Peroxisome Proliferators and Signal Transduction[a]

MIGUEL BRONFMAN,[b] LILIANA NUÑEZ, ARIEL ORELLANA,[c] VERÓNICA BURZIO, AND PERLA HIDALGO

Departamento de Biología Celular y Molecular
Facultad de Ciencias Biológicas
P. Universidad Católica de Chile
Casilla 114-D
Santiago, Chile

Hypolipidemic peroxisome proliferators (HPPs), as plasticizers, agrochemicals, hypolipidemic drugs, and other structurally unrelated xenobiotics are known to induce a pleiotropy of similar short- and long-term effects in treated animals.[1–3]

Increased synthesis of specific mRNAs for peroxisomal and microsomal enzymes, which is followed by an increased number and size of hepatic peroxisomes and induction of specific cytochrome P450, increased amounts of liver fatty acid and fatty acyl-CoA binding proteins (FABP and ACBP), and acyl-CoA hydrolases are well known examples of short-term HPP effects.[1–3] On the other hand, long-term treatment of susceptible laboratory animals results in the development of hepatic tumor by non-genotoxic mechanisms.[3,4]

HPP-INDUCED CHANGES IN THE INTRACELLULAR LEVELS OF CoASH AND ACYL-CoA

HPP effects are not restricted to the induction of cell proteins and organelles. They also affect the intracellular content of several metabolites related to lipid metabolism. Among them, an increased content of CoASH and acyl-CoAs in the liver of HPP-treated animals[5–8] might be related to the pleiotropic effects induced by HPPs.

The scheme of FIGURE 1 illustrates the main metabolic pathways in which fatty acids and fatty acyl-CoA are involved. HPPs can influence the intracellular concentration of both fatty acids and fatty acyl-CoAs at several points. First, at the level of fatty acyl-CoA synthesis, the formation of thioesters of CoASH with carboxylate-containing HPPs will sequestrate CoASH and generate non-metabolizable acyl-CoAs,[9,10] which in turn can alter cell functioning. Furthermore, HPP-CoAs may inhibit both mitochondrial and peroxisomal β-oxidation,[10] increasing both fatty acids and fatty acyl-CoA levels.

[a]This work was supported by grants from the Fondo Nacional de Ciencias, Chile.
[b]Address for correspondence: Dr. Miguel Bronfman, P. Universidad Católica de Chile, Facultad de Ciencias Biológicas, Casilla 114-D, Santiago, Chile.
[c]Present address: Facultad de Ciencias, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile.

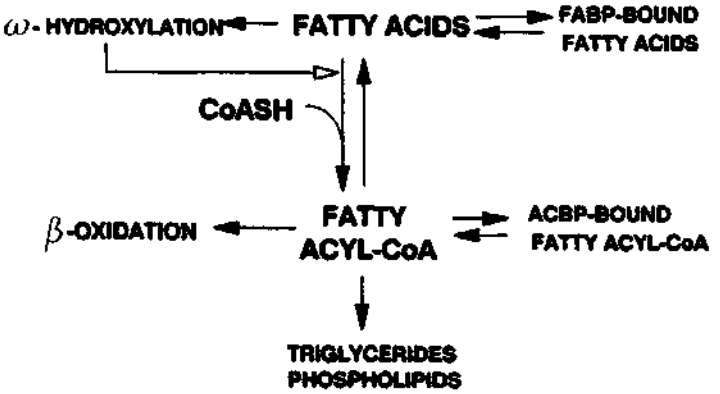

FIGURE 1. Main metabolic pathways involving free and bound fatty acids and fatty acyl-CoAs. FABP: fatty acid binding protein; ACBP: Acyl-CoA binding protein.

Therefore, an alteration of CoASH metabolism (CoASH sequestration) by the metabolic transformation of carboxylic acid group–containing HPPs in acyl-CoA esters in probably one of the first events after liver cell exposure to HPPs which precedes the synthesis of specific mRNAs for peroxisomal and microsomal enzymes. High concentration of HPP-CoAs are attained in isolated liver cells in less than 30 minutes exposure to HPPs.[9] On the other hand several noncarboxylic acid–containing HPPs are known inhibitors of mitochondrial β-oxidation, and presumably they will also increase fatty acid and long-chain acyl-CoA levels shortly after HPP exposure.[11]

Whether increased levels of acyl-CoAs and HPP-CoAs is causal in the HPP-induced pleoiotropic effects is not yet known. It is noteworthy that peroxisomal proliferation is also induced by physiological or dietary factors, such as high-fat diets[12,13] or diabetes,[14] which raise the level of intracellular fatty acids and fatty acyl-CoAs.

