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ΕΡΓΑΣΤΗΡΙΟ ΒΙΟΧΗΜΕΙΑΣ

Διδακτορική διατριβή

ΜΗΧΑΝΙΣΜΟΙ ΜΕΤΑΓΡΑΦΙΚΗΣ ΡΥΘΜΙΣΗΣ ΤΟΥ ΓΟΝΙΔΙΟΥ SR-BI ΤΟΥ ΑΝΘΡΩΠΟΥ

Σοφία Μαυρίδου

Επιβλέπων Καθηγητής: Δ. Καρδάσης

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Στον άντρα μου, Σταύρο

και στον γιό μας, Φίλιππο.

Μετά από μία πορεία έξι χρόνων στο εργαστήριο του Δημήτρη Καρδάση, όπου πραγματοποίησα την μεταπτυχιακή μου εργασία και την διδακτορική μου διατριβή, μόνο καλά πράγματα έχω να θυμάμαι. Η πορεία μου αυτή δεν θα ήταν τόσο ουσιώδης, επιμορφωτική και ευχάριστη χωρίς την συμβολή κάποιων ανθρώπων τους οποίους οφείλω να ευχαριστήσω.

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Contents

ABSTRACT

Scavenger receptor class B type I (SR-BI) facilitates the reverse transport of excess cholesterol from peripheral tissues to the liver via high-density lipoproteins. In steroidogenic tissues, SR-BI supplies cholesterol for steroid hormone production. In the present thesis, we focused on the role of hormone nuclear receptors in human SR-BI gene regulation in hepatic, adrenal and ovarian cells. We show that the transcription of the human SR-BI gene is subject to feedback inhibition by glucocorticoids in adrenal and ovarian cells. SR-BI mRNA levels were increased in adrenals from corticosterone-insufficient mice, whereas corticosterone replacement by oral administration inhibited SR-BI gene expression in these mice. SR-BI mRNA levels were increased in adrenals from wild-type mice treated with metyrapone, a drug that blocks corticosterone synthesis. Experiments in adrenocortical H295R and ovarian SKOV-3 cells using cycloheximide and siRNA-mediated gene silencing revealed that glucocorticoid-mediated inhibition of SR-BI gene transcription requires *de novo* protein synthesis and the glucocorticoid receptor (GR). No direct binding of GR to the SR-BI promoter could be demonstrated *in vitro* and *in vivo*, suggesting an indirect mechanism of repression of SR-BI gene transcription by GR in adrenal cells. Deletion analysis established that the region of the human SR-BI promoter between nucleotides -201 and -62 is sufficient to mediate repression by

glucocorticoids. This region contains putative binding sites for transcriptional repressors that could play a role in SR-BI gene regulation in response to glucocorticoids.

In hepatic cells, SR-BI is regulated by an interplay of multiple hepatocyte-specific transcription factors such as Hepatocyte Nuclear Factor 3β (HNF-3β or FOXA2) and the orphan nuclear receptor Hepatocyte Nuclear Factor 4 (HNF-4). Using the human hepatoblastoma HepG2 cells and a model system, we showed that the activity of the hSR-BI promoter is subject to negative regulation by HNF-4. These data are in agreement with previous studies which had shown that inactivation of the HNF-4 gene in the mouse is associated with a drastic increase in hepatic SR-BI levels. Silencing of the endogenous HNF-4 gene in HepG2 cells by shRNA increased the SR-BI mRNA levels whereas overexpression of HNF-4 repressed endogenous SR-BI gene expression. Promoter deletion analysis established that the region of the human SR-BI promoter between nucleotides -201 and -62 is sufficient to mediate repression by HNF-4. By *in vitro* and *in vivo* protein-DNA interaction assays we demonstrated recruitment of HNF-4 to multiple sites on the hSR-BI promoter, suggesting a direct mechanism of repression of SR-BI gene transcription by HNF-4 in the liver.

In summary, this is the first report showing that glucocorticoids suppress SR-BI gene expression in steroidogenic tissues suggesting that these tissues maintain steroid hormone homeostasis by prohibiting SR-BI-mediated high-density lipoprotein cholesterol uptake when the endogenous levels of glucocorticoids are elevated. This is also the first report showing that HNF-4, a known transcriptional activator of apolipoprotein gene expression in the liver, acts as a direct negative regulator of SR-BI activity via a direct mechanism.

Understanding in depth the molecular mechanisms that regulate SR-BI gene expression in steroidogenic tissues and in the liver and the role of specific transcription factors might offer new therapeutic avenues for the prevention or treatment of atherosclerosis and might be useful in assessments of risk factors and drug efficacy.

ΠΕΡΙΛΗΨΗ

Ο υποδοχέας εκκαθαριστής τάξης Β τύπου Ι (SR-BI) διαμεσολαβεί στην αντίστροφη μεταφορά της περίσσειας χοληστερόλης από τους περιφερικούς ιστούς στο ήπαρ μέσω των λιποπρωτεϊνών υψηλής πυκνότητας (HDL). Στους στεροειδογενείς ιστούς ο SR-BI παρέχει χοληστερόλη για την σύνθεση στεροειδών ορμονών. Στην παρούσα διατριβή επικεντρωθήκαμε στον ρόλο των ορμονικών πυρηνικών υποδοχέων στην ρύθμιση του γονίδιου SR-BI του ανθρώπου σε ηπατικά, επινεφριδιακά κύτταρα και κύτταρα ωοθηκών. Δείξαμε ότι η μεταγραφή του γονιδίου SR-BI του ανθρώπου υπόκειται σε ανάστροφη καταστολή από τα γλυκοκορτικοειδή σε επινεφριδιακά κύτταρα και κύτταρα ωοθηκών. Τα επίπεδα mRNA του SR-BI αυξήθηκαν στα επινεφρίδια ποντικών με ανεπάρκεια κορτικοστερόνης, ενώ η από του στόματος αποκατάσταση της κορτικοστερόνης κατέστειλε την έκφραση του γονιδίου του SR-BI σε αυτούς τούς ποντικούς. Τα επίπεδα mRNA του SR-BI αυξήθηκαν στα επινεφρίδια αγρίου τύπου ποντικών στους οποίους είχε χορηγηθεί μετυραπόνη, ένα φάρμακο το οποίο παρεμποδίζει την σύνθεση της κορτικοστερόνης. Πειράματα σε επινεφριδιακά κύτταρα H295R και σε κύτταρα ωοθηκών SKOV-3 με τη χρήση κυκλοεξιμίδης και με γονιδιακή αποσιώπηση μέσω siRNA, έδειξαν ότι η καταστολή της μεταγραφής του γονιδίου SR-BI από τα γλυκοκορτικοειδή χρειάζεται *de novo* πρωτεϊνοσύνθεση, καθώς και τον υποδοχέα των γλυκοκορτικοειδών (GR). Σε αναλύσεις *in vitro* και *in vivo* αλληλεπίδρασης πρωτεΐνης-DNA δεν δείχθηκε άμεση πρόσδεση του υποδοχέα των γλυκοκορτικοειδών στον υποκινητή του γονιδίου SR-BI, γεγονός που

υποδεικνύει έναν έμμεσο μηχανισμό καταστολής της μεταγραφής του από τα γλυκοκορτικοειδή σε επινεφριδιακά κύτταρα. Η ανάλυση ελλειμματικών μορφών του υποκινητή του γονιδίου SR-BI έδειξε ότι η περιοχή μεταξύ των νουκλεοτιδίων -201 και -62 επαρκεί για την καταστολή από τα γλυκοκορτικοειδή. Αυτή η περιοχή περιέχει πιθανές θέσεις πρόσδεσης μεταγραφικών καταστολέων οι οποίοι πιθανώς να διαδραματίζουν ρόλο στην ρύθμιση του γονιδίου SR-BI του ανθρώπου ως απόκριση στα γλυκοκορτικοειδή.

Σε ηπατικά κύτταρα, ο SR-BI ρυθμίζεται από αλληλεπιδράσεις μεταξύ πολλαπλών ηπατο-ειδικών μεταγραφικών παραγόντων, όπως ο Hepatocyte Nuclear Factor 3β (HNF-3β/FOXA2) και ο ορφανός πυρηνικός υποδοχέας Hepatocyte Nuclear Factor 4 (HNF-4). Χρησιμοποιώντας τα κύτταρα ηπατοβλαστώματος ανθρώπου HepG2 ως σύστημα μοντέλο, δείξαμε ότι η ενεργότητα του υποκινητή του γονιδίου hSR-BI υπόκειται σε αρνητική ρύθμιση από τον HNF-4. Αυτά τα ευρήματα είναι σε συμφωνία με προηγούμενες μελέτες οι οποίες έδειξαν ότι η απενεργοποίηση του γονιδίου του HNF-4 σε ποντικούς σχετίζεται με την δραματική αύξηση στα επίπεδα mRNA του γονιδίου SR-BI στο ήπαρ. Η αποσιώπηση του ενδογενούς γονιδίου HNF-4 σε κύτταρα HepG2 με shRNA αύξησε τα επίπεδα mRNA του γονιδίου SR-BI, ενώ η υπερέκφραση του HNF-4 κατέστειλε την έκφραση του γονιδίου SR-BI του ανθρώπου. Η ανάλυση των ελλειμματικών μορφών του υποκινητή του γονιδίου SR-BI έδειξε ότι η περιοχή του μεταξύ των νουκλεοτιδίων -201 και -62 επαρκεί για την καταστολή από τον HNF-4. Με *in vitro* και *in vivo* αναλύσεις αλληλεπίδρασης πρωτεΐνης-DNA δείξαμε ότι ο HNF-4 στρατολογείται σε πολλαπλές θέσεις στον υποκινητή

του γονιδίου SR-BI, υποδεικνύοντας έναν άμεσο μηχανισμό καταστολής της μεταγραφής του γονιδίου SR-BI από τον HNF-4 στο ήπαρ.

