Celecoxib Decreases Fatty Acid Synthase Expression *via* Down-Regulation of c-Jun N-Terminal Kinase-1

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are inhibitors of cyclooxygenase (COX). Our previous observations that celecoxib, a COX-2-specific inhibitor, not only inhibits rat mammary carcinogenesis, but also decreases fat deposition in rats fed a high-fat diet, prompted us to determine whether celecoxib affects lipid metabolism. At 57 days of age, two groups of 10 female Sprague Dawley rats were pair-fed a high-fat diet with or without 1500 ppm celecoxib for 15 weeks. Compared with controls, celecoxib-treated rats had 44.4% less hepatic triglycerides and 22.6% less intra-abdominal adipose tissue mass. In the liver and adipose tissue of several genes involved in fat metabolism and mobilization that we measured, only fatty acid synthase (FAS) was significantly down-regulated by celecoxib treatment. There were no differences in the level of prostaglandin E_2 in these tissues, indicating that celecoxib decreases fat accumulation by down-regulating FAS through a COX-2–independent mechanism. Among the potential molecular targets by which celecoxib may regulate FAS expression, only c-Jun N-terminal kinase-1 (JNK1) was significantly down-regulated. Furthermore, a known inhibitor of JNK suppressed FAS expression in rat hepatocytes. Our observations suggest that celecoxib suppresses FAS expression and decreases fat accumulation by down-regulating JNK1. Exp Biol Med 232:643–653, 2007

Key words: celecoxib; fatty acid synthase; c-Jun N-terminal kinase-1; rat; high-fat diet

Introduction

Cyclooxygenase (COX) is the rate-limiting enzyme in the synthesis of prostaglandins from arachidonic acid (1). There are two COX isoforms, a constitutive gene, COX-1, and an inducible gene, COX-2 (2). Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit COX activity and are widely used for the treatment of rheumatoid arthritis and osteoarthritis (3). Recently, a COX-1 variant designated COX-3 has been discovered that is highly expressed in the cerebral cortex (4). The inhibition of COX-3 in the central nervous system could be a mechanism by which NSAIDs decrease pain and possibly fever (4). In addition to COX, however, a series of new molecular targets for NSAIDs have recently been identified, including 15-lipoxygenase-1 (5), extracellular signal-regulated kinase 1/2 signaling (6), NF-κB (7), p70S6 kinase (8), $p21^{ras}$ signaling (9), and 3-phosphoinositide-dependent protein kinase-1 (PDK1) (10). These new molecular targets suggest new biological roles for NSAIDs.

Some NSAIDs, such as indomethacin, fenoprofen, and ibuprofen have been shown to activate the peroxisome proliferator-activated receptors (PPAR) α and γ (11). Since the PPARα activator fenofibrate reduces adiposity in C57BL/6 mice fed a high-fat diet (12), while the PPARγ activator rosiglitazone lowers serum triglyceride levels in *db/db* mice (13), NSAIDs may play a role in regulating lipid metabolism. Indeed, a recent study has shown that high doses of salicylates reverse hyperglycemia, hyperinsulinemia, and dyslipidemia in obese rodents by sensitizing insulin signaling (14).

Recently, celecoxib, an NSAID that is a much more potent inhibitor of COX-2 than COX-1, has been shown by us and others to possess strong chemopreventive activity against mammary, colon, and skin carcinogenesis in rodents (15–18). In our study, celecoxib-treated rats not only had lower mammary tumor incidence and tumor multiplicity, but also reduced body weight gain, less abdominal adipose tissue accumulation, and lower serum triglyceride levels compared with untreated controls (16). We administered the

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celecoxib, however, in a high-fat diet rich in n-6 polyunsaturated fatty acids, while other studies not showing any effect of celecoxib on body weight gain used low-fat diets (15, 17).

Generally, high-fat diets induce an increase in fatty acid oxidation (19) and a decrease in *de novo* fatty acid synthesis (20, 21). After prolonged exposure to a high-fat diet, however, *de novo* fatty acid synthesis can be induced by hyperinsulinemia in insulin-resistant obese animals (22). Several molecular targets have been shown to affect obesity and insulin resistance induced by high-fat diets. For example, in C57BL/6 mice, PPARα activators improve insulin sensitivity and reduce adiposity (12). In a recent study of the role of c-Jun N-terminal kinase 1 (JNK1) in obesity and insulin resistance, Hirosumi *et al*. (23) observed that the body weight gain of JNK1 knockout mice was similar to that of wild type mice when they were fed a low-fat diet, but lower when they were fed a high-fat diet. Osei-Hyiaman *et al*. (21) have demonstrated that anandamide acting at hepatic CB_1 receptors contributes to diet-induced obesity by increasing basal rates of fatty acid synthesis. These various results prompted us to investigate whether celecoxib plays a role in lipid metabolism, thereby decreasing fat accumulation induced by a high-fat diet.

Materials and Methods

Animals and Diets. All animal treatment protocols were reviewed by and were in compliance with the Animal Care and Use Committee of the University of Toronto. Fifty-day-old female Sprague Dawley rats, purchased from Charles River Laboratories (St. Constant, Quebec, Canada), were housed at 22 ± 2°C, 50% humidity with 12:12-hr light:dark cycle. Tap water was provided *ad libitum* throughout the experiment. The rats were acclimatized for 1 week on an AIN-93G diet. At 57 days of age, they were divided into two groups (10/group). The control group was fed a modified AIN-93G diet containing 18% safflower oil and 3% soybean oil at the expense of carbohydrate. The experimental group was fed the same diet supplemented with 1500 ppm celecoxib (supplied by Pharmacia, Skokie, IL). The control group was pair-fed to the mean daily food intake of the experimental group. Body weights were recorded weekly. After 15 weeks, all rats were fasted overnight and killed the next morning. Immediately after blood collection by cardiac puncture, livers, intra-abdominal adipose tissue (including retroperitoneal, parametrial, and mesenteric adipose) and gastrocnemius muscle were removed, weighed, immediately frozen in liquid nitrogen, and stored at –80°C.

