Arachidonic acid and PGE_2 regulation of hepatic lipogenic gene expression

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Abstract **N–6 polyunsaturated fatty acids (PUFA) suppress hepatic and adipocyte de novo lipogenesis by inhibiting the transcription of genes encoding key lipogenic proteins. In cultured 3T3-L1 adipocytes, arachidonic acid (20:4,n–6) suppression of lipogenic gene expression requires cyclooxygenase (COX) activity. In this study, we found no evidence to support a role for COX-1 or -2 in the 20:4,n–6 inhibition of hepatocyte lipogenic gene expression. In contrast to L1 preadipocytes, adipocytes and rat liver, RT-PCR and Western analyses did not detect COX-1 or COX-2 expression in cultured primary hepatocytes. Moreover, the COX inhibitor, flurbiprofen, did not affect the 20:4,n–6 regulation of lipogenic gene expression in primary hepatocytes. Despite the absence of COX-1 and -2 expression in primary hepatocytes, prostaglandins (PGE_2 and $PGF_{2\alpha}$) suppressed fatty acid synthase, L-pyruvate kinase, and the S14 protein mRNA, while having no effect on acyl-CoA oxidase or CYP4A2 mRNA. Using PGE_2 receptor agonist, the PGE_2 effect on lipogenic gene expression was linked to EP3 receptors. PGE_2 inhibited S14CAT activity in transfected primary hepatocytes and targeted the S14 PUFA-response region located −220 to −80 bp upstream from the transcription start site. Taken together, these studies show that COX-1 and COX-2 do not contribute to the n–6 PUFA suppression of hepatocyte lipogenic gene expression. However, cyclooxygenase products from non-parenchymal cells can act on parenchymal cells through a paracrine process and mimic the effects of n–6 PUFA on lipogenic gene expression.**—Mater, M. K., A. P. Thelen, and D. B. Jump. **Arachidonic acid and PGE_2 regulation of hepatic lipogenic gene expression.** ***J. Lipid Res.*** **1999.** 40: **1045–1052.**

Supplementary key words S14 protein • fatty acid synthase • l-pyruvate kinase • cyclooxygenase • gene transcription

The effect of dietary n–6 polyunsaturated fatty acids (PUFA) on hepatic de novo lipogenesis was first reported by Allman and Gibson (1) while studying essential fatty acid deficiency. Those studies showed that removal of n–6 PUFA from the diet led to a rise in hepatic de novo lipogenesis. In subsequent studies, Flick, Chen, and Vagalos (2) reported that in vivo administration of indomethacin failed to block the n–6 PUFA suppression of fatty acid synthase (FAS) activity. N–6 PUFA are precursors of prostaglandins and indomethacin inhibits prostaglandin synthesis. These observations led investigators to suggest that n–6 PUFA regulation of hepatic lipogenesis did not involve prostaglandins.

However, a recent study examining the effects of n–6 PUFA on lipogenic gene expression in cultured adipocytes implicated the involvement of cyclooxygenase (3). Briefly, treatment of 3T3-L1 adipocytes with 20:4,n–6 suppressed ($ED_{50} \sim 50$ μm) mRNAs encoding two proteins involved in lipogenesis, FAS and the S14 protein (S14). The 20:4,n–6 effect on lipogenic gene expression was blocked by flurbiprofen, a non-specific cyclooxygenase inhibitor. PGE_2 and $PGF_{2\alpha}$ also suppressed $mRNA_{S14}$ ($ED_{50} < 10$ μm) and the PGE_2 effect on adipocyte gene expression was blocked by pertussis toxin, implicating a G_i-linked regulatory pathway.

Like adipocytes, n–6 PUFA suppression of hepatic lipogenic gene transcription leads to a decline in de novo lipogenesis (4–7). The liver contains Kupffer and endothelial cells, cells known to generate prostanoids under appropriate stimulation (8). Moreover, primary hepatocytes respond to prostanoids through specific G-protein-linked receptors leading to changes in carbohydrate metabolism and DNA synthesis (8–13). In this report, we have reexamined the requirement for cyclooxygenase in the n–6 PUFA regulation of hepatic lipogenic gene expression and have extended this analysis to include effects of prostaglandins on lipogenic gene expression. We report that 20:4,n–6 suppression of lipogenic gene expression in hepatic parenchymal cells does not involve metabolism through a cyclooxygenase-dependent pathway. However, PGE_2 and $PGF_{2\alpha}$ suppress mRNAs encoding FAS, S14, and l-pyruvate kinase (L-PK) as well as the activity of S14CAT reporter genes in transfected primary hepatocytes. These studies suggests that PUFA and prostanoids may utilize a common mechanism at the genomic level to control lipogenic gene transcription.

Abbreviations: PUFA, polyunsaturated fatty acids; PPAR, peroxisome proliferator activated receptor; CYP, cytochrome P450; AOX, acyl-CoA oxidase; FAS, fatty acid synthase; S14, S14 protein; COX, cyclooxygenase; RT-PCR, reverse transcriptase-polymerase chain reaction; CAT, chloramphenicol acetyl transferase; nt, nucleotide.

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MATERIALS AND METHODS

Primary hepatocyte preparation, transfection, and treatments

Primary hepatocytes were prepared as described previously (4), with the following modifications. Male rats (180–300 g) were anesthetized by inhalation of methoxyflurane rather than injection of pentabarbitol. The perfusion buffer contained either collagenase (at 100 mg/400 ml) or Liberase™ (at 7 mg/400 ml) (Boehringer Mannheim, Indianapolis, IN) for liver digestion. Hepatocytes were plated at 3×10^6 per 60 mm Primaria plate for CAT assays or 10^7 per 100 mm Primaria plate for RNA analysis. Cells were plated in Williams E containing 10 mm lactate, 10% fetal bovine serum, 200 nm insulin, and 10 nm dexamethasone. After a 4–6 h cell attachment period, media were removed and replaced with Williams E media without serum.

