Endocrine (1995) 3, 695-703

Reduced hepatic LDL-receptor, 3-hydroxy-3-methylglutaryl coenzyme A reductase and sterol carrier protein-2 expression is associated with pregnancy loss in the diabetic rat

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Hepatic low density lipoprotein receptor (LDLR), 3-hydroxy-3-methylglutaryl coenyzme A reductase (HMGR), cholesterol 7α-hydroxylase, and sterol carrier protein-2 are important proteins associated with the uptake, synthesis, degradation and transport of cellular cholesterol. Since cholesterol is critically important for steroid hormone synthesis and is an essential component in membrane biosynthesis, this study investigated whether these proteins are altered in the normal pregnant and streptozotocin (STZ)-induced diabetic pregnant rat. The goal of these experiments was to determine whether diabetic reproductive dysfunction is associated with a significant change in maternal cholesterol homeostasis. Diabetic animals were grouped based on their ability or inability to maintain pregnancy up to day 15 post-conception. LDLR and HMGR mRNA levels were significantly reduced in animals which did not maintain pregnancy whereas diabetic animals with fetuses had normal LDLR and HMGR mRNA levels. Hepatic LDLR, HMGR, and SCP2 protein levels were examined in normal pregnant and diabetic pregnant animals by Western blot analysis. SCP2 levels were reduced in all diabetic animals, particularly in the diabetic animals which lost their fetuses. The decline in SCP2 was associated with an increase in sterol carrier protein-X (SCPx), a protein related to SCP2. SCPx has been shown to have thiolytic activity and is thought to have a role in β-oxidation of fatty acids. HMGR was also significantly reduced in diabetic animals which lost their fetuses. Cholesterol 7α-hydroxylase mRNA was slightly, but not significantly, reduced in all diabetic animals. Serum very low density lipoprotein (VLDL) +LDL cholesterol increased significantly in the STZ-treated diabetic rats while the HDL cholesterol levels declined in these animals. Reduced hepatic LDLR and HMGR mRNA levels were consistently associated with reduced serum progesterone and an inability to maintain pregnancy. The results of this study suggest that the maintenance of maternal cholesterol metabolism is a critical factor directly associated with successful pregnancy outcome in the diabetic rat.

Keywords: LDL-receptor; HMG-CoA reductase; cholesterol 7α-hydroxylase; SCP2; cholesterol; diabetic rat

Introduction

Reproductive dysfunction is a common occurrence in the diabetic female. Reproductive problems associated with this disease include ovarian atrophy (Lawrence & Cantopoulos, 1960), impaired folliculogenesis (Garris *et al.*, 1982), anovulation (Kirchick *et al.*, 1978), insufficiency in corpus luteum progesterone output (Garris *et al.*, 1982; Vomachka & Johnson, 1982), uterine involution, and associated problems with the maintenance of pregnancy (Angervall, 1959; Garris & Smith, 1983; Garris, 1984). Reproductive complications associated with diabetes in humans are directly related to the severity of the disease (Olofsson *et al.*, 1984). Although insulin therapy can reverse many of these problems, the etiology and underlying mechanism of reproductive dysfunction in the diabetic remains unresolved. To date, several studies have examined altered ovarian function in association with diabetes, however, less attention has been given to the alterations in maternal hepatic cholesterol metabolism as an associated factor in reproductive demise. Since maternal triglyceride and very low density lipoprotein (VLDL) levels have been reported to rise 2.5-fold during normal pregnancy (Knopp *et al.*, 1986) it is important to study what effect diabetes may have on altered hepatic cholesterol metabolism and reproductive function. Whether altered maternal cholesterol metabolism is a contributing factor in altered ovarian steroid production (Garris *et al.*, 1982; Vomachka & Johnson, 1982), embryo growth retardation (Kawaguchi *et al.*, 1994; Pedersen & Molsted-Pedersen, 1979) or fetal demise (Angervall, 1959; Garris & Smith, 1983; Garris, 1984) remains to be clarified. It is also unclear what role maternal hepatic cholesterol homeostasis plays in the reproductive state.

Concomitant with the change in reproductive function, cholesterol metabolism is profoundly altered in the diabetic condition (O'Meara *et al.*, 1991; Feingold *et al.*, 1982). Since cholesterol is a major constituent of cell membranes and is the essential precursor molecule in the synthesis of steroid hormones, the alteration in cholesterol metabolism will most likely have a direct bearing on reproductive capacity and embryonic growth. Hepatic cholesterol uptake (Howard, 1987), synthesis (Ness *et al.*, 1994a–d), and degradation (Devery *et al.*, 1987) are severely altered in diabetes. Thus, the possibility exists that the maintenance of pregnancy may be compromised by an alteration in maternal cholesterol homeostasis. Furthermore, accumulating evidence indicates that poorly controlled maternal diabetes during pre-pregnancy and the initial stage of pregnancy is associated with an increased risk of congenital anomalies in the offspring (Mills, 1982; Pedersen & Pedersen, 1985; Tanigawa *et al.*, 1991).

The expression of several key hepatic proteins associated with cholesterol metabolism have been shown to be altered in diabetes. These include the LDL-receptor (LDLR) (Howard, 1987), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) (Ness *et al.*, 1994a), and cholesterol 7α-hydroxylase (Devery *et al.*, 1987), proteins associated with the uptake, synthesis, and degradation of cellular cholesterol, respectively. The mRNA levels of LDLR (Howard, 1987) and HMGR (Ness *et al.*, 1994a) appear attenuated in the liver of animals which have been treated with streptozotocin (STZ), a known pancreatic beta cell cytotoxin (Takasu *et al.*, 1991). Cholesterol 7α-hydroxylase activity is reported to be elevated in the diabetic state (Devery *et al.*, 1987).

Hepatic cholesterol synthesis and LDL-mediated cholesterol uptake generate significant amounts of sterol in the cytosolic, peroxisomal, and lysosomal compartments. Since cholesterol is insoluble in the cell, it must be bound to

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Received 10 May 1995; accepted 22 June 1995

a protein to facilitate its transport between these compartments for further processing. Sterol carrier protein-2 (SCP2) is a 13.2 kDa protein which has been implicated in intracellular cholesterol transport. SCP2 is reported to be involved in *de novo* cholesterol biosynthesis (Vahouny *et al.*, 1983), cholesterol esterification and storage (Gavey *et al.*, 1981) and free cholesterol generation from cholesterol esters (Vahouny *et al.*, 1993). It is also reported to be an activator of the cholesterol 7α-hydroxylase enzyme involved in bile acid synthesis (Seltman *et al.*, 1985). Although diabetes related hyperlipidemia and altered hepatic cholesterol metabolism have been studied extensively (Howard, 1987; Ness *et al.*, 1994a; Devery *et al.*, 1987), the expression of SCP2 in a hyperglycemic state has only recently been addressed (McLean *et al.*, 1995a,b).

