrine is known to exert its action through the stimulation of phospholipase C (PLC-β) *via* α_1-adrenergic receptor/Gq-protein. PLC-β catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG). Generation of IP_3 leads to the mobilization of calcium ions from intracellular stores, whereas DG activates different isozymes of the PKC family.[43]

As shown in Fig. 3, PDGF-induced hepatocyte ERK2 activity was enhanced by phenylephrine (10^{-6} M), an α_1-adrenergic receptor agonist and an indirect PKC activator (Figs. 3, 4). In contrast, phenylephrine alone had no significant effect on hepatocyte ERK activity in the absence of PDGF (Fig. 4). The enhancing effects of phenylephrine in the presence of PDGF were inhibited by both the PLC inhibitor U-73122 and the PKC inhibitor GF109203X (10^{-7} M), thus suggesting the involvement of PLC and PKC in the potentiating effects of phenylephrine (Fig. 4). In support of this notion, phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 10^{-7} M), is a direct activator of PKC, and can either directly or indirectly stimulate PDGF-induced ERK2 phosphorylation.[13,44] The α_1-adrenergic receptor-mediated signals may interact with upstream signal transducers, such as the MEK, raf or ras pathways.[45,46]

However, as the specific PKC inhibitor GF109203X alone had no significant effect on PDGF-induced ERK2 phosphorylation (Fig. 2), GF109203X-sensitive PKC does not appear to be involved in the PDGF-induced ERK2 phosphorylation. Taken together, these results suggest that PLC and PKC are involved in the PDGF pathway, but these enzyme subtypes are apparently different from one another. Accordingly, it has been reported that some PLC-γ-mediated cellular responses have been shown to be independent of the PLC-β-mediated pathway.[43,47] PLC-γ, but not PLC-β may be activated by the phosphorylation of growth factor receptor tyrosine kinase *via* the Src homology (SH) domain.[48,49]

Accordingly, PDGF-induced hepatocyte DNA synthesis and proliferation were almost completely blocked by AG1296, LY294002, PD98059, U-73122 and rapamycin (Fig. 7). Moreover, the involvement of PKC in the effects of phenylephrine is supported by the present results, indicating that phenylephrine- or TPA-induced potentiation of hepatocyte DNA synthesis and proliferation in the presence of PDGF (10 ng/ml) was completely inhibited by the PKC inhibitor GF109203X (10^{-7} M) treatment (Fig. 7). These results support the notion that ERK2 play a key role in cross-talk between PDGF receptor/PLC-γ/ERK and α_1-adrenergic receptor/PLC-β/PKC pathway (Fig. 8). On the other hand, the PDGF-induced hepatocyte ERK2 activity was not enhanced by a maximal dose of U.K.14304 (10^{-6} M), α_2-adrenergic receptor agonist (indirect adenylate cyclase inhibitor) and/or metaproterenol (10^{-6} M), a β_2-adrenergic receptor agonist (indirect adenylate cyclase activator) or the cell-permeable cAMP analog, 8-bromo cAMP (10^{-7} M, Fig. 5). These results indicate that the α_2- or β_2-adrenergic receptor/cAMP pathway was not involved in cross-talk with the PDGF receptor/ERK pathway to potentiate hepatocyte growth.

A possible scheme for cross-talk between the PDGF receptor/PLC-γ/ERK pathway and the α_1-adrenergic receptor/PLC-β/PKC pathway is shown in Fig. 8. However, more detailed mechanisms for cross-talk between the PDGF-signaling pathway and the α_1-adrenergic receptor/PLC/PKC pathway remain to be explored.

In conclusion, the present study demonstrates that the cross-talk signals by extracellular α_1-adrenoceptor agonists, such as phenylephrine, potentiate the PDGF-induced ERK2 activity in primary cultured adult rat hepatocytes, and that

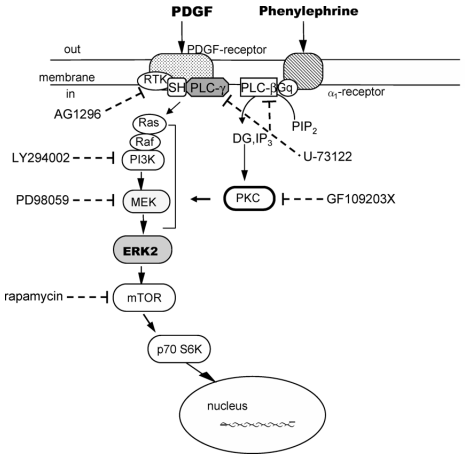

Fig. 8. Possible Model for Cross-Talk between PDGF Receptor/ERK Pathway and α_1-Adrenergic Receptor-Mediated Pathways