Cells were transfected with chloramphenicol acetyltransferase (CAT) reporter vectors (2 μg/plate) and thyroid hormone receptor β1 expression vector (MLV-TRβ1, 1 μg/plate) using Lipofectin™ (6.6 μl/μg DNA) (Life Technologies, Gaithersburg, MD). Sixteen hours later, cells were treated for up to 48 h with Williams E containing 25 mm glucose, 1 μm insulin, 1 μm T_3, 10 nm dexamethasone and fatty acid or prostanoids. Fatty acids (18:1,n–9; 20:4, n–6, or 20:5,n–3) were used at 250 μm at a 5:1 ratio with fatty acid-free albumin. Prostanoids (PGE_2, $PGF_{2\alpha}$, and receptor-specific agonists) were purchased from Cayman Chemicals (Ann Arbor, MI) and dissolved in DMSO.

RNA analysis

Total RNA was isolated from preadipocytes, adipocytes, rat liver, and primary hepatocytes then transferred to nitrocellulose for hybridization with [^{32}P]cDNA for S14, FAS, L-PK, AOX, or CYP4A2 as described previously (3–5). Note: our CYP4A2 probe also detects CYP4A3 (93% homologous to CYP4A2).

Cyclooxygenase (COX-1 and COX-2) mRNAs were measured by RT-PCR using the "Superscript RT-PCR One-Step" kit from Life Technologies. The RT-PCR reaction used 1 μg total RNA extracted from preadipocytes, adipocytes, rat liver, or cultured primary hepatocytes. The reverse transcription reaction conditions were: 30 min at 50°C followed by 2 min at 94°C. The PCR cycling conditions were: 1 min at 94°C; 1 min at 55°C, and 1 min at 72°C. Samples were collected after 25, 30, or 40 cycles. The primers used for COX-1 were : sense 5′ GCCGAGGATGTCATCAAGGAG TCCC and antisense 5′ ACGAAATCTCAAGATGGGCCCCGAC. RT-PCR amplifies the region between 1400 and 1579 nt of the COX-1 mRNA to generate a 179 bp fragment. The primers used for COX-2 were: sense 5′ CTTGTACGTCAGATTGCTGCCGTAG and antisense 5′ CGAACCGAACACTGAAACCGTCCGA. RT-PCR amplifies the region between 2705 and 2959 nt of the COX-2 mRNA to give a 254 bp fragment. The S14 primers [sense 5′ TAT GCAAGTGCTAACGAAACGCTACCC; antisense 5′ TCCTACAGG ACCTGCCCCGTCATTTCC] amplify the protein coding region extending from 19 to 474 bp to give a fragment of 455 bp. The CYP4A2 primers [sense 5′ CCACATGGGACCACCTGG; antisense 5′ GCTGGGAAGGTGTCTGGAGT] amplify the DNA region extending from 7370 to 12650 bp (genomic coordinates) to yield a 550 bp fragment (5). All sequence data were obtained from Genbank (www.ncbi.nlm.nih.gov/ PubMed).

Plasmid construction

The construction of S14CAT124, S14CAT155, S14CAT156, S14CAT158, TKCAT222, TKCAT224, RSVCAT116, RSVCAT117, RSVCAT119, RSVCAT100 were described previously (4, 6). RSVCAT132 was constructed using PCR to amplify the −35 to −115 bp region of the HSV thymidine kinase promoter. This region contains a Y-box binding NF-Y flanked by two Sp1 sites. The sense and antisense primers were: 5′: ATATAAGCTT^{-115} GACAC AAACCCCGCCAGCG and 5′: TATACTGCAG^{-38} ACCTGGGAC CGCGCCG and the template was TKCAT. The primers have artificial HindIII and PstI sequences that were used to insert the 90 bp fragment directionally into the RSVCAT119 plasmid. The RSVCAT119 plasmid was previously treated with HindIII and PstI to remove the S14 −220 to −80 bp region. The resultant plasmid, RSVCAT132, contains the RSV TATA box, the thymidine kinase Y-box and Sp1 sites, and the S14 thyroid hormone response region.

Assays

Cells were assayed for CAT activity and protein (5). CAT units: [^{14}C]butylated chloramphenicol CPM/100 μg protein per h. Hepatocellular cAMP was assayed before and after addition of 10 μm PGE_2 using the cAMP enzyme immunoassay kit (Amersham Pharmecia Biotec). The effect of PGE_2 on DNA synthesis in primary hepatocytes was measured using [^{3}H]thymidine (11). Each experiment was done in triplicate and repeated at least three times. Western analysis for COX-1 and -2 expression was performed as described (14) using 80 μg of primary hepatocyte microsomal protein separated by SDS-polyacrylamide gel electrophoresis. After electrophoretic transfer, blots were exposed to anti-human COX-1 or -2 antibodies that cross-react with rat COX-1 and COX-2. The antibody concentration in the assay was 50 ng/ml and the detection system used the Renaissance CDP-Star Western chemiluminescence kit from NEN-Life Science Products.

