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Effects of the Peroxisome Proliferator Ciprofibrate and Prostaglandin $F_{2\alpha}$ Combination Treatment on Second Messengers in Cultured Rat Hepatocytes

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Peroxisome proliferators induce hepatic peroxisome proliferation and hepatic tumors in rodents. These chemicals increase the expression of the peroxisomal β-oxidation pathway and the cytochrome P-450 4A family, which metabolizes lipids, including eicosanoids. Peroxisome proliferators transiently induce increased cell proliferation *in vivo*. However, peroxisome proliferators are weakly mitogenic and are not co-mitogenic with epidermal growth factor (EGF) in cultured hepatocytes. Earlier study found that the peroxisome proliferator ciprofibrate is comitogenic with eicosanoids. In order to study possible mechanisms of the comitogenicity of peroxisome proliferator ciprofibrate and eicosanoids, we hypothesized that the co-mitogenicity may result from synergistic or additive increases of second messengers in mitogenic signal pathways. We therefore examined the effect of the peroxisome proliferator ciprofibrate, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and the combination of ciprofibrate and $PGF_{2\alpha}$ with or without growth factors on the protein kinase C (PKC) activity, and inositol-1, 4, 5-triphosphate (IP_3) and intracellular calcium ($[Ca^{2+}]_i$) concentrations in cultured rat hepatocytes. The combination of ciprofibrate and $PGF_{2\alpha}$ significantly increased particulate PKC activity. The combination of ciprofibrate and $PGF_{2\alpha}$ also significantly increased EGF, transforming growth factor-α (TGF-α) and hepatic growth factor (HGF)-induced particulate PKC activity. The combination of ciprofibrate and $PGF_{2\alpha}$ greatly increased $[Ca^{2+}]_i$. However, the increases of PKC activity and $[Ca^{2+}]_i$ by ciprofibrate and $PGF_{2\alpha}$ alone were much smaller. Neither ciprofibrate or $PGF_{2\alpha}$ alone nor the combination of ciprofibrate and $PGF_{2\alpha}$ significantly increased the formation of IP_3. The combination of ciprofibrate and $PGF_{2\alpha}$, however, blocked the inhibitory effect of TGF-β on particulate PKC activity and formation of IP_3 induced by EGF. These results show that co-mitogenicity of the peroxisome proliferator ciprofibrate and eicosanoids may result from the increase in particulate PKC activity and intracellular calcium concentration but not from the formation of IP_3.

Key words : Peroxisome proliferator, Ciprofibrate, Prostaglandin $F_{2\alpha}$, Second messengers

INTRODUCTION

Peroxisome proliferators induce hepatic peroxisome proliferation and hepatocellular carcinomas when administered to rodents (Reddy & Lalwani, 1983; Rao *et al.*, 1988). The mechanism by which these agents cause hepatocellular carcinoma is not known, but is likely related to biochemical changes or other changes in gene expression induced by peroxisome proliferators, since most studies have not shown them to be directly genotoxic (Gupta *et al.*, 1985; Elliott & Elcombe, 1987). Peroxisome proliferators induce increased expression of several genes, including the peroxisomal β-oxidation pathway and the cytochrome P-450 4A family, which metabolize lipids, including eicosanoids and their precursor fatty acids (Capdevila *et al.*, 1992; Brass & Ruff, 1991). Peroxisome proliferator also induce increased cell proliferation *in vivo*. Cell proliferation by peroxisome proliferators is transiently increased early after their administration (Reddy and Lalwani,1983). At later times, cell proliferation has been found to decrease to basal levels in rats receiving some peroxisome proliferators, including ciprofibrate, whereas the peroxisome proliferator Wy-14,643 increases cell proliferation for an extended period (Marsman *et al.*, 1988; Eacho *et al.*, 1991; Yeldandi *et al.*, 1989; Chen *et al.*, 1994). However, peroxisome proliferators are weakly mitogenic and are not comitogenic with epidermal growth factor (EGF) in cultured hepatocytes (Marsman

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et al., 1993; Bennett and Williams,1993; Bieri *et al.*, 1984; Muakkassah-Kelly *et al.*, 1988).

It was found that the eicosanoids prostaglandin E_2 and $F_{2\alpha}$ (PGE_2, $PGF_{2\alpha}$), and leukotriene C_4 (LTC_4) are comitogenic with the peroxisome proliferator ciprofibrate in cultured hepatocytes (Hong and Glauert, 1996). The possible mechanisms of the comitogenicity are not clear, but may be related to the effects of these chemicals on mitogenic signal transduction pathways.

