BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **248,** 174–179 (1998)
ARTICLE NO. RC988935

Polyenylphosphatidylcholine Inhibits PDGF-Induced Proliferation in Rat Hepatic Stellate Cells

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Received June 2, 1998

Polyenylphosphatidylcholine (PPC), a polyunsaturated phospholipid extract from soy beans, prevents the development of liver cirrhosis in animal models. Its mechanism of action is unknown. Based on the hypothesis that PPC might act by decreasing hepatic stellate cell proliferation, we studied the effect of PPC and its main components, dilinoleoylphosphatidylcholine (DLPC) and palmitoyl-linoleoylphosphatidylcholine (PLPC), on PDGF-induced stellate cell proliferation and intracellular signal transduction. Normal rat hepatic stellate cells in tissue culture were serum-starved, and incubated with 10ng/ml PDGF in the absence or presence of phospholipids. Cell proliferation was measured by ^{3}H-thymidine incorporation. P44^{MAPK} activation was determined by kinase assay, and AP-1 binding by electrophoretic mobility shift assay. PPC (200ng/ml) significantly inhibited PDGF-induced proliferation ($p < 0.05$; ANOVA, $n = 3$) and antagonized PDGF-induced P44^{MAPK} activation and AP-1 binding. This effect was mimicked by DLPC but not by PLPC. Neither DLPC nor PLPC prevented PDGF receptor activation. We conclude that PPC exerts a previously unrecognized effect on mitogen-induced stellate cell proliferation which may be mediated by DLPC. Inhibition of this cascade represents a potential mechanism for the inhibitory effect of PPC on hepatic fibrogenesis.

Liver fibrosis, characterized by the excessive deposition of extracellular matrix materials in the liver, is a disease with high morbidity and mortality and only limited treatment options. Hepatic stellate cells are the main source of the abnormal extracellular matrix material that represents the biochemical hallmark of the disease process (1-4). In response to hepatic injury, stellate cells undergo a phenotypic conversion that has been termed "activation" (5). Stellate cell activation includes the increased expression of matrix protein genes, changes in cell morphology, and increased proliferation. Data from several laboratories indicate that the proliferative response of stellate cells to PDGF is mediated through the mitogen-activated protein kinase (MAPK) cascade (6-9). This signalling pathway mediates the activation of the transcription factor AP-1 that regulates the transcription of the procollagen Iα1 gene (10,11).

In recent years, polyenoylphosphatidylcholine (PPC) has emerged as a promising agent for the treatment and prevention of liver cirrhosis. Interest in this polyunsaturated phospholipid stems from the observation that dietary supplementation with PPC prevents the development of alcoholic liver cirrhosis in baboons (12). These findings have been confirmed in rat models of cirrhosis induced by the administration of heterologus albumin and by carbon tetrachloride (13). Collectively, these data suggest that PPC interferes with a fundamental step in hepatic fibrogenesis. The mechanism(s) by which PPC exerts its antifibrotic effects are presently under investigation. Evidence from *in vivo* and *in vitro* studies suggests that PPC may increase collagen breakdown by stimulating collagenase activity in stellate cells (14). PPC has also shown biological activity in acute models of *in vitro* stellate activation by acetaldehyde (15). Furthermore, it has been argued that PPC may have antioxidant properties since alcohol or carbon tetrachloride-mediated lipid peroxidation was prevented in PPC-treated hepatocytes (16-18).

PPC is a mixture of seven phospholipid species. Current evidence suggests that dilineoyl-phosphatidyl choline (DLPC), which accounts for approximately 50% (w/w) of PPC, may be the biologically active species in liver disease. DLPC mimicked the effect of PPC on hepatic fibrogenesis in the baboon cirrhosis model (12) and on collagenase secretion by

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0006-291X/98 $25.00

activated stellate cells *in vitro* (14). In contrast, no such biological activity has been attributed to palmitoyl-lineleoylphosphatidyl-choline (PLCP), the second most abundant ingredient of PPC (12).

The present study was designed to determine whether PPC might affect the mitogen-mediated proliferative response of stellate cells. Experiments are presented to determine whether PPC affects the activation of the PDGF receptor or the proliferation-associated signal transduction cascade. We show that PPC and DLPC inhibit PDGF-mediated proliferation *in vitro* via a postreceptor mechanism, by inhibiting MAP kinase cascade and the activation of the transcription factor AP-1. Our data are the first to demonstrate inhibition of mitogen-induced signalling by PPC, and raise the possibility that the antiproliferative effect of PPC may contribute to the clinical efficacy of the drug.

