Dexamethasone enhanced by insulin, but not by thyroid hormones stimulates apolipoprotein B mRNA editing in cultured rat hepatocytes depending on the developmental stage

Axel Lorentz, Dietmar Plonné, Hans-Peter Schulze, Rolf Dargel*

Institute of Pathobiochemistry, Department of Medicine, Friedrich-Schiller University, D-07740 Jena, Germany

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Abstract The increase of hepatic apolipoprotein B (apoB) mRNA editing during rat development was not affected by hypothyroidism. Furthermore, the addition of 3,3′,5-triiodothyronine (T_3) to cultured hepatocytes taken from fetal, neonatal and adult rats had no effect on apoB mRNA editing. In contrast, dexamethasone markedly stimulated apoB mRNA editing in hepatocytes taken from neonates. This effect was enhanced by the addition of insulin. For the first time our data provide evidence that glucocorticoids together with insulin are important for the regulation of apoB mRNA editing during postnatal development, whereas thyroid hormones are not critical for this process.

Key words: apoB; mRNA editing; Rat development; Hepatocytes; Dexamethasone

1. Introduction

Apolipoprotein B (apoB) is synthesized in the liver and small intestine of mammals as the structural component of triglyceride-rich very low density lipoproteins (VLDL) and chylomicrons. VLDL are the metabolic precursor of low density lipoproteins (LDL), the main carrier of cholesterol in humans. ApoB plays a crucial role in the secretion and catabolism of these lipoproteins. It exists in two forms, the full-length translation product apoB-100 and the shorter form apoB-48 which is identical to the amino-terminal 48% of apoB-100. ApoB-48 is the result of apoB mRNA editing, a posttranscriptional site-specific cytidine deamination that changes CAA for Gln-2153 in apoB-100 mRNA to a UAA stop codon [1]. In humans, apoB-48 is produced by the small intestine whereas the liver produces only apoB-100. In other mammals, like the rat and mouse, the liver is also able to synthesize apoB-48. Several components have been suggested to be required for the editing of apoB mRNA. The catalytic subunit of the postulated apoB editing complex, apobec-1, has been cloned [2] whereas the other protein factors are not yet identified.

ApoB mRNA editing was demonstrated to undergo developmental regulation in the intestine of all mammals including humans as well as in rat and mouse liver [3–6]. Generally, a low level of apoB mRNA editing of 10–20% was found in the neonatal rat liver. The editing rate increased between days 13 and 30 of postnatal life to adult levels of 70% [7–10]. Addition of insulin for a longer period increased the apoB mRNA editing in primary rat hepatocytes in culture [8], just like supplementation of 3,3′,5-triiodothyronine (T_3) in hypothyroid rats [9]. However, the factors governing the increase in hepatocellular apoB production and apoB mRNA editing up to the weaning period are still unknown.

In this study we show the changes in total apoB serum concentration observed during the perinatal development of rats. Furthermore, we analysed the effect of hypothyroidism on hepatic apoB mRNA editing during the suckling and weaning period and investigated the effect of dexamethasone, insulin and T_3 on apoB mRNA editing and apoB-48 synthesis in cultured hepatocytes taken from fetal, neonatal and adult rats.

2. Materials and methods

2.1. Animals

Fetal (22nd day of gestation), neonatal (10th day of postnatal life) and adult (3 months old) Wistar rats of the strain Han-Wist were used in the experiments. Adult rats were housed under standardized conditions and fed a standard laboratory chow diet (Altromin) with water ad libitum. Neonatal rats were allowed free access to maternal milk until they were killed. Hypothyroidism of neonates was induced according to Gallo et al. [11] by addition of 0.1% 2-mercapto-1-methylimidazole (MMI) to the drinking water of the dams beginning on day 22 of gestation. The serum concentrations of T_3 and thyroxin (T_4) were measured by means of a commercial radioimmunoassay (Dynotest, Brahm's Diagnostica, Berlin). ApoB in the serum of rats was determined by means of rocket immunoelectrophoresis [12].

