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Reduced hepatic sterol carrier protein-2 expression in the streptozotocin treated diabetic rat

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While a strong relationship between the hypercholesterolemia of diabetes and premature atherosclerosis is established, the etiology for the elevation in serum cholesterol in this disease is unknown. To determine whether diabetic hypercholesterolemia may be related to alterations in hepatic cholesterol transport capacity, sterol carrier protein-2 (SCP2) expression was examined in rats treated with streptozotocin (SZT). Furthermore, this study examined whether 17β-estradiol and insulin confer a protective effect on liver cholesterol homeostasis by maintaining hepatic SCP2 levels. SCP2 protein and mRNA expression were examined 13 days following SZT-induced diabetes onset and in diabetic rats treated with estradiol (1 cm silastic implant) or insulin (12 units/day). Data indicate that SCP2 protein levels were significantly reduced in the diabetic animals and that SCP2 protein expression in the liver was inversely related to the level of serum cholesterol in the diabetic animals. In contrast, SCP2 mRNA levels examined by slot blot, ribonuclease protection assay, and Northern blot analysis were significantly elevated. Both insulin and estradiol were able to enhance the expression of SCP2 protein in the liver following SZT treatment. The results of this investigation clearly indicate that hepatic SCP2 protein levels are significantly altered in the diabetic state suggesting that cholesterol transport capacity is reduced in the SZT-treated diabetic rat. The inverse relationship between serum cholesterol and hepatic SCP2 protein content suggests that the reduction in this protein may be a contributing factor in diabetic hypercholesterolemia.

Keywords: SCP2; cholesterol; diabetic rat; live; streptozotocin

Introduction

The hypercholesterolemia associated with diabetes mellitus is thought to be an underlying factor for accelerated atherogenesis in this disease (Barrett-Connor & Orchard, 1985; Tomkin & Owens, 1991). Despite major advances in clinical therapy, coronary heart disease and peripheral vascular disease remain the major causes of death among patients with diabetes mellitus. Although hyperphagia is reported to be a significant factor in regulating cholesterogenesis and diabetic hypercholesterolemia (Young *et al.*, 1983), recent studies indicate that other factors are also important in the regulation of cholesterol metabolism in diabetes (Jiao *et al.*, 1988). One potential intracellular element which is directly involved with cell cholesterol metabolism and which may be altered during diabetes is the intracellular cholesterol transport protein, sterol carrier protein-2 (SCP2).

Hepatic cholesterol synthesis and LDL-uptake generate significant amounts of cholesterol in the cytosolic, peroxisomal, and lysosomal compartments. Since cholesterol is insoluble in the cell, it is thought to be associated with a protein which facilitates its transport between these compartments for further processing. SCP2 is a 13.2 kDa protein which has been implicated in intracellular cholesterol trafficking. 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.*, 1983). SCP2 is also an activator of the cholesterol 7α-hydroxylase enzyme involved in bile acid synthesis (Seltman *et al.*, 1985). Since the liver plays an important role in regulating circulating LDL concentrations via the LDL-receptor pathway (Garg, 1992), reduced processing of cholesterol following LDL internalization due to altered SCP2 expression may thereby affect serum cholesterol levels. Although diabetes related hyperlipidemia and altered hepatic cholesterol metabolism have been extensively studied (Tomkin & Owens, 1991), intracellular cholesterol movement during hyperglycemic states has not been examined.

While feedback regulation of LDL-receptor gene expression by cholesterol has been closely examined (Goldstein & Brown, 1990; Smith *et al.*, 1990), less information exists concerning the regulation of hepatic LDL-receptor and SCP2 expression by hormones. It has been reported that thyroid hormone (Ness & Zhao, 1994) and ethynylestradiol caused an increase in hepatic LDL-receptor activity and mRNA levels (Kovanen *et al.*, 1979; Rudling *et al.*, 1992). Furthermore, the administration of growth hormone was found to enhance the stimulation of hepatic LDL receptor mRNA by estrogen (Rudling *et al.*, 1992). Estradiol's beneficial influence to reduce hyperglycemia and glycosuria in diabetes has also been demonstrated (Goodman & Hazelwood, 1974; Paik *et al.*, 1982), but little is known regarding the effect of this steroid on hepatic cholesterol transport capacity. Estradiol's anti-diabetic action can alter the course of experimental diabetes (Paik *et al.*, 1982) and reduce cholesterol synthesis by suppressing hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in the rat (Mukherjee & Bhose, 1968). No information is available, however, regarding the effect of estradiol administration on SCP2 expression in the liver.

Because of the important role SCP2 may play in cellular cholesterol metabolism and movement, this study was undertaken to examine possible alterations in SCP2 expression in the streptozotocin (SZT)-treated rat liver and to determine whether altered SCP2 expression might be associated with diabetic hypercholesterolemia. This study also examined whether insulin and estradiol mediate hepatic SCP2 expression following diabetes onset.

Results

To demonstrate that the SZT-treated rat model mimics the physiological changes typical of a diabetic condition, average daily water (Figure 1A) and food consumption (Figure 1B) and serum glucose levels (Figure 1D) were monitored. All animals treated with SZT displayed symptoms of insulin-

