Hepatic Cytochrome P450 4a Expression Level In A Rat Model Of Microvesicular Steatosis

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Hepatic Cytochrome P450 4a Expression Level In A Rat Model Of Microvesicular Steatosis

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Abstract

Microvesicular steatosis in general plays an important role in the pathogenesis of drug, disease and alcohol induced liver damage. Such an event is manifested by the accumulation of lipid within the hepatocytes. Initial experiments conducted previously showed that short-term intake of diets containing Orotic acid (OA) produced rapid and extensive steatosis that follows a microvesicular distribution (Su, Sefton et al. 1999). The present study evaluated the hepatic cytochrome P450 4A expression level in a rat model of microvesicular steatosis that was marked by the intake of diets containing 1% of the Orotic acid for a time period of 21 days. The animals were sacrificed on the 22nd day and the hepatic microsomal P450 A4 protein level was quantitated using immunoblot analysis. The result of the investigation showed a significant induction of CYP 4A expression level in groups that were fed with diets containing the 1% OA. The CYP4A in the test group livers was found to have been up regulated by 53% when compared to the control group.

Introduction

Steatosis is a disease state that occurs due to accumulation of lipid within hepatocytes. This is often due to the intake of alcohol and drugs such as corticosteroids, tetracycline and some non-steroidal anti-inflammatory agents. Factors such as inherited metabolic disorders and diseases with hepatic involvement can also cause such a state (Farrell 1994; Burt, Mutton et al. 1998). The advent of steatosis by OA is thought to be mediated via mitochondrial injury, resulting in the impaired β-oxidation of fatty acids (Berson, De Beco et al. 1998; Burt, Mutton et al. 1998), however the detailed effects of lipid accumulation on hepatic gene expression are still unclear.

In the rat, OA has been shown to cause severe fatty infiltration of the liver when administered in concentrations of 0.2% or higher in a purine-deficient diet (Standerfer and Handler 1955). The accumulation of lipids (mainly triglycerides) is due to inhibition of lipoprotein synthesis. The lipoprotein precursors, i.e. the apoprotein and lipid moieties, are synthesised, but the apoprotein is deficient in N-acetylglucosamine, D-galactose and N-acetyl neuraminic acid (Martin, Biol et al. 1982) and the conjugation of the two moieties in the liver is impeded (Roheim, Switzer et al. 1965), resulting in a progressive increase in liver fat with a concomitant reduction in serum concentration of very low density lipoproteins and triglycerides (Hay, Fleming et al. 1988). The mechanism however, is not yet fully understood.

Previous study showed a significant increase in cholesterol, triglycerides and phospholipids levels in rat liver after feeding of experimental diets containing OA for 21 days (Su, Sefton et al. 1999). Fatty acid overload is accompanied by an increased capacity for oxidation of fatty acids by the mitochondrial peroxisomal and microsomal pathways of liver and heart (Mannaerts and Van Veldhoven 1992). The peroxisomal β-oxidation is only a minor pathway for fatty acid oxidation relative to the mitochondrial counterpart, its role is more important for the β-oxidation of very long-chain fatty acids (Osmundsen, Bremer et al. 1991). Diets containing long chain fatty acids have been shown to induce peroxisomal and microsomal oxidation enzymes and also the expression of the cytoplasmic liver-type fatty acid-binding protein (L-FAB) (Issemann, Prince et al. 1992; Reddy and Mannaerts 1994).

The L-FAB has been shown to have marked affinity for long-chain fatty acids and is considered to have a significant role in mediating the cellular uptake and intracellular targeting of long-chain fatty acids (Glatt and van der Vusse 1996). Therefore, in the metabolism and transport of long-chain fatty acids during fatty acid overload, the activity of peroxisomal, microsomal enzymes and the expression of L-FAB are of great importance.

