Effect of estrogen on scavenger receptor BI expression in the rat

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Abstract

High-dose 17α-ethinyl estradiol treatment is associated with increased adrenal and decreased hepatic levels of scavenger receptor class B type 1 (SR-BI) in rats. In this paper we explored the mechanisms responsible for the differential regulation of SR-BI by estrogen in these two tissues. Previously it was shown that estrogen-treated rats are profoundly hypolipidemic due to increased hepatic low density lipoprotein receptor (LDLR) activity, and that this effect is not maintained with hypophysectomy. To determine if the reduction in hepatic SR-BI was a direct or indirect effect of estrogen, we treated hypophysectomized rats with high-dose estrogen; the levels of SR-BI expression did not change in the livers or adrenals of these animals. To determine if the absence of response to estrogen in the adrenals of hypophysectomized animals was due to the absence of adrenocorticotropic hormone (ACTH), we examined the effect of estrogen treatment on SR-BI expression in animals treated with dexamethasone, which inhibits endogenous ACTH production. The administration of dexamethasone completely inhibited the increase in SR-BI expression in the adrenals of estrogen-treated rats. From these studies we conclude that estrogen does not have a direct effect on SR-BI expression in either the liver or the adrenals. In the liver, the decrease in SR-BI is dependent on the estrogen-induced increase in LDLR activity, and in the adrenal glands, ACTH is required for the estrogen-associated increase in expression of SR-BI.

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Introduction

High-dose estrogen treatment of rats causes a reduction in circulating levels of lipoproteins (Boyd & McGuire 1956) due to increased expression of low density lipoprotein receptors (LDLR) in the liver (Kovanen *et al.* 1979, 1980). The increase in hepatic LDLR activity results in accelerated clearance of circulating low density lipoprotein (LDL) and the subset of high density lipoproteins (HDL) containing apolipoprotein (apo) E (Chao *et al.* 1979). The lipid-lowering effect of estrogen requires an intact pituitary gland (Steinberg *et al.* 1967), and growth hormone is necessary for this response (Norstedt *et al.* 1981, Rudling *et al.* 1992; for review see Angelin & Rudling 1994).

In rodents, where HDL is the predominant cholesterol-carrying lipoprotein, the scavenger receptor, class B, type I (SR-BI) mediates the selective transfer of cholesterol and cholesteryl esters between lipoproteins and tissues (Acton *et al.* 1996) and plays a major role in the transport of cholesterol from peripheral tissue to the liver (Glass *et al.* 1983, Acton *et al.* 1994, 1996, Landschulz *et al.* 1996, Krieger 1999). SR-BI is the major conduit by which lipoprotein-associated cholesterol is supplied to rodent adrenal glands, testes and ovaries for steroidogenesis (Temel *et al.* 1997).

Modulation of hepatic SR-BI expression is associated with significant changes in plasma levels of HDL-cholesterol (C) (Kozarsky *et al.* 1997, Rigotti *et al.* 1997, Arai *et al.* 1999). Inactivation of SR-BI in mice results in a ∼two- to threefold increase in plasma levels of HDL-C (Rigotti *et al.* 1997) and, conversely, mice expressing high levels of recombinant SR-BI in the liver have low plasma cholesterol levels (Arai *et al.* 1999). Efforts to alter hepatic SR-BI expression using dietary manipulations, metabolic perturbations and pharmacological agents have modest effects on SR-BI levels (for review see Krieger 1999). The most dramatic change in hepatic SR-BI expression is seen with estrogen treatment of the rat. Hepatic SR-BI expression levels fall >95% after 5 days administration of high dose 17α-ethinyl estradiol (Landschulz *et al.* 1996).

In contrast to the liver, the level of immunodetectable SR-BI in the rat adrenal increases fourfold in response to estrogen treatment (Landschulz *et al.* 1996). Targeted inactivation of either SR-BI or apoAI, the major apolipoprotein of HDL, results in cholesterol depletion of the adrenal glands, indicating that HDL is the major source of cholesterol in this tissue and that SR-BI is the major conduit by which HDL-C enters the gland (Andersen & Dietschy 1978, Plump *et al.* 1996, Wang *et al.* 1996, Rigotti *et al.* 1997). The regulatory stimulus for the

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increased expression of SR-BI in the hypolipidemic animal remains unclear. Adrenocorticotropic hormone (ACTH), which is secreted from the pituitary gland in response to low circulating levels of glucocorticoids, is associated with increased adrenal SR-BI expression (Rigotti *et al.* 1996, Wang *et al.* 1996, Liu *et al.* 1997, Cao *et al.* 1999). ACTH also stimulates the expression of multiple other genes encoding proteins involved in the steroid biosynthetic pathway. ACTH mediates its effects by protein kinase A and the orphan nuclear hormone receptor, steroidogenic factor-1 (SF-1) (for review see Parker & Schimmer 1997). SF-1 binds in a sequence-specific manner to the promoter of human SR-BI (Cao *et al.* 1997), and we have provided evidence that SF-1 also regulates the expression of SR-BI *in vivo* (Cao *et al.* 1999).