The association of fatty acids and fatty acyl-CoAs to fatty acid and fatty acyl-CoA binding proteins can also be affected by HPPs and HPP-CoAs. HPPs have been shown to bind to fatty acid binding protein (FABP).[11] This fatty acid transport protein has been involved in HPP-induced mitogenesis.[15] On the other hand, we have recently found that HPP-CoAs can also compete with natural fatty acyl-CoAs for intracellular fatty acyl-CoA binding sites.[16] Increased levels of fatty acids and fatty acyl-CoAs may result in both cases. Whether these increased levels are involved in HPP-induced short-term effects mediated by PPAR (peroxisome proliferator activated receptor) is not known. Acyl-CoA has been shown to modulate the nuclear thyroid hormone receptor,[17] which is a heterodimerization partner for PPAR.[18]

LONG-TERM HPPS-EFFECT ON PROTEIN KINASE C ACTIVATION

Acyl-CoAs and HPP-CoAs have been shown to potentiate the *in vitro* activity of protein kinase C[19–21] (Ca^{+2}-activated, phospholipid-dependent protein kinase C, termed *PKC*). PKC phosphorylates seryl and threonyl residues of endogenous pro-

teins, and it is thought to be a central factor in signal transduction, including cellular differentiation and carcinogenesis.[22] By affecting PKC activity, HPP-CoA and acyl-CoAs might affect the intracellular signaling network involving this kinase.

Incubation of ^{32}P-loaded isolated hepatocytes with the HPP ciprofibrate results in an increased phosphorylation of the epidermal growth factor receptor (EGF-R), as determined by immunoprecipitation.[23] This receptor protein, a known PKC substrate, itself displays a tyrosine kinase activity regulated by EGF[24] that is also involved in processes of cellular differentiation, proliferation, and tumorigenesis. Phosphorylation of purified EGF-R by PKC is stimulated by ciprofibroyl-CoA, but not by ciprofibrate, while receptor autophosphorylation induced by EGF is insensitive to both ciprofibrate and ciprofibroyl-CoA.[24] *In vitro* phosphorylation of the EGF receptor was also stimulated by palmitoyl-CoA. These observations suggest that an increased phosphorylation of the EGF receptor might result from PKC stimulation induced by metabolically produced ciprofibroyl-CoA. In this context, the carcinogenic properties of HPPs might be related to HPPs-CoA and/or acyl-CoA-mediated activation of PKC.[20,21]

MECHANISM OF PROTEIN KINASE C ACTIVATION BY LONG-CHAIN FATTY ACYL-CoAs AND HPP-CoA

PKC is reversibly activated by phospholipids, particularly phosphatidylserine, and Ca^{2+} Diacylglycerol, a second messenger product of polyphosphoinositol lipid breakdown by phospholipase C, increases the affinity of the enzyme for Ca^{2+} and renders it fully active at the normal intracellular Ca^{2+} concentration.[25]

Several molecular species of PKC have been defined.[26] These proteins are derived both from multiple genes and from alternative splicing of a single RNA transcript, and possess a primary structure containing a high degree of sequence homology. The enzyme subspecies show subtle differences in sensivity to $Ca,^{2+}$ in catalytic activity towards endogenous substrates, and in their activation by unsaturated fatty acids.

