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Growth Failure in Cholestatic Rats: The Effect of Malnutrition on Insulin-Like Growth Factor I[1]

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ABSTRACT. Low IGF-I levels are found in patients with chronic liver disease, but it is not known whether these reductions in IGF-I are secondary to hepatic dysfunction or to malnutrition. To determine whether malnutrition associated with hepatic dysfunction causes the decrease in these levels, serum and liver IGF-I concentrations and liver IGF-I mRNA content were compared in three groups of Sprague-Dawley rats: 15 rats underwent bile duct obstruction; 10 rats were sham-operated and pair-fed with operated rats to control for nutritional status; and 12 rats were sham-operated controls fed *ad libitum*. In addition, IGF-I peptide and mRNA were compared with food intake, crude nitrogen balance, total wt gain, tail length increase and leg muscle wt. All the parameters were lower in cholestatic and nutritionally deprived animals than in control animals ($p < 0.001$). There was no difference in serum and hepatic IGF-I and liver IGF-I mRNA values between the 10 cholestatic and pair-fed animals, despite lower crude nitrogen balance, tail length gains, and leg muscle wt in the bile duct-obstructed animals. These studies suggest that in chronic bile duct obstruction, the low serum and hepatic IGF-I levels are primarily due to decreased food intake and appear unrelated to cholestatic liver disease itself. However, factors in addition to suboptimal nutrition and decreased IGF-I levels must also contribute to cholestasis-induced growth failure. (*Pediatr Res* 26: 410–414, 1989)

Abbreviations

OP, operated (bile duct obstruction)
PF, pair-fed
CON, control
HYPOX, hypophysectomized
GH, growth hormone
ANOVA, analysis of variance

Most children with chronic cholestatic liver disease suffer from malnutrition and growth failure (1–3). It has been suspected that this poor growth is related to depressed somatomedin levels because the liver is a major source of circulating somatomedins (4), and either circulating or local IGF-I is believed to mediate most of the growth-promoting actions of GH on peripheral tissues (5). Reductions in somatomedins measured by a variety of methods have been observed in studies on adults with chronic liver disease. Wu *et al.* (6) initially demonstrated depressed somatomedins (measured as sulfation factor) in adults with cirrhosis, and this finding was confirmed in additional studies using radioreceptor assays (7) and RIA (8, 9). Unfortunately, the role of malnutrition in these patients was not considered. Malnutrition alone also results in depression of somatomedins (measured as sulfation factor) (10–12). Deficient IGF-I production might thus contribute to the growth failure observed in children with chronic cholestatic liver disease, either because of intrinsic liver dysfunction or malnutrition, or both. In addition, GH levels are elevated both in patients with chronic liver disease (13) and with malnutrition (11, 12). It has been proposed that malnutrition, in the absence of hepatic failure, results in a GH postreceptor defect (14, 15). Preliminary studies in children also suggest that short stature in chronic liver failure may be due to a relative GH resistance and further that this may be independent of nutritional status (16). Thus, abnormal IGF-I levels associated with chronic liver disease could be the result of decreased hepatic synthesis of IGF-I or be the direct result of a defect in response to GH caused by either malnutrition or intrinsic hepatic dysfunction.

The following studies were designed to determine the effects of liver disease and malnutrition on growth and IGF-I production. We used a model of bile duct obstruction to induce cholestatic liver disease and growth failure in young rats. To assess the effects of nutrition, we pair-fed the cholestatic animals with sham-operated rats. Serum IGF-I levels, liver IGF-I content and liver IGF-I mRNA were assessed in all groups.