Cell Culture and Treatment of Rat Hepatocytes. Rat hepatocytes (Cat. No. CRL-1439, ATCC, Rockville, MD) were cultured in Ham's F12K medium with 10% fetal bovine serum and kept in at 37°C humidified incubator with a mixture of 95% air and 5% CO_2. Because regenerating liver overexpresses FAS and JNK1 (24, 25), to simulate the nondividing hepatocytes in resting liver, we treated confluent cells with 20, 40 μ*M* celecoxib, or 10, 20 μ*M* JNK inhibitor SP600125 (Biomol Research Laboratories, Inc., Plymouth Meeting, PA) for various time periods. DMSO was used as vehicle with a final concentration of 0.1% in all cases. After treatments, hepatocytes were washed with ice-cold phosphate-buffered saline followed by incubation in cold lysis buffer (50 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* EGTA, 0.5 m*M* Na_3VO_4, 10 m*M* glycerophosphate, 5 m*M* NaF, 1 m*M* phenylmethylsulfonyl fluoride, and 40 μg/ml each of pepstatin A, aprotinin, and leupeptin). Whole cell lysates were stored at –80°C for Western analysis.

Biochemical Analysis. The following were analyzed in serum: triglycerides, measured enzymatically using Sigma Diagnostics Triglyceride (GPO-Trinder) reagent (Sigma, St. Louis, MO); insulin, measured by an insulin ^{125}I RIA kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA); free fatty acids (FFA), measured by an acyl-CoA synthetase (ACS)-acyl-CoA oxidase (ACOD) method (Wako Chemicals USA, Inc., Richmond, VA); γ-glutamyl transferase (γ-GT), measured by a γ-glutamyltransferase kit (Sigma). Liver and muscle triglyceride levels were measured as previously described (26). Briefly, tissue samples (200~250 mg) were extracted in 2 ml isopropanol. After centrifugation at 12,000 *g* for 10 mins, 10 μl of supernatant were used to measure the triglyceride concentration. Prostaglandin E_2 (PGE_2) levels in liver and adipose tissue were measured by an ELISA kit (Cedarlane Laboratories Limited, Hornby, Canada), using positive and negative controls that we previously described (27).

Gene Expression Analysis. Total RNA from livers, retroperitoneal adipose, and muscle tissues was isolated using TRI REAGNET (Sigma), and poly(A)$^+$ mRNA was purified using GenElute mRNA miniprep kits (Sigma). About 10 μg poly(A)$^+$ mRNA were elecrophoresed in 1% formaldehyde-agarose gels, transferred onto nylon membranes and probed with ^{32}P-labeled cDNA probes for fatty acid synthase (FAS), acyl-CoA oxidase (ACO), carnitine palmitoyltransferase I (CPT-I) (liver and muscle isoforms), hormone sensitive lipase (HSL), and lipoprotein lipase (LPL). cDNA probes were prepared by PCR using cDNAs from rat liver, retroperitoneal adipose tissue, or muscle as templates with the following primer pairs: FAS, 5′-AGAGGCTGTTCTCAAGGAAGG-3′, 5′-AGGGTACATCCCAGAGGAAGT-3′; ACO, 5′-CACTGCCTATGCCTTCCACT-3′, 5′-GGCCAAGAAGTGAGCCAAGT-3′; CPT-I liver isoform, 5′-CTGGATGATCCCTCAGAGCC-3′, 5′-CTCCATGGCTCAGACAATAC-3′; CPT-I muscle isoform, 5′-GATTCTCTGGAACTGCATCT-3′, 5′-CTGAGACACATCTACCTGTC-3′; HSL, 5′-CTGCGCATAGACTCCGTAAG-3′, 5′-GCCATAGACCCAGAGTTGCGT-3′; LPL, 5′-TCGTGCGAGCACTTCACCAG-3′, 5′-TCTGTGTCTAACTGCCACTT-3′. cDNA probes of β-actin and glyceraldehyde phosphate dehydrogenase (GAPDH) (Oncogene Research Products, La

Table 1. Body, Liver, and Intra-Abdominal Adipose Tissue Weights and Liver Triglyceride Levels in Rats Fed a High-Fat Diet With or Without 1500 ppm Celecoxib for 15 Weeks[a]

Group	Body weight (g)	Liver (% body weight)	Visceral adipose tissue (% body weight)	Liver triglycerides (mg/g)
Celecoxib	371.4 ± 12.1	2.8 ± 0.1**	10.6 ± 0.4**	5.9 ± 0.4*
Control	386.9 ± 15.7	2.2 ± 0.1	13.3 ± 0.5	8.3 ± 0.9

[a] Values are mean ± SEM; $n = 10$ per group.
* $P < 0.05$; ** $P < 0.01$ compared with controls.

Jolla, CA) were used to adjust for mRNA loading. Bands were quantified by electronic autoradiography (InstantImager, Packard Instrument Company, Meriden, CT).

Western Blot Analysis. Liver and retroperitoneal adipose tissue were homogenized on ice in a glass-glass tissue grinder with PBS containing 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin. The homogenates were centrifuged at 16,000 *g* for 15 mins, and the supernatants were stored at –80°C for Western analysis. The proteins from livers, retroperitoneal adipose tissue, and hepatocytes were separated by 10% SDS-PAGE. Proteins were transferred onto PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, CA) using a semi-dry blotter (C.B.S. Scientific Co., Del Mar, CA). Membranes were blocked with 5% nonfat dried milk in TTBS (10 m*M* Tris-HCL, pH 8.0, 100 m*M* NaCl, and 0.05% Tween 20) overnight at 4°C, then incubated for 2 hrs at room temperature with one of the following antibodies: mouse monoclonal antihuman FAS at 1:500 dilution (BD Transduction Laboratories, Mississauga, Canada); rabbit polyclonal antihuman JNK1 at 1:500 dilution; mouse monoclonal antihuman pJNK at 1:2000 dilution; rabbit polyclonal antihuman phospho-p38 MAP kinase at 1:1000 dilution; rabbit polyclonal antihuman phospho-Erk1/2 at 1:1000 dilution; mouse monoclonal antimouse pAkt/PKB (pSer473), rabbit polyclonal antimouse pAkt/PKB (pThr308) at 1:1000 (New England Biolabs, Ltd., Pickering, Ontario, Canada); rabbit polyclonal antihuman phospho-cAMP response element binding protein (pCREB) at 1:1000 dilution (Upstate Biotechnology, Lake Placid, NY). The membranes were then incubated with horseradish peroxidase-conjugated goat antimouse or antirabbit secondary antibodies (Santa Cruz Biotechnology) at 1:2000 dilution for 45 mins at room temperature. Membranes were stripped in 62.5 m*M* Tris pH 6.7, 100 m*M* β-mercaptoethanol, and 2% SDS, for 30 min at 50°C. β-Actin was used as a loading control. Bands were quantified using a FluorChem digital imager (Alpha Innotech Corp., San Leandro, CA).