RESULTS

Expression of cyclooxygenase in rat liver and primary hepatocytes

Northern analysis failed to detect significant levels of COX-1 or -2 mRNA in rat liver or primary hepatocytes. Consequently, RT-PCR was used to examine the expression of COX-1 and COX-2 in 3T3-L1 preadipocytes and adipocytes, rat liver, and primary hepatocytes. **Figure 1** illustrates the results of 30 cycles of RT-PCR. Measurement of S14 and CYP4A mRNA by RT-PCR was included for comparison.

After 30 cycles of RT-PCR, COX-1 expression was detected in preadipocytes, adipocytes, and rat liver, but not primary hepatocytes. COX-2 was not detected in any RNA after 30 cycles. After 40 cycles, both COX-1 and COX-2 mRNAs were detected in all RNA preparations (not shown). The presence of COX-1 and -2 transcripts after 40 cycles of RT-PCR probably represents residual contamination of the hepatocyte preparation with Kupffer and endothelial cells. $mRNA_{S14}$ was detected in adipocytes, liver, and hepatocytes, but not preadipocytes after 30 cycles of RT-PCR. CYP4A2 was only detected in rat liver and primary hepatocytes after 30 cycles. The cell-specific expression of S14 and CYP4A2 measured by RT-PCR is consistent with previous studies using Northern analyses (3–5).

Efforts to detect COX-1 or -2 protein by Western analysis using COX-1 and -2 specific antibodies that cross-react with rat (14) failed to detect any COX-1 or -2 in microsomal preparations of primary hepatocytes (not shown). In a final effort to assess the contribution COX makes to the PUFA regulation of hepatocyte lipogenic gene expression, we compared the effect of flurbiprofen on the 20:4,n–6 suppression of S14 gene expression in L1 adipocytes and pri-

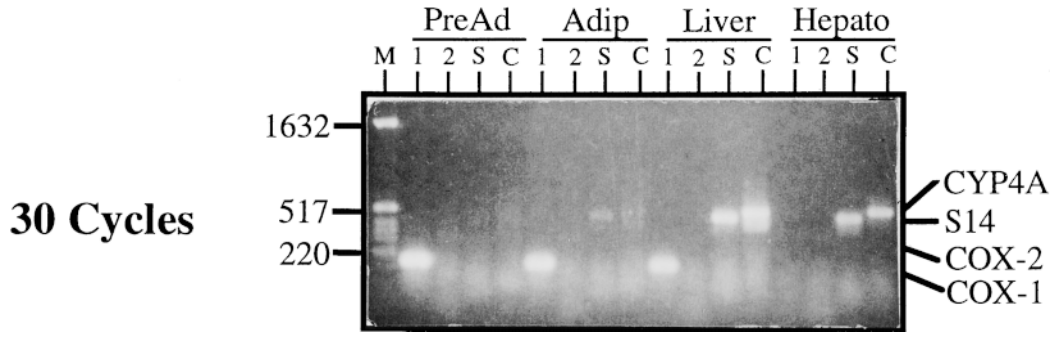

Fig. 1. RT-PCR analysis of COX1, COX2, S14, and CYP4A2 mRNAs in L1 preadipocytes and adipocytes and rat liver and primary hepatocytes. RT-PCR analysis was performed on total RNA from L1 preadipocytes and adipocytes, rat liver, and cultured primary hepatocytes. The RT-PCR and primers used to amplify fragments from COX 1 (1), COX 2 (2), S14 (S), and CYP4A (C) mRNA are described in Materials and Methods. After electrophoresis, reaction products were separated in 1% agarose with 0.09 m Tris-borate–EDTA, pH 8.3, as buffer and stained with ethidium bromide. Results of 30 cycles of RT-PCR reaction are presented in the figure. Markers (M) are pBR322 digested with HinfI; location of the 1632, 517, and 220 bp fragments are indicated. Fragment size: COX1, 179 bp; COX2, 254 bp; S14, 450 bp and CYP4A, 550 bp and their location is shown on the figure. The results presented here are representative of at least 3 different measurements.

mary hepatocytes transfected with a S14CAT reporter gene (**Fig. 2**). When compared to fatty acid-free or 18:1, n–9-treated cells, 20:4,n–6 suppressed CAT activity by 50% and 70% in adipocytes and hepatocytes, respectively. While treatment of L1 adipocytes with flurbiprofen blocked the 20:4,n–6 suppression of S14CAT, a similar treatment of primary hepatocytes had no effect on 20:4,n–6 inhibition of S14CAT activity.

The presence of COX-1 mRNA in preadipocytes and adipocytes supports our previous report on the role of this enzyme in the n–6 PUFA regulation of lipogenic gene expression in these cells (3). The presence of COX-1 and possibly COX-2 mRNA in the intact liver suggests that liver has the capacity to generate prostanoids (8–13, 15, 16). However, no COX-1 or COX-2 mRNA was detected in primary hepatocytes after 30 cycles. Moreover, no COX-1 or -2 protein was detected using a sensitive Western analysis (14). Finally, flurbiprofen did not block the 20:4,n–6-mediated inhibition of S14CAT in cultured primary hepatic parenchymal cells. Taken together, these results indicate that neither COX-1 or COX-2 is required for the 20:4,n–6-mediated inhibition of hepatic lipogenic gene expression. Moreover, it is also highly unlikely that hepatic parenchymal cells generate physiologically significant levels of prostanoids.

