Exogenous administration of eicosapentaenoic acid (EPA) improves insulin sensitivity, but its precise mechanism remains unknown. Here we show that EPA stimulates the intracellular insulin signaling pathway in hepatoma cells. Exposure of these cells to EPA caused up-regulation of several insulin-induced activities including tyrosine phosphorylation of insulin receptor substrate-1, insulin receptor substrate-1-associated phosphatidylinositol 3-kinase, and its downstream target Akt kinase activity as well as down-regulation of gluconeogenesis. In contrast, EPA decreased mitogen-activated protein kinase activity and inhibited cell proliferation. These findings underscore the possibility that EPA up-regulates metabolic action of insulin and inhibits cell growth in humans.

Eicosapentaenoic acid (EPA) is one of the dietary n-3 polyunsaturated fatty acids (n-3 PUFAs) present in fish oils. Accumulating evidence in animal and human studies indicates that administration of n-3 PUFAs improves insulin sensitivity (1–4). On the other hand, leptin can improve insulin sensitivity and glucose disposal independently, apart from its suppressive effect on food intake (5–7). We recently reported that EPA up-regulates leptin mRNA expression and its secretion through the hexosamine biosynthetic pathway in 3T3-L1 adipocytes (8), which indicate this fact. In the present study, the study is aimed at clarifying whether EPA directly affects insulin signaling in target tissues via the other novel mechanism ameliorating insulin sensitivity as well as cell proliferation.

Experimental Procedures

Materials—Horseradish peroxidase-conjugated monoclonal anti-phosphotyrosine antibody (RC20) and anti-SH2 were purchased from Transduction Laboratories. Anti-insulin receptor substrate-1 (IRS-1), phosphotyrosine antibody (RC20) and anti-adapter molecule growth factor receptor-bound protein 2 (GRB2), anti-insulin receptor β, and horseradish peroxidase-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-IRS-2 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). EPA was from Mochida Pharmaceutical Co. (Tokyo, Japan). Clofibrate, carbaprostacyclin (cPGI2), 6-diazo-5-oxo-l-norleucine (DON), oleic acid sodium salt, cis-4,7,10,13,16,19-docosahexaenoic acid ethyl ester, palmitic acid sodium salt, arachidonic acid ethyl ester, and cycloheximide were purchased from Sigma. The p44/42 mitogen-activated protein kinase (MAPK) assay kit and Akt kinase assay kit were purchased from New England Biolabs. CellTiter 96 AQueous One Solution Cell Proliferation Assay was purchased from Promega. The Gene Images random prime labeling module and Gene Images CDP-star detection module were purchased from Amersham Pharma Biotech.

Cell Culture—Human hepatocellular carcinoma cell line HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin (200 units/ml), and streptomycin (50 µg/ml). All treatments were started after maintaining the cells for 12 h in Dulbecco’s modified Eagle’s medium with 0.1% bovine serum albumin (BSA). Then cells were cultured in Dulbecco’s modified Eagle’s medium containing 0.1% BSA with varying concentrations of test substances and collected for Western blot analysis, MAPK assay, and Akt kinase assay. H4-II-E cells were maintained in α-modified Eagle’s medium containing 10% fetal bovine serum, penicillin (200 units/ml), and streptomycin (50 µg/ml). All treatments were started after maintaining the cells for 12 h in α-modified Eagle’s medium with 0.1% BSA. Then cells were cultured in α-modified Eagle’s medium containing 0.1% BSA with varying concentrations of test substances and collected for Western blot analysis and Northern blot analysis.