PKC has been established as the major intracellular receptor of phorbol esters,[27] potent tumor promoters that can substitute for the endogenous PKC modulator, diacylglycerol.[28] Sustained activation by phorbol esters has been proposed as one possible mechanism for their tumor-promoting action, since they are far more stable in the cell than diacylglycerol.[22] This sustained activation results in a depletion of PKC by down-regulation elicited by the action of calpains on the activated enzyme.[26]

PKC is present mainly in the cytosolic fraction, whereas its hydrophobic activators, phosphatidylserine and diacylglycerol, are present in the membrane.[29] Stimulation of cells with hormones that increase diacylglycerol concentration in membranes, or with phorbol esters, induces translocation from the cytosol to the particulate fraction.[30] In contrast with the metabolically stable phorbol esters, diacylglycerol is found only transiently in membranes.[31] Since sustained activation of PKC is a prerequisite for inducing long-term physiological response such as cell proliferation and differentiation,[31] other mechanisms might also be regulating the long-term effects of PKC.[32] In fact, generation of diacylglycerol through phospholipase C action on phosphatidylcholine or through the combined action of phospholipase D plus phosphatidate phosphohydrolase on phosphatidylcholine has been suggested as an alternative mechanism of diacylglycerol generation.[33] Lipid PKC mediators and activators pro-

duced through the action of phospholipase A2 (fatty acids and lysophospholipids) have also been proposed as being involved in sustained activation of PKC.[33]

Long-chain acyl-CoAs and HPP-CoAs might also be involved in PKC long-term effects. In contrast with the activating effect of phorbol esters, the activation of PKC by acyl-CoAs requires the simultaneous presence of phosphatidylserine, diacylglycerol, and low physiological Ca^{2+} concentration.[19,21] FIGURE 2 shows the activation of αPKC from rat brain, which presents the highest sensitivity of the cPKC subgroups (α,β and γ subspecies) to acyl-CoAs.

Two main isozymes of PKC, α and β, are present in rat liver.[34,35] As shown in FIGURE 3, both isozymes are sensitive to palmitoyl-CoA activation.

HPP-CoA have the same stimulating effect observed with long-chain acyl-CoAs on PKC activity from rat liver and brain, while the free drugs are comparatively inactive.[20,21] An HPP-CoA potentiating effect would also require the simultaneous presence of phosphatidylserine, diacylglycerol and Ca^{2+} HPP-CoA, at concentrations within the range observed in the liver of drug-treated rats or in isolated hepatocytes, induce a PKC activation similar to the one observed with palmitoyl-CoA.[20,21] Basal PKC activity (measured in the absence of phosphatidylserine and diacylglycerol) is also higher with HPP-CoAs than with palmitoyl-CoA.[10] The *in vitro* enhancement of PKC by acyl-CoAs and HPP-CoAs is due to a decrease in the phosphatidylserine re-

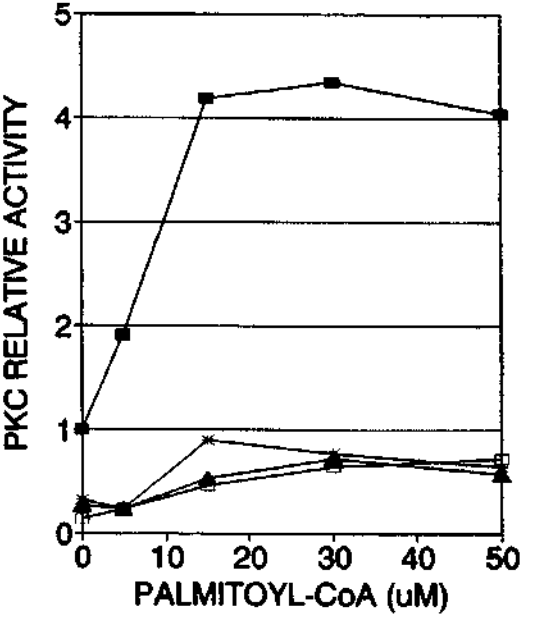

FIGURE 2. Effect on increasing concentration of palmitoyl-CoA on αPKC activity. Assays were performed as described by Bronfman *et al.*[19] in the presence of 1 μM Ca^{2+} and phosphatidylserine and diolein (■-■), of phosphatidylserine (✱-✱), of diolein (□-□), and 0.5 mM EGTA (▲-▲). αPKC was purified by hydroxylapatite chromatography according to Kosaka *et al.*[34] from a crude mixture of the isozymes prepared according to Bronfman *et al.*[19]