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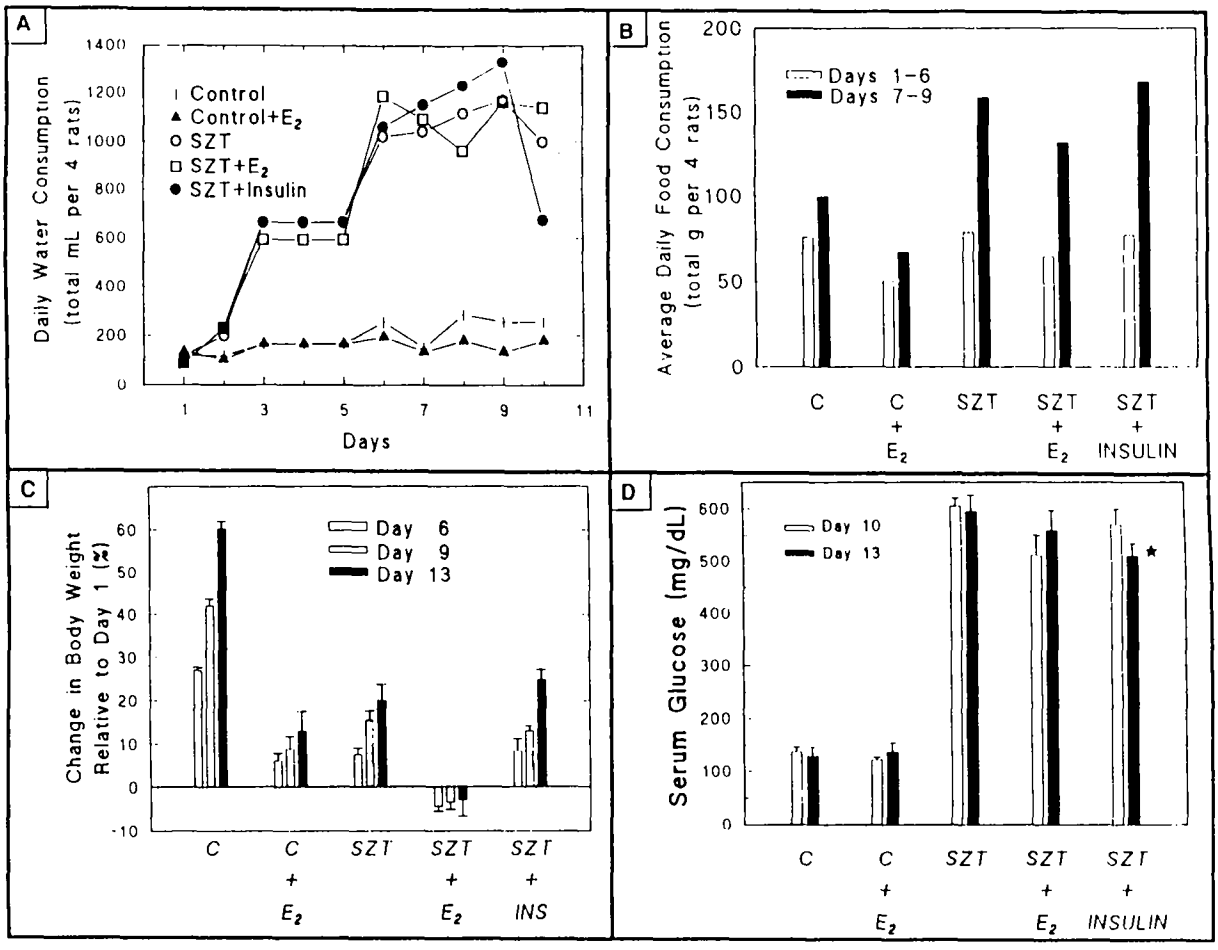

Figure 1 (**A**) Daily water consumption following vehicle (control) or SZT-injection. Data represents the total water consumed per 4 rats ($n = 4$/treatment). Rats were fed and received water *ad libitum* 24 h/day. (**B**) Average daily food consumption during the first 6 days of the experiment (▭) and on days 7–9 post-vehicle or SZT-injection (▨). Diabetic rats display hyperphagia during days 7–9 of the experiment. The SZT + insulin bars represent the food consumed prior to insulin injection and the data presented verifies that the animals to be treated with insulin were hyperphagic. (**C**) The percentage change in body weight expressed relative to the rat weights on day 1 of the experiment. Weights were examined on the days indicated. (**D**) Serum glucose levels in control and SZT-treated diabetic rats on days 10 and 13 of the experiment. As expected, serum glucose levels were significantly ($P<0.001$) elevated in the SZT-treated animals. Insulin treatment reduced glucose levels ($P<0.01$). The serum sample collected for the insulin treated group was taken prior to the insulin injection (8 am)

dependent diabetes, i.e. polyphagia, polydipsia, polyurea, and a reduced rate of body growth (Figure 1C). Estradiol treatment had no effect on water consumption but appeared to reduce food intake in both the non-diabetic and diabetic rats. As shown in Figure 1C, control rats displayed an increase in weight throughout the treatment period. In contrast, smaller weight gains were noted in the diabetic rats during the experiment. The estradiol treated control rats showed smaller weight gains than normal, while estradiol-treated diabetic rats displayed a decrease in total body weight. The reduced weight gain of the SZT-treated rats appears to be related to the diabetic condition, whereas the reduction in weight gain in the estradiol-treated control animals was due to reduced food consumption. The weight loss in the estradiol-treated diabetic rats was most likely due to the compounding effects of the diabetic condition and the reduction in food consumption by these animals (Figure 1B). Figure 1D shows the serum glucose levels in the control and diabetic animals. Glucose levels were significantly ($P<0.001$) elevated in the SZT treated rats, further demonstrating the efficacy of this chemical in promoting a diabetic state in this investigation. Estradiol administration provided little protection from hyperglycemia on days 10 or 13 of the acute diabetic study. Insulin treatment, as expected, reduced serum glucose levels in the diabetic rats. Within 48 h of insulin treatment, serum glucose levels in the diabetic rats, examined at mid day, were reduced to 99.3 + 25 mg/dL. Glucose levels were normalized following insulin administration in all diabetic animals examined in these experiments. The apparent lack of complete glucose normalization noted in Figure 1D is related to the time of insulin injection. If the blood sample is taken too soon following insulin administration, animals appear hypoglycemic. This was observed in our experiment when animals received insulin at 8 am and the serum glucose was measured 1 h later at the time of tissue removal. To avoid this, the serum glucose levels reported in Figure 1D were examined prior to the final insulin injection. When examined at this time point, glucose levels were significantly ($P<0.01$) lower in the diabetic rats treated with insulin, but not to the control glucose level.

Serum cholesterol levels (Figure 2) were significantly ($P<0.01$) elevated in the SZT-treated animals on days 10 and 13 in the acute diabetic experiment relative to age matched non-diabetic controls ($n = 4$/treatment). Insulin treatment reduced serum cholesterol to normal values. Estradiol appeared to have little effect on serum cholesterol levels in the SZT-treated rats.