Effects of PGE_2 on mRNAs encoding proteins involved in hepatic fatty acid synthesis and oxidation

Although 20:4,n–6 inhibition of S14CAT activity in hepatic parenchymal cells does not require cyclooxygenase conversion to a prostanoid, numerous reports have indicated that hepatic parenchymal cells possess prostanoid receptors that affect hepatocellular function (8–13, 15, 16). Moreover, intact liver contains cyclooxygenase (COX-1) (Fig. 1). In order to determine whether prostanoids affected lipogenic gene expression, primary hepatocytes were treated with PGE_2. mRNAs encoding proteins involved in fatty acid synthesis (FAS, S14, and l-pyruvate ki-

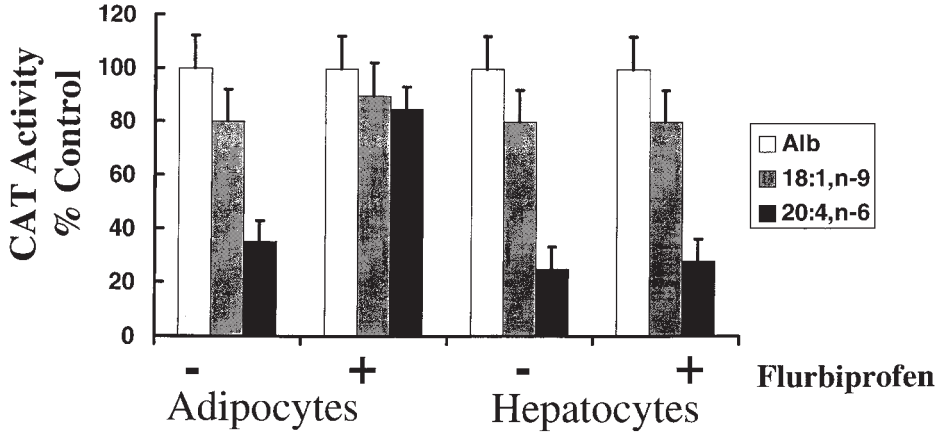

Fig. 2. Effect of flurbiprofen on the 20:4,n–6 regulation of S14CAT activity in adipocytes and hepatocytes. Adipocytes and hepatocytes were transfected with S14CAT124 and treated with 50 μm albumin, 250 μm 18:1,n–9 or 20:4,n–6 in the absence or presence of flurbiprofen (100 μm) for 48 h. CAT activity was determined and normalized to the albumin treatment. Mean ± SD, n = 9.

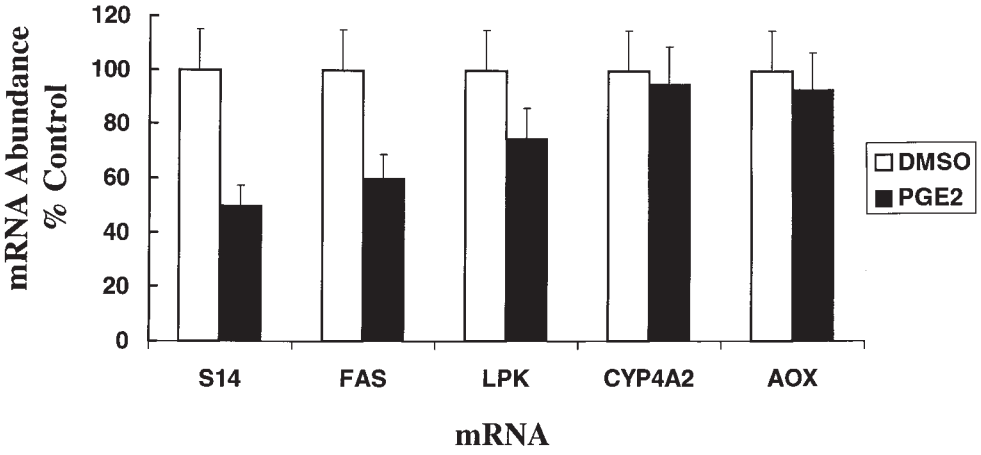

Fig. 3. Regulation of FAS, S14, AOX, and CYP4A2 mRNA by PGE_2. Cultured primary hepatocytes were treated for 48 h with DMSO or 10 μm PGE_2. The relative abundance of mRNAs encoding FAS, S14, AOX, and CYP4A2 was measured by Northern analysis. Results are normalized to the level of mRNA expression in DMSO-treated cells. Mean ± SD, n = 8.

nase [L-PK]) and non-mitochondrial fatty acid oxidation (acyl-CoA oxidase, AOX; cytochrome P450 4A2, CYP4A2) were examined by Northern analysis (**Fig. 3**).

Treatment of hepatocytes with PGE_2 (10 μm) for 48 h suppressed $mRNA_{S14}$, $mRNA_{FAS}$, and $mRNA_{LPK}$ by 50, 50, and 25%, respectively (Fig. 3). At 1 μm, PGE_2 gave only modest inhibition (<10%) and lower doses were ineffective. In contrast, PGE_2 (at 10 μm) had no consistent effect on mRNAs encoding AOX or CYP4A2. These results indicate that mRNAs encoding proteins involved in lipid synthesis are suppressed by PGE_2, while mRNAs encoding proteins involved in non-mitochondrial fatty acid oxidation (AOX and CYP4A) are not sensitive to PGE_2 regulation.

PGE_2 inhibits S14 gene transcription in primary hepatocytes

To examine the effects of PGE_2 on parenchymal cell gene transcription, primary hepatocytes were transiently transfected with either an S14CAT reporter gene (S170) or RSVCAT (R100). S170 contains the S14 promoter extending from +19 bp to −2.8 kb. This promoter contains a proximal promoter region required for proper initiation of gene transcription and two enhancers (4, 6). One enhancer located between −1.6 and 1.4 kb is a target for insulin and glucose induction of S14 gene transcription. A second enhancer located between −2.8 and −2.5 kb contains three thyroid hormone response elements (TRE) that are targets for thyroid hormone receptors (TR) binding in association with the retinoid X receptor (RXR).

