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Research Article [Araştırma Makalesi]

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Isolation of biliary lipid carrying vesicles, analysis of their biochemical properties and hepatic ABCG5 mRNA expression in diosgenin-fed rats

[Diosgenin ile beslenmiş sıçanlarda safra kökenli lipid taşıyan veziküllerin izolasyonu, biyokimyasal analizleri ve hepatik ABCG5 mRNA ekspresyonu]

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ABSTRACT

Objective: An attempt was made to isolate vesicles, rich in biliary-type lipid from livers of rats-fed with diosgenin, which is reported to increase cholesterol carrying vesicles in hepatocytes, and to determine their lipid, alkaline phosphodiesterase I, protein status and hepatic ATP-binding cassette half-transporter genes 5/8 (ABCG5/ABCG8) expression.

Material and Methods: The livers were removed, homogenized, subjected to centrifugation, and the microsomal fraction was obtained. This microsomal fraction was then loaded onto a 12% self generating gradients of OptiPrep™ in a Beckman Vti 65 vertical-tube rotor and centrifuge at 350000 x*g* for 90 min at 4°C. The gradient fractions were analysed for total phospholipid, cholesterol, protein and alkaline phosphodiesterase I (PDE).

Results: There were no differences in total phospholipid, cholesterol and protein contents of the subcellular fractions of livers from control and diosgenin fed rats. However, PDE activity was significantly lower in some subcellular fractions in diosgenin-fed rats compared to controls. The gradient fractions with density 1.05-1.07 g/ml had increased amounts of cholesterol, PDE activity and some phospholipids, but contained very little protein. However, the gradient fractions with density 1.09-1.23 g/ml were significantly rich in cholesterol, phospholipids and protein, but not PDE activity, suggesting another population of vesicles. Hepatic ABCG5 mRNA expression was increased in diosgenin-fed rats compared with the control group, although, no significant difference was found in hepatic ABCG8 mRNA expression between control and diosgenin-fed rats.

Conclusion: These findings suggested that ABCG5 is involved in diosgenin induced biliary cholesterol secretion and may provide an insight into mechanisms involved in biliary lipid transport.

Key Words: ABCG5; ABCG8; biliary lipid; diosgenin; biliary cholesterol

Conflict of interest: No conflicts of interest are declared by the authors.

ÖZET

Amaç: Hepatositlerde kolesterol taşıyıcı vezikülleri arttırdığı bildirilmiş olan diosgenin ile beslenen sıçan karaciğerlerinden, safra kökenli lipid yönünden zengin vezikülleri izole etmek ve onların lipid, alkalin fosfodiesteraz I, protein ile hepatik ATP-bağlayan kaset yarım taşıyıcı genleri 5/8 (ABCG5/ABCG8) ekspresyonlarının düzeylerinin saptanması amaçlandı.

Gereç ve Yöntem: Karaciğerler alınarak, homojenize edildikten sonra santrifüjlenerek mikrozomal fraksiyonları elde edildi. Daha sonra bu mikrozomal fraksiyon, Beckman Vti 65 vertikal-tüp rotor içindeki, % 12'lik kendiliğinden gradiyent oluşturan OptiPrep™'in üzerin konuldu ve 4°C'de, 350000 x*g* devirde, 90 dakika süreyle santrifüj edildi. Gradiyent fraksiyonlarının toplam fosfolipid, kolesterol, protein ve alkalin fosfodiesteraz I (PDE) için analizleri yapıldı.

Bulgular: Kontrol ve diosgeninle beslenmiş sıçanlar arasında, subsellüler fraksiyonların toplam fosfolipid, kolesterol ve protein içerikleri yönünden farklılıklar bulunmadı. Bununla birlikte, diosgenin ile beslenen sıçanların, bazı subsellüler fraksiyonlarının PDE aktivitelerinin, kontrol ile karşılaştırıldığında, belirgin olarak daha düşük olduğu tespit edildi. 1.05-1.07 g/ml dansiteli gradiyent fraksiyonlarındaki kolesterol, PDE aktivitesi ve biraz da fosfolipid içeriği, daha yüksek düzeylerde bulunmalarına rağmen, çok az bir miktarda protein içeriyorlardı. Buna karşılık, 1.09-1.23 g/ml dansiteli gradiyent fraksiyonları; kolesterol, fosfolipidler ve protein miktarları yönünden yüksek olmalarına karşılık, PDE aktivitesinde böyle bir artış olmaması, bu fraksiyonlarda diğer bir vezikül popüsyonunu olduğunu düşündürmektedir. Hepatik ABCG5 mRNA ekspresyonu, kontrol grubuyla karşılaştırıldığında, diosgeninle beslenen sıçanlarda istatistiksel anlamda yükselmesine rağmen, hepatik ABCG8 mRNA ekpresyonunda, kontrol ve diosgeninle beslenen sıçanlar arasında belirgin bir farklılık bulunmamıştır.