To study the possible relationship between altered maternal hepatic cholesterol metabolism and pregnancy outcome in diabetes, a STZ-diabetic rat model was utilized and hepatic LDLR, HMGR, cholesterol 7α-hydroxylase and SCP2 expression were examined in normal pregnant animals and in pregnant diabetic rats which were able to maintain pregnancy and those which were not.

Results

The design of this study was such that diabetic rats were grouped based on their ability or inability to maintain pregnancy following chemical induction of a diabetic state. The diabetic animals which maintained pregnancy (+Ft; $n = 6$) and those which did not (−Ft, $n = 6$), were grouped and compared to normal pregnant animals on day 15 of pregnancy ($n = 12$). In this study, 40% of the pregnant diabetic animals lost their fetuses by day 15 of pregnancy ($n = 32$). The effect of STZ on maternal serum glucose is indicated in Figure 1A. The normal range for control rats was 60–150 mg/dL and for diabetic rats it was 300–600 mg/dL. Serum glucose levels were significantly ($P < 0.001$) elevated 8.6-fold in the STZ-treated rats ($n = 6$ group) demonstrating the efficacy of this chemical in promoting a diabetic state in this investigation. No difference in the degree of hyperglycemia was observed between the diabetic animals +/− fetuses. In contrast, serum cholesterol levels in the diabetic animals which did not maintain pregnancy were significantly elevated relative to both the control ($P < 0.01$) and diabetic animals with fetuses ($P < 0.05$) (Figure 1B). The normal range for serum cholesterol in control rats was 60–120 mg/dL and for diabetic rats it was 140–300 mg/dL. Although the diabetic animals with fetuses had elevated serum cholesterol levels, the values were not significantly greater than the control animals. As expected, the rise in serum cholesterol was due to an increase in the VLDL + LDL serum cholesterol fraction (Figure 2A). The HDL cholesterol levels were significantly ($P < 0.003$) reduced in the diabetic animals (Figure 2B). Interestingly, the VLDL + LDL levels in the diabetic animals without fetuses were significantly ($P < 0.04$) higher than in the control or diabetic animals with fetuses. Serum β-HBA levels were not detectable in the control animals while the levels of β-HBA in the diabetic animals were elevated significantly ($P < 0.004$). The β-HBA levels in animals with fetuses (20.1 ± 5.5 mg/dL) were not, however, significantly different from the animals in which the fetuses regressed (18.3 ± 3.2 mg/dL). These results indicate that the pregnancy outcome in this experiment was not related to differences in ketoacidosis in the diabetic groups.

Consistent with previous reports, serum progesterone levels were significantly lower in the diabetic pregnant animals (68.1 ± 9.5 ng/ml, $n = 6$) compared to day 15 normal pregnant animals (113.6 ± 6.5 ng/ml, $n = 6$). The lower steroid level in the diabetic rats was associated with reduced fetal weight (162 ± 16 mg *vs* 241 ± 25 mg) relative to the control animals. Gross appearance of the fetuses from the diabetic animals suggested that fetal development lagged 1–2 days behind the control animals. The diabetic animals which were unable to maintain pregnancy had profoundly lower serum progesterone levels (7.1 ± 0.8 ng/ml; $P < 0.001$). At autopsy, sites of implantation were confirmed in all animals. In one diabetic animal, regressing fetuses were observed. This indicates that embryos had implanted in these animals and argues against the possibility that STZ-treatment may have prevented implantation in the diabetic animals without fetuses.

To examine possible mediators of maternal cholesterol homeostasis which might be altered in the diabetic animals in association with their inability to maintain pregnancy, hepatic LDLR, HMGR and cholesterol 7α-hydroxylase steady-state mRNA levels were examined along with hepatic LDLR, HMGR and SCP2 protein levels. As shown in Figure 3A, LDLR Northern blot analysis indicated that diabetic animals which maintained pregnancy had normal LDLR

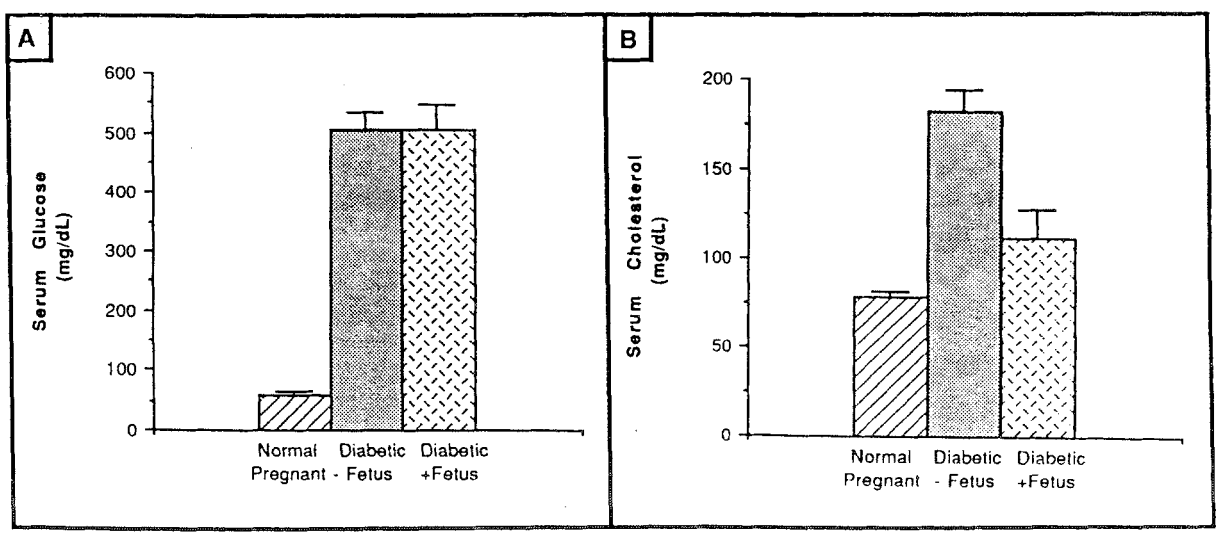

Figure 1 Serum glucose and cholesterol levels in control (normal pregnant rats) and STZ-treated diabetic rats which maintained their pregnancy (+ Fetus) and those which did not (− Fetus). (**A**) indicates the serum glucose levels in control and STZ-treated diabetic animals. Serum glucose levels were significantly ($P < 0.0001$) elevated in both diabetic groups. (**B**) indicates the serum cholesterol levels in control and STZ-treated diabetic rats. Serum cholesterol was elevated in both diabetic treatment groups, however, only the diabetic group without fetuses had significantly ($P < 0.05$) elevated cholesterol levels

levels while diabetic animals which did not maintain pregnancy had significantly ($P<0.05$) lower LDLR mRNA levels. LDLR mRNA levels for each group are summarized in Figure 3B. The fact that LDLR levels in diabetic animals with fetuses were similar to the control animals is consistent with the possibility that the maintenance of maternal cholesterol metabolism in the diabetic animals is an essential component for the normal reproductive process.