To examine possible alterations in the hepatic tissue content of SCP2 following SZT-treatment, SCP2 Western blot analysis was performed (Figure 3). A 13.2 kDa band was detected in the liver samples from all treatment groups. A reduction in hepatic SCP2 protein content was consistently

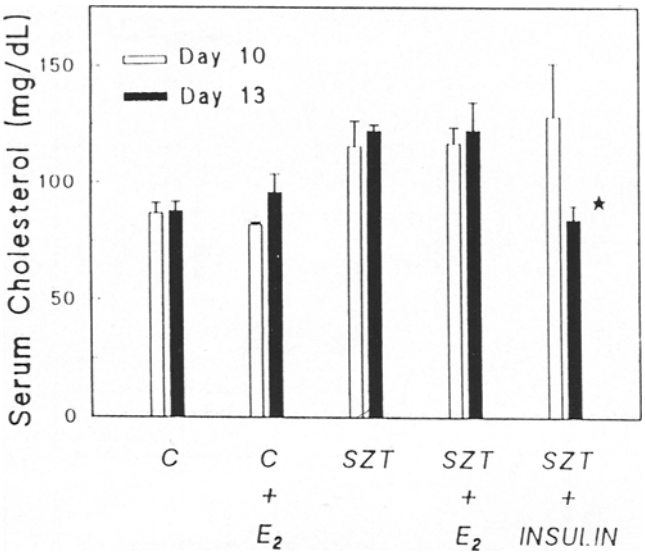

Figure 2 Serum cholesterol levels in the control (±estradiol) and SZT-treated diabetic rats. Serum cholesterol was examined on day 10 (□) and on day 13 (■) of the experiment. Serum cholesterol was significantly elevated in the SZT-treated animals on days 10 and 13 compared to the control animals. Insulin treatment significantly reduced serum cholesterol levels when given on days 9–12 of the experiment. The ★ indicates a $P<0.01$ relative to the SZT-treated animals

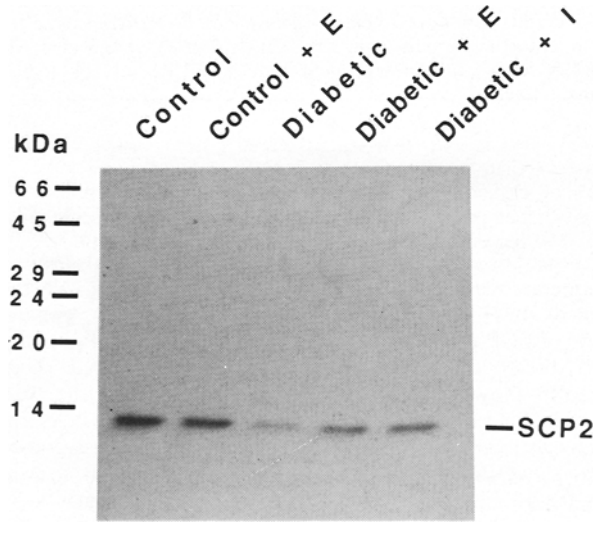

Figure 3 SCP2 Western blot analysis of control and SZT-treated (Diabetic) rats. Fifty micrograms of liver homogenate was loaded per lane. Equal loading was verified by fast green staining prior to immunoblot analysis. Hepatic SCP2 protein levels were determined using a polyclonal antiserum to rat SCP2 in a 2 h incubation. The protein-antisera complex was visualized by autoradiography using ^{125}I-labeled protein A. Blot exposure time was 12 h. Liver SCP2 was significantly reduced ($P<0.001$) in the diabetic rats

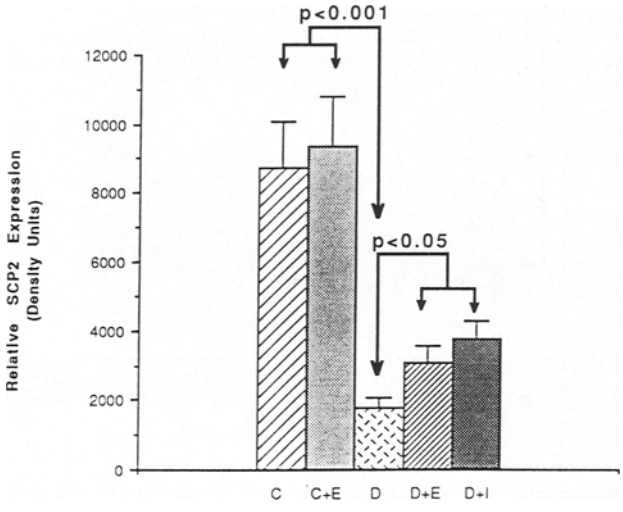

Figure 4 Densitometric analysis of hepatic SCP2 protein levels in control (**C**), control rats treated with estradiol (**C + E**). SZT-treated diabetic rats (**D**) and diabetic rats treated with estradiol (**D + E**) or insulin (**D + I**) ($n = 4$/treatment). Equal protein loading was ascertained prior to immunoblotting in each experiment. SCP2 protein levels were significantly ($P<0.001$) reduced in the diabetic rats. Estradiol and insulin were able to enhance significantly ($P<0.05$) SCP2 expression in diabetic rats, but not in control levels

associated with the diabetic state. Densitometric analysis of the SCP2 Western blots (four animals per treatment; acute diabetic study) indicates that SCP2 was reduced by 80% in the SZT-treated diabetic rats ($P<0.001$) (Figure 4). In this study estradiol treatment for 12 days and insulin treatment for 3 days were able to enhance significantly hepatic SCP2 levels in the diabetic animals relative to the non-hormone treated diabetic rats ($P<0.05$ each). SCP2 levels were maintained at 35 and 42% of the control levels in the SZT-injected animals treated with estradiol and insulin, respectively. These results indicate that both insulin and estradiol can influence liver SCP2 content in the SZT-treated diabetic rat model. Western blot results from a parallel study in which animals were examined following six days of insulin treatment (day 15-post SZT injection), indicate that insulin treatment will restore SCP2 protein levels (diabetic + insulin: 5612.5 ± 400 density units; $n = 4$) to the control level of SCP2 expression (non-diabetic: 5197.5 ± 437 density units; $n = 4$). Consistent with the animals examined on day 13 post-SZT, hepatic SCP2 levels in the 15 day diabetic animals were significantly reduced (diabetic: 1270.1 ± 283 density units; $n = 4$, $P<0.02$). In this experiment, the SCP2 protein levels in the diabetic rats treated with insulin were significantly ($P<0.01$) elevated relative to the SCP2 levels within the untreated diabetic animals. These data indicate that a period of six days of insulin treatment was required to fully restore the hepatic SCP2 protein content in the diabetic rat to control levels.

Although SCP2 expression was not completely eliminated in the acute SZT-treated diabetic animals, SCP2 protein levels were significantly reduced. Furthermore, the reduction in SCP2 was not due simply to the acute effects of SZT in these animals since SZT-treated animals in the long-term diabetic study also displayed significant ($P<0.002$) reductions in SCP2 protein levels (Figure 5). SCP2 Western blot densitometric results (Figure 6) from the long-term diabetic study confirm that the livers from diabetic rats have lower hepatic SCP2 content ($n = 5$) relative to the age matched control animals ($n = 3$).

