LY295427, a novel hypocholesterolemic agent, enhances [^{3}H]25-hydroxycholesterol binding to liver cytosolic proteins

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Abstract LY295427, (3α, 4α, 5α)-4-(2-propenylcholestan-3-ol), acts through an unknown mechanism to derepress the transcription of the low density lipoprotein (LDL) receptor in the presence of 25-hydroxycholesterol (25-OH chol). Preincubation with LY295427 in Chinese hamster ovary (CHO) cells increased uptake of 25-OH chol in a time-dependent manner, suggesting that the drug interfered with the negative feedback mechanism of 25-OH chol on LDL receptor expression. To explore the mechanism by which LY295427 inhibited the suppressive actions of 25-OH chol, the radioactive ligand [^{3}H]25-OH chol and specific antibodies to the oxysterol binding protein (OSBP) were used to identify possible drug:protein interactions. After separation by anion exchange chromatography, protein fractions from hamster liver cytosol were found to selectively bind [^{3}H]25-OH chol with high affinity. In fractions in which 25-OH chol binding was evident, and in other distinct fractions that lacked specific binding, addition of LY295427 increased [^{3}H]25-OH chol binding 2- to 5-fold. LY306039, the 3β-isomer of LY295427, failed to derepress the LDL receptor in CHO cells, and it had no effect on [^{3}H]25-OH chol binding. Analysis of Western blots using polyclonal antibodies to OSBP showed that specific [^{3}H]25-OH chol binding in the absence of LY295427 was present only in fractions containing OSBP. However, enhanced [^{3}H]25-OH chol binding in the presence of LY295427 was evident in distinct fractions after immunodepletion of both the 90–100 kDa form of OSBP and a 170 kDa protein; and specific binding of a radioiodinated analog of LY295427 was detected in select fractions lacking [^{3}H]25-OH chol binding in the absence of LY295427. Moreover, LY295427 did not displace or enhance [^{3}H]25-OH chol binding to OSBP purified to near homogeneity. These data suggest that LY295427, while not dependent on the presence of oxysterol binding protein, binds to cytosolic protein(s) that interact with 25-hydroxycholesterol and other oxysterols, thus preventing the repression of the LDL receptor.—**Bowling, N., W.F. Matter, R.A. Gadski, D.B. McClure, T. Schreyer, P.A. Dawson, and C.J. Vlahos.** LY295427, a novel hypocholesterolemic agent, enhances [^{3}H]25-hydroxycholesterol binding to liver cytosolic proteins. *J. Lipid Res.* 1996. **37:** 2586–2598.

Supplementary key words low density lipoprotein receptor • oxysterol binding protein • cholesterol lowering • atherosclerosis

Cholesterol, an essential component of mammalian cells, is obtained through low density lipoprotein (LDL) receptor-mediated endocytosis and from intracellular synthesis. Cellular cholesterol homeostasis is achieved through complex feedback mechanisms that control the activities of several enzymes in the cholesterol biosynthetic pathway and in the expression of the LDL receptor. Physiologically, the major regulator of this system is cholesterol derived from plasma LDL. After entering cells, LDL is delivered to lysosomes, where its cholesteryl esters are hydrolyzed and free cholesterol becomes available. In humans and animals, failure to adequately remove circulating LDL results in vascular deposition of cholesterol and appearance of atherosclerotic plaques (1, 2).

Selective stimulation of LDL receptor expression presents a novel and effective mechanism for lowering serum cholesterol by increasing uptake of serum LDL. When cholesterol accumulates in cells, endogenous synthesis is reduced by decreasing the number of LDL receptors, and cholesterol esterification is increased through the activation of acyl-CoA:cholesterol acyltransferase (ACAT). In addition to cholesterol, oxygenated sterols (oxysterols) also act at the transcriptional and post-transcriptional levels to regulate cholesterogenic enzymes and the LDL receptor, and may serve as the intracellular regulators of cholesterol biosynthesis (3–5). When added in solvents to cultured cells, cholesterol itself is not active; however, the addition of 25-hy-

Abbreviations: LDL, low density lipoprotein; CHO, Chinese hamster ovary; ACAT, acyl-coenzyme A:cholesterol acyltransferase; 25-OH chol, 25-hydroxycholesterol; OSBP, oxysterol binding protein; PMSF, phenylmethyl-sulfonyl fluoride; SREBP-1 and SREBP-2, sterol regulatory element-binding protein 1 and 2.

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droxycholesterol (25-OH chol) results in the transcriptional repression of the genes encoding the enzymes HMG-CoA reductase, HMG-CoA synthase, and the LDL receptor (6, 7).

LY295427, originally discovered in a transcription-based screening assay, was found to derepress LDL receptor gene expression in the presence of 25-OH chol (8). The compound lowered serum cholesterol in hypercholesterolemic hamsters and rabbits without inhibiting cholesterol synthesis (9). In addition, LY295427 inhibited microsomal ACAT activity (R.A. Gadski, unpublished results). Therefore, the efficacy of LY295427 could not be attributed to a single transcription factor such as the sterol regulatory element binding protein (SREBP-1) (10). One of the proposed mechanisms by which oxysterols regulate intracellular cholesterol is through their binding to specific proteins (1, 3, 4, 11–13). These binding proteins may then directly participate in the SREBP-mediated regulatory pathway or may indirectly regulate cholesterol metabolism by influencing intracellular cholesterol trafficking.

One such protein, designated "oxysterol binding protein" (OSBP), has been isolated and purified from mouse fibroblasts and hamster liver cytosol (14, 15). The OSBP cDNA was cloned from rabbit (14) and human (16) sources, and the predicted amino acid sequence showed 98% identity between the two species. Although its role in maintaining cholesterol homeostasis is unknown, OSBP is widely expressed in different tissues and will bind oxysterols with affinities that strongly correlate with their ability to repress HMG-CoA reductase activity in cultured cells (17). Whereas there is no evidence that OSBP enters the nucleus or binds to DNA, 25-OH chol binding to OSBP results in the protein's translocation from perinuclear vesicles to the Golgi apparatus (18).

This study was undertaken to determine whether LY295427 interacts with OSBP and/or other protein(s) that bind endogenous oxysterols. Protein fractions from hamster liver subjected to ammonium sulfate precipitation and Q-Sepharose column chromatography, [^{3}H]25-OH chol binding, direct radioligand binding by ^{125}I-labeled LY296480 (a radioiodinated analog of LY295427), and polyclonal antibodies to OSBP were used to characterize the protein(s) that bind to LY295427. In fractions containing partially purified OSBP, LY295427 enhanced [^{3}H]25-OH chol binding in a concentration-dependent manner. In some fractions lacking specific [^{3}H]25-OH chol binding, addition of LY295427 or LY296480 unmasked a second pool of [^{3}H]25-OH chol binding. Specific ^{125}I-labeled LY296480 binding was detected in a single fraction from this second pool. LY295427 had no direct effect on [^{3}H]25-OH chol binding to OSBP, purified to near homogeneity. These data support the idea that LY295427 interacts with oxysterol-binding protein(s), independent of OSBP, to increase their oxysterol binding efficiency. LY295427 may be involved in a novel mechanism to regulate cellular oxysterol pools and ultimately reduce total cellular cholesterol.