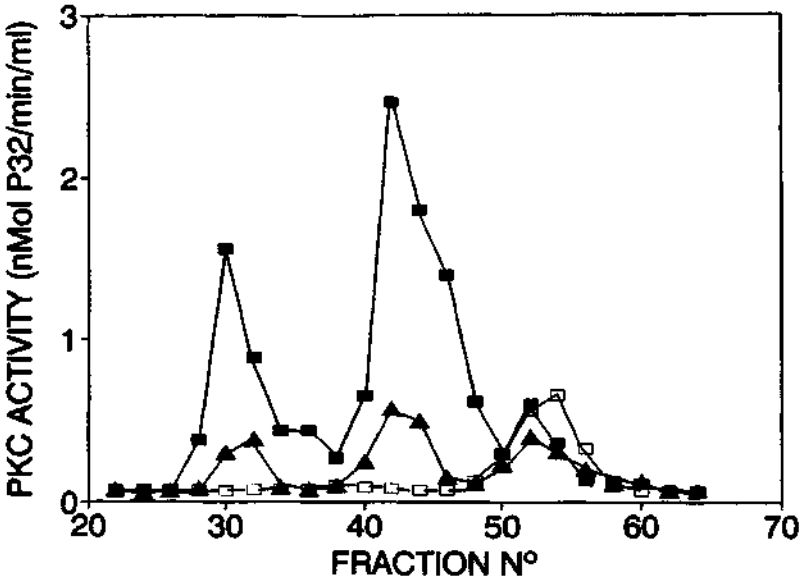

FIGURE 3. Separation of αPKC and βPKC from rat liver by hydroxylapatite chromatography. Separation was performed according to Kosaka *et al.*[34] Fractions were assayed for PKC activity[19] in the presence of 0.5 mM EGTA (□-□) or 1 μM Ca^{2+}, phosphatidylserine and diolein in the absence (▲-▲) and presence (■-■) of 20 μM palmitoyl-CoA.

quirement of the enzyme,[21] while no relative changes are observed in the apparent enzyme-dependence for ATP, diacylglycerol, or Ca^{2+} By using a purified rat brain enzyme and histone H1 as substrate, it was found that 20 μM palmitoyl-CoA or 25 mM ciprofibroyl-CoA decreased the kinase dependence for phosphatidylserine by 6- and 4-fold respectively. Similar results were observed using two different assay systems; sonicated dispersion of the lipids or mixed micelles with Triton X-100. Furthermore, the acyl-CoA induced decrease in phosphatidylserine requirement was also observed using the human platelet enzyme and a physiological substrate of platelet PKC.[21]

On the basis of these results, and since phosphatidylserine is in the membrane, it was proposed that acyl-CoAs might be facilitating the interaction of PKC with membranes under *in vivo* conditions. In agreement with this proposal, we have found that palmitoyl-CoA induces the binding of purified PKC to erythrocyte ghosts (FIGURE 4). Further support of this hypothesis comes from experiments with digitonin-permeabilized human platelets. Incubation of platelets with increasing concentration of digitonin results in almost 95 percent release of PKC and lactic dehydrogenase to the supernatant fluid. Low concentration of palmitoyl-CoA (10 μM) or of phorbol ester (100 nM) decreases the digitonin-induced release of PKC to 55 percent and 70 percent of the control (FIGURE 5), while no effect was observed on lactic dehydrogenase release. Simultaneously, an increase of PKC in the particulate fraction was observed by immunoblotting (not shown).

These results support the possibility that high levels of acyl-CoAs or HPP-CoAs might result in increased binding of PKC to membranes, leading to increased phosphorylation of PKC substrates after the phosphatidylinositol transduction pathway is activated and diacylglycerol is released. This mechanism of overstimulation of PKC would be different from the one induced by tumor promoters, such as phorbol esters, which activate PKC permanently, leading to down-regulation. Calpain I cleaves PKC in the presence of phosphatidylserine plus diacylglycerol or phorbol esters, implying