SR-BI levels are increased both by ACTH and by cholesterol depletion in cultured corticoadrenal cells (Rigotti *et al.* 1996, Cao *et al.* 1999, Sun *et al.* 1999); the relative role of these two stimuli *in vivo* is still not known. In wildtype and apoAI knockout mice, adrenal SR-BI levels tend to correlate with plasma levels of ACTH which, in turn, are inversely related to the lipid content of the adrenal gland (Sun *et al.* 1999). To determine if the increase in expression of SR-BI in estrogen-treated animals is a direct effect of estrogen or an indirect effect due to cholesterol depletion of the adrenal gland and/or stimulation by ACTH, we examined SR-BI levels in estrogen-treated animals with and without the addition of dexamethasone, which potently decreases ACTH secretion and thereby corticosterone production. The effect of estrogen on hepatic SR-BI expression was also examined. These studies revealed that estrogen has an indirect, rather than a direct effect on hepatic and adrenal SR-BI expression in the rat.

Materials and Methods

Materials

One- to three-month-old male Sprague Dawley rats (150–250 g) and hypophysectomized male rats (225–250 g) were obtained from Harlan Laboratories (Gilmore, CA, USA). 17α-Ethinyl estradiol, dexamethasone and propylene glycol were purchased from Sigma Chemical (St Louis, MO, USA). Plasma ACTH and corticosterone levels were measured by radioimmunoassay (Analytics Inc., Gaitherburg, MD, USA).

Animals

A light-darkness cycle of 12 h (light cycle: 0600 to 1800 h) was maintained in the animal facility in which the rats were housed. They were provided with water and rodent chow (Teklad Premier Laboratories Diets, Madison, WI, USA) *ad libitum*. After two weeks of acclimatization, rats were injected subcutaneously with 17α-ethinyl estradiol (5 mg/kg) in propylene glycol (10 mg/ml) or vehicle alone for the indicated times. Estrogen treatment of hypophysectomized rats was initiated one week after surgery. Dexamethasone (0·2 mg) in ethanol (2 mg/ml) was injected subcutaneously at 0800 h and 2000 h. Animals were killed at 0900 h using halothane (Halocarbon Laboratories, River Edge, NJ, USA). The group size was three in almost all experiments.

Immunoblot analysis

Livers and adrenal glands were harvested from anesthetized rats and frozen immediately in liquid nitrogen. The tissue was pulverized and membranes were isolated as described previously (Landschulz *et al.* 1996). The protein extracts were size-fractionated on a 6·5% SDS-polyacrylamide gel and transferred to nitrocellulose (Hybond-C extra, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Immunoblot analysis was performed using IgG-SRBI, a rabbit polyclonal anti-peptide antibody directed against the carboxy-terminal 14 amino acids of SR-BI (Landschulz *et al.* 1996), IgG-638, a rabbit polyclonal antibody against bovine LDLR (Herz *et al.* 1990), and IgG-692, a rabbit polyclonal antibody against the rat receptor associated protein (RAP) (Herz *et al.* 1991). Filters were washed, and bound antibody was detected using horseradish peroxidase-coupled donkey anti-rabbit IgG and enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech). Filters were exposed to Kodak Dupont NEF 496 film (Wilmington, DE, USA) at room temperature for the indicated times.

RNase protection assay

RNase protection assays were performed as previously described (Cao *et al.* 1999). Briefly, a 307-basepair fragment encoding sequences corresponding to amino acids 397 to 499 of the rat SR-BI was amplified from hepatic RNA using reverse transcriptase (RT) and polymerase chain reaction (PCR) (Stratagene, La Jolla, CA, USA), and subcloned into pGEM-T Easy Vector (Promega, Madison, WI, USA); a rat actin cDNA fragment (Ambion, Austin, TX, USA) was used to generate an internal control probe. Radiolabeled probes were made using the Riboprobe *in vitro* Transcription System (Promega) (Cao *et al.* 1999). A HybSpeed kit (Ambion) was used for the RNase protection assay. A total of 1×10^5 c.p.m. of each probe was incubated with 10 μg total cellular RNA isolated using RNA STAT (Tel-Test, Friendswood, TX, USA). Protected fragments were size-fractionated on a 6% denaturing polyacrylamide gel. The gel was dried and then exposed to film.

Immunocytochemistry

Tissues were removed, fixed in 4% paraformaldehyde and paraffin embedded. Sections were deparaffinized with xylene and then incubated in AR-10 Retrieval Solution

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Table 1 Hepatic cholesterol levels of 17α-ethinyl estradiol-treated rats. Male rats (n=3) were injected subcutaneously with 17α-ethinyl estradiol (5 mg/kg; Estrogen) or vehicle alone (Sham) every 24 h and then killed at the indicated times. The livers were frozen in liquid nitrogen and the hepatic cholesterol levels were quantitated as described in the Materials and Methods section. Results are means ± S.E.M. The experiment was repeated twice and similar results were obtained.

	Hepatic cholesterol (mg/g)				
	0 h	3 h	24 h	48 h	120 h
Treatment					
Sham	2·20 ± 0·06	2·18 ± 0·06	2·52 ± 0·13	2·60 ± 0·15	2·50 ± 0·15
Estrogen		3·26 ± 0·20**	3·63 ± 0·17**	3·63 ± 0·26**	3·15 ± 0·42**

**P<0·005 compared with Sham group.

according to the manufacturer's instructions (Biogenex, San Ramon, CA, USA). Slides were blocked with universal blocking agent (Biogenex) and incubated with IgG-SRBI or with a mouse anti-rat ED1 antibody (Serotec, Raleigh, NC, USA) overnight. ED1 is an antigen expressed on Kupffer cells but not hepatocytes (Armbrust & Ramadori 1996). Sections were rinsed and incubated with biotinylated anti-rabbit or anti-mouse IgG (Jackson Immunochemical, West Grove, PA, USA) for 1 h, and rinsed again prior to addition of streptavidin-conjugated horseradish peroxidase (Research Genetics, Huntsville, AL, USA). Sections were developed using DAB (Research Genetics), counterstained with hematoxylin, and then viewed using a photomicroscope (Nikon, New York, NY, USA).