MATERIALS AND METHODS

Animals. Female retired breeder Sprague-Dawley rats (Harlan, Indianapolis, IN) were given food and water ad libitum, and were quarantined for at least two days prior to stellate cell isolations. The animals were handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal experiments were approved by the Animal Care Committee at the Saint Louis University Health Sciences Center.

Reagents. Unless otherwise specified, reagents were obtained from Sigma Pharmaceuticals (St. Louis, MO). Dilineoylphosphatidylcholine (DLPC) and palmitoyl-lineoyl-phosphatidylcholine (PLPC) were obtained from Avanti (Alabaster, AL). Polyenylphosphatidylcholine (PPC) was obtained through Natterman and Company (Cologne, Germany). Antibodies for the MAP kinase assay (anti-ERK1 polyclonal antibody) and oligonucleotides for AP-1 analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Phospholipids. Phospholipid stock solutions were prepared in 100% ethanol under sterile conditions. For cell culture studies, aliquots of the stock solutions were transferred to teflon-coated glass vials using sterile glass pipettes, and dried under a stream of nitrogen. Appropriate amounts of culture media were added, and the phospholipid suspensions were sonicated for approximately 2 min to achieve a homogenous-appearing suspension. The final concentration of the phospholipids was 20ng/ml or 200 ng/ml.

Stellate cell cultures. Hepatic stellate cells were isolated from Sprague-Dawley male rats by previously described methods and subcultured on plastic tissue culture flasks (7). Experimental manipulations were performed with cells at passage 2-3 maintained on 24-well plates under subconfluent conditions. Cellular quiescence was induced by 24 hour culture in 0.4% fetal calf serum.

Cell proliferation assays. Stellate cells (in groups of 4 parallel wells) were cultured on 24-well plates under subconfluent conditions in 0.4% fetal calf serum. The media were then replaced with fresh medium containing PDGF (10ng/ml) with or without phospholipids. Eight hours later, the cultures were pulsed with ^{3}H-thymidine, and incubated for an additional 16 hours. These conditions were previously found to be optimal for determining the HSC response (9). The proliferation assays were repeated three times. Cellular protein concentrations were determined as described by Bradford (19).

MAP kinase assay. HSC maintained in media containing 0.4% fetal calf serum were treated with 10ng/ml PDGF in the absence or presence of phospholipids for 10 min. The cells were washed and lysates were prepared as described previously (9). $P44^{MAPK}$ was immunoprecipitated from aliquots of cell lysates. After three washes in lysis buffer and two washes in kinase buffer (10mM HEPES pH7.4, 5mM $MgCl_2$, 1mM $MnCl_2$, 10mM *p*-nitrophenyl phosphate), the immune complexes were incubated in kinase buffer (50μl) containing myelin basic protein (MBP, 10μg/reaction), 25μM ATP and 2μCi ^{32}P-ATP (3min, 30°C). The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis on a 14% gel. The gels were fixed, dehydrated in acetone, dried, and exposed for autoradiography.

Electrophoretic mobility shift assay for AP-1. HSC were maintained in media with 0.4% fetal calf serum for 48 hours, followed by a 10 min incubation with 10ng/ml PDGF in the absence or presence of phospholipids. The media overlying the cells were removed and replaced with ice-cold PBS. Cells were harvested by scraping, and concentrated by centrifugation at 1000×g for 2 min. Nuclear extracts were prepared using a previously published protocol (20). Binding reactions were established in 25μl binding buffer as previously described, using 3μg of extracted nuclear protein per reaction. In the binding reaction, nuclear extracts were incubated for 15min at 20°C with 0.1ng (10,000cpm) of ^{32}P-end labeled double-stranded AP-1 consensus oligonucleotide. Samples were electrophoresed through a 4% polyacrylamide gel at a constant voltage of 155V. The gels were dried and exposed for autoradiography.