Similar to the LDLR mRNA levels which were maintained near control values in the diabetic animals with continuing pregnancies, HMGR levels were also maintained in the liver of diabetic female rats in which fetuses were observed (Figure 4A). HMGR mRNA levels in the diabetic animals which were unable to maintain pregnancy were significantly ($P<0.018$) lower relative to the control animals. Densitometric analysis of steady-state HMGR mRNA levels indicated near normal levels of hepatic HMGR mRNA in the diabetic animals with fetuses (Figure 4B). The decline in hepatic HMGR mRNA together with the reduction in LDLR mRNA in the diabetic animals which had no fetuses on day 15 of pregnancy clearly indicates an association between altered maternal hepatic cholesterol synthesis and uptake and the ability of the diabetic animals to maintain pregnancy.

To determine whether the expressed protein levels for LDLR and HMGR paralleled the steady state mRNA levels, Western blot analysis was conducted using antisera specific for LDLR (Ness *et al.*, 1994a) and HMGR (Ness *et al.*, 1994b). The immunoreactive protein levels for both LDLR and HMGR are represented graphically in Figure 5A. Hepatic tissue from diabetic rats with fetuses had slightly lower levels of the LDLR protein relative to the control animals. HMGR protein levels (Figure 5B) were markedly ($P<0.05$) reduced in the diabetic animals without fetuses but normal in diabetic animals with fetuses.

In addition to endogenous synthesis and cellular uptake of cholesterol, the intracellular transport of cholesterol derived from either of these sources may be critical to cellular cholesterol metabolism. Since the capacity of intracellular cholesterol transport is thought to be determined by the hepatic SCP2 levels, we measured hepatic SCP2 by Western blot analysis. As shown in Figure 6, hepatic SCP2 levels were reduced by 40% ($P<0.01$) in diabetic rats with fetuses. SCP2 levels were further reduced by 75% ($P<0.001$) in diabetic rats without viable fetuses when compared to the normal pregnant animals. Densitometric analysis of SCP2 Western blots clearly demonstrates that hepatic levels were significantly altered by the diabetic condition (Figure 6B). Concomitant with the reduction in hepatic SCP2 detected by

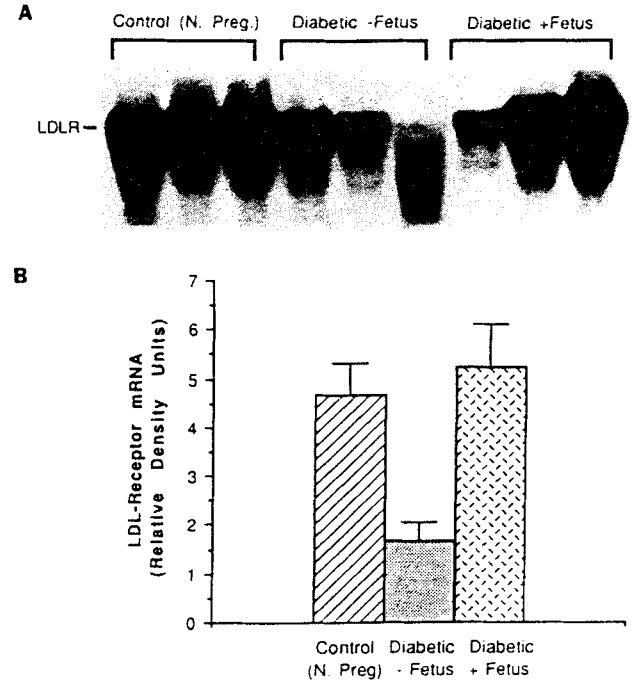

Figure 3 Northern blot and densitometric analyses of liver LDL-receptor (LDLR) levels in normal pregnant (control) and STZ-treated diabetic rats which maintained their pregnancy (+ Fetus) and rats which did not (– Fetus). (**A**) indicates the Northern blot for LDLR using hepatic mRNA from control and diabetic animals. Ten μg of poly(A)$^+$ RNA was loaded per lane. (**B**) indicates the densitometric analysis of LDLR mRNA levels in normal and diabetic rats. Any difference in RNA loading was corrected for based on the β-actin mRNA signal noted in Figure 4. LDLR was significantly ($P<0.05$) reduced in the diabetic animals which did not maintain pregnancy. The steady-state LDLR mRNA level in the diabetic animals with fetuses was similar to the LDLR mRNA level in the normal pregnant rat