In contrast to the reduction in hepatic SCP2 protein levels following SZT-treatment, SCP mRNA levels were significantly elevated. Slot blot analysis of hepatic SCP mRNA (Figure 7) indicates that diabetic rats had elevated SCP mRNA levels (2-fold increase) relative to the control animals. Densitometric analysis of the SCP mRNA levels from the slot blot experiments shown in Figure 7 indicates that the SCP mRNA levels were significantly ($P<0.01$) elevated in the SZT-treated rats. The elevation in SCP mRNA in the diabetic animals was confirmed using a ribonuclease protection assay (Figure 8). The SCP2 RPA analysis clearly indicates a significant ($P<0.01$) increase in the 429 bp SCP-specific mRNA fragment in the diabetic animals. Estradiol treatment alone had little effect on the SCP mRNA levels. SCP mRNA levels in the diabetic rats

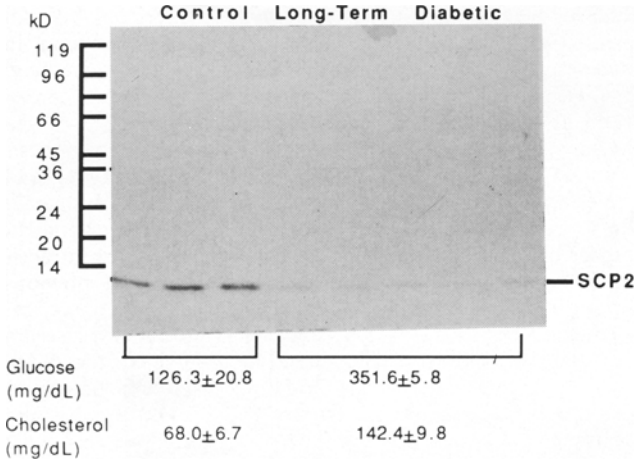

Figure 5 SCP2 Western blot analysis of control and long-term SZT-treated diabetic (16 weeks) rats. Proteins were analysed as in Figure 3. Corresponding serum glucose and cholesterol levels are indicated

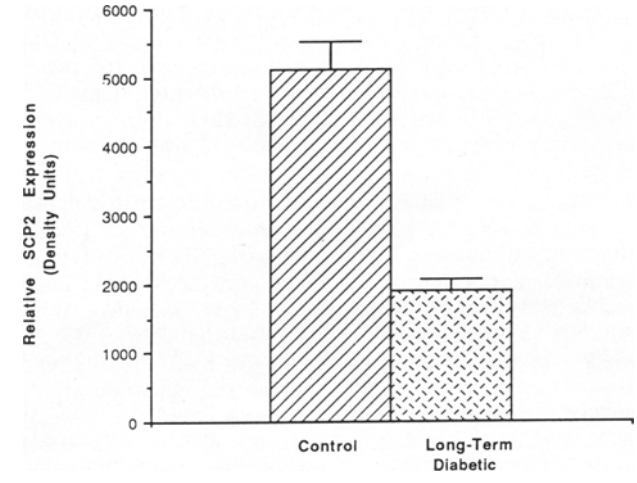

Figure 6 Densitometric analysis of hepatic protein levels in control and long-term diabetic animals. SCP2 protein levels were significantly ($P<0.02$) reduced in the diabetic animals. Serum cholesterol varied inversely with the level of hepatic SCP2 expressed in these animals

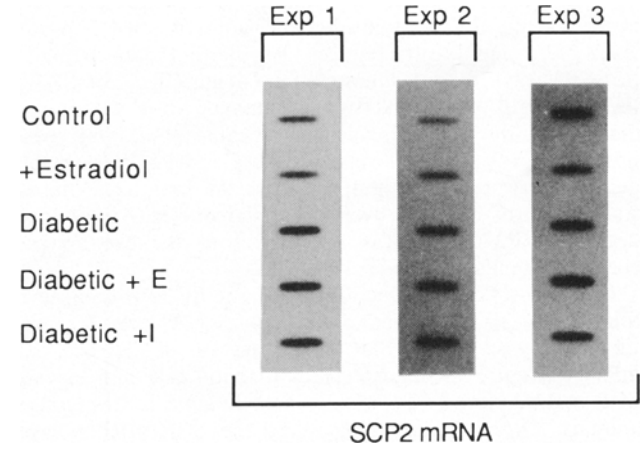

Figure 7 Determination of SCP2 mRNA in control and hormone treated rats. Total RNA (10 μg/well) was transferred onto a nylon membrane (0.45 μm pore) in a vacuum manifold at 7.5 cm Hg psi. RNA was uv crosslinked to the membrane which was then hybridized with a ^{32}P-labeled SCP2 cDNA probe. The SCP2 mRNA signal was identified by autoradiography. Each well represents a separate animal. This experiment was repeated three times as indicated. Equal RNA loading was verified by probing the membrane with a β-actin cDNA (not shown)

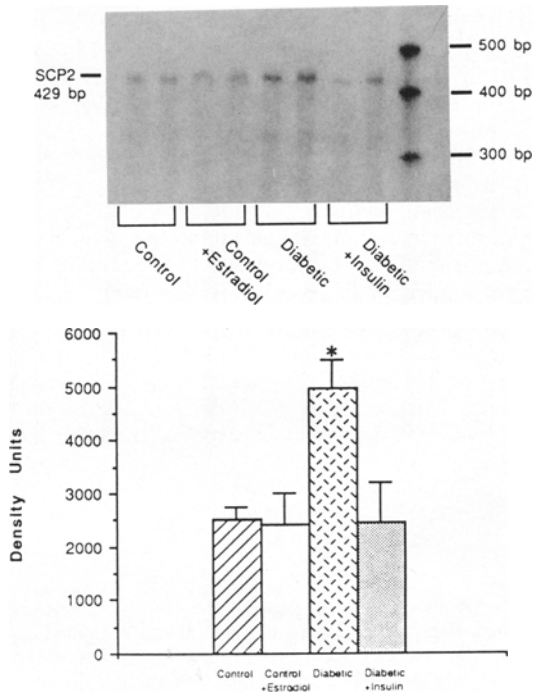

Figure 8 SCP2 ribonuclease protection assay of hepatic RNA isolated from control and SZT-diabetic rats. The top panel indicates the SCP2 specific 429 bp protected fragment identified following hybridization. RNA markers are indicated. The lower panel indicates the densitometric analysis of SCP2 mRNA levels in control and diabetic rats ($n = 4$/treatment) as determined by RPA. Hepatic tissue from diabetic animals had significantly ($P<0.01$) elevated SCP2 mRNA levels. Insulin treatment reduced SCP2 mRNA levels to control levels