Protein kinase C (PKC), inositol triphosphate (IP_3) and intracellular calcium are major mediators of mitogenic signals, and are implicated in cell proliferation (Armato *et al.*, 1994; Pardee, 1989; Moolenaar *et al.*, 1984; Kaneko *et al.*, 1992; Poenie *et al.*, 1985). Prostaglandins, including PGE_2 and $PGF_{2\alpha}$, have been found to increase cellular concentrations of inositol triphosphate and free calcium in hepatocytes (Anderis *et al.*, 1981; Melien *et al.*, 1988; Athari and Jungermann, 1989). Ciprofibrate was also found to increase PKC activity and free cytosolic calcium in hepatocytes (Bennett and Williams, 1992; Bojes and Thurman, 1994). Synergistic effects of second messengers were proposed to increase cell proliferation by several tumor promoters (Nishizuka, 1988).

In this study, we examined whether the peroxisome proliferator ciprofibrate and $PGF_{2\alpha}$ synergistically increase the second messengers PKC, IP_3 and intracellular calcium to bring about the comitogenicity of the peroxisome proliferator ciprofibrate and eicosanoids in cultured rat hepatocytes. $PGF_{2\alpha}$ was chosen for study since it was found to increase DNA synthesis more than other eicosanoids (Hong and Glauert,1996).

MATERIALS AND METHODS

Animals and materials

Male Sprague-Dawley rats weighing 200~250 g were used as the source of hepatocytes. Ciprofibrate was a gift from Sanofi-Winthrop Inc. (Collegeville, PA, USA). Collagenase type I and soybean trypsin inhibitor were obtained from Worthington Biochemical Co. (Freehold, NY, USA). Leibovitz's L-15 medium, EGF, TGF-β, PKC assay kit and newborn calf serum were obtained from Gibco (Grand Island, NY, USA). TGF-α and HGF were purchased from Collaborative Biomedical Products (Bedford, MA, USA). $PGF_{2\alpha}$ were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Type I collagen was obtained from Upstate Biotechnology Incorporated (Lake Placid, NY, USA). [^{3}H] IP_3 and [γ-^{32}P] ATP were purchased from Dupont (Boston, MA, USA). Fura-2 /AM calcium probe was purchased from Molecular Probes Inc. (Eugene,. OR, USA). Proline, glutamic acid, insulin, transferrin, selenium, glucagon, and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation of hepatocytes and culture in collagen gels

Hepatocytes were isolated from rat liver by a two-step collagenase perfusion method (Seglen, 1976; Hong *et al.*, 1995). Collagen gels were prepared as described by Michalopoulos and Pitot (1975). The isolated hepatocytes were plated at a cell density of 6.5×10^4 viable cells/ cm^2 in 3 ml of L-15 medium (Mitaka et al., 1991) supplemented with 5% newborn calf serum, insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), albumin (2 mg/ml), glucagon (3.55 ng/ml), dexamethasone (40 ng/ml), and gentamycin (10 μg/ml) on 60 mm Falcon tissue culture dishes containing collagen gels. A medium change was routinely performed 2~3 h after plating (designated time 0) to remove unattached cells. Ciprofibrate and $PGF_{2\alpha}$ were dissolved in ethanol, and were added at time 0. The same concentration of ethanol, which did not exceed 0.1% in the medium, was used in the control group.

Assay of particulate PKC activity

6.5×10^4 viable cells/ cm^2 were cultured for 24 h in 3 ml of L-15 serum free medium and then were exposed by ciprofibrate, $PGF_{2\alpha}$ or the combination of ciprofibrate and $PGF_{2\alpha}$ with or without growth factors for 30 minutes. This time was used because it produced the highest levels of DNA binding activity of AP-1 (unpublished data). PKC activity was determined by phosphorylation of the substrate using acetylated myelin basic protein (MBP) as described by other (Brockenbrough *et al.*, 1991) and product company description (Gibco BRL, Gaithersburg, MD). In brief, the harvested hepatocytes were first washed with PBS. The cell pellets were resuspended in extraction buffer [20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 1 mM bezamidine hydrochloride, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% soybean trypsin inhibitor, 0.01% (w/v) leupeptin, and 50 mM 2-mercaptoethanol] sonicated for 10 seconds, and then allowed to stand on ice for 30 minutes. The cell suspension were then centrifuged at $100,000\times g$ for 60 min. The pellets were dissolved in 20 mM Tris-HCl buffer (pH 7.5, 0.2 mM EDTA, 1 mM benzamidine hydrochloride, 1 mM PMSF, 0.1% soybean trypsin inhibitor, 0.01% (w/v) leupeptin, 50 mM 2-mercaptoethanol, 10 mM EGTA and 1% (v/v) nonidet P-40) and set on ice for 60 min with vortexing. The suspension was centrifuged again at $100,000\times g$ for 60 min and the supernatant was used to measure particulate PKC activity after purification. To purify the protein, the soluble supernatants were applied into a diethylaminoethyl-cellulose column equilibrated with washing buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol). The soluble fraction was eluted with 0.2 M NaCl elution buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol). For the PKC acti-

