Nutritionally Induced Changes in the Peroxisome Proliferator-Activated Receptor-α Gene Expression in Liver of Suckling Rats Are Dependent on Insulinaemia

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It was previously found that the expression of peroxisome proliferator-activated receptor-α (PPARα) was markedly augmented in the liver of suckling rats, in comparison to the fetuses and most notably to adult rats and it paralleled similar changes in hepatic lipid concentration. To determine whether these changes could be related to the high lipid content of the maternal milk and/or to hormonal status, the role of changes in nutrient availability and in plasma insulin concentration on liver expression during the perinatal stage *in vivo* in the rat was studied. When suckling rats were weaned on day 17, instead of on day 20, the level of hepatic PPARα mRNA decreased earlier than in rats weaned later. When 10-day-old rats were force-fed with either glucose or Intralipid or a combination of both diets, it was found that, at similar low levels of plasma insulin, a high level of FFA stimulated PPARα expression, whereas, at similar high plasma FFA concentrations, an elevated insulin level attenuated the increase in PPARα expression. It is proposed that both the high lipid intake and decreased plasma insulin level are responsible for the high PPARα expression detected in rat neonates. © 2001 Academic Press

Key Words: PPARα; mRNA levels; suckling rats; lipids; insulin.

Peroxisome proliferator-activated receptors (PPAR)[2] are members of the steroid nuclear receptor superfamily, which is a large class of ligand-activated transcription factors regulating gene expression. After binding peroxisome proliferator compounds, fatty acids or their metabolites, these receptors are activated and regulate the expression of genes related to lipid metabolism (1, for a review, 2), such as peroxisomal β-oxidation, gluconeogenesis, lipid transport, and ketogenesis. So far, three PPAR subtypes have been identified in the rat: PPARα, PPARβ, and PPARγ. The isoform α is the best-characterized. It is primarily expressed in tissues that have a high level of fatty acid catabolism such as liver (3) and appears to play a crucial role in the modulation of fatty acid oxidation.

To date, the hormonal regulation of PPARα expression has not been investigated in detail, and only glucocorticoids have been shown to have a clear effect (4). Furthermore, most studies have been carried out *in vitro* and conflicting results have been reported regarding the regulation of the expression of PPARα by either insulin (5–7) or fatty acids (6, 8). Physiological conditions of modified plasma insulin and fatty acid levels are therefore desirable in order to test whether these factors control PPARα expression *in vivo*.

During perinatal development, both the transition from fetal to neonatal period and from suckling to weaning stage are known to modify endocrine and nutritional conditions that may influence the expression of PPARα, as has already been shown for PPARα related genes, like phosphoenolpyruvate carboxykinase (PEPCK) (9). We have previously reported similarities in the changes in PEPCK and PPARα mRNA expression during development (10), suggesting a common mechanism of regulation, in which the high lipid intake of the suckling rat may be involved. The present study therefore focused on characterizing the *in vivo* regulation of the PPARα expression by nutrients and hormones at neonatal stage.

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[2] Abbreviations used: PPAR, peroxisome proliferator-activated receptor; FFA, free fatty acids; ACO, peroxisomal acyl-CoA oxidase; TLC, thin layer chromatography.

MATERIAL AND METHODS

Animals. Female Sprague–Dawley rats weighing 160–190 g were fed ad libitum standard rat chow (B&K Universal, Barcelona, Spain) and mated. The experimental protocol was approved by the Animal Research Committee of the University San Pablo-CEU (Madrid, Spain). Mothers and their fetuses were sacrified by decapitation at different post-fecundation times: 19, 20, and 21 days (estimated by the appearance of spermatozoids in vaginal smears). Other dams were allowed to deliver and on the day of birth, each suckling litter was reduced to nine pups per mother. On the 20th day after delivery, the lactating mothers were removed from the cages to stop the suckling period and pups were killed at different ages. In some experiments rats were weaned on day 17.