Phosphorylation analysis of the PDGF receptor. Cells were quiesced for 48 hours in 0.4% fetal calf serum and then stimulated with PDGF (10ng/ml) with our without simultaneous addition with PPC, DLPC (200ng/ml), or PLPC (200ng/ml) for 10 minutes. At the end of the incubation, the cells were lysed in KLB lysis buffer (1% Triton X-100, 0.05% SDS, 10mM Na Phosphate pH 7.5, 0.3M NaCl, 5 μM EDTA, 0.21 μM Na-ortho-vanadate, 0.3μg/μl leupeptin, 5μg/μl aprotinin, 0.3μg/μl benzimidate). Normalized protein lysates were immunoprecipitated with a polyclonal antibody directed against the PDGF receptor. Immune complexes were precipitated with protein A-agarose, followed by the detection of phosphorylated PDGF receptor by phosphotyrosine Western blotting (21).

Statistical analysis. Experiments were performed in quadruplicate unless stated otherwise. Statistical comparisons were done by analysis of variance (ANOVA). Differences between means were determined by Dunnett multiple comparisons test. A p value of <0.05 was considered significant.

RESULTS

Stellate cell proliferation. PDGF is a powerful stellate cell mitogen *in vitro,* and plays an important role in stellate cell proliferation during hepatic fibrogenesis *in vivo.* Since proliferation represents the fundamental biological response to mitogens, we asked whether PPC might affect the proliferative response of the cells *in vitro.* Stellate cells were quiesced by 72 hours of serum starvation, and PDGF-mediated proliferation was determined by ^{3}H-thymidine incorporation. As shown in Table 1, PDGF at a concentration of 10ng/ml significantly stimulated stellate cell proliferation. PPC at a concentration of 20ng/ml had no effect on this response, but was significantly inhibitory at 200ng/ml ($p<0.01$). A similar effect was observed with DLPC, which significantly ($p<0.01$) inhibited PDGF-induced proliferation at a concentration of 200ng/ml. PLPC in similar concentrations had no significant effect on the PDGF response, consistent with previous observations that it is without biologic effect (12). These data suggest that

TABLE 1

Effect of Phospholipids on PDGF-Mediated Stellate Cell Proliferation

Condition	^{3}H thymidine incorporation (DPM/mg protein)
Negative control	105 ± 7
PDGF alone (10 ng/ml)	378 ± 28*
PDGF + PPC (20 ng/ml)	312 ± 32*
PDGF + PPC (200 ng/ml)	249 ± 21**
PDGF + DLPC (20 ng/ml)	334 ± 33*
PDGF + DLPC (200 ng/ml)	224 ± 26**
PDGF + PLPC (20 ng/ml)	439 ± 26*
PDGF + PLPC (200 ng/ml)	340 ± 26*

Note. Passaged stellate cells under subconfluent conditions were made quiescent with 0.4% FCS and incubated in the absence (negative control) or presence of PDGF (10 ng/ml) for 8 hours. ^{3}H-thymidine was added and incorporation measured after 16 hours as described in Materials and Methods. The data are presented as the mean ± S.D of 3 independent sets of experiments. (* = $p < 0.01$ vs. negative control.; ** = $p < 0.01$ vs. PDGF alone).

PPC and its putative active ingredient, DLPC, can inhibit activation by blocking the proliferative response of the cells.

Effect of polyunsaturated phospholipids on PDGF-induced MAP kinase activation. Activation of the MAP kinase signal transduction cascade occurs rapidly in response to PDGF stimulation. To determine whether the inhibition of PDGF-induced proliferation involves a downregulation of this pathway, we tested the effect of PPC on the MAP kinase response of PDGF-stimulated stellate cells. Similarly, we compared the effect of PPC to that of its two main constitutents, DLPC and PLPC. P44^{MAPK} activities were low in quiescent cells, and were stimulated by addition of PDGF. PPC at a concentration of 200ng/ml significantly inhibited the PDGF-induced response (Fig. 1A). DLPC, the component of PPC reported to possess antifibrogenic activity, similiarly reduced the MAP kinase response and mimicked the effect of PPC (Fig. 1B), whereas PLPC was without effect. These data demonstrate an inhibitory effect of PPC on MAP kinase signalling in stellate cells that may be mediated by DLPC.