The peroxisome proliferator activated receptors (PPARs; NR1C) belong to the steroid/thyroid/retinoid receptor superfamily. They are nuclear lipid-activatable receptors that control a variety of genes in several pathways of lipid metabolism, including fatty acid transport, uptake by the cells, intracellular binding and activation, as well as catabolism (β-oxidation and ω-oxidation) or storage. Three related isotypes of PPAR have been identified in rat and human, they are PPAR α (NR1C1), PPAR β (NR1C2) and PPAR γ (NR1C3) (Issemann and Green...
1990; Dreyer, Krey et al. 1992; Gottlicher, Widmark et al. 1992; Schmidt, Endo et al. 1992; Chen, Law et al. 1993; Sher, Yi et al. 1993; Ziu, Alvares et al. 1993; Kliewer, Forman et al. 1994; Amri, Bonino et al. 1995; Aperlo, Pognonec et al. 1995; Greene, Blumberg et al. 1995; Xing, Zhang et al. 1995). PPARs belong to the TR/RAR subfamily that recognize preferentially the core hexanucleotide motif AGGTCA in the up stream region of the target genes and are also characterized by the ability to form heterodimers with the 9-cis-retinoic acid receptor, RXR (NR2B). PPARs bind to DNAAs heterodimerically with RXR as their binding partner. The PPAR: RXR heterodimeric protein binds to the PPAR response elements (PPRE), a direct repeat of two core recognition motifs AGGTCA spaced by one nucleotide (also known as DR1)(Kliewer, Umesono et al. 1992). The PPRE was first located in the promoter region of the acyl-CoA oxidase gene (Dreyer, Krey et al. 1992; Tugwood, Issemann et al. 1992). PPAR interacts with the upstream-extended core hexamer of the DR1, whereas RXR occupies the downstream motif (Jipenber, Jeannin et al. 1997). It has been demonstrated that the carboxy-terminal extension (CTE) regions of both receptors is indeed responsible for the recognition of the 5'-flank of the DR1 in PPREs (Hsu, Palmer et al. 1998). Interestingly, it was observed that the expression of RXR abolished PPARα stimulation of the PRL promoter in pituitary GH4C1 cells (Tolon, Castillo et al. 1998) which suggests that stimulation of the PRL promoter by PPARα was mediated by protein-protein interaction rather than binding of PPAR:RXR to the promoter. It has been proposed that the mechanism of this phenomenon is a ligand-dependent association of PPARα with the transcription factor GHF-1 that stimulates transcription (Tolon, Castillo et al. 1998). This also implies that PPARα would act similarly to a co-activator. It is postulated that in the event of over-expression of RXR, the stimulatory effect of PPARα can be suppressed as RXR might compete for its association with GHF-1.

Materials and Methods

Materials

The Hyperfilm-MP and reagents for enhanced chemiluminescence were acquired from Amersham Australia (North Ryde, NSW, Australia). Retinal, retinyl acetate and α-tocopherol acetate were purchased from Sigma Chemical Co. (St. Louis, MO). Chemicals used for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad laboratories (Richmond, CA). Constituents for the rat experimental diets were purchased from ICN Biochemicals (Seven Hills, NSW, Australia). Other biochemicals were procured from Sigma Chemical Co. (Castle Hill, NSW, Australia) The rabbit anti-CYP4A1 IgG was a gift from Prof. G.G. Gibson, University of Surrey.

Animal Treatments

The research work was carried out according to the guidelines endorsed by the Australian National Health and Medical Research Council and was approved by the University of New South Wales and Central Area Health Service Animal Care and Ethics Committees. 12 male Wistar rats of approximately 3 weeks old with weight range of 237-282 g were divided equally into 2 groups with 3 rats per cage. Each group received basal diets of a high sucrose-containing semi-purified (SP) diet with each kilogram consisting of sucrose (600g), casein (200 g), cellulose (110 g), corn oil (40 g), ICN salt mixture 4179 (40 g), ICN vitamin diet fortification mixture (10 g), α-tocopherol (20 mg), and retinyl acetate (8.7 mg). The rat group that had 1% Orotic Acid in their diet was labelled as OA+ group and the group without any Orotic Acid (the control group) was labelled as OA- . The rats were held on the diet for 21 days and had free access to water. All the rats were individually labelled and their weights recorded throughout the 21 days and all the animals were sacrificed on the 22nd day under anaesthesia.