MATERIALS AND METHODS

Animals. Prior approval for all procedures was obtained from the institutional committee for the use of animals in research (Research Animal Resources, University of Minnesota, MN). Three groups of weanling Sprague Dawley rats (Bio-Laboratory, St. Paul, MN) were studied at 21 d of age: 15 rats (eight males, seven females) OP; 10 rats (five males, five females) were sham-operated and PF with OP rats to control for the contribution of nutritional intake; 12 rats (six males, six females) were sham-operated CON and were fed *ad libitum*. In brief, the surgery for the bile duct obstruction included three ligatures tied securely around the common bile duct. The bile duct was transected between the two sutures most distal from the liver. Sham-operated animals underwent an identical procedure without ligation and transection. The animals were returned to the cage with litter mates for 1 d and then roomed individually in metabolic cages. Rats were fed with Purina Rodent Laboratory Chow 5001 (St. Louis, MO). Each PF rat was PF with another OP rat.

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The amount of food ingested by the OP rats was measured daily and the same amount of food was given to the corresponding PF rats unless the PF rat was bigger. When the PF rat was bigger than the OP, the same amount calculated per gram of body wt was given. Total food intake was recorded and urine collected for each animal. Wt and tail length measurements were recorded on the day of surgery and at time of sacrifice. The animals were killed at 41 d of age by exsanguination after sodium pentobarbital anesthesia. Serum was frozen for bilirubin, albumin, and IGF-I assays. The liver was perfused with chilled saline until it became pale to eliminate peripheral blood IGF-I contamination of liver IGF-I. After weighing, a portion was reserved for histologic examination. The major portion was frozen in liquid nitrogen and stored at −70°C for IGF-I peptide and IGF-I mRNA measurements. Aliquots were saved for determination of total protein (17). Gastrocnemius muscle was removed and weighed as another parameter of growth. The same parameters were also determined in an additional 10 HYPOX rats (five males, five females) aged 41 ± 3 d (Taconic Laboratories, Germantown, NY). Hypophysectomy was performed by the supplier at 28 ± 3 d of age.

Serum IGF-I. Serum IGF-I levels were assayed in duplicate using a RIA kit purchased from Nichols Institute Diagnostics (San Juan Capristano, CA) and a polyclonal antibody which recognizes rat IGF-I (gift of Dr. L. Underwood, University of North Carolina, Chapel Hill, NC). Serum samples were subjected to acid-ethanol extraction before assay to free IGF-I from binding proteins as described (18). The intraassay coefficient of variation was 3.42%. The interassay coefficient of variation of an adult rat pool was 11.4%. When IGF-I in the form of extracted adult rat pool serum was added to the samples, recovery was 101–131%.

Liver IGF-I. Acetic acid extraction of liver was performed as described (19). Extracts were frozen at −20°C for IGF-I RIA (within 6 wk). All studies were performed on liver perfused with chilled saline to minimize the contribution of circulating IGF-I on the measurement of tissue levels, as we found that in four (41 d old) rats, saline perfusion of liver decreased the IGF-I content by 12.6% in comparison with values obtained in three rats of the same age without liver perfusion. This estimate of peripheral blood contribution to liver IGF-I was similar to the 17 ± 2.4% estimate of D'Ercole *et al.* (20) calculated by measuring liver hemoglobin content. Recovery of IGF-I in the form of extracted adult rat pool serum added to liver extracts averaged 62.8–65.8%.

Liver IGF-I mRNA. Dot blot hybridization. Liver IGF-I mRNA was measured after guanidine-HCl extraction of total hepatic RNA (21). Total IGF-I mRNA was measured by dot-blot hybridization (21) with an IGF-I cDNA insert. The cDNA probe used in this study was a 2.2-kb fragment obtained by screening a λgt 11 adult rat liver cDNA library (Dr. H. Towle, Minneapolis, MN) with a human IGF-I cDNA (Dr. K. Gabbay, Houston, TX). The identity of the probe was established by cDNA sequencing with comparison of these data to published sequences for this gene (data not shown). Sequence analysis confirmed that this probe is like the IGF-IA cDNA reported by Roberts *et al.* (22). The probe was labeled using the oligo labeling kit from Pharmacia (Piscataway, NJ). Typical specific activities of labeled cDNA were 5–9 × 10^8/μg DNA. After hybridization, the dot-blots were washed four times for 10 min in 2 × SSC (1 × SSC = 0.15M NaCl and 0.015M Na citrate, pH 7.0), 0.1% SDS at 25°C and three times in 0.2 × SSC, 0.1% SDS at 60°C twice for 45 min and once for 30 min. The papers were blotted, and exposed to Kodak XAR-5 x-ray film (Eastman Kodak, Rochester, NY) for autoradiography, typically overnight at 23°C. The intensity of the signal was determined by videodensitometry (23), and expressed as a percentage of IGF-I mRNA observed in adult rat pooled RNA. Samples were blotted at four concentrations and the OD/μg RNA was determined by linear regression. For all samples, r for the regression of concentration *versus* intensity was more than 0.98.

