Linoleic acid stimulates gluconeogenesis via Ca$^{2+}$/PLC, cPLA$_2$, and PPAR pathways through GPR40 in primary cultured chicken hepatocytes

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Suh HN, Huong HT, Song CH, Lee JH, Han HJ. Linoleic acid stimulates gluconeogenesis via Ca$^{2+}$/PLC, cPLA$_2$, and PPAR pathways through GPR40 in primary cultured chicken hepatocytes. Am J Physiol Cell Physiol 295: C1518–C1527, 2008. First published October 8, 2008; doi:10.1152/ajpcell.00368.2008.—Fatty acids serve vital functions as sources of energy, building materials for cellular structures, and modulators of physiological responses. Therefore, this study examined the effect of linoleic acid on glucose production and its related signal pathways in primary cultured chicken hepatocytes. Linoleic acid (double-unsaturated, long chain) increased glucose production in a dose ($\geq$10$^{-3}$ M)- and time (3 h)-dependent manner. Both oleic acid (monounsaturated, long chain) and palmitic acid (saturated, long chain) also increased glucose production, whereas caprylic acid (saturated, short chain) failed to increase glucose production. Linoleic acid increased G protein-coupled receptor 40 (GPR40; also known as free fatty acid receptor-1) protein expression and glucose production that was blocked by GPR40-specific small interfering RNA. Linoleic acid increased intracellular calcium concentration, which was blocked by EGTA (extracellular calcium chelator)/BAPTA-AM (intracellular calcium chelator), U-73122 (phospholipase C inhibitor), nifedipine, or methoxysterapamil (L-type calcium channel blockers). Linoleic acid increased cytosolic phospholipase A$_2$ (cPLA$_2$) phosphorylation and the release of [3H]-labeled arachidonic acid. Moreover, linoleic acid increased the level of cyclooxygenase-2 (COX-2) protein expression, which stimulated the synthesis of prostaglandin E$_2$ (PGE$_2$). The increase in PGE$_2$ production subsequently stimulated peroxisome proliferator-activated receptor (PPAR) expression, and MK-886 (PPAR-α antagonist) and GW-9662 (PPAR-δ antagonist) inhibited glucose-6-phosphatase and phosphoenolpyruvate carboxykinase. In addition, linoleic acid-induced glucose production was blocked by inhibition of extracellular and intracellular calcium, cPLA$_2$, COX-2, or PPAR pathways. In conclusion, linoleic acid promoted glucose production via Ca$^{2+}$/PLC, cPLA$_2$/COX-2, and PPAR pathways through GPR40 in primary cultured chicken hepatocytes.

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DIETARY FAT IS AN IMPORTANT macronutrient for growth and development of all organisms. It provides substrates for energy metabolism, membranes, and signaling molecules and regulates gene expression. Also, the effects of fatty acids have been attributed to their intracellular metabolism to long-chain acyl-CoA esters. Free fatty acid (FFA) levels are often increased in obese individuals in both the fed and fasted states and have been implicated as critical players in the progression of obesity to type II diabetes (1–3, 16, 19, 23). Among FFAs, linoleic acid is abundant in the daily diet and belongs to an essential fatty acid that is essential for survival of humans and other mammals. The liver plays a major role in regulating blood glucose levels by maintaining the balance between storage and release of glucose (20). Gluconeogenesis in the liver is the process of de novo synthesis of glucose from nonhexose carbohydrate precursors. Although FFAs have been proposed to directly regulate hepatic gluconeogenesis independent of hormones in several ways (9, 21), the components of the signaling cascade through which FFAs operate have not been systematically investigated and remain largely unknown. The intermediary protein kinase Akt2/protein kinase B (PKB)-β, PGC-1 (24), p38 MAPK (9), and AMP-activated protein kinase (AMPK) (10) all promote gluconeogenesis in the liver. Also, FFAs can directly modulate the activity of transcription factors, including peroxisome proliferator-activated receptors (PPARs), which are involved in physiological issues such as energy balance, lipid metabolism, and glucose control (22). Therefore, we hypothesize that PPARs may play an important role in gluconeogenesis in primary cultured chicken hepatocytes. However, the functions of PPARs in gluconeogenesis in hepatocytes exposed to FFAs such as linoleic acid are largely unknown.