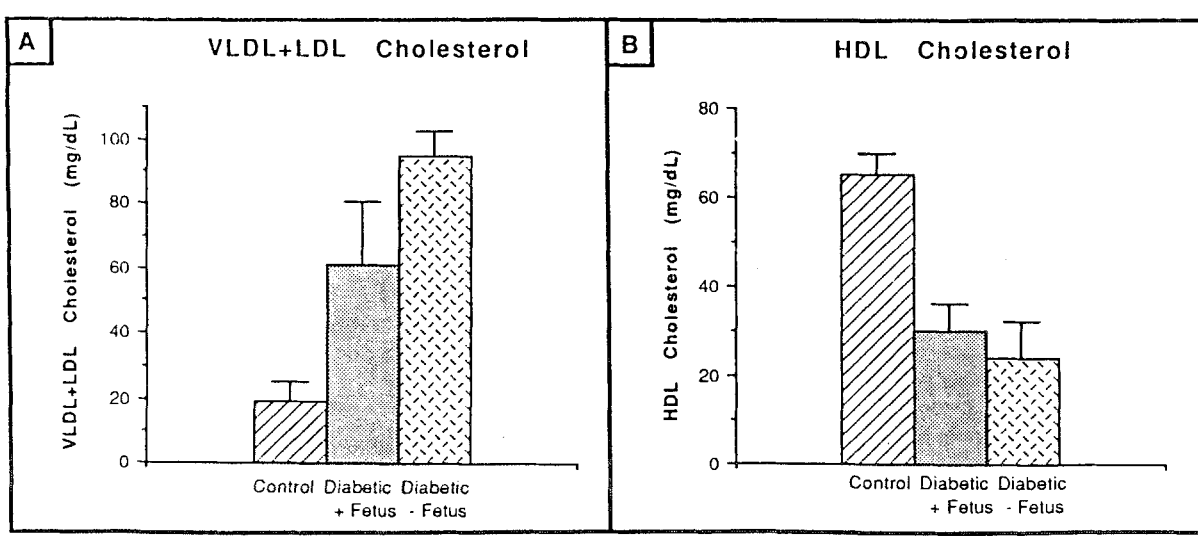

Figure 2 Serum VLDL + LDL cholesterol and HDL cholesterol levels in control (normal pregnant rats) and STZ-treated diabetic rats which maintained their pregnancy (+ Fetus) and those which did not (– Fetus). (A) indicates that VLDL + LDL cholesterol levels were elevated in both diabetic groups. Only the diabetic group without fetuses had significantly ($P<0.04$) elevated VLDL + LDL cholesterol levels. (**B**) indicates the HDL cholesterol levels in control and STZ-treated diabetic rats. Serum HDL cholesterol declined in both diabetic treatment groups

Western blot analysis, the 58 kDa SCP2 related protein, SCPx was increased (Figure 6A). Hepatic SCP2 levels were consistently reduced when SCPx expression was elevated in the diabetic animals. Densitometric analysis of SCP2 and SCPx immunoreactive proteins (Figure 6B and C) clearly demonstrated the differential expression of these sterol carrier proteins; SCPx levels were increased 3- to 3.5-fold ($P<0.001$) in the diabetic animals. These data indicate an inverse relationship in the expression of SCP2 and SCPx in the diabetic animals. These results suggest that cholesterol transport is profoundly affected in the diabetic animals and demonstrate that the maternal livers with the lowest SCP2 levels correspond with the animals which were unable to maintain pregnancy. These data clearly indicate a correlation between altered maternal hepatic cholesterol transport capacity and fetal demise in the pregnant rat.

To further examine alterations in maternal cholesterol metabolism, hepatic cholesterol 7α-hydroxylase mRNA levels were examined by Northern blot analysis. The level of cholesterol 7α-hydroxylase was slightly reduced in both diabetic groups suggesting a decline in bile acid production in the diabetic rats (Figure 7). The cholesterol 7α-hydroxylase mRNA levels declined independent of whether the animals were able to maintain their pregnancy. The 30–40% decrease in cholesterol 7α-hydroxylase, although consistent in all diabetic animals, did not represent a significant reduction in cholesterol 7α-hydroxylase mRNA levels in the diabetic animals relative to the non-diabetic control animals.

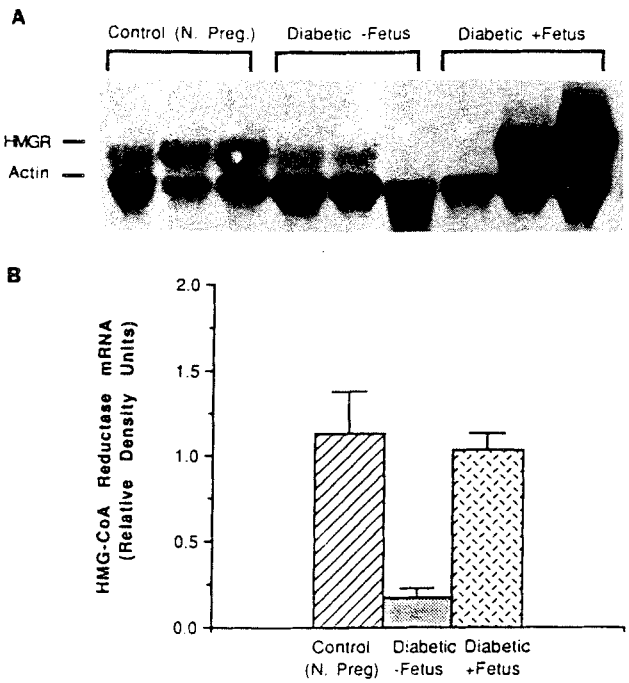

Figure 4 Northern blot and densitometric analyses of liver HMG-CoA Reductase (HMGR) and β-actin mRNA levels in normal pregnant (control) and STZ-treated diabetic rats which maintained their pregnancy and rats which did not. (**A**) indicates the Northern blot for HMGR using hepatic mRNA from control and diabetic animals. Ten μg of poly(A)$^+$ RNA was loaded per lane. HMGR and β-actin cDNAs were added simultaneously to the hybridization reaction. (**B**) indicates the densitometric analysis of HMGR mRNA levels in normal and diabetic rats. Any difference in RNA loading was corrected for based on the β-actin mRNA signal. HMGR was significantly ($P<0.018$) reduced in the diabetic animals which did not maintain pregnancy. The steady-state HMGR mRNA level in the diabetic animals with fetuses was similar to the HMGR mRNA level in the normal pregnant rat

Discussion

High dose STZ-treatment of pregnant rats has been used by numerous investigators as a model that simulates severe diabetes in pregnancy (Pitkin & van Orden, 1974; Aerts & van Assche, 1977; Cuezva *et al.*, 1982). Although the STZ model does not completely mimic severe diabetes in the human, similar reductions in fetal growth are observed. The data presented in this study show for the first time that fetal loss in the diabetic pregnant rat is consistently associated with a significant disruption in maternal cholesterol metabolism. Pregnancy loss was associated with a significant decline in maternal LDLR and HMGR mRNA levels as well as SCP2 protein levels. In contrast, hepatic LDLR and HMGR mRNA levels were normal in diabetic females which maintained their pregnancy. Furthermore, the results of this investigation indicate that the diabetic alteration in maternal cholesterol metabolism was associated with a significant reduction in hepatic SCP2 cholesterol transport capacity, while a related sterol carrier protein, SCPx, increased significantly. The decline in hepatic cholesterol 7α-