Northern gel analysis. Liver IGF-I mRNA species were examined using Northern gels under formaldehyde denaturing conditions (24). The gels were electrotransferred in 0.75 × TAE (10 × TAE: 100 mM Tris Base, 50 mM ammonium acetate, 5 mM EDTA) using a Transblot apparatus (Bio-Rad Laboratories, Richmond, CA) on Zetabind (Cuno, Inc., Meriden, CT) at 80 V for 5 h. Filters were air-dried and baked for 2 h at 80°C under vacuum. Hybridization was performed by the above procedure except the hybridization solution contained 2 × Denhardt's solution, 0.1% SDS, and 0.1 mg/mL sheared salmon sperm DNA.

Albumin and Total Bilirubin. Serum albumin was measured using the colorimetric methodology of the Kodak Ektachem Clinical Chemistry Slides (ALB) (Eastman Kodak Company, Rochester, NY) by the bromcresol green dye.

Total serum bilirubin was measured using colorimetric methodology of the Kodak Ektachem Clinical Chemistry Slides (TBIL) (Eastman Kodak Company, Rochester, NY) by the diazonomium salt.

Urine Nitrogen and Estimated Nitrogen Balance. Urine from each animal was pooled and total urine nitrogen was measured using chemiluminescent total nitrogen analysis (25). Estimated nitrogen balance was calculated from the second postoperative day until death by subtracting urine nitrogen excretion from intake.

Histology. A portion of the liver was fixed in buffered formalin and processed for histologic examination. Hematoxylin and eosin stained microscopic sections were evaluated in a blinded fashion by one of the authors (H.L.S.).

Statistical Analysis. Mean measurements were compared across the three study groups using ANOVA. Where an overall difference among group means was found, the Scheffé method for multiple comparisons was used to test for the differences between OP and CON groups, between PF and CON groups, and between HYPOX and the other groups. Comparisons between measurements for the subset of 10 pair-rats of the OP and PF groups were performed using Student's t test for paired values. Pearson product-moment correlation coefficient was used to assess the relationship between serum IGF-I and bilirubin in OP rats.

RESULTS

All bile duct-ligated rats were clinically jaundiced at the time of death. Bilirubin was greater than 42.75 in all OP rats (mean ± SD: 68.23 ± 21.72 μM), and was normal in the other rats (PF: 3.08 ± 2.05 μM, CON: 3.25 ± 2.39 μM). In OP rats, histologic examination showed ductular cholestasis, bile duct proliferation, and intermittent bridging fibrosis between portal tracts. In PF rats, the liver was normal. In some CON rats, hepatocytes were vacuolated with microvesicular fat. The successful implementation of the PF was demonstrated by the similarity of the total food intake between the subset of matched OP and PF animals and the significant differences between these groups and the CON group (Fig. 1). Serum albumin concentrations were not significantly different among the three groups (CON: 27.0 ± 2.6, OP: 29.1 ± 5.2, PF: 30.2 ± 5.1 g/L). However, estimated nitrogen balance was higher in CON than in either OP or PF rats ($p < 0.001$) and higher in PF than in OP rats ($p < 0.05$) (Fig. 1).