Western Blot Analysis—Cells were lysed in lysis buffer (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml aprotinin) for 30 min on ice and cleared by centrifugation for 20 min at 4°C. Immunoprecipitation was performed by incubating the supernatant fraction with antibodies specific for IRS-1, insulin receptor β, GRB2, p85 P13K, IRS-2, or SHC overnight at 4°C, followed by binding to protein A-Agarose (Amersham Pharmacia Biotech). After a 2-h incubation at 4°C, the incubation was centrifuged briefly at 10,000 g, and the agarose was washed with 20 mM Hepes, pH 7.0, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl, 1 mM sodium orthovanadate and recentrifuged. This was repeated three times after which Laemmli medium was added and the agarose was heated at 100°C for 5 min. Immunoprecipitated proteins were fractionated on 7.5% acrylamide gel and blotted to polyvinylidene difluoride membrane for 30 min. Immunoblotting was blocked by incubation in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% skim milk for 1 h. Blots were incubated with horseradish peroxidase-conjugated anti-phosphotyrosine antibodies RC 20 for 1 h to detect tyrosine-phosphorylated IRS-1, IRS-2, and SHC. Blots were incubated with...
anti-IRS-1 for 1 h and then horseradish peroxidase-conjugated anti-rabbit IgG for 1 h to detect association of GRB2 or PI3K with IRS-1. A chemiluminescent peroxidase substrate (ECL, Amersham Pharmacia Biotech) was applied according to the manufacturer’s instructions, and the membranes were exposed briefly to x-ray film.

**MAPK in Vitro Kinase Assays**—MAPK activity of HepG2 cells was measured by using MAPK assay kit according to the manufacturer’s instructions. In short, the cells were washed with PBS, lysed in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycero-phosphate, 1 mM Na3VO4, 1 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride), scraped off the dishes, transferred to microcentrifuge tubes, and microcentrifuged at 4°C for 10 min at 4°C. The supernatant was used for immunoprecipitation of activated MAPKs. The supernatant was incubated with immobilized phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibody for 4 h and microcentrifuged for 30 s at 4°C. The pellets were washed twice with ice-cold lysis buffer and twice with kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2). The pellets were incubated with 200 μM ATP and 2 μg of Elk-1 fusion protein for 30 min at 30°C. The reaction was terminated by 25 μl of 3× SDS sample buffer (187.5 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol, 150 mM dithiothreitol, 0.3% (w/v) bromophenol blue). Samples were boiled, separated by electrophoresis through a 10% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membranes for 30 min. Immunoblotting was done by incubation in TBST (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) containing 5% skim milk at room temperature for 1 h and then probed with a 1:1000 dilution of anti-phospho-Elk-1 antibody. The membranes were washed three times in TBST and incubated with a 1:1000 dilution of horseradish peroxidase-linked anti-rabbit IgG antibody and then reacted with LumiGLO reagent according to the manufacturer’s instructions. The membranes were exposed briefly to x-ray film.

**Akt in Vitro Kinase Assays**—Akt kinase activity of HepG2 cells was measured by using Akt kinase assay kit according to the manufacturer’s instructions. In short, the cells were washed with PBS, lysed in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycero-phosphate, 1 mM Na3VO4, 1 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride), scraped off the dishes, transferred to microcentrifuge tubes, and microcentrifuged at 4°C for 10 min. The supernatant was used for immunoprecipitation of activated Akt kinase. The supernatant was incubated with immobilized Akt antibody for 2–3 h and microcentrifuged for 30 s at 4°C. The pellets were washed twice with cold lysis buffer and twice with kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2). The pellets were incubated with 200 μM ATP and 1 μg of GS3-kinase fusion protein for 30 min at 30°C. The reaction was terminated by 25 μl of 3× SDS sample buffer (187.5 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol, 150 mM dithiothreitol, 0.3% (w/v) bromophenol blue). Samples were boiled, separated by electrophoresis through a 12% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane for 30 min. Immunoblotting was done by incubation in TBST (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) containing 5% skim milk at room temperature for 1 h and then probed with a 1:1000 dilution of anti-phospho-GS3-kinase antibody. The membranes were washed three times in TBST and incubated with a 1:1000 dilution of horseradish peroxidase-linked anti-rabbit IgG antibody and then reacted with LumiGLO reagent according to the manufacturer’s instructions. The membranes were exposed briefly to x-ray film.

**Reverse Transcribease-Polymerase Chain Reaction**—Total RNA was prepared from H4-II-E cells using Trizol. Twenty μg of total RNA was heat-denatured for 10 min at 70°C in buffer containing 50% formamide, 2.4 M formaldehyde, 1 × 0.02 M MOPS, 50 mM sodium acetate, 10 mM Na3EDTA, and then loaded onto a 1.2% agarose/formaldehyde gel made up in 1× TBE, pH 7.4, 50 V for 3–4 h. After electrophoresis, DNA was transferred onto a Hybond N membrane by capillary action and fixed onto the filters by ultraviolet light cross-linking. The blot was probed with either a rat PECK or rat β-actin probe labeled by the Gene Images random prime labeling module and detected by the Gene Images CDP-star detection module according to the manufacturer’s instructions, and the membranes were exposed to x-ray film for 30–60 min.