Συνοψίζοντας, αυτή είναι η πρώτη αναφορά η οποία δείχνει ότι τα γλυκοκορτικοειδή μειώνουν την έκφραση του γονιδίου SR-BI σε στεροειδογενείς ιστούς υποδηλώνοντας ότι αυτοί οι ιστοί διατηρούν την ομοιόσταση των στεροειδών ορμονών με την παρεμπόδιση της πρόσληψης χοληστερόλης (από την HDL) όταν τα ενδογενή επίπεδα των γλυκοκορτικοειδών είναι υψηλά. Επίσης, αυτή είναι η πρώτη αναφορά η οποία αποδεικνύει ότι ο HNF-4, γνωστός μεταγραφικός ενεργοποιητής της έκφρασης των γονιδίων των απολιποπρωτεϊνών στο ήπαρ, ενεργεί ως άμεσος αρνητικός ρυθμιστής της ενεργότητας του υποκινητή του γονιδίου SR-BI.

Η σε βάθος κατανόηση των μοριακών μηχανισμών που ρυθμίζουν την έκφραση του γονιδίου SR-BI στους στεροειδογενείς ιστούς και στο ήπαρ και του ρόλου συγκεκριμένων μεταγραφικών παραγόντων πιθανώς να προσφέρει νέες θεραπευτικές οδούς για την πρόληψη ή την θεραπεία της αθηροσκλήρωσης και πιθανώς να είναι χρήσιμη στην εκτίμηση των παραγόντων κινδύνου και της δραστικότητας των φαρμάκων.

Introduction

ATHEROSCLEROSIS

Background

Coronary Artery Disease (CAD) accounts for approximately fifty per cent of deaths of the population in Europe. In the US, Cardiovascular Diseases ranked first as a cause of mortality for the period of 1985-1996. The underlying cause of CAD is atherosclerosis, the gradual accumulation of cholesterol in the arterial wall that appears in areas of disturbed blood which leads to the development of atherosclerotic plaques that limit blood flow and cause myocardial infarction (MI). This disease develops relatively silently through late adolescence and early adulthood, and clinical symptoms are often not evident until after the age of 45 years [1].

Atherosclerotic disease risk factors are cholesterol, blood pressure, cigarette smoking, lifestyle and behavioral factors. Also, age, sex and family history have been considered as important risk factors for CVD. In more than 300.000 middle-aged men screened for the Multiple Risk Factor Intervention Trial (MRFIT), higher cholesterol levels were associated with an increased risk of CHD death [2]. Using a cholesterol level of 200 mg/dl as a base line, a cholesterol level of 250 mg/dl was associated with a twofold risk of CVD death and a cholesterol level of 300 mg/dl with a threefold risk [3].

Lowering of low-density lipoprotein cholesterol (LDL-C) can probably save 30% of patients with CAD. For the remaining 70% new targets for therapeutic intervention need to be urgently identified. Various epidemiological

and clinical studies as well as studies in animal models of atherosclerosis indicated that High-density lipoprotein cholesterol (HDL-C) in plasma are a strong risk factor of CAD, independent of other risk factors such as high LDL-C levels. HDL-C levels are reversely associated with the risk for developing atherosclerosis. These studies suggested that increasing HDL-C levels will reduce CAD risk. Prospective population studies showed that for every 1mg/dl increase in HDL-C the risk of having CHD event is reduced by 2% in men and 3% in women. However, llifestyle interventions as well as therapeutic interventions with existing drugs increase HDL-C by 5 to 30% indicating that more effective regimens are needed. Furthermore, data from both inborn errors of metabolism, genetic animal models and HDL-modulating drugs indicate that the concentration of HDL cholesterol is not a suitable marker to monitor the benefit of a therapeutic intervention. Thus, functional markers and standardized functional assays are needed to better assess and monitor the CAD risk associated with disturbances or therapies of HDL metabolism.

Lipoproteins

It is remarkable that a common polycyclic lipid such as cholesterol, the primary function of which is to maintain the permeability and fluidity of cell membranes, can cause more death and disability than all types of cancer combined. Cholesterol is an essential compound involved in many biological functions such as maintaining cell membranes, producing hormones and manufacturing vitamin D on surface of the skin, among others. Maintaining optimal cellular cholesterol concentration is essential for proper cell function and viability, as excess cholesterol concentration is toxic to cells. Cholesterol as a lipid is insoluble in water and in circulation it requires a transport vesicle to protect it from the aqueous nature of plasma. The transport of plasma lipids i.e. free cholesterol, cholesteryl esters, triglycerides, phospholipids is facilitated by the water-soluble lipoproteins. Plasma lipoproteins have a polar outer shell of protein and phospholipids and an inner core of neutral lipid. They can be either spherical with a core consisting of cholesteryl esters (CE) and triglycerides (TG) and coats of phospholipids, cholesterol and proteins or discoidal consisting of polar lipids and proteins in a bilayer conformation (Figure 1).

Lipoproteins are grouped into five major classes based on their density: chylomicrons, very-low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) [4-5].

Chylomicrons are the largest lipoproteins and are synthesized in the intestine. Following food uptake, dietary lipids assemble with apolipoprotein apoB-48 to form these lipoproteins. Then they are secreted in the lymph with the help of the microsomal triglyceride transfer protein (MTP) and after their entrance into the bloodstream their core triglycerides are hydrolyzed by lipoprotein lipase (LPL). Triglyceride hydrolysis, which results in the release of excess surface components such as phospholipids, unesterified cholesterol and apolipoproteins and their transfer to other circulating lipoproteins, especially HDL, converts chylomicrons to chylomicron remnants rich in cholesteryl esters.

VLDL is synthesized by hepatocytes, where apoB assembles intracellularly with lipids by the action of MTP, and secreted into the plasma. Triglycerides of VLDL are hydrolyzed by LPL giving rise to smaller intermediate-density lipoprotein (**IDL**), which is further converted to **LDL** through the action of hepatic lipase (HL). LDLs are the major carriers of cholesterol in humans. IDL and LDL are recognized and removed from the circulation by the LDL receptor, through endocytosis, where LDL is internalized and delivered to lysosomes for degradation.

HDL is a macromolecular complex of lipids synthesized mainly in the liver and to a smaller degree in the small intestine. HDL originates as discoidal particles which consist of two or more apolipoprotein molecules. The major apolipoproteins of HDL are apoA-I (about 70% of the total HDL protein) essential for the biogenesis and the functions of HDL and apoA-II (about 20% of the total). Other components of HDL are apoA-II, apoE, apoA-IV, apoC and apoD[6]. HDLs can be divided in a number of discrete subpopulations. There are three major classes of HDL particles with respect to shape: 1) small, lipid-poor apoA-I/phospholipid particles, called

pre-β2 HDL, 2) discoidal particles, consisting of phospholipids and apoA-I that exist only transiently, before being converted into spherical particles, and 3) the most abundant, large spherical particles, α-HDL (alpha migrating), with neutral lipid cores and two or more apolipoproteins in the outer surface (Figure 1). On the basis of apolipoprotein composition, α-HDL can be separated into two subpopulations: one comprises HDL containing apoA-I but no apoA-II and the other comprises particles containing both apoA-I and apoA-II. On the basis of density there are two major subpopulations of HDL: HDL_2 and HDL_3 (Figure 1) and on the basis of particle size into five subpopulations: HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b} and HDL_{3c} (not shown). Changes in HDL levels more closely reflect variations in the HDL_2 subfraction rather than HDL_3 [7]. Several studies have shown that low levels of HDL_2 and HDL_3 are associated with increased progression of atherosclerosis and risk of cardiovascular disease [8]. Since HDL and apoA-I are the major receptors of cholesterol in the cholesterol efflux, increasing HDL levels may increase cholesterol efflux and reverse cholesterol transport (RCT, discussed later) contributing to reduced cardiovascular disease risks.