Liver Enzyme Activity Analysis. FAS activity of livers (adipose tissue was not analyzed because of limited sample size) was measured by using a previously described method (28). Briefly, livers were homogenized on ice in 20 m*M* Tris-HCl, pH 7.5 containing 1 m*M* DTT, and 1 m*M* EDTA using a glass-glass tissue grinder. Homogenates were centrifuged at 12,000 *g* for 10 mins, and the supernatants were used for measuring FAS activity. One-hundred μg protein in a volume of 20 μl was added to 125 μl of 100 m*M* potassium phosphate, pH 7.0, containing 100 m*M* KCl and 0.5 m*M* NADPH. The reaction mixtures were prewarmed for 15 mins at 37°C, and reactions were started by the addition of 4.5 μl of a substrate mixture containing 25 nmol of acetyl-CoA and 25 nmol of malonyl-CoA together with 0.05 μCi (5 μl) of [2-^{14}C] malonyl-CoA (47.0 mCi/mmol; NEN Life Sciences Products, Inc., Boston, MA). Reactions were carried out at 37°C for 10 mins and were stopped by the addition of 1 ml of ice-cold 1 *N* HCl/methanol (6:4, v/v). Fatty acids were extracted with 1 ml of petroleum ether (Sigma), and incorporation of radioactivity into the fatty acids was assessed by scintillation counting.

Statistical Analysis. All data were expressed as mean ± SEM. The differences between the two groups were analyzed by Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Celecoxib-Treated Rats Accumulate Less Intra-Abdominal Adipose Tissue and Hepatic Triglycerides. In our previous carcinogenesis study (16), from 57 days of age, female Sprague Dawley rats had *ad libitum* access to a high-fat diet (modified AIN-93G diet containing 18% safflower oil and 3% soybean oil) containing 1500 ppm celecoxib, a dose that had been used by others with no adverse effects (15, 17). After 14 weeks, our rats had decreased body weight gain and lower serum triglyceride levels than controls not given celecoxib. There was no evidence of toxicity caused by the drug. In that study, however, we did not control food intake in the two groups. To be sure there would be no differences in intake levels, in the present experiment we pair-fed the treated and control rats. In the first 3–4 days of the experiment, celecoxib-treated rats had a lower food intake than controls, presumably due to reduced palatability, but the food intakes were not different between the two groups thereafter. After 15 weeks, there were no differences in the body weights of the two groups (Table 1). The weight of intra-abdominal adipose tissue, however, was significantly lower in the celecoxib-treated rats compared with controls (Table 1). Liver weights were slightly higher in the treated group (Table 1), but there were no differences in serum levels of γ-GT (Table 2), indicating that celecoxib did not induce

Table 2. Serum Levels of γ-Glutamyl Transferase (γ-GT), Insulin, Triglycerides, and Free Fatty Acids (FFA) in Rats Fed a High-Fat Diet With or Without 1500 ppm Celecoxib for 15 Weeks[a]

Group	γ-GT (units/ml)	Triglycerides (mg/dl)	Insulin (μIU/ml)	FFA (mEq/L)
Celecoxib	0.5 ± 0.1*	72.3 ± 8.9**	60.6 ± 5.7	0.8 ± 0.1
Control	0.7 ± 0.1	120.0 ± 35.3	57.7 ± 2.9	0.7 ± 0.1

[a] Values are mean ± SEM; $n = 10$ per group.
* $P < 0.48$; ** $P < 0.23$ compared with controls.

hepatotoxicity. Serum levels of triglycerides, insulin, and FFA were not different in the two groups after the 15-week period of this experiment (Table 2), but the celecoxib-treated animals had significantly lower levels of hepatic triglycerides than the controls (Table 1). Muscle triglyceride levels were similar in the two groups (data not shown).

FAS in Liver and Retroperitoneal Adipose Tissue Is Down-Regulated in Celecoxib-Treated Rats. To determine the metabolic pathway(s) by which celecoxib affects lipid metabolism in these animals, we measured the expression in liver, retroperitoneal adipose and muscle tissues of several genes involved in fatty acid metabolism. In the celecoxib-treated rats, hepatic FAS mRNA was significantly reduced to about 70% of controls