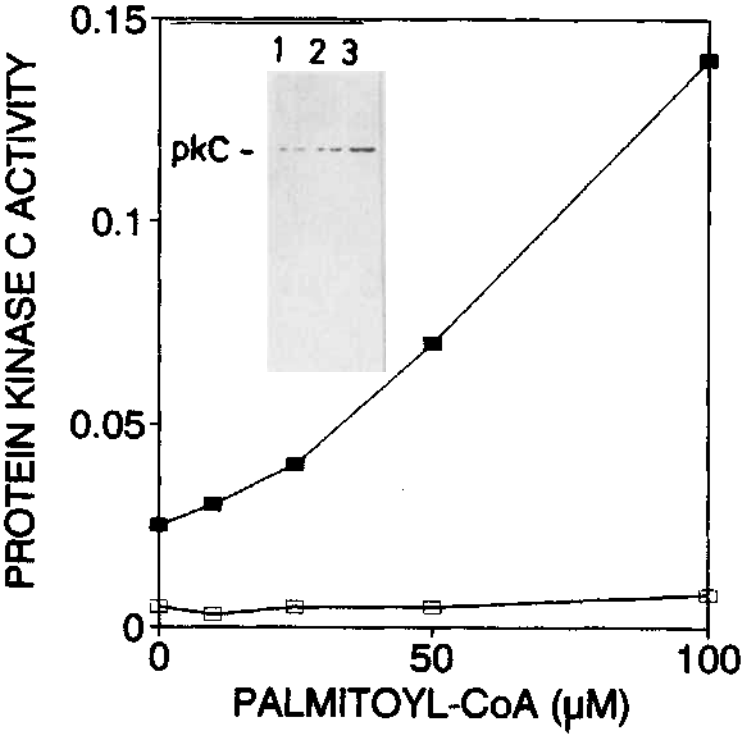

FIGURE 4. Effect of palmitoyl-CoA on PKC binding to erythrocyte membranes. A rat brain preparation[19] of the cPKC isozymes (α,β and γ) was used. PKC binding to inverted erythrocyte ghosts was performed according to Wolf *et al.*[38] in 150 μL of incubation medium containing 10 mM Tris-HCl pH 7.6, 1 mM 2-mercaptoethanol, fatty acid free bovine serum albumin (30 μg/mL), 3 mM $MgCl_2$, 100 μM EGTA, 150 μM $CaCl_2$, 1 μg/mL of diolein, 0.1 % Triton-X100, 10 μl of PKC (450 μg protein/mL), erythrocyte ghosts (3.6 μg of protein), and various palmitoyl-CoA concentrations. After 10 min incubation at 25°C, the suspension was centrifuged (40,000 g, 20 min) and PKC activity was determined[19] in the pellet. Activity from PKC incubated in the presence (■-■) and absence (□-□) of erythrocyte membranes is presented. *Inset:* immunoblotting of SDS-PAGE of pellets corresponding to PKC incubated with membranes in the absence of palmitoyl-CoA (1) and in the presence of 50 μM (2) and 100 μM (3) palmitoyl-CoA (the MC-5 monoclonal antibody against PKC from Amersham Corporation was used for immunoblotting).