MATERIALS AND METHODS

Materials

Golden Syrian male hamsters were purchased from Charles River. Dextran T-500, Q-Sepharose, and Protein A Sepharose were acquired from Pharmacia LKB Biotechnology Inc. Unlabeled 25-hydroxycholesterol (5-cholestene-3β,25-diol), pepstatin A, leupeptin, aprotinin, and phenylmethyl-sulfonyl fluoride (PMSF) were obtained from Sigma. Electrophoresis gels and buffers were purchased from Novex. The Immun-Lite assay kit was a product of Bio-Rad. [^{3}H]25-OH chol (77.0–84.5 Ci/mmol) was from DuPont New England Nuclear. The OSBP antibodies AB21 and W980 were as described in Ridgway et al. (18). LY295427, LY306039, and LY296480 were synthesized at Lilly Research Laboratories as described in Lin et al. (8). Bradford protein reagent was purchased from Bio-Rad. Milli-Q water (Millipore Corp.) was used for all aqueous solutions. All other reagents were of the highest quality commercially available.

Uptake of 25-OH chol in CHO (clone S27B30) cells after pretreatment with LY295427 or LY306039

CHO S27B30 cells (Chinese hamster ovary cells, in which a DNA segment containing the promoter and regulatory control elements of the LDL receptor gene fused to the firefly luciferase reporter gene, S27B30 referring to the subclone) seeded in 24-well dishes were grown to confluency in complete medium (GibcoBRL-DMEM/F-12, 3:1) supplemented with 20 mM HEPES, 50 μg/ml tobramycin, and 5% fetal bovine serum. After washing cells with serum-free medium (5 mg/ml Fraction V bovine serum albumin replacing fetal bovine serum), cells were incubated for 24 h in serum-free medium with no drug (control) or with LY295427 (20 μg/ml) or LY306039 (20 μg/ml). After 24 h pretreatment, cells were washed with serum-free medium and again incubated in serum-free medium containing 25-OH chol (0.5 μg/ml) and [^{3}H]25-OH chol (2×10^5 cpms/well). At indicated times, cells were washed in phosphate-buffered saline (without Ca^{2+} or Mg^{2+}) and solubilized in buffer containing 1% Triton X-100, 1 mM ATP, 25 mM glycylglycine (pH 7.8), 4 mM EGTA, 15 mM

$MgSO_4$, 15 mM KPO_4 (pH 7.8) and 1 mM dithiothreitol. Aliquots were added to liquid scintillation fluid (Ready Protein, Beckman) and radioactivity was counted. Data were expressed as cpm/μg protein. Protein was determined by the method of Bradford (19).

Preparation of hamster liver lysates

Fresh livers from 50 hamsters were rinsed in cold buffer containing 10 mM Tris-HCl, pH 8.3, 5 mM β-mercaptoethanol, 1 mM EDTA, 0.6 μM PMSF, 50 μM leupeptin, 0.5 μg/ml pepstatin A, 0.5 μg/ml aprotinin, and 10% glycerol (buffer A), and blotted dry. The livers were homogenized in 3 volumes (w/v) of buffer A for 30 sec at 4°C using a Tekmar Tissuemizer at high speed. The homogenate was centrifuged at 10,000 *g* for 2 h at 4°C. The supernatant was collected and further fractionated by a 15–50% ammonium sulfate precipitation, followed by centrifugation for 1 h, 4°C at 10,000 *g*. A tight pellet was collected and resuspended with buffer A and diluted to a final conductivity equal to 0.1 M KCl. This preparation was designated "liver cytosol." Protein was determined by the method of Bradford (19).

Anion exchange column chromatography

The liver cytosol preparation was loaded onto a 300 ml Q-Sepharose column. The column was washed with buffer A, and fractions were collected over a linear gradient run from 0 to 1 M NaCl. [^{3}H]25-OH chol binding activity and protein concentrations were determined; fractions containing specific [^{3}H]25-OH chol binding activity in the absence or presence of LY295427 were then subjected to immunoblotting analysis using anti-OSBP antibodies. Fractions that bound [^{3}H]25-OH chol only in the presence of drug were pooled for additional protein purification by column chromatography. For some experiments OSBP was purified to near homogeneity (approximately 3000-fold purification) using the protocol of Dawson et al. (20) through step 6.

Assay for [^{3}H]25-OH cholesterol binding and ^{125}I-labeled LY296480 binding

Specific binding was determined by modifying the dextran-charcoal assay (13). [^{3}H]25-OH chol binding was assayed in 110 μl of buffer A containing 10–100 μg protein and the indicated concentration of [^{3}H]25-OH chol (0.21–300 nM, 85.6 Ci/mmol), in the presence or absence of 10 μM LY295427. Nonspecific binding was determined by incubating a duplicate sample with 1 μM unlabeled 25-OH chol. After incubation at room temperature for 4 h or at 4°C for 12 h, aliquots (85 μl) of the binding reaction were transferred to a new 1.5-ml Eppendorf tube and 50 μl of the 5% dextran/charcoal solution was added and incubated for 30 min at room temperature. After centrifugation at 10,000 *g* in a microfuge for 5 min, 85-μl aliquots were added to scintillation cocktail and quantitated by liquid scintillation spectroscopy. Specific [^{3}H]25-OH chol binding was defined as radioactivity remaining after subtraction of nonspecific binding (binding in the presence of unlabeled 25-OH chol). In routine assays of Q-Sepharose fractions, a 100-μl sample and 24 nM [^{3}H]25-OH chol were used to determine binding activity, and protein was not determined. ^{125}I-labeled LY296480 (a radioiodinated analog of LY295427) binding was assessed in four preparations, using this latter protocol described for [^{3}H]25-OH chol with 100 μl sample and approximately 1 nM ^{125}I-labeled LY296480. Ten μM of either unlabeled LY295427 or LY296480 was used for determination of nonspecific binding. Radioligand binding affinity and receptor density were determined from saturation isotherm data using the nonlinear regression analysis program Lundon-1 (21).

Western blot to OSBP

Western blots were performed on fractions throughout the Q-Sepharose column elution using the polyclonal antibody AB21, raised against amino acids 737–755 of rabbit OSBP. Fractions from the Q-Sepharose column were resolved by 8% SDS-PAGE, and transferred onto a Bio-Rad Immun-Lite blotting membrane. The membranes were blocked with a 5% non-fat dry milk solution (Bio-Rad) and incubated overnight with the AB21 antibody (2.7 μg/ml). After washing the membrane, the AB21 antibody was visualized using an alkaline-phosphatase goat anti-rabbit antibody and a chemiluminescent detection system (Bio-Rad).

Anti-OSBP immunoprecipitation

Q-Sepharose column fractions were incubated with 8.6 μg/ml of W980 IgG, a rabbit polyclonal anti-OSBP antibody (18), for 1 h at 4°C on a rocker platform. The samples were then incubated with Protein A-Sepharose (10% suspension in buffer A) for 1 h at 4°C, transferred to a new 1.5-ml Eppendorf tube, and washed five times with 1 ml of ice-cold buffer A. After the last wash the Protein A-Sepharose was resuspended in 100 μl of Tris-glycine SDS running buffer + 0.5% β-mercaptoethanol (Novex #LC2675-4), boiled for 5 min, and resolved by SDS-PAGE on 8% acrylamide gels. After electrophoresis, the proteins were transferred onto a Bio-Rad Immun-Lite blotting membrane and subjected to immunoblotting as described above.