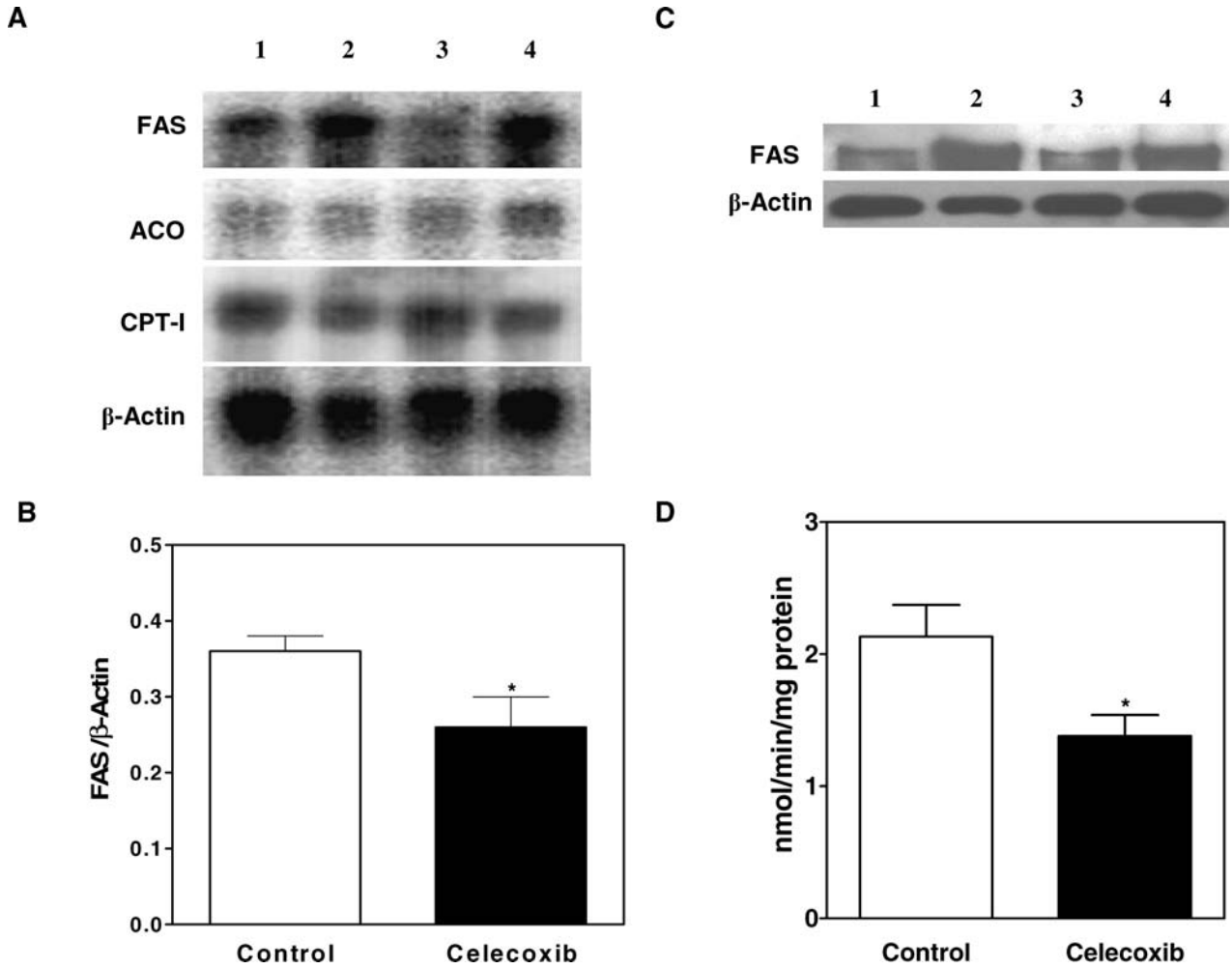

Figure 1. Celecoxib decreases hepatic FAS expression in rats fed a high-fat diet. (A) About 10 μg poly $(A)^+$ mRNA were subjected to Northern blotting followed by hybridization with the indicated cDNA probes. A cDNA probe for β-actin was used as a loading control. Lanes 1 and 3 are representative samples from celecoxib-treated rats; Lanes 2 and 4 are from control rats. (B) Quantification of FAS expression by densitometry in celecoxib-treated and control rats. Values are mean ± SEM ($n = 9$; * $P < 0.05$). (C) Protein levels of FAS in celecoxib-treated and control rats. Four μg protein were subjected to Western blot analysis as described in Materials and Methods. β-Actin was used as a loading control. Lanes 1 and 3 are representative samples from celecoxib-treated rats; Lanes 2 and 4 are from control rats. (D) Hepatic FAS activity in celecoxib-treated rats and control rats. Activity was assessed by ^{14}C-malonyl CoA incorporation into fatty acids. Values are mean ± SEM ($n = 9$; * $P < 0.05$).