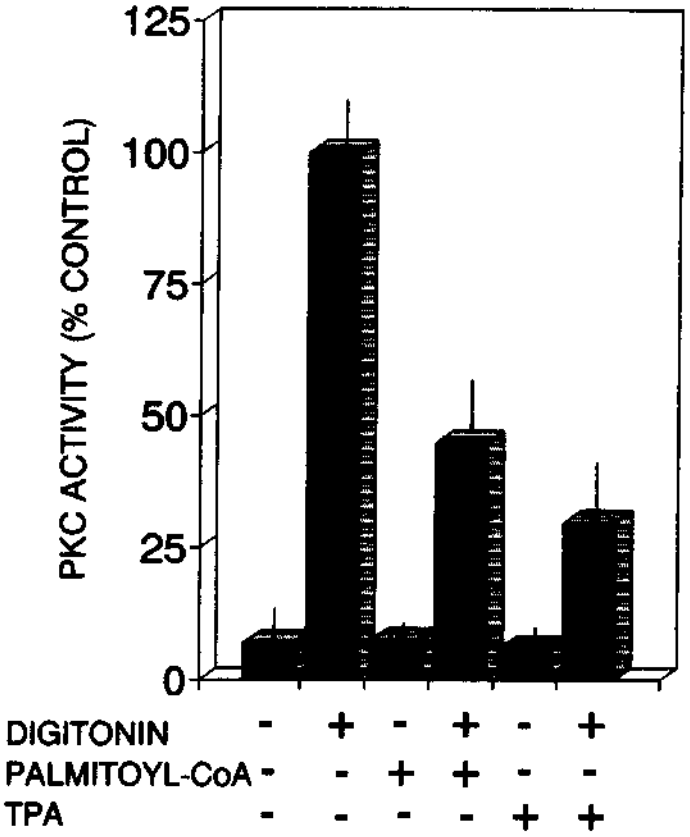

FIGURE 5. Effect of palmitoyl-CoA and phorbol 12-myristate 13-acetate (TPA) on digitonin-induced release of PKC from human platelets. Human platelets (5×10^7 cells in 50 μL) were incubated 1 min at 37°C in the presence and absence of 0.25 mg/mL of digitonin in a medium containing no supplementary additions, 10 μM palmitoyl-CoA, and 100 nM TPA. After incubation the cells were centrifuged for 2 min at 13,000 g, and PKC was determined in the supernatant and pellet. Using 0.25 mg/mL of digitonin, 100 % of PKC and lactic dehydrogenase are present in the supernatant. Activities of PKC in the supernatant relative to the control (0.25 mg/mL digitonin) are presented. Recoveries of PKC activity (pellet+supernatant) were in the range 80–110%. One representative experiment out of three is presented. Under the same experimental conditions, palmitoyl-CoA and TPA were without effect on lactic dehydrogenase release (not shown).

that the activated form of PKC is a target of the calpain action.[36] Thus, the extent of down-regulation induced by high levels of acyl-CoAs would be different from that induced by phorbol esters.

Whether acyl-CoAs induce increased PKC binding, PKC activity, or both, under *in vivo* conditions, remains to be established. Under *in vitro* conditions, diacylglycerol and phorbol esters, although potent activators of PKC, had little effect on the

binding of PKC to phospholipid vesicles or on the Ca^{2+} requirement for binding.[32] Under the *in vitro* assay conditions of PKC, increased binding of PKC to lipids would result in increased enzyme activity, which indeed is what it is observed.[21] On the other hand, it has been shown that the factor limiting PKC binding to phospholipid vesicles is the availability of phosphatidylserine and acidic phospholipids.[32] Since acyl-CoAs decrease the requirement for acidic phospholipids, less phosphatidylserine in the membrane should be required for PKC binding to particulate fractions in the presence of acyl-CoAs. Moreover, at least 20 percent of phosphatidylserine in the phospholipid vesicles is required for maximal binding of PKC.[32] With natural membranes, containing only a few percent of phosphatidylserine, this phospholipid content would be clearly insufficient to induce PKC binding by itself, and acyl-CoAs levels might be modulating the effect.

On the other side, if acyl-CoAs or HPP-CoAs are inducing an abnormally high proportion of bound PKC under *in vivo* conditions, it is possible that PKC becomes activated at diacylglycerol or phorbol ester concentrations which under normal conditions would not be high enough to fully activate the enzyme.

Recent studies on ciprofibrate-induced differentiation of HL-60 human promyelocytic leukemia cells support this hypothesis. Treatment of HL-60 with phorbol esters results in growth-arrest of the cells; they become adherent and differentiate into macrophage-like cells. Activation of βPKC is necessary and sufficient for phorbol ester-induced differentiation.[37] We have found that 10 nM PMA induces complete differentiation of HL-60 cells in 24 hours. Ciprofibrate up to 0.5 mM induces less than one percent of adherent cells, relative to culture containing 10 nM PMA (100 % differentation). However, in the presence of 0.1 nM PMA, which by itself does not induce differentiation, 0.5 mM ciprofibrate induced a reproducible and significant differentiation of HL-60 cells (30 % of adherent cells, relative to the culture in the presence of 10 nM PMA).

These results strengthen the view that the tumor-promoting action of HPPs might result from increased levels of particulate PKC and overstimulation of PKC substrate phosphorylation when diacylglycerol is released. This proposal suggests that the tumor-promoting activity of HPPs, the formation of HPPs CoAs, and the increased concentrations of acyl-CoAs induced by HPPs are correlated with the activity of an enzyme involved in cell proliferation and carcinogenesis.

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