In another experiment, 10-day-old suckling rats were separated from their mothers, maintained at 37°C, in high humidity, and subjected to oral force-feeding three times (at 10:00 a.m., 11.00 a.m., and 13.00 p.m.) with 10–20 μl/g of body weight of a solution containing either 25% glucose, 10% Intralipid (emulsion of soybean oil), glucose plus Intralipid or the medium alone (bidistillated water). The animals were decapitated two hours after the last administration. In all cases, the liver was immediately removed, placed in liquid nitrogen and stored at −70°C until analysis. Blood was collected from the neck into EDTA-containing receptacles and after centrifugation, plasma was kept at −20°C until processed for insulin and free fatty acid (FFA) analysis using commercial kits (DiaSorin, USA and Wako, Germany, respectively).

RNA analysis. Total cellular RNA was prepared either from the liver of individual animals or from liver pools of three animals of the same litter in the case of fetuses. Total RNA was isolated by a modification of the guanidium isothiocyanate method using Ultraspec RNA according to the manufacturer's instructions (Biotecx Labs, Houston, TX). Total RNA concentration was determined by absorbance measurement at 260 nm. The 260/280 absorption ratio of all samples was between 1.8 and 2.0. Ten, 20, and 30 μg of total RNA was denatured, spotted, and fixed to a nylon membrane. The cDNA probes for dot blot analysis were labeled with [α-^{32}P]dCTP using the random primer DNA labeling kit provided by Amersham. Probes used were: 1.1 kb *Pst*I fragment of rat peroxisomal acyl-CoA oxidase (ACO) cDNA (kindly supplied by Dr. H. N. Sorensen, University of Oslo, and with the permission of Dr. T. Osumi, Humeji Institute of Technology, Hyogo, Japan), 1.6 kb *Eco*RI fragment of rat PPARα cDNA (a generous gift from Dr. J-Å. Gustafsson, Karolinska Institute, Huddinge, Sweden) and a fragment of rat β-actin cDNA (kindly supplied by Dr. D. Langin, INSERM U317, Toulouse, France). Blots were prehybridized at 42°C for 2 h and hybridized overnight at the same temperature. Washing conditions were 2× SSC, 0.1% SDS at 42°C for 15 min twice for PPARα, and 0.1× SSC, 0.1% SDS at 53°C for 15 min three times for the other probes. Membranes were then exposed to Kodak X-OMAT films at −70°C from 1 h to 3 days. Filters were stripped of label at 75–80°C for 1 h with 0.1× SSC, 0.5% SDS, 0.1% tetrasodium pyrophosphate, and then rehybridized. Linearity of the relationship between signal intensity and RNA concentration was confirmed for each sample and probe.

An aliquot of hepatic total RNA from representative rats of different ages was subjected to RT-competitive PCR for quantification of PPARα mRNA. Briefly, it consisted of the reverse transcription of the specific mRNA and then, the co-amplification of the target cDNA with known amounts of the specific competitor DNA in the same tube (11). The competitor DNA used and the conditions and validation of the RT-competitive PCR assays have been reported in detail elsewhere (12). The sequence of the sense primer was: 5′-GAATATGT-GGGGACAAGGCCTC-3′ and the antisense primer: 5′-TCGTGGAT-TCTCTTGGCCAG-3′. The size of the PCR products obtained was: 330 base pairs for rat PPARα mRNA and 261 base pairs for the competitor.

Hepatic lipid composition analysis. Frozen liver aliquots were used for lipid extraction (13), and aliquots of lipid extracts were quantified after image analysis and separation by one-dimensional TLC (14) using the G5-700 BIOIMAGE TLC scanner of Bio-Rad (U.S.A.). Spots were quantified as integrated optical densities against an internal standard of cholesteryl formate and calibration curves of triglyceride and phospholipid standards.

Statistical analysis. Data are expressed as mean ± standard error. Results were subjected to a one-way analysis of variance (ANOVA), differences in mean values among groups were tested using the Tukey multiple range test, and were considered statistically different at $P < 0.05$. For results of plasma insulin levels, the Mann–Whitney U test was used.