Sonuç: Bu bulgular, ABCG5'in, diosgenin tarafından uyarılan safra kökenli kolesterol sekresyonuyla ilgili olduğunu göstermektedir ve safra lipid transportunda yer olan mekanizmalar hakkında yeni bir fikir sağlayabilir.

Anahtar Kelimeler: ABCG5; ABCG8; safra kökenli lipid; diosgenin; safra kökenli kolesterol

Çıkar çatışması beyanı: Yazarlar tarafından hiçbir çıkar çatışmasının bulunmadığı beyan edilmiştir.

Introduction

Phospholipids and cholesterol are synthesised in the hepatocytes and are thought to be transferred into bile by vesicular and non-vesicular mechanisms. Biliary lipids mainly consist of cholesterol and phospholipids and their secretion into bile is effected by secretion of bile salts. Hepatocytes acquire biliary lipid by three pathways namely (i) biosynthesis, (ii) lipoproteins and existing lipid molecules drawn from intracellular membranes and (iii) newly synthesised biliary lipids; these account for less than 20% of the total lipids [1].

The pathways involved in transhepatic cholesterol trafficking into bile are still not fully understood. A number of intracellular transport proteins such as Niemann Pick C1 and 2, sterol carrier protein 2 and caveolins have been identified, but their importance is unknown [2]. ABCG5 and ABCG8 are half-size ATP-binding cassette (ABC) transporter proteins that function together as a heterodimer (ABCG5/ABCG8) and have an essential role in biliary cholesterol secretion and intestinal absorption of sterols [3-6]. After arrival at the canalicular membrane of the hepatocyte, most of biliary cholesterol secretion is mediated by ABCG5 and ABCG8. The remaining cholesterol which contributes between 10% to 30% of total bilary cholesterol secretion is independent of ABCG5 and ABCG8 and is thought to be transferred into bile by vesicular mechanism [7-10]. Several studies [9, 11-12] have shown the hepatic expression of ABCG5/ABCG8 induced cholesterol secretion into bile, although contradictory results have also been reported [13, 14]. A similar function to that of the heterodimer ABCG5/8 has also been shown for ABCB4, which mediates the translocation and secretion of phosphatidylcholine into bile [15-17].

Diosgenin, a plant-derived sapogenin structurally similar to cholesterol, has been shown to stimulate biliary cholesterol secretion and to decrease cholesterol absorption in mice and rats without altering biliary phospholipid and bile salt secretion [37]. In this study, diosgenin was fed to rats and its effects on biliary phospholipid and cholesterol secretion is reported as is its effect on total protein levels, Alkaline phosphodiesterase I (PDE) activity and the expression of hepatic ABCG5/ABCG8. The aim of the study was to isolate biliary lipid carrying vesicles in hepatocytes by using a novel gradient centrifugation technique and to identify lipid carrying vesicles by measuring cholesterol with gas liquid chromatography.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma Chemical Co., Poole, Dorset, UK, except for cannulation tubing PP10 (internal diameter 0.28 mm) which was from Portex Ltd., Hythe, UK.

Animals and treatments

Animals used throughout this study, were male Wistar rats (250-300 g), bred within Liverpool John Moores University, Life Services Support Unit. Protocol number of the Animal Ethics Committee is 2004/48. Animals were allowed free access to standard laboratory diet in powdered form for 3 days. One group of rats received 1.0% diosgenin (w/w) incorporated into their standard laboratory diet for 7 days. Diosgenin was dissolved in chloroform and sprayed over the crushed chow diet and mixed thoroughly. The solvent was allowed to evaporate at room temperature for 48 hours in a fume cupboard. Corresponding control groups received standard diet only as previously described [18, 19].