**Cell Proliferation Assays**—Approximately 48–72 h before the assay, cells were seeded into 24-well plates at 1–3 × 103 cells/well. When cells reached 60–70% confluence, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 0.1% BSA, and then EPA, docosahexaenoic acid, arachidonic acid, oleic acid, or palmitic acid was added at the indicated concentration. The cultures were incubated in a CO2 incubator for 72 h at 37°C. One day later, the cells were washed with phosphate-buffered saline and stripped with 0.05% trypsin, 0.53 mM EDTA. The cell number was then determined using a cell counter. All samples were assayed in duplicate, and each experiment was repeated at least three times. Representative data are presented.

**Statistical Analysis**—The data were expressed as the mean ± S.E. unless noted otherwise. Statistics were analyzed by one-way repeated measures analysis of variance with a significant level of 0.05.

**RESULTS**

The effect of EPA on the tyrosine-phosphorylated cellular proteins was studied in HepG2 cells by immunoblot analysis with antibodies to phosphotyrosine (anti-Tyr(P)). Treatment for 24 h with 100 μM EPA caused a significant increase in tyrosine-phosphorylated IRS-1 levels in HepG2 cells compared with those with vehicle treatment (455 ± 44 versus 105 ± 6% basal level, p < 0.01, n = 5) (Fig. 1A). When HepG2 cells were incubated with 1–1000 μM EPA for 24 h, EPA caused a significant and dose-dependent increase in tyrosine-phosphorylated IRS-1 levels in the cells as shown in Fig. 1B. HepG2 cells were treated with 100 μM EPA for 24 h, followed by stimulation with 100 nM insulin for 1 min. EPA and insulin significantly increased tyrosine-phosphorylated IRS-1 levels up to 362 ± 34 and 618 ± 35 densitometric units (DU), respectively, compared with vehicle alone (73 ± 6 DU).

Combined stimulation with EPA and insulin resulted in a further increase of tyrosine-phosphorylated IRS-1 levels up to 714 ± 34 DU (p < 0.01, n = 5) (Fig. 1C). The effect of EPA on IRS-1 phosphorylation is not mediated via insulin receptor (IR), because tyrosine phosphorylation of the insulin receptor β chain was not different be-
The amount of IR was determined in the same blot by anti-IR. Statistical significance is shown by asterisks. An arbitrary value of 100 was assigned to the basal level before treatment. Statistical significance is shown by asterisks. All values are expressed as the mean ± S.E. of tyrosine phosphorylation of IRS-1 after densitometric analysis (n = 5). HepG2 cells were treated with 100 μM EPA for 24 h followed by 100 nM insulin for 1 min. Cell extracts were immunoprecipitated (IP) with antibodies to IRS-1 and immunoblotted with anti-Tyr(P) (Anti-pTyr). The representative result is shown in the upper panel. The lower panel, all values are expressed as the mean ± S.E. of tyrosine phosphorylation of IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks. * p < 0.01 versus control; ** p < 0.01 versus insulin-treated control. B, HepG2 cells were treated for 24 h with vehicle alone (C), 100 μM EPA, 100 μM docosahexaenoic acid (DHA), 100 μM arachidonic acid (AA), 100 μM oleic acid (OA), and 100 μM palmitic acid (PA). Control cells (lane 1) gave 85 ± 13 DU. EPA-treated cells (lane 2) gave 329 ± 6 DU. Cells treated with docosahexaenoic acid (lane 3) gave 76 ± 9 DU. Cells treated with arachidonic acid (lane 4) gave 81 ± 9 DU. Cells treated with oleic acid (lane 5) gave 82 ± 10 DU. The representative result is shown in the upper panel. In the lower panel, all values are expressed as the mean ± S.E. of tyrosine phosphorylation of IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks. * p < 0.01 versus control. The amount of IR was determined in the same blot by anti-IR.