RESULTS AND DISCUSSION

Developmental Changes in PPARα mRNA and Lipid Levels in Liver of Suckling Rats

PPARα mRNA in liver was clearly detectable by RT-competitive PCR in fetuses close to birth (10.9 ± 1.4 amol/μg of total RNA at day 20 of intrauterine life). This value turned out to be higher than the one obtained in adult rats (4.2 ± 1.0 amol/μg of total RNA at two months of age) and parallels the high amount of PPARα receptor previously found in fetal liver (10), a change that may contribute to the adequate handling of the enormous quantity of lipids that are ingested by the neonates just after birth. In this way, PPARα could coordinately control (or determine) the fate of lipids arriving at the liver during suckling by means of regulating the expression of its putative target genes. In fact, just after birth, the expression of these genes became enhanced, as has already been shown for mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (15), carnitine palmitoyltransferase I (CPT-I) (16), phosphoenolpyruvate carboxykinase (PEPCK) (9, 10), lipoprotein lipase (LPL) (17), and even acyl-CoA oxidase (10, 18).

The change of the expression of some of these genes during the suckling period has been shown to have a characteristic bell-profile, that is, higher mRNA level during the suckling period than during the fetal and adult stages. This profile has been related to the hormonal status and the high lipid intake (9, 16, 19) and is thought to be mediated by PPARα (20). Interestingly, we also found a bell-shaped curve for the hepatic PPARα expression during the suckling period (17.5 ± 2.9 and 19.5 ± 2.5 amol/μg of total RNA, at 1 and 15 days of age, respectively, in comparison to the values given above for fetuses and adult rats). These results could indicate a common mechanism controlling the expression of all these genes during the suckling period. In this process, PPARα could be participating in such control, by means of a coordinated regulation of its own expression and the expression of its target genes, in response to lipids arriving in the liver (20). Thus, it would further represent an *in vivo* example of the PPARα autoregulation previously described in
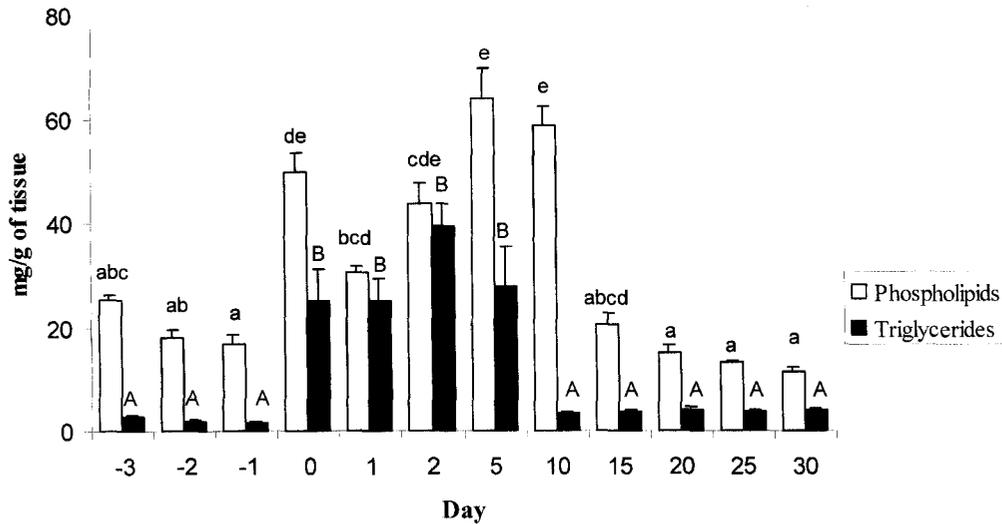
FIG. 1. Changes in the hepatic lipid concentration throughout development in rat neonates. Values are means ± SE of 5 rats/group. Capital letters (for triglycerides) and small letters (for phospholipids) correspond to the statistical comparisons between the time-points throughout the development for each parameter (values not sharing a common letter are significantly different at $P < 0.05$). Day 0 represents the day of delivery and day 20 when the neonates were weaned.

brown adipocytes (21). A similar regulatory mechanism has been previously described by Brun *et al.* (22) for the expression of PPARα and uncoupling protein-3 (UCP-3) in the murine skeletal muscle of neonates, where the induction of PPARα expression preceded that of its target gene UCP-3, and then both genes presented a similar expression profile during the perinatal period. Whether the bell-shaped profile of PPARα target gene expression during the suckling period results from the induction of PPARα mRNA and protein (10), and/or from an increased PPARα ligand activation, as has been proposed under other conditions (23), remains to be established.