Primary hepatocyte cultures have been used in many physiological studies of liver function because they retain many liver-specific functions and respond to various hormones through the expression of liver-specific genes (37). The primary chicken hepatocytes culture system used in the present study also retains an in vitro differentiated phenotype that is typical of the liver, including albumin expression (17), P450 1A induction (17), tyrosine aminotransferase expression (29), and ascorbate recycling (30). Therefore, the present study examined the effect of linoleic acid on gluconeogenesis and its related pathways in primary cultured chicken hepatocytes.

MATERIALS AND METHODS

Materials. Two-week-old male White Leghorn chickens were obtained from Dae Han Experimental Animal (Chungju, Korea). All procedures for animal management were performed according to the standard protocols at Seoul National University. The Institutional Review Board at Chonnam National University approved the research proposal as well as the relevant experimental procedures including.
animal care. The experimental samples and quality control of the laboratory facility and equipment were appropriately managed and maintained. GW-9662 and L-165041 were purchased from Calbiochem (La Jolla, CA). Fetal bovine serum (FBS) was supplied by Gibco (Rockville, MD). Linoleic acid, EGTA, BAPTA-AM, U-73121, nifedipine, metoxyverapamil, mepacrine, A23187, and indomethacin were obtained from Sigma Chemical (St. Louis, MO). G protein-coupled receptor 40 (GPR40), phosphorylated cytosolic phospholipase A2 (p-cPLA2), p-cPLA2, cyclooxygenase-1 (COX-1), COX-2, PPAR-α, PPAR-δ, lamin, and goat anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were of the highest purity commercially available.

Primary culture of chicken hepatocytes. The chicken hepatocytes were prepared and maintained as a monolayer culture, as described elsewhere (17). Briefly, chicken hepatocytes were isolated by perfusion of a liver that had been starved for 24 h with 0.05% collagenase. Hepatocytes with >90% viability, as verified by a trypan blue exclusion test, were used for subsequent plating. The hepatocytes were plated (5.0 × 10^5 cells/60-mm collagen-coated dish) in incubation medium (Williams’s medium) containing 75 U/ml penicillin and 75 U/ml streptomycin, 1 μg/ml insulin, 10^{-12} M dexamethasone, 5 μg/ml transferrin, 10^{-8} M T_{3}, and 5% FBS, and incubated for 4 h at 37°C in a 5% CO_{2} atmosphere. The medium was then replaced with fresh incubation medium, and the hepatocytes were incubated for a further 20 h to achieve the monolayer culture. Cells were cultured 2 more days, and the experiments were then carried out. When we started primary cultured chicken hepatocyte studies, we also checked for hepatocyte-specific markers such as albumin, α-fetoprotein, and α-antitrypsin.

Measurement of glucose production. Glucose production from primary cultured chicken hepatocytes was measured as previously described (9). Briefly, cells were washed three times with warm phosphate-buffered saline (PBS) to remove glucose, followed by treatment with 10^{-4} M linoleic acid for 12 h in glucose-free medium (Gibco 11966-025) containing gluconeogenic substrates (20 mM sodium lactate and 2 mM sodium pyruvate). Glucose concentration was determined with a glucose assay kit from Roche Applied Science (catalog no. 0716251) and was normalized to the cellular protein concentration.

Measurement of intracellular calcium concentration. The changes in intracellular calcium concentration ([Ca^{2+}],) were monitored with fluo-3 AM, which was initially dissolved in dimethylsulfoxide and stored at −20°C. Chicken hepatocytes in 35-mm culture dishes were rinsed twice with bath solution [140 mM NaCl, 5 mM KCl, 1 mM CaCl_{2}, 0.5 mM MgCl_{2}, 10 mM glucose, and 5.5 mM HEPES (pH 7.4)]. They were then incubated in bath solution containing 3 μM fluo-3 AM in a 5% CO_{2}-95% O_{2} atmosphere at 37°C for 40 min, rinsed twice with bath solution, mounted on a fluorescence microscope chamber, and scanned at 1-s intervals using confocal microscopy (×400) (Fluoview 300, Olympus). The fluorescence was excited at 488 nm, and the emitted light was observed at 515 nm. All analyses of [Ca^{2+}] were processed in a single cell, and the results are expressed as the fluorescence intensity (F/F_{0}, arbitrary unit). A-23187 (calf ionophore) was used as the positive control.