2.2. Primary hepatocyte culture

Hepatocytes from adult and neonatal rats were isolated by a collagenase digestion method according to Zimmerman et al. [13]. Hepatocytes from 10 day old rats were isolated by retrograde perfusion via the vena cava inferior. Hepatocytes from fetal rats were isolated using the method of Fry [14]. Generally, the vitality of the hepatocytes used for cultivation was higher than 85% when measured with the trypan blue exclusion technique. Cells were resuspended at a density of 1×10^6 cells/ml (fetal and neonatal rats) or 0.6×10^6 cells/ml (adult rats) in William's medium E supplemented with 10% fetal calf serum, 26 mM $NaHCO_3$ and 50 μg/ml gentamycin. 3.3 ml of the cell suspension was plated on collagen coated 50 mm plastic culture dishes and maintained at 37°C in a water saturated atmosphere with 5% CO_2. After 2 h, the plating medium and nonadherent cells were aspirated and replaced with 3 ml feeding medium per plate. The feeding medium consisted of William's medium E supplemented with 10% fetal calf serum, 26 mM $NaHCO_3$ and 50 μg/ml gentamycin and, if indicated, with 100 nM dexamethasone, 50 nM T_3 and 10 nM insulin, each alone or in combination with the others. Medium was replaced with fresh one daily.

2.3. Measurement of [^{35}S]methionine incorporation into secreted apoB-48 and apoB-100

Following 72 h of culture the medium was replaced with 2 ml of fresh one and 40 μCi/ml [^{35}S]methionine was added. After 6 h of

*Corresponding author. Fax: (49) (3641) 631318.

Abbreviations: apoB, apolipoprotein B; VLDL, very low density lipoprotein; LDL, low density lipoprotein; Apobec-1, apolipoprotein B mRNA editing enzyme catalytic polypeptide 1

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incubation the medium was removed, centrifuged for removal of any tissue debris and set aside for analysis of apoB secretion. Cells were harvested and frozen in liquid nitrogen and stored at −80°C for RNA preparation. The analysis for measuring the incorporation of [^{35}S]methionine into secreted apoB was done according to Plonné et al. [15]. After flotation of the medium at $d = 1.21$ g/ml the lipoprotein fraction was subjected to SDS-PAGE and the radioactivity of the excised apoB-100 and apoB-48 bands was measured by β-counting.

2.4. Primer extension analysis for determination of apoB mRNA editing

The relative amounts of apoB-100 and apoB-48 mRNA were determined by primer extension analysis following reverse transcription and polymerase chain reaction (RT-PCR) amplification of total cellular RNA [3]. Total RNA from liver and from hepatocytes was prepared by acid guanidinium isothiocyanate-phenol-chloroform extraction [16]. RNA samples were treated with DNase to remove genomic DNA. RT-PCR was performed with Tth DNA polymerase (Promega) and oligonucleotides ABT1 (nt 6614–6639) and ABT2 (nt 6765–6740). This reaction resulted in products of 152 bp flanking the edited base [17]. ApoB cDNA was purified from oligonucleotides using QIAquick columns (Qiagen) and annealed to an antisense [γ-^{32}P]ATP end labeled oligonucleotide (nt 6708–6674). Primer extension was carried out with AMV reverse transcriptase (Promega) according to Driscoll et al. [18]. The reaction products indicative of apoB-100 and apoB-48 mRNA were separated on an 8% polyacrylamide-urea gel, exposed to a GS-250 Imaging Screen B-I and analysed with the GS-250 Molecular Imager (Bio-Rad).

Statistical differences were calculated by Student's t-test and considered significant at $P < 0.05$.