Fast performance liquid chromatography (FPLC) analysis of plasma lipoproteins

Blood was collected from the vena cava at the time of death and plasma was isolated by centrifugation and stored at − 80 °C. Plasma cholesterol and triglyceride levels were measured using the cholesterol/HP system pack kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). Fresh plasma from a subset of the rats was subjected to Superose 6 gel filtration by FPLC; cholesterol and triglyceride contents of the fractions were estimated using spectrofluorometry as described (Ishibashi *et al.* 1993). Total hepatic cholesterol and triglyceride levels were measured (Carlson & Goldfarb 1977). The hepatic and adrenal cholesterol/cholesteryl ester content was analyzed as described by Turley *et al.* (1994). For validation of the assay a control standard was used, the maximum deviation was 5%.

Statistical analysis

The results are expressed as means ± S.E.M. and multiple comparisons of treatments were evaluated using analysis of variance followed by Student's t-test.

Results

Estrogen treatment of rats results in reciprocal changes in the levels of SR-BI and the LDLR in the liver

Administration of 17α-ethinyl estradiol (5 mg/kg) to rats for 5 days results in a dramatic reduction in SR-BI and increase in LDLR in the liver (Landschulz *et al.* 1996). To examine the temporal relationship between the reduction in SR-BI and the increase in hepatic LDLR and how these changes are related to the associated alterations in plasma and tissue cholesterol levels, we performed a time-course experiment in the estrogen-treated animals (Fig. 1A). The amount of hepatic SR-BI decreased ∼20% within 2 h and fell to very low levels within 24 h. The levels of LDLR increased twofold by the 3-h time point and then rose steadily to reach a maximum at 48 h, at which time the levels were ∼fivefold higher than those of the sham-injected animals. The plasma cholesterol level fell progressively to very low levels within the first 48 h of the experiment (Fig. 1B). Estrogen had little effect on plasma triglyceride levels until the 48-h time point, at which time the mean plasma triglyceride levels increased in the sham-injected animals (from 49 to 73 mg/dl) and decreased in the estrogen-treated groups (from 54 to 26 mg/dl) (data not shown). These levels remained unchanged over the ensuing 3 days (data not shown).

As expected, the increase in LDLR activity in the estrogen-treated animals resulted in an increase in hepatic cholesterol content (Table 1). Hepatic cholesterol concentrations progressively increased until 24 h, at which time they were ∼50% higher in the estrogen-treated animals than in the sham-injected controls. The hepatic triglyceride levels did not change significantly during the experiment in the sham-injected animals, but fell ∼25% in the estrogen-treated animals, which may be due to the anorexic effects of estrogen administration (Weinstein *et al.* 1986).

Thus, estrogen treatment was associated with directly divergent alterations in the levels of SR-BI and LDLR in

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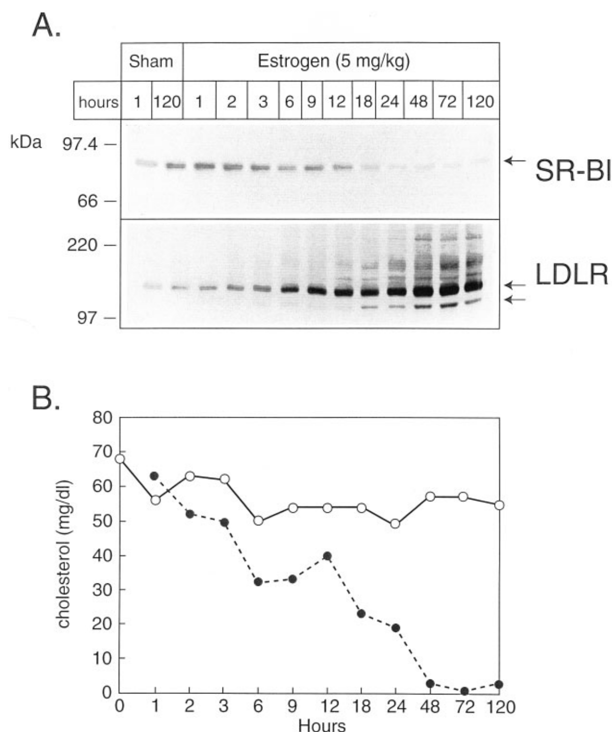

Figure 1 Immunoblot analysis of hepatic SR-BI and the LDL R (A) and plasma lipid levels (B) in estrogen-treated rats. Male rats were given 17α-ethinyl estradiol (5 mg/kg) in propylene glycol via subcutaneous injection. Control rats were given an equal volume of propylene glycol. At each time point rats (n=2) were killed and tissues collected. Membrane proteins were isolated from rat livers as described in Materials and Methods. A total of 50 µg membrane protein was size-fractioned on a 6·5% SDS-polyacrylamide gel and transferred to nitrocellulose. Immunoblot analysis was performed using rabbit anti-SRBI and anti-LDLR polyclonal antibodies. Subsequently, the filters were incubated with a horseradish peroxidase-coupled donkey anti-rabbit secondary antibody. The filters were developed using the ECL system and exposed to Kodak Dupont NEF 496 film. (B) Plasma cholesterol levels in the sham-injected and estrogen-treated rats. Plasma was isolated from the rats treated with estrogen (●) or vehicle alone (○) for the indicated time periods. The plasma cholesterol levels were measured as described in Materials and Methods and the mean data are provided. This experiment was performed three times with similar results.

the liver, with the increase in levels of LDLR being associated with a progressive decrease in levels of SR-BI.