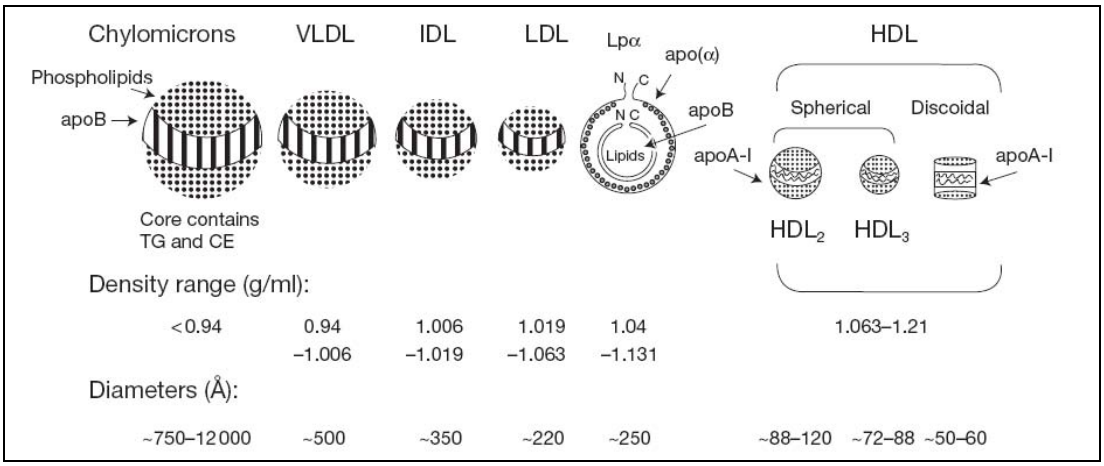


Figure 1| ***Schematic representations of plasma lipoproteins [4]***

Pathophysiology of atherosclerosis

Our ability to understand better the role of apolipoproteins, plasma enzymes, lipid transfer proteins, and lipoprotein receptors in the homeostasis of cholesterol and other lipids and their contribution to atherogenesis was assessed first in human studies. This knowledge has been greatly enhanced during the last 15 years by the generation of animal models in which one or more protein(s) have been altered by the addition or deletion of the corresponding gene(s). Following alterations of one or more gene(s) of the pathway of interest, the parameters analyzed are the lipid and lipoprotein profile, the pathogenesis of atherosclerosis, or other physiological changes.

The initiation of atherosclerosis elicits in response to proatherogenic conditions, such as those created by hypercholesterolemia [4]**.** This involves monocyte binding to adhesion molecules on the endothelial cell surface and migration to the subendothelial space**,** where they differentiate to macrophages (**Figure 2**). Induction of adhesion molecules is promoted by proinflammatory stimuli [9]. Recruitment and migration of monocytes into the subendothelial space is promoted by oxidized LDL, as well as by monocyte chemoattractant protein 1 (MCP-1) (2), which binds to the MCP-1 receptor CCR2 [10]. These proteins are expressed in endothelial cells, smooth muscle cells, and monocyte/ macrophages, and are induced in hypercholesterolemia [11]. Macrophages can be loaded with cholesteryl ester with the help of scavenger receptors (SRAI, SRAII, and CD36) [12-13] and possibly other processes and are converted to

foam cells which are later deposited in the site of lesions and contribute to the development of the atherosclerotic plaque [14].

The initial lesion created by macrophages is called the fatty streak, and is reversible [15-17]. The lesions may progress with the recruitment of additional monocytes and T cells and migration into the intima (**Figure 2**) [18]. Signals secreted by the blood-borne cells, as well as by the activated endothelial cells, promote migration of smooth muscle cells from the media into the intima, which subsequently proliferate and synthesize matrix components such as collagen and proteoglycans [14, 19]. As the development of lesions progresses, cholesteryl ester-laden monocytes/macrophages and smooth muscle cells in the plaque die. This leads to the creation of the necrotic core with extracellular cholesterol clefts, which characterize the advanced lesions [20]. The luminal face of the lesion often forms a fibrous cap consisting of smooth muscle cells, matrix components, and calcium deposits. This cap is produced by the smooth muscle cells and stabilizes the plaque. In humans, a clinical event, such as myocardial infarction or stroke, may occur as a result of rupture of unstable plaques that are enriched in lipid-filled macrophages and have weak fibrous caps, or from intra-plaque hemorrhage that leads to the generation of a thrombogenic event that will occlude the plaque [21-22]. It was shown recently that in late stages of necrosis or in plaques with thin caps, the region containing cholesterol clefts is enriched in glycophorin A and iron deposits. The findings suggest that erythrocytes contribute to cholesterol deposition, macrophage infiltration, enlargement of the necrotic core, and destabilization of the plaque [23].

It is believed that atherogenic lipoproteins, such as LDL and lipoprotein remnants, promote atherosclerosis whereas antiatherogenic lipoproteins such as

HDL protect from atherosclerosis. It has been proposed that when the concentration of LDL and of other atherogenic lipoprotein particles is high, they enter the subendothelium. The retention of LDL in the vessel wall depends on their interaction with extracellular proteoglycans. Transgenic mice overexpressing and apoB-100 mutant that is defective in binding to glycosaminoglycans have substantially less initial atheroma[24]. The tendency of LDL to adhere to proteoglycans increases with phospholipase A2-treated LDL. This procedure generates small, dense LDL which is atherogenic [25]. Phospholipase A2 transgenic mice on a high or a low-fat diet have decreased HDL and paraoxonase levels, and slightly increased LDL levels, and develop more aortic lesions than do control mice [26-27].

Modification of LDL is mediated by products of lipid peroxidation in plasma and in the subendothelial space. Oxidized LDL is taken up by the scavenger receptors SRAI, SRAII and CD36 leading to the accumulation of cholesteryl esters in these cells[12, 28].

Mice are generally resistant to the development of atherosclerosis, and the genetic background of the mouse strain influences their susceptibility [29-30]. Mice can, however, become susceptible to atherosclerosis on normal diets or atherogenic western-type diets by mutations in the apoE or the LDL receptor genes, by overexpression of apoB-100, or by crosses with atherogenic mouse lines [31-34]. In addition, atherosclerosis-susceptible strains can become resistant by transfer into susceptible strains of an atherosclerosis resistance gene locus derived from a resistant strain [35].

Analysis of atherosclerotic lesions of normocholesterolemic children aged 1–13 who died of trauma or other causes indicated that maternal

hypercholesterolemia during pregnancy may influence the susceptibility to atherosclerosis of the offspring [36-37]. Similar observations were made in normo cholesterolemic offspring of New Zealand White rabbits [38] and LDLr–/– mice. Offspring of LDLr–/– mice showed that maternal hypercholesterolemia altered patterns of gene expression in the non-atherosclerotic descending aorta of the offspring [37]. Although not rigorously proven, it is possible that hypercholesterolemia reprograms the expression of proatherogenic genes in the offspring and that these changes may persist after birth and predispose to atherogenesis. In various mouse models the extent of atherosclerosis is assessed by measuring the area of aortic root lesions at the fatty streak stage in advanced lesions [15, 39].

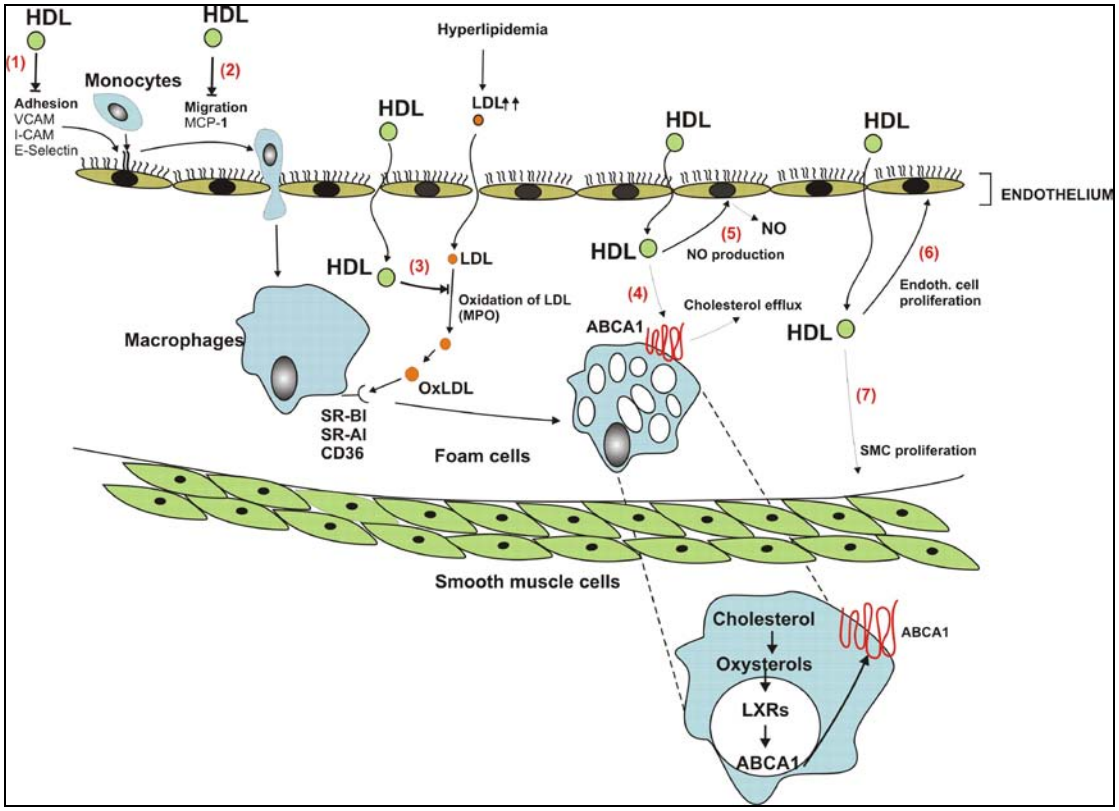


Figure 2| ***Schematic representation of the cascade of events which promote the initiation and progression of atherosclerosis and the various atheroprotective functions of HDL. Adapted from [4].*** See text for details.