RESULTS

25-OH cholesterol uptake into S27B30 CHO cells

Control experiments in the absence of drug pretreatment showed that maximal uptake of [^{3}H]25-OH chol

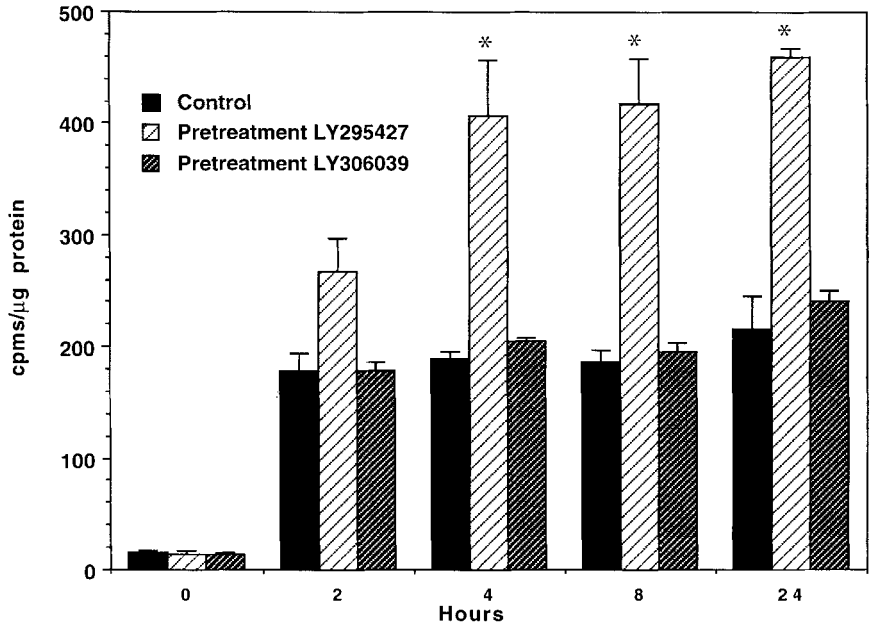

Fig. 1. Comparison of [^{3}H]25-OH cholesterol influx into CHO cells after pre-treatment with LY295427 or LY306039. Confluent CHO cells were incubated in serum-free medium for 24 h with no drug (Control), 20 μg/ml LY295427, or 20 μg/ml LY306039 (the β-isomer of LY295427) as described in Materials and Methods. Compounds were removed, and cells were incubated for 24 h in serum-free medium containing 25-OH cholesterol (0.5 μg/ml) plus [^{3}H]25-OH cholesterol (2×10^5 cpm/well). At indicated times, cells were washed and processed to determine cell-associated protein and radioactivity. Each value represents the mean and standard error of triplicate determinations. $^*P < 0.05$, indicates significant difference from Control cells, determined by unpaired Student's *t* test.

into S27B30 CHO cells was achieved by 2 h and remained constant for 24 h. As shown in **Fig. 1,** pretreatment with LY295427 resulted in significant increases in [^{3}H]25-OH chol uptake at all time points measured. Maximal uptake in pretreated cells was evident by 4 h and remained constant for 24 h. 25-OH chol uptake in cells with LY306039, the β-isomer of LY295427, was not significantly different from uptake in control cells at any time point.

25-OH cholesterol binding in protein fractions separated by Q-Sepharose column chromatography

[^{3}H]25-OH chol binding activity could not be accurately measured in hamster liver cytosolic extracts prior to ammonium sulfate fractionation because of high nonspecific binding. Analysis of liver cytosolic fractions precipitated with increasing percentages of ammonium sulfate indicated the greatest specific [^{3}H]25-OH chol binding was in the 15–50% precipitate. The 15–50% ammonium sulfate precipitate was then chromatographed on a Q-Sepharose column, and these fractions were used for subsequent [^{3}H]25-OH chol binding and immunoblotting studies. The results are shown from one of four separate hamster liver preparations subjected to Q-Sepharose column chromatography (**Fig. 2**). The fractions that displayed [^{3}H]25-OH chol binding activity and bound anti-OSBP antibody were very similar in all four preparations.

A chromatograph from a representative study is shown in Fig. 2A. Fractions were assayed for [^{3}H]25-OH chol binding activity. As indicated in Fig. 2B, specific [^{3}H]25-OH chol binding was evident only in certain fractions: 25, 30, 33, and 36. Unlabeled 25-OH chol inhibited binding in these fractions in a concentration-dependent manner with an IC_{50} of 0.1 ± 0.03 μM (n = 5), identical to the concentration of 25-OH chol found by Dawson et al. (20) to inhibit by 50% binding of [^{3}H]25-OH chol to partially purified OSBP. In contrast to the unlabeled 25-OH chol, LY295427 increased specific [^{3}H]25-OH chol binding in fractions 25–36. Whereas no [^{3}H]25-OH chol binding was evident in fractions 1–24 and 39–48 in the absence of LY295427, addition of 10 μM LY295427 resulted in increasingly detectable [^{3}H]25-OH chol binding in fraction 20 and fractions 39, 42, and 45. No specific [^{3}H]25-OH chol binding in the absence or presence of LY295427 was detected in fractions 51–90. The [^{3}H]25-OH chol bind-