Assay of [3H]arachidonic acid release. Arachidonic acid (AA) release from the cultures was determined by modification of the method of Xing et al. (40). To summarize, confluent monolayers of chicken hepatocytes were incubated for 24 h in basal medium containing 0.5 μCi [3H]AA/ml, as well as the three growth supplements. The monolayers were washed three times with Williams’s medium (pH 7.4) and incubated (at 37°C) for 1 h in incubation medium containing specified agents. At the end of the 1-h incubation period, the incubation medium was removed and transferred to ice-cold tubes containing 55 mM EGTA and EDTA (final concentration, 5 mM each). The incubation medium was then centrifuged at 12,000 g to eliminate cell debris. To determine radioactive levels, aliquots of the samples were placed in scintillation vials containing scintillation fluid, and the radioactivity was counted using a liquid scintillation counter. The cells, which remained attached to the plates, were scraped into 1 ml of 0.1% SDS, and 900 μl of the resulting cell lysate was used for scintillation counting. The remaining 100 μl of the cell lysate was used for protein determinations. The quantity of [3H]AA released in each condition (determined as described above) was then standardized with respect to protein. Subsequently, this standardized level of released [3H]AA was compared percentage-wise to the level of total [3H]AA that was incorporated into the cells at the start of the 1-h incubation period with the specified agents (equivalent to the quantity of total released radioactivity plus the total cell-associated radioactivity at the end of the 1-h incubation period).

Prostaglandin E_{2} assay. Chicken hepatocytes plated on 60-mm culture dishes were grown in FBS-free medium for 24 h and divided into groups according to the experimental protocol. The prostaglandin E_{2} (PGE_{2}) concentration in the culture medium was measured using a prostaglandin E_{2} enzyme-linked immunosorbent assay (ELISA) with a Prostaglandin E_{2} High Sensitivity Immunoassay kit (R&D Systems, Minneapolis, MN).

Small interfering ribonucleic acid transfection. Chicken hepatocytes were grown in 60-mm culture dishes until they reached 75% confluence. Cells were then transfected for 24 h with either a SMART pool of the small interfering (si)RNAs specific for GPR40 (200 pmol) or a nontargeting siRNA (as negative control; 200 pmol; Dharmacon, Lafayette, CO) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Real-time reverse transcriptase-polymerase chain reaction. Total RNA was extracted from cells treated with each designated agent using STAT-60 (Tel-Test, Friendswood, TX). The real-time quantification of RNA targets was then performed in the Rotor-Gene 6500 real-time thermal cycling system (Corbett Research, NSW, Australia) using the Quantitect SYBR Green RT-PCR kit (Qiagen). The reaction mixture (20 μl) contained 200 ng total RNA, 0.5 μM of each primer, appropriate amounts of enzymes, and fluorescent dyes as recommended by the supplier. The Rotor-Gene 6500 cyclers were programmed as follows: 30 min at 50°C for reverse transcription; 15 min at 95°C for DNA polymerase activation; 45 cycles of 15 s at 94°C, 30 s at 55°C, 30 s at 72°C. The data were collected during the extension step (30 s at 72°C). The PCR reaction was followed by a melting curve analysis to verify the specificity and identity of the RT-PCR products; this analysis can distinguish specific PCR products from the nonspecific PCR product resulting from primer-dimer formation. The primers used are described in Table 1.

### Table 1. Primers used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence 5′-3′</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6Pase</td>
<td>GTGAAATTACCAAGACATCCAGG</td>
<td>305</td>
</tr>
<tr>
<td>Sense</td>
<td>GGCACCATGCTGCGGACAGGGG</td>
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</tr>
<tr>
<td>Antisense</td>
<td>CTGTGTTCCGAAAGACAGAAA</td>
<td>350</td>
</tr>
<tr>
<td>PEPCK</td>
<td>GCCGAGACTGCTCTCTCTAT</td>
<td>374</td>
</tr>
<tr>
<td>Sense</td>
<td>CTGGCCAGGCTCTCTCTCTAT</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>GAGCAATGCGCAAGATCTGGAGAG</td>
<td>483</td>
</tr>
<tr>
<td>PPAR-δ</td>
<td>GCCGTCTTGTGTTGCTGAGGC</td>
<td>314</td>
</tr>
<tr>
<td>Sense</td>
<td>ATCCGCAGATGGGCTGTAC</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>AAGAGCTGCAACAATGGATTG</td>
<td>230</td>
</tr>
<tr>
<td>β-Arin</td>
<td>TCCATTCTGGAAAGGCTGTCG</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>ACCGATCAGTACGAGCATCC</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>CTTCATGCTGTTGCTGTTGAA</td>
<td></td>
</tr>
</tbody>
</table>

G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PPAR, peroxisome proliferator-activated receptor.
The temperature of PCR products was elevated from 65°C to 99°C at a rate of 1°C/5 s, and the resulting data were analyzed using the software provided by the manufacturer.

Preparation of cytosolic and total membrane fractions. The medium was removed, and the cells were washed twice with ice-cold PBS, scraped, harvested by microcentrifugation, and resuspended in buffer A (137 mM NaCl, 8.1 mM NaH2PO4, 2.7 mM KCl, 1.5 mM KH2PO4, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, and 10 μg/ml leupeptin, pH 7.5). The resuspended cells were lysed mechanically on ice by trituration using a 21.1-gauge needle. The lysates were first centrifuged at 1,000 g for 10 min at 4°C. The supernatants were further centrifuged at 100,000 g for 1 h at 4°C to prepare the cytosolic and total particulate fractions. The particulate fraction containing the membrane fraction was washed twice and resuspended in buffer A containing 1% Triton X-100. The protein level in each fraction was quantified by the Bradford procedure (4).

Western blot analysis. The cell homogenates (40 μg protein) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The blots were then washed with TBST [Tris-buffered saline and Tween 20: 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.05% Tween 20]. The membrane was blocked with 5% skim milk for 1 h and incubated with the appropriate primary antibody at the dilution recommended by the supplier. The membrane was then washed, and the primary antibodies were detected with goat anti-rabbit IgG conjugated to horseradish peroxidase. The antibodies were incubated at 4°C. The bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Statistical analysis. The results are expressed as means ± SE. All experiments were analyzed by analysis of variance. In some experiments, the treatment means were compared with the control using a Bonferroni-Dunn test. \( P < 0.05 \) was considered significant.

RESULTS

Effect of FFA on gluconeogenesis. The effective treatment time and concentration of linoleic acid for glucose production were determined by observing the chicken hepatocytes at various times (0–12 h) with various concentrations (0–1 M) of linoleic acid. Linoleic acid increased the level of glucose production in a dose (≥10⁻⁴ M)- and time (≥8 h)-dependent manner (Fig. 1, A and B). Therefore, 10⁻⁴ M linoleic acid for 12 h was selected as the treatment condition for the present study. To compare the effect of linoleic acid on gluconeogenesis with that of other fatty acids, the cells were treated with 10⁻⁴ M linoleic acid [double-unsaturated, long chain; 18:2(n-6) carbon chain], oleic acid [monounsaturated, long chain; 18:1(n-9) carbon chain], palmitic acid (PA), or caproic acid (CA; 10⁻⁴ M) for 12 h; glucose production was then measured. Values represent means ± SE of four independent experiments with triplicate dishes. * \( P < 0.05 \) vs. control. C: cells were incubated with linoleic acid, oleic acid (OA), palmitic acid (PA), or caproic acid (CA; 10⁻⁴ M) for 12 h; glucose production was then measured. Values represent means ± SE of four independent experiments with triplicate dishes. * \( P < 0.05 \) vs. control. D: cells were treated with linoleic acid for 0–12 h and then harvested. The total protein was extracted and blotted with G protein-coupled receptor 40 (GPR40) or β-actin antibodies. Values represent means ± SE of three independent experiments as determined from densitometry relative to β-actin. * \( P < 0.05 \) vs. control. E: cells were transfected for 24 h with either a SMART pool of GPR40 small interfering (si)RNAs (200 pmol) or a nontargeting (Nt) control siRNA (200 pmol) using LipofectAMINE 2000 before linoleic acid treatment for 12 h; glucose production was then measured. Values represent means ± SE of three independent experiments. * \( P < 0.05 \) vs. control. ** \( P < 0.05 \) vs. linoleic acid. F: cells were transfected for 24 h with either a SMART pool of GPR40 siRNAs (200 pmol) or a nontargeting control siRNA (200 pmol) using Lipofectamine2000 before linoleic acid treatment for 12 h. The total protein was extracted and blotted with GPR40 or β-actin antibodies. Each sample shown is representative of three experiments.
18:1 (cis-9) carbon chain), palmitic acid [saturated, long chain; 16 carbon chain], or caproic acid [saturated, short chain; 6 carbon chain] for 12 h, and then glucose production was determined. Linoleic acid, oleic acid, and palmitic acid promoted glucose production, whereas caproic acid failed to promote glucose production (Fig. 1C). FFAs can signal directly via the FFA receptor, GPR40, which is also known as free fatty acid receptor-1. To determine the involvement of GPR40 activation in linoleic acid-induced gluconeogenesis, Western blot analysis was performed. Linoleic acid increased GPR40 protein expression (Fig. 1D). Also, GPR40-specific siRNA blocked linoleic acid-induced glucose production, whereas nonspecific siRNA had no effect (Fig. 1E). The level of GPR40 protein expression was significantly blocked in GPR40-specific siRNA-transfected cells (Fig. 1F). Therefore, we confirmed that linoleic acid induced gluconeogenesis via GPR40.

**Involvement of \([\text{Ca}^{2+}]_i\) in linoleic acid-induced gluconeogenesis.** To assess the involvement of \([\text{Ca}^{2+}]_i\) in linoleic acid-induced gluconeogenesis, first we confirmed that linoleic acid increases the \([\text{Ca}^{2+}]_i\). Linoleic acid increased \([\text{Ca}^{2+}]_i\) followed by a decline (Fig. 2A). Linoleic acid-induced \([\text{Ca}^{2+}]_i\) increase was completely blocked by EGTA/BAPTA-AM mixture (extracellular/intracellular calcium chelator) (Fig. 2B). To determine the involvement of phospholipase C (PLC) or L-type calcium channels (LTCC) on linoleic acid-induced \([\text{Ca}^{2+}]_i\), cells were treated with PLC inhibitor or LTCC blockers and then \([\text{Ca}^{2+}]_i\) was measured. U-73122 (PLC inhibitor), nifedipine, or methoxyverapamil (LTCC blockers) inhibited linoleic acid-induced increase of \([\text{Ca}^{2+}]_i\) (Fig. 2, C–E). Moreover, linoleic acid increased glucose production, which was blocked by EGTA/BAPTA-AM mixture (Fig. 2F). These results suggest that linoleic acid-induced glucose production requires influx of \(\text{Ca}^{2+}\) and is likely to involve stimulation of LTCC and PLC.

**Involvement of cPLA2, COX-2, and PGE2 in linoleic acid-induced gluconeogenesis.** Linoleic acid increased the level of cPLA2 phosphorylation (Fig. 3A). To determine the relationship between \([\text{Ca}^{2+}]_i\) and cPLA2, cells were treated with extracellular/intracellular calcium chelators, and then cPLA2 Western blotting was performed. EGTA/BAPTA-AM blocked cPLA2 phosphorylation (Fig. 3B). In addition, cells were treated with cPLA2 inhibitors, and \([^{3}\text{H}]\text{AA}\) release was then measured. AACCOCF3 or mepacrine (cPLA2 inhibitors) significantly blocked the linoleic acid-induced increase of \([^{3}\text{H}]\text{AA}\) release (Fig. 3C). Therefore, linoleic acid-induced \([\text{Ca}^{2+}]_i\) stimulates cPLA2 and then AA release. As shown in Fig. 3D, AACCOCF3 or mepacrine significantly blocked the linoleic acid-induced increase of glucose production, which suggests involvement of cPLA2.

The cyclooxygenase-(1, 2) [COX-(1, 2)] expression was examined to determine whether linoleic acid induced the expression of the COX-(1, 2) proteins. The results showed that linoleic acid increased the level of COX-2 expression but had no effect on COX-1 (Fig. 4A). To determine upstream signaling molecules, cells were treated with cPLA2 inhibitors. Linoleic acid-induced COX-2 protein expression was inhibited by...
either AACOCF₃ or mepacrine (Fig. 4B). Indeed, as shown in Fig. 4C, linoleic acid increased PGE₂ production, which was blocked by AACOCF₃ or mepacrine and indomethacin heptyl ester (COX-2 inhibitor). Therefore, we confirmed that COX-2 is downstream of cPLA₂ and subsequently stimulates PGE₂ production. To determine the involvement of COX-2 in linoleic acid-induced gluconeogenesis, cells were treated with AACOCF₃ or mepacrine for 30 min before treatment with linoleic acid for 12 h; [³²P]arachidonic acid ([³²P]AA) release was then measured. Values represent means ± SE of three independent experiments with triplicate dishes. *P < 0.05 vs. control, **P < 0.05 vs. linoleic acid. D: cells were treated with AACOCF₃ or mepacrine for 30 min before treatment with linoleic acid for 12 h; glucose production was then measured. Values represent means ± SE of three independent experiments with triplicate dishes. *P < 0.05 vs. control, **P < 0.05 vs. linoleic acid.

Involvement of PPARs and gluconeogenic enzymes in linoleic acid-induced gluconeogenesis. The expression of PPAR isotypes was detected by real-time RT-PCR to determine whether the PPARs are regulated by linoleic acid. As shown in Fig. 5A, linoleic acid increased PPAR-α and PPAR-δ mRNA expression level more than fourfold relative to control but PPAR-γ less than other isoforms. PPAR-α is highly expressed in hepatocytes (12) and is involved in gluconeogenesis (28). However, although PPAR-δ is ubiquitously expressed (12), its function is not well known. Thus, we chose PPAR-α and -δ as experimental isotypes in the present study. Indeed, linoleic acid increased the level of PPAR-α and -δ protein expression (Fig. 5B), which was blocked by AACOCF₃, mepacrine, and indomethacin heptyl ester (Fig. 5C). Each inhibitor had no effect itself (glucose production and cell injury). These results show that linoleic acid stimulates PPARs and that COX-2 is upstream of PPARs. Linoleic acid increased glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (gluconeogenic enzymes) mRNA levels (Fig. 6A). These results indicate that linoleic acid can directly elevate levels of gluconeogenic gene transcripts in hepatocytes.

Fig. 3. Involvement of cytosolic phospholipase A₂ (cPLA₂) in linoleic acid-induced gluconeogenesis. A: primary cultured chicken hepatocytes were treated with linoleic acid for 0–240 min and then harvested. The total protein was extracted and blotted with phospho-cPLA₂ or cPLA₂ antibodies. Values represent means ± SE of three experiments for each condition as determined from densitometry relative to cPLA₂. *P < 0.05 vs. control. B: cells were treated with a mixture of EGTA/BAPTA-AM for 30 min before treatment with linoleic acid for 1 h. The total protein was extracted and blotted with the phospho-cPLA₂ or cPLA₂ antibodies. Values represent means ± SE of three experiments for each condition as determined from densitometry relative to cPLA₂. **P < 0.05 vs. control, ***P < 0.05 vs. linoleic acid. C: cells were treated with AACOCF₃ or mepacrine for 30 min before treatment with linoleic acid for 12 h; [³²P]arachidonic acid ([³²P]AA) release was then measured. Values represent means ± SE of three independent experiments with triplicate dishes. *P < 0.05 vs. control, **P < 0.05 vs. linoleic acid. D: cells were treated with AACOCF₃ or mepacrine for 30 min before treatment with linoleic acid for 12 h; glucose production was then measured. Values represent means ± SE of three independent experiments with triplicate dishes. *P < 0.05 vs. control, **P < 0.05 vs. linoleic acid.
determine whether PPARs are involved in linoleic acid-induced gluconeogenesis, the cells were treated with MK-886 (PPAR-α antagonist), GW-9662 (PPAR-δ antagonist), fenofibrate (PPAR-α agonist), and L-165041 (PPAR-δ agonist), and then gluconeogenic gene mRNA expression and glucose production were measured. PPAR antagonists blocked linoleic acid-induced gluconeogenic gene mRNA expression (Fig. 6B) and glucose production (Fig. 6C), whereas PPAR agonists increased them.

**DISCUSSION**

The present study demonstrated that linoleic acid promotes glucose production via Ca²⁺/PLC, cPLA₂, and PPAR pathways through the GPR40 in primary cultured chicken hepatocytes. In the present study, we showed that linoleic acid (double-unsaturated, long chain), oleic acid (monounsaturated, long chain), and palmitic acid (saturated, long chain) increased glucose production, whereas caproic acid (saturated, short chain) did not. Linoleic acid also increased glucose production via the GPR40, whereas oleic and palmitic acids did not. These results suggest that linoleic acid may promote glucose production through the GPR40 pathway in primary cultured chicken hepatocytes.
chain) did not increase glucose production. This result suggests that long-chain FFAs (saturated or unsaturated) can stimulate glucose production. Linoleic acid is one of the most abundant FFAs in blood (7). Linoleic acid also acts through membrane lipids, as ligands for receptors and transcription factors that regulate gene expression, as precursor for eicosanoids, in cellular communication, and through direct interactions with proteins. We observed that linoleic acid increased glucose production in a dose-dependent manner from 10^{-4} M. On the other hand, excessive fatty acid disrupts cellular environments and alters structure and function (18). Therefore, 10^{-4} M linoleic acid was selected for further experiments in this study. However, it is difficult to say correspond concentration in vivo due to wide range of concentration. GPR40 is a member of the G protein-coupled receptor superfamily (31) and is activated by fatty acids with carbon chains longer than six carbons. Binding affinity of GPR40 for the fatty acids that are predominant in plasma (oleate, palmitate, stearate, linoleate, and linolenate) is near physiologically relevant concentrations (5). The alpha-subunit of the G_{q} family of G proteins (G_{q}alpha) is known to stimulate PLC activity (34, 38). Therefore, GPR40 of chicken hepatocytes is linked to PLC activation via G proteins, particularly G_{q}alpha, in response to linoleic acid stimulation. Also, GPR40 is related to glucose metabolism. We observed GPR40-
Fig. 6. Involvement of PPARs in gluconeogenic enzymes. A: primary cultured chicken hepatocytes were treated with linoleic acid for 6 h, and glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels were analyzed by real-time RT-PCR. Values represent means ± SE of three independent experiments. *P < 0.05 vs. control. B and C: cells were treated with MK-886 or GW-9662 for 30 min before treatment with linoleic acid for 6 h, or cells were treated with fenofibrate or L-165041 for 6 h. The G6Pase and PEPCK mRNA levels were analyzed by real-time RT-PCR, and glucose production was measured. Values represent means ± SE of three independent experiments. *P < 0.05 vs. control, **P < 0.05 vs. linoleic acid.
specific siRNA blocked gluconeogenesis. Steneberg et al. (36) reported that feeding GPR40−/− mice a high-fat diet (HFD) led to a dramatic increase in lipid storage in livers and led to hepatic gluconeogenesis, whereas feeding GPR40+/− mice a HFD resulted in a more modest increase in lipid storage, because GPR40−/− mice are protected from increased hepatic glucose output in a HFD.

In the present study, linoleic acid-induced [Ca2+]i increases were inhibited by the PLC inhibitor and LTCC blockers. This result suggests that the linoleic acid-stimulated, GPR40-mediated increase in [Ca2+]i, depends on PLC activation and LTCC-mediated pathways, which was similar to a previous study (13). However, Shapiro et al. (32) reported that GPR40 signaling is not sufficient for LTCC opening, but acts on preactivated LTCC, thereby increasing Ca2+ permeation. In the present study, a mixture of extracellular and intracellular Ca2+ chelators inhibited linoleic acid-induced cPLA2 phosphorylation. This result suggests that activation of cPLA2 depends on extracellular and intracellular Ca2+. However, Caro and Cederbaum (8) reported that PLA2 depends not on intracellular calcium but rather on influx of extracellular calcium in HepG2. In addition, Nolan et al. (27) reported that calcium-independent PLA2 was involved in activating free fatty acid receptor-1 in β-cells. This disparity is due to different cell type (primary hepatocytes vs. HepG2 vs. β-cells). PLA2 enzyme catalyzed the hydrolysis of cellular phospholipids at the sn-2 position to liberate AA (26). In the present study, linoleic acid led to AA release, which was consistent with a previous study (14). Martins et al. (25) showed that PLA2 increased gluconeogenesis in rat liver. Moreover, we observed that cPLA2 inhibitor blocked linoleic acid-induced gluconeogenesis, which confirms the involvement of PLA2 in linoleic acid-induced gluconeogenesis. Also, linoleic acid significantly increased the COX-2 protein expression and PGE2 concentration. This result was consistent with a previous study in which linoleic acid increased the expression of COX-2 as well as PGE2 in retinal pigment epithelial cells (26). COX, which includes the COX-1 and COX-2 isomers, is the rate-limiting enzyme that catalyzes the conversion of AA into endoperoxide intermediates, which are ultimately converted by specific synthases to prostanoids, including PGE2 (15, 33). In the present study, COX-2 inhibitor blocked linoleic acid-induced gluconeogenesis, which suggests that linoleic acid-induced gluconeogenesis is probably mediated by eicosanoid synthesis. Similarly, Martins et al. (25) reported that inhibition of COX-2 synthesis by indomethacin blocked gluconeogenesis in rat liver.

We also observed that linoleic acid upregulated PPAR expression. Oxidative metabolites of linoleic and AA are natural ligands of PPAR-β/δ and downregulate PPAR-β/δ (42). Ram (28) reported that PPAR-α plays a role in the regulation of nutrient metabolism and gluconeogenesis, whereas PPAR-β/δ has been found to be effective in controlling dyslipidemia. However, in the present study, linoleic acid increased gluconeogenesis via PPAR-α and PPAR-δ. Recently, Sprecher (35) reported that activation of PPAR-δ, like PPAR-α, enhanced energy production. These results suggest that crosstalk occurs between PPAR-α and PPAR-δ. We reproduced this observation using PPAR-α and PPAR-δ agonist as a mediator of gluconeogenesis. These data suggest a model in which activation of PPARs may alter the linoleic acid signaling required for gluconeogenesis. It is well known that both G6Pase and PEPCK are rate-controlling enzymes in gluconeogenesis. In the present study, the expression of both G6Pase and PEPCK was stimulated by linoleic acid, which is dependent on PPARs; this finding is in agreement with previous studies (11, 39, 41). Presence of elevated FFAs may have an allosteric stimulatory effect on G6Pase, since this enzyme catalyzes the final step leading to glucose production. However, PEPCK may play a more important role in the regulation of gluconeogenesis because it is the earliest rate-controlling enzyme in the process of gluconeogenesis. Our current studies show that PPARs play a critical role in the regulation of hepatic gluconeogenesis induced by linoleic acid. Therefore, future studies on the signaling pathways by which linoleic acid activates PPARs may shed new light into understanding of glucose homeostasis. Figure 7 is a hypothetical model depicting the signaling mechanisms of linoleic acid-induced gluconeogenesis. In conclusion, linoleic acid induced gluconeogenesis via Ca2+/PLC, cPLA2/COX-2 pathways and PPAR pathways through GPR40.

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