Effect of polyunsaturated phospholipids on PDGF-induced AP-1 binding. Having established an inhibitory effect of PPC on the MAP kinase response to PDGF, we asked whether this effect was transmitted to the downstream elements of the MAP kinase pathway. We focused on the activator protein-1 (AP-1), which is activated in many cells following the PDGF-induced translocation of MAP/ERK into the nucleus (22,23). Electrophoretic mobility shift assays were performed to assess AP-1 nuclear activation in nuclear lysates from quiescent and PDGF-activated stellate cells. AP-1 binding activity was low in quiescent cells, and was stimulated upon addition of PDGF. This response was partially inhibited by PPC (Fig. 2A). In similar experiments using DLPC and PLPC (200ng/ml), DLPC reduced PDGF-induced AP-1 binding, mimicking the effect of PPC (Fig. 2A), whereas PLPC was without effect. These data suggest that PPC may affect the PDGF-induced AP-1 response through its putative active component, DLPC.

Effect of polyunsaturated phospholipids on PDGF receptor autophosphorylation. Our data suggest that PPC inhibits the PDGF signalling cascade upstream of the MAP kinase activation step. The initial step of this cascade involves the ligand-induced autophosphorylation of the PDGF receptor. In order to determine whether PPC interfered with the function of the PDGF receptor, we measured its autophosphorylation by immunoprecipitation with a PDGF receptor antibody, followed by phosphotyrosine Western blotting as described in the Method Section. Autophorphorylation was absent or minimal in quiescent cells. The addition of PDGF was followed by the appearance of a phosphorylated species of approximately 185 kB, the predicted molecular weight of the PDGF receptor dimer (Fig. 3). Receptor phosphorylation was unaffected by PPC, DLPC, or PLPC, suggesting a post-receptor site of action of PPC and DLPC. Furthermore, these data raise the possibility that the phospholipid effect may not be limited to PDGF.

DISCUSSION

PPC is a phospholipid compound that has shown promise in the prevention of hepatic fibrosis. It is currently undergoing clinical testing in patients with alcoholic liver disease. Its striking properties in animal models of liver disease have stimulated investigations into its mechanism of action and its cellular targets. PPC has been tested in a number of *in vitro* models, and appears to have pleiotropic effects on different hepatic cell types (for review, see 24). In this report, we present data to show that PPC inhibits proliferation and signal transduction ion PDGF-activated hepatic stellate cells. This pathway is biologically relevant since stellate cell proliferation and the expansion of the hepatic stellate cell pool are fundamental features of hepatic fibrogenesis (5). Furthermore, evidence from *in vivo* and *in vitro* studies suggests that PDGF, a potent stellate cell mitogen, is likely to be a mediator of the increased proliferation of the cells during the hepatic wound healing response (6,7).

Our data suggest that PPC acts at an intracellular site(s) by inhibiting the PDGF-dependent signalling cascade. The PDGF signalling pathway has previously been characterized in detail, and involves the activation of the mitogen-activated protein kinase/extracellular signal-related protein kinase (MAPK/ERK) (25,26).