vity assay, the reaction mixtures containing 25 μl of enzyme fraction, 5 μl lipid preparation (10X=100 mM phorbol 12-myristate 13-acetate, 2.8 mg/ml phosphatidyl serine, Triton X-100) and 10 μl water were incubated for 20 minutes at room temperature followed by another 5 min incubation with 10 μl of a specific substrate acetylated myelin basic protein solution [250 μM acetyl-MBP, 100 mM ATP, 5 mM $CaCl_2$, 100 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5 containing [γ-^{32}P] ATP (20~25 μCi/ml)] at 30°C. In the parallel microcentrifuge tube, 25 μl of enzyme fraction, 5 μl water, 10 μl of a specific substrate and 10 μl inhibitor (100 mM PKC, 20 mM Tris-HCl, pH 7.5 as negative control) were incubated. A 25 μl reaction mixture was blotted on the phosphocellulose disc paper. The disc paper was washed with 0.75% phosphoric acid for 5 min and then washed with double distilled water for 5 min. The papers were transferred to scintillation vials and then the radioactivity was counted. PKC activity was determined as the difference between the radioactivity in the lipid-stimulated versus the inhibitor-containing reactions.

Measurement of inositol-1,4,5-triphosphate formation

IP_3 formation was determined as described by Bellomo *et al.* (1987). In brief, isolated hepatocytes were precultured for 24 h on collagen gel plates at a density of 6.5×10^4 cells/cm^2 in serum free L-15 medium. After a 30 minute incubation with the medium containing 1 μM of myo-2-[^{3}H]-inositol (15 μCi/ml), the cells were subsequently washed with PBS three times to remove the un-incorporated [^{3}H]-inositol. Cells were then incubated for 15 minutes in L-15 medium supplemented with 10 mM LiCl before treatment with ciprofibrate or $PGF_{2\alpha}$, or the ciprofibrate-$PGF_{2\alpha}$ combination with or without the growth factors. After a 30 min incubation, cells were harvested and washed with PBS. The cell suspension was treated by ice-cold 10% trichloroacetic acid (TCA) (1 ml of cell suspension; 1 ml of 10% TCA) and allowed to stand on ice for 30 minutes. After removal of the acid-precipitated protein by centrifugation for 10 min at 3000 RPM using a Mistral 3000i centrifuge equipped with a 257-341 4-place windshielded swing-out rotor (Sanyo Fisher Service Co., Leicester, UK), the supernatant was extracted with 5 ml diethyl ether two times and then neutralized to pH 7.2 with 0.1 ml of 10 mM sodium tetraborate. Separation of inositol phosphate was done by anion-exchange chromatography on Dowex-1-X-8 columns (Bio-Rad). A mixture of the same volume of 0.1 M ammonium formate and 0.1 M formic acid was used to elute IP_3. The radioactivity of [^{3}H]-IP_3 was determined by a scintillation counter.

Measurement of intracellular calcium level

Hepatocytes (2×10^5 cells/cm^2) were plated onto poly-L-lysine coated round glass coverslips and preincubated for 2 h in L-15 medium with 5% newborn calf serum. After removal of unattached cells, cells were incubated for 1 h in Hanks solution (pH 7.4; NaCl, 137 mM; KCl, 5.4; $NaHCO_3$, 4.2 mM; KH_2PO_4, 0.44 mM Na_2HPO_4, 0.33 mM; glucose, 10 mM; $CaCl_2$, 1 mM) followed by another 45 min incubation with Fura-2/AM (5 μM) to load the cells as described by Baffy *et al.* (Baffy *et al.*, 1992). Fura-2/AM loaded cells were then washed with Hanks solution and were treated with ciprofibrate or $PGF_{2\alpha}$ or the combination of ciprofibrate and $PGF_{2\alpha}$. The intracellular calcium concentration in a single cell was recorded. Fluorescence excitation wavelengths were 345 and 380 nm, and the emission wavelength 510 nm was recorded using a dual wavelength spectrofluorometer (Deltascan, Photon Technology, Inc.) as described elsewhere (McCoy *et al.*, 1993). Calculations of intracellular calcium concentration were done according to the method of Grynkiewicz *et al.* (1985).

Statistics

Data were analyzed using one-way analysis of variance followed by Tukey's test (Gill,1978). Differences were considered significant at $p<0.05$.