Anti-atherogenic properties of HDL

HDLs have various properties that might contribute to their anti-atherogenicity. The most well-established mechanism by which HDL protects against atherosclerosis is the transport of excess cholesterol from extra-hepatic tissues to the liver for excretion into bile and faeces. This process is termed "Reverse cholesterol transport" (RCT). The removal of excess cholesterol from macrophage prevents or reverts overloading of these cells with cholesterol, their subsequent death and thereby the extracellular deposition of cholesterol in the artery wall. Also, HDL might protect against atherosclerosis by inhibiting LDL oxidation and thus protecting cells from the deleterious plaque-promoting properties of oxidized LDL [40]. Furthermore, HDL might function as an acceptor, transporter and inactivator of oxidized LDL lipids [41].

In addition, HDLs possess anti-thrombotic and anti-inflammatory properties. HDLs promote the activation of nitric-oxide synthase 3 (NOS3 or eNOS), thus increasing the bioavailability of nitric oxide [42]. Also, these lipoproteins bind to lipopolysaccharide (LPS), a component of bacterial cell wall, and protect mice from lipopolysaccharide-induced mortality. All these mechanisms may contribute to atheroprotection by HDL through pathways that are poorly understood at the present time [43-45].

Findings in humans with HDL deficiency syndromes have however also been inconclusive, and defects in major HDL genes such as apoA-I, LCAT and

ABCA1 in individual subjects, have not consistently been translated into the expected increased risk of atherosclerosis[46-48].

HDL metabolism and SR-BI

The delivery of cholesterol from peripheral tissues, via HDL, into the liver for recycling or secretion, either as cholesterol or as bile acids (products of hepatic cholesterol metabolism) is crucial for body sterol balance. The rate-limiting step in this pathway is cholesterol efflux which is mediated by the ABCA1 lipid transporter.

HDLs are generated by the intestine and the liver through the secretion of lipid –free apoA-I. ApoA-I acquires phospholipids and free cholesterol via its interaction with ATP Binding Cassette Transporter A1 (ABCA1) (**Figure 3**). ABCA1 is a member of a large family of ABC transporters facilitating translocation of ions, lipids, steroids, pigments and drugs. Mutations in ABCA1 cause Tangier Disease which is characterized by very low plasma HDL levels and premature atherosclerosis (ref). The acquisition of cellular phospholipids and cholesterol by apolipoprotein A-I (apoA-I) leads to the formation of the nascent (pre-β) HDL particles, and this protects apoA-I from being rapidly degraded by the kidneys. Nascent HDL particles, through a series of intermediate steps that are poorly understood, are further lipidated and form discoidal HDL particles. The latter are converted to the large spherical particles α-HDL (alpha migrating) through esterification of cholesterol by the plasma enzyme Lecithin: Cholesterol Acyl

Transferase (LCAT) to generate cholesteryl ester enriched mature particles. LCAT is synthesized in the liver and catalyzes the formation of cholesteryl esters by transferring the 2-acyl group of lecithin to the hydroxyl group of cholesterol [49].

Cholesteryl esters are extremely hydrophobic and accumulate in the center (core) of HDL particles, causing a change in geometry from discoidal to large spherical HDL particles that predominate in normal human plasma. This process is crucial for maintaining a concentration gradient favoring addition of free cholesterol to lipoproteins including HDL [50-51]. The discoidal and the spherical particles interact with the HDL receptor, SR-BI, which has a bidirectional role in promoting selective lipid uptake and cholesterol efflux. SR-BI directly mediates the selective uptake of cholesteryl esters from HDL into the liver and steroidogenic tissues for steroidogenesis without internalizing HDL proteins [52]. As internalization of the entire particle does not occur, mature α-HDL is quickly recycled back into lipid-poor pre-β-HDL and RCT can start again.

In the late steps of the HDL pathway (**Figure 3**), HDL cholesteryl esters are transferred by the cholesteryl-ester transfer protein (CETP) to the triglyceride rich VLDL lipoproteins for eventual catabolism by the LDL receptor. Finally, phospholipids can be transferred from VLDL/LDL back to HDL by the action of phospholipid transfer protein (PLTP). Accumulation of cholesterol in the arterial wall is a pathologic hallmark of atherosclerosis as mentioned earlier. Thus, distortion of reverse cholesterol transport can favor the deposition of cholesterol within the arterial wall and thereby contribute to the development of atherosclerosis.

Cholesterol is the precursor to all steroid hormones and therefore a constant supply must be available to the steroidogenic tissues. Adrenal cholesterol for steroidogenesis is obtained by three mechanisms: a) lipoprotein-derived uptake, b) hydrolysis of intracellular cholesterol esters, and c) de novo synthesis. Steroidogenic cells store cholesterol esters in lipid droplets and can synthesize endogenous cholesterol, but the SR-BI-mediated selective uptake pathway accounts for more than 80% of adrenal cholesterol.

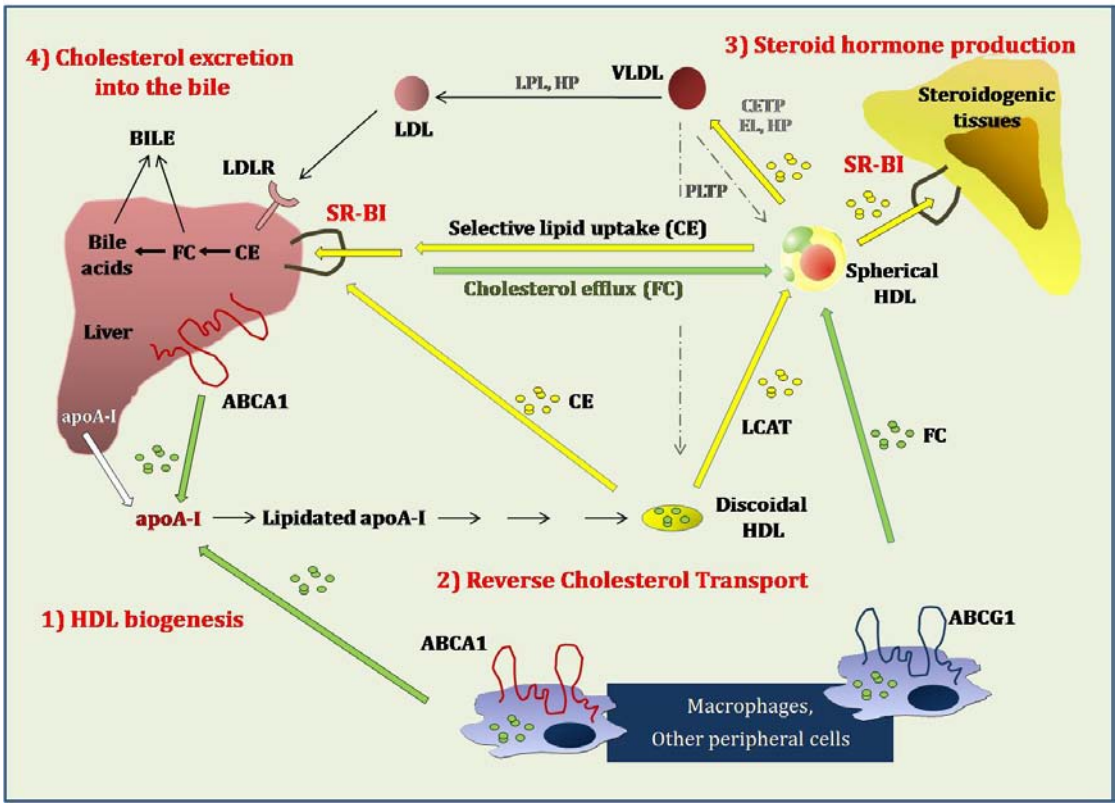


Figure 3| ***Schematic representation of HDL metabolism and the role of SR-BI.*** SR-BI plays a role in 1) HDL biogenesis, 2) reverse cholesterol transport, 3) steroid hormone production and 4) cholesterol excretion into the bile. ABCA1, ATP-binding cassette A1, ABCG1, ATP-binding cassette transporter, LCAT, lecithin;cholesterol acyl transferase, CETP, cholesteryl ester transfer protein, HP, hepatic lipase, EL, endothelial lipase, LPL, lipoprotein lipase, PLTP, phospholipid transfer protein, VLDL, very low density lipoprotein, LDL, low density lipoprotein, LDLR, low density lipoprotein receptor. Green arrows indicate free cholesterol and phospholipid transfer, yellow arrows indicate cholesteryl ester transfer.