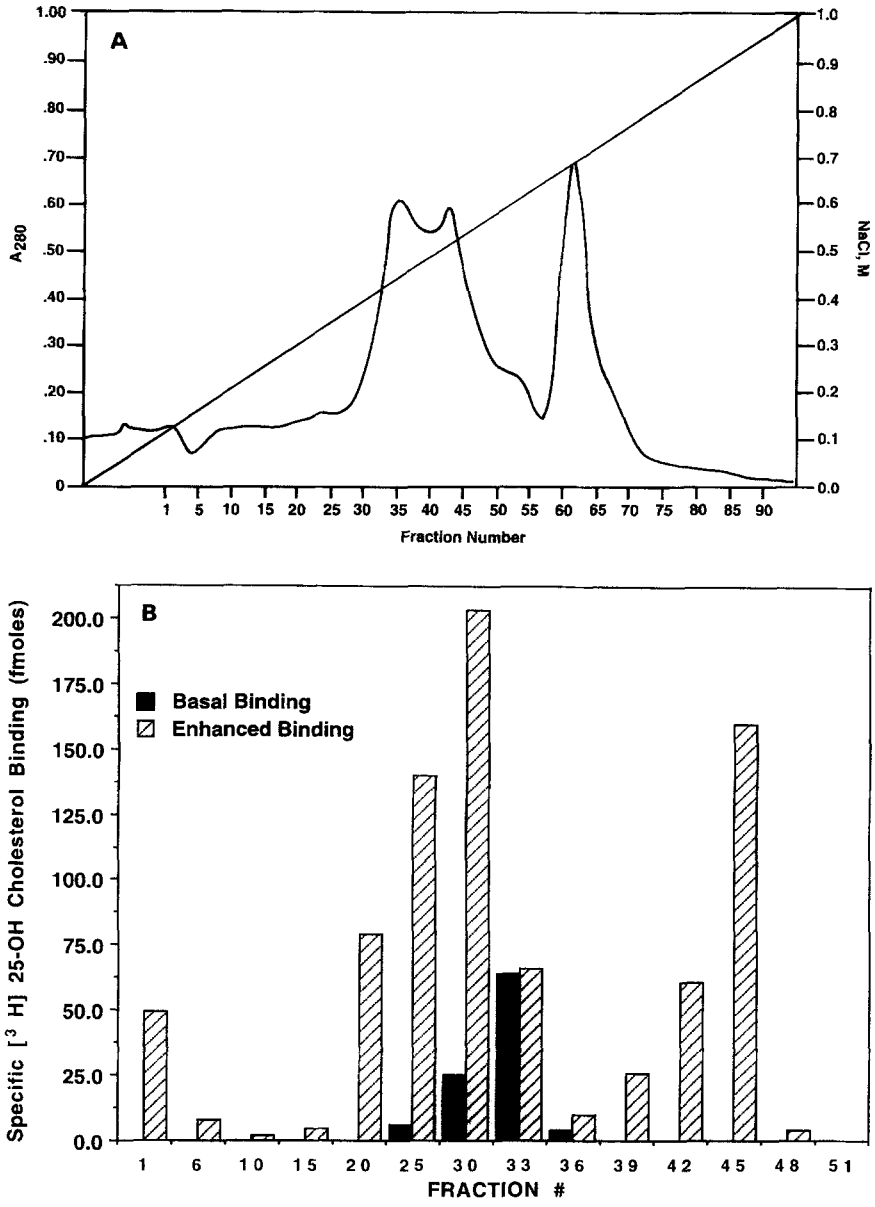

Fig. 2. A: Elution profile of protein fractions from Q-Sepharose anion exchange column. Liver cytosol preparations were applied to an anion exchange column as described in Materials and Methods. Fractions were collected for subsequent determination of [^{3}H]25-OH cholesterol binding activity. Solid line indicates NaCl molarity gradient of 0.0–1.0 M. B: [^{3}H]25-OH cholesterol binding activity in protein fractions in the presence and absence of LY295427. Protein fractions eluted from the Q-Sepharose column (shown in A) were assayed for [^{3}H]25-OH cholesterol binding activity using the dextran/charcoal assay. Each assay tube contained 100 µl protein and 20 nM [^{3}H]25-OH cholesterol in the presence or absence of 1 µM unlabeled 25-OH cholesterol or 10 µM LY295427. Basal binding represents specific [^{3}H]25-OH cholesterol binding in the absence of LY295427; enhanced binding represents specific [^{3}H]25-OH cholesterol binding in the presence of LY295427. No basal [^{3}H]25-OH cholesterol binding was detected in fractions 1–24, and no basal or enhanced binding was detected in fractions 51–90. [^{3}H]25-OH cholesterol binding in the presence of LY295427 was detected in two distinct pools, occurring in fractions with and without specific [^{3}H]25-OH cholesterol binding activity in the absence of drug [referred to in text as (+) fractions and (−) fractions, respectively].

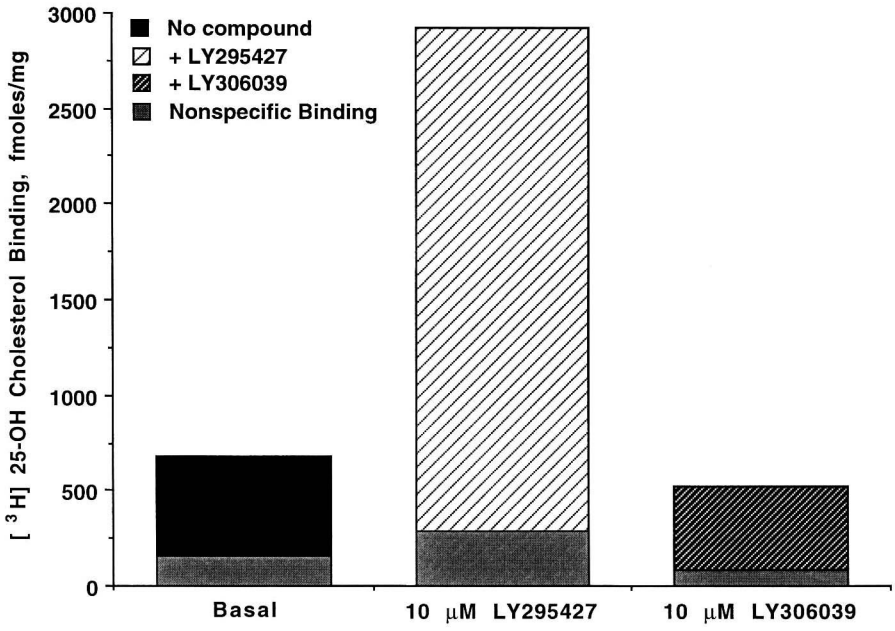

Fig. 3. Stereospecificity of enhanced [^{3}H]25-OH cholesterol binding in the presence of LY295427 and LY306039. Aliquots from a Q-Sepharose column fraction containing specific [^{3}H]25-OH cholesterol binding [(+) fractions] were assayed in the absence (Basal) or presence of 10 μM LY295427 or 10 μM LY306039, the β-isomer of LY295427. To determine specific [^{3}H]25-OH cholesterol binding, each assay tube contained 100 μg protein, 20 nM [^{3}H]25-OH cholesterol, and vehicle or compound. Nonspecific binding was determined with 1 μM unlabeled 25-OH cholesterol as described in Materials and Methods. Values are the means of 3 to 5 experiments.

ing observed in the presence of LY295427 in fraction 1 was likely due to protein in the column wash.

Characteristics of enhanced [^{3}H]25-OH cholesterol binding in the presence of LY295427

After separation by Q-Sepharose anion exchange chromatography, specific [^{3}H]25-OH chol binding activity in the absence of LY295427, referred to as "basal binding", consistently appeared in fractions collected early from the first peak. After addition of LY295427, [^{3}H]25-OH chol binding in these fractions was enhanced; the fractions were designated (+) fractions. Incubation of a (+) fraction, containing specific basal [^{3}H]25-OH chol binding, with 10 μM LY295427 resulted in up to 5-fold increases in binding activity (Fig. 2B and **Fig. 3**). However, [^{3}H]25-OH chol binding in the presence of LY295427 was revealed in a second distinct pool of fractions. These fractions, designated (−) fractions, exhibited [^{3}H]25-OH chol binding only in the presence of drug and were without basal [^{3}H]25-OH chol binding activity (measured in the absence of drug).

Enhanced [^{3}H]25-OH chol binding in the presence of LY295427 was stereospecific. LY306039, the β-isomer of LY295427 that did not increase [^{3}H]25-OH chol uptake into CHO cells (Fig. 1), had no effect on [^{3}H]25-OH chol binding. As shown in Fig. 3, only LY295427 increased [^{3}H]25-OH chol binding above basal levels in (+) fractions. Addition of 10 μM LY306039 to (−) fractions, in which there was no basal [^{3}H]25-OH chol binding and binding activity was detectable only in the presence of LY295427, did not result in any specific [^{3}H]25-OH chol binding (data not shown).

Increases in [^{3}H]25-OH chol binding activity in the presence of LY295427 that were evident in (−) fractions, as well as in (+) fractions, were dependent on concentration of drug, and all binding activity was destroyed by boiling samples. As shown in **Fig. 4,** [^{3}H]25-OH chol binding in pooled fractions 30–31 [(+) fractions, with both basal and enhanced [^{3}H]25-OH chol binding] and fractions 44–45 [(−) fractions, with binding only in the presence of drug] in the presence of 0.001–10 μM LY295427 were similar. Within the sample variation seen between fractions from different preparations, maximum binding was achieved at 0.5–10 μM LY295427. Concentrations of LY295427 that increased binding by 50% in (+) and (−) fractions were 34 ± 1 nM (n = 4) and 36 ± 16 nM (n = 4), respectively. [^{3}H]25-OH chol binding in both the absence and presence of LY295427 was protein dependent. The linearity of [^{3}H]25-OH chol binding in the presence of increasing concentrations of protein is shown in **Fig. 5.**