Cells transfected with S170 and treated with the insulin (1 μm), T_3 (1 μm), glucose (25 mm), and dexamethasone (10 nm) express high levels of CAT activity (~1500 CAT units). R100 contains the RSV promoter/enhancer fused to CAT and cells transfected with this plasmid constitutively express high levels of CAT activity (~10,000 CAT units). Under these culture conditions, PGE_2 (10 μm) suppressed S170 CAT activity by ~50% (**Fig. 4**). Treatment of hepatocytes with $PGF_{2\alpha}$ or the PGE_2 agonist dimethyl PGE_2 at 10 μm also suppressed S14CAT activity (not shown). In contrast, PGE_2 (10 μm) had no effect on CAT activity in RSVCAT-transfected cells. These results are consistent with PGE_2 acting at the transcriptional level to suppress $mRNA_{S14}$ (Fig. 3). The lack of a PGE_2 effect on R100 indicates the promoter-specificity of PGE_2 action on hepatic gene transcription.

PGE_2 regulates hepatic S14 gene transcription through an EP3 receptor

PGE_2 action on cells has been linked to G-proteins that either increase cellular cAMP, decrease cAMP, or activate phospholipase C (PLC) leading to a change in intracellular inositol 1,4,5-phosphate (IP_3) and calcium (17). In 3T3-L1 adipocytes, PGE_2 suppressed S14 and FAS gene expression through a pertussis toxin-sensitive G_i-linked receptor (3). Because pertussis toxin inhibited hepatocyte S14CAT activity and suppressed $mRNA_{S14}$ (not shown), we were unable to use this approach to assess the contribu-

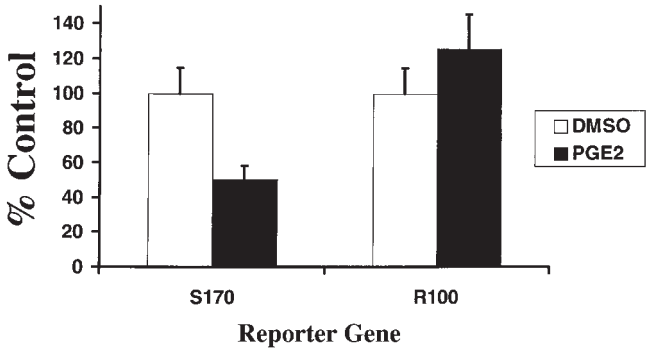

Fig. 4. Effect of PGE_2 on S14CAT activity. Hepatocytes were transfected with either S14CAT170 (S170) or RSVCAT100 (R100) and treated with DMSO or 10 μm PGE_2 for 48 h. CAT activity was determined and results were normalized to the level of CAT activity in DMSO-treated cells. Mean ± SD, n = 6.

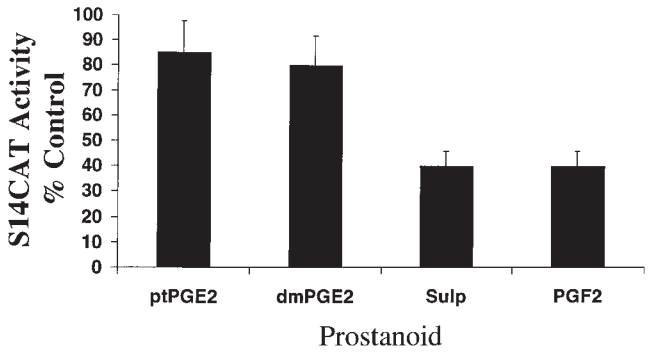

Fig. 5. Effects of receptor subtype-specific agonists on S14CAT activity. Hepatocytes were treated with the PGE_2 receptor agonists or $PGF_{2\alpha}$ at 10 μm for 48 h and CAT activity was determined. The receptor agonists were:17-phenyl trinor PGE_2 (ptPGE2), 11-deoxy-16,16 dimethyl PGE_2 (dmPGE2), and sulprostone (Sulp). Results were normalized to the level of CAT activity in DMSO-treated cells. Mean ± SD, n = 9.

tion of a specific G-protein to the PGE_2 regulation of S14 gene expression.

A second strategy to define the PGE_2 signal transduction mechanism used PGE_2 receptor agonists. The EP1 agonist, 17-phenyl trinor PGE_2, activates a IP_3/calcium pathway while the EP2 agonist, 11-deoxy,16-16 dimethyl PGE2, induces cAMP formation. The EP3 agonist, sulprostone, activates PLC leading to changes in intracellular calcium or cAMP levels. $PGF_{2\alpha}$ activates PLC leading to changes in intracellular IP_3/calcium (17, 18). Of these prostanoids, only sulprostone and $PGF_{2\alpha}$ suppressed S170 CAT activity (**Fig. 5**). This result suggests the prostanoid effect on lipogenic gene expression is mediated by EP3 and FP receptors. The suppression of S14 gene expression by EP3 and PGF2α implicates changes in hepatocellular IP3/calcium as likely mediators for genomic effects on hepatic gene transcription.