RESULTS

We first determined the time course effect of EGF on the formation of IP_3. The formation of IP_3 was increased by the time, and peaked at 30 min after treatment, then was gradually decreased (Fig. 1). We next determined the formation of IP_3 by the peroxisome proliferator ciprofibrate or $PGF_{2\alpha}$ alone, or the combination of ciprofibrate and $PGF_{2\alpha}$ in the absence or pre-

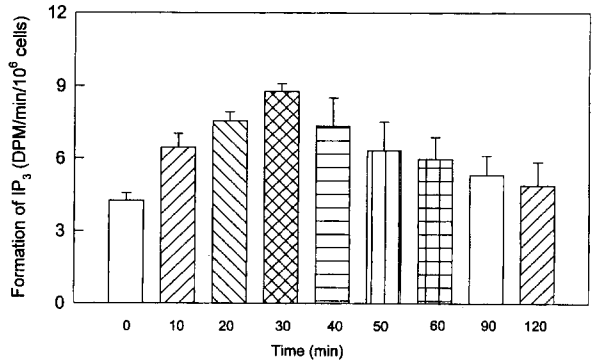

Fig. 1. Time course effect of EGF on the the formation of IP_3. Hepatocytes were cultured in serum free medium for 24 h, and then were exposed to EGF (40 ng/ml), for 10, 20, 30 , 40, 50, 60, 90, 120min. The formation of IP_3 was determined by the method described in materials and methods. Data represent means±SE of five experiments, with duplicates of each experiment.

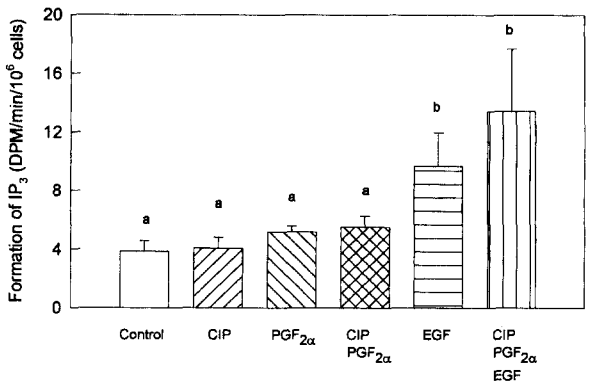

Fig. 2. Effect of ciprofibrate (CIP), $PGF_{2\alpha}$, the combination of CIP and $PGF_{2\alpha}$ or EGF on the formation of IP_3. Hepatocytes were cultured in serum free medium for 24 h, and then were exposed to EGF (40 ng/ml), CIP (400 μM) or $PGF_{2\alpha}$ (3 μM) alone or the combination of CIP with $PGF_{2\alpha}$ in the absence or presence of EGF for 30 min. The formation of IP_3 was determined by the method described in materials and methods. Data represent means±SE of five experiments, with duplicates of each experiment. Values with different superscripts are significantly different.

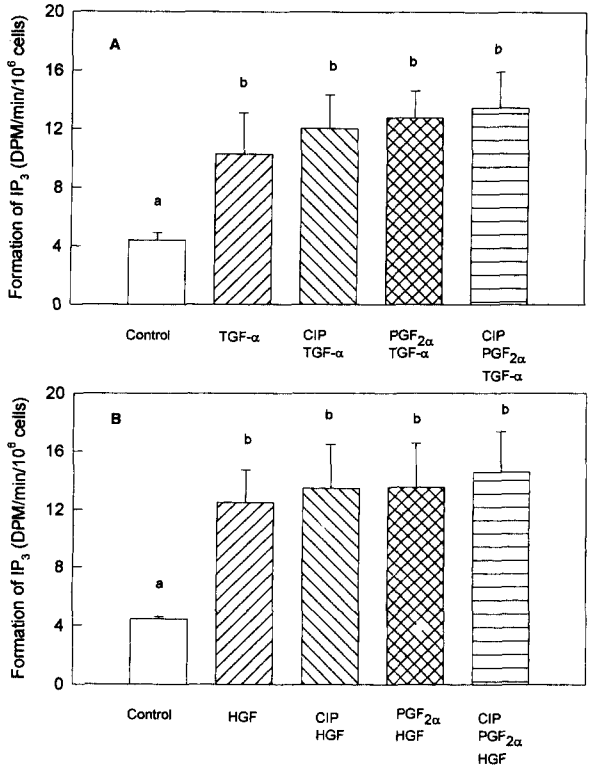

Fig. 3. Effect of ciprofibrate (CIP), $PGF_{2\alpha}$, or the combination of CIP and $PGF_{2\alpha}$ on the formation of IP_3 in the presence of TGF-α (A) or HGF (B). Hepatocytes were cultured in serum free medium for 24 h, and were exposed to CIP (400 μM) or $PGF_{2\alpha}$ (3 μM) alone or the combination of CIP with $PGF_{2\alpha}$ in the absence or presence of TGF-α (50 ng/ml) or HGF (50 ng/ml) for 30 min. The formation of IP_3 was determined by the method described in materials and methods. Data represent means±SE of five experiments, with duplicates of each experiment. Values with different superscripts are significantly different.