It has been suggested (10, 22, 24) that, in addition to hormonal regulation (insulin and/or glucagon), the change in nutrient supply around birth (from placental transfer of maternal circulating nutrients to lipid-rich milk) could be directly responsible for the concordant changes in the expression of enzymes and receptors involved in fatty acid utilization in the liver. In agreement with this hypothesis, as shown in Fig. 1, the concentration of both phospholipids and triglycerides in rat liver rapidly increase after birth, continue to increase up to the middle of the suckling period and later decline to values which, at the end of the lactation period (day 20), do not differ from those seen in 30-day-old rats. This bell-shaped change of liver lipids is therefore quite similar to that previously described for the expression of some fatty acid metabolizing enzymes and for PPARα (10, 15, 16). The decline in the concentration of liver phospholipids that takes place in the suckling pups occurs around day 15 of life (Fig. 1), which corresponds to the time when it is known that

rat neonates begin to mix milk and pellets (with a higher content of glucose and lower amount of lipids) (25). However, the profile obtained for the concentration of liver triglycerides was somehow different from those of liver phospholipids. After the dramatic increase just after birth, triglyceride concentration shows a sharp reduction around the 10th day of life (Fig. 1). This change could be related to the enhanced liver expression of apolipoprotein CIII, previously seen at this specific time-point (10, 18), which would indicate an increased capability of the liver to release lipoproteins into the blood. However, since liver triglycerides have also been related to the induction of LPL activity taking place at this specific life stage (26), the role of the progressive reduction of liver lipoprotein lipase activity and expression which is known to take place from birth until weaning (17, 27) cannot be excluded.

