Induction of Hepatic Insulin-Like Growth Factor Binding Protein-1 (IGFBP-1) in Rats by Dietary n-6 Polyunsaturated Fatty Acids

(44561)

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Abstract. **The insulin-like growth factors (IGFs) are mitogenic polypeptides that have been linked to a variety of normal physiological processes as well as neoplasia. Overexpression of several components of the IGF system is associated with hepatocarcinogenesis in humans and rodents. In rat liver, diets rich in n-6 polyunsaturated fatty acid (PUFA) enhance the development of preneoplastic lesions and tumors. Therefore, the objective of this study was to determine the effect of these dietary fatty acids on the hepatic expression of the various components of the IGF system. The mRNA levels of IGF-1 and the type 1 receptor were not different in livers of rats fed a diet containing 20% corn oil (CO) compared with those fed 5% CO. Analysis of the IGF binding proteins revealed that insulin-like growth factor binding protein-1 (IGFBP-1) levels were altered by the amount and type of dietary fat. A 2.5-fold induction of IGFBP-1 mRNA occurred within 1 week after the animals were fed the 20% corn oil diet compared with those fed 5% CO and was further enhanced to over 6-fold after 1 month. Furthermore, IGFBP-1 protein was only detectable in the livers of animals fed the 20% CO diet. Induction of IGFBP-1 mRNA (4.5-fold) also occurred in rats fed a high-fat diet containing safflower (rich in n-6 PUFAs) compared with those fed a high-fat diet containing menhaden oil (rich in n-3 PUFAs). The induction of IGFBP-1 mRNA was independent of serum insulin levels and the development of insulin resistance. Since IGFBP-1 mRNA is upregulated in regenerating liver, we reasoned that the induction of IGFBP-1 mRNA may be associated with an increase in cell proliferation; however, no difference was observed in the hepatic labeling index of rats fed the 20% CO compared with the 5% CO diet. In summary, these studies show a striking induction by dietary n-6 PUFAs of hepatic IGFBP-1, a protein that has been implicated in liver cancer development.** [P.S.E.B.M. 2000, Vol 225:128–135]

There is abundant evidence that the content and type of fat in the diet influences the development of spontaneous and carcinogen-induced tumors in experimental animals. In general, diets rich in n-6 polyunsaturated fatty acids (PUFAs) enhance tumor development compared with diets containing mainly n-3 PUFAs or saturated fatty acids (1–3). In the breast and colon, dietary fat appears to exert its major effect on the promotion phase of carcinogenesis (1, 3). In the liver, diets rich in n-6 PUFAs enhance the development of tumors and preneoplastic lesions (4, 5), but are believed to act primarily at the initiation stage (6–8). These fatty acids, however, enhance the action of liver tumor promoters such as phenobarbital (9) or a diet deficient in choline (10).

Diets with a high n-6/n-3 PUFA ratio are associated with the development of insulin resistance and hyperinsulinemia (11). In humans, hyperinsulinemia has been linked to an increase in the risk of breast (12, 13) and colon (14, 15) cancer, whereas in mice it may contribute to the devel-

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opment of diethylnitrosamine-induced liver tumors (16). It has been proposed that the increased levels of insulin and/or insulin-like growth factors may stimulate tumor development (12–16). In particular, the role of the insulin-like growth factors (IGFs) in carcinogenesis has been the subject of intensive investigation over the past decade. The IGFs are mitogenic polypeptides that have been implicated in a variety of normal physiological processes as well as in neoplasia (17).

The biological functions of the IGFs occur after their interaction with cell-surface receptors. This interaction can be modulated by at least six high-affinity binding proteins (IGFBPs) that are synthesized by most tissues including cancer cells (18, 19). IGFBPs are capable of inhibiting or enhancing IGF-mediated actions depending on the cellular context (18, 19). Although many effects of IGFBPs involve interactions with IGFs and their receptors, evidence is accumulating that IGFBPs are able to exert biological actions in an IGF-independent manner (20).