Abbreviations: PDGF, platelet-derived growth factor; TK, receptor tyrosine kinase; SH, Src homology domain; PLC, phospholipase C; PI3K, phosphatidylinositol 3-kinase; MEK, MAP kinase kinase; ERK, extracellular-signal regulated kinase; mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal protein S6 kinase; PKC, protein kinase C; PIP_2, phosphatidylinositol 3,4-bisphosphate; DG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate. →; stimulation, – – –|; inhibition.

the cross-talk element may be present downstream of PDGF receptor tyrosine kinase, and upstream of MEK (*i.e.*, ras, raf and fos), PI3K, mTOR and p70 S6K (Fig. 8). The present findings support the notion that endogenous catecholamine-induced potentiation of hepatocyte DNA synthesis and proliferation in the presence of PDGF is mediated by α_1-adrenergic receptors and plays an important role in the potentiation of the ERK cascade during liver regeneration after partial hepatectomy or liver necrosis caused by toxic chemicals *in vivo*.[6—8)]

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REFERENCES

1) Bornfeldt K. E., Raines E. W., Graves L. M., Skinner M. P., Krebs E. G., Ross R., *Ann. N.Y. Acad. Sci.*, **766**, 416—430 (1995).
2) Mycrs M. G., Cheatham B., Fisher T. L., Jachna B. R., Kalln C. R., Baker J. M., White M. F., *Ann. N.Y. Acad. Sci.*, **766**, 369—387 (1995).
3) Bornfeldt K. E., Raines E. W., Nakano T., Graves L. M., Krebs E. G., Ross R., *J. Clin. Invest.*, **93**, 266—1274 (1994).
4) Lechuga C. G., Hernández-Nazara Z. H., Hernández E., Bustamante M., Desierto G., Cotty A., Dharker N., Choe M., Rojkind M., *Am. J. Physiol. Gastrointest. Liver Physiol.*, **291**, G1051—G1061 (2006).
5) Patt L. M., Houck J. C., *Kidney Int.*, **23**, 603—610 (1983).
6) Michalopoulos G. K., *FASEB J.*, **4**, 176—187 (1990).
7) Michalopoulos G. K., *J. Cell. Physiol.*, **213**, 286—300 (2007).
8) Cantley L. C., Auger K. R., Carpenter C., Duckworth B., Graziani A., Kapeller R., Soltoff S., *Cell*, **64**, 281—302 (1991).
9) Ulhich A., Schlessinger J., *Cell*, **61**, 203—212 (1990).
10) Haluska P., Adjei A. A., *Curr. Opin. Investig. Drugs*, **2**, 280—286 (2001).
11) Kimura M., Ogihara M., *Jpn. J. Pharmacol.*, **76**, 165—174 (1998).
12) Si H. F., Lv X., Guo A., Jiang H., Li J., *Cytokine*, **42**, 24—31 (2008).
13) Yu J., Ustach C., Kim H. R., *J. Biochem. Mol. Biol.*, **36**, 49—59 (2003).
14) Moriuchi A., Hirono S., Ido A., Ochiai T., Nakama T., Uto H., Hori T., Hayashi K., Tsubouchi H., *Biochem. Biophys. Res. Commun.*, **280**, 368—373 (2001).
15) Seger R., Krebs E. G., *FASEB J.*, **9**, 726—735 (1995).
16) Nakamura T., Tomomura A., Kato S., Noda C., Ichihara A., *J. Biochem.*, **96**, 127—136 (1984).
17) Dajani O. F., Sandnes D., Melien O., Rezvani F., Nilssen L. S., Thoresen G. H., Christoffersen T., *J. Cell. Physiol.*, **180**, 203—214 (1999).
18) Refsnes M., Sandnes D., Melien O., Sand T. E., Jacobsen S., Christoffersen T., *FEBS Lett.*, **164**, 291—298 (1983).
19) Seglen P. O., *Methods Cell Biol.*, **13**, 29—83 (1975).
20) Towbin H., Staehelin T., Gordon J., *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350—4354 (1979).