Fig. 1. EPA up-regulates tyrosine phosphorylation of IRS-1. A, time-dependent effects of EPA on tyrosine phosphorylation of IRS-1. Serum-starved HepG2 cells were treated with 100 μM EPA for 0, 6, 12, and 24 h. Cell extracts were immunoprecipitated (IP) with antibodies to IRS-1 and immunoblotted with anti-Tyr(P) (Anti-pTyr). The representative result is shown in the upper panel. In the lower panel, all values are expressed as the mean ± S.E. of tyrosine-phosphorylated IRS-1 after densitometric analysis (n = 5). HepG2 cells were treated with 100 μM EPA (○) or untreated (●). An arbitrary value of 100 was assigned to the basal level before treatment. Statistical significance is shown by asterisks. * p < 0.01 versus vehicle control. B, dose-dependent up-regulation by EPA of tyrosine phosphorylation of IRS-1. HepG2 cells were treated with 1–1000 μM EPA for 24 h. Control cells (lane 1) gave 66 ± 6 DU. Cells treated with 1, 10, 100, and 1000 μM EPA gave 86 ± 18, 197 ± 16, 269 ± 20, and 286 ± 19 DU, respectively. A representative result is shown in the upper panel. In the lower panel, all values are expressed as the mean ± S.E. of tyrosine phosphorylation of IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks. * p < 0.01 versus vehicle control. C, serum-starved HepG2 cells were treated with 100 μM EPA for 24 h followed by 100 nM insulin for 1 min. Cell extracts were immunoprecipitated with antibodies to IRS-1 and immunoblotted with anti-Tyr(P). The representative result is shown in the upper panel. In the lower panel, all values are expressed as the mean ± S.E. of tyrosine phosphorylation of IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks. * p < 0.01 versus control; ** p < 0.01 versus insulin-treated control. B, HepG2 cells were treated for 24 h with vehicle alone (C), 100 μM EPA, 100 μM docosahexaenoic acid (DHA), 100 μM arachidonic acid (AA), 100 μM oleic acid (OA), and 100 μM palmitic acid (PA). Control cells (lane 1) gave 85 ± 13 DU. EPA-treated cells (lane 2) gave 329 ± 6 DU. Cells treated with docosahexaenoic acid (lane 3) gave 76 ± 9 DU. Cells treated with arachidonic acid (lane 4) gave 81 ± 9 DU. Cells treated with oleic acid (lane 5) gave 82 ± 10 DU. Cells treated with palmitic acid (lane 6) gave 82 ± 10 DU. The representative result is shown in the upper panel. In the lower panel, all values are expressed as the mean ± S.E. of tyrosine phosphorylation of IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks. * p < 0.01 versus control. The amount of IR was determined in the same blot by anti-IR.

Fig. 2. EPA up-regulates tyrosine phosphorylation of IRS-1 in H4-II-E cells and down-regulates tyrosine phosphorylation of SHC in HepG2 cells. A, serum-starved H4-II-E cells were treated with 100 μM EPA for 24 h followed by 100 nM insulin for 1 min. Cell extracts were immunoprecipitated (IP) with antibodies to IRS-1 and immunoblotted with anti-Tyr(P) (Anti-pTyr). Control cells (lane 1) gave 52 ± 5 DU. Insulin-treated cells (lane 3) gave 284 ± 22 DU. Cells treated with EPA and insulin (lane 4) gave 371 ± 30 DU. Cells treated with EPA alone (lane 2) gave 202 ± 21 DU. The representative result is shown in the upper panel. In the lower panel, all values are expressed as the mean ± S.E. of tyrosine phosphorylation of IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks. * p < 0.01 versus vehicle control; ** p < 0.01 versus insulin-treated control. B, HepG2 cells were treated with 100 μM EPA for 0, 6, 12, and 24 h. Cell extracts were immunoprecipitated (IP) with antibodies to IRS-1 and immunoblotted with anti-Tyr(P). Control cells (lane 1) gave 58 ± 6 DU. Insulin-treated cells (lane 3) gave 239 ± 39 DU. Cells treated with EPA and insulin (lane 4) gave 248 ± 29 DU. Cells treated with EPA alone (lane 2) gave 64 ± 10 DU. The representative result is shown in the upper panel. In the lower panel, all values are expressed as the mean ± S.E. of tyrosine phosphorylation of IRS-1 after densitometric analysis (n = 5). Statistical significance is shown by asterisks. * p < 0.01 versus control. The amount of IR was determined in the same blot by anti-IR.