Changes in PPARα mRNA Levels Induced by Weaning

In order to test the influence of lipid intake on the expression of PPARα during suckling, we carried out an experiment in which the natural day of weaning was anticipated. As shown in Fig. 2A, just 24 h after an anticipated weaning (on day 17), there is a marked decline in the level of PPARα mRNA in liver as compared to pups of the same age but maintained suckling. In fact, the neonates lactating for a longer time (up to 20 days of age) presented higher levels of PPARα mRNA for the same time-points (18, 19, and 20 days old) than those that were suckling for a shorter time
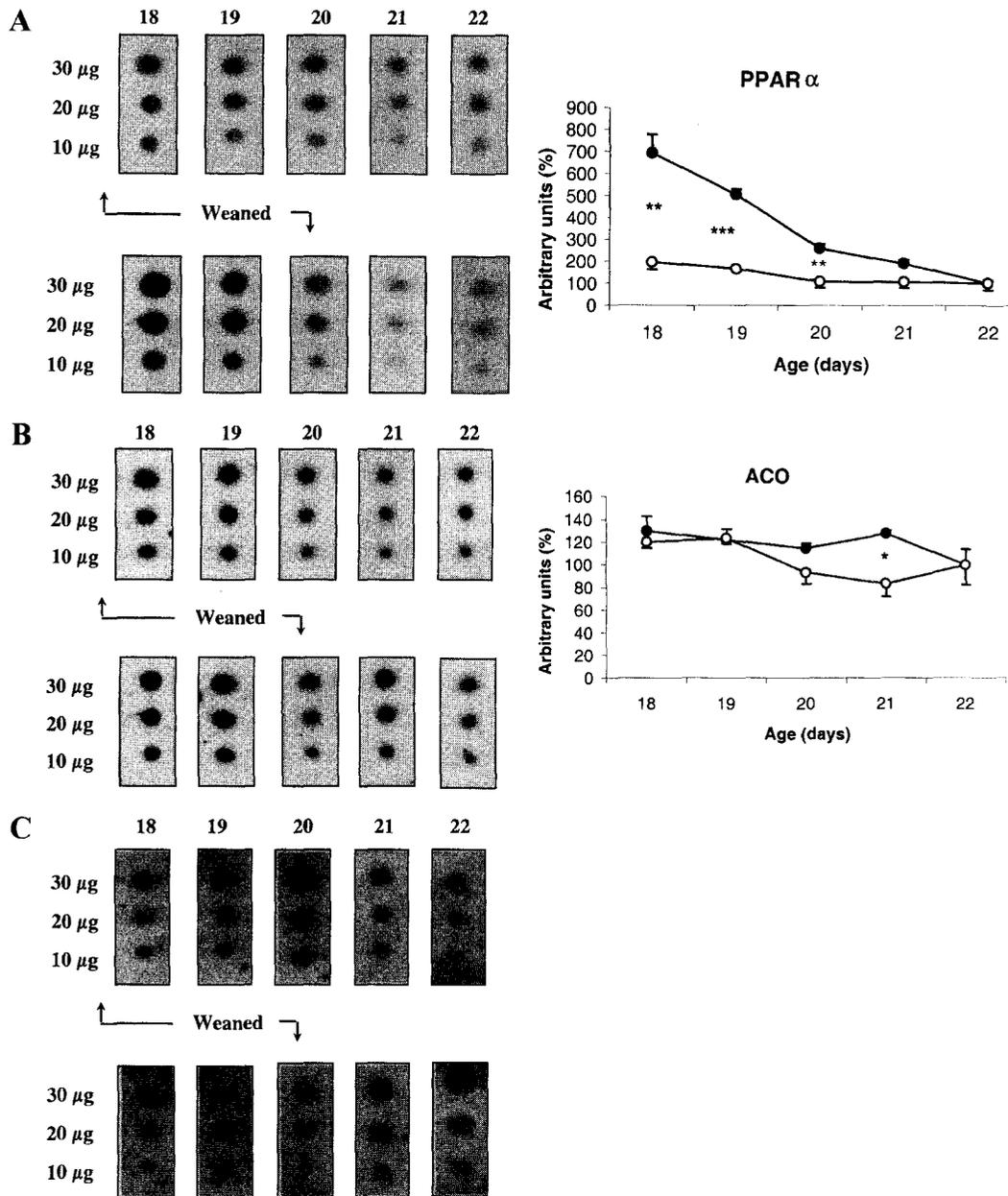
FIG. 2. Expression of PPARα and ACO in the liver of weaned rats. (A) Left, dot blot assays of mRNAs for hepatic PPARα from weaned rats either at the normal weaning day (Day 20, closed circles) or 3 days earlier (Day 17, open circles). Representative dot blot analysis of RNA prepared from livers of rats from each group are shown. The amount of RNA spotted is indicated on the left of each panel and the developmental time-points considered are also indicated. Right, relative amount of liver PPARα obtained by densitometric scanning of the blots and normalized against actin was represented using arbitrary units, representing the mean value in the liver of the 22-day-old animals as the 100% value. (B) Left, dot blot assays of mRNAs for hepatic acyl-CoA oxidase (ACO). Right, relative amount of liver ACO obtained by densitometric scanning of the blots. (C) Dot blot assays of mRNAs for β-actin. Statistically (t test) significant differences between values of the same age are indicated by asterisks (*P < 0.05; **P < 0.01; ***P < 0.001). Each value represents the mean ± SE of four animals.

(Fig. 2A). Therefore, it appears that an early interruption of lipid intake provokes an anticipated reduction in the level of PPARα mRNA. Similar nutritionally induced changes have been previously found in rat intestine for PEPCK (20), CPT-I (16), and HMG-CoA synthase (15) expression, as well as in rat liver for LPL

mRNA expression and activity (M. I. Panadero, E. Herrera, and C. Bocos, unpublished results).

On the other hand, as shown in the Fig. 2B, weaning did not produce any clear effect in the mRNA levels of ACO, a known PPARα target gene (28), independently of whether weaning was anticipated or occurred at the

TABLE I

Effect of Force-Feeding with Either Glucose, Intralipid, Glucose Plus Intralipid or the Medium (Bidistillated Water) on Plasma Insulin and FFA Levels in 10-Day-Old Rats

Oral treatment	Insulin (μU/ml)	FFA (μM)
Glucose	8.9 ± 2.3^a	375 ± 88^a
Intralipid	4.1 ± 1.4^b	884 ± 123^b
Glucose + Intralipid	27.3 ± 5.1^c	818 ± 92^b
Medium	$7.1 \pm 2.1^{a,b}$	772 ± 73^b

Note. Values are means $\pm$ SE of 7–10 rats/group. The superscript letters correspond to the statistical comparisons between the different nutritional oral treatments for each parameter (values not sharing a common superscript letter are significantly different at $P <$ 0.05).

normal time. This result was, however, in line with the previously described stable pattern of ACO expression in suckling and weaned rats (10, 18). These findings could be related to those we reported previously (10), suggesting that rat liver is able to efficiently oxidize milk fatty acids by the activation of the expression of ACO in the first days of life and, subsequently, the expression of 17β-hydroxysteroid dehydrogenase type IV, an enzyme also implicated in the peroxisomal fatty acid β-oxidation.