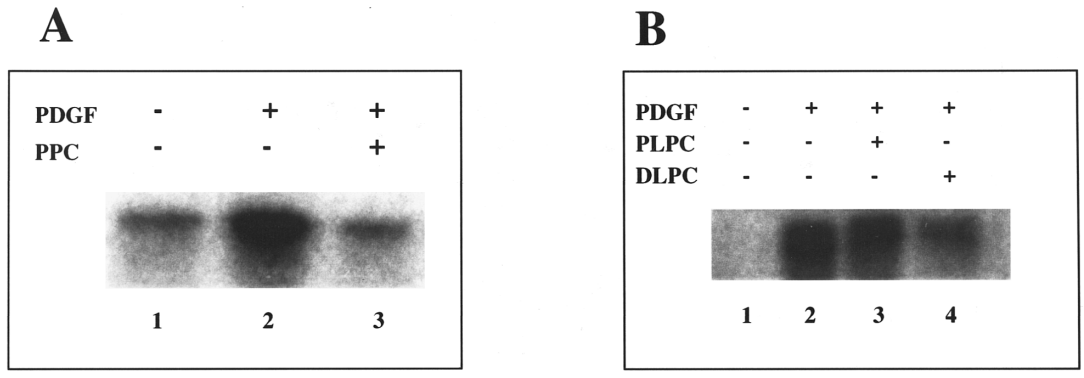

FIG. 1. Inhibition of PDGF-stimulated $P44^{MAPK}$ activity by PPC and DLPC in stellate cells. Stellate cells were stimulated for 10 minutes with 10ng/ml PDGF in the presence or absence of PPC (A) or its components, PLPC or DLPC (200ng/ml) (B). Cell lysates (600 μg/plate) were immunoprecipitated with an ERK1 polyclonal antibody, and an immune complex kinase assay was performed with MBP as the phosphoacceptor protein. The immunoprecipitated material was resolved by SDS/PAGE on a 14% gel, fixed, dried, and visualized by autoradiography. The band represents ^{32}P-labelled MBP. The figure is representative of four separate experiments. MAP kinase activity was low or non-measurable in the absence of PDGF (lane 1), and was stimulated by PDGF (lane 2). The effect of PDGF was inhibited by PPC (A). DLPC but not PLPC mimicked the effect of PPC (B), suggesting that DLPC is the active moiety of PPC in this assay.

Activated MAPK/ERK translocates into the nucleus to activate a number of transcription factors, including c-Fos and C-Jun, by phosphorylation of the activator protein-1 (AP-1) (22,23). Our data demonstrate that PPC diminishes the PDGF-induced activation of the MAPK/ERK isoenzyme, p44ERK1 (Figure 1). Consistent with this inhibition, the observed a decrease of AP1 activation in both PPC- and DLPC-treated cells (Figure 2). In contrast, PPC or DLPC had no effect on PDGF receptor autophosphorylation, one of the initial steps of the PDGF signalling cascade (Figure 3). Collectively, these data suggest that PPC acts at an intracellular site downstream of the PDGF receptor but upstream of MAPK/ERK.

The rapidity with which PPC inhibited the PDGF response indicates a direct effect on cell signalling, and argues against delayed effects on cellular protein synthesis or membrane turnover. The specific intracellular targets of PPC's action are unknown at present, but are likely to include previously identified members of the PDGF signalling cascade, such as Grb2, SOS, RAS, and c-Raf (25). Furthermore, our data would predict that PPC's action may extend to mitogens other than PDGF, since its effect occured downstream from its specific cellular receptor.

Our data support previous reports that 18:2-18:2 dilineoyl-phosphatidylcholine (DLPC), the main component of PPC, is responsible for the biological activity of the parent compound (12). DLPC, which accounts for approximately 50% of the chemical mass of PPC, mimicked the effect of PPC in our experimental assays, whereas PLPC, a structurally similar molecule in

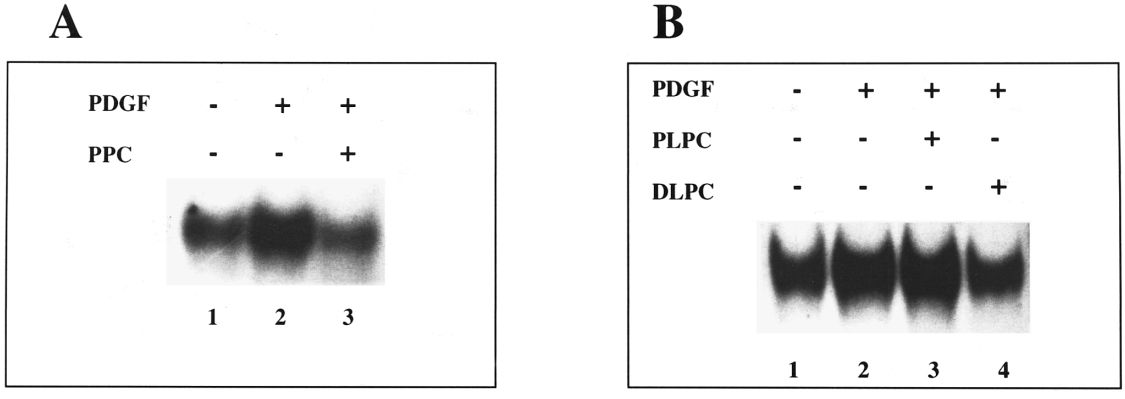

FIG. 2. Inhibition of PDGF-stimulated AP-1 binding by PPC and DLPC in stellate cells. Stellate cells were incubated for 10 minutes with PDGF (10ng/ml) in the presence or absence of PPC (A) or its main components, PLPC or DLPC (200ng/ml) (B). Nuclear extracts were prepared, and AP-1 binding was quantitated by electrophoretic mobility shift assay. The results shown are representative of four separate sets of experiments. (A) AP-1 binding is present in untreated, passaged cells (lane 1), and is increased following incubation with PDGF (lane 2). The PDGF-induced increase in binding activity is inhibited by PPC (A). DLPC but not PLPC mimicked the inhibitory effect of PPC, suggesting that the effect of PPC is mediated through DLPC (B).