In the liver, the IGFs could act in an autocrine and/or paracrine fashion to modulate the development of tumors. Indeed, overexpression of IGF-2 in the livers of IGF-2 transgenic mice results in a significant increase in the number of spontaneous hepatocellular carcinomas (21). Increased levels of IGF-2 mRNA have been observed in human hepatocellular carcinomas (22, 23) and in chemically induced rat liver tumors (24). Elevated levels of IGF-1 receptor mRNA occur in hepatocellular carcinomas induced by chronic hepatitis B virus infection (25). Cultured rat hepatoma cells overproduce IGFBP-1 protein (26) and recently, *IGFBP-1* was identified as one of eight genes expressed at higher levels in human hepatocellular carcinoma than in normal liver tissue (27). The objective of this study was to determine in rats the effect of diets rich in n-6 PUFAs on the hepatic expression of the various components of the IGF system and to relate these changes to the efficacy of tumor enhancement by these fatty acids.

Materials and Methods

Animals and Diets. Pathogen-free, 43-day-old female Sprague-Dawley rats were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). Female lean (*FA/FA*) and obese (*fa/fa*) Zucker rats at 5 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). All rats were housed at 25°C and 50% humidity, with a 12:12-hr light:dark cycle and were acclimatized for 7 days with food and water provided *ad libitum*. The Sprague-Dawely rats were randomized into four groups and were fed one of four experimental diets for 1–4 weeks (Table I). Ten rats/group were fed either the 5% or 20% corn oil (CO) diets and five rats/group were fed the 18% safflower oil (SO) or 18% menhaden oil (MO) diets. The corn oil diets were chosen for our preliminary studies because they have been commonly used in previous experiments on hepatocarcinogenesis (4, 6). To test more specifically the effects of high-fat diets containing n-6 or n-3 PUFAs on *IGFBP-1* induction, we chose to use SO that contains high levels of n-6 PUFAs and MO that contains high levels of n-3 PUFAs. Because SO is deficient in linolenic acid, and MO is deficient in linoleic acid, 3% soybean oil that contains adequate amounts of these two essential fatty acids was added to each of the diets. To assess the impact of insulin resistance and hyperinsulinemia, groups of three lean and obese Zucker rats were fed a pelleted standard AIN-93M diet (Dyets, Bethlehem, PA) provided *ad libitum* for 13 weeks. Blood samples were collected by cardiac puncture under light halothane anesthesia, placed on ice, and then centrifuged at 3000*g* to isolate the serum for insulin analysis. All animals were sacrificed at the same time of day by cervical dislocation, and the livers were frozen immediately in liquid nitrogen and stored at –80°C or fixed in 5% formalin.

Table I. Composition (%) and Sources of Fat in Experimental Diets

Ingredient	5% CO[a]	20% CO	18% SO[b]	18% MO[c]
Cornstarch	15	11.5	28	28
Casein	20	20	23	23
Dex-CS	50	38.5	9	9
Sucrose	0	0	7	7
Alphacel	0	0	6	6
Cellulose	5	5	0	0
Mineral mix	3.5	3.5	4	4
Vitamin mix	1	1	1.2	1.2
L-Cystine	0	0	0.4	0.4
DL-Methionine	0.3	0.3	0	0
Choline bitartrate	0.2	0.2	0.29	0.29
Soybean oil	0	0	3	3
Corn oil	5	20	0	0
Safflower oil	0	0	18	0
Menhaden oil	0	0	0	18
Energy (kcal/100g)	385	460	416	416
n-6:n-3	59	59	50	0.38

[a] CO, corn oil
[b] SO, safflower oil
[c] MO, menhaden oil

Northern Blot Analysis. Total RNA (20–25 μg) from the livers of individual rats was isolated by TRIzol reagent (Life Technologies, Grand Island, NY) and subjected to Northern blot analysis (28) using Zeta-Probe nylon membranes (Bio-Rad, Hercules, CA). The hybridization solution contained 100ng of the probe labeled with [α^{32}P]dCTP using random hexamer priming (Ambion Co., Austin, TX) to a specific activity of $\approx 10^9$ cpm/μg. The following rat-specific probes were used: IGF-1 (generously provided by Dr. P. Rotwein, Washington University School of Medicine, St. Louis, MO), a 182-base pair *Bam*HI-*Eco*RI fragment of exon 4 from plasmid pGEM1 (29); IGF-2 (generously provided by Dr. M. Rechler, NIADDKD), a 551-base pair *Pst*I-*Bam*HI fragment from plasmid pG3-rIGF-II-7a (30); IGF-1 receptor (generously provided by Dr. D. LeRoith, NIADDKD), a 265-base pair *Eco*RI-*Rsa* fragment from plasmid pGEM-3 (31); *IGFBP-1* (generously provided by Dr. L. Murphy, University of Manitoba, Winnipeg,