High-dose estrogen treatment results in decreased SR-BI expression in hepatocytes

The cell type-specific changes in the expression level of SR-BI were examined by performing immunocytochemistry in the livers of estrogen-treated rats (Fig. 2). Intense staining was evident on sinusoidal surfaces of hepatocytes in liver sections from the sham-injected animals (Fig. 2C and G). A significant decrease in the hepatocellular staining was seen in the estrogen-treated animals (Fig. 2D and H). No staining was seen in association with the bile canalicular membranes in the control or in the estrogen-treated livers. Fluiter *et al.* (1998) reported that estrogen treatment of rats is associated with increased levels of expression of SR-BI in Kupffer cells. Kupffer cells were identified using ED1, a Kupffer cell-specific antigen (Armbrust & Ramadori 1996) (Fig. 2E, F, I and J). There appeared to be no changes in the number of Kupffer cells with estrogen treatment. No increase in SR-BI staining was seen associated with the Kupffer cells in association with estrogen treatment (Fig. 2G and H).

Evidence for post-translational regulation of hepatic SR-BI expression

To determine if the reduction in levels of hepatic SR-BI in the estrogen-treated animals was due to a reduction in SR-BI mRNA, we analyzed the amount of SR-BI mRNA in these tissues. The SR-BI transcript is differentially spliced at the 3′ end, producing two transcripts, SR-BI and SR-BII (Webb *et al.* 1997). An RNase protection assay was used to distinguish the two spliced forms of SR-BI. Only a trace amount of the SR-BII transcript was detected in livers of these animals, the level of SR-BI mRNA fell ∼50% (Fig. 3). Northern blot analysis was also performed and a similar reduction in SR-BI mRNA was detected (data not shown). The magnitude of the reduction in the level of hepatic SR-BI mRNA was significantly less than that seen for the protein (compare data in Fig. 3 with that in Fig. 1), which suggests that there is post-transcriptional or translational regulation of SR-BI under these conditions.

To determine if the apparent post-transcriptional regulation of SR-BI was due to the release of the protein from the hepatocyte cell surface into either the blood or bile, immunoblot analysis was performed on both bile and blood from the estrogen-treated rats using polyclonal anti-SR-BI and anti-LDLR antibodies. No immunodetectable SR-BI was detected in either body fluid (data not shown), but with the LDLR antibody two immunoreactive peptides (∼130 and ∼100 kDa) appeared in the plasma of the estrogen-treated rats (data not shown) and circulated in the apoAI-containing plasma fractions on fast performance liquid chromatography (data not shown).

Figure 2 Immunolocalization of SR-BI in the liver of sham-injected (A, C, E, G, I) and 17α-ethinyl estradiol-treated (B, D, F, H, J) rats. Sections were incubated with pre-immune serum (A, B), anti-SR-BI antibody (C, D, G, H) or anti-ED1 (E, F, I, J) followed by DAB staining, as described in Materials and Methods. The nuclei were counterstained with hematoxylin. Magnification: 200 ×, panels A–F; 400 ×, panels G–J. Immunohistochemistry was performed on three different experiments, giving similar results.