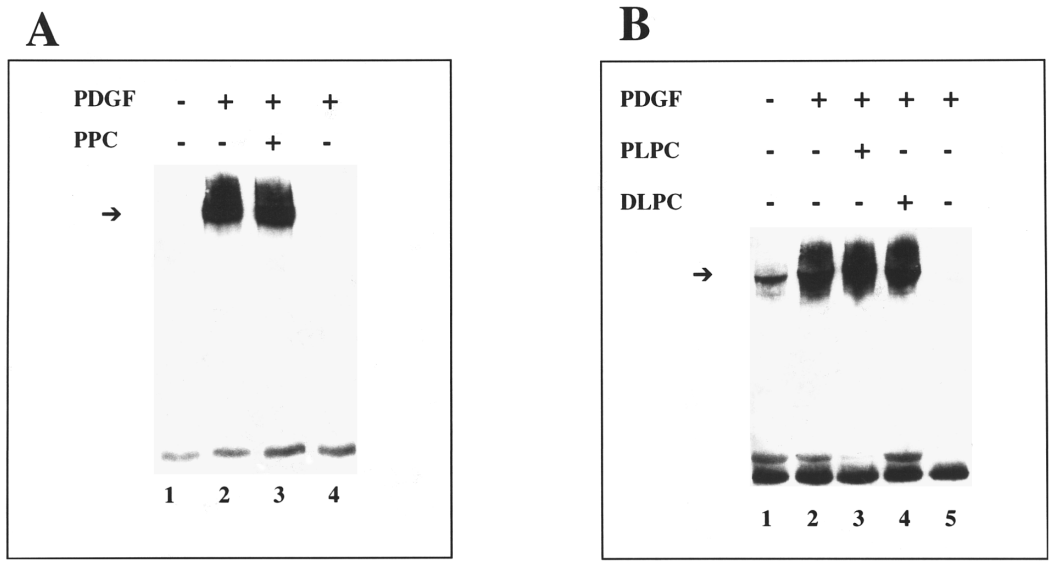

FIG. 3. PDGF receptor autophosphorylation is unaffected by polyunsaturated phospholipids. Passaged stellate cells were incubated for 10 min with PDGF (10ng/ml) in the presence or absence of PPC or its major components, PLPC or DLPC. PDGF receptor autophosphorylation was measured by receptor immunoprecipitation and phosphotyrosine Western blotting as described in Materials and Methods. The expected molecular mass of the phosphorylated PDGF receptor (185kD) is marked by the arrow. The autoradiograms shown are representative of three separate experiments. In the absence of PDGF, receptor phosphorylation was undetectable or minimal. Incubation with PDGF was followed by the appearance of a prominent signal of the antibipated molecular weight. This signal was not diminished by simultaneous incubation with PPC (A, lane 3), PLPC (B, lane 3), or DLPC (B, lane 4). The specificity of the immunoprecipitation was confirmed by performing mock reactions in the absence of the anti-PDGF receptor antibody (A, lane 4, and B, lane 5).

which one lineoyl side chain has been substituted with a palmitoic acid chain had no such activity. The presence of two lineoylic acid chains on the phosphatidyl choline backbone appears to be critical for its biological effect in the liver, since substitutions of one of the fatty acid chains resulted in a loss of biological activity in all assay systems tested to date (12). The biochemical basis for this exquisite specificity is unknown. We cannot exclude the possibility that additional components of PPC may contribute to its antiproliferative effect, but this appears unlikely.

In conclusion, our data suggest a novel antiproliferative mechanism of action for PPC in hepatic stellate cells. PPC exerts its antiproliferative effect through its principal component, DLPC, on the intracellular signalling cascade. The inhibition of stellate cell proliferation by PPC and its active metabolite represents an attractive avenue for the development of novel therapies in chronic liver disease.

ACKNOWLEDGMENTS

Supported by grants from the American Liver Foundation to L.M.B., from the National Institute of Diabetes, Digestive and Kidney Diseases to E.S.F. (DK-44305), and from the Fleur de Lis Foundation to E.S.F.

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