Activation of PGE_2 receptors in primary hepatocytes has been reported to suppress cAMP levels and increase DNA synthesis (10–13). We measured these parameters and found that under the culture conditions used in this study, cAMP is low (~1 pmol/3 × 10^6 hepatocytes). However, after 8 h of PGE_2 treatment, hepatocellular cAMP levels were suppressed to 0.5 pmol/3 × 10^6 hepatocytes. Using [^{3}H]thymidine incorporation into DNA as a measure of DNA synthesis, PGE_2 consistently induced DNA synthesis by 50%. Based on these results, PGE_2 activation of EP3 receptors in primary hepatocytes leads to a decline in lipogenic gene expression and cAMP and an increase in DNA synthesis.

Location of the *cis*-regulatory targets for 20:4,n–6 and PGE_2 in the S14 promoter

The *cis*-regulatory targets for PGE_2 action were localized using a series of S14CAT plasmids with successive promoter deletions (**Fig. 6**). Primary hepatocytes were transfected with S14CAT fusion genes S170, S155, S156, or S158. S170 contains the full-length promoter extending from −2800 to +19 bp fused to CAT. S155, S156, and S158 contain the S14 proximal promoter elements with 5′ end points at −220, −120 and −80 bp, respectively. The S14 TRR (−2.8 to −2.5 kb) fused upstream from the proximal promoter elements was used to enhance CAT activity. A plasmid containing the S14 TRR in the context of the thymidine kinase promoter (T222) was used to determine whether the TRR was a target for PGE_2 control.

Hepatocytes transfected with S170 express high levels of CAT activity (2,500 CAT units) in response to T_3. 20:4,n–6

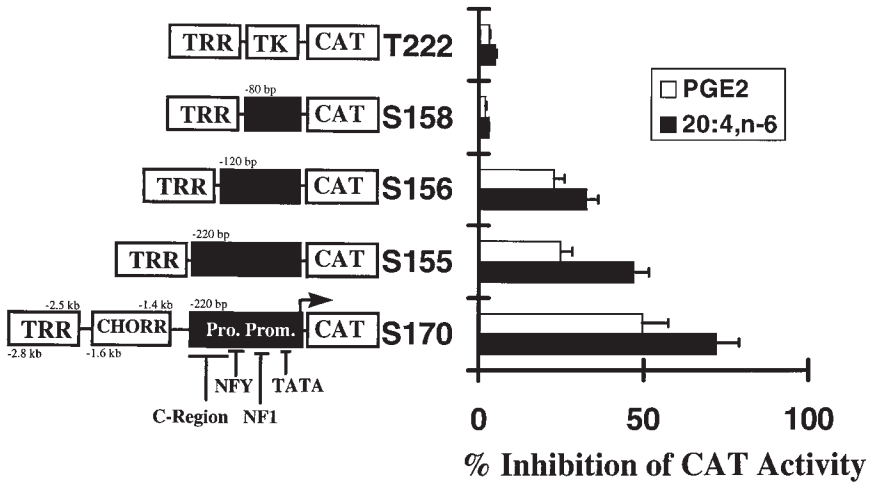

Fig. 6. Deletion analysis of the S14 promoter. Primary hepatocytes were transfected with S170, a plasmid containing the full-length S14 promoter. The location of the TATA, NF-1, and NF-Y sites are illustrated. The C-region binds several undefined transcription factors. The location of the two upstream enhancers, TRR and CHORR are also indicated. Plasmids with proximal promoter deletions include (S155, S156, S158) constructs. These plasmids have 5′ promoter endpoints at −220, −120, and −80 bp, respectively (see Materials and Methods). The T222 plasmid contains the S14 TRR upstream from the thymidine kinase promoter and CAT. After transfections, cells were treated for 48 h with T_3 and either 250 μm 20:4,n–6 or 10 μm PGE_2. The corresponding controls were 18:1,n–6 plus albumin or DMSO, respectively. Results are presented as % inhibition by 20:4,n–6 or PGE_2 mean ± SD, n = 9.

and PGE_2 inhibited CAT activity by 75% and 50%, respectively. Hepatocytes transfected with T222 also express high levels of CAT activity (3,500 CAT units) in response to T_3. The expression of this plasmid was marginally affected (<15% inhibition) by 20:4,n–6 or PGE_2. Like 20:4,n–6, PGE_2 effects on S14CAT activity are not due to interference with T3-mediated regulation of S14 gene transcription.

Deletion of the promoter region extending from −2.5 to −0.22 kb, which includes the insulin/carbohydrate response region, led to a reduction in the inhibition of CAT activity from 75 to 50% for 20:4,n–6 and from 50 to 25% for PGE_2, respectively. Deleting the region between −220 and −120 bp had no effect on PGE_2 inhibition but decreased 20:4,n–6 inhibition to 35%. Deleting the region between −120 to −80 bp resulted in a complete loss of inhibition of CAT activity by both 20:4,n–6 and PGE_2. These results show that the *cis*-regulatory targets for PGE_2 regulation of S14 gene transcription converge with the PUFA-response region, i.e., between −220 and −80 bp. The region between −120 and −80 bp is the minimal element retaining sensitivity to both 20:4,n–6 and PGE_2.

Promoter context dictates the response to PUFA and PGE_2

Nearly 70% of the inhibitory effect of 20:4,n–6 and 50% of the PGE_2 effect is directed at the S14 proximal promoter PUFA-RR, i.e., −220 to −80 bp. Moreover, the minimal component retaining sensitivity to both PUFA and PGE_2 control is a region between −120 and −80 bp (Fig. 6). A Y-box (−104 to −99 bp) binding the transcription factor, NF-Y, resides within this minimal component. This factor is required for the function of the upstream enhancers. Any mutation within the Y-box disrupting NF-Y binding inhibits S14 gene transcription (6). One explanation for the inhibitory effect of 20:4, n–6 and PGE_2 on S14 gene transcription may involve regulation of NF-Y binding or action.