The weaning-induced diminution of PPARα expression without parallel down-regulation of ACO expres-

sion could be related to the situation described during the cold acclimatization in brown adipose tissue (29). It could also be in consonance with the fact that PPARα-null mice contain normal levels of peroxisomal enzymes (28, 30), suggesting that constitutive expression of ACO may be independent of PPARα (28).

Nutritionally Induced Changes in Hepatic PPARα mRNA Levels and Dependency of the Hormonal Status

To directly test the role of nutritional and hormonal factors in the regulation of PPARα expression during the neonatal period, 10-day-old suckling rats were given orally either glucose, Intralipid, both, or fasted for the 5-h period (receiving just the medium, water). As shown in Table I, plasma FFA were higher in rats receiving Intralipid, glucose plus Intralipid, or fasted (medium treated), as compared to those receiving only glucose. Plasma insulin levels were significantly higher in the glucose plus Intralipid group than in any of the other conditions studied (Table I). As shown in Fig. 3, the expression of hepatic PPARα was higher when the animals were fed with a high lipid diet (Intralipid) as compared to those fed glucose. This finding contrasts with the reported lack of effect of unsaturated fatty acid induction of PPARα mRNA in adult rat jejunum (31), but is in accordance with the positive effect of fatty acids seen in isolated hepatocytes (6), suggesting a tissue-specific effect. In the animals
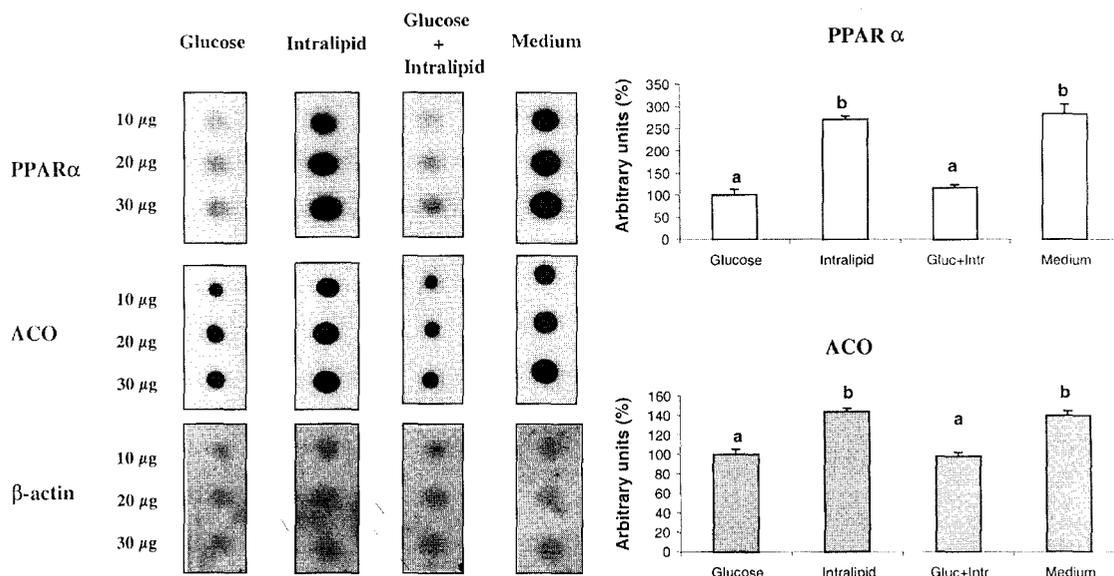
FIG. 3. Effect of force-feeding with either glucose, Intralipid, glucose plus Intralipid, or medium (bidistillated water) on hepatic expression of PPARα and ACO in 10-day-old rats. Left, representative dot blot analysis of mRNA of PPARα, ACO and β-actin, prepared from livers of rats from each group are shown. The amount of RNA spotted is indicated on the left of each panel. Right, relative amount of liver PPARα (upper panel) and ACO (lower panel) obtained by densitometric scanning of the blots and normalized against actin using arbitrary units, considering 100% the mean value in liver of glucose fed neonates. Each value represents the mean $\pm$ SE of four animals. Comparison between groups was performed with one-way analysis of variance (ANOVA), and a different letter means differences statistically significant at $P <$ 0.05.