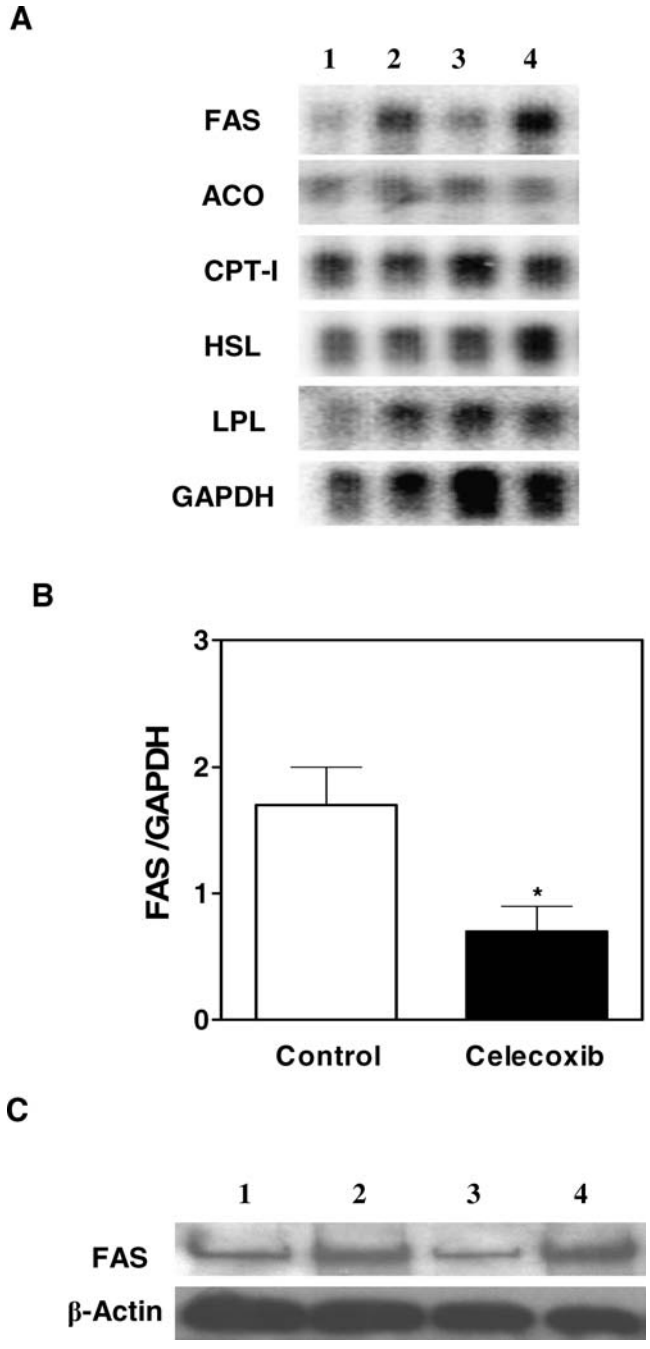

Figure 2. Celecoxib decreases FAS expression in retroperitoneal adipose tissue in rats fed a high-fat diet. (A) About 10 μg poly $(A)^+$ mRNA were subjected to Northern blotting followed by hybridization with the indicated cDNA probes. A cDNA probe for GAPDH was used as a loading control. Lanes 1 and 3 are representative samples from celecoxib-treated rats; Lanes 2 and 4 are from control rats. (B) Quantification of FAS expression by densitometry in celecoxib-treated and control rats. Values are mean ± SEM ($n = 4$; * $P < 0.05$). (C) Protein levels of FAS in celecoxib-treated and control rats. Six micrograms protein were subjected to Western blot analysis as described in Materials and Methods. β-Actin was used as a loading control. Lanes 1 and 3 are representative samples from celecoxib-treated rats; Lanes 2 and 4 are from control rats.

(Fig. 1A), while in the retroperitoneal fat pad, expression of FAS was reduced to about 50% of controls (Fig. 2A). FAS protein levels were also reduced in liver and retroperitoneal adipose tissue as shown by Western blot analysis (Figs. 1C and 2C, respectively). Furthermore, hepatic FAS enzyme activity was lower in celecoxib-treated rats than in controls (Fig. 1D). The hepatic expression of ACO and CPT-I (Fig. 1A) and the expression of ACO, CPT-I, HSL, and LPL in the retroperitoneal fat pad (Fig. 2A) were not different between the two groups. In muscle, there were no differences in expression of any these genes between the two groups (data not shown). To determine whether the changes in lipid metabolism in celecoxib-treated rats were mediated by inhibition of COX-2, we measured PGE_2 levels in liver and retroperitoneal adipose tissue. There were no differences, however, in PGE_2 levels between the two groups (Fig. 3).

JNK1 Is Down-Regulated in the Livers of Celecoxib-Treated Rats. Celecoxib has been shown to inhibit 3-phosphoinositide-dependent kinase 1 (PDK1) activity (10). This kinase phosphorylates Akt/PKB at Thr308 (29). To determine whether celecoxib down-regulates FAS by this mechanism in rat liver, we measured the levels of pAkt/PKB (pThr308). pThr308 levels, however, were not different in the livers of celecoxib-treated rats compared with untreated controls (data not shown). Akt/PKB is also a substrate of PDK2 that phosphorylates Ser473, and Ser473 phosphorylation plays an important role in the activation of Akt. Therefore, we examined the activity of Akt by Western blot with phosphoserine 473-specific antibody (30). Figures 4A and 4B show that levels of pSer473 were higher in celecoxib-treated rats than controls. The levels of total Akt/PKB did not differ between the two groups (data not shown). Celecoxib also has been demonstrated to decrease the phosphorylation of p38 and Erk1/2 (p44/42) in osteoarthritic chondrocytes (31), and both Erk1/2 and p38 participate in the activation of CREB (32, 33). The activation of this transcription factor has been shown to play an essential role in the insulin stimulation of FAS in HepG2 cells (34). However, we did not observe any difference between controls and celecoxib-treated rats in the hepatic levels of pErk1/2, pp38, or pCREB (Fig. 4A).

It has been shown that feeding rats a high-fat diet leads to the activation of hepatic JNK1, a stress-activated protein kinase (35), and that JNK1 knockout mice are resistant to obesity induced by a high-fat diet (23). Furthermore, down-regulation of JNK signaling has been shown to mediate the suppression of FAS expression in the livers of rats treated with pu-erh tea (36). Therefore, we explored whether the effect of celecoxib in our study is mediated by this molecular target. As shown in Figures 4A and 4B, the levels of JNK1 and pJNK1 were significantly lower in the livers of celecoxib-treated rats than those of controls.

In Rat Hepatocytes Celecoxib Decreases Levels of FAS and JNK1, and JNK Inhibitor Down-

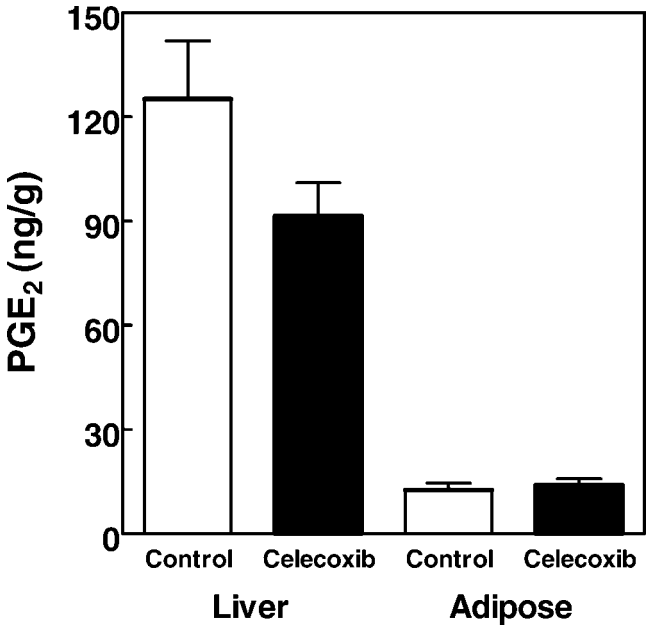

Figure 3. Effect of celecoxib on prostaglandin 2 (PGE_2) levels in liver and intra-abdominal adipose tissue in rats fed a high-fat diet. About 200~250 mg of liver and adipose tissue were used to measure PGE_2 levels using an ELISA kit as described in Materials and Methods. Values are mean ± SEM ($n = 8$; $P = 0.21$ for liver; $n = 6$ for adipose).