sence of growth factors. Ciprofibrate or $PGF_{2\alpha}$ alone did not increase the formation of IP_3 (Fig. 2). The addition of $PGF_{2\alpha}$ along with ciprofibrate also only slightly increased IP_3 formation. The combination of ciprofibrate and $PGF_{2\alpha}$ did not significantly increase EGF-induced IP_3 formation (Fig. 2). The combination of ciprofibrate and $PGF^{2\alpha}$ also did not significantly increase TGF-α and HGF- induced IP_3 formation (Fig. 3A and B). We next examined the effect of the combination of ciprofibrate and $PGF_{2\alpha}$ in the presence of TGF-β (Fig. 4). TGF-β alone did not increase the formation of IP_3, but inhibited EGF-induced IP_3 formation. The combination of ciprofibrate and $PGF_{2\alpha}$ significantly blocked the inhibitory effect of TGF-β on EGF-induced IP_3 formation.

We next examined particulate PKC activity in cultured rat hepatocytes. The combination of ciprofibrate and $PGF_{2\alpha}$ significantly increased PKC activity (Fig. 5). Ciprofibrate and $PGF_{2\alpha}$ alone did not significantly increase PKC activity, but the increase of PKC by ciprofibrate was about 1.6 fold and that by $PGF_{2\alpha}$ about 1.8 fold over control. The combination of ciprofibrate and $PGF_{2\alpha}$ also significantly increased TGF-α and HGF-induced PKC activity. $PGF_{2\alpha}$ alone also significantly enhanced HGF-induced PKC activity (Fig. 6A and B). TGF-β significantly inhibited EGF-induced PKC activity. $PGF_{2\alpha}$ alone and the combination of ciprofibrate and PGF2α significantly blocked the inhibitory effect of TGF-β on PKC activity (Fig. 7).

We next examined the intracellular calcium concentration ($[Ca^{2+}]_i$). Ciprofibrate or $PGF_{2\alpha}$ alone slightly increased $[Ca^{2+}]_i$, whereas the combination of cipro-

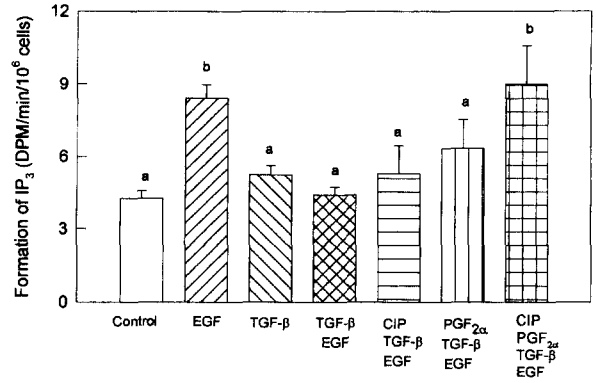

Fig. 4. Effect of the ciprofibrate (CIP), $PGF_{2\alpha}$ or the combination of CIP and $PGF_{2\alpha}$ on the inhibition by TGF-β on EGF-induced formation of IP_3. Hepatocytes were cultured in serum free medium for 24 h, and then were exposed to EGF (40 ng/ml), TGF-β (1 ng/ml), CIP (400 μM) or $PGF_{2\alpha}$ (3 μM), or the combination of CIP and $PGF_{2\alpha}$ in the presence of EGF and TGF-β and HGF for 30 min. The formation of IP_3 was determined by the method as described in materials and methods. Data represent means±SE of three experiments, with duplicates of each experiment. Values with different superscripts are significantly different.

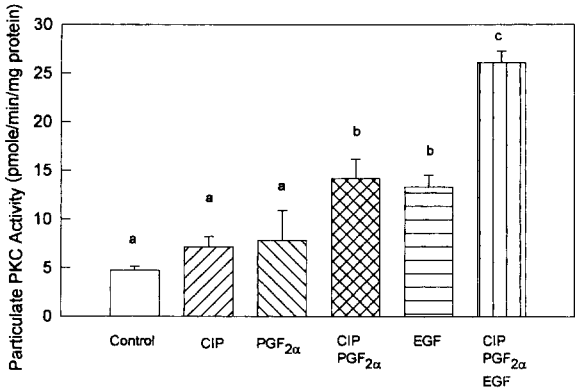

Fig. 5. Effect of ciprofibrate, $PGF_{2\alpha}$ and EGF on particulate PKC activity. Hepatocytes were cultured in serum free medium for 24 h, and were exposed to EGF (40 ng/ml), ciprofibrate (CIP, 400 μM), $PGF_{2\alpha}$ (3 μM), or the combination of CIP and $PGF_{2\alpha}$ in the absence or presence of EGF for 30 min. PKC activity was determined in the particulate fraction as described in materials and methods. Data represent means±SE of three experiments, with duplicates of each experiment. Values with different superscripts are significantly different.