treated with estradiol (data not shown) appear similar to the mRNA levels in the SZT-treated animals. In contrast, insulin treatment reduced the amount of the SCP mRNA protected fragment detected in this assay to control levels. Northern blot analysis was utilized to determine whether the enhanced level of SCP mRNA noted in the slot blot and RPA analysis was due to the increase in a specific SCP mRNA species. Results shown in Figure 9 indicate that both the SCP2-specific 0.8 kb and the 2.1 kb SCPx-specific mRNA transcripts were increased in the diabetic animals. Densitometric analysis of the 0.8 kb and 2.1 kb mRNA species is shown in Figure 10. The 2.1 kb mRNA was increased 360% in the diabetic rats ($P<0.008$) while the 0.8 kb mRNA was increased 42% ($n = 8$/treatment). While the increase in the 0.8 kb SCP2 mRNA transcript was consistent in these experiments, it was not significantly enhanced relative to the control animal transcript level. Although the SCP transcripts appear to change to different degrees in the control and diabetic animals, both SCPx and SCP2 mRNAs appear to increase simultaneously. The decline in hepatic SCP2 protein levels in the diabetic animals (Figures 3–6) must result from either altered SCP2 mRNA transcription, translation or from rapid post-translational catabolism of SCP2.

Discussion

While diabetic hypercholesterolemia and altered cholesterol metabolism have been associated with altered cholesterol synthesis (Young *et al.*, 1982a; Feingold *et al.*, 1984), LDL-uptake (Lopes-Virella *et al.*, 1982; Garg, 1992), and hyperphagia (Young *et al.*, 1982b, 1983; Feingold, 1989), little attention has been focused on the potential alterations in intracellular cholesterol transport as a contributing factor in altered diabetic cholesterol homeostasis. The results of the

present study establishes for the first time that hepatic SCP2 content is profoundly reduced in the SZT-treated diabetic rat model. This investigation further demonstrates that plasma cholesterol levels vary inversely with the level of hepatic SCP2 expression. The use of both acute and long-term SZT-diabetic animal models clearly indicate that the reduction in SCP2 is associated with the diabetic condition. Furthermore, these experiments demonstrate that the reduction in hepatic SCP2 in the acute study is not simply an artifact of SZT-injection nor is this observation due to selective SZT toxicity in the rat model. In the rodent, SZT is reported to have a biological half-life of 5 min (Schein & Loftus, 1968) and thus, the reduction in SCP2 in the long-term diabetic study cannot be attributed to the residual effect of SZT. Since SCP2 is believed to function in intracellular cholesterol trafficking and metabolism, a decline in the expression of this protein in diabetes may be a contributing factor in the alterations in cholesterol metabolism noted in this disease.

The liver plays an important role in serum LDL-cholesterol removal (Billheimer *et al.*, 1984; Brown & Goldstein, 1986). A reduction in LDL-receptor expression in diabetes (Lopes-Virella *et al.*, 1982; Garg, 1992) in association with an alteration in SCP2 expression may act synergistically to reduce cholesterol processing and clearance. Given the suggested role that SCP2 plays in cholesterol biosynthesis (Vahouny *et al.*, 1983), storage (Gavey *et al.*, 1981) and cholesterol 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. Consistent with previous reports of elevated cholesterol levels in the SZT-treated rat (Young *et al.*, 1982a, b; Feingold *et al.*, 1982, 1984), serum cholesterol levels were significantly elevated in the SZT-treated animals in the acute and long-term diabetic experiments relative to age matched non-diabetic controls. Insulin treatment reduced serum cholesterol to normal values while estradiol appeared to have little effect on serum cholesterol levels in the SZT-treated rats.

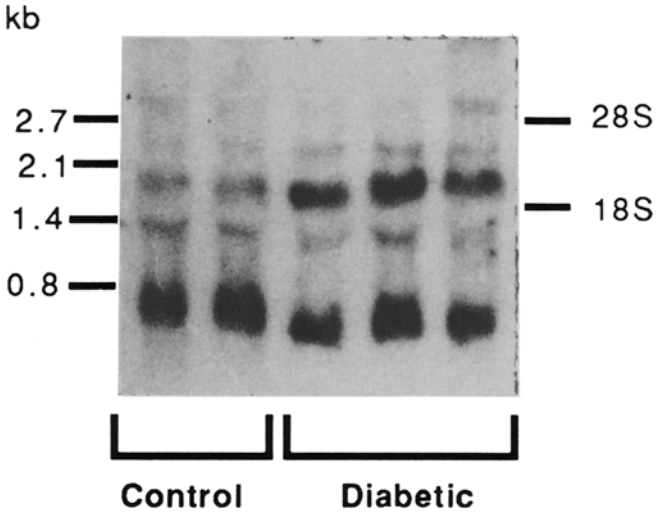

Figure 9 Total RNA (20 μg) was size fractionated, transferred to a nylon membrane and probed with a ^{32}P-labeled rat SCP2 cDNA probe. SCP2 mRNA levels in control and diabetic rats are indicated in this Northern blot. In contrast to the decrease in hepatic SCP2 protein content in the diabetic rats, SCP2 mRNA levels were elevated in the diabetic animals

In the study reported here, we confirmed earlier observations that experimental diabetes in the rat is associated with hyperphagia and hypercholesterolemia (Young *et al.*, 1982b, 1983; Feingold, 1989). Our results are also consistent with the findings of Jiao *et al.* (1988) which suggested that factors other than hyperphagia may control diabetic hypercholesterolemia. This concept is clearly demonstrated in the present study by the fact that estradiol-treated diabetic animals had elevated serum cholesterol levels while food consumption by this group was reduced compared to the non-hormone treated diabetic animals. This finding supports the possibility that the decline in intracellular cholesterol transport capacity coupled with a reduction in hepatic LDL uptake (Lopes-Virella *et al.*, 1982; Garg, 1992) may provide a significant regulatory mechanism, which, when altered by the hyperglycemic state, results in hypercholesterolemia. Interestingly, estradiol has been shown to increase hepatic LDL-receptor activity and mRNA levels (Kovanen *et al.*, 1979; Rudling *et al.*, 1992) and reduce 3-hydroxy-3-methylglutaryl coenzyme A reductase activity (Mukherjee & Bhose, 1968). Although the findings of these previous studies would suggest that estradiol should have a protective action to reduce serum cholesterol, the results of our investigation do not support this action of estradiol in the acute diabetic study. While our results indicate that estradiol can enhance hepatic SCP2 levels to a limited degree, the alteration in cholesterol metabolism associated with diabetes may exceed estradiol's limited protective action in the acute SZT-diabetic rat model. Our