Manitoba, Canada), a 1.5-kb *Eco*RI insert from plasmid pSVLJ (32); and *IGFBP-4* (generously provided by Dr. S. Shimasaki, Whittier Institute), a 444-base pair *Sma*I-*Hin*dIII fragment from plasmid pRBP4-SH (33). Following hybridization and autoradiography, membranes were rehybridized to a ^{32}P-labeled-28S human ribosomal probe (Cedarlane Labs, Hornby, Ontario, Canada) to verify equal RNA loading. Autoradiograms were scanned with an Ultrascan XL laser densitometer (LKB, Washington, DC), and signals were integrated and normalized to the corresponding level of the 28S ribosomal band.

Immunoprecipitation of Liver Homogenates. Livers were homogenized in 0.25*M* sucrose, 10m*M* EDTA, pH7.6 to yield a 20% homogenate (w/v). Aliquots of homogenate were incubated with 2.0μg of anti-rat–IGFBP-1 (Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. The immunocomplex was captured by adding 100μl of Protein A agarose bead slurry (Santa Cruz Biotechnology, Santa Cruz, CA) and incubating at 4°C for 2 hr.

SDS PAGE and Immunoblot Analysis. The washed agarose beads were mixed with Laemmli sample buffer without β-mercapatoethanol, heated at 60°C for 10 min, and microfuged, and the supernatants were subjected to 15% nonreduced SDS PAGE (34). Proteins were transferred to 0.2 μm polyvinylidene difluoride membranes using a semidry apparatus. The membranes were blocked with 3% (w/v) nonfat dry milk, 10 m*M* Tris-buffer (pH 7.4, 150 m*M* NaCl and 0.1% (v/v) Tween) overnight at 4°C and then probed with 1 μg/ml of anti-rat–IGFBP-1 for 3hr at room temperature. Proteins were visualized using a goat–anti-rabbit secondary antibody conjugated with horseradish peroxidase and a chemiluminescence detection system according to the manufacturers specifications (Santa Cruz Biotechnology, Santa Cruz, CA).

Determination of Hepatic Labeling Index. Rats (five/group) were fed a 5% or 20% corn-oil diet (Table I) *ad libitum* for 13 days. On Day 7, newly synthesized DNA was labeled by continuously infusing 20 mg/ml bromodeoxyuridine (BrdU) dissolved in phosphate-buffered saline for 6 days using osmotic minipumps (Alzet model 2ML1, Alza Co., Palo Alto, CA) that were implanted subcutaneously under halothane anesthesia. On Day 13, the hepatic labeling index was determined by immunohistochemistry as previously described (35) but modified to include pepsin digestion for formalin-fixed sections.

Serum Insulin Analysis. Serum insulin was measured by radioimmunoassay (RIA) (ICN Pharmaceuticals, Costa Mesa, CA) according to the manufacturer's protocol.

Serum IGF-1 Analysis. Serum IGF-1 was measured by radioimmunoassay (RIA) (DSL, Webster, TX) according to the specifications described by the manufacturer. Briefly, 50 μl of IGF-1 standards or extracted and neutralized serum samples were added to 100 μl of rat IGF-1 [I^{125}] and 100 μl of rat IGF-1 antiserum and incubated at room temperature for at least 3 hr. The samples were precipitated with 1.0 ml of donkey anti-goat gamma globulin and counted on a gamma counter. Concentrations of IGF-1 in the serum samples were determined graphically from a standard curve obtained from the results of the IGF-1 standards.

Results

Effects of Dietary PUFAs on the IGF System in the Liver. The levels of the major (8-kb) and minor (2- and 1-kb) hepatic IGF-1 transcripts were not significantly different in rats fed diets containing 5% or 20% CO for 1 month (data not shown). This finding was reflected in the serum IGF-1 levels that were also unaffected by the amount of fat in the diet. IGF-2 mRNA was undetectable in the livers of rats fed either of the experimental diets, consistent with findings of extremely low levels of IGF-2 in adult rat liver (24). Furthermore, dietary fat had no effect on the mRNA levels of the type 1 IGF receptor.