Liver homogenization

Rats were anaesthetised with sodium pentobarbitone (6 mg/100 g body weight, intraperitoneal) before cannulating the bile duct. Once bile collection was complete, the rats were sacrificed and liver of each rat was removed and weighed. The livers were transferred to 3 vol. (w/v) of ice-cold buffered sucrose (0.25 M containing 1 mM HEPES pH 7.4.). They were then cut into several large pieces and swirled around in the buffer to remove as much blood as possible. The livers were minced finely with a sharp scissors and transferred to ice-cold homogenising vessel and were finally homogenised with about six strokes of the pestle at full speed. Finally, the homogenate was made up to 4 vol. (w/v) with sucrose buffer solution.

Fractionation of liver homogenate

The homogenate from the liver was used to produce subcellular fractions based on the method of Ford and Graham [20]. A sample of homogenate (3-4 ml) was removed for analysis and the remainder was centrifuged in a fixed angle roto at 4°C for 10 min at 1000 xg to pellet the nuclei and heavy mitochondrial. The pellet was suspended in sucrose buffer and stored frozen at -20°C.

Further centrifugtion was performed at 4000 xg for 10 min to produce the mitochondrial raction, followed by 15000 xg for 20 min to produce the light mitochondrial and lysosome fraction. A final cetrifugation step at 100000 xg for 45 min was then performed and the microsomal fraction was obtained. All fractions were assayed for cholesterol, phospholipids, protein and PDE.

Purification of vesicles from microsomal fraction

A microsomal pellet was obtained as described above. The pellet was dissolved in sucrose buffer solution up to 8 ml and then loaded onto 2 ml of OptiPrep™ (1.32 g/ml) in a Beckman Vti 65 vertical tube roor and centrifuged at 350000 xg for 90 min at 4ºC. After centrifugation, the gradient was fractioned by upward displacement into 10 x 1 ml fractionation and the fractions were analysed for cholesterol, phospholipids, protein and PDE.

Lipid Analysis

Cholesterol was analysed as trimethylsilyl ether derivatives as described by Zak et al. [21]. The method is useful for detecting low quantities of cholesterol which cannot be detected satisfactorily by other methods. Aliquots (10-50 µl) of the fraction of liver samples placed in a glass stoppered centrifuge tube containing 10 µl of mM 5α-cholestane as internal standard, and diluted with 0.8 ml of 80% (v/v) ethanol. This solution was extracted twice with 3 ml of light petroleum (b.p 40-60°C) and the combined light-petroleum extracts were evaporated to dryness. Samples were kept overnight in an evacuated dessicator. Trimethylsilyl esters were produced by adding 50 µl of Sigma Sil-A (trimethylchlorosilane, hexamethyl disilazane, pyridine (1:3:9 v/v) and the tubes were incubated at 50°C for 15 min. Aliquots (0.5-1 µl) of the derivatized mixture or standard were injected to the Gas Liquid Chromatography (GLC). The GLC system was a Pye-Unicam series-400 chromatograph equipped with a flame-ionization detector and the column (152 cm x 3 mm) was 1.5% (w/w) SE30 on diatomite CQ (80-100 mesh). The operating conditions were as follows; injection temperature 250°C, column temperature 235°C, detector temperature 290°C, carrier gaz (N_2) flow rate 50 ml/min. The cholesterol concentration (mM) was calculated by peak-area ratios of cholesterol to the internal standard 5α-cholestane.

Phospholipid was extracted from fraction of liver samples by a method based upon that of Bligh and Dyer [38] in which lipid is extracted into a chloroform-methanol-water mixture. Addition of further chloroform and water forms a biphasic system with non-lipids passing into the methanol-water phase. Phospholipid in the chloroform phase was then assayed by the method of Bartlett [22] in which organic phosphate is digested and the resulting ortophosphate is determined by converting it to phosphomolybdic acid. This is reduced to a blue complex allowing spectrophotometric measurement at 830 nm.

Alkaline Phosphodiesterase I (PDE) Analysis

PDE (EC 3.1.4.1) was measured at 37°C by the methods of Trams and Lauter [23]. Units of the enzyme activity are µmol of substrate hydrolyzed/h. The following were added to each well of a microplate: 133 µl of 50 mM glycine, pH 9.7, 13 µl of 150 mM $MgCl_2$ and 40 µl of substrate. p-nitrophenyl phosphothymidine (15 mM, 13 µl) was then added and after a lag period of 1 min, the change in absorbance was determined at 405 nm at 5 min intervals for 30 min in a Titertek Multiskan MCC/340 platereader.