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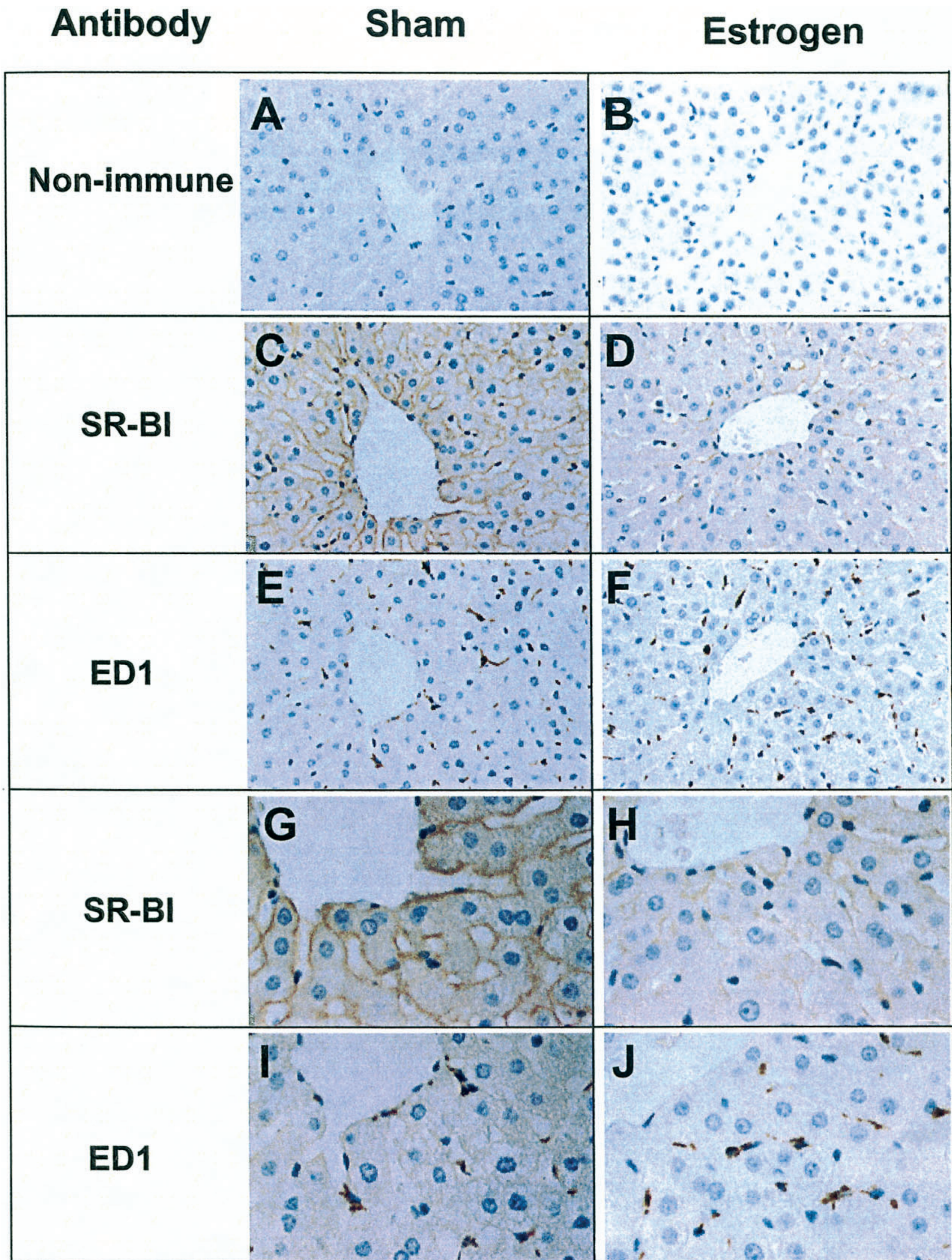

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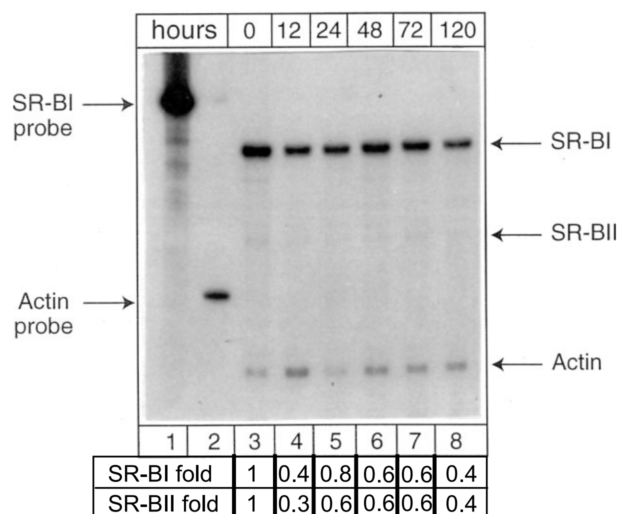

Figure 3 Effect of estrogen treatment on the levels of SR-BI and SR-BII mRNA in male rats. Animals were treated with estrogen as described in Fig. 1. An RNase protection assay was performed using 10 µg total hepatic RNA as a template and a 307 bp fragment from the 3′-end of the rat SR-BI cDNA as a probe, as described in Materials and Methods. A rat actin probe was used as an internal standard. Protected fragments were analyzed on a 6% denaturing polyacrylamide gel. The gel was dried and exposed to film. The signals were quantified for rat SR-BI, SR-BII and actin using a phoshoimager (Fuji Photo Film Co. Ltd). The relative intensities of the signals were determined by dividing the SR-BI or SR-BII signal by actin at each time point.

Hypophysectomy blocks effect of estrogen on hepatic and adrenal SR-BI expression

To examine further whether reduction in hepatic SR-BI expression in the estrogen-treated rats was a direct or indirect effect on SR-BI, hepatic SR-BI levels were examined in hypophysectomized rats ($n=4$) treated with high-dose estrogen for 5 days (Fig. 4). In the estrogen-treated rats there was a decrease in hepatic SR-BI and a dramatic increase in LDLR after 5 days of treatment (Fig. 4A). No increase in hepatic LDLR or decrease in mean plasma cholesterol (64 to 70 mg/dl) was seen with estrogen treatment in hypophysectomized animals, which is similar to previously reported results (Rudling *et al.* 1992, Angelin & Rudling 1994). The levels of hepatic SR-BI also did not change in the estrogen-treated hypophysectomized rats. The lack of reduction of SR-BI in the estrogen-treated hypophysectomized animals suggests that the reduction in SR-BI may be mechanistically tied to the increase in LDLRs. Although less likely, it is also possible that other factor(s) expressed in the pituitary gland are necessary for estrogen to down-regulate SR-BI expression.