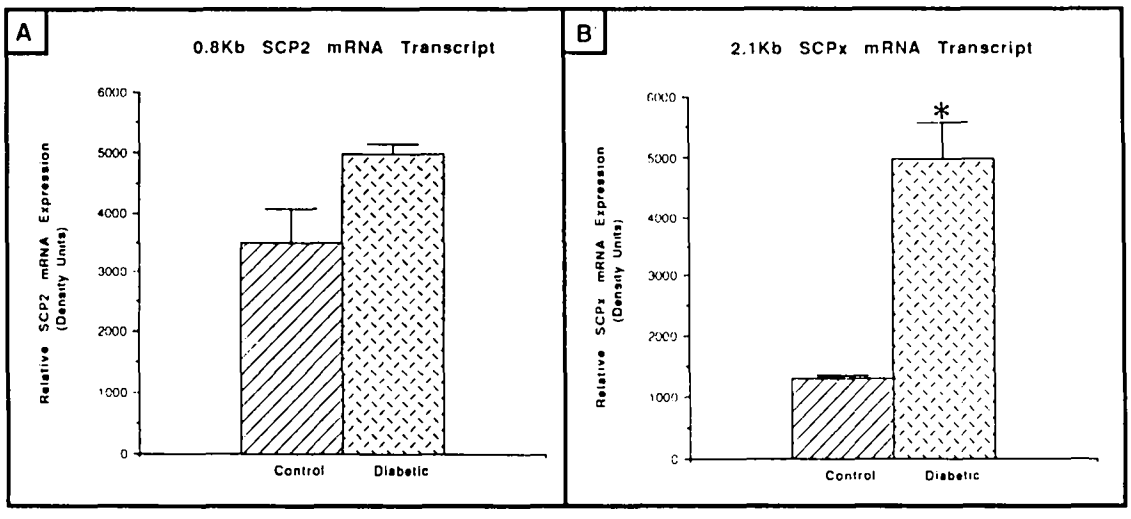

Figure 10 Densitometric summary of the major SCP mRNA transcripts in hepatic tissue from control and diabetic animals ($n = 8$/treatment). Results of this analysis indicate that diabetic animals had significant 3.75-fold increase in the 2.1 kb SCPx-specific mRNA transcript (**B**) and a 1.4-fold increase in the 0.8 kb SCP2-specific mRNA transcript (**A**) relative to the control animal mRNA levels (* = $P<0.008$)

results suggest that the estradiol mediated SCP2 increase cannot compensate for the increased very low density lipoprotein (VLDL) secretion which is associated with enhanced hepatic fatty acid synthesis in the diabetic state. The fact that insulin increased SCP2 protein levels in the liver is consistent with its role in maintaining cholesterol removal. Insulin appears to decrease fatty acid synthesis and VLDL secretion and enhance SCP2 levels in the liver as well as maintain hepatic LDL receptors (Mazzone *et al.*, 1984). Whether insulin acts directly to enhance SCP2 expression or whether this occurs indirectly via the action of insulin on some other aspect of cholesterol processing requires additional study.

Recent investigations revealed the existence of larger SCP2-related proteins at 30 and 58 kDa (Trzeciak *et al.*, 1987; van Amerongen *et al.*, 1987; Fujiki *et al.*, 1989; van Heusden *et al.*, 1990). These findings suggest that SCP2 belongs to a family of related proteins which have a common C-terminal region homologous with SCP2. Several studies have now indicated the existence of multiple SCP2 mRNA transcripts. (Billheimer *et al.*, 1990; Moncecchi *et al.*, 1991; Seedorf & Assmann, 1991). In the liver, four mRNA species of 0.8, 1.4, 2.1 and 2.7 have been detected. The 0.8 and 1.4 kb transcripts have been reported to encode the pro-SCP2 with a 20 amino acid prepeptide that is post-translationally removed to generate the mature SCP2 (Trzeciak *et al.*, 1987; Fujiki *et al.*, 1989). The 2.1 and 2.7 kb transcripts are thought to encode SCPx (Seedorf & Assmann, 1991). Ossendorp *et al.* (1991) and Seedorf & Assmann (1991) have demonstrated that the different mRNA species are generated from one gene, first by differential splicing and then by alternative polyadenylation. These transcripts appear to encode SCP2 and a related protein, sterol carrier protein-X (SCPx). Since the fragment used to detect SCP mRNA by RPA in our study interacts with the common C-terminus of all SCP transcripts, Northern blot analysis was utilized to determine whether the enhanced level of SCP mRNA noted in the slot blot and RPA analysis was due to the increase in a specific SCP mRNA species. The difference noted between slot blot and RPA analyzed SCP mRNA levels following insulin treatment most likely reflects differences in assay sensitivity. The RPA is a more sensitive means by which to assess the change in SCP mRNA levels compared to the slot blot assay. The observation in the present study that mRNA transcripts for both SCP2 and SCPx were enhanced in diabetes is intriguing. The fact that SCPx was not detected in the Western blot analysis in these experiments is most likely related to the brief (2 h) incubation with the SCP2 antisera. The reactivity of the antiserum to the SCP2 related proteins (ie. 30 and 58 kDa forms) is dependent on the antibody incubation period and antiserum incubation times greater than four hours appear to be required to detect SCPx when using [^{125}I]-protein A for antibody-protein detection (McLean *et al.*, 1989; unpublished observation). Differential regulation of SCP2 and SCPx mRNAs has been reported (He *et al.*, 1991; Rennert *et al.*, 1991) and Fujiki *et al.* (1989) suggest that different promoters may influence the transcription of the SCP2 and SCPx mRNAs. A recent preliminary report by Ohba *et al.* (1995) indicate that the human SCPx/SCP2 gene has two promoters. One promoter appears to direct the transcription of SCPx and the other controls SCP2 transcription. Such a mechanism might explain how the SCP mRNA species in the present study might be differentially regulated. Alterations in translational efficiency, mRNA stability, or post-translational catabolism cannot, however, be ruled out.

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 expression in diabetes would be consistent with an increase in fatty acid β-oxidation which is enhanced in diabetes. The extent to which the 2.1 kb SCPx mRNA is translated in the diabetic state is currently under investigation.

The findings in this study that estradiol-treatment can enhance SCP2 levels in the liver without affecting SCP mRNA levels is consistent with a previous report by McLean *et al.* (1994). In this study, estradiol had no effect on corpus luteum SCP2 mRNA levels but rather altered post-translational processing of SCP2 and the SCP2-related proteins. This is consistent with the findings of Baum *et al.* (1993) which suggested that SCP2 is regulated both transcriptionally and post-transcriptionally in non-steroidogenic tissue of the rat and via a post-translational process in the heart and lung of this animal. Clearly the regulation of SCP2 is complex and the potential mechanism for estradiol mediated post-translational processing in the liver will require additional experiments.