Microsome preparation

The rat livers harvested were perfused with cold saline solution followed by snap freezing in liquid N2 before storage at -70o C. The hepatic microsomes were prepared according to methods established earlier (Murray, Zaluzny et al. 1986). The liver sections from storage were thawed on ice in microsome preparation buffer (pH 7.4) containing 0.01M K2HPO4, 1mM EDTA and 0.25 M sucrose. The samples were homogenised and centrifuged at 10 000g for 25 minutes at 4o C under vacuum. The pellets were discarded and the supernatant was subjected to ultracentrifugation under vacuum at 35 000rpm for 1 hour 10 minutes at 4 oC. The supernatant was transferred into another tube and stored at -70 oC and the remaining pellet was covered with preparation buffer and homogenised for 5 passes followed by further ultracentrifugation at 4 oC at 35 000rpm for 35 minutes under vacuum. All the supernatant was discarded and the final microsomal pellet was resuspended in 50mM potassium phosphate buffer (pH 7) containing 1 mM EDTA and 20% glycerol and homogenised for 5 passes before being snapped frozen in liquid N2 and stored at -70°C.

Immunoblotting for CYP 450 4A Apoproteins in Rat Hepatic Microsomes.

The rat hepatic microsomes protein concentration was
determined and standardised using Lowry Protein quantification assay prior to Western Blot analysis. 5 mg / lane of each sample was subjected to electrophoresis on 7.5% acrylamide running gel and 3% polyacrylamide stacking gel (Towbin, Staehelin et al. 1979; Murray, Zaluzny et al. 1986). Proteins were transferred to nitrocellulose paper via electrophoresis gel transfer operation. The nitrocellulose paper was left to incubate in anti-rabbit IgG 4A antibody followed by anti-sheep IgG antibody and the immunoreactive proteins were detected by enhanced chemiluminescence on Hyperfilm-MP, and the resultant signals were analyzed by densitometry (Bio-Rad, Richmond, CA).

Results

Effects of intake of Orotic Acid containing diets on rat body weight

Table 1 shows that the group of rats that were fed with diets containing Orotic acid (OA+) had lower body weights when compared with control (OA-). Student’s t-test indicated that the mean values of the two population body mass were significantly different from each other (P< 0.05). The rats also had a lower rate of weight gain when compared to the control, but the total food consumed per cage over the 21 days period was not different as shown by Table 2. The OA- group consumed 1706+65 g and the OA+ group consumed 1655+152g of rat diet. On Day 21, the OA- group had a mean body mass of 357+15 g and the OA+ group had a mean mass of 296+14g.

The chart in Figure 2 is a graphical representation of the results shown in Figure 1. The data represents the total amount of Cyp4A in liver microsomes from OA+ group and OA- rats in densitometry units. The OA+ group had a value of 1.560+0.232 unit and the OA- group had a value of 1.0173+0.2463 unit. The student’s t-test showed a significant difference between the values of the two groups (P

References


Illustrations

Illustration 1

Table 1: Change in rat body mass in during 21 days of dietary conditioning.

<table>
<thead>
<tr>
<th>RAT BODY MASS (g)</th>
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Illustration 2

Table 2: Food consumption during the 21 days period

| MASS OF DIET CONSUMED (g) | DAY | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | Total (Kg) |
|---------------------------|-----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----------|
| CAGE 1 (-OA 1,2,3)        |     | 48 | 48 | 100| 100| 83 | 86 | 82 | 68 | 77 | 77 | 81 | 82 | 89 | 85 | 91 | 84 | 97 | 100| 91 | 91 | 92 | 1752     |
| CAGE 2 (-OA 4,5,6)        |     | 48 | 48 | 100| 100| 90 | 87 | 90 | 70 | 79 | 67 | 70 | 76 | 78 | 73 | 83 | 77 | 83 | 86 | 90 | 83 | 82 | 1660     |
| CAGE 3 (+OA 1,2,3)        |     | 48 | 48 | 100| 80 | 63 | 74 | 85 | 65 | 82 | 85 | 88 | 92 | 91 | 87 | 98 | 99 | 99 | 99 | 99 | 98 | 1762     |
| CAGE 4 (+OA 4,5,6)        |     | 48 | 48 | 100| 87 | 63 | 73 | 65 | 38 | 61 | 65 | 62 | 58 | 78 | 81 | 100| 82 | 93 | 81 | 97 | 71 | 99 | 1546     |
Illustration 3

Figure 1: Western Blot of Cyp4A level between rat groups

Illustration 4

Figure 2: Cyp4A level between OA+ group and OA-group.

Chart showing the Cyp450 Protein level in OA- and OA+ group.
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