Hypophysectomy was associated with the near absence of SR-BI expression in the adrenal gland (Fig. 4B). Levels of SR-BI in the adrenals of the hypophysectomized rats were less than 10% of the intact animals, presumably due to the absence of ACTH in these animals. Also LDLR fell to very low levels. The levels of SR-BI did not increase

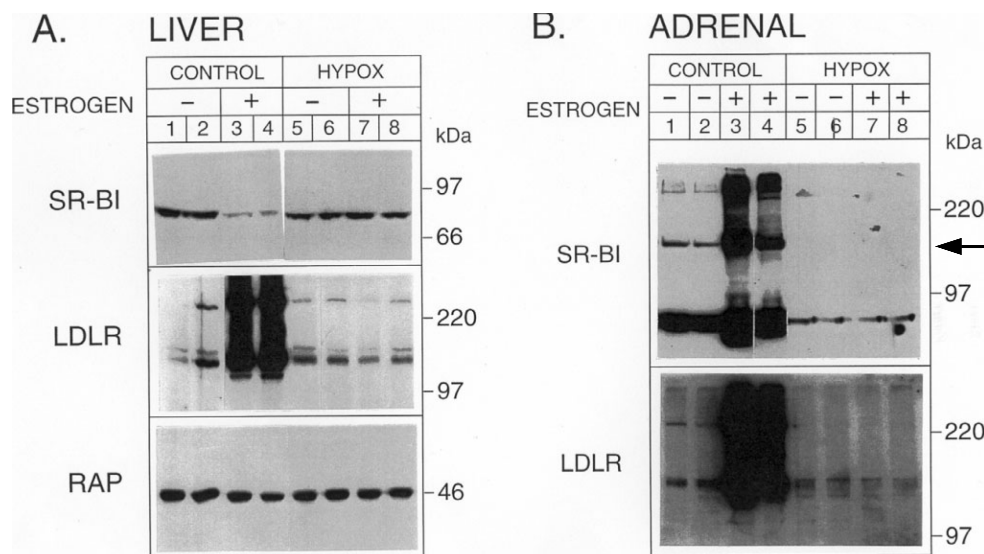

Figure 4 Effect of hypophysectomy on the levels of immunoreactive SR-BI in the liver (A) and adrenal (B) of estrogen-treated rats. Control or hypophysectomized (HYPOX) rats were injected with vehicle alone or 17α-ethinyl estradiol (5 mg/kg) for 5 days. Solubilized hepatic and adrenal membrane proteins were isolated and aliquots of hepatic (50 µg) or adrenal (30 µg) membrane protein were subjected to immunoblot analysis using a rabbit anti-mouse SR-BI antibody (IgG-SRBI) (upper panels), a rabbit anti-bovine LDLR antibody (IgG-638) (A, middle panel, B, lower panel) or a rabbit anti-human receptor associated protein (RAP) polyclonal antibody (IgG-692) (A, lower panel); the latter served as a control for the experiment. The blots were incubated with horseradish peroxidase-conjugated sheep anti-rabbit IgG and developed using an ECL detection system. The arrow on panel B marks the 164 kDa band of the SR-BI dimer.

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with estrogen treatment in the hypophysectomized animals (Fig. 4B). In contrast, estrogen treatment of intact rats resulted in a fivefold increase in adrenal SR-BI and the appearance of a 164 kDa band that likely represents a dimer of SR-BI, as has been proposed previously (Landschulz *et al.* 1996).

Dexamethasone inhibits an increase of adrenal SR-BI in estrogen-treated rats

Cholesterol is required to maintain adrenal steroidogenesis. Depletion of intracellular cholesterol results in increased ACTH secretion by the pituitary, which stimulates increased SR-BI expression. Both cholesterol depletion and ACTH up-regulate SR-BI expression in cultured adrenal cells (Rigotti *et al.* 1996), but which signal predominates *in vivo* is still not clear. To address this question, we suppressed endogenous ACTH expression using dexamethasone, a potent corticosteroid. Dexamethasone and estrogen were co-administered to male rats for 2 or 5 days. No significant changes in body weight were apparent at 2 days in rats treated with estrogen alone or estrogen plus dexamethasone. A significant decrease in body weight was seen in all animals treated with estrogen at the 5-day time point (Table 2, $P<0·05$), which is similar to previous reports (Hornstein *et al.* 1992).

Estrogen treatment was associated with a 2·8- and 2·1-fold increase in hepatic cholesterol ester content after 2 and 5 days respectively (Table 2). Co-administration of dexamethasone with estrogen increased hepatic cholesterol esters further (by 3·5-fold at 2 days and 4·8-fold at 5 days). The levels of hepatic SR-BI were similarly decreased in animals treated with estrogen alone or with estrogen plus dexamethasone (data not shown). Thus, co-administration of dexamethasone did not change the effect of estrogen on hepatic SR-BI.

In contrast to the liver, dexamethasone had a profound effect on the levels of SR-BI in the adrenal gland of the estrogen-treated animals. Adrenal SR-BI, as well as LDLR, levels fell dramatically with dexamethasone treatment and co-administration of estrogen did not influence the magnitude of this effect (Fig. 5). To determine if the reduction in SR-BI levels was due to a fall in ACTH levels or an increase in intracellular cholesterol, we measured the levels of circulating ACTH and intracellular lipids in these animals. Mean ACTH levels fell within 2 days of initiation of dexamethasone treatment and this was associated with an increase in adrenal free cholesterol and cholesteryl ester content. Thus, although animals treated with estrogen and dexamethasone were hypolipidemic (plasma cholesterol levels fell to ∼15% of control levels), this did not result in a reduction in adrenal free cholesterol or cholesteryl esters (Fig. 5), presumably because sufficient sterol is available to support steroidogenesis. These data suggest that ACTH is required for the increased expression

Table 2 Mean hepatic and adrenal lipid levels and plasma chemistries in male rats (n=3) treated with 17α-ethinyl estradiol (5 mg/kg/day) with or without dexamethasone (200 μg/day) for 2 and 5 days. Sham-injected (Control), estrogen-treated (EE), dexamethasone-treated (Dex) and estrogen plus dexamethasone-treated (EE+Dex) male rats were treated for the indicated times as described in the legend to Fig. 1. Hepatic and adrenal free cholesterol (FC) and cholesteryl ester (CE) and plasma cholesterol, triglycerides, ACTH and corticosterone levels were measured as described in Materials and Methods. Results are means ± S.E.M.