Protein Estimation

Protein estimation was determined by the method of Winterbourne and all samples were assayed in duplicate [24]. A sheet of 3 mm Whatman chromatography paper was divided into 1 cm by 1 cm squares. Standard protein concentration ranged from 0.5- 8 µl aliquots of the 1 mg/ml protein solution. The standards were prepared by spotting corresponding amounts into the centre of the squares on the sheet. 3 µl of sample was also carefully spotted onto the centre of individual squares and blank squares were left for the determination of background staining. The standards and samples were then left to dry and were later fixed by immersing into 10% Trichloracetic (TCA) solution for 15 min. The sheet was then transferred to a working dye solution (0.04% w/v Coomassie blue, 25% v/v ethanol and 12% v/v acetic acid) and left to stain for 1 hour. The sheet was then destained by immersing in three changes of destaining solution 10% (v/v) methanol and 5% (v/v) glacial acetic acid for 10 minutes each. This was then left to dry in the oven at 80 ºC. The grid was cut into its individual component squares and were placed into small plastic vials containing 1 ml eluent 1 M potassium acetate in 70% (v/v) ethanol for 1 hour. The absorbance of the eluted dye solution was then read at 590 nm against the background dye measurements.

RNA isolation and PCR detection

Total RNA was isolated from approximately 30 mg of frozen liver tissue by using RNeasy Mini Kit (Qiagen, Maryland, USA) following the homogenization of the tissue. The RNA precipitate was dissolved in 75 µL of RNAse free water.

First-strand cDNA synthesis

Total RNA was used for the synthesis of single-strand complementary DNA (cDNA) by using antisenses and 100 units avian myeloblastosis virus *reverse transcriptase* (Q-Biogene, Irvine, Canada) for 60 minutes at 42ºC in a final volume of 20 µL. This cDNA pool was used as control for the qualitative detection of Real-Time PCR.

Real-Time PCR

The expression of ABCG5 and ABCG8 was determined by Real-Time PCR, using Lightcycler Instrument (Roche Diagnostics, Mannheim, Germany). A previously described method using a SYBR green I dye was used after making some modification for Real-Time PCR [24]. Briefly, Fast Start DNA Master SYBR Green mix (Roch Diagnostics, Mannheim, Germany) containing hot start *Taq DNA polymerase*, 5 µL cDNA, 0.5 µM of each gene specific ABCG5: sense 5'-GAG GTT ACT TAA TAG CCT ACG-3', antisense: 5'-GAA CAC CAA CTC TCC GTA AG-3'; ABCG8: sense 5'-GCT CAG TTC AAG TTA CCG TG-3', antisense: 5'-GTC AAG TCC ACG TAG AAG TC-3' and 3 mM magnesium chloride in a final volume of 20 µL in glass capillaries were run in duplicate. The reaction mixture was preheated at 95 ºC for 10 minutes, followed by 45 cycles at 95 ºC for 15 second and at 60 ºC for 1 minute. Glyceraldehyde-3-phophate dehydrogenase (GAPDH) a housekeeping gene was also amplified in each sample by using GAPDH: sense 5'-ACC ACA GTC CAT GCC ATC AC-3', antisense: 5'-

TCC ACC ACC CTG TTG GTG TA-3' [13]. All cycle threshold (Ct) values of studied gene expressions were adjusted according to the Ct value of GAPDH expression profiles.

Statistical analysis

Data was subjected to 2-tailed paired t-test and P values ≤0.05 were considered as statistically significant.

Results

Effect of diosgenin on biliary lipid output

Biliary cholesterol output was significantly increased 3-fold in rats fed diosgenin for 7 days whereas no effect on biliary phospholipid secretion was obsereved when compared to the control rats (Tab. 1).

Table 1. Effect of diosgenin feeding on biliary lipid secre

	Control	Diosgenin
Cholesterol (mM)	0.677 ±0.14	3.04 ±0.78**
Phospholipid (mM)	3.384 ±0.25	3.054 ±0.29

Analyses of biliary cholesterol and phospholipid in control and diosgenin-treated rats. The values are expressed as means ±SEM of 6 animals. Control rats received powdered diet and diosgenin-treated rats received 1% diosgenin (w/w) incorporated into the diet for 7 day.
** P <0.001 significantly different from control.