Two CAT reporter plasmids were constructed to test this hypothesis (**Fig. 7**). R119 contains a TATA box, the S14 −220/−80 bp region which binds NF-Y and the S14 TRR fused upstream. In R132, the thymidine kinase (TK) proximal promoter region (−115 to −38 bp) replaces the S14 proximal promoter region in R119. The TK (−115/−38 bp) region contains a Y-box flanked by GC-rich regions (19). The Y-box binds NF-Y while the GC-rich regions bind Sp1.

Transfection of hepatocytes with the R119 and R132 plasmids and treatment with T_3 yields high levels of CAT activity, i.e., 2500 and 2200 CAT units, respectively. Interestingly, CAT activity in R119-transfected cells is inhibited by ~50% after PGE_2 and PUFA (20:4,n–6 and 20:5,n–3) treatment. In cells transfected with R132, CAT activity was not suppressed by these treatments. Thus, NF-Y alone is not the sole determinant for 20:4,n–6, 20:5,n–3, or PGE_2 inhibition of lipogenic gene transcription. These results suggest that the promoter context in which NFY resides dictates the response to PUFA and PGE_2. Preliminary studies indicate that factors binding upstream of the S14 Y-box (at −104/−99 bp) are critical for S14 gene transcription. Efforts are underway to identify these factors and evaluate their role in PUFA and PGE_2 control of S14 gene transcription.

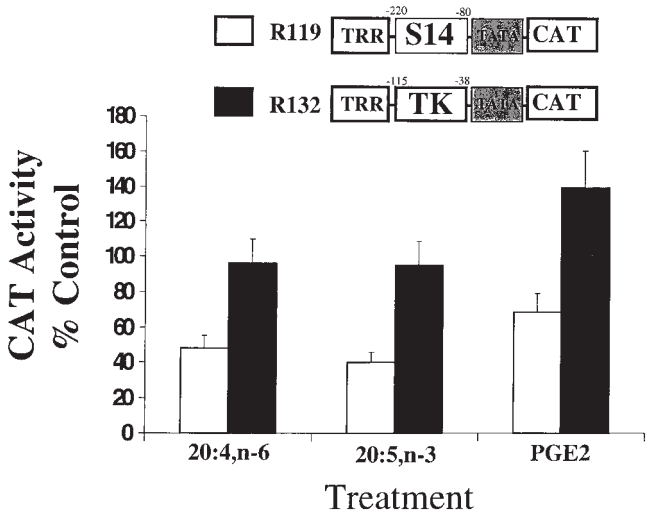

Fig. 7. Does NF-Y confer PUFA and PGE_2 control to the S14 proximal promoter? Primary hepatocytes were transfected with R119 or R132. Both R119 and R132 contain the RSV TATA box and the S14TRR located upstream of CAT. R119 contains the S14 PUFA-RR (−220/−80 bp) inserted between the TRR and the TATA box. R132 contains a region from the HSV thymidine kinase promoter that binds NF-Y and Sp1. Primary hepatocytes were transfected with these plasmids and treated with 250 μm 20:4,n–6 or 20:5,n–3 or 10 μm PGE_2 for 48 h. CAT activity was measured and normalized to the control treatment (18:1,n–9 and albumin for PUFAs or DMSO for PGE_2). Mean ± SD, n = 9.

DISCUSSION

Our evaluation of prostanoid regulation of hepatic lipogenic gene expression was prompted by recent studies showing that 20:4,n–6 suppression of lipogenic gene expression in 3T3-L1 adipocytes required cyclooxygenase (3). In contrast to preadipocytes, adipocytes, and rat liver, no COX-1 or COX-2 mRNA (Fig. 1) or protein was detected in primary hepatocytes. Moreover, the cyclooxygenase inhibitor, flurbiprofen, did not block the 20:4,n–6-mediated inhibition of hepatic lipogenic gene expression (Fig. 2). Finally, HPLC analysis of media derived from primary hepatocytes treated for 24 h with 250 μm 20:4,n–6 did not contain detectable PGE_2 or $PGF_{2\alpha}$ (not shown). Based on these results, cyclooxygenase does not contribute to the 20:4,n–6 inhibition of lipogenic gene expression in primary hepatocytes. Moreover, it is unlikely that hepatic parenchymal cells generate prostanoids. However, we do not exclude the possibility that some other 20:4,n–6 metabolites generated within parenchymal cells activate a signalling pathway that affects lipogenic gene expression. Clearly, cytochrome P450-mediated fatty acid metabolism can potentially generate PUFA metabolites that activate signalling cascades affecting gene transcription (20). Stud-

ies are currently in progress to evaluate the role these pathways play in dietary PUFA regulation of hepatic lipogenic gene expression.

Despite the absence of physiologically significant levels of COX-1 or COX-2 in hepatic parenchymal cells, our results and those of others indicate that 20:4,n–6 cyclooxygenase products from non-parenchymal cells can act in a paracrine fashion on parenchymal cells (8–13, 15, 16). Prostaglandins produced by Kupffer cells in response to injury, sepsis, or other stimuli act on surrounding hepatocytes to alter specific functions (8). Glucagon interaction with Kupffer cells induces PGE_2, PGD_2, and $PGF_{2\alpha}$ synthesis and release from Kupffer cells. PGE_2 lowers cAMP and lipogenic gene expression (Figs. 2, 3, and 5), but increases hepatocyte proliferation, DNA synthesis, and glycogen breakdown through the EP3 receptors (10, 12, 13, 15, 16). EP3 receptors have been linked to G-proteins that regulate adenylyl cyclase and PLC. Lipogenic gene expression is inhibited by an increase in cAMP (3, 21), not a decline in cAMP. Thus, the effect of PGE_2 on cAMP in primary hepatocytes is probably not linked to the control of lipogenic gene expression. Activation of PLC leads to increased levels of IP3 and calcium. The calcium ionophore, A23187, inhibits S14 gene expression in primary hepatocytes (21). Based on this reasoning, we speculate that the PGE_2 and $PGF_{2\alpha}$-mediated suppression of hepatic lipogenic gene expression may require PLC activation leading to changes in intracellular free calcium. However, additional studies will be required to verify this regulatory mechanism.