In rats fed the 20% CO diet for 1 month, however, there was approximately a 6.5-fold increase in hepatic IGFBP-1 transcripts compared with animals fed the 5% CO diet (Figs. 1a & 1b). IGFBP-4 mRNA was highly expressed in these samples, but no significant differences were seen between the dietary groups (Figs. 1a & 1b). Western analysis of liver

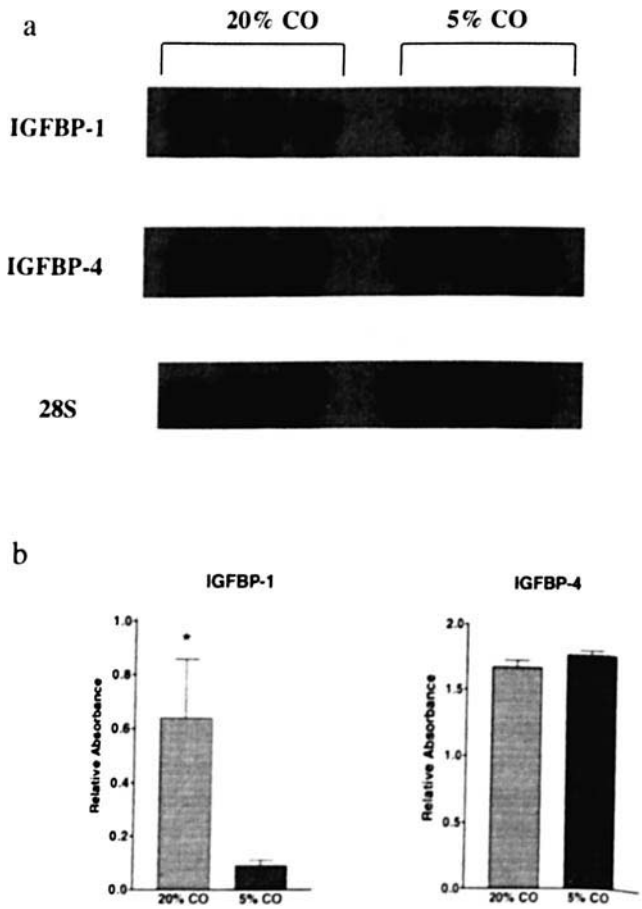

Figure 1. (a) Northern blot analysis of IGFBP-1 and IGFBP-4 mRNA in the livers of rats fed a 5% or 20% corn-oil diet for 30 days. (b) The transcripts were normalized to the 28S ribosomal band. Data are the means ± SEM for 10 animals. *indicates a significant difference ($P < 0.05$) using the Student's *t* test.

homogenates revealed that IGFBP-1 was only detectable in the animals fed the 20% CO diet (Fig. 2). The induction of hepatic IGFBP-1 mRNA in the groups fed the 20% CO diet could be detected within 1 week and at this time was about 2.5-fold higher than the 5% CO group (Figs. 3a & 3b).

The induction of hepatic IGFBP-1 mRNA was dependent not only on the amount of dietary fat, but also its type. In an experiment in which rats were fed for 3 weeks diets containing either 18% safflower (SO) that is rich in n-6 PUFAs or 18% menhaden (MO) oil that is rich in n-3 PUFAs, there was approximately a 4.5-fold increase in hepatic IGFBP-1 transcripts in the SO compared with the MO group (Figs. 4a & 4b). The body weights of animals in these groups did not differ at the end of the feeding period.

Effects of Insulin Levels and Insulin Resistance on Hepatic IGFBP-1 mRNA Levels. Since insulin is known to be a potent transcriptional inhibitor of the