Changes in physical parameters of growth are demonstrated in Figure 2. Significant differences ($p < 0.001$) were found in wt gain, tail length increase, leg muscle wt, and liver wt when expressed as g liver/g body wt. Wt gain was higher in CON than in OP or PF rats ($p < 0.001$). The higher wt gain in 10 matched OP than in PF rats ($p < 0.05$) was attributed to significant ascites and increased liver wt. Tail length increase was greater in CON than in OP or PF rats ($p < 0.001$). However, tail length increase was higher in PF than in OP rats ($p < 0.001$). Leg muscle wt was higher in CON than in OP or in PF rats ($p < 0.001$), and in PF than in OP rats ($p < 0.01$). Liver wt when expressed as percentage of g/g body wt was similar in CON and in PF rats

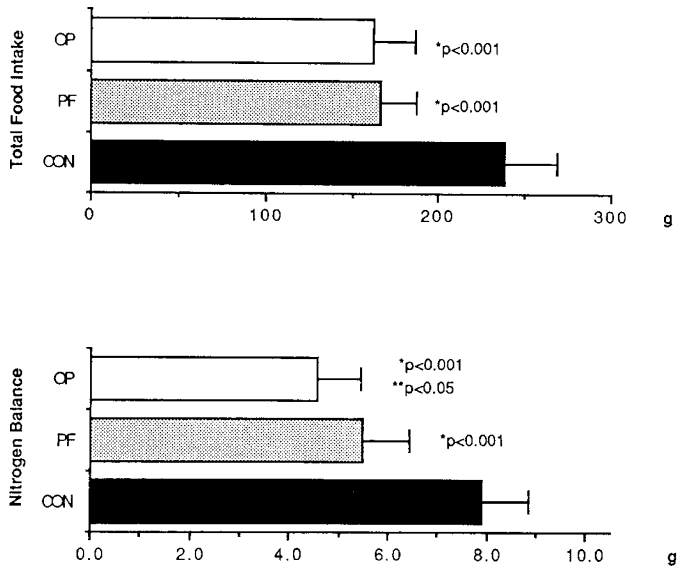

Fig. 1. Total food intake (g) and estimated nitrogen balance (g) in 12 CON, 10 PF, and 15 OP rats. *statistical comparison of PF or OP *versus* CON; **statistical comparison of OP *versus* PF. Values shown are means ± SD. The differences in the groups for estimated nitrogen balance were significant ($p < 0.01$, $F_{2,34} = 45.49$).