3. Results and discussion

The changes in VLDL and LDL apoB serum concentration and hepatic apoB mRNA editing during the development of rats are shown in Fig. 1. During the suckling period the serum LDL apoB concentration was high and the apoB mRNA editing was low. During the weaning period, apoB mRNA editing increased to the adult value of 70%, which was reached on day 30 of life. This alteration ran parallel with the developmental changes observed for VLDL apoB. The data reveal an inverse relationship between the LDL apoB concentration in the serum and the hepatic apoB mRNA editing. Obviously due to the low apoB mRNA editing in the suckling period, the liver mainly secretes apoB-100 containing lipoproteins resulting in an increase in the serum concentration of LDL apoB. On the other hand, at the high rate of apoB mRNA editing observed after the weaning period the liver mainly produces apoB-48 containing lipoproteins (VLDL) which do not function as precursors for LDL and which are eliminated much more quickly from the serum than lipoproteins containing apoB-100 [19–21]. This may lead to the lower LDL concentration in the postweaning phase when compared with that measured in the suckling period.

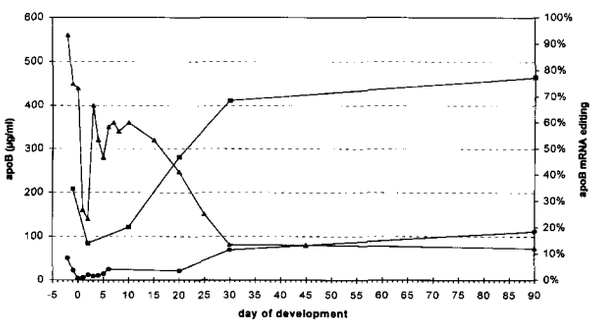

Fig. 1. Changes in VLDL (●) and LDL apoB (▲) serum concentrations ($n = 7$) and hepatic apoB mRNA editing (■) ($n = 3$) during rat development. For clear arrangement SD is not shown in the figure.

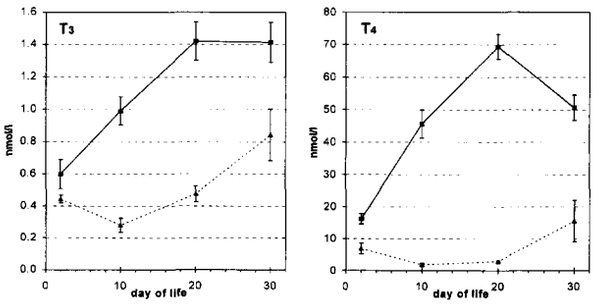

Fig. 2. Concentrations of T_3 and T_4 in the serum of hypothyroid (▲) and control (■) animals during postnatal development ($n = 3$), values are given as means ± SD.

Different nutritional and hormonal conditions might be responsible for these changes during the perinatal development of rats. The serum T_3 concentration, for instance, is low in the fetal and suckling state and increases after the second week of life, peaking 4–5 weeks after birth [22]. Since T_3 is known to stimulate apoB mRNA editing in the adult rat liver [23], we asked the question whether the increase in the T_3 concentration might be responsible for the observed increase in hepatic apoB mRNA editing. To test this hypothesis hepatic apoB mRNA editing was measured in hypothyroid animals. As shown in Fig. 2, MMI treatment effectively reduced the concentration of T_3 and T_4 in the serum when compared with control animals. However, no differences in hepatic apoB mRNA editing were observed between the control and hypothyroid animals during the postnatal development. The proportion of edited apoB mRNA increased from about 13% (2nd day of life) to about 22% (10th day of life), 47% (20th day of life) and 68% (30th day of life) in both groups. This result provides evidence that the thyroid hormones do not trigger the changes in apoB mRNA editing during the suckling and weaning period.