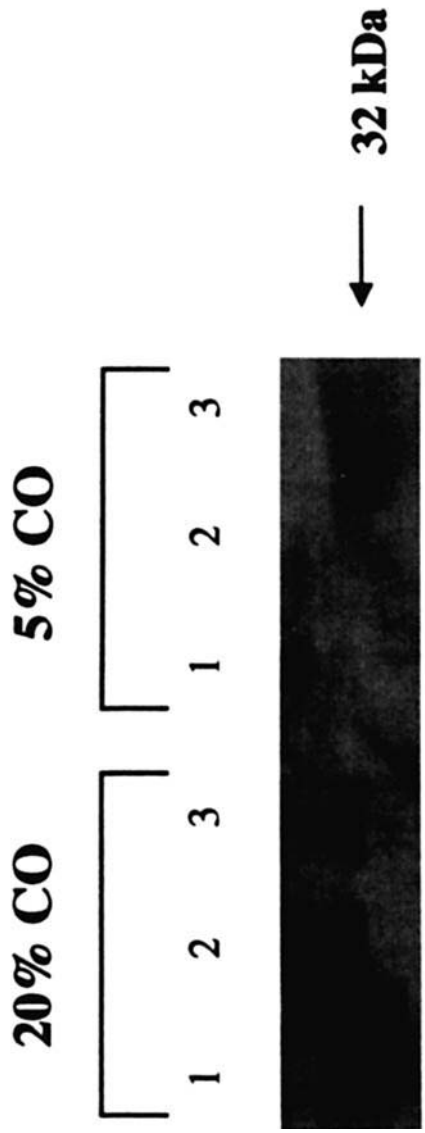

Figure 2. Western Blot analysis of IGFBP-1 protein in the livers of rats fed a 5% or 20% corn-oil diet for 1 month.

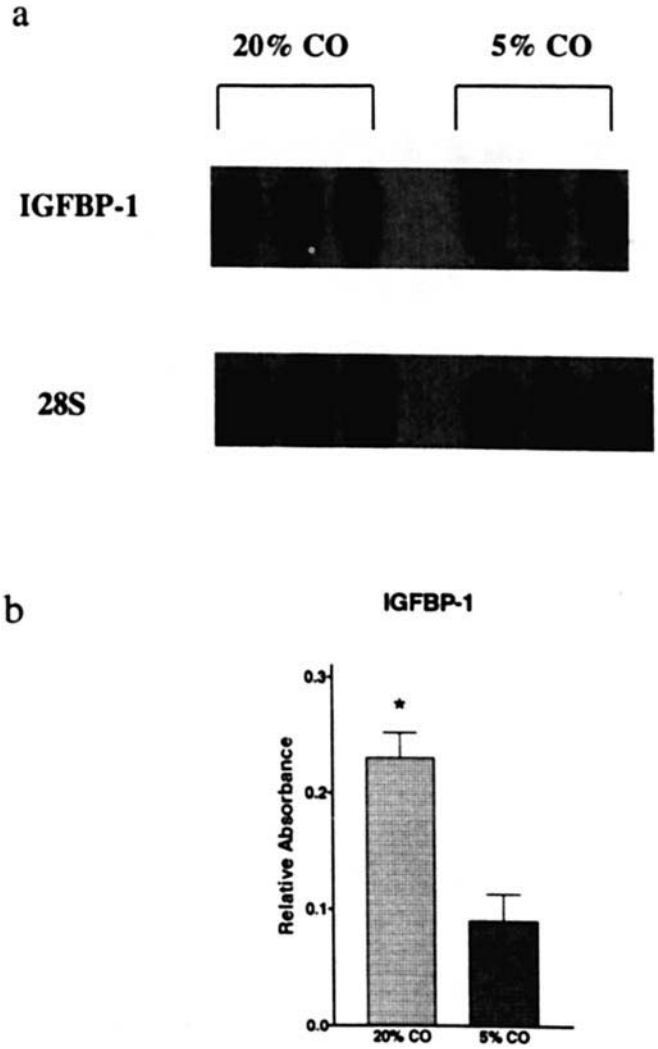

Figure 3. (a) Northern blot analysis of IGFBP-1 mRNA in the livers of rats fed a 5% or 20% corn-oil diet for 7 days. (b) Quantitation of IGFBP-1 transcripts relative to the 28S ribosomal band. Data are the means ± SEM for five animals. *indicates a significant difference ($P < 0.05$) using the Student's t test.

IGFBP-1 gene (36), serum insulin levels were determined in animals fed the 20% or 5% CO diets for 1 month. The mean serum insulin values were somewhat higher for the rats fed the 20% CO diet compared with those fed the 5% CO diet, but the differences did not reach significance (65.3 ± 13.6 vs 36.4 ± 14.1μIU/ml, respectively).