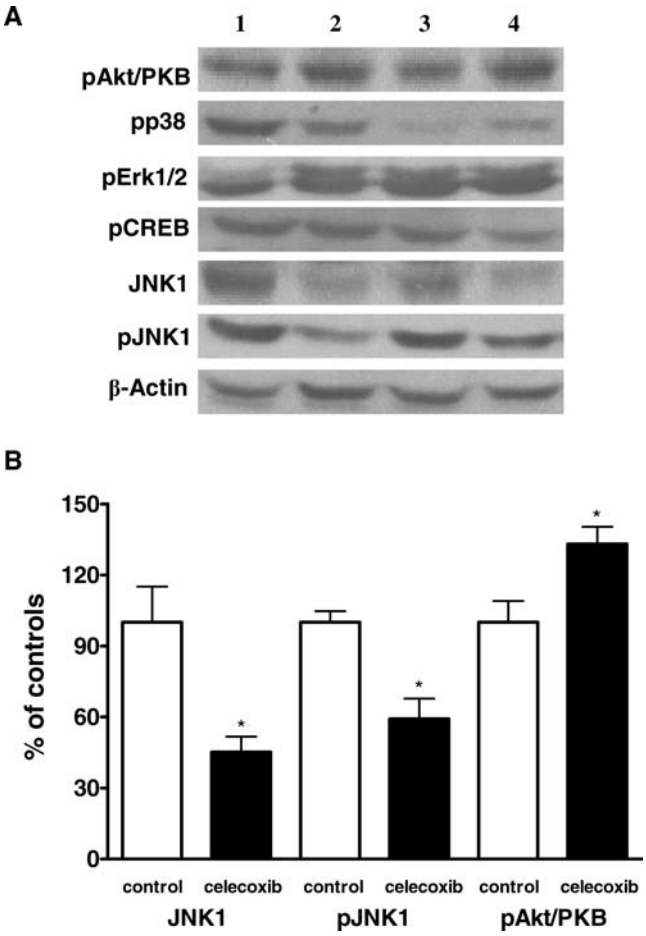

Figure 4. Celecoxib decreases levels of JNK1/pJNK1 but increases levels of pAkt/PKB (pSer473) in the livers of rats fed a high-fat diet. (A) Sixty μg protein was subjected to Western blot analysis as described in Materials and Methods. β-Actin was used as a loading control. Lanes 1 and 3 are representative samples from control rats; Lanes 2 and 4 are from celecoxib-treated rats. (B) Quantification by densitometry of JNK1, pJNK1, and pAkt/PKB in celecoxib-treated and control rats. Values are mean ± SEM ($n = 3$; * $P < 0.05$).

Regulates FAS Expression. To further explore the relationship between celecoxib, FAS, and JNK1, we treated rat hepatocytes with celecoxib at concentrations of 20 and 40 μ*M in vitro* for 24, 48, or 72 hrs. These concentrations of celecoxib are within the range of serum concentrations we measured previously when rats were administrated 1500 ppm of the drug in the diet (16), the same level as used in the present experiment. As expected, celecoxib decreased the expression of both FAS and pJNK1. Levels of pJNK1 were significantly down-regulated by 24 hrs (Fig. 5A and C, respectively). Inhibition of FAS expression, however, was not apparent until 72 hrs after celecoxib treatment (Fig. 5A and B, respectively). As in the *in vivo* study, celecoxib increased the levels of pAkt/PKB (pSer473) (Fig. 5D and E, respectively) but did not alter the levels of pErk1/2, pp38, and pCREB (Fig. 5D). To investigate further whether the down-regulation of JNK1 is causally related to the suppression of FAS expression, we treated rat hepatocytes with the known JNK inhibitor SP600125 (37). Figures 6A and 6B show that the JNK inhibitor significantly suppressed the expression of FAS.

Discussion

The purpose of this study was to determine whether the COX-2 inhibitor celecoxib affects lipid metabolism in rats fed a high-fat diet. After pair-feeding controls and celecoxib-treated rats for 15 weeks, the celecoxib-treated rats accumulated significantly less intra-abdominal fat and lower levels of hepatic triglycerides. To determine whether these effects were related to alterations of enzymes involved in fatty acid metabolism, we analyzed the expression in liver, retroperitoneal adipose, and muscle tissues of several important genes involved in fatty acid synthesis, fatty acid oxidation, and fat mobilization and distribution. FAS, a central enzyme in the pathway of *de novo* lipogenesis, catalyzes all of the steps in the conversion of malonyl-CoA, the product of acetyl-CoA carboxylase, to palmitate. FAS is abundant in liver and adipose tissue and is known to be regulated primarily at the level of transcription (38). Acyl-CoA oxidase (ACO) catalyzes the first step of peroxisomal long-chain fatty acid β-oxidation, while carnitine palmitoyltransferase I (CPT-I) is the rate-limiting enzyme of mitochondrial β-oxidation. Northern and Western analysis showed a clear down-regulation of FAS in both the liver and the retroperitoneal fat pad in celecoxib-treated rats compared with untreated controls. As expected, hepatic FAS activity was also lower in celecoxib-treated rats. Celecoxib