Treatment	Time (days)	Body weight (g)	Liver weight (g)	Liver cholesterol (mg/g) FC	Liver cholesterol (mg/g) CE	Adrenal cholesterol (mg/g) FC	Adrenal cholesterol (mg/g) CE	Plasma cholesterol (mg/dl)	Plasma triglycerides (mg/dl)	Plasma ACTH (pg/ml)	Plasma corticosterone (ng/ml)
Control	2	263 ± 3	9·2 ± 0·3	2·22 ± 0·08	0·48 ± 0·03	2·96 ± 0·17	26·12 ± 3·93	82·7 ± 4·4	40·9 ± 6·1	405 ± 193	35 ± 10
	5	302 ± 13	11·7 ± 0·9	2·08 ± 0·06	0·42 ± 0·07	2·96 ± 0·18	25·96 ± 3·62	59·6 ± 4·2	82·7 ± 10·0	402 ± 66	120 ± 138
EE	2	268 ± 5	11·6 ± 0·7**	2·26 ± 0·03	1·36 ± 0·24**	2·62 ± 0·21	12·45 ± 4·44*	9·9 ± 5·7**	33·5 ± 38·8	302 ± 129	114 ± 76
	5	272 ± 13*	13·1 ± 0·5	2·26 ± 0·08*	0·88 ± 0·33	2·98 ± 0·22	4·78 ± 0·93**	4·8 ± 1·8**	16·7 ± 3·8**	267 ± 17*	57 ± 64
Dex	2	239 ± 7*	10·7 ± 0·2	2·00 ± 0·05*	0·67 ± 0·14	4·27 ± 0·23**	33·51 ± 4·38	87·9 ± 11·7	105·6 ± 7·8**	92 ± 7*	3 ± 4*
	5	220 ± 12**	9·2 ± 1·1*	2·07 ± 0·08	0·87 ± 0·36	3·83 ± 0·33	31·94 ± 9·67	69·5 ± 11·1	125·3 ± 31·8	80 ± 19**	3 ± 2
EE Dex	2	254 ± 13	13·2 ± 1·4**	1·95 ± 0·04*	1·70 ± 0·38**	4·85 ± 0·81*	40·09 ± 5·65*	11·0 ± 6·2**	34·9 ± 28·7	101 ± 19*	0 ± 0**
	5	229 ± 7**	13·5 ± 1·2	1·88 ± 0·11*	2·00 ± 0·58*	4·69 ± 0·99*	35·52 ± 10·68	9·0 ± 2·6**	30·5 ± 11·1**	64 ± 11**	0 ± 0

*$P<0·05$; **$P<0·005$.

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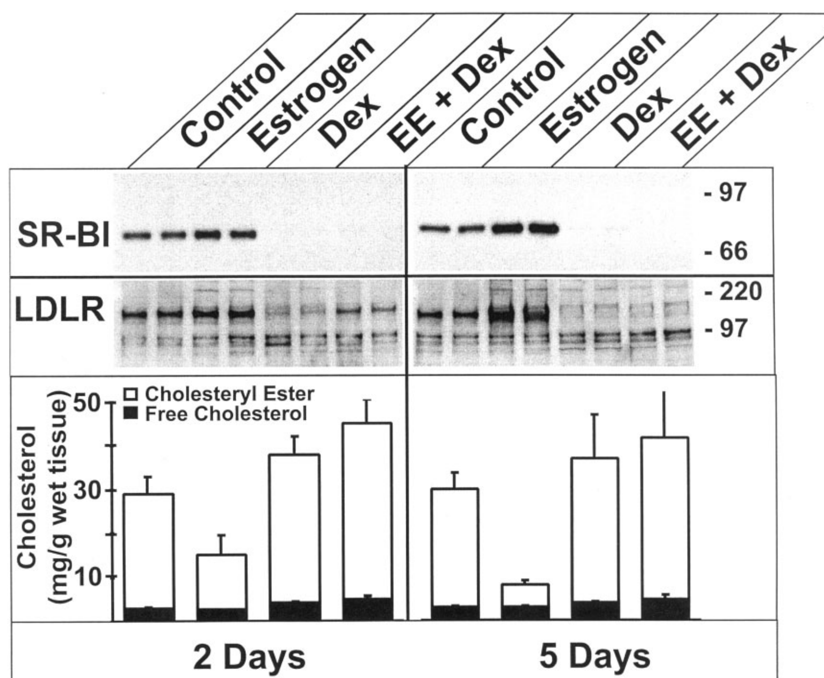

Figure 5 Effect of dexamethasone on the levels of SR-BI and LDLR in the adrenals of estrogen-treated rats (n=3). Immunodetectable SR-BI protein and LDLR were analyzed by immunoblotting using adrenal membrane fractions in control rats and those treated with 17α-ethinyl estradiol (5 mg/kg), dexamethasone (Dex; 200 µg/day) or both drugs for 2 or 5 days. Immunoblotting was performed as described in the legend to Fig. 1. The corresponding tissue free cholesterol and cholesteryl ester content (mg/g wet tissue) was determined as described in Materials and Methods (bottom panel). This experiment was performed three times with similar results.

of SR-BI in the adrenal gland in response to estrogen treatment in the rat.