between the presence and absence of EPA (Fig. 1D). EPA also increased tyrosine phosphorylation of IRS-1 in H4-II-E cells (Fig. 2A). Furthermore, we tested the effect of other n-3 PUFAs, docosahexaenoic acid, n-6 PUFAs, arachidonic acid, monounsaturated fatty acid, oleic acid, saturated fatty acid, and palmitic acid on the tyrosine phosphorylation of IRS-1.
When HepG2 cells were pretreated with 100 nM EPA for 3 min and insulin for 3 min, the amount of GRB2--associated IRS-1 (basal, 118 ± 16 DU) was increased either by EPA (214 ± 26 DU, p < 0.05) or by insulin (298 ± 37 DU, p < 0.01, n = 5) as well as by combined treatment with EPA and insulin (307 ± 30 DU, p < 0.01, n = 5) (Fig. 3A). No additive increase of GRB2-associated IRS-1 was noticed when the cells were treated with both EPA and insulin (Fig. 3A). Similarly treated cells were analyzed for the association of PI3K with IRS-1. The amount of PI3K was determined by the amount of phosphorylated Elk1 fusion protein. The representative result is shown in the upper panel. In the lower panel, all values are expressed as the mean ± S.E. (Fig. 3A). Statistical significance is shown by asterisks. *, p < 0.05; **, p < 0.01 versus control. The amount of PI3K was determined by the amount of phosphorylated Elk1 fusion protein after densitometric analysis (n = 5). Statistical significance is shown by asterisks. *, p < 0.05 versus control. **, p < 0.01 versus insulin-treated cells.

MAPK activity in vehicle-treated cells (162 ± 10 DU) was increased either by EPA (251 ± 11 DU, p < 0.01) or by insulin (290 ± 25 DU, p < 0.01) as well as by combined treatment with EPA and insulin (476 ± 58 DU, p < 0.01 versus basal; p < 0.01 versus insulin-treated cells (Fig. 3B). An additive increase of PI3K associated with IRS-1 was noticed by combined treatment with EPA and insulin (Fig. 3B).

Furthermore, since MAPKs and Akt are downstream substrates for GRB2 and PI3K, respectively, we tested whether MAPKs and Akt are involved in cellular responses to EPA. Phosphorylated active MAPK was collected from cell lysates using anti-phospho-MAPK antibody, and its activity was determined by the amount of phosphorylated Elk1 fusion protein. The representative result is shown in the upper panel. In the lower panel, all values are expressed as the mean ± S.E. of the amount of phosphorylated Elk1 fusion protein after densitometric analysis (n = 5). Statistical significance is shown by asterisks. *, p < 0.01 versus control. **, p < 0.01 versus insulin-treated cells. **, p < 0.01 versus insulin-treated cells (Fig. 4A). These findings were incompatible with the association of PI3K with IRS-1 but compatible with tyrosine phosphorylation of SHC. We also measured the Akt kinase activity determined by the amount of phosphorylated glycogen synthase kinase-3 fusion protein. Similarly treated cells were analyzed for the Akt kinase activity. Akt kinase activity in vehicle-treated cells (162 ± 10 DU) was increased either by EPA (251 ± 11 DU, p < 0.01) or by insulin (290 ± 25 DU, p < 0.01) as well as by combined treatment with EPA and insulin (476 ± 58 DU, p < 0.01 versus basal; p < 0.01 versus insulin-treated cells (Fig. 4B).
treated cells, \( n = 5 \) (Fig. 4B). These findings were correlated with PI3K-associated IRS-1.

Next, we examined the mechanism by which EPA stimulates the tyrosine phosphorylation of IRS-1. EPA is a natural ligand for peroxisome proliferator-activated receptors (PPARs) and activates all three PPARs (PPAR-\( \alpha \), PPAR-\( \delta \), and PPAR-\( \gamma \)) with the highest affinity to PPAR-\( \alpha \). We tested the effect of other PPAR ligands on the tyrosine phosphorylation of IRS-1 in HepG2 cells. The effective dose of PPAR-\( \alpha \)-specific ligand (clofibrate), PPAR-\( \delta \)-ligand (cPGI\(_2\)), or PPAR-\( \gamma \)-specific ligand (troglitazone) was chosen based on recent studies (20–22). None of these ligands affected the amount of tyrosine-phosphorylated IRS-1 (Fig. 5A).