21) Laemmli U. K., *Nature* (London), **227**, 680—685 (1970).
22) Kimura M., Osumi S., Ogihara M., *Endocrinology*, **142**, 4428—4440 (2001).
23) Lee M. B., Paxman S., *Anal. Biochem.*, **47**, 184—192 (1972).
24) Peterson R. T., Beal P. A., Comb M. J., Schreiber S. L., *J. Biol. Chem.*, **275**, 7416—7423 (2000).
25) Morley C. G. D., Kingdon H. S., *Anal. Biochem.*, **45**, 298—305 (1972).
26) Adachi T., Nakashima S., Saji S., Nakamura T., Nozawa Y., *Hepatology*, **21**, 1668—1674 (1995).
27) Alessi D. R., Cuenda A., Cohen P., Dudley D. T., Saltiel A. R., *J. Biol. Chem.*, **270**, 27489—27494 (1995).
28) Kovalenko M., Gazit A., Böhmer A., Rorsman C., Rönnstrand L., Heldin C. H., Waltenberger J., Böhmer F. D., Levitzki A., *Cancer Res.*, **54**, 6106—6114 (1994).
29) Thompson A. K., Mostafapour S. P., Denlinger L. C., Bleasdale J. E., Fisher S. K., *J. Biol. Chem.*, **266**, 23856—23862 (1991).
30) Vlahos C. J., Matter W. F., Hui K. Y., Brown R. F., *J. Biol. Chem.*, **269**, 5241—5248 (1994).
31) Chung J., Kuo C. J., Crabtree G. R., Blenis J., *Cell*, **69**, 1227—1236 (1992).
32) Toullec D., Pianetti P., Coste H., Bellevergue P., Grand-Perret T., Ajakane M., Baudet V., Boissin P., Boursier E., Loriolle F., Duhamel L., Charon D., Kirilovsky J., *J. Biol. Chem.*, **266**, 15771—15781 (1991).
33) Kajiyama Y., Ui M., *Cell. Signal.*, **10**, 241—251 (1998).
34) Liu C., Hermann T. E., *J. Biol. Chem.*, **253**, 5892—5894 (1978).
35) Cambridge D., *Eur. J. Pharmacol.*, **72**, 413—415 (1981).
36) Ogihara M., *Biol. Pharm. Bull.*, **19**, 752—757 (1996).
37) Derubertis F. R., Zenser T., *Biochim. Biophys. Acta*, **428**, 91—103 (1976).
38) Zuscik M. J., Puzas J. E., Rosier R. N., Gunter K. K., Gunter T. E., *Arch. Biochem. Biophys.*, **315**, 352—361 (1994).
39) Cárcamo-Orive I., Tejados N., Delgado J., Gaztelumendi A., Otaegui D., Lang V., Trigueros C., *Exp. Cell Res.*, **314**, 1777—1788 (2008).
40) Frémin C., Ezan F., Boisselier P., Bessard A., Pagès G., Pouysségur J., Baffet G., *Hepatology*, **45**, 1035—1045 (2007).
41) Ma L., Chen Z., Erdjument-Bromage H., Tempst P., Pandolfi P. P., *Cell*, **121**, 179—193 (2005).
42) Chen J., Lu G., Wang Q. J., *Mol. Pharmacol.*, **67**, 152—162 (2005).
43) Kim T. J., Han H. J., Hong S. S., Hwang J. H., Hwang B. Y., Yoo H. S., Jin Y. R., Lee J. J., Yu J. Y., Lee K. H., Kang B. W., Yun Y. P., *Biol. Pharm. Bull.*, **30**, 805—809 (2007).
44) Berridge M. J., *Biochim. Biophys. Acta*, **1793**, 933—940 (2009).
45) Rao Y. P., Studer E. J., Stravitz R. T., Gupta S., Qiao L., Dent P., Hylemon P. B., *Hepatology*, **35**, 307—314 (2002).
46) Carloni V., Defranco R. M., Caligiuri A., Gentilini A., Sciammetta S. C., Baldi E., Lottini B., Gentilini P., Pinzani M., *Hepatology*, **36**, 582—591 (2002).
47) Rhee S. G., Bae Y. S., *J. Biol. Chem.*, **272**, 15045—15048 (1997).
48) Falasca M., Logan S. K., Lehto V. P., Baccante G., Lemmon M. A., Schlessinger J., *EMBO J.*, **17**, 414—422 (1998).
49) Buckley C. T., Sekiya F., Kim Y. J., Rhee S. G., Caldwell K. K., *J. Biol. Chem.*, **279**, 41807—41814 (2004).