Total phospholipids and total cholesterol in subcellular fraction of liver homogenate

Total phospholipids and total cholesterol in subcellular fractions of liver homogenate from control and diosgenin fed rats are shown in Fig. 1. Results are means ±SEM of 6 animals.

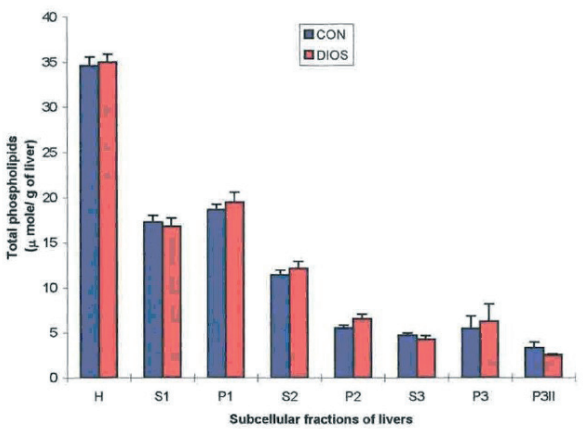

Figure 1. Total phospholipids in subcellular fractions of liver homogenate from control and diosgenin-fed rats. Results are means ±SEM of 6 animal. Symbols are (H) homogenate, (S1) supernatant 1, (P1) pellet 1, (S2) supernatant 2, (P2) pellet 2, (S3) supernatant 3, (P3) pellet 3 and (P3II) pellet 3 diluted with sucrose and KCI. Significant differences from controls were assessed by student's t-test and are indicated by *(P<0.05).

The total lipids have been expressed as µ mole/g of liver due to the differences in liver weight of the animals. No significant differences were found in the total phospholipids in subfraction of the control andiosgenin-fed rats (Fig 1). The amount of cholesterol was lower in the total homogenate of the liver from diosgenin-fed rats when compared to control rat, however, this was not statisly significant (Fig. 2). In the subcellular fraction, no significant differences between control and diosgenin-fed rats were observed.

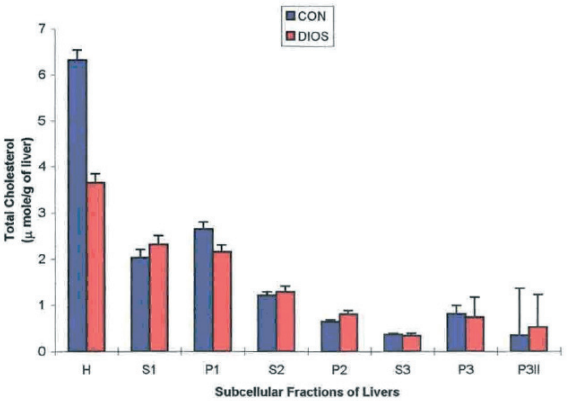

Figure 2. Total cholesterol in subcellular fractions of liver homogenate from control and diosgenin-fed rats. Results are means ±SEM of 6 animal. Symbols are (H) homogenate, (S1) supernatant 1, (P1) pellet 1, (S2) supernatant 2, (P2) pellet 2, (S3) supernatant 3, (P3) pellet 3 and (P3II) pellet 3 diluted with sucrose and KCI. Significant differences from controls were assessed by student's t-test and are indicated by *(P<0.05).

Total protein and PDE activity in subcellular fraction of liver homogenate

Total protein and PDE activity in subcellular fraction of liver homogenate from control and diosgenin-fed are depicte Fig. 3. Fig. 4.