Drugs currently used for increasing HDL-C

The drugs that are currently used for increasing the levels of HDL-C include fibrates (increase by 20%), statins (increase by 5-10%) and niacin (increase by 30%). Fibrates activate the transcription factor peroxisome proliferator-activated receptor α (PPARα) and its target genes including apoA-I. Statins are inhibitors of HMG-CoA reductase, a key enzyme in the de novo cholesterol biosynthesis pathway. Niacin (nicotinic acid) has unknown modes of action, although it has been shown that it increases the synthesis of both apolipoproteins apoA-I and apoA-II.

New strategies for fighting atherosclerosis

New generation drugs must be developed in order to increase plasma HDL cholesterol levels to sufficient levels to protect from atherosclerosis. This new generation of drugs is based on RCT. Compounds that would increase the rate of RCT in plasma would be protective in hyperlipidaemic patients. Several new strategies and molecules tested to increase RCT include:

a) intravenous injections of reconstituted HDLs (rHDLs) containing a variant of apoA-I (apoA-I_{milano}) combined with phospholipids. This strategy reduced the amount of atheroma in the coronary artery within only six weeks [53],

b) administration of apoA-I mimetics (18 amino acid peptides). These peptides reduced atherosclerosis and increased the anti-oxidative functions of HDL without increasing plasma HDL levels. These peptides can be administered orally, making the use of these drugs very easy,

c) increase of LXR agonists, in order to stimulate the expression of the two membrane transporters ABCA1 and ABCG1, which function in the efflux and transport of cholesterol from cells to discoidal and mature HDL respectively,

d) CETP inhibitors. Japanese individuals with a genetic deficiency in the cholesteryl ester transfer protein (CETP) had extremely high HDL cholesterol concentration. This observation led to the development of CETP inhibitors, torcetrapid and JTT-705. Torcetrapid was terminated as it almost doubled HDL cholesterol levels but it increased mortality and cardiovascular events.

Other HDL-raising strategies under development include stimulators of the transcription factor, RVX208, which increase apoA-I production and preβ HDL concentration. Inhibitors of endothelial lipase are also of interest.

SCAVENGER RECEPTOR CLASS B TYPE 1

The scavenger receptor family is comprised of cell-surface transmembrane proteins that function as multiligand receptors. There are many classes of scavenger receptors but in *vivo* experiments implicated scavenger receptor class A types I and II (SR-AI/II) and the class B (SR-B) scavenger receptors in atherogenesis [54]. Class B receptors, are members of the cluster determinant 36 (CD36) superfamily of membrane proteins which include among others, the mammalian proteins CD36 and CLA-1 and lysosomal integral membrane protein II (LIMPII), croquemort (a *Drosophila* hemocyte/macrophage receptor that recognizes apoptotic cells) and SnmP-1 (a silk moth olfactory neuron membrane protein). Most CD36 family members share 30% sequence identity.

SR-BI was recognized in 1996 during the study of a novel scavenger activity in Chinese hamster ovary cells [55]. The gene encoding CLA-1, the human homologue of SR-BI (now called hSR-BI) is ~75kb in length, contains 13 exons and 12 introns and is located on the long arm of chromosome 12. The cDNA for CLA-1, which stands for CD36 and LIMPII Analogous-I is 2566 nucleotides in length and encodes a 509 amino acid long protein [56].

Structure of SR-BI

SR-BI is a 82kDa glycoprotein of a horselike-shoe like structure with a large extracellular loop (~403 residues), two internal hydrophobic, putative membrane-

spanning domains (~ 28 and ~ 25 residues) adjacent to short cytoplasmic amino- and carboxy-terminal tails (~8 and ~45 residues) (**Figure 4**). SR-BI is a highly N-glycosylated protein and contains a set of similarly spaced conserved cysteine residues. Glycosylation of some or all of these putative sites in the ~57 kDa polypeptide core of SR-BI results in the mature form of the 82kDa SR-BI glycoprotein[54]. SR-BI undergoes fatty acylation in the form of palmitoylation at the cysteine residues 462 and 470, but this does not appear to influence SR-BI expression or function [54, 57]. An alternatively spliced form of SR-BI, SR-BII, differs from SR-BI in that 40 entirely different residues replace 42 of the 45 C-terminal residues in the cytoplasmic domain of SR-BI [58]. This produces a protein that is shortened by three amino acids compared to SR-BI. Both isoforms bind HDL with the same affinity but SR-BII mediates selective cholesterol uptake with lower efficiency than SR-BI [59].

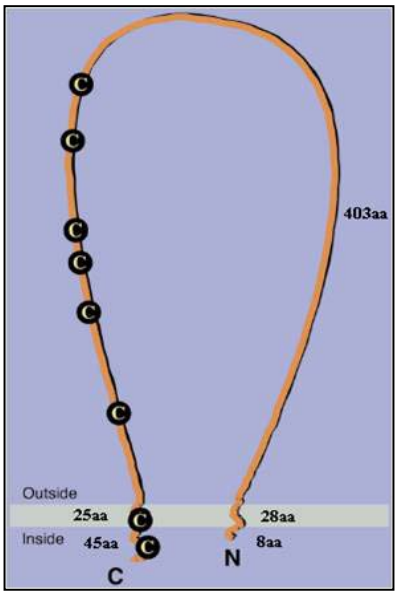


Figure 4| ***Schematic representation of the HDL receptor SR-BI [60].***

Localization of SR-BI expression

SR-BI is highly expressed in the liver and steroidogenic tissues such as adrenals, ovaries and testis [52, 61]. Other sites of lower SR-BI expression include the intestine, yolk sac and placenta during intra-uterine development and uterine endometrium, as well as lungs, macrophages and endothelial cells [62-68]. The liver expresses the highest levels of total tissue SR-BI protein and under basal conditions, most hepatic SR-BI expression is in parenchymal cells. SR-BI protein expression has also been reported in Kupffer and liver endothelial cells [66]. In the adrenal gland, immunohistochemical methods showed that SR-BI is expressed primarily on the surfaces of steroidogenic parenchymal cells, such as in the zona fasciculata and zona reticularis cells of the adrenal cortex [61, 69]. In preovulatory rodent and bovine ovary, SR-BI mRNA and protein are found in the theca interna cells and in granulosa cells during the formation of corpus luteum [70-71]. Leydig cells and to a lesser extent sertoli cells, show the most SR-BI protein expression in the testis [61, 72].

SR-BI expression in the liver is dependent on PDZK1 protein for its intracellular transport, expression, stability and activity. PDZK1 (or CLAMP) is a soluble PDZ-domain-containing protein with four PSD-95/Dlg/ZO-1 (PDZ) domains and associates with the C terminus of SR-BI through its N-terminal first PDZ domain [73]. PDZK1 is expressed in the liver, kidney and small intestine, but not in steroidogenic organs where SR-BI expression is high [74]. Kocher *et al.* showed that PDZK1 KO mice exhibit a ~95% reduction in SR-BI protein expression in the liver, as well as increased plasma cholesterol levels and abnormally large

HDL particles [75-76]. This study indicated that hepatic SR-BI expression is up-regulated by PDZK1 at the protein level *in vivo.*

In cultured cells, SR-BI is localized in caveolae, specialized plasma membrane micro-domains. Recent studies suggested that the main protein component of many caveolae, caveolin-1, does not affect SR-BI-mediated selective uptake [58, 77-79], so that localization in caveolae is not necessary for SR-BI-mediated selective cholesteryl ester uptake. The sites of SR-BI mediated selective uptake in cultured cells and steroidogenic tissues *in vivo* seem to be structures called microvillar channels which are formed upon SR-BI expression [80-81].

Ligand binding and oligomerization of SR-BI

SR-BI is a multiligant glycoprotein which binds to a variety of ligands with very different structures [54, 82-83]. These ligands include modified low density lipoproteins (acetylated or oxidized), maleylated bovine serum albumin, anionic phospholipids, advanced glycation end-products and apoptotic cells [55, 84]. The receptor also binds native lipoproteins such as HDL, LDL and VLDL [55]. The first native lipoprotein shown to bind to SR-BI with high affinity ($K_d \sim 10nM$) was LDL [85]. SR-BI was the first molecularly well defined and physiologically relevant HDL receptor to be identified [52]. It binds spherical α-HDL whereas pre-β-HDLs are poor substrates of the receptor [86]. Reconstituted discoidal complexes of phospholipid/cholesterol/apolipoprotein (apoA-I, apoA-II, apoE, or apoC-III) bind more tightly to SR-BI than native spherical α-HDLs [86]. A study by Williams *et al.*

[87] reported that amphipathic helices play critical roles in the binding of apolipoproteins to SR-BI and this may explain the ability of SR-BI to bind to multiple lipoproteins. Besides cholesteryl esters, SR-BI facilitates selective transport of HDL-derived free cholesterol and triglycerides [88-89]. Overall, SR-BI is a multi-lipoprotein receptor and not just a multi-apolipoprotein receptor.