It has been reported that high intracellular glucosamine concentrations increase leptin production in adipose tissue (9). We also reported that EPA up-regulates leptin mRNA expression and its secretion through hexosamine biosynthetic pathway in 3T3-L1 adipocytes (8). Increased inflow of free fatty acid to HepG2 cells produces increased amounts of fatty acyl-CoA, causing the inhibition of glycolysis, and subsequently increases glucose 6-phosphate, which increases the substrate for glucosamine:fructose-6-phosphate amidotransferase, which plays a critical role as the rate-limiting enzyme in glucosamine biosynthesis. Glutamine:fructose-6-phosphate amidotransferase enhances the production of glucosamine 6-phosphate and its metabolites formed by subsequent acetylation and uridylation of glucosamine 6-phosphate (9, 11, 12). EPA would increase intracellular glucosamine 6-phosphate through this mechanism. DON, a well-established inhibitor of glutamine:fructose-6-phosphate amidotransferase (23), however, did not affect EPA-induced tyrosine phosphorylation of IRS-1 (Fig. 5A).

To assess whether the step of protein synthesis is involved in stimulation by EPA of the tyrosine phosphorylation of IRS-1, HepG2 cells were treated with 100 \( \mu \)M EPA in the absence or presence of 20 \( \mu \)g/ml cycloheximide (CHX), a protein synthesis inhibitor, for 24 h, and IRS-1 was analyzed by immunoblot analysis with antibodies to phosphotyrosine. CHX blunted EPA-induced tyrosine phosphorylation of IRS-1 (Fig. 5C), although the amount of IRS-1 was decreased by CHX treatment.

We also investigated whether EPA affects glucose homeostasis in cell culture. Hepatic and renal gluconeogenesis is a major factor in maintaining glucose homeostasis. The rate-limiting enzyme of gluconeogenesis is PEPCK. This enzyme has no critical role as the rate-limiting enzyme in glucosamine biosynthesis. Glutamine:fructose-6-phosphate amidotransferase, which plays a critical role as the rate-limiting enzyme in glucosamine biosynthesis, is up-regulated by EPA (24–26). The amount of PEPCK mRNA in HepG2 cells was decreased by EPA treatment and, in contrast, increased by arachidonic acid treatment as compared with control cells (Fig. 6). This observation is compatible with the result that EPA stimulated the IRS-1-PI3K pathway.

Next, we tested the effects of EPA on cell proliferation, considering the difference between EPA and insulin with regard to the regulation of MAPK activity in HepG2 cells. We found that EPA inhibited proliferation of HepG2 cells in a time- and dose-dependent manner (Fig. 7, A and B). The number of HepG2 cells was decreased by \(-30\%\) following a 72-h incubation with 100 \( \mu \)M EPA (Fig. 7A). The phenotypic features were not changed. To exclude the possibility that EPA acted as a death factor and accelerated cell death of HepG2 cells, we counted the numbers of live and dead cells. The ratio of dead cells to the total cell number of EPA-treated cells was not significantly different from that in vehicle-treated cells (Table I). Furthermore, to investigate whether the decrease in the cell number was linked to apoptosis, we tested the DNA fragmentation showing the typical oligosomal “ladder” configuration by ApoLadder EX (Takara Biomedicals). There were no ladder formation in the cells (data not shown). EPA-inhibited cell proliferation was dose-dependent (Fig. 7B), and the result of the cell proliferation assay using MTS solution (Fig. 7C) was correlated well with that of the decrease in cell number induced by EPA.
DU, lane 1 basal amount of cytoplasmic PEPCK mRNA was increased by 100 H9262 and H11021 *isks p shown by reblotting the membrane with a probe corresponding to rat vehicle (lane 1 mRNA. The basal amount of cytoplasmic PEPCK mRNA (1167 H11006 cytoplasmic RNA with a DNA probe corresponding to rat PEPCK with vehicle alone (lane 1), 100 μm arachidonic acid (AA) (lane 4), 100 μm oleic acids (OA) (5), and 100 μm palmitic acid (PA) (6). Northern blot analysis was done on cytoplasmic RNA with a DNA probe corresponding to rat PEPCK mRNA. The basal amount of cytoplasmic PEPCK mRNA (1167 ± 74 DU, lane 1) was reduced by 100 μm EPA (92 ± 20 DU, p < 0.01, lane 2) and by 10 μm EPA (831 ± 25 DU, p < 0.01, lane 3). In contrast, the basal amount of cytoplasmic PEPCK mRNA was increased by 100 μm arachidonic acid (2024 ± 148 DU, p < 0.01, lane 4). One hundred μm oleic acid (1207 ± 104 DU, lane 5) as well as 100 μm palmitic acid (1207 ± 104 DU, lane 6) did not change significantly compared with vehicle (lane 1). The same amount of RNA was present in each lane, as shown by reblotting the membrane with a probe corresponding to rat actin mRNA (lower panel). Statistical significance is shown by asterisks. * p < 0.01 versus vehicle control.