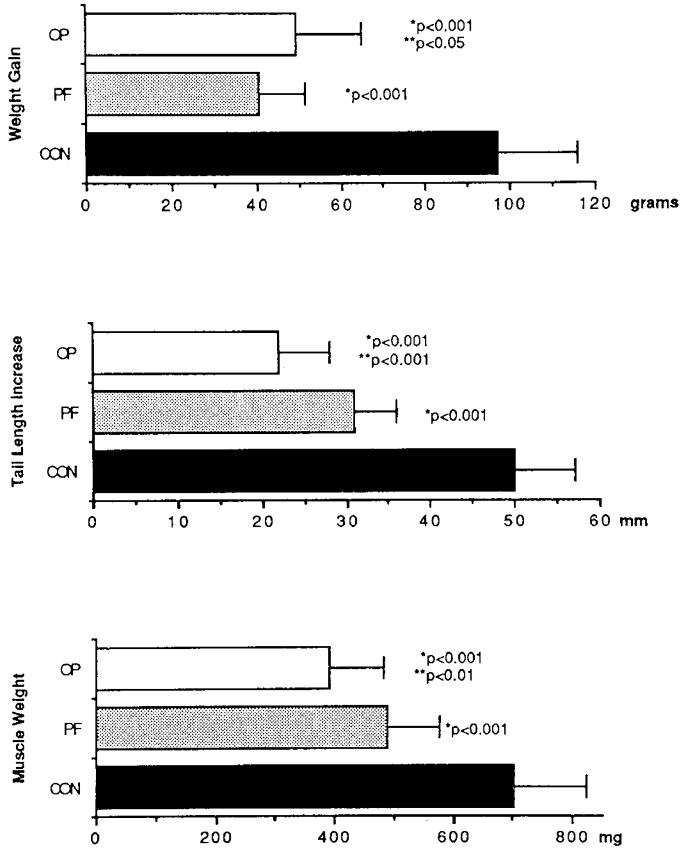

Fig. 2. Wt gain (g), tail length increase (mm), leg muscle wt (mg) in 12 sham-operated CON rats fed *ad libitum*, 10 sham-operated and PF rats, and 15 OP rats (*OP*). *statistical comparison of PF or OP versus CON; **statistical comparison of OP *versus* PF. Values shown are means ± SD. Significant differences ($p < 0.001$) are found in wt gain ($F_{2,34} = 43.54$), tail length increase ($F_{2,34} = 66.72$), and leg muscle wt ($F_{2,34} = 32.24$).

(4.7 ± 0.6 *versus* 4.5 ± 0.9% g/g) and higher in OP rats (7.7 ± 0.8% g/g, $p < 0.001$).

Serum and tissue IGF-I values by RIA and tissue IGF-I mRNA measurements are summarized in Figure 3. Serum IGF-I levels were 3.5 times higher in CON rats than either OP or PF rats ($p < 0.001$), but not different between PF and OP rats. In 10 HYPOX rats, serum IGF-I concentrations were significantly lower than in all the other groups ($p < 0.001$). No correlation was found between serum IGF-I levels and bilirubin concentrations in the OP rats. Liver IGF-I levels, expressed per g of liver protein, were 2.9 times higher in CON compared to OP rats and 2.1 times those observed in PF animals ($p < 0.001$ for both), and not different between PF and OP rats. In 10 HYPOX rats, liver IGF-I concentrations per g of protein were again significantly lower than in the other groups ($p < 0.001$). Liver IGF-I mRNA levels were essentially twice as high in CON animals compared to either OP or PF rats (1.8- and 2.1-fold changes, respectively), but not different between PF and OP rats. In HYPOX rats, the autoradiogram signal was insufficient for quantitation. Changes in tissue IGF-I levels measured by RIA closely paralleled changes observed in IGF-I mRNA in terms of fold change.

Hybridization of IGF-I cDNA containing sequences common to all IGF-I mRNA to Northern blots of RNA from OP, PF, and CON rat liver demonstrated the presence of the major species of hepatic IGF-I mRNA [0.8–1.2 kb, 1.7 kb, and 7.5 kb (26)] in all three groups of rats (Fig. 4). Shown are representative lanes from one gel. Bile duct ligation and malnutrition did not alter the distribution of the RNA hybridizing to these major bands, but resulted in a uniform diminution of all hybridizing RNA species.

DISCUSSION

Children with cholestatic disease, especially with extrahepatic biliary atresia, constitute the largest group of pediatric patients requiring liver transplantation, as this is the only therapy available for the end stage of this disease. Improvement of nutritional status is associated with increased survival although normal linear growth is not achieved in all patients even after liver transplantation and improved nutrition (2, 3). In our model of cholestasis in juvenile rats, we found decreased levels of serum IGF-I. However, we found no correlation between serum IGF-I levels and the degree of cholestasis as judged by serum bilirubin levels. Liver IGF-I content and serum IGF-I levels were decreased to a similar degree suggesting impaired IGF-I synthesis rather than decreased release of liver IGF-I. The low levels of liver IGF-I mRNA found in cholestatic rats are consistent with an IGF-I synthesis defect, and suggest an abnormality at the pretranslational level. Serum levels of IGF-I were four times higher in control than in bile duct-obstructed or malnourished animals, whereas liver IGF-I mRNA levels were 2-fold different. Although this fold difference is small, it may also reflect posttranslational modulation of IGF-I serum levels, or a difference in the metabolic clearance rate of the circulating peptide. Low circulating IGF-I levels may be responsible for the high GH levels observed in chronic liver disease (12) because of a decrease in the negative feedback inhibition of GH secretion (27).