High-fat diets can induce insulin resistance (11, 37) such that target tissues lose their ability to respond to the metabolic actions of insulin such as glucose uptake. It is possible that insulin may have lost its ability to suppress IGFBP-1 transcription in the livers of the rats fed the high-CO or SO diets. To test this notion, we measured the levels of hepatic IGFBP-1 transcripts in obese Zucker rats, a well-established model for the development of hyperinsulinemia and insulin resistance (38). Groups of obese rats and lean controls were fed a standard AIN-93M diet for 13weeks. At this time, serum insulin levels were markedly higher in the obese rats compared with the lean controls (1200 ± 215 vs

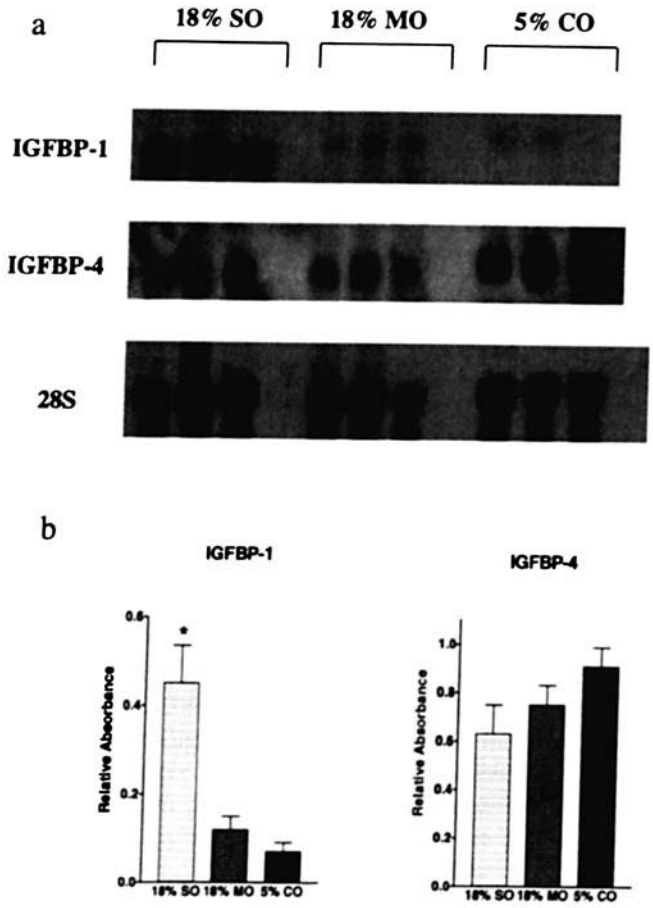

Figure 4. (a) Northern blot analysis of IGFBP mRNA in the livers of rats fed an 18% safflower-, 18% menhaden-, or 5% corn-oil diet for 3–4 weeks. (b) Quantitation of IGFBP transcripts relative to the 28S ribosomal band. Data are the means ± SEM for five animals. *indicates a significant difference ($P < 0.05$) using the Student's *t* test.