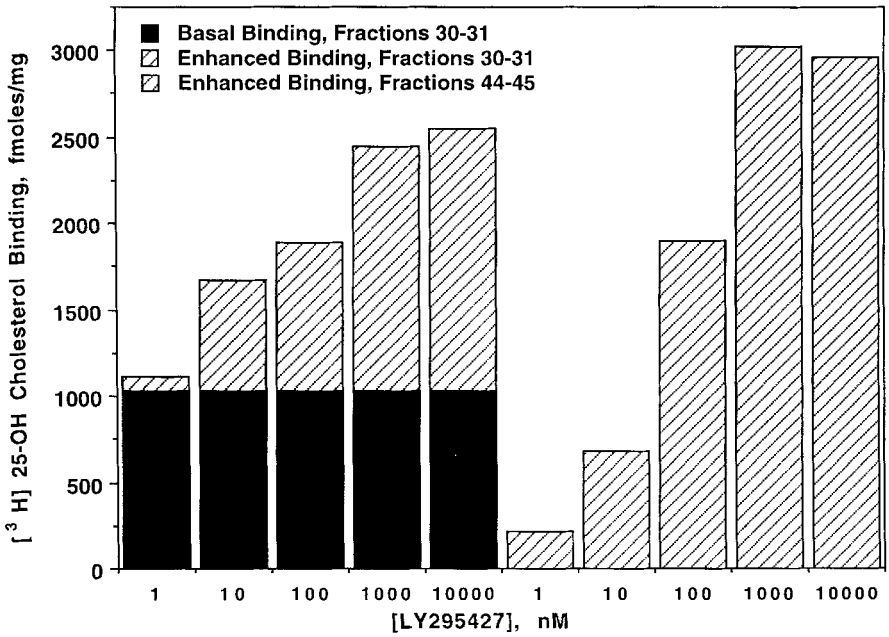

Fig. 4. Effects of increasing concentrations of LY295427 on enhancement of [^{3}H]25-OH cholesterol binding. Aliquots from fractions 30 and 31 [(+) fractions, which exhibit [^{3}H]25-OH cholesterol binding in the absence of LY295427] and from 44 and 45 [(−) fractions, which exhibit no [^{3}H]25-OH cholesterol binding in the absence of LY295427] from a representative study (Fig. 2B) were used to assess [^{3}H]25-OH cholesterol binding in the presence of the indicated concentrations of LY295427. Protein concentrations were adjusted to 1 mg/ml; each assay tube contained 50 μg protein, 20 nM [^{3}H]25-OH cholesterol, and vehicle or LY295427 in the absence or presence of 1 μM unlabeled 25-OH cholesterol (dextran/charcoal assay described in Materials and Methods). Total specific [^{3}H]25-OH cholesterol binding in the presence of LY295427 in the (+) fractions is represented on the left hand side of the graph as the sum of basal binding and enhanced binding. [^{3}H]25-OH cholesterol binding in the presence of LY295427 in the (−) fraction is represented on the right hand side of the graph. There is no basal [^{3}H]25-OH cholesterol binding in the (−) fraction.

Saturation binding experiments using [^{3}H]25-OH chol were done using appropriate pooled fractions with radioligand concentrations of 0.1–200 or 300 nM. Each experiment represents fractions from a single hamster cytosol preparation. Saturation isotherms and Scatchard analysis from representative experiments of [^{3}H]25-OH chol binding in the absence and presence of LY295427 are presented in **Fig. 6.** Lundon-1 analysis of (+) fractions exhibiting specific [^{3}H]25-OH chol binding in the absence of LY295427 determined that a one-site model provided the best fit for binding, with a K_d = 37 ± 20 nM and B_{max} = 4.5 ± 1.8 pmol/mg (n = 3). In these (+) fractions, addition of LY295427 increased both the K_d and B_{max} to 119 ± 37 nM and 20.3 ± 4.4 pmol/mg (n = 3), respectively, with a single-site model representing the best fit. (−) Fractions in which [^{3}H]25-OH chol binding was evident only in the presence of LY295427 displayed complex binding parameters. Radioligand concentrations of 0.1–310 nM were used to estimate binding parameters. Under these conditions, an apparent non-saturable low affinity site was detected in two of the five experiments. A K_d of 143 ± 43 nM and B_{max} of 49.7 ± 15 pmol/mg was determined for the single high affinity [^{3}H]25-OH chol binding site of the (−) fractions. Compared to [^{3}H]25-OH chol binding in the absence of LY295427, increase in binding site density in the presence of drug was significant ($P < 0.05$) in both (−) and (+) fractions. Whereas the lower binding affinity of [^{3}H]25-OH in the presence of drug in both (−) and (+) fractions was apparent, statistical significance did not reach <0.05 compared to the K_d values obtained in the absence of drug.

Interaction of Q-Sepharose fractions with OSBP antibodies

To investigate whether fractions exhibiting specific [^{3}H]25-OH chol binding activity contained OSBP, samples from fractions were incubated with antibody AB21, a polyclonal antibody raised against the COOH terminus region of the protein containing the oxysterol binding region (18). Depending on phosphorylation state, OSBP appears as a singlet or a doublet of 96 kDa and 101 kDa (18, 20). The fractions with [^{3}H]25-OH chol binding activity shown in Fig. 2 were used in one such

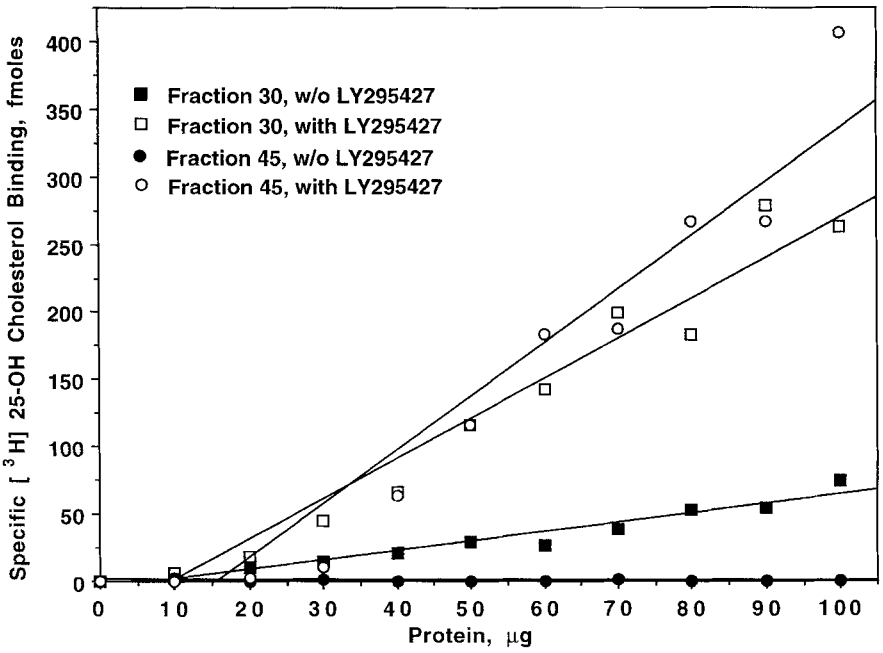

Fig. 5. Effects of increasing protein concentrations on [^{3}H]25-OH cholesterol binding in the absence and presence of LY295427. Aliquots from a (+) fraction (which exhibits [^{3}H]25-OH cholesterol binding in the absence of LY295427) and a (−) fraction (which exhibits no [^{3}H]25-OH cholesterol binding in the absence of LY295427) (#30 and #45; see Fig. 2B), containing 10–100 µg protein were incubated with 20 nM [^{3}H]25-OH cholesterol in the absence or presence of 10 µM LY295427 and 1 µM 25-OH cholesterol. Specific [^{3}H]25-OH cholesterol binding increased linearly with increasing amounts of protein in fractions 30 and 45 in the presence of LY295427 and in fraction 30 in the absence of compound. No specific [^{3}H]25-OH cholesterol binding was evident in fraction 45 in the absence of LY295427.

experiment. As shown in **Fig. 7,** proteins corresponding to one or both OSBP bands were present in fractions 25, 30, 33, 36, 48, and faintly in 42 and 45.