The HDL receptor exhibits nonreciprocal cross competition, where one ligand can block the binding of another ligand, but the reverse does not apply [83]. Thus HDL can effectively block binding of LDL to SR-BI, whereas LDL can not block HDL binding to its receptor, as it binds poorly to SR-BI receptor [52].

Different research groups reported that SR-BI in rodent adrenals exists as oligomers (dimers, tetramers and possibly higher order oligomers) [61, 90-92]. Fluorescence resonance energy transfer (FRET) spectroscopy in live cells revealed that SR-BI self-associates at the plasma membrane and homo-oligomerises via interactions of the carboxy-terminal of SR-BI monomers [91].

SR-BI and atherosclerosis

In order to answer the question "Is SR-BI good or bad?" a series of *in vitro* and *in vivo* studies were carried out over the past 14 years. First of all, these studies provided evidence that SR-BI is the physiologically relevant HDL receptor. Studies of the activity, expression and regulation of SR-BI, as well as, studies in mice where the levels of SR-BI in liver have been manipulated either by

adenovirus-mediated gene transfer or by transgenesis and gene targeting revealed the important role of SR-BI in HDL metabolism [93-95].

SR-BI overexpression in the liver leads to decreased plasma levels of HDL cholesterol and apoA-I, increased cholesteryl ester clearance, and a concurrent increase in the cholesterol concentration in hepatic bile [93-94, 96]. Adenovirus-mediated gene transfer of SR-BI in LDL receptor-deficient (LDLr-/-) mice with early or advanced atherosclerotic lesions, reduced plasma apoA-I and HDL levels and protected the mice from atherosclerosis [97-98]. A study using two lines of SR-BI transgenic mice with high (10-fold increases) and low (2-fold increases) SR-BI expression in an inbred mouse background hemizygous for a human apolipoprotein (apo) B transgene revealed that only mice with moderately elevated hepatic SR-BI expression were found to be protected against diet-induced atherosclerosis [99]. It is unclear why high levels of hepatic SR-BI expression failed to protect against atherosclerosis. This study indicated that very high, non-physiological, levels of SR-BI may not always be beneficial.

SR-BI -/- mice exhibit elevated plasma HDL cholesterol concentrations[95], reduced selective HDL cholesterol clearance [48], reduced amounts of cholesterol content in steroidogenic tissues and apoE-enriched HDL particles. These SR-BI deficient mice have also decreased levels of biliary cholesterol concentration and secretion without alterations in bile acid secretion, bile acid pool size or fecal bile secretion [95, 100]. Complete disruption of the SR-BI gene in both LDLr-/- or apoE-/- mice dramatically accelerated the onset of atherosclerosis [94, 101-102]. These studies suggested that SR-BI facilitates cholesterol transfer from HDL to bile for secretion and supplies cholesterol for steroid production to steroidogenic tissues.

SR-BI -/- x apoE-/- mice develop premature occlusive coronary atherosclerosis, spontaneous myocardial infarction, have cardiac hypertrophy and die within 8 weeks of birth. Their premature death is associated with substantial cardiac dysfunction. These defects resemble those found in human patients with coronary heart disease. Treatment of SR-BI -/- x apoE-/- mice with the antioxidant, lipid-lowering drug, probucol extended their life-span by up to 60 weeks, and lipid and lipoprotein profiles as well as most of the cardiac and red blood cell pathologies were reversed [103]. Overall, expression of SR-BI in the mouse seems to be cardioprotective, whereas absence of SR-BI is atherogenic.

The above studies indicated that SR-BI is important for maintaining normal plasma HDL-cholesterol levels. Interactions of SR-BI with HDL control the structure and composition of plasma HDL [60, 96, 104] , the cholesterol contents of HDL, the adrenal gland, the ovaries, the bile [95, 100] and protect mice from atherosclerosis [94, 101].

Other SR-BI functions

SR-BI plays an important role in **female fertility**. The observation that female SR-BI KO mice are infertile supports the role of SR-BI in normal ovarian function. It may influence oocyte maturation and development either directly or indirectly, by blocking cholesterol influx or efflux or by leading to accumulation of abnormal plasma lipoproteins respectively [101]. SR-BI may contribute to **embryogenesis and fetal development,** as it is expressed during murine

embryonic development. It has been reported that SR-BI may play a role in cholesterol uptake and delivery from maternal lipoproteins into extraembyonic tissues and within the developing embryo itself [105].

Studies of SR-BI overexpression in cultured cells showed that this receptor can mediate the cellular uptake of α-tocopherol from HDL [100, 106]. Thus, SR-BI receptor play a role in **Vitamin E transport** as α-tocopherol is the form of vitamin E preferentially absorbed and accumulated in humans. It is believed that the reproductive pathologies observed in SR-KO mice result from defective tissue uptake of Vitamin E, which is an anti-oxidant essential for the protection of the premature rupture of fetal membranes [107-108].

Although there are conflicting data about the role of SR-BI in **cholesterol gallstone disease** it may be involved in the pathogenesis of the disease, as it controls biliary cholesterol secretion and gallbladder bile cholesterol content [93-94, 100-101, 109].

A study by Altmann *et al.* [110] showed that the **intestinal cholesterol absorption** inhibitor, ezetimide, binds with high affinity to SR-BI and blocks cholesterol uptake by the receptor. SR-BI expression is not essential for cholesterol intestinal absorption observed in SR-BI deficient mice, but these mice may have compensatory, SR-BI independent, mechanisms for cholesterol absorption.

Holm *et al.* [111] observed that hypercholesterolemic mice with abnormal lipoproteins, due to SR-BI gene inactivation, have unexpected defect in late **erythroid maturation**, as well as, abnormal erythrocyte morphology and anemia.

The HDL receptor may play a role in **NO metabolism.** Nitric oxide has protective effects in the vascular system including prevention of lipoprotein oxidation, monocyte adhesion to the endothelium and down-regulation of inflammatory mediators [112-113]. SR-BI and eNOS transfected Chinese hamster ovary cells revealed that SR-BI facilitates the activation of eNOS by HDL and the subsequent production of nitric oxide and requires the presence of native HDL particles [114]. Studies on HDL particles isolated from mice or humans revealed that female, but not male HDL, is able to stimulate eNOS because of the estradiol associated with this lipoprotein [115].

SR-BI mediates binding of β-amyloid to transfected chinese hamster ovary cells [116] and cultures of microglia and brain vascular smooth muscle cells [117-118], suggesting a potential **role in Alzheimer's disease**. SR-BI may have a role as **hepatitis C virus receptor** as it has been shown that it binds the hepatitis C virus receptor [119].

Furthermore, SR-BI is required for microvillar channel formation and has an effect on microvillar membrane structure. Adrenocortical cells of SR-BI -/- mice show disorganized microvilli, no microvillar channels, and no binding of HDL to the cell surface. Also, microvillar membranes are thinner than those of normal littermates and this may be due to less cholesterol accumulation as SR-BI deficiency causes a 70% reduction in CE accumulation in the adrenal gland [80, 95]. In insect cell membranes, it was shown that SR-BI expression caused a decrease in phosphatidylcholine (PC) species with shorter fatty acyl chains and an increase in PC with longer fatty acyl chains [120] and was observed in PC species with mono- and poly-unsaturated acyl chains. Phospholipids with long,

unsaturated acyl chains make membranes more fluid. Together the above indicate that SR-BI expression **affect membrane fluidity** and in nature and may allow free cholesterol desorption[121].

Overall, the SR-BI receptor may be atheroprotective due to its role in the following mechanisms: 1) can stimulate reverse cholesterol transport by mediating the hepatic uptake and biliary secretion of HDL cholesterol, 2) may prevent accumulation of plasma atherogenic lipoproteins, 3) may contribute to α-tocopherol-mediated atheroprotection, 4) may mediate the HDL-dependent NO synthase activation, 5) its expression may influence arterial oxygen supply by controlling red blood cell maturation and preventing anemia and 6) prevents cholesterol accumulation in macrophages, thereby prevents their conversion to foam cells.

TRANSCRIPTIONAL REGULATION OF SR-BI

The regulation of SR-BI gene expression is exerted mainly at the level of transcription. The promoters of human and rat SR-BI gene of approximately 2.2kb, contain consensus-DNA sequences that bind several positively-acting transcription factors in response to exogenous and endogenous stimuli. These factors are:

Sterol regulatory element binding protein 1a (SREBP-1a) is a ubiquitously expressed transcription factor that is activated following cholesterol depletion. This protein is synthesised as 125kDa precursor that is attached to the endoplasmic reticulum membrane and the nuclear envelope. Upon decreased cholesterol availability, a two step proteolytic process releases the smaller mature NH-2-terminal segment, which translocates to the nucleus, where it binds to sterol regulatory elements (SRE) and eventually cause activation of genes involved in cholesterol uptake and biosynthesis [122]. SREBP-1a positively regulates SR-BI through two SREs that are present in the SR-BI promoter and requires the presence of a coactivator like Sp-1 [123].