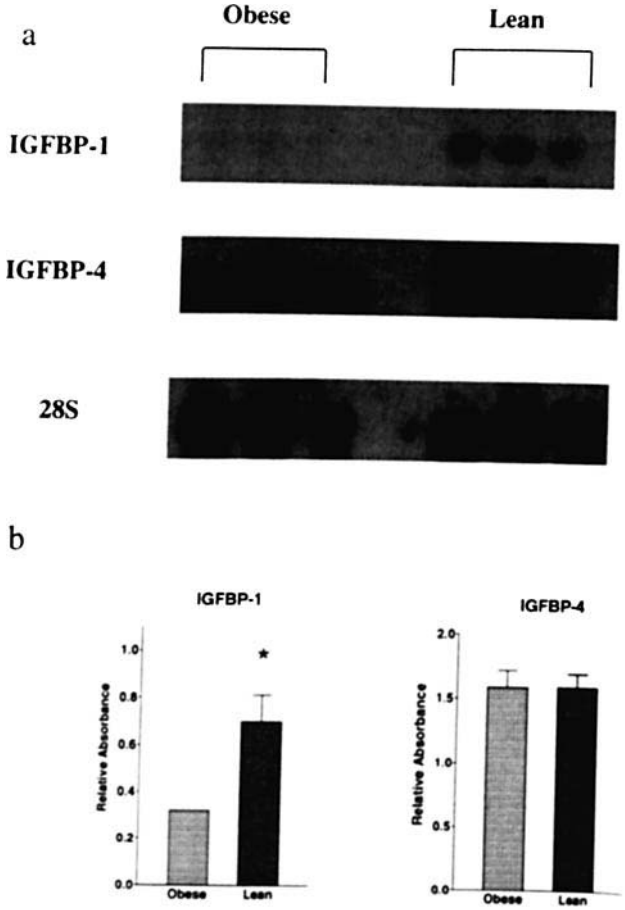

Figure 5. (a) Northern blot analysis of IGFBP mRNA in the livers of Zucker obese and lean rats. (b) Quantitation of IGFBP transcripts relative to the 28S ribosomal band. Data are the means ± SEM for three animals. *indicates a significant difference ($P < 0.05$) using the Student's *t* test.

50 ± 11 μIU/ml, respectively, $P < 0.0001$). The obese animals also showed significantly higher levels of serum glucose than the controls, indicating that they had developed insulin resistance (184 ± 9 vs 156 ± 11 mg/dl, respectively, $P < 0.05$). Despite the development of hyperinsulinemia and insulin resistance in the obese rats, hepatic IGFBP-1 transcripts were ≈ 2-fold lower compared with the lean controls (Figs. 5a & 5b), suggesting that insulin was still able to suppress IGFBP-1 transcription.

Effects of Dietary PUFAs on Hepatocyte-Labeling Index. IGFBP-1 mRNA has been shown to be rapidly and highly induced in regenerating liver and *IGFBP-1* has been characterized as an immediate–early growth response gene (3941). Because of its potential role in hepatocyte proliferation, we reasoned that the induction of IGFBP-1 mRNA by dietary fat may be associated with an increase in cell proliferation. To test this possibility, we measured the effect of feeding the 20% CO diet on the hepatic labeling index (LI). The 20% CO diet was fed for 2 weeks and during the second week the rats were implanted with miniosomotic pumps containing BrdU. Under these conditions, we found no significant difference in the LI between the 20% and 5% CO groups (2.78 ± 0.51% vs 2.71 ± 0.48%, respectively).

Discussion

Our results show that dietary n-6 PUFAs significantly increase hepatic IGFBP-1 transcripts and protein levels. Induction of IGFBP-1 occurred in Sprague-Dawley rats fed a 20% CO diet compared with a 5% CO diet. A 2.5-fold induction in mRNA levels occurred within 1 week that increased to 6.5-fold after 1 month. Furthermore, IGFBP-1 protein was detected in the livers of only those animals fed the high-fat diet. Upregulation of IGFBP-1 mRNA was caused by diets containing either CO or SO that are rich in n-6 PUFAs, whereas a diet containing a similar level of MO that is rich in n-3 PUFAs did not lead to upregulation. In contrast to IGFBP-1, hepatic mRNA levels of IGFBP-4 were not different among the various dietary groups. IGFBP-4 has been previously shown to be expressed constitutively in the liver (18).

Caloric restriction has been shown to induce hepatic IGFBP-1 (42); however, in our experiments induction of IGFBP-1 mRNA and protein occurred in the 20% CO diet

that had a somewhat higher caloric content than the 5% CO diet (Table I). Furthermore, the SO diet induced IGFBP-1 mRNA relative to the MO diet even though these diets were strictly isocaloric (Table I), and the weight gains of the animals were identical. Thus, our experiments demonstrate that the upregulation of the *IGFBP-1* gene by n-6 PUFAs occurs independently of any caloric effects of the diets.

It is known that the proliferative stimulus of a partial hepatectomy leads to a marked induction of *IGFBP-1* (39–41); therefore, we reasoned that the induction of *IGFBP-1* might be secondary to an increase in hepatocyte proliferation caused by the diets rich in n-6 PUFAs. Alternatively, since IGFBP-1 plays a role in regulating IGF-1–induced mitogenesis (18, 19), changes in *IGFBP-1* expression might cause a change in hepatocyte proliferation. However, when we measured the hepatocyte labeling index, it was not different between the rats consuming the 20% or 5% CO diets. Furthermore, there were no significant differences in the levels of either hepatic IGF-1 transcripts or the serum levels of IGF-1. These results suggest that induction of *IGFBP-1* by n-6 PUFAs is not secondary to an increase in hepatocyte proliferation, nor does the induction of this gene alter the level of hepatocyte proliferation. These findings are in agreement with those of Liu *et al.* (43) who demonstrated that despite the overexpression of IGFBP-1 mRNA in the livers of *IGFBP-1* transgenic mice, hepatic regeneration was not enhanced after partial hepatectomy.