Discussion

In this paper we examined the mechanism responsible for the discordant effects of high-dose estrogen treatment on SR-BI expression in the liver and adrenal glands of rats. Time-course experiments revealed that the decrease in SR-BI expression mirrored the increase in LDLR expression in the estrogen-treated animals. The magnitude of reduction in hepatic SR-BI protein was greater than that for the SR-BI mRNA, suggesting that the effect of estrogen is post-transcriptional or post-translational. Hypophysectomy, which completely abolishes the estrogen-stimulated increase in hepatic LDLR expression in the rat (Rudling *et al.* 1992), resulted in no change in hepatic SR-BI levels with estrogen treatment. In contrast to the liver, SR-BI expression in the adrenal glands of the hypophysectomized animals was reduced, and did not increase with estrogen treatment. The administration of dexamethasone had a similar effect as hypophysectomy on the expression of SR-BI in rat liver and adrenal glands. These results are consistent with the estrogen-associated changes of SR-BI in the liver and adrenal glands being an indirect rather than a direct effect of the hormone, and with ACTH being the major physiological stimulus for SR-BI expression in the adrenal gland.

The LDLR stimulatory effect of estrogen in rat liver is mediated via the estrogen receptor (Parini *et al.* 1997) and requires growth hormone (Rudling *et al.* 1992). No increase in hepatic LDLR occurs in hypophysectomized rats and therefore these animals have normal plasma cholesterol levels. In this paper we demonstrated that hepatic SR-BI levels also did not change in the estrogen-treated, hypophysectomized rats. The lack of reduction in hepatic SR-BI in the estrogen-treated hypophysectomized rats is most likely linked to the lack of any increase in LDLR activity. Thus, hepatic SR-BI reflects somewhat the plasma cholesterol levels.

The rat SR-BI gene has a sterol responsive element in its 5′-flanking sequence and it has been suggested that SR-BI expression is regulated by sterol regulatory element binding protein (SREBP) (Lopez & McLean 1999). SREBPs activate LDLR as well as numerous other genes involved in lipid metabolism in response to reductions in intracellular sterols (for review see Brown & Goldstein

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1999). It is unlikely that SREBP plays a significant role in hepatic SR-BI regulation in the estrogen-treated animal since the SR-BI and LDLR genes are regulated in opposite directions. Moreover, SR-BI protein levels are not elevated, and in fact are modestly reduced, in mice expressing constitutively active, truncated forms of SREBP (Shimano *et al.* 1996; H Stangl & H H Hobbs, unpublished data).

The discrepancy between the magnitude of the reduction in SR-BI mRNA and protein in the liver suggests a post-transcriptional effect of estrogen on SR-BI expression. The influx of cholesterol into the liver with estrogen treatment alters membrane morphology and fluidity (Rosario *et al.* 1988). Normally, the sinusoidal membrane surface has an extensive array of microvillar extensions. Estrogen treatment is associated with a flattening of the microvilli, resulting in a 43% decrease in the sinusoidal surface area and a 50% decrease in sinusoidal volume (Hornstein *et al.* 1992). Moreover, high-dose estrogen treatment is associated with a 15-fold increase in circulating bile acids (Hornstein *et al.* 1992), which also may affect the lipid composition and fluidity of hepatocyte membranes. Since much of the hepatic SR-BI resides in the plasma membrane, its half-life may be affected by these changes. We found no evidence that SR-BI was 'shed' into either the plasma or the bile (S Turley, unpublished observation (UT Southwestern Medical Center)) of estrogen-treated animals. It remains possible that the chemical and morphological changes in hepatocyte membranes associated with estrogen treatment alters the conformation of SR-BI so that it no longer binds the detecting antibody.

Over-expression of SR-BI in the liver is associated with immunodetectable SR-BI on the canalicular membranes (Kozarsky *et al.* 1997). We saw no immunoreactive SR-BI associated with the bile canaliculi. It is likely that the very high levels of expression of SR-BI associated with gene transfer result in promiscuous trafficking of the receptor to the apical (canalicular) surface (Kozarsky *et al.* 1997), although it is possible that the levels of SR-BI associated with the bile canaliculi may be below the detection limits of our immunodetection methods.

The results of our experiments suggest that ACTH is the major physiological stimulus for SR-BI expression in the adrenal gland. In the hypophysectomized animal, which secretes no ACTH, the amount of SR-BI in the adrenal gland of estrogen-treated rats falls to almost undetectable levels. The fivefold increase in the adrenal SR-BI expression with estrogen treatment was completely inhibited by co-administration of dexamethasone. However, we were unable to uncouple the effect of changes in intracellular cholesterol content from the plasma ACTH levels on SR-BI expression to determine which is the predominant stimulus for up-regulation of SR-BI in the estrogen-treated animal. Our results are most consistent with ACTH and cholesterol depletion acting in concert to increase SR-BI expression in the adrenal gland of the estrogen-treated rat, which is similar to what has been observed in cultured adrenal cells (Rigotti *et al.* 1996).

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