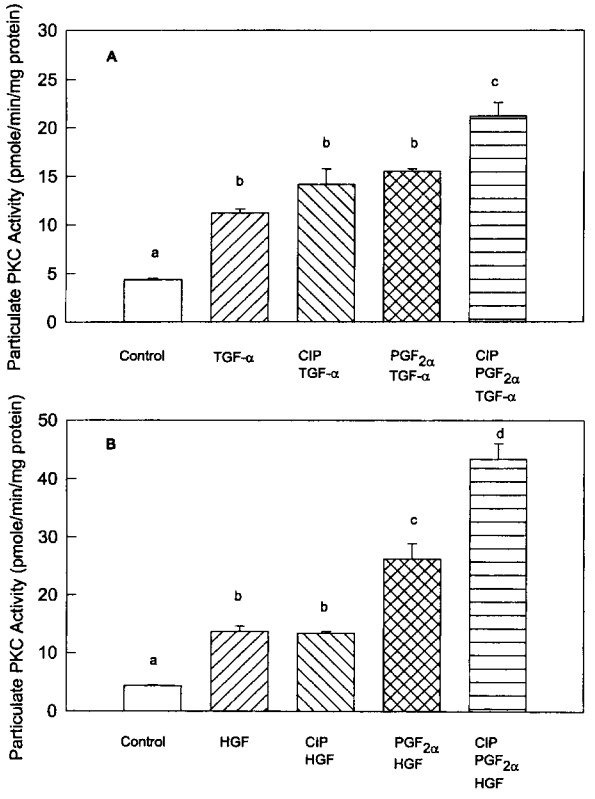

Fig. 6. Effect of the ciprofibrate (CIP), $PGF_{2\alpha}$ or the combination of CIP and $PGF_{2\alpha}$ on particulate PKC activity in the presence of TGF-α (A) or HGF (B). Hepatocytes were cultured in serum free medium for 24 h, and then were exposed to CIP (400 μM), $PGF_{2\alpha}$ (3 μM) or the combination of ciprofibrate and $PGF_{2\alpha}$ in the presence of TGF-α (50 ng/ml) or HGF (50 ng/ml) for 30 min. PKC activity was determined in the particulate fraction as described in materials and methods. Data represent means±SE of three experiments, with duplicates of each experiment. Values with different superscripts are significantly different.

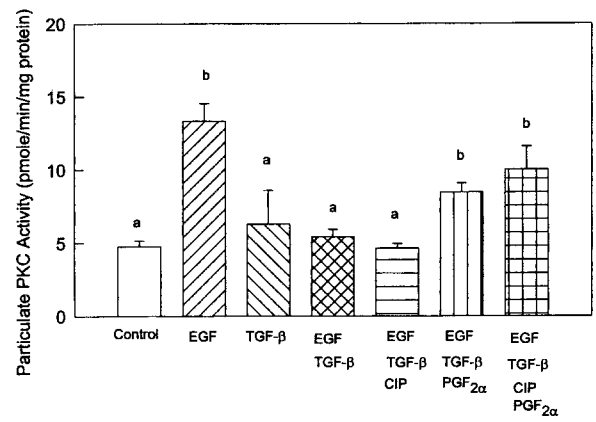

Fig. 7. Effect of ciprofibrate (CIP) and $PGF_{2\alpha}$ combination on the inhibition of PKC activity by TGF-β. Hepatocytes were cultured in serum free medium for 24 h, and then were exposed to EGF (40 ng/ml), TGF-β (1 ng/ml), CIP (400 μM) or $PGF_{2\alpha}$ (3 μM) alone, or the combination of CIP and $PGF_{2\alpha}$ in the presence of EGF and TGF-β for 30 min. PKC activity was determined in the particulate fraction described in methods and materials. Data represent means±SE of three experiments, with duplicates of each experiment. Values with different superscripts are significantly different.