This observation is compatible with the finding that EPA inhibited MAPK activity in HepG2 cells. We also tested the effect of other fatty acids, docosahexaenoic acid, arachidonic acid, oleic acid, and palmitic acid on proliferation of HepG2 cells. Other fatty acids, except EPA, did not inhibit cell proliferation (Fig. 7D). Furthermore, we tested the effects of EPA on cell proliferation in other cell lines, rat L6 myocytes, mouse 3T3-L1 preadipocytes, and the human gastric adenocarcinoma cell line, MKN 45. EPA did not inhibit cell proliferation of these cells (Fig. 7E).

**DISCUSSION**

We demonstrated for the first time that EPA treatment for 24 h caused stimulation of both the basal and insulin-induced IRS-1-Pi3K pathway in HepG2 cells as well as reduction of the amount of PEPCK mRNA expression in H4-II-E cells. Furthermore, it is of great interest that tyrosine phosphorylation of IRS-1 is specific for EPA, since other fatty acids cannot induce tyrosine phosphorylation of IRS-1. These findings are consistent with recent reports that EPA improves insulin sensitivity (1–4). Moreover, it is our surprise that EPA inhibited MAPK activity as well as proliferation of HepG2 cells.

We focused on clarifying the mechanism by which EPA stimulated tyrosine phosphorylation of IRS-1. First, we examined the possibility of an involvement of PPARs. PPARs are nuclear receptors for fatty acids that regulate glucose and lipid homeostasis. The hypolipidemic actions of the fibrates appear to be primarily mediated through PPAR-α, whereas the glucose-lowering effects of the thiazolidinediones are mediated through PPAR-γ (27). EPA binds efficiently all three PPARs and activates these receptors in cell-based assays (20, 22). However, neither PPAR ligands mimicked the tyrosine phosphorylation of IRS-1 by EPA, indicating that PPARs probably are not involved in this mechanism, although the involvement of PPAR-δ is not completely ruled out. Human PPAR-δ-specific ligands have not been identified so far, and cPGI 2, a ligand of PPAR-δ and PPAR-α, failed to stimulate tyrosine phosphorylation of IRS-1. Moreover, neither the function nor the array of genes regulated by PPAR-δ is completely known. Therefore, the role of PPAR-δ in the tyrosine phosphorylation of IRS-1 is not conclusive in our study. EPA has lipid-lowering effects and enhances insulin sensitivity in human and rodents, which is possibly mediated through PPAR-α and PPAR-γ, since these effects are similar to those of fibrates and thiazolidinediones. We speculate that EPA can improve insulin sensitivity and dyslipidemia possibly through its stimulation of the insulin-induced tyrosine-phosphorylated IRS-1 as well as PPAR activation in accordance with dietary EPA intake. The second
results, we propose that EPA may suppress cell proliferation, not inhibit cell proliferation in other cell lines, L6 myocytes, EPA, did not inhibit proliferation of HepG2 cells, and EPA did not inhibit cell proliferation in other cell lines, L6 myocytes, 3T3-L1 preadipocytes, and MKN 45 cells. On the basis of these results, we propose that EPA may suppress cell proliferation, at least in HepG2 cells, by inhibiting the MAPK pathway.