In conclusion, this study indicates that SCP2 is reduced in the SZT-diabetic rat and has demonstrated that the expression of this protein varies inversely with the level of serum cholesterol. This investigation also demonstrates that estradiol and insulin treatment can influence hepatic SCP2 expression. In contrast to the reduction in hepatic SCP2 protein in the SZT-diabetic animals, SCP mRNA levels were increased substantially. Since both SCPx and SCP2 mRNAs appear to increase simultaneously, the decline in hepatic SCP2 protein levels in the diabetic animals most likely results from either altered SCP2 mRNA transcription, translation or from rapid post-translational catabolism of SCP2. While a role for the enhanced expression of the larger SCPx transcript and its translation into a protein involved in β-oxidation of fatty acids is envisioned, what controls differential SCP2 and SCPx mRNA translation and/or protein catabolism in diabetes will require further study.

Material and methods

Chemicals and cDNA probes

Phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA), ethylene glycolbis (β-aminoethyl ether) N,N,N′, N′-tetraacetic acid (EGTA) and bovine serum albumin (BSA; fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{125}I]-Protein A (specific activity = 47.2 mCi/mg) was purchased from ICN Biochemicals (Irvine, CA), [α-^{32}P]-dCTP (3000 Ci/mmol) was from Amersham (Arlington Heights, IL) and [^{32}P]-UTP (800 Ci/mmol) and [^{35}S]-ATP (1000–1500 Ci/mmol) were from DuPont/NEN (Wilmington, DE). Acrylamide was obtained from Schwarz/Mann Biotec (Cleveland, OH) and XAR-5 and BioMax films were purchased from Eastman Kodak (Rochester, NY). Nitrocellulose and nylon membranes were obtained from Schleicher and Schuell (Keene, NH) and SeaKem agarose was from the FMC Corporation (Rockland, ME). TRI-Reagent, Background Quencher, and Formazol were from Molecular Research Center Inc. (Cincinnati, OH). The random primed DNA labeling kit and all restriction enzymes were from Boehringer Mannheim (Indianapolis, IN). Insulin (human lente insulin) was from Eli Lilly (Indianapolis, IN) and 17β-Estradiol was obtained from Steraloids (Wilton, NH). All other chemicals were reagent grade from Sigma Chemical Co. or Fisher Scientific (Orlando, FI). The SCP2 cDNA sequence has been reported previously (Billheimer *et al.*, 1990). The β-actin cDNA was a gift from Dr. Randal Jaffe (University of Illinois at Chicago, IL).

Animals

Acute diabetic study Male Wistar rats (200g) were purchased from Charles River (Wilmington, MA). Rats were divided into five groups: (1) control animals, (2) estradiol treated animals; and diabetic animals which received: (3) no other treatment, (4) an estradiol implant or (5) insulin treatment. The acute diabetic study lasted 13 days during which

all animals had free access to food and water and were on a 12 h dark and 12 h light cycle. Diabetic rats were injected intravenously (jugular puncture) with 60 mg/kg streptozotocin dissolved in 20 mM sodium acetate (pH 4.5)/0.15 M NaCl on day 0 of the experiment. Control rats were injected intravenously with 20 mM sodium acetate (pH 4.5)/0.15 M NaCl (vehicle injection) on day 0 of the experiment. Estradiol treated rats received a 1 cm silastic implant placed subcutaneously in the neck region (McLean *et al.*, 1989) at the time of the SZT or vehicle injection. Insulin treated rats received 6 units/200 g body weight of human lente insulin injected subcutaneously twice a day at 8 am and 4 pm for the final 3 days of the experiment (injections started on day 9 post-SZT). In one experiment, control and diabetic rats were treated ± insulin ($n = 4$/treatment) and maintained until day 15 post-STZ. Hepatic tissue was examined on day 15 in these animals following six full days of insulin administration. Throughout the experiments, animal food and water consumption were monitored and rat weights were recorded. On day 11 post-SZT (12–1 pm), serum samples were taken by jugular puncture to verify that serum glucose levels were normalized by insulin injection. On days 13 or 15 of these experiments (between the hours of 9 and 11 am), rat livers were removed 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 assayed.

Long-term diabetic study: Fasted male Wistar rats (Charles River, Wilmington, MA) weighing 130–150 grams were made diabetic by SZT injection (50 mg/kg). Seven and 14 days after SZT administration, a tail vein blood glucose level >300 mg/dL was used as a criteria to identify animals with chemically induced diabetes. Diabetic rats were maintained on 1 unit of human lente insulin per 200 g body weight per week for 16 weeks. Age matched control rats (no SZT) were maintained during the 16 week experiment. At the end of the 16 week period, serum samples were obtained and the livers were removed and immediately frozen in liquid nitrogen. Serum samples were stored at −20°C until glucose and cholesterol assays were conducted.

Glucose and cholesterol assays

Serum glucose was determined by means of the glucose oxidase oxygen consumption method (Raabo & Terkildsen, 1960) using a Beckman Glucose Analyzer 2. This procedure measures the oxygen consumed in the enzymatic (glucose oxidase) conversion of glucose to gluconic acid. In this reaction, the rate of oxygen consumption is directly proportional to the concentration of the glucose in the serum. Glucose levels were expressed as mg/dL. The normal range for control rats was 60–150 mg/dL and for diabetic rats it was 300–600 mg/dL.

Serum cholesterol was determined using a Cholesterol 50 Assay from Sigma Chemical (St. Louis, MO). In this assay, serum cholesterol was oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide produced was then coupled with the chromogen, 4-aminoantipyrine and p-hydroxybenzenesulfonate, in the presence of peroxidase to yield a quinoneimine dye which has an absorbance maximum of 500 nm. When examined spectrophotometrically at 500 nm, the intensity of the color produced is directly proportional to the total cholesterol concentration in the serum. Serum cholesterol was expressed as mg/dL. The normal range for control rats was 60–120 mg/dL and for diabetic rats was 140–300 mg/dL.