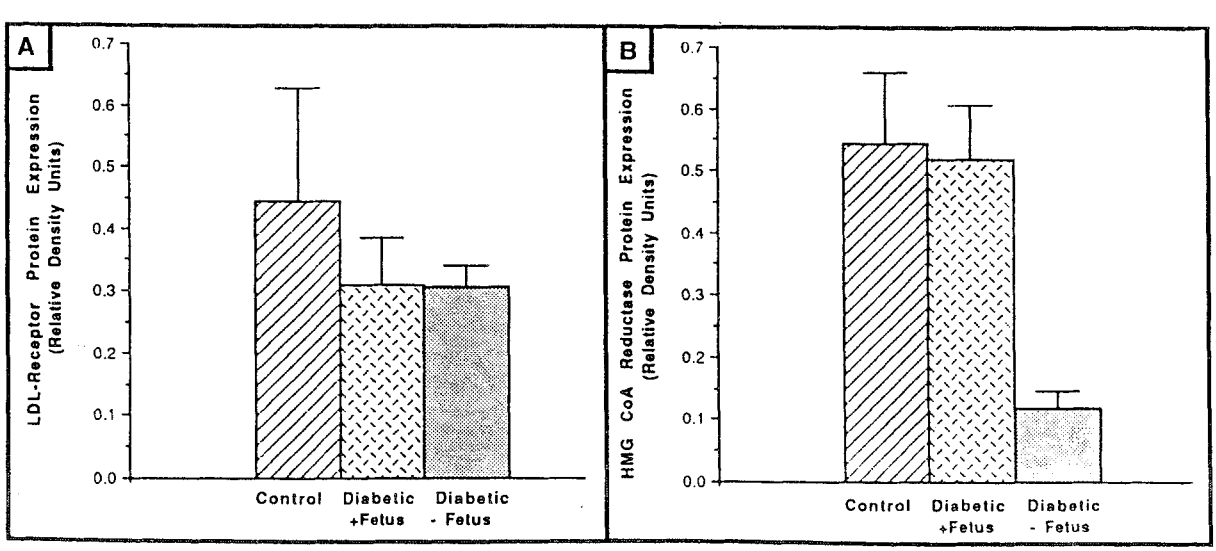

Figure 5 Densitometric analysis of LDLR and HMGR protein levels in normal pregnant and diabetic pregnant rats. (**A**) indicates the LDLR protein levels were reduced in the diabetic animals. (**B**) shows the HMGR protein levels in hepatic tissue of control and diabetic animals. HMGR levels were significantly ($P<0.05$) reduced in the diabetic animals which did not maintain pregnancy, whereas the HMGR protein levels in the diabetic group which maintained pregnancy were near control values

hydroxylase mRNA in the diabetic rats may also contribute to the alteration in maternal cholesterol levels, but the decrease did not appear to be an underlying determinant in fetal survival in this study.

In our study STZ was given 2 days prior to the time of blastocyst implantation which typically occurs on day 5 of pregnancy (Eriksson *et al.*, 1989). The timing of this injection avoided direct STZ toxicity to the fetus since implantation had not occurred. Implantation sites were observed in all diabetic animals confirming that STZ did not alter embryo implantation. Furthermore, direct embryo toxicity is doubtful, since STZ has a half-life of 5 min in the rodent (Schein & Loftus, 1968) and would most likely be cleared or degraded prior to blastocyst implantation.

Our results are consistent with the suggestion by Atkins *et al.* (1994) that factors other than altered glucose availability may limit fetal growth or determine reproductive outcome in the STZ model. Although fetal growth retardation and demise have been linked to acidosis in severe maternal diabetes (Chartrel *et al.*, 1990), this condition does not appear to be the cause of fetal loss by the animals in this study. In contrast to the implications put forth in other studies (Eriksson & Borg, 1991; Horton & Sadler, 1983; Sadler *et al.*, 1989), elevated serum glucose and β-HBA levels were not significantly different in the pregnant diabetic rats +/− fetuses, and thus glucose and β-HBA levels do not appear to be the underlying determinants for altered pregnancy outcome in the STZ-diabetic rat. This finding suggests that another factor(s) may determine reproductive competence in the diabetic animals.

The liver plays an important role in serum LDL-cholesterol removal (Brown & Goldstein, 1986; Bilheimer *et al.*, 1984); therefore, a reduction in LDL-receptor expression in diabetes (Lopes-Virella *et al.*, 1982), in association with an alteration in SCP2 expression, may act coordinately to reduce cholesterol processing. In those animals which were unable to maintain their pregnancy, striking decreases in hepatic LDL-receptor and HMG-CoA reductase gene expression were observed. This pattern of expression is similar to that seen in male diabetic rats (Ness *et al.*, 1994c). Given the suggested role that SCP2 plays in cholesterol biosynthesis (Vahouny *et al.*, 1983), storage (Gavey *et al.*, 1981), and metabolism (Seltman *et al.*, 1985), a decline in SCP2 expression would be expected to have a significant impact on the regulatory mechanisms which control intracellular sterol balance in the liver. It appears that a decrease in hepatic lipoprotein removal in insulin-deficient diabetes intensifies with the duration of insulin deficiency (Reaven & Reaven, 1974). An important and novel finding of our study is the fact that the reduction in the hepatic SCP2 protein level was clearly associated with a rise in the level of serum cholesterol. Our previous study using male diabetic animals demonstrated that the decline in SCP2 was a gradual process following diabetes onset (McLean *et al.*, 1995b). These studies also