To obtain greater insight into the regulation of hepatic apoB mRNA editing during development the effect of hormones on cultured hepatocytes was studied. The apoB mRNA editing measured in freshly isolated hepatocytes from fetal, neonatal and adult rats amounted to 50%, 28% and 85%, respectively (Figs. 3 and 4). These values of apoB mRNA editing were somewhat higher than those found for RNA isolated from liver tissues (compare Fig. 1). Without addition of hormones to the medium (see control in Fig. 4), the apoB mRNA editing decreased, independently of the initial value, to about 20% in the cells of all developmental stages after 72 h of cultivation. T_3 did not show any effect on apoB mRNA editing in cultured hepatocytes from fetal, neonatal and adult rats. This finding, however, does not rule out an effect of T_3 in vivo as reported by Davidson et al. [23] for adult rats, because thyroid hormones have been shown to modulate gene expression of growth hormone [24] known to affect the hepatic apoB mRNA editing [7].

When the cells were cultured in the presence of dexamethasone, the apoB mRNA editing was significantly higher than in the corresponding controls without hormones (Figs. 3 and 4). After the addition of dexamethasone alone to hepatocytes from adult rats apoB mRNA editing remained at a relatively high level of 50%. The combined addition of dexamethasone

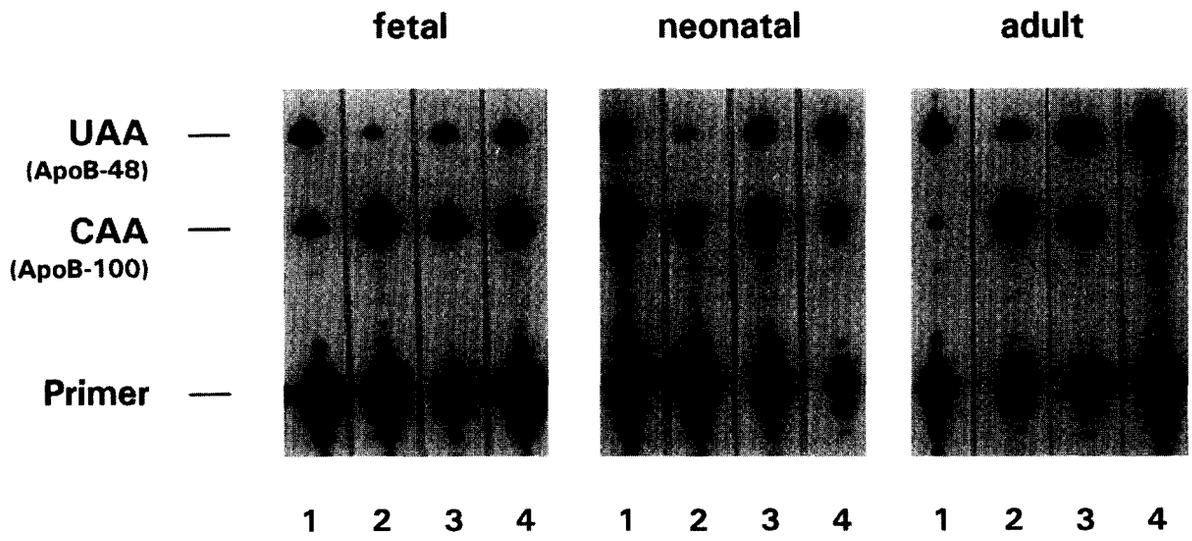

Fig. 3. Representative autoradiographs of primer extension analysis for determining the proportion of edited (UAA) and unedited (CAA) apoB mRNA. Total RNA was prepared from freshly isolated hepatocytes (1) and from cells cultured for 72 h without hormones (2) or with 100 nM dexamethasone (3) or with 100 nM dexamethasone plus 10 nM insulin plus 50 nM T_3 (4).

plus insulin, however, was still more efficient, leading to an apoB mRNA editing of about 71%, which was almost as high as the rate found in freshly isolated cells. Interestingly, the combined effect of insulin and dexamethasone was actually more pronounced than expected on a simple additive level.