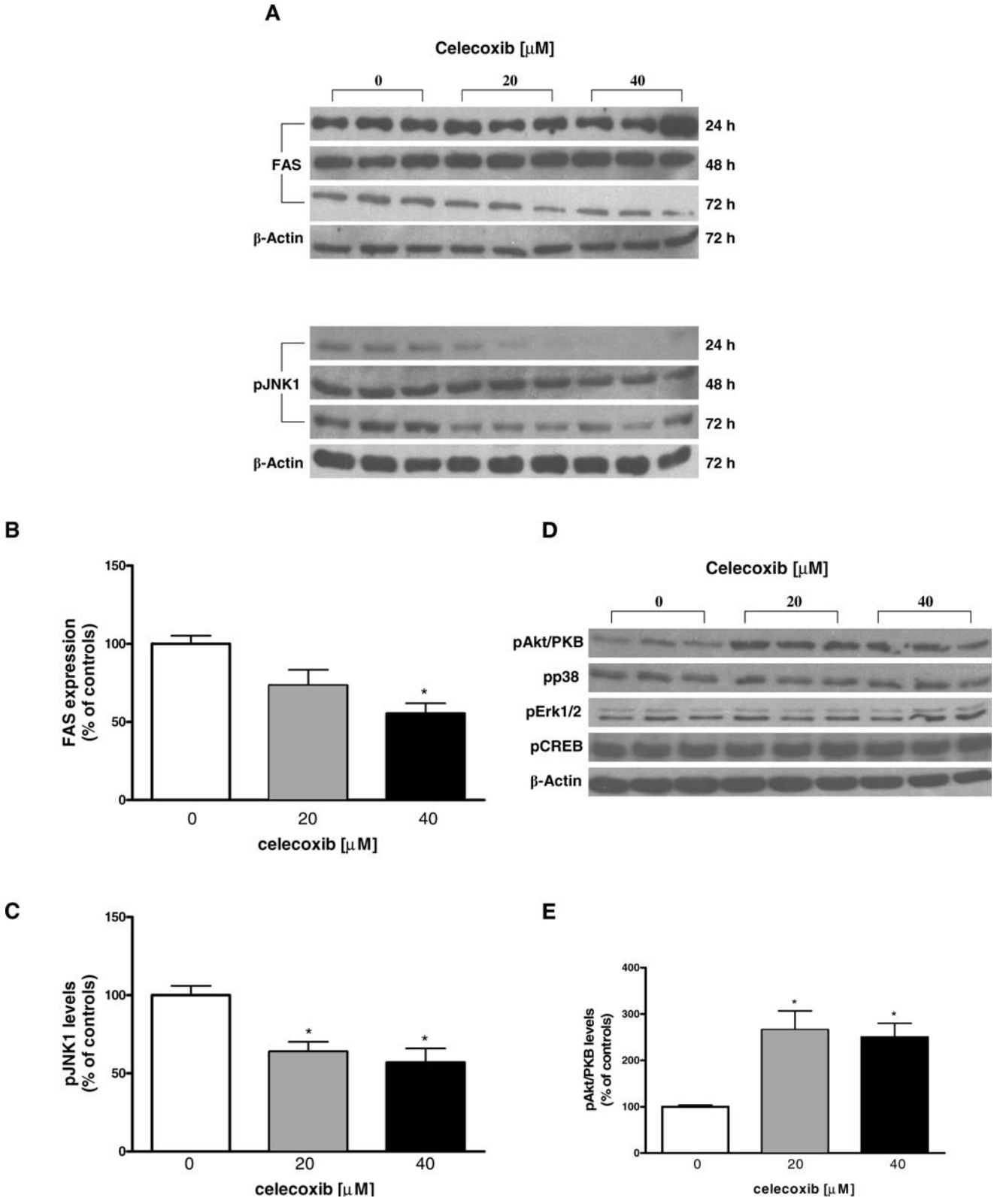

Figure 5. Celecoxib treatment decreases levels of FAS and pJNK1 but increases levels of pAkt/PKB (pSer473) in rat hepatocytes. (A) Rat hepatocytes were treated with celecoxib for 24, 48, or 72 hrs. (B) and (C) Quantification by densitometry of FAS and pJNK1 in celecoxib-treated and control rat hepatocytes at 72 hrs. (D) Rat hepatocytes were treated with celecoxib for 72 hrs. Ten micrograms (for FAS analysis) or 60 μg (for pJNK1 analysis) of protein were subjected to Western blot analysis as described in Materials and Methods. β-Actin was used as a loading control. (E) Quantification by densitometry of pAkt/PKB in celecoxib-treated and control rat hepatocytes at 72 hrs. Values are mean ± SEM ($n = 3$; * $P < 0.05$, versus controls).

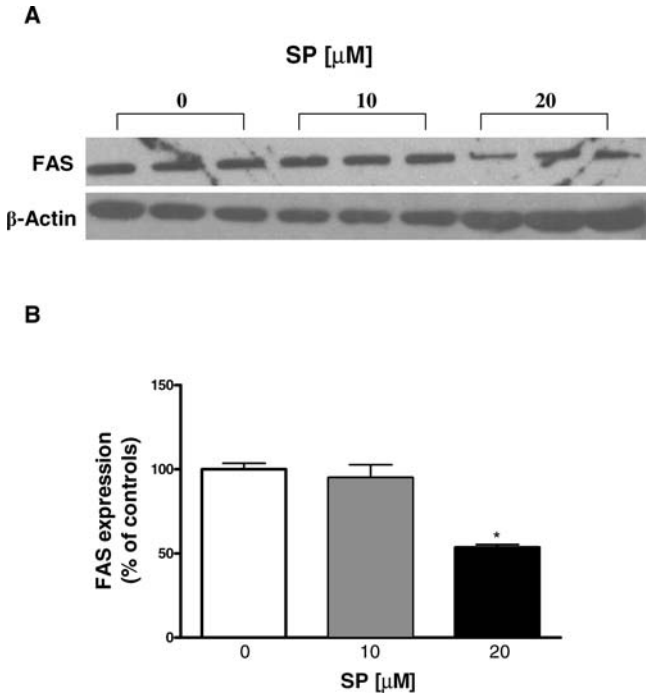

Figure 6. JNK inhibitor SP600125 (SP) decreases FAS expression in rat hepatocytes. (A) Ten μg protein was subjected to Western blot analysis as described in Materials and Methods. β-Actin was used as a loading control. (B) Quantification by densitometry of FAS in SP-treated and control rat hepatocytes. Values are mean ± SEM ($n = 3$; * $P < 0.01$, versus controls).

had no effect on the expression of ACO and CPT-1 in liver, retroperitoneal fat pad, or muscle. Since ACO and CPT-1 are transcriptionally induced by PPARs (39, 40), our results suggest that the effects of celecoxib on lipid metabolism are unlikely to be mediated by PPAR activation. However, the down-regulation of FAS by celecoxib may lead to elevated levels of malonyl-CoA, a known inhibitor of CPT-1 (41), thereby leading to reduced activity of this enzyme.