Whereas fractions 30 and 33 exhibited specific [^{3}H]25-OH chol binding in the absence of LY295427, fractions 25, 36, 42, 45, and 48 exhibited little or no binding in the absence of LY295427. Similar results were obtained with three other hamster liver cytosolic preparations. Thus, after Q-Sepharose chromatography, OSBP always coincided with specific [^{3}H]25-OH chol binding in the absence of LY295427; however the appearance of OSBP was not limited to those fractions and may be present in fractions exhibiting [^{3}H]25-OH chol binding only in the presence of LY295427 (enhanced binding).

Several other higher molecular weight bands were evident on the autoradiogram. Of particular interest was a band of approximately 170 kDa, which appeared in fractions 42, 45, and 48, all fractions that had no detectable [^{3}H]25-OH chol binding in the absence of LY295427. Whereas fractions 42 and 45 exhibited [^{3}H]25-OH chol binding activity in the presence of LY295427, fraction 48 displayed only minimal binding in the presence of drug. In contrast, fraction 10, which like fraction 48 exhibited minimal binding in the presence of LY295427 and no binding in the absence of drug, did not contain the 170 kDA band. Also, enhanced [^{3}H]25-OH chol binding was easily measurable in fractions 1, 25, and 30 in the presence of drug, and these fractions did not contain the 170 kDa band.

Two fractions were selected, one that contained [^{3}H]25-OH chol binding activity in the absence and presence of LY295427 (#30) and one that displayed binding only in the presence of drug (#42). Half of each fraction was subjected to OSBP immunodepletion using the polyclonal antibody W980 (18, 20). The immunodepleted and the non-precipitated samples were resolved by SDS-PAGE and analyzed by immunoblotting using the anti-OSBP polyclonal antibody AB21 (18, 20).

As shown in **Fig. 8,** immunoblot analysis of fraction 30 using antibody AB21 revealed a single band (molecular mass 90–100 kDa) corresponding to OSBP. This band was undetectable in fraction 30 after immunodepletion with antibody W980. Whereas a 90–100 kDa band corresponding to OSBP was not detectable in fraction 42, a higher molecular weight band of approximately 170 kDa was observed. This 170 kDa band was removed after immunoprecipitation with antibody W980. Thus, both

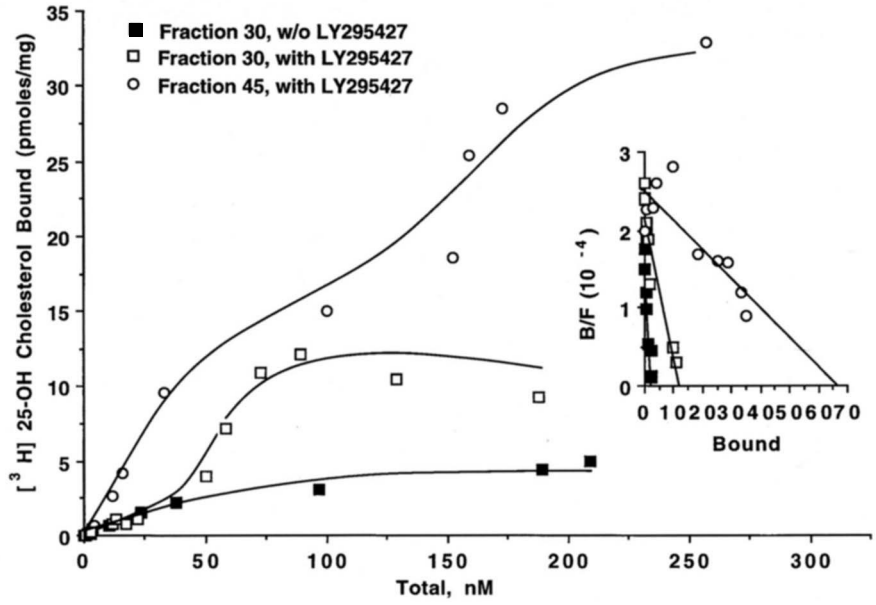

Fig. 6. Binding isotherms and Scatchard analysis of [^{3}H]25-OH cholesterol binding data from 3 representative experiments. Aliquots from (−) fractions (which exhibit no [^{3}H]25-OH cholesterol binding in the absence of LY295427) and (+) fractions (which exhibit [^{3}H]25-OH cholesterol binding in the absence of LY295427) were assayed for specific [^{3}H]25-OH cholesterol binding in the absence or presence of LY295427. In order to compare binding activity in the absence and presence of drug, (+) fraction samples were assayed in parallel. Each reaction tube contained 100 µg protein, 0.2–300 nM [^{3}H]25-OH cholesterol, and vehicle, 10 µM LY295427, or 1 µM cold 25-OH cholesterol (dextran/charcoal assay described in Materials and Methods). The K_d and B_{max} for a (+) fraction (fraction 30) without LY295427 were 21.4 nM and 2.4 pmol/mg, respectively. In the presence of LY295427, the K_d and B_{max} for the (+) fraction (fraction 30) were 53.6 nM and 11.4 pmol/mg, respectively. The K_d and B_{max} values for a (−) fraction (fraction 45), in the presence of LY295427, were 245.2 nM and 66.6 pmol/mg, respectively. Scatchard analysis indicated a possible non-saturated low affinity binding site.

the 90–100 kDA and 170 kDa proteins contained sequences that cross-reacted with antibodies raised against OSBP; but only fraction 30, containing the 90–100 kDa OSBP, exhibited [^{3}H]25-OH chol binding activity in the absence of LY295427.

After immunodepletion with antibody W980, the supernatants from fractions 30 and 42 were assayed for [^{3}H]25-OH chol specific binding in the absence or presence of LY295427 (**Table 1**). After depleting OSBP from fraction 30, no [^{3}H]25-OH chol binding was observed in the absence of LY295427, and approximately 90% of [^{3}H]25-OH chol binding in the presence of drug in fraction 42 was lost. However, [^{3}H]25-OH chol binding was still detected in the presence of drug in both fractions, supporting the idea that there was specific binding to protein(s) not precipitated by anti-OSBP antibody.

To determine whether LY295427 directly affected the binding of [^{3}H]25-OH chol to OSBP, the protein was purified to near homogeneity and used for competition

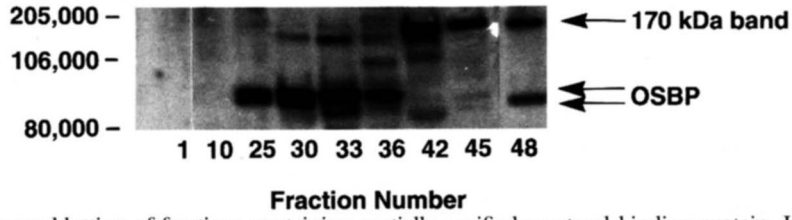

Fig. 7. Immunoblotting of fractions containing partially purified oxysterol binding protein. Hamster liver cytosol fractionated by ammonium sulfate precipitation and Q-Sepharose chromatography was assayed for specific [^{3}H]25-OH cholesterol binding activity (see Fig. 2). Aliquots from the indicated fractions (5–15 µg protein per lane) were subjected to 8% SDS-polyacrylamide gel electrophoresis, transferred to blotting membrane, and incubated with antibody AB21. The AB21 antibody was detected using alkaline phosphatase-conjugated goat anti-rabbit antibody. After incubation with chemiluminescent substrate, the immunoblot was exposed on Kodak XAR film for 15 min. The 96/101 kDa OSBP doublet is denoted by arrows.