Sp1 belongs to the Sp family of transcription factors (Sp1, Sp2, Sp3 and Sp4) that are ubiquitously expressed and required for the constitutive and inducible expression of a variety of genes. Sp1 and Sp3 bind to several GC-rich boxes present on the proximal SR-BI promoter and have been shown to be important for the basal activity as well as the SREBP-1a mediated transactivation

of the SR-BI promoter. Sp1 acts as a stabilizing component of the SREBP-1a/SRE complex [123].

Positively acting transcription factors also include nuclear hormone receptors such as the **liver X receptors α and β** (**LXRα and LXRβ**). These NRs are sensors of cholesterol metabolism and lipid biosynthesis. **LXRα** is highly expressed in liver, kidney, intestine and adipose tissues and is activated by oxysterols. LXRs heterodimerize with retinoid X receptor (RXR) and bind to a distal LXRE on the human and rat SR-BI promoter and regulate the expression of the SR-BI gene [124].

The peroxisome proliferator activated receptor α (PPARα) is activated by fibrates, drugs that are currently used for the treatment of CAD. PPARα is expressed in tissues with high metabolic rates such as liver, heart, and intestinal mucosa and in the adrenal gland [125-129]. PPARα dimerizes with RXRα and binds to a proximal PPARE on the rat SR-BI promoter and activate the expression of the SR-BI gene in response to fibrates [128]. Another member of the PPAR family, **PPARγ**, induces hepatic SR-BI expression upon dimerization with RXR and binding to a proximal PPARE on the human SR-BI promoter [130].

The estrogen receptors α and β (**ERα ,ERβ)** are found in most cell types within the adrenal gland, and the ovary, but only ERα subtype is expressed in the liver [131-132]. Transcriptional factors that have been shown to interact with the ER include SF-1, Sp1, NF-Y, and activator protein AP-1 [133-137]. Landschulz *et al.* reported that estrogen treatment increased SR-BI expression in steroidogenic tissues but dramatically decreased hepatic SR-BI gene expression demonstrating tissue specific regulation (15). Estrogen receptors bind to three

different estrogen response elements on the rat SR-BI promoter and regulate its activity in response to estrogens in cooperation with SREBP-1a [138].

Steroidogenic factor 1 (SF-1) is involved in development, differentiation and function of the hypothalamus, pituitary, adrenal gland and gonads. It regulates many genes such as cholesterol side-chain cleavage cytochrome P450 gene (CYP11A) and steroidogenic acute regulatory protein (StAR). SF-1 exerts its effects through interactions with coactivators or corepressors and is modified by phosphorylation, SUMOylation and acetylation. SF-1 binds and activates the human and the rat SR-BI promoters and seems to be the key mediator of the cAMP-dependent regulation of the SR-BI gene in response to steroidogenic hormones [139].

The liver receptor homolog 1 (**LRH-1)** is a nuclear orphan receptor that binds as a monomer to DNA. It plays a critical role during development by controlling the expression of α-fetoprotein and a number of transcription factors critical for hepatic and pancreatic development (HNF-3β, HNF-4α, HNF-1α) [140]. The physiological role of LRH-1 is linked to the control of cholesterol and bile acid homeostasis. Also, it regulates the expression of cholesterol ester transfer protein (CETP) [141]. It has been shown that LRH-1 binds to a proximal response element on the human SR-BI promoter and regulates its expression in liver and ovary [142]and to a distal response element [143].

It has been shown that bile acids, which are the natural ligands of the farnesoid x receptor (**FXR)**, increase significantly the liver mRNA and protein levels of SR-BI in mice [144]. However, FXR responsive elements on the SR-BI promoter have not been identified thus far. In another study, it was reported that

bile acids inhibit SR-BI gene expression in liver of mice and reduce the SR-BI promoter activity. It was proposed that this inhibition was due to the FXR-mediated activation of small heterodimer partner (SHP), which repressed the activity of LRH-1 that binds to the proximal SR-BI promoter [143].

In addition to the positively acting transcription factors described above (except the conflicting data about FXR), the SR-BI promoter is also regulated by negatively-acting factors. The orphan nuclear receptor **dorsal-sensitive sex adrenal hypoplasia congenital critical region on the X chromosome gene 1 (DAX-1)**, is a protein that plays an important role in adrenal development. DAX-1 functions as a transcriptional corepressor via heterodimeric interactions with other NRs, including the androgen (AR), estrogen (ER) and progesterone receptor (PR), in addition to SF-1, and LRH-1 [145-149]. DAX-1 represses the rat SR-BI promoter by directly binding to and inhibiting SF-1 and SREBP-1a-mediated transactivation of the SR-BI promoter [139].

Pregnane X receptor (PXR) is a nuclear xenobiotic receptor, expressed in the liver and intestine. It is activated by several compounds including rifampicin, the anti-glucocorticoids pregnelonone 16$^{\alpha}$-cardonitrile (PCN), endogenous steroids (estrogens, corticosteroids) and by the synthetic glucocorticoid dexamethasone. Also, PXR is activated by lithocholic acid (LCA), a potent bile acid that can cause liver damage and other pathological changes. PXR heterodimerizes with retinoid X receptor (RXR) and regulates the expression of potentially harmful compounds from liver and intestine. SR-BI gene expression was down-regulated in HepG2 cells by the PXR activators rifampicin and LCA, and by PCN in primary hepatocytes [150].

Yin Yang 1 zinc finger transcription factor (YY1) can function in the absence of any known YY1 binding sites, and contains distinct domains that regulate either by transactivation, or transrepression, the expression of many genes. YY1 binds to the 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA), farnesyl diphosphate, and LDL promoters, and result in displacement of NF-Y factor. This nuclear factor represses the activity of the SR-BI promoter through direct binding to two distal sites on the rat SR-BI promoter or by disrupting the SREBP-1a binding to this promoter [123].

A summary of the regulatory elements and transcription factors that were identified on the human and rat SR-BI promoter is shown schematically in **Figure 5**.

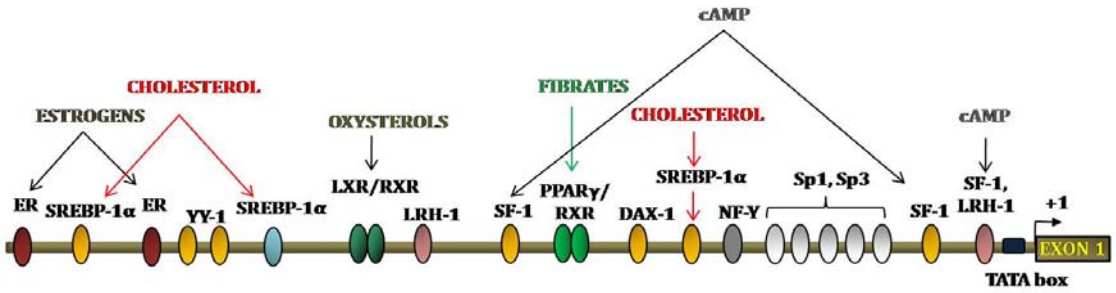


Figure 5| ***Transcription factors that regulate the activity of rat and human SR-BI gene.*** Yellow ovals indicate previously characterized binding sites of the rat SR-BI promoter. ER, estrogen receptor, YY-1, Yin Yang 1 zinc finger transcription factor, SREBP-1a, Sterol regulatory element binding protein 1a, LXR, Liver X receptor, RXR, Retinoid X receptor, PPAR γ, peroxisome proliferator activated receptor γ, LRH-1, Liver receptor homolog 1, DAX-1, dorsal-sensitive sex adrenal hypoplasia congenital critical region on the X chromosome gene 1, NF-Y, nuclear factor-Y, SF-1, Steroidogenic factor 1.

NUCLEAR HORMONE RECEPTORS

Nuclear hormone receptors are a class of proteins that are responsible for sensing the presence of small lipophilic molecules such as steroid and thyroid hormones or retinoids and vitamin D, and play an important role in growth, differentiation, metabolism, development and homeostasis of the organism [151-152]. Nuclear receptors act as ligand-induced transcription factors. The ligands that bind to these receptors can be generated in three ways: 1) synthesized in an endocrine organ and enter the cell, 2) generated within the target cell and 3) may be a metabolite synthesized within the target cell. Nuclear receptors have been historically divided in three types.

Type I nuclear receptors, include the classical hormone receptors, which translocate to the nucleus upon ligand binding and bind DNA as monomers recognizing inverted repeats. Type II receptors, which are ligand-independent, include the thyroid hormone receptor (THR) and retinoic acid (RAR), reside in the nucleus, and bind DNA as heterodimers with RXR in direct repeats. Type III receptors, have still unidentified regulatory ligands and are called "Orphan receptors''. The orphan receptors can be constitutively active and others can be activated by other means, i.e. phosphorylation [151-152].