In hepatocytes from neonates the apoB mRNA editing was increased to 58% after incubation in the presence of insulin plus T_3 plus dexamethasone. This value was significantly higher than the low value of 28% found in freshly isolated hepatocytes from the 10th day of life, indicating that apoB mRNA editing could be stimulated by hormone treatment. T_3 and insulin alone had no significant effect but enhanced the stimulation of apoB mRNA editing by dexamethasone. The hormonal stimulation of apoB mRNA editing in vitro might be relevant for the in vivo situation, since the insulin/glucagon ratio is low during the suckling period but high after weaning [22]. The same is true of the glucocorticoids. Their level is extremely low during the suckling period and increases strikingly after day 12–14 of life [25,26]. The missing effect of exogenous glucocorticoid administration to neonatal rats on hepatic apoB mRNA editing [4] indicates that a synergistic effect of different hormones on hepatic apoB mRNA editing might be important also in vivo.

In comparison with the findings in hepatocytes from adult and neonatal rats, the effect of hormones on apoB mRNA editing was much less pronounced in those from fetal rats. Even the addition of insulin, dexamethasone and T_3 in combination led to apoB mRNA editing of only about 35%, clearly less than the initial level of 50% determined in freshly isolated cells.

In order to examine the editing process at the translational level, the incorporation of [^{35}S]methionine into apoB-100 and apoB-48 secreted by the cultured hepatocytes was measured. Basically, the changes of apoB mRNA editing, found after hormone treatment in cultured hepatocytes from fetal, neonatal and adult rats, were reflected at the protein level (data not shown). No significant differences were observed between the corresponding ratio of edited apoB mRNA to edited plus unedited apoB mRNA and the ratio of secreted apoB-48 radioactivity to secreted apoB-48 plus apoB-100 radioactivity. Therefore, the editing is the major determinant of the form of apoB secreted not only in adult rats [7,8,27], but also during the developmental period.

Our findings, that insulin alone had no significant effect on apoB mRNA editing but acted in a synergistic manner together with dexamethasone, are noteworthy with regard to the results of others [8,28]. Pan and Koontz [28] analysed the effect of insulin on glucocorticoid receptor-mediated transcription using minimal artificial promoters. Insulin alone had virtually no effect on the expression of these promoters but it acted in a synergistic manner with glucocorticoids.

Since an accumulation of apobec-1 mRNA is associated with an increase in apoB mRNA editing [29,30], one may be led to speculate that dexamethasone stimulates the expression of the apobec-1 gene via a glucocorticoid response element (GRE) in the promoter and that insulin enhances this stimulation possibly through a component of the basic transcriptional apparatus.

In summary, the data shown here demonstrate that T_3 is

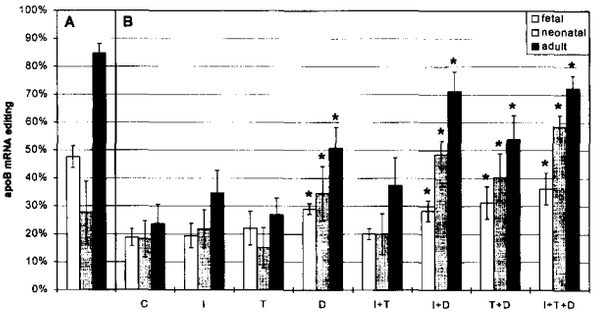

Fig. 4. ApoB mRNA editing of hepatocytes taken from fetal ($n = 3$), neonatal ($n = 5$) and adult ($n = 5$) rats before (A) and after cultivation for 72 h (B). Cells were cultured without hormones (C) or with 10 nM insulin (I), 50 nM T_3 (T) or 100 nM dexamethasone (D), alone or in combination with each of the others. Values are given as means ± SD. The star indicates significant differences at $P < 0.05$ vs. the corresponding control (C).

not critical for increasing hepatic apoB mRNA editing during the postnatal development of the rat. Rather, glucocorticoids together with insulin seem to be important for the regulation of this process. The details of the mechanism of the stimulation of hepatic apoB mRNA editing, e.g. whether there exists a GRE-mediated control of apobec-1 gene expression, are still unknown and deserve further investigation.

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