This study was performed to evaluate if the role of nutrition could be dissociated from the role of other factors in liver disease-associated growth failure. Although low IGF-I levels were found in animals with cholestasis, the levels were comparable to similarly malnourished animals that did not have impaired liver function. Thus, IGF-I levels are more likely to be related to decreased food intake than to cholestatic liver disease. Malabsorption is well documented in chronic liver disease especially in cholestasis (28). Metabolism of all three macronutrients (fat, protein, and carbohydrate) is affected by hepatic dysfunction (29). The association between malnutrition and reduced IGF-I levels has been reported previously in rats as a decrease in

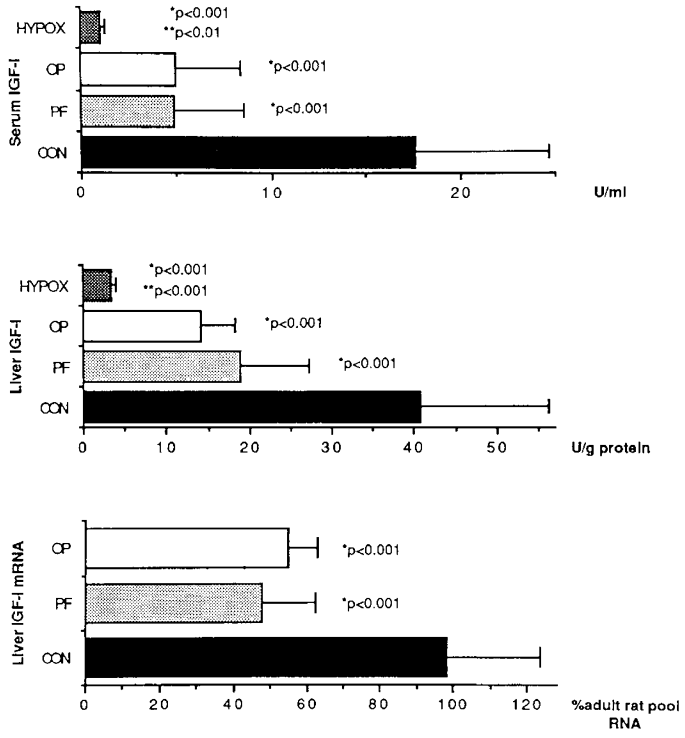

Fig. 3. Serum and liver IGF-I concentrations and hepatic IGF-I mRNA in 12 CON, 10 PF, 15 OP rats, and 10 HYPOX rats. Liver IGF-I mRNA content is expressed as a percentage of IGF-I mRNA observed in adult rat pooled RNA. *statistical comparison of PF or OP *versus* CON; **statistical comparison of HYPOX *versus* OP, PF, CON. Values shown are means ± SD. Significant differences were present for both serum ($F_{2,34} = 26.74$) and liver ($F_{2,34} = 23.88$) IGF-I by RIA and for liver IGF-I mRNA measurements ($F_{2,34} = 30.36$).

somatomedin bioactivity (30). Low somatomedin levels noted in children with protein-calorie malnutrition were improved by refeeding (10, 11). Normalization of somatomedin levels has been documented during refeeding in malnourished rat pups (15), fasted adult rats (31), human volunteers (32), and in chronic diseases associated with malnutrition (33). However, a direct relationship between IGF-I levels and growth velocity is not consistently demonstrated.

Although both the cholestatic and sham-operated, PF animals were clearly undernourished, albumin concentrations were not significantly different when compared to control rats fed *ad libitum*. This supports the hypothesis of others that a reduction in circulating IGF-I levels is a more sensitive indicator of malnutrition than traditional indices, such as serum albumin or transferrin (34).