Moreover, some steroid receptors are cytoplasmic in the absence of a ligand and form multicomplexes with chaperones (Glucocorticoid receptor). Upon ligand binding the complex is dissociated and the activated receptor translocates to the nucleus (discussed in more detail below). Once in the nucleus the receptors regulate transcription by binding to specific DNA sequences

referred to as hormone response elements (HREs) normally located in the regulatory regions of target genes.

HREs are present in the 5' flanking regions of target genes and sometimes in enhancer regions. The consensus motifs that have been identified are the sequences AGAACA and AGG/TTCA. However, HREs can show significant variation. Most receptors bind as homo-or heterodimers to HREs through two core hexameric motifs, although some monomeric receptors bind to a single hexameric motif. The half-sites for dimeric HREs are configured as palindromes (Pal), inverted palindromes (IPs) and direct repeats (DRs).

	Receptor	Subtype	Denomination	Ligand	Response Element	Monomer, Homodimer, or Heterodimer
Class I	TR	α, β	Thyroid hormone receptor	Thyroid hormone (T_3)	Pal, DR-4, IP	H
	RAR	α, β, γ	Retinoic acid receptor	Retinoic acid	DR-2, DR-5 Pal, IP	H
	VDR		Vitamin D receptor	1-25$(OH)_2$ vitamin D_3	DR-3, IP-9	H
	PPAR	α, β, γ	Peroxisome proliferator activated receptor	Benzotriene B4; Wy 14.643 Eicosanoids; thiazolidinediones (TZD_S); 15-deoxy-12,41-prostaglandin J_2; polyunsaturated fatty acids	DR-1	H
	PXR		Pregnane X receptor	Pregnanes; C21 steroids	DR-3	H
	CAR/MB67	α, β	Constitutive androstane receptor	Androstanes; 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene	DR-5	H
	LXR	α, β	Liver X receptor	Oxysterols	DR-4	H
	FXR		Farnesoid X receptor	Bile acids	DR-4, IR-1	H
	RevErb	α, β	Reverse ErbA	Unknown	DR-2, Hemisite	M, D
	RZR/ROR	α, β, γ	Retinoid Z receptor/retinoic acid-related orphan receptor	Unknown	Hemisite	M
	UR		Ubiquitous receptor	Unknown	DR-4	H
Class II	RXR	α, β, γ	Retinoid X receptor	9-*Cis*-retinoic acid	Pal, DR-1	D
	COUP-TF	α, β, γ	Chicken ovalbumin upstream promoter transcription factor	Unknown	Pal, DR-5	D, H
	HNF-4	α, β, γ	Hepatocyte nuclear factor 4	Fatty acyl-CoA thioesters	DR-1, DR-2	D
	TLX		Tailles-related receptor	Unknown	DR-1, Hemisite	M, D
	PNR		Photoreceptor-specific nuclear receptor	Unknown	DR-1, Hemisite	M, D
	TR2	α, β	Testis receptor	Unknown	DR-1 to DR5	D, H
Class III	GR		Glucocorticoid receptor	Glucocorticoids	Pal	D
	AR		Androgen receptor	Androgens	Pal	D
	PR		Progesterone receptor	Progestins	Pal	D
	ER	α, β	Estrogen receptor	Estradiol	Pal	D
	ERR	α, β, γ	Estrogen-related receptor	Unknown	Pal, Hemisite	M, D
Class IV	NGFI-B	α, β, γ	NGF-induced clone B	Unknown	Pal, DR-5	M, D, H
Class V	SF-1/FTZ-F1	α, β	Steroidogenic factor 1 Fushi Tarazu factor 1	Oxysterols	Hemisite	M
Class VI	GCNF		Germ cell nuclear factor	Unknown	DR-0	D
Class 0	SHP		Small heterodimeric partner	Unknown		H
	DAX-1		Dosage-sensitive sex reversal	Unknown		

Table 1| ***Subfamilies of mammalian nuclear receptors***. M, monomer, D, homodimer, H, heterodimer, DR, direct repeat, Pal, palindrome, IP, inverted palindrome. Underlined are receptors discussed in more detail in this chapter [153].

Domain structure of NRs

Nuclear receptors consist of a variable NH_2-terminal region (A/B), a conserved DNA-binding domain (DBD) or region C, a linker region D, and a conserved E region which contains the ligand binding domain (LBD) (**Figure 6**) [151-152]. Region F is a COOH-terminal region of unknown function present in some receptors. The A/B region contains the activation function 1 (AF-1), a region required for transcriptional activation, and whose action is independent of the presence of ligand. The A/B region is the most variable in size and sequence among nuclear receptors. It shows promoter and cell-specific activity and is the domain that mediates the phosphorylation of the receptor. The DBD is of the zinc-finger type and is responsible for recognition of target sequences. It is the most conserved domain of nuclear receptors and contains nine cysteines required for high-affinity DNA binding. The D domain serves as a hinge region between the DBD and LBD, allowing rotation of the DBD, and influences intracellular trafficking and subcellular distribution. The LBD is moderately conserved in sequence and highly conserved in structure between the various nuclear receptors [154]. In addition to ligand binding, it mediates homo-and heterodimerization and interaction with heat shock proteins. It contains the signature motif or Ti and the COOH-terminal activation function AF-2 motif responsible for ligand-dependent transcriptional repression. Region F is present in some receptors, such as HNF-4 and it has been shown that it blocks binding of coactivators GRIP-1 and SRC-1[155].

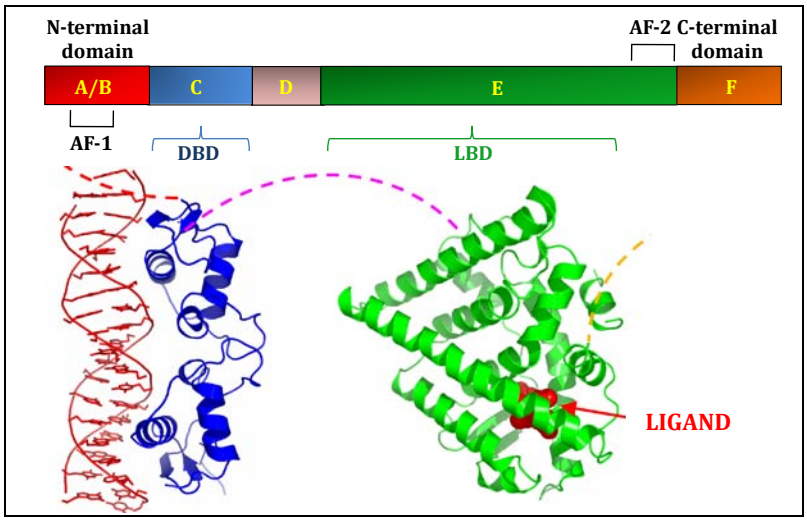


Figure 6| ***Schematic representation of the domain structure of nuclear hormone receptors.*** The N-terminal domain (A/B) is shown in red, the DNA binding domain (DBD) in blue, the hinge region (D) in pink, the ligand binding domain in green and the COOH-terminal region F in orange. The structures of the DBD and LBD are shown below.

Transactivation and transrepression by NRs

Nuclear hormone receptors (NRs) can bind to HREs and activate the transcription of target genes. However, NRs can also mediate negative regulation by ligands. Negative HREs have been identified for glucocorticoids in the proopiomelanocortin gene (POMC) [156-157]. Ligand-independent transcriptional repression is another mechanism whereby the receptor binds to a positive HRE and represses transcription in the absence of ligand. This is accomplished via corepressors which bind to the ligand free receptors. In the case of heterodimeric receptors, COUPs act as transcriptional repressors that antagonize activation mediated by a different receptor, competing for DNA binding. [158]. Phosphorylation of nuclear receptors provides an opportunity for cross talk with other signaling pathways including MAPK or cell cycle dependent kinases (CDKs) pathways among others [159]. Moreover, NRs can activate or inhibit gene transcription without binding to an HRE, but by interfering with the activity of other transcription factors [160]. For instance, the glucocorticoid receptor or the receptors for retinoic acid (RAR) and T3 (TR) act as ligand-dependent transrepressors of AP-1 (Activating Protein 1) activity and reciprocally, AP-1 inhibits transactivation by nuclear receptors. [161].

Coactivators of nuclear receptors are members of the p160 family, which includes the SRC-1/NCoA-1, SRC2/TIF2 and SRC3 coactivators, and possess histone acetyltransferase activity [162]. SRC-1 functions as a coactivator of the glucocorticoid receptor and the nonsteroid receptors PPAR, RXR or TR. SRC-3 enhances among others the transcriptional activity of signal transducers and

activators of transcription (STAT-1) and cAMP response element binding protein (CREB) [163]. Other nuclear receptor coactivators include: the PPARγ coactivator -