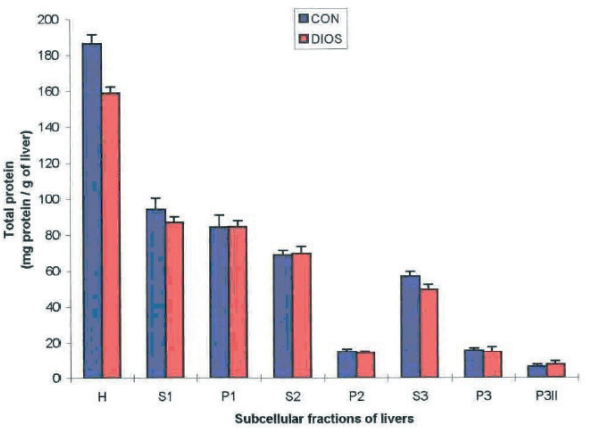

Figure 3. Total protein in subcellular fractions of liver homogenate from control and diosgenin-fed rats. Results are means ±SEM of 6 animal. Symbols are (H) homogenate, (S1) supernatant 1, (P1) pellet 1, (S2) supernatant 2, (P2) pellet 2, (S3) supernatant 3, (P3) pellet 3 and (P3II) pellet 3 diluted with sucrose and KCI. Significant differences from controls were assessed by student's t-test and are indicated by *(P<0.05).

No significant differences were found in total proteins in the liver from control andsgenin- fed rats (Fig.3). PDE, decreased in all subcelllular fractions except S3 and P3 from diosgenin-fed rats, although it was significant only in sactions P1 and P2 (Fig.4).

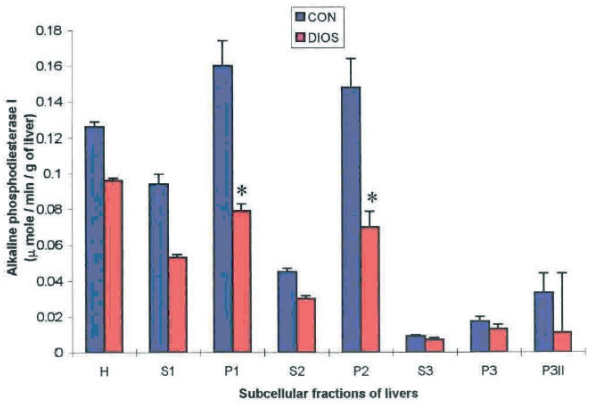

Figure 4. Alkaline phosphodiesterase I activity in subcellular fractions of liver homogenate from control and diosgenin-fed rats. Results are means ±SEM of 6 animal. Symbols are (H) homogenate, (S1) supernatant 1, (P1) pellet 1, (S2) supernatant 2, (P2) pellet 2, (S3) supernatant 3, (P3) pellet 3 and (P3II) pellet 3 diluted with sucrose and KCI. Significant differences from control assessed by students' t-test and are indicated by *(P<0.05).

Analysis of the subfractions of the microsomal fraction

The gradient fractions were analyzed for total phospholipids, total cholesterol, total protein and PDE activity and results were presented in Fig. 5. Fractions 1 and 2 (ρ = 1.05 g/ml-1.07 g/ml) had very low amount of phospholipids and proteins (Figs. 5a and 5c). However, the same fractions had much higher amounts of cholesterol and the membrane bound enzyme PDE (Figs.5b and 5d). In fractions 3-7, relatively low values were observed for all parameters. Fraction 7-10 (ρ = 1.09 g/ml-1.23 g/ml) were all enriched in total phospholipids, cholesterol and proteins but were depleted in PDE (Figs.5a, 5b, 5c and 5d). In addition, different profiles of PDE were observed in diosgenin-fed rats.

Expression of hepatic ABCG5 and ABCG8 mRNA

In diosgenin-fed rats, the level of hepatic ABCG5 mRNA expression was significantly higher than control. In contrast, no significant differences were found in hepatic ABCG8 mRNA expression between controls and diosgenin-fed rats. All data were standardized by GAPDH expression and values are expressed as means ±SEM of 6 animals, compared with control. ABCG5, ABCG8 and GAPDH were determined by Real-Time PCR as described in the experimental section.