PGE_2 effects on primary hepatic parenchymal cell gene expression were specifically directed at lipogenic genes, FAS, S14, and L-PK. We found no evidence for PGE_2 regulation of gene expression involved in non-mitochondrial fatty acid oxidation, i.e., AOX and CYP4A2 (Fig. 3). The transfection studies indicated that the mechanism of 20:4,n–6 and PGE_2 inhibition of S14 was transcriptional (Fig. 4) and the *cis*-regulatory targets for PGE_2 action converged with the S14 PUFA-RR (−220/−80 bp). Unlike peroxisome proliferator-activated receptors (22), neither 20:4, n–6 nor PGE_2 had any effect on thyroid hormone regulation of gene expression (Fig. 6).

A key transcription factor within the PUFA/PGE_2 response region (−220 to −80 bp) is NF-Y. NF-Y is a heterotrimeric transcription factor that binds a Y-box at −104/−99 bp and is required for the functioning of the two upstream enhancers (6). Any mutation or substitution of this element essentially abrogates S14 gene transcription. Thus, factors controlling NF-Y action impact on the transcriptional capacity of the S14 gene. The identification of the −220/−80 bp region as the target of PUFA/PG suppression implicated NF-Y in the 20:4,n–6/PGE_2-mediated suppression of S14CAT activity. A test to determine whether NF-Y alone was required for this inhibition indicated that promoter context dictates sensitivity of NF-Y to PUFA/PGE_2 control (Fig. 7). In the context of the S14 PUFA-RR, NF-Y is sensitive to PUFA/PG control. However, when NF-Y is in the context of the thymidine kinase (TK) promoter, it is not sensitive to either PUFA or PGE_2 control. The difference between these two promoters is that the Y-box in the TK promoter is flanked by two GC-rich regions binding Sp1 (19). NF-Y has been reported to interact functionally with Sp1 to augment transcription (23). While there are no Sp1 sites near the S14 Y-box, a number of other transcription factor binding sites just upstream from the S14 Y-box may bind factors that interact with NF-Y. Studies are in progress to identify these factors and evaluate their role in the PUFA/PG suppression of S14 gene transcription.

In summary, our studies have shown that 20:4,n–6 acts directly on hepatic parenchymal cells to suppress lipogenic gene transcription (**Fig. 8**). There is no requirement for cyclooxygenase activity for this control mechanism. However, specific prostanoids can act on the these cells through EP3 and FP receptors. In vivo, these prostanoids arise from non-parenchymal cells and act in a paracrine fashion on parenchymal cells to affect carbohydrate and lipid metabolism. Taken together with our previous studies on PPARs (5), fatty acids can regulate liver lipogenic gene transcription through three distinct pathways: one requires PPARα, another is prostanoid-dependent, and a third pathway is independent of PPARα and prostanoids. This latter pathway is likely to be the operative pathway in-

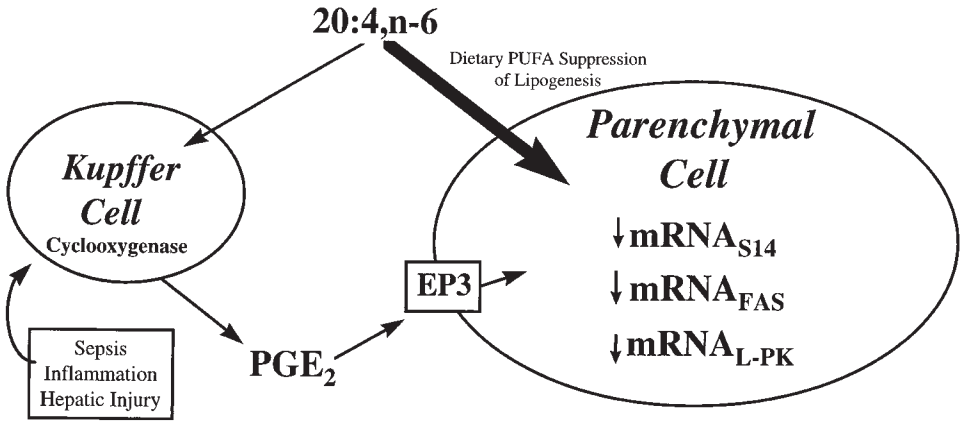

Fig. 8. Direct and paracrine effects of n–6 PUFA on hepatic lipogenic gene expression. See description in text.

volved in the suppression of hepatic lipogenic gene expression by dietary n–6 PUFA.

The research was supported by grants from the National Institutes of Health (DK 43220), the USDA (98-35200-6064), and the Michigan Agriculture Experiment Station. The authors thank Drs. A. Spencer, D. L. DeWitt, and W. Smith for their help in the Western blot analyses of COX1 and 2 in microsomal protein from primary hepatocytes.

Manuscript received 10 November 1998 and in revised form 17 February 1999.

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