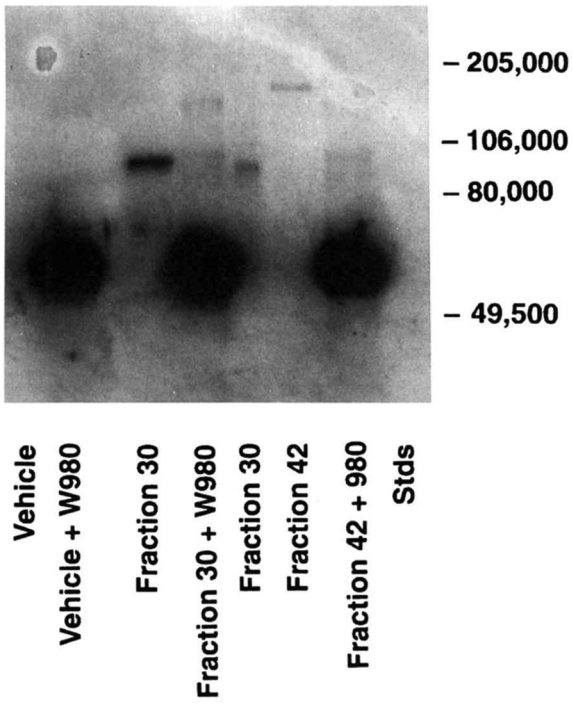

Fig. 8. Immunoblot of proteins reacting with OSBP antibody AB21 after incubation with OSBP immunoprecipitating antibody W980. Fractions 30 and 42 (see Fig. 2) were selected to further explore the reactivity of OSBP with proteins eluted from the Q-Sepharose column. Aliquots (15 μg protein per lane) from fraction 30 (specific [^{3}H]25-OH cholesterol binding in the absence and presence of LY295427) and fraction 45 (specific [^{3}H]25-OH cholesterol binding only in the presence of LY295427) and a buffer (vehicle) without protein were immunoprecipitated using the anti-OSBP antibody W980. Samples and aliquots of fractions 30, 45, and vehicle that had not been immunoprecipitated were analyzed by 8% SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-OSBP antibody AB21. The AB21 antibody was detected using alkaline phosphatase-conjugated goat anti-rabbit antibody. After incubation with chemiluminescent substrate, the immunoblot was exposed to Kodak XAR film for 15 min. One band, corresponding to the 90–100 kDa molecular weight of purified OSBP, was evident in fraction 30 (not immunoprecipitated). One band of molecular weight of approximately 170 kDa was evident in fraction 42 (not immunoprecipitated). In both fractions 30 and 42, after immunoprecipitation with antibody W980, no bands are evident.

binding studies. As shown previously (14), unlabeled 25-OH chol competed effectively with partially purified OSBP for [^{3}H]25-OH chol binding. After separation by Q-Sepharose column chromatography, hamster cytosol fractions containing OSBP exhibited enhanced [^{3}H]25-OH chol binding in the presence of LY295427 (Fig. 4). With further purification of OSBP, the ability of unlabeled 25-OH chol to compete for [^{3}H]25-OH chol binding was not diminished, but LY295427's ability to enhance [^{3}H]25-OH chol binding was lost (**Fig. 9**).

Addition of a phenyl ring to LY295427 allowed for the synthesis of an iodinated analog (LY296480). LY296480 increased [^{3}H]25-OH chol binding to the same extent as LY295427, but only in the (−) fractions (data not shown). Radioiodination of LY296480 allowed for direct binding to cytosolic protein(s) in the individual (−) fractions comprising the second pool of enhanced [^{3}H]25-OH chol binding activity (comparable to fractions 40–48 in Fig. 2). In each of four separate preparations, specific ^{125}I-labeled LY296480 binding was limited to one of the (−) fractions, and there was no correlation between ^{125}I-labeled LY296480 binding and extent of enhanced [^{3}H]25-OH chol binding in that fraction. Moreover, no ^{125}I-labeled LY296480 binding was detected in a (+) fraction containing [^{3}H]25-OH chol binding in the absence of LY295427. **Table 2** summarizes these binding data from the fractions in which both enhanced [^{3}H]25-OH chol binding and ^{125}I-labeled LY296480 binding were detected. Further isolation and purification of the protein to which ^{125}I-labeled LY296480 bound has been undertaken.

DISCUSSION

In this study, we report that LY295427, a 3α-hydroxysterol, selectively enhances binding of [^{3}H]25-OH chol in hamster liver cytosolic extracts. The [^{3}H]25-OH chol binding in the presence of LY295427 was protein dependent, saturable, and stereospecific, thus confirming that the drug's effects were not due to nonspecific binding of endogenous or exogenously added oxysterols.

As determined from immunoblot analysis, the Q-Sepharose fractions that exhibited specific [^{3}H]25-OH chol binding in the absence of LY295427 contained one or both of the 90–100 kDa forms of OSBP; however, OSBP was also present in protein fractions that lacked detectable [^{3}H]25-OH chol binding (Fig. 7, fraction 48). The reason for lack of binding activity is not clear. One possibility is that this fraction contained OSBP that was already bound to endogenous oxysterols. As demonstrated in earlier purification studies (20), 25-OH chol bound to OSBP exchanges very slowly in vitro. In fractions that bound 25-OH chol in the absence of LY295427, several lines of evidence suggest that the [^{3}H]25-OH chol binding was due to OSBP. The binding site density and affinity for [^{3}H]25-OH chol were in close agreement with values previously reported by Dawson et al. (20). In addition, immunodepletion using anti-OSBP antibody W980 resulted in complete loss of [^{3}H]25-OH chol binding activity in the supernatant from these fractions (see Table 1, fraction 30). In contrast, after precipitation of 90–100 kDa forms of OSBP, [^{3}H]25-OH chol binding in the presence of LY295427 was not affected (Table 1, fraction 30), supporting the

TABLE 1. [^{3}H]25-OH cholesterol binding from two Q-Sepharose-eluted fractions subjected to OSBP precipitating antibody W980

	[^{3}H]25-OH Cholesterol Binding[a]			
	− W980[b]		+ W980[c]	
Fraction	− LY295427	+ LY295427	− LY295427	+ LY295427
	dpm		*dpm*	
30[d] (+ OSBP)	24424	30191	nb	5098
42[e] (− OSBP)	nb	53731	nb	5132

[a]Binding in the absence (−) and presence (+) of LY295427 was determined before and after interaction with antibody W980, using approximately 25 nM [^{3}H]25-OH cholesterol, as described in Materials and Methods. Nonspecific binding was determined from radioactivity remaining in the presence of 1 μM 25-OH cholesterol; nb, no specific binding.

[b][^{3}H]25-OH cholesterol binding before immunoprecipitation with W980.

[c][^{3}H]25-OH cholesterol binding in supernatants of immunoprecipitated samples after removal of OSBP with W980.

[d]Fraction 30 contained the 90–100 kDa form of OSBP and exhibited [^{3}H]25-OH cholesterol binding in the absence of LY295427.

[e]Fraction 42 did not contain the 90–100 kDa form of OSBP and did not exhibit [^{3}H]25-OH cholesterol binding in the absence of LY295427.

idea that LY295427 bound to cytosolic protein(s) other than OSBP. Moreover, when OSBP was purified to near homogeneity, [^{3}H]25-OH chol binding was not significantly increased or displaced in the presence of LY295427 (Fig. 9).