treated with water, which were fasted for 5 h, we also found a significant increase in the hepatic mRNA PPARα content as compared to animals receiving glucose (Fig. 3). This finding is in agreement with previous reports (23) in adult rats subjected to a longer period of fasting (24 h), supporting again the role of high FFA levels, enhancing hepatic mRNA PPARα expression. However, surprisingly, Intralipid treatment did not stimulate the liver expression of PPARα when 10-day-old rats were simultaneously treated with glucose (Fig. 3). This counteracting effect of glucose in the presence of enhanced FFA levels could be a consequence of enhanced insulin levels. Therefore, it may be proposed that the high insulin level seen in the glucose plus Intralipid group has been able to overcome the positive effect of the enhanced FFA levels on the mRNA expression of PPARα. These findings could be related to those previously described by Arias et al. (19) for liver CPT I and mitochondrial HMG-CoA synthase in 12-day-old suckling rats, where fasting enhanced their mRNA level while insulin treatment caused significant declines. Thus, a common regulatory mechanism may be claimed for the regulation of these genes in liver, since their expressions respond to an overload of lipids and an increase in the plasma insulin level in a similar way. Furthermore, these results would be consistent with the existence of a cis-element of response to insulin in the PPARα gene, as suggested elsewhere (6).

To our knowledge this is the first time that the down-regulatory effect of insulin on the fatty acid-induced expression of PPARα has been demonstrated in vivo. This effect has previously been described in vitro by Steineger et al. (6) in rat hepatocytes, and by Inoue et al. (7) in human vascular endothelial cells, although not observed by others (5) in rat hepatoma cells.

In order to check whether the changes observed with PPARα expression in the liver of 10-day-old pups subjected to force-feeding with different nutrients were reflected in its target genes, the mRNA expression of ACO was also determined in the same blots. As shown in Fig. 3, ACO mRNA expression was characterized by trends similar in response to the nutritional changes as those found with PPARα expression. Thus, the presence of high plasma insulin levels appeared to prevent the increase in ACO expression provoked by the elevated FFA level. This result supports the notion that PPARα and its target gene could be affected in a similar manner by insulin and fatty acids, as was previously proposed (6). These findings are also in accordance with the observed changes in ACO expression in hepatocytes and hepatoma cell lines (32). However, they contrast with a recent work in which, using cultured cardiomyocytes of neonatal rats, an increased expression of putative PPARα target genes was found in the presence of glucose plus fatty acids (24). In that study, however, the potential role of insulin was not tested, whereas present findings strongly indicate that this hormone, in connection with free fatty acids, plays in vivo a key role in the expression of liver mRNA PPARα and related genes.

The response of ACO expression to Intralipid force-feeding seemed to be in contradiction with the lack of change found in the anticipated weaning experiment. This could be due to an unidentified factor in milk (16, 20, 33), which may modulate PPARα expression but not ACO. Alternatively, the different fatty acid composition of milk as compared to Intralipid could also be involved in the difference in gene expression. In any case, unparalleled changes in the expression of PPARα and peroxisomal β-oxidation enzymes have also been reported elsewhere (5, 28, 34) and attributed to the fact that inducibility, but not constitutive expression of these genes, is dependent on PPARα (28, 34). The findings observed here for the ACO expression would be in consonance with the idea that a fat-overload of the liver is necessary to obtain a considerable effect on the expression of these fatty acid-metabolizing enzymes (23).

In summary, present results show that liver PPARα mRNA expression during the perinatal period is under the control of both hormonal and nutritional factors in vivo in rats, with crucial roles of lipid intake and plasma insulin concentration. Further, given that PPARα is already found in fetuses, whereas the expression of its putative target genes generally increase at birth (10, 15, 16, 22), it could be proposed that the putative participation of PPARα in the regulation of these gene expressions would be mediated by its ligand-activation rather than by the increase in its own expression.

ACKNOWLEDGMENTS

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