Growth failure, as measured by tail length and muscle mass changes, was greater in cholestatic rats than in those with malnutrition alone despite similar decreases in IGF-I. Thus, growth failure in chronic liver disease might not be explained solely by poor nutritional intake and low serum IGF-I levels. The lower nitrogen balance found in cholestatic rats was comparable to sham operated animals of equal nutritional status, suggesting either less efficient utilization of nutrients or increased catabolism. Patients with liver disease may have decreased cortisol clearance (35) that might contribute to poor muscle mass and low nitrogen balance. Somatomedin inhibitors may be produced by liver and are reportedly elevated in fasted animals (36, 37) and may decrease after improved nutrition. Somatomedin inhibitor levels have not been reported in chronic liver disease but might contribute to the growth failure.

In conclusion, the low IGF-I levels observed in chronic liver disease are most likely a function of inadequate nutrition. Unknown factors, in addition to decreased IGF-I and inadequate nutrition, must also contribute to the growth failure observed in chronic liver disease.

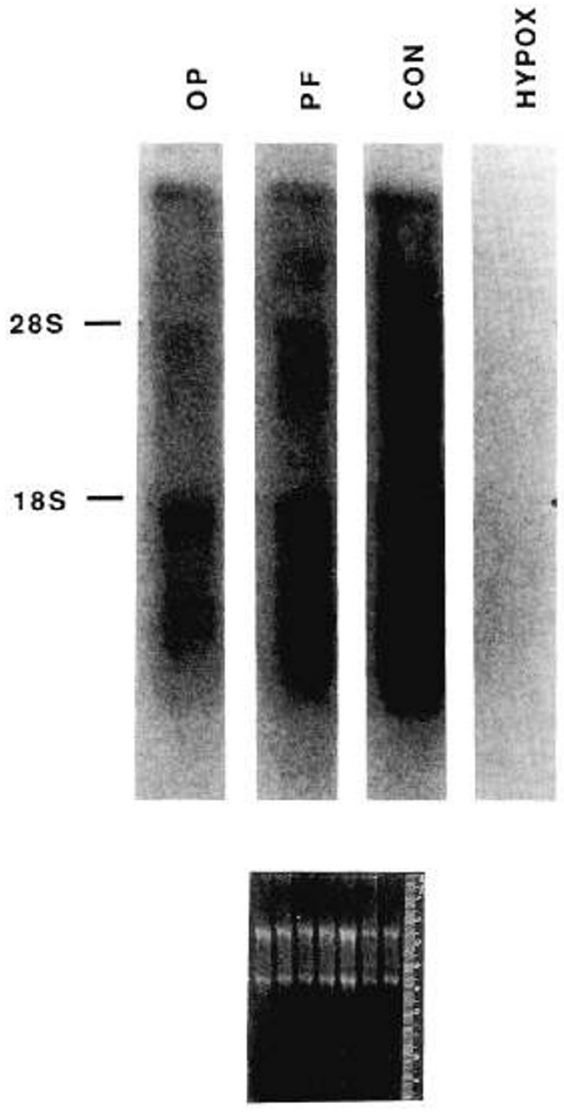

Fig. 4. Northern blot of total RNA extracted from representative OP, PF, CON, and HYPOX rats. Size markers are 18 and 28 S RNA species. Shown as an *inset* is the ethidium bromide stained gel from which these samples were transferred, demonstrating approximately equal intensities of major ribosomal species for all lanes. Lanes shown in the figure above are 1 (OP), 3 (PF), 5 (CON), and 7 (HYPOX). OP and PF rats have lower levels of all RNA species hybridizing to this IGF-I cDNA when compared to CON. No signal was observed in RNA from HYPOX animals at this exposure. Similar results for each experimental group were seen on additional gels, and on other lanes on this gel (not shown).

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