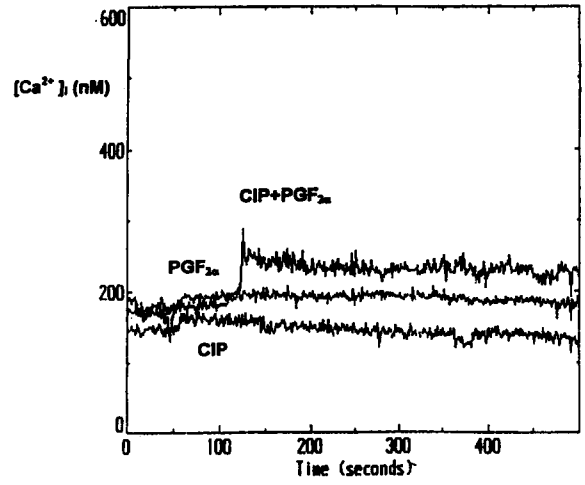

Fig. 8. Effect of ciprofibrate, $PGF_{2\alpha}$ and the combination of ciprofibrate and $PGF_{2\alpha}$ on intracellular calcium concentrations. Hepatocytes were cultured in serum free medium on poly-L-lysine glass bottom overslips. Intracellular calcium was measured in single cell by the methods as described in materials and methods. CIP; ciprofibrate (400 μM), $PGF_{2\alpha}$; prostagladin $F_{2\alpha}$ (3 μM).

fibrate and $PGF_{2\alpha}$ increased it much greatly, and maintained elevated level for more than 8 minutes (Fig. 8).

DISCUSSION

In this study, we found that the combination of ciprofibrate and $PGF_{2\alpha}$ increased PKC activity and intracellular calcium concentrations, but did not increase the formation of IP_3. In contrast, ciprofibrate or PGF 2α alone did not increase PKC activity or $[Ca^{2+}]_i$.

We previously found that the peroxisome proliferator ciprofibrate and eicosanoids are comitogenic in cultured hepatocytes (Hong and Glauert, 1996). However, possible mechanisms of the comitogenicity of ciprofibrate and eicosanoids are currently unclear. We hypothesized that the comitogenicity of the peroxisome proliferator ciprofibrate and eicosanoids may result from additive or synergistic increases of second messengers since second messengers such as inositol triphosphate, intracellular calcium, and PKC play an important role in cell proliferation (Bennett and Williams, 1992; Andreis *et al.*, 1981; Melien *et al.*, 1988; Athari and Jungermann, 1989). Moreover, synergistic effects of second messengers were proposed to increase cell proliferation by tumor promoters (Nishizuka, 1988).

Several peroxisome proliferators have previously been found to increase $[Ca^{2+}]_i$. In the presence of extracellular calcium (1.2 mM), nafenopin (160 mM) increased $[Ca^{2+}]_i$ about 105 nM in cell suspensions (Shackleton *et al.*, 1995). Ciprofibrate also increased it about 58 nM (for 100 mM) and 126 nM (for 200 mM) in the presence of 2 mM extracellular calcium in cell suspensions (Bennett and Williams, 1992), and about 72 nM by 500 mM in the presence of 1.3 mM extracellular calcium, also in cell suspensions (Shackleton *et al.*, 1995). The peroxisome proliferator Wy-14,643 increase $[Ca^{2+}]_i$ about 31 nM in the same study (Shackleton *et al.*, 1995). The smaller increase (about 30 nM) in $[Ca^{2+}]_i$ in this study may be due to the difference of extracellular calcium concentration (1 mM in our study) and/or the difference in methodologies in measuring $[Ca^{2+}]_i$ (single cell versus cell suspensions). The effect on $[Ca^{2+}]_i$ by $PGF_{2\alpha}$ has also been studied in hepatocytes (Refsnes *et al.*, 1995; Dajani *et al.*, 1996). Refsnes *et al.* (1995) found that 1 mM of $PGF_{2\alpha}$, increased about 0.4 ratio of 345/385 fluorescence in single cell cultured in Hepes medium. 100 mM of $PGF_{2\alpha}$, was found that 100% of cells responsed to increase $[Ca^{2+}]_i$ (Dajani *et al.*, 1996). The comparison of the increase of $[Ca^{2+}]_i$ by prostaglandins between our data and that of other studies is impossible since data are expressed differently.

We found that ciprofibrate and $PGF_{2\alpha}$, and the combination of ciprofibrate-$PGF_{2\alpha}$ did not increase IP_3 formation. The inability of ciprofibrate to induce the formation of IP_3 is consistent with other data which show that nafenopin did not increase formation of IP_3 (Ochsner *et al.*, 1990). However, prostaglandin $F_{2\alpha}$ was found in other studies to increase the formation of IP_3: about 4.5 fold at 25 mM (Altin and Bygrave, 1988), 2. 3 fold at 100 mM of $PGF_{2\alpha}$ (Refsnes *et al.*, 1995), and 2 fold at 25 mM (Dajani *et al.*, 1996). This difference may be partially related to the difference in culture conditions and concentration of $PGF_{2\alpha}$. We used collagen gel plates to culture hepatocytes, whereas the other studies used collagen coated plates.