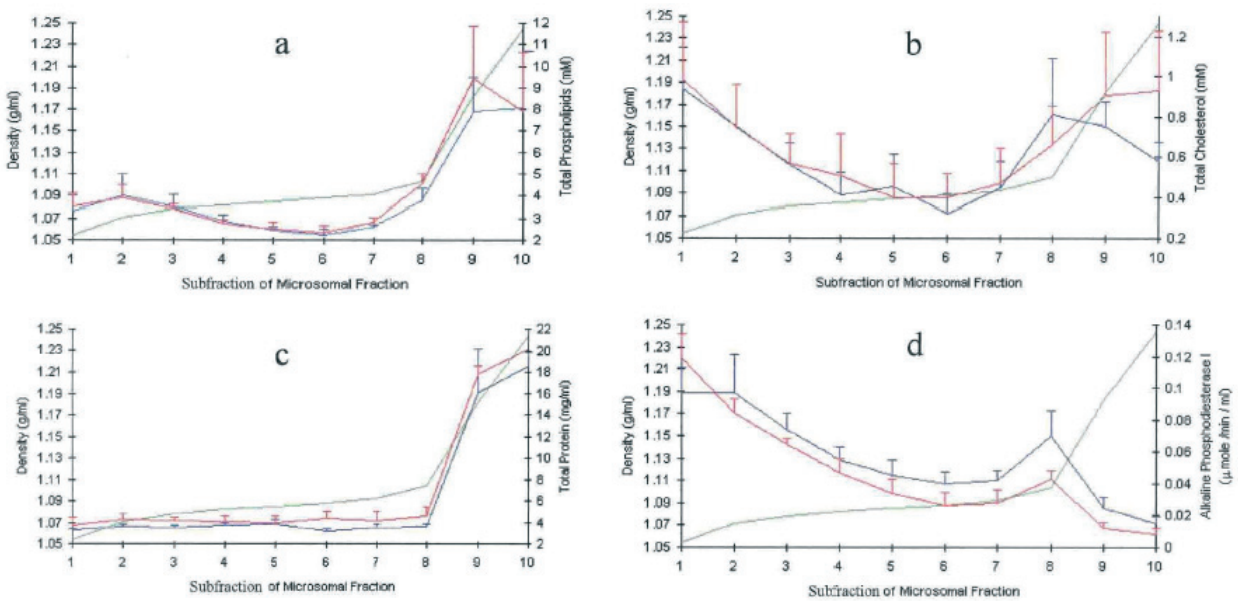

Figure 5. Analysis of the subfractions of the microsomal fraction in diosgenin treatment and control rats. Animals were sacrificed and liver removed. Liver were then subjected to centrifugation and the microsomal fraction obtained. This was then subjected to self generating gradients of OptiPrep, centrifuged at 350000 xg for 90 min at 4°C. After centrifugation the gradient was fractionated into 10 x 1 ml fractions. The fractions were analysed for total phospholipids, total cholesterol, total protein and PDE. —— Control; —— Diosgenin (a) Total phospholipid, (b) Total cholesterol, (c) Total protein, (d) Alkaline phosphodiesterase I; —— Gradient density. Results are means ± SEM of 6 animals.

Due to lack of positive control of these genes; the cycle threshold values having the earliest fluorescent increase were considered as references. Naturally, the mRNAs of higher expressed genes are expected to be higher as well; therefore, their maximum fluorescents values could be obtained by the lower cycle numbers. This indicates that a reverse relationship exists between the values of gene expressions and the cycle numbers which gives a maximum fluorescence value.

Table 2. Expression of Hepatic ABCG5 and ABCG8 mRNA in Control and Diosgenin-Fed Rats

	Control Cycle threshold (Ct)	Diosgenin Cycle threshold (Ct)
ABCG5/GAPDH	1.29 ±0.07	0.9 ±0.07*
ABCG8/GAPDH	0.723 ±0.04	0.86 ±0.11

Analyses of hepatic ABCG5, ABCG8 and GAPDH mRNAs in control and diosgenin-treated rats. ABCG5, ABCG8 and GAPDH were determined by RT-PCR. All data were standardized for GAPDH and values are expressed as means ±SEM of 6 animals, *P <0.05 significantly different from control.

Discussion

Diosgenin has previously been shown to increase biliary cholesterol secretion without altering biliary phospholipid and bile salt secretion. Previous publications have shown that feeding with diosgenin for 7 days leads to cholesterol saturation in bile [12, 19, 40]. In our study, diosgenin increased biliary cholesterol over 3 fold (Tab. 1), which suggests that diosgenin had been effected on biliary cholesterol mechanisms. Hence, in the present study diosgenin was successfully used to increase biliary cholesterol. Since biliary cholesterol is transported in cholesterol-carrier vesicles it was envisaged that these would increase in the hepatocytes of doisgenin-fed rats. Separation of a vesicle population, from diosgenin-fed rats as identified by increased cholesterol content compared to controls, may therefore be an alternative strategy to obtain putative biliary lipid vesicles. Attempts to isolate vesicles containing biliary type phosphatidylcholine and cholesterol have largely been unsuccessful in hepatocytes [26]. However, Gilat and Somjen [27] and Somjen et al, [28] have identified three form of biliary lipid carriers in bile such as unilamellar vesicles, stacked lamellae and micelles.