LPL-mediated fatty acid uptake and HSL-mediated lipolysis also affect adipose accumulation. A recent clinical study showed that adipose tissue loss induced by a low-calorie diet in obese patients was associated with a significant decrease in LPL expression and a significant increase in HSL expression in adipose tissue, and a decrease in HSL expression but no change in LPL expression in muscle tissue (42). Adipose tissue loss was also associated with a large increase in serum FFA levels (42). In our study, however, there were no differences in the expression of HSL and LPL in retroperitoneal adipose tissue or muscle tissue or in serum FFA levels between the celecoxib-treated and control rats. These results indicate that the effect of celecoxib on lipid metabolism in our animals is unlikely to be caused by changes in the mobilization and redistribution of triglycerides in muscle and adipose tissue. Taken together, our results suggest that celecoxib inhibits triglyceride accumulation in liver and intra-abdominal adipose tissue of rats fed a high-fat diet by down-regulating FAS in these tissues.

Next we investigated whether the effect of celecoxib on lipid metabolism is mediated by inhibition of COX-2 activity. COX-1 and COX-2 are mainly expressed in nonparenchymal cells (such as Kupffer cells in liver and endothelial cells in adipose tissue), and COX products act on parenchymal cells through a paracrine process (43, 44). By acting *via* the EP_3 receptor to increase cellular cAMP levels, PGE_2, the main product of COX, has been shown to decrease lipogenic gene expression, including FAS, in hepatocytes and adipocytes (43, 45). To determine whether decreased fat accumulation in celecoxib-treated rats was mediated by inhibition of COX-2, we measured PGE_2 levels in liver and retroperitoneal adipose tissue. There were no differences between the two groups suggesting that COX-1 in these tissues is the major source of PGE_2. Indeed, studies have shown that COX-1 is expressed at much higher levels than COX-2 in rat liver (43) and mouse adipose tissue (46). Yuan *et al.* (14) have shown that homozygous or heterozygous deletion of either COX-1 or COX-2 has no effect on lipid metabolism in insulin-resistant mice. Therefore, our results, together with previous studies, suggest that celecoxib down-regulates FAS in livers and abdominal adipose tissue in rats fed a high-fat diet by a mechanism that is independent of COX-2.

Under physiological conditions, insulin is a major factor that regulates FAS expression (38). Conceivably, modification of the insulin signaling pathway may lead to an alteration in FAS expression. The serine/threonine kinase Akt/PKB, one of the major elements in the insulin signaling pathway, is known to mediate stimulation of FAS expression by insulin (47) and is a downstream target of PDK1 (10). PDK1 phosphorylates Akt/PKB on Thr308 (29). Arico *et al.* (10) have reported that celecoxib is an inhibitor of PDK1. In the livers of our celecoxib-treated rats, however, levels of pAkt/PKB (pThr308) were not different from those in the control animals. Although Thr308 phosphorylation is necessary and sufficient for Akt/PKB activation (48), maximal activation requires additional phosphorylation at Ser473 by PDK2 (30). In both our *in vivo* and *in vitro* experiments, celecoxib treatment caused the up-regulation of hepatic pAkt/PKB (pSer473), further suggesting that the down-regulation of FAS by celecoxib is not mediated through the Akt/PKB pathway.

Although celecoxib is a COX-2–specific inhibitor, recent studies have shown that this drug affects multiple molecular targets (7, 10). In human osteoarthritic chondrocytes, celecoxib has been shown to decrease the phosphorylation of p38 and Erk1/2, members of the mitogen-activated protein (MAP) kinase pathway (31). Both p38 and Erk1/2 have been shown to participate in the phosphorylation of CREB (32, 33). Insulin stimulates the phosphorylation of CREB at serine 133, and the activation of CREB plays an essential role in the insulin activation of FAS (34). Herzig *et al.* (49), on the other hand, have shown that mice

deficient in CREB activity have a fatty liver and display elevated hepatic expression of lipogenic genes, including FAS. We measured the levels of pp38, pErk1/2, and pCREB to test the potential involvement of these pathways in the regulation of hepatic FAS by celecoxib. The drug, however, did not significantly alter the levels of the phosphorylated proteins either *in vivo* or *in vitro*. Our observations indicate that down-regulation of FAS by celecoxib in the liver is not mediated through alteration of p38, Erk1/2, or CREB and, in view of the results in human osteoarthritic chondrocytes (31), suggest that the effects of celecoxib on signaling pathways may be species or cell-type specific.

Activation of JNK1, a stress-activated protein kinase, is associated with hepatic triglyceride accumulation and insulin resistance in rats fed a high-fat diet (35). Furthermore, JNK1 knockout mice are resistant to the obesity induced by a high-fat diet and have improved insulin sensitivity (23). Therefore, we measured the levels of both JNK1 and pJNK1 to explore the possibility that the effects of celecoxib in lipid metabolism are mediated by this kinase. In both our *in vivo* and *in vitro* studies, celecoxib significantly decreased the levels of JNK1 and pJNK1. Furthermore, down-regulation of JNK1 occurred prior to inhibition of FAS expression in celecoxib-treated rat hepatocytes. To determine whether the down-regulation of JNK1 is causally related to the suppression of FAS expression, we treated rat hepatocytes with a known JNK inhibitor. This inhibitor significantly decreased the expression of FAS. These observations demonstrate that JNK1 is directly involved in the regulation of FAS by celecoxib. In a similar manner, celecoxib has been reported to decrease endothelial tissue factor expression in human aortic endothelial cells by down-regulating the activation of JNK1 without affecting levels of pp38 or pErk1/2 (50). The molecular mechanism by which celecoxib regulates FAS expression *via* JNK1 signaling needs further investigation.

In summary, we have demonstrated that celecoxib reduces fat accumulation in rats fed a high-fat diet by decreasing FAS expression *via* down-regulation of JNK1. Since JNK1 is involved in the development of inflammation (51), obesity, insulin resistance (23, 52), and cancer (53), as well as in maintaining normal cardiovascular (54) and neural function (55), our observations may contribute to understanding why celecoxib has a variety of biological effects, including the recent finding of an increased cardiovascular risk caused by this drug (56, 57).

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