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

Hepatic tissues (150 mg) obtained from control and SZT 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 DDT, 10.5 μM leupeptin and 10 μM pepstatin A (homogenizing buffer). Liver homogenates were assayed for protein concentrations by the method of Bradford (1975) using bovine serum albumin as the standard. Hepatic proteins (50 μg protein) were denatured at 100°C in 10% glycerol, 4.5% SDS, 5% β-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), 1.5 mM EDTA and 0.01% bromphenol blue for 10 min and subjected to electrophoresis on 7.5–18% gradient SDS-polyacrylamide gels (20 mA/gel, 5 h separation) according to the method of Laemmli (1970). Appropriate molecular weight markers (Sigma Chemical Co., St. Louis, MO) were included in all gels. 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. 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 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% non-fat dry milk for 1 h at room temperature (RT). Buffer was replaced with TB-NaCl containing a specific rabbit polyclonal antibody to SCP2 (1:2000 dilution) (McLean *et al.*, 1989) in 3% milk for 1 h at 4°C. This antiserum is specific for the sterol carrier protein family and does not cross react with other proteins (McLean *et al.*, 1989). 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 cpm/ml) for 1 h at RT. Differences in band density were quantified on autoradiograms densitometrically with a Hoefer scanning densitometer (San Francisco, CA) for statistical analysis (Zar, 1974).

RNA isolation and electrophoresis

Total RNA was isolated from liver using a modification of the Chomczynski and Sacchi method (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 TRI-Reagent with a polytron homogenizer and centrifuged at 12 000 *g* for 15 min at 4°C. RNA was precipitated from the aqueous phase with isopropanol and the RNA pellet washed in 75% ethanol and resuspended in Formazol (Molecular Research Center, Inc. Cincinnati, OH). RNA was quantified by absorbance at 260 nm in a Beckman DU-70 spectrophotometer (Palo Alto, CA).

RNA (20 μg) was denatured at 65°C (15 min) and loaded onto 1% agarose gels containing 3% formaldehyde. Ethidium bromide staining of the gel confirmed that the ribosomal RNAs (18S and 28S subunits) were intact and determined whether equal amounts of RNA were loaded in each lane. Following size fractionation, RNA was blotted on to nylon membrane (0.45 μm pore) by capillary transfer and RNA was fixed to the membrane by uv crosslinking (0.3 J/cm^2).

Northern and slot blot analysis

SCP2 Northern and slot blot analyses were performed using a 1.4 kb Eco RI rat SCP_2-cDNA fragment (Billheimer *et al.*, 1990). This cDNA has been shown to hybridize with RNA from numerous rat tissues (Billheimer *et al.*, 1990). β-Actin mRNA was identified using a 2 kb PstI chicken β-actin cDNA. The cDNA inserts were labeled with [^{32}P]-dCTP using the random primed DNA labeling method (Feinberg & Vogelstein, 1983). Northern and slot blots were prehybridized

at 62°C for 3 h in 1 M NaCl, 1% SDS solution and 1X Background Quencher (Molecular Research Center, Cincinnati, OH). Hybridization was completed in a High Efficiency Hybridization Solution (Molecular Research Center, Cincinnati, OH) containing the ^{32}P-labeled probe (1×10^6 dpm/ml; specific activity = 2×10^8dpm/μg DNA) at 62°C for at least 16 h. Blots were washed three times at RT (10 min) in 1 × SSC/1% SDS and twice at 60°C (15 min) in 0.1 × SSC/1% SDS. RNA:cDNA hybrids were visualized on XAR-5 film using two intensifying screens and a 12–48 h exposure period. Resulting autoradiograms were quantitated with a Hoefer scanning densitometer (San Francisco, CA) for statistical analysis (Zar, 1974). Densitometric analysis was performed on the 18S ribosomal band or the β-actin transcript for the standardization of RNA loading.

For slot blots, total RNA (10 μg) was vacuum blotted on to a nylon membrane (0.45 μm pore), washed with 10 × SSC (20 × SSC contains 3 M NaCl and 0.3 M sodium citrate, pH 7), and RNA was fixed to the slot blot membrane by uv crosslinking. Following hybridization to the SCP2 cDNA, slot blots were stripped and reprobed with the β-actin cDNA to correct for differences in loading.

SCP2 ribonuclease protection assay

A 429 bp SCP2 specific probe was generated from the pro-SCP2 cDNA sequence (Billheimer *et al.*, 1990). This region was amplified by polymerase chain reaction (PCR) using oligonucleotide primers with a plasmid containing the SCP2 cDNA (Billheimer *et al.*, 1990). The 429 bp PCR product was then cloned into the TA vector (Invitrogen, San Diego, CA) and subcloned into the high copy number plasmid, Bluescript-SK, generating pSK-proSCP2. The correct DNA sequence was verified by sequencing using the dideoxy chain termination method (Sanger *et al.*, 1977) with the Sequenase 2.0 protocol (Amersham, Arlington Heights, IL). The SCP2 RNA probe necessary for ribonuclease protection assay (RPA) analysis was then synthesized from pSK-proSCP2 using the MAXIscript *In Vitro* Transcription kit (Ambion, Austin, TX). For this, pSK-proSCP2 was digested with XbaI approximately 500 bp downstream of the T7 transcription promoter. The linearized plasmid was subjected to proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. The DNA template (200 ng) was combined with ribonucleotides using UTP as the limiting nucleotide and 50 mCi [α^{32}P]-UTP. The polymerase was added and the reaction incubated for 60 min at RT. DNase was added to remove the template and the reaction continued for 15 min at 37°C. RPAs were performed using the Promega Ribonuclease Protection Assay kit (Madison, WI). Total cellular RNA (5 μg) was incubated with 50 000 cpm of probe in hybridization buffer overnight at 42°C. RNase was added to remove the unhybridized RNA and incubated at 37°C for 30 min. The duplex RNA was precipitated with ethanol, resuspended in loading buffer, denatured at 95°C for 5 min and electrophoresed on a 4% polyacrylimide/ 8M urea gel. The gel was dried and exposed to Kodak BioMax film for 2–12 h. Equal RNA loading was verified using a 125 bp actin control riboprobe (Ambion, Austin, TX). The band densities were quantified by densitometry. The protected fragment size was compared to a 100–500 bp RNA marker (Ambion, Austin, TX) made by transcription.

Data analysis

SCP2 Western, Northern, slot blot and RPA autoradiographs were quantitatively analyzed using a Hoefer Densitometer (Hoefer Scientific Instruments, San Francisco, CA) in the transmittance mode. The scan data was expressed in arbitrary density units. Correction for protein loading was not required in the Western blots since equal protein staining was required prior to immunoblot analysis. In the Northern and slot blots, minor variations in RNA loading were corrected using the β-actin cDNA. Serum glucose and cholesterol levels were expressed as the mean ± SEM. Densitometric data was expressed as arbitrary density units and compared by analysis of variance (ANOVA) or by Mann-Whitney U test (non-parametric analysis) if variation between experiments (i.e. variations in blot exposures) made parametric ANOVA impractical. Following ANOVA, data was analyzed 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.

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