The results showed that cholesterol secretion into bile increased in diosgenin fed rats (Tab. 1), however the cholesterol and phospholipids content of liver of diosgenin fed rats were similar to that observed in the subcellular fractions of the control rats (Figs. 1 and 2). This is in agreement with the results of Roman et al. [8]. Furthermore, diosgenin stimulated the hepatic expressions of ABCG5, whereas the hepatic expression of ABCG8 was unchanged when compared to the controls.

In the experiments reported in this study, the isolation of biliary type vesicles was achieved by using the novel gradient medium, Iodixanol. This is nonionic medium which has an advantage over sucrose that it rapidly forms self-generated gradients in vertical or near-vertical rotor [30]. Increased lipid transfer vesicles might be present in the microsomal fraction however, subfraction of microsomal fraction on density gradient with Iodixanol failed to identify biliary transfer vesicles.

No significant differences were found in total protein of the liver between control and diosgenin fed-rats (Fig. 3). PDE is a membrane bound enzyme and would be expected to be in membrane vesicles, however, PDE activity decreased in some of diosgenin-treated rat liver subfractions (Fig. 5d). The inhibition effect on PDE is unlikely due to diosgenin and this may suggest a cholesterol-related change in the normal distribution or function of some membrane constituents destined for biliary excretion. The decrease in this enzyme activity may also be involved in the formation or exocytosis of phospholipid/cholesterol vesicles from the canalicular membrane to bile; this is in agreement with the observations of Thewles et al [19].

The microsomal fraction which was expected to contain putative biliary type vesicles was subselected for density gradient centrifugation. The analysis of the fraction revealed that it may have two different population vesicles (Figs.5a,b,c and d). First putative vesicles appeared in fractions 1-2 (ρ = 1.05 g/ml-1.07 g/ml) that had higher cholesterol level and PDE activity but had lower total phospholipid and protein concentration. Another putative vesicles population was present in fractions 7-10 (ρ = 1.09 g/ml-1.23 g/ml) which had much higher total phospholipid and protein compared to fractions 1-2, however, PDE activity was depleted. On the density gradient, there were no differences between control and diosgenin-fed rats in biliary lipid, protein and PDE activity. Fractions 3-7 (ρ = 1.75 g/ml-1.09 g/ml) had a relatively small amount of phospholipid, cholesterol, protein concentration and PDE activity (Figs.5a,b,c and d).

Cholesterol/phospholipid ratio, the fatty acid composition saturated/unsaturated ratio (S/U) and types of phosphatidylcholine, all determine canalicular fluidity. An increase in membrane fluidity enhances the activity of various membrane transport, including sinusoidal transporters and canalicular transporters. Yamaguchi et al. [31] and Amigo et al. [2] have reported that canalicular membrane fluidity was increased by diosgenin. In contrast, some studies indicated that feeding rats with diosgenin had no effect on the cholesterol content of canalicular membranes [31], suggesting that the effect of diosgenin may involve increased delivery of cholesterol through the canalicular membranes. This is in agreement with our results that no significant differences were found in cholesterol and phospholipid contents of the microsomal fractions between control and diosge-

nin treated rats. Crawford et al. [33, 34] reported that vesicles are secreted from the outer leaflet of the canalicular membrane by ABCB4 transporter. Subsequently, bile salt/phospholipid micelles in bile extract cholesterol from these vesicles. Vesicular secretion is compatible with the function of ABCG5/ABCG8. Several studies [33, 35-37] suggested that vesicular secretion of cholesterol is one of the mechanisms by which sterols appear in bile.

According to the results of this study no significant changes in biliary lipid carrying vesicles were observed, however, significantly different profiles of PDE and expression of ABCG5 were seen. Diosgenin exerts its effects via ABCG5; however, more detailed studies are required to validate this hypothesis.

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The work cited in this article was included in the author's Ph.D and M.Phil studies. Some part of this article results were presented at the 31.FEBS congress as a poster.

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