The combination of ciprofibrate and $PGF_{2\alpha}$ increased $[Ca^{2+}]_i$ more than additively. However, the increase in $[Ca^{2+}]_i$ induced by the combination of ciprofibrate and $PGF_{2\alpha}$ did not coincide with the increase in IP_3 formation, suggesting that increase of in$[Ca^{2+}]_i$ by the combination ciprofibrate and $PGF_{2\alpha}$ was not related to the formation of IP_3. Because the formation of IP_3 has been thought a common pathway to increase $[Ca^{2+}]_i$ via release from endoplamic reticulum (ER) (Exton, 1988), the mechanism of increased $[Ca^{2+}]_i$ concentration without an increase of IP_3 is not clear. Other mechanisms, such as activation of a calcium channel, or release from calcium-bound protein or from mitochondrial calcium pool, may be involved in the increase of $[Ca^{2+}]_i$. Ciprofibrate was found to increase $[Ca^{2+}]_i$, but it was not changed by endoplamic reticulum Ca^{2+}-ATPase inhibitor thapsigargin, suggesting that inositol triphosphate pool may not be related to increase of $[Ca^{2+}]_i$ concentration by ciprofibrate (Skackleton *et al.*, 1995). It is noteworthy that $PGF_{2\alpha}$ increased $[Ca^{2+}]_i$ synergistically with the cAMP agonist glucagon in liver, furthermore mitochondria calcium fraction was increased about 10 fold by the combination treatment, but only 30~40% increase in ER fraction (Dajani *et al.*, 1996). The mechanism by which the combination of the peroxisome proliferator and $PGF_{2\alpha}$ increase $[Ca^{2+}]_i$ is not clear. However, it is possible that the increase in $[Ca^{2+}]_i$ may increase play a role in the comitogenicity of ciprofibrate and $PGF_{2\alpha}$. A recent study showed that the calcium mobilizing agents vasopressin and angiotensin II increased DNA synthesis additively in hepatocytes, but the formation of IP_3 was not increased (Dajani *et al.*, 1996).

We also found that the combination of ciprofibrate and $PGF_{2\alpha}$ significantly increased particulate PKC activity. The PKC activity was increased about 1.6 fold by ciprofibrate alone, similar to other results (Bojes and Thurman, 1994). The elevation of $[Ca^{2+}]_i$ by the combination of ciprofibrate and $PGF_{2\alpha}$ corresponds to the increase of PKC activity. PKC is a Ca^{2+}- and phospholipid-dependent protein kinase (Blumberg, 1991). For its activation, PKC requires Ca^{2+} and phospholipid, particularly phosphophatidyl serine. Since the acyl-CoA of ciprofibrate was found to increase PKC activity by decreasing the phosphatidylserine requirement of enzyme (Orellana *et al.*, 1990), it is possible that the decrease in requirement for phosphatidylserine by ciprofibrate may potentiate $PGF_{2\alpha}$-induced PKC activity. $PGF_{2\alpha}$ has been found to activate PKC in MC3T 3-E1 osteoblasts (Quarles, 1993); however, no studies in cultured hepatocytes have been published. The increase in PKC activity may also play a role in the comitogenicity from the ciprofibrate-$PGF_{2\alpha}$ combination. PKC and $[Ca^{2+}]_i$ have been found to be implicated in cell proliferation and oncogene activation (Blumberg, 1991). Furthermore, PKC and $[Ca^{2+}]_i$ synergistically in-

crease cell proliferation by tumor promoters (Nishizuka, 1988). Therefore, costimulation of PKC and $[Ca^{2+}]_i$ by the combination of ciprofibrate-$PGF_{2\alpha}$ may play a role in the comitogenicity of these chemicals.

This study showing that the combination of ciprofibrate and $PGF_{2\alpha}$ costimulate PKC activity and increase intracellular calcium concentration more than additively implies the significance of eicosanoids on mitogenic signaling induced by the peroxisome proliferator ciprofibrate. Calcium dependent PKC has been found to facilitate and promote the clonal expansion of initiated hepatocytes and to be activated in an early stage of malignant progression in diethylnitrosamine-induced rat hepatocarcinogenesis (La Porta and Comolli, 1994; La porta *et al.*, 1993). Therefore, our previous findings that the peroxisome proliferator ciprofibrate decreased hepatic eicosanoid concentrations (Wilson *et al.*, 1995; Leung & Glauert, 1995; Hong *et al.*, 1995) may be important in the peroxisome proliferator ciprofibrate-induced cell proliferation and in the process of hepatic tumor promotion.

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