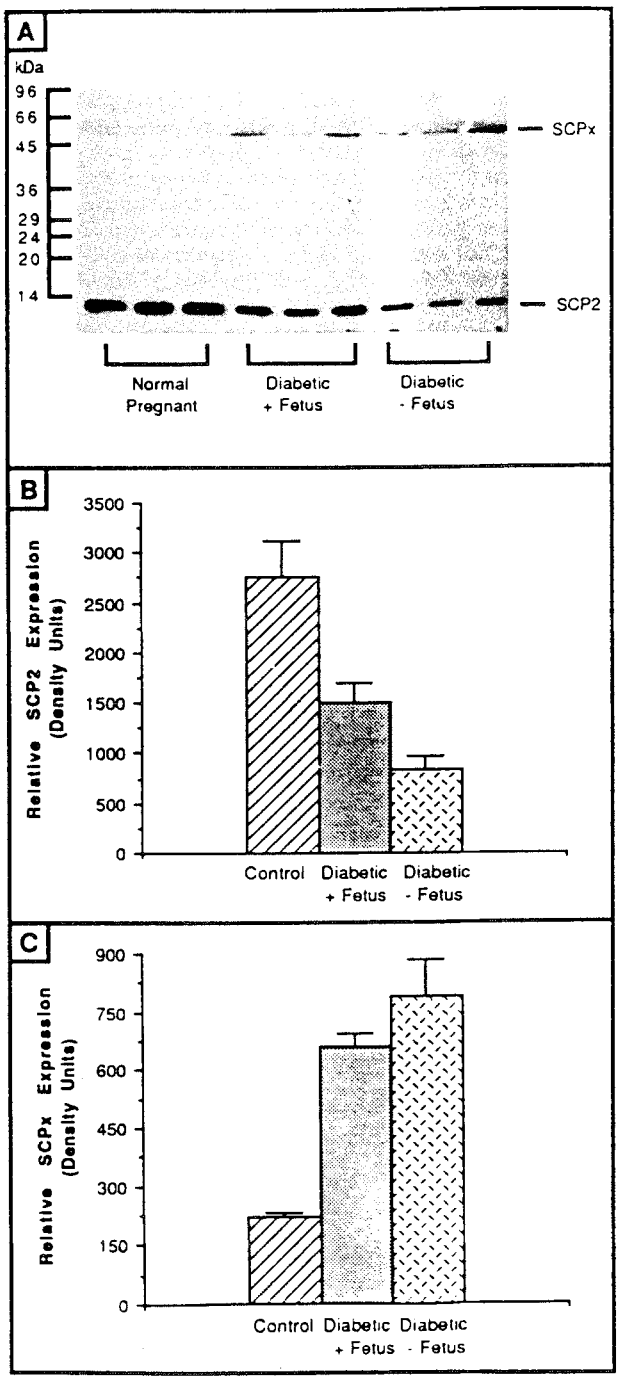

Figure 6 SCP2 Western blot and densitometric analyses of hepatic tissue from normal pregnant and diabetic rats +/− fetuses. (**A**) indicates a Western blot for SCP2. Fifty μg of liver homogenate were loaded per lane and SCP2 protein levels were determined using a polyclonal antibody to rat SCP2. The protein-antisera complex was visualized by autoradiography using ^{125}I-labeled protein A. (**B**) indicates the densitometric analysis of SCP2 protein levels in normal and diabetic rats. Equal protein loading was ascertained prior to immunoblotting. SCP2 protein levels were significantly ($P<0.05$) reduced in the diabetic animals. The largest decrease was observed in the animals which did not maintain pregnancy. (**C**) indicates the densitometric analysis of SCPx in control and diabetic animals

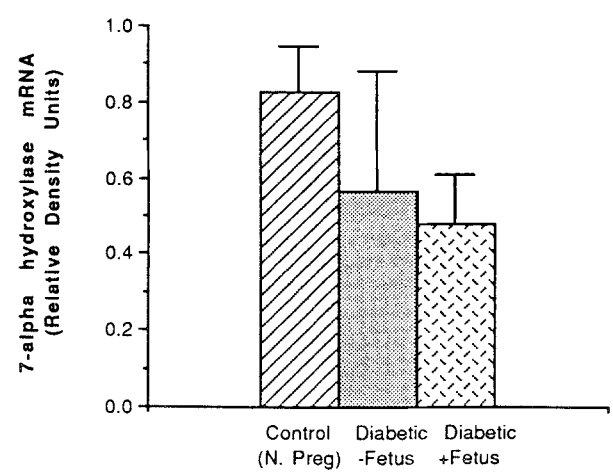

Figure 7 Densitometric analysis of hepatic cholesterol 7α-hydroxylase mRNA levels in normal and diabetic rats. Any difference in RNA loading was corrected for based on the β-actin mRNA signal (Figure 4). Cholesterol 7α-hydroxylase mRNA levels were reduced in all diabetic animals

demonstrated that the increase in SCPx preceded the decline in SCP2 which occurred 4 to 5 days after SCPx levels were significantly increased. Recently, SCPx has been reported to have peroxisomal 3-oxoacyl coenzyme A thiolase activity (Seedorf *et al.*, 1994), suggesting that it may function in the β-oxidation of fatty acids. An increase in SCPx in diabetes would be consistent with an increase in fatty acid β-oxidation which is known to be enhanced in this disease (Dunn, 1992). The increase in SCPx following diabetes onset is consistent with the enhanced expression of other hepatic peroxisomal enzymes following their induction by the peroxisome proliferator, clofibrate (Kaikaus *et al.*, 1993). A role for SCPx in peroxisomal lipid metabolism is further supported by the finding that rats treated with clofibrate, a drug which stimulates peroxisomal β-oxidative enzymes, have been reported to have increased SCPx protein and mRNA levels (Fujiki *et al.*, 1989). Diabetic animals have higher rates of lipolysis and increased free fatty acid levels (Howard, 1987; Dunn, 1992). The increase in lipolysis may provide the fatty acid substrate which promotes the expression of enzymes with fatty acid oxidative capacity, such as SCPx.

The steady-state concentration of LDL cholesterol in the plasma is determined primarily by the lipoprotein production rate and rate of LDL removal from the circulation by receptor-dependent transport of LDL cholesterol. The magnitude of the two processes was shown to be affected by the type of fatty acids in the diet (Woollett & Dietschy, 1994). The fibrate drugs used to reduce serum fatty acid levels are known to enhance peroxisome proliferation. These drugs do so by stimulating peroxisome proliferator activated receptors (PPARs) (Green, 1992). PPARs are also modulated by fatty acids, which may be the endogenous ligands for these receptors (Gottlicher *et al.*, 1992). Since SCPx is increased and SCP2 is decreased when exposed to the peroxisome proliferator clofibrate, it is possible that fatty acids may also regulate the expression of these proteins. In this regard, Ohba *et al.*, 1994) have recently reported that the human SCP2/SCPx gene contains an imperfect DR1 element which may provide a potential binding region for PPARs (Green *et al.*, 1993; Mukherjee *et al.*, 1994). Given the fact that fatty acids may be the endogenous ligands for the PPAR, their action on PPARs may provide a mechanism for the altered SCP2/SCPx expression noted in this study. A mechanism such as this would also support the hypothesis that some fatty acids and triacylglycerols alter the distribution of excess intracellular cholesterol (Daumerie *et al.*, 1992; Spady & Dietschy, 1985).

In general the pregnant diabetic rat had somewhat elevated VLDL + LDL serum cholesterol levels, whereas those animals that lost their fetuses had serum VLDL + LDL cholesterol levels which were elevated significantly ($P < 0.04$). The marked elevation in serum cholesterol was consistently associated with fetal loss. The likely source of this cholesterol is from the mobilization of adipose cholesterol esters via activation of hormone sensitive lipase since hepatic HMG-CoA reductase is reduced and the animals are on an essentially cholesterol free diet. Despite the elevated serum VLDL + LDL cholesterol levels, the availability of cholesterol to support steroid hormone production may have been limited. Insulin is thought to increase VLDL-receptor gene expression and uptake of VLDL by the placenta (Desoye *et al.*, 1987). Since placental cells utilize VLDL to provide cholesterol substrate, the reduced capacity of the placental cells to take up VLDL, in the absence of insulin, would contribute to a rise in serum cholesterol, as well as, fetal demise. In the animals without fetuses, VLDL levels increased significantly suggesting the possibility that the loss of placental VLDL uptake may contribute to the rise in serum cholesterol levels. We must, however, acknowledge that non-pregnant (diabetic) rats were compared to pregnant (control and diabetic) rats and the differences in various indices of cholesterol metabolism described in this investigation may have been influenced by this fact.