Immunoblot analysis of some Q-Sepharose fractions that bound [^{3}H]25-OH chol only in the presence of LY295427 revealed a 170 kDa band but not the 90–100 kDa monomeric form of OSBP. This 170 kDa band was also precipitated with OSBP antibody W980 (Fig. 8). After immunodepletion with the anti-OSBP antibody, greater than 90% of [^{3}H]25-OH chol binding activity (in the presence of LY295427) was abolished (Table 1, fraction 44), suggesting that the 170 kDa band was inter-

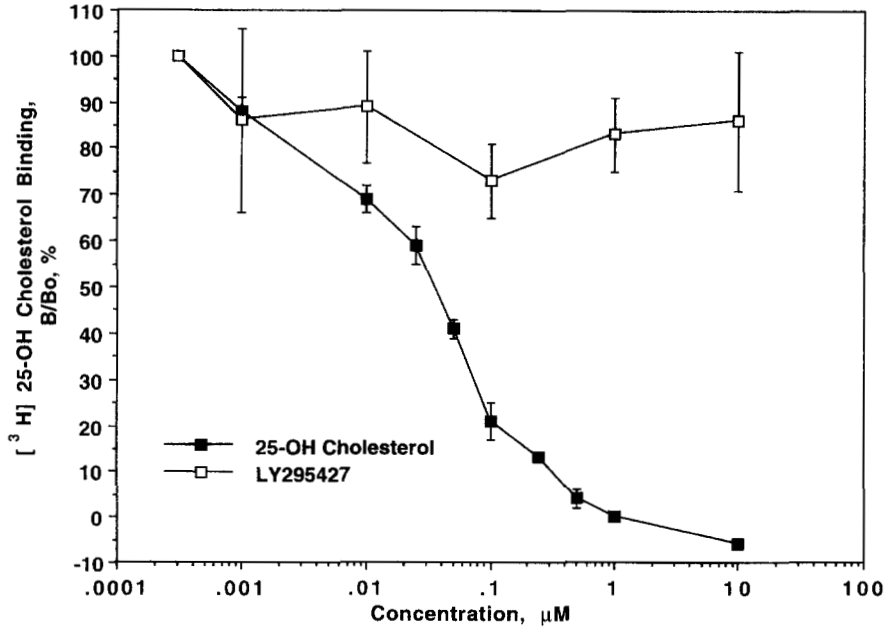

Fig. 9. Effect of LY295427 on specific [^{3}H]25-OH cholesterol binding activity to OSBP. OSBP was isolated using ion exchange chromatography as described under Materials and Methods and assayed for [^{3}H]25-OH cholesterol binding with increasing concentrations of unlabeled 25-OH cholesterol or LY295427. Each assay tube contained 50 μg protein, 20 nM [^{3}H]25-OH cholesterol, and vehicle, or increasing concentrations of unlabeled 25-OH cholesterol or LY295427 (0.0003–10 μM). [^{3}H]25-OH cholesterol binding activity in the absence of competing ligand was set at 100%. Values are the means ± SEM of 3 experiments.

TABLE 2. Specific [^{3}H]25-OH cholesterol binding and ^{125}I-labeled LY296480 binding from four Q-Sepharose-eluted fractions

Fraction[a]	[^{3}H]25-OH Cholesterol[b]		^{125}I-Labeled LY296480[c]
	− LY295427	+ LY295427	
	dpm		*dpm*
1 (− OSBP)	nb	7984	4253
2 (− OSBP)	nb	3039	2102
3 (− OSBP)	nb	43940	2606
4 (− OSBP)	nb	30511	3483

[a]Arbitrary numeral designations for hamster liver cytosol fractions (obtained from Q-Sepharose anion exchange chromatography) contained both [^{3}H]25-OH cholesterol and ^{125}I-labeled LY296480 binding.

[b][^{3}H]25-OH cholesterol binding in the absence (−) and presence (+) of LY295427 was determined using approximately 25 nM [^{3}H]25-OH cholesterol, as described in Materials and Methods. Nonspecific binding was determined from radioactivity remaining in the presence of 1 μM 25-OH cholesterol; nb, no [^{3}]25-OH cholesterol binding detected in the absence of LY295427.

[c]^{125}I-labeled LY296480 binding was determined using approximately 1 nM ^{125}I-labeled LY296480, as described in Materials and Methods. Nonspecific binding was determined from radioactivity remaining in the presence of 10 μM LY295427 or LY296480.

acting with LY295427 in some way to increase binding activity. While all proteins subjected to PAGE were reduced, it is possible that incomplete reduction of an OSBP dimer, oligomer, or heterologous complex might result in a 170 kDa band. With gel filtration, the 90–100 kDa form of OSBP (derived from hamster liver) forms an oligomer of 280 kDa (20). In the mouse, OSBP exhibits a molecular weight of 236 kDa that dissociates to 169 kDa (22). Srinivasan, Patel, and Thompson (23) also reported that the human OSBP, overexpressed in baculovirus, was present in a large molecular weight form that may contain nonsterol binding components. In this regard, OSBP may be similar to the complex of the inactive glucocorticoid receptor and the 90 kDa heat shock protein (hsp90) (24, 25). Thus, it is possible that OSBP is contained within the 170 kDa protein in some fractions that exhibit enhanced [^{3}H]25-OH chol binding only in the presence of LY295427. The lower binding affinity and higher binding capacity for [^{3}H]25-OH chol in the presence of the drug are consistent with the unmasking of new binding sites either in an OSBP-containing complex or in other cytosolic protein(s). In this regard, the role of other cytosolic protein(s) is further supported by the following observations. *1*) Neither the 90–100 kDa form of OSBP or the 170 kDA protein was detected in some fractions that still bound [^{3}H]25-OH chol in the presence of LY295427 (Fig. 2B and Fig. 7, fraction #1). *2*) Specific binding of ^{125}I-labeled LY296480 was not evident in all (−) fractions (those exhibiting [^{3}H]25-OH chol binding only in the presence of LY295427), suggesting that the compound was binding to a particular protein isolated from a single fraction.

LY295427 acts through mechanisms that derepress the LDL receptor in the presence of 25-OH chol (8) and ultimately increases LDL receptor mRNA (R.A. Gadski, unpublished results). Recently, two proteins that bind to the sterol regulatory element in the promoter region of the LDL receptor gene have been identified (10, 26, 27). Sterol regulatory element binding protein 1 and 2 (SREBP-1, SREBP-2) are novel members of the basic-helix-loop-helix-leucine zipper family of transcription factors. The SREBPs are synthesized as larger precursors that are embedded in the membrane of the endoplasmic reticulum. In the absence of sterols the SREBPs are cleaved, releasing the amino-terminal basic-helix-loop-helix-leucine zipper domain that activates LDL receptor transcription (5). This proteolytic processing is under the negative control of sterols (such as 25-OH chol), thereby blocking the activation of LDL receptor gene transcription. Pretreatment with LY295427, which resulted in significantly increased 25-OH chol influx into these cells, did not translate into the expected decrease in LDL receptors, but rather resulted in derepression of transcription of the LDL receptor promoter (8). The observation that LY295427 was effective in blocking the effects of exogenously added oxysterol argues that the compound is acting at a step between oxysterol production and the SREBPs and/or other factors that regulate transcription.

In summary, LY295427, a compound that effectively lowers serum cholesterol, was found to inhibit repression of the LDL receptor in the presence of exogenously added 25-OH chol and to interact with liver cytosolic proteins to increase the binding of [^{3}H]25-OH chol. Specific protein fractions, separated by Q-Sepharose chromatography, exhibited enhanced [^{3}H]25-OH chol binding in the presence of LY295427. Enhanced [^{3}H]25-OH chol binding activity in the presence of drug was still evident in distinct fractions after immunodepletion of the 90–100 kDa form of OSBP and a 170 kDa protein by an OSBP-specific antibody. However,

increased [^{3}H]25-OH chol binding in the presence of LY295427 was not limited to fractions containing the monomeric forms of OSBP or the 170 kDa protein. These data suggest that LY295427 acts through mechanisms that involve select cytosolic proteins that ultimately regulate gene transcription of the LDL receptor.

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