Insulin appears to play a key role in controlling the levels of IGFBP-1 by inhibiting hepatic *IGFBP-1* expression in rodents at the level of gene transcription (36). We reasoned therefore that the induction of *IGFBP-1* by n-6 PUFAs might be secondary to changes in insulin levels. In the present study, however, there was no significant difference in serum insulin levels between rats fed either the 20% or 5% corn oil diet for 1 month. Indeed, it is well established that long-term feeding of a high-fat diet induces hyperinsulinemia (37) that would lead to downregulation of *IGFBP-1* expression, the opposite of what we observed. Since consumption of a high-fat diet also induces insulin resistance (37), we thought it was possible that insulin may have lost its ability to suppress transcription of the *IGFBP-1* gene in the animals fed 20% CO. To test this hypothesis, we examined IGFBP-1 mRNA levels in the livers of obese Zucker rats that are well known to develop insulin resistance in the absence of a high-fat diet (38). In these rats, however, that we showed to be insulin-resistant, the level of hepatic *IGFBP-1* transcripts was about 2-fold lower than in lean Zucker controls. These results suggest that in animals that are insulin-resistant, insulin is still capable of suppressing the transcription of the *IGFBP-1* gene. The obese Zucker rats were also hyperinsulinemic, and our results are consistent with those of Lewitt *et al.* (44) who reported about 3-fold lower serum IGFBP-1 levels in obese Zucker rats compared with lean controls. In the same study, Lewitt *et al.* found that consumption of an undefined high-fat diet for 1 month resulted in about 5-fold lower serum IGFBP-1 in Wistar rats. In their experiments, however, the fat (unspecified) comprised 59% of the calories of the diet and induced hyperinsulinemia. In our experiments with Sprague-Dawley rats, the PUFAs comprised no more than 40% of the calories of the diet and did not induce hyperinsulinemia in the 1 month of feeding.

Our results suggest that induction of *IGFBP-1* expression by n-6 PUFAs does not depend on the caloric content of the diet, the induction of cell proliferation, or changes in insulin levels or its effects. It seems likely that the n-6 PUFAs induce the expression of the *IGFBP-1* gene either by increasing the stability of the mRNA or by increasing the rate of gene transcription, possibly *via* a *cis*-acting PUFA responsive element in the promoter region of the gene. Both of these mechanisms have been described in studies on the regulation by PUFAs of a number of other genes (45, 46).

Taken together with the reports of high levels of IGFBP-1 in rat hepatoma cells (26) and human liver tumors (27), our results suggest that this protein may be involved in hepatocellular carcinogenesis. That we observed no concomitant increases in the expression of IGF-1 or IGF-2 by dietary n-6 PUFAs suggests that IGFBP-1 may act in an IGF-independent manner to promote cancer development. This type of mechanism has recently been suggested to explain the increased tumorigenic potential of adrenocortical tumor cells overexpressing IGFBP-2 (47).

We observed no changes in hepatocyte proliferation in the livers that overexpressed IGFBP-1. It has been suggested that upon entering the cell cycle, hepatocytes pass through two phases termed competence and progression (48–51). Growth signals associated with competence include upregulation of *IGFBP-1*, as well as several proto-oncogenes such as c-*myc*, c-*fos*, and c-*jun* (48, 49). Competent cells can then respond to progression signals and eventually commit to DNA synthesis (48, 49). Our results suggest that the induction of *IGFBP-1* expression by dietary n-6 PUFAs may render hepatocytes competent. Although not yet committed to replication, these cells may be more susceptible to mitogenic stimuli during the formation and subsequent clonal expansion of initiated cells.

In summary, our studies show a striking induction of hepatic IGFBP-1 by dietary n-6 PUFAs. Further studies are needed to determine whether induction of IGFBP-1 plays a causative role in hepatocellular carcinogenesis.

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