The fetoplacental unit is also important for steroidogenesis and the fetus itself is essential for steroid metabolism in pregnancy. The loss of this tissue would clearly alter maternal steroid levels. In addition to the change in placental cholesterol uptake, the alteration in ovarian progesterone production noted in this study, would also have profound effects on fetal development since this hormone is essential for the maintenance of pregnancy in the rat (Gibori & Keyes, 1978). Since the rat ovary appears to utilize high density lipoproteins (HDL) as a preferred cholesterol source for steroid production (Khan *et al.*, 1985), the diabetes associated increase in VLDL which coincides with the marked decline in hepatic HDL production noted in this and other diabetic rat studies (Howard, 1987) may limit ovarian (corpus luteum) steroid production. Although the corpus luteum can synthesize cholesterol *de novo* (Azhar *et al.*, 1985), the magnitude of the lipoprotein imbalance in diabetes with the significant decrease in HDL-derived cholesterol may exceed the synthetic capacity of the ovary to generate endogenous cholesterol. Also, since the placenta relies on the ovary for an aromatizable androgen substrate for placental estradiol production (Sridaran *et al.*, 1981), this tissue would also be unable to maintain steroid production in the absence of the ovarian synthesized androgen during early pregnancy (Durkee *et al.*, 1992).

Alterations in fatty acid levels (Kahn *et al.*, 1991) or the decline in available HDL derived cholesterol may directly contribute to fetal growth retardation or fetal loss in STZ-induced diabetes during pregnancy. The alteration in maternal cholesterol homeostasis with the decline in serum HDL and reduced placental VLDL uptake, may directly impair fetal development. Our study suggests that the magnitude of the alteration in maternal cholesterol homeostasis is directly associated with the diabetic animals' ability to maintain pregnancy. These results suggest that the maintenance of maternal cholesterol metabolism may be a critical factor directly associated with successful pregnancy outcome in the diabetic rat.

Material and methods

Chemicals, antisera and cDNA probes

Phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA), ethylene glycolbis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), bovine serum albumin (BSA; fraction V) and streptozotocin were purchased from Sigma Chemical Co. (St. Louis, MO). [^{125}I]Protein A (specific activity = 47.2 mCi/mg) was obtained from ICN Biochemicals (Irvine, CA). [α^{32}P]dCTP (3000 Ci/mmol) was from Amersham (Arlington Heights, IL) and [1,2,6,7-^{3}H(N)]progesterone (104.1 Ci/mmol) was from DuPont-New England Nuclear (Wilmington, DE). Acrylamide was obtained from Schwartz/Mann Biotec (Cleveland, OH) and XAR-5 film was purchased from Eastman Kodak (Rochester, NY). Nitrocellulose was obtained from Schleicher & Schuell (Keene, NH) and SeaKem agarose was obtained from the FMC Corporation (Rockland, ME). TRI Reagent and Formazol were obtained from Molecular Research Center Inc. (Cincinnati, OH). The nick translation kit was obtained from Boehringer Mannheim (Indianapolis, IN). All other chemicals were reagent grade from Fisher Scientific (Orlando, FL). The SCP2 specific polyclonal antisera to rat SCP2 was a gift from Dr Jeffrey T. Billheimer (DuPont/Merck Pharmaceuticals, Wilmington, DE) (Tanaka *et al.*, 1984). The preparation and charcterization of the rabbit polyclonal antisera to the LDLR (Ness & Zhao, 1994) and HMGR (Ness *et al.*, 1994b) were reported previously. The cDNA probe for human β-actin, HHCI89, was purchased from American Type Culture Collection (Rockville, MD). A cDNA probe for rat liver HMG-CoA reductase was prepared by PCR as described previously (Ness *et al.*, 1994b).

The probe for rat liver cholesterol 7α-hydroxylase, 7α6 (Li *et al.*, 1990), was a gift from Dr J.Y.L. Chiang (Northeastern Ohio University, Rootstown, OH) and the rat LDL-receptor cDNA (Lee *et al.*, 1989) was a gift from Dr R.D. Tanaka (Bristol-Meyers Squibb, Princeton, NJ).

Animals

Pregnant Sprague-Dawley rats (200 *g*) were purchased from Harlan Industries (Madison, WI). All procedures for STZ injection and tissue and blood sampling were approved by the University of South Florida Animal Care Committee. Rats were made diabetic by intravenous injection (jugular puncture) of 60 mg/kg streptozotocin (STZ) dissolved in 20 mM sodium acetate (pH 4.5)/0.15 M NaCl (50 μl volume) on day 3 of pregnancy. Control rats were also injected on day 3 of pregnancy but with an intravenous injection of 20 mM sodium acetate (pH 4.5)/0.15 M NaCl (vehicle injection). The study lasted 12 days during which all animals had free access to food and water and were on a 12 h dark and 12 h light cycle. A serum sample was obtained prior to the STZ or vehicle injection using jugular puncture. Pregnancy was confirmed in all animals based on an elevation in serum progesterone on day 3 of pregnancy (jugular puncture). Non-pregnant animals were not included in this study.

Twelve days following STZ or vehicle injection (day 15 of pregnancy) rat livers were removed (between the hours of 9 and 11 am) with the animal under ether anesthesia and immediately frozen in liquid nitrogen. Serum samples were obtained by cardiac puncture at the time of tissue removal and serum was stored at −20°C until it was assayed. Rats were euthanized by clipping the diaphragm while under anesthesia.

Serum glucose total cholesterol, HDL & VLDL + LDL cholesterol, β-hydroxybutyrate and progesterone assays

Serum glucose was determined by means of the glucose oxidase oxygen consumption method using a Beckman Glucose Analyzer 2 (Raabo & Terkildsen, 1960) as described previously (McLean *et al.*, 1995a).

Total serum cholesterol and fractionated lipoprotein cholesterol were determined using a Cholesterol 50 Assay from Sigma Chemical (St Louis, MO) as described previously (McLean *et al.*, 1995a). HDL cholesterol was determined following precipitation of the HDL fraction using phosphotungstic acid (30.3 mmol/L) and magnesium chloride (100 mmol/L) according to the method of Marz & Gross (1986). The cholesterol associated with the VLDL + LDL fraction was measured in the supernatant following HDL precipitation.