Finally, the evidence that EPA caused not only up-regulation of IRS-1-P13K pathway as well as down-regulation of gluconeogenesis but also inhibition of both the MAPK pathway and cell proliferation warrants further study to identify unknown mediators involved in EPA action cross-talking with the insulin signaling pathway.

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REFERENCES
1. Storlien, L. H., Kraegen, E. W., Chisholm, D. J., Ford, G. L., Bruce, D. G., and Pascoe, W. S. (1987) Science 237, 885–888
2. Popp-Snijders, C., Schouten, J. A., Heine, R. J., van der Meer, J., and van der Veen, E. A. (1987) Diabetes Res. 4, 141–147
3. Storlien, L. H., Jenkins, A. B., Chisholm, D. J., Pascoe, W. S., Khouri, S., and van der Veen, E. A. (1991) Diabetes 40, 260–269
4. Mori, Y., Murakawa, Y., Katoh, S., Yokoyama, J., Tajima, N., Ikeda, Y., Nobukata, H., Ishikawa, T., and Shibutani, Y. (1997) Metabolism 46, 1458–1464
5. Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995) Science 269, 540–543
6. Chen, G., Koyama, K., Yuan, X., Lee, Y., Zhou, Y. T., O’Doherty, R., Newgard, C. B., and Ungar, R. H. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 14795–14799
7. Shimomura, I., Hammer, R. E., Ikemoto, S., Brown, M. S., and Goldstein, J. L. (1999) Nature 401, 73–76
8. Murata, M., Kaji, H., Takahashi, Y., Iida, K., Mizuno, I., Okimura, Y., Abe, H., and Chihara, K. (2000) Biochem. Biophys. Res. Commun. 270, 343–348
9. Wang, J., Liu, R., Hawkins, M., Barzilai, N., and Rossetti, L. A. (1998) Science 282, 684–688
10. Rodent, M., Price, T. B., Perseghin, G., Petersen, K. F., Rothman, D. L., Cline, G. W., and Shuman, G. I. (1996) J. Clin. Invest. 97, 2859–2865
11. Hawkins, M., Barzilai, N., Liu, R., Hu, M., Chen, W., and Rossetti, L. (1997) J. Clin. Invest. 99, 2173–2182
12. Hawkins, M., Angelov, I., Liu, R., Barzilai, N., and Rossetti, L. (1997) J. Biol. Chem. 272, 4889–4895
13. Willet, W. C. (1994) Science 264, 532–537
14. Carroll, K. K., Braden, L. M., Bell, J. A., and Kalamepsilon;ehmagham, R. (1986) Cancer 58, 1818–1825
15. Rose, D. P., Boyar, A. P., and Wynder, E. L. (1986) Cancer 58, 2263–2271
16. Carroll, K. K. (1997) Curr. Opin. Lipidol. 8, 53–56
17. Rajas, F., Croset, M., Zitoun, C., Montano, S., and Mithieux, G. (2000) Biochem. Biophys. Res. Commun. 272, 343–348
18. Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) Proc. Natl. Acad. Sci. U.S.A. 93, 5796–5801
19. Forman, B. M., Chen, J., and Evans, R. M. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 4312–4317
20. Granner, D., Andreone, T., Sasaki, K., and Beale, E. (1983) Nature 305, 549–551
21. Xing, L., and Quinn, P. G. (1993) Mol. Endocrinol. 7, 1484–1494
22. Cahill, D. A., Novick, D., and Rubinstein, M. (1996) Science 274, 1185–1188
23. Willson, T. M., and Wahl, M. (1997) Curr. Opin. Chem. Biol. 1, 235–241
24. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) Nature 352, 73–77
25. Sun, X. J., Haag, B. L., Zhang, Y., Chuang, L. M., Zhang, Y., Yang-Feng, T. White, M. F., and Kahn, C. R. (1993) Diabetes 42, 1041–1054
26. Sun, X. J., Forman, B. M., Myers Jr, M. G., Miralpeix, M., and White, M. F. (1996) Mol. Cell. Biol. 16, 7418–7428
27. Skoink, E. Y., Lee, C. H., Batzer, A., Vicentini, L. M., Zhou, M., Daly, R., Myers, M. J., Jr., Backer, J. M., Ulbrich, A., White, and Schlessinger, J. (1993) EMBO J. 12, 1529–1538
28. Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 19443–19450
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