Serum β-hydroxybutyrate (β-HBA) was measured using a β-HBA Assay from Sigma Chemical (St. Louis, MO) which quantitatively measures β-HBA in serum using a spectrophotometer at 340 nm (Williamson *et al.*, 1962).

Progesterone was measured by RIA using [1,2,6,7-^{3}H]progesterone. This assay followed the methods described previously (McLean *et al.*, 1989) and used the progesterone antibody GDN 337 kindly provided by Dr G.D. Niswender (Colorado State University, Fort Collins, CO). The specificity, validity, and reliability of this RIA have been reported previously (Gibori *et al.*, 1984).

RNA isolation and northern blot analysis

RNA was prepared from liver using a modification of the Chomczynski and Sacchi method (Chomczymski & Sacchi, 1987) (TRI Reagent Method: Molecular Research Center, Cincinnati, OH). This method consistently yields 5–10 μg RNA/mg tissue. Frozen tissue (<200 mg) was homogenized in 4 ml of TRI Reagent with a polytron homogenizer and centrifuged at 12 000 *g* for 15 min at 4°C. Poly(A)$^+$ RNA was selected using an oligo (dT)-cellulose column as previously described (Aviv & Leder, 1972) Poly(A)$^+$-enriched RNA was precipitated from the aqueous phase with isopropanol and the RNA pellet washed in 75% ethanol and resuspended in diethyl pyrocarbonate-treated water or Formazol (Molecular Research Center, Inc, Cincinnati, OH). RNA was quantified by absorbance at 260 nm in a Beckman DU-70 spectrophotometer (Palo Alto, CA). Poly(A)$^+$ RNA (10 μg) was denatured at 65°C (15 min) and electrophoresed in 1% agarose gels containing 0.02 M borate, pH 8.3, 0.2 mM EDTA and 3% formaldehyde. The separated RNAs were transferred to GeneScreen *Plus* membranes by capillary blotting and the RNA was fixed by baking under vacuum for 2 h at 80°C. The cDNA probes were labeled with [^{32}P]dCTP to specific activities ranging from 2×10^7 to 4×10^9 c.p.m./μg by nick translation. The hybridizations were carried out as previously described (Ness & Zhao, 1994; Ness *et al.*, 1991, 1994c,d). Typically 5 ml of hybridization solution containing $2-4 \times 10^8$ c.p.m. and 1–2 μg of ^{32}P-labeled cDNA probe were incubated in 72 × 215 mm glass tubes. Washing conditions were as described previously (Ness & Zhao, 1994; Ness *et al.*, 1991, 1994c,d). The washed membranes were exposed to Kodak XAR-5 film with Cronex intensifying screens at −70°C for 4–16 h. The autoradiograms were scanned with an LKB ultrascan laser densitometer to determine relative mRNA levels. The value for the actin mRNA transcript was used as an internal control. All values are presented graphically as means ± SEM. Membranes were stripped using boiling 0.01% sodium dodecyl sulfate (SDS) in 0.15 mM sodium citrate and 1.5 mM sodium chloride, pH 7.0 and then reprobed.

Tissue SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransfer

Hepatic tissue (150 mg) obtained from control and STZ diabetic animals were homogenized in a 1.5 ml ice cold solution of 50 mM Tris-HCl buffer (pH 7.4) containing 250 mM sucrose, 2 mM EDTA, 1 mM PMSF, 1 mM DTT, 10.5 μM leupeptin and 10 μM pepstatin A (homogenizing buffer).

Liver homogenates were assayed for protein concentration by the method of Bradford (1976) using bovine serum albumin as the standard. Hepatic proteins (50 μg protein) were denatured at 100°C in loading buffer and subjected to electrophoresis on 7.5–18% gradient SDS–polyacrylamide gels according to the method of Laemmli (1970) as described previously (McLean *et al.*, 1995a). After electrophoresis, samples were electroblotted onto nitrocellulose (0.2 μm pore) in buffer containing 20% methanol, 20 mM Tris-base (pH 8.3), and 150 mM glycine for 16 h at 4°C as described previously (McLean *et al.*, 1989). To verify equal protein loading, nitrocellulose sheets were stained with 0.01% fast green (in 20% methanol and 7% acetic acid) and destained in the same solution without fast green.

SCP2, LDL-receptor and HMG-CoA reductase western blot analysis

Hepatic SCP2 content was estimated by incubating transferred proteins in a 20 mM Tris-base buffered (pH 7.5) sodium chloride (500 mM) solution (TB-NaCl) with 3% nonfat dry milk for 1 h at room temperature (RT). Buffer was replaced with TB-NaCl containing a specific rabbit polyclonal antibody to SCP2 (Tanaka *et al.*, 1984) (1:2000 dilution) in 3% milk for 16 h at 4°C (McLean *et al.*, 1995a). Nitrocellulose blots were washed in TB-NaCl containing 0.01% Tween-20 and then incubated in TB-NaCl containing 3% milk and ^{125}I-labeled protein-A (200 000 c.p.m./ml) for 1 h at RT. Differences in the antisera-protein A band density were quantified on autoradiograms densitometrically with a Hoefer scanning densitometer (Hoefer Instruments, San Francisco, CA) for statistical analysis (Zar, 1974).

Hepatic LDLR and HMGR protein content were estimated using specific antisera diluted 1:1500 and 1:500,

respectively, as described previously (Ness & Zhao, 1994; Ness *et al.*, 1994b). The immunoreactive bands were identified using the enhanced chemiluminescent method (Isacsson & Wettermark, 1974). Differences in immunoreactive band density were quantified on XAR-5 film densitometrically with an LKB ultra scan laser densitometer.

Data analysis

Northern and Western blots were quantitatively analysed using densitometry. All scan data were expressed in arbitrary density units. Correction for protein loading was not required since equal protein staining was required prior to immunoblot analysis. Minor variations in RNA loading were corrected for using a β-actin cDNA probe as an internal marker. Densitometric data were compared by analysis of variance followed by Student-Newman-Keuls multiple comparison test when applicable (Zar, 1974). All analysis was completed using the Statview program (Abacus Concepts, Berkeley, CA) on a Macintosh IIci computer. A $P<0.05$ value was considered significant for all tests.

Acknowledgements
This work was supported by an American Health Assistance Foundation (National Heart Foundation) Research Award to MPM and by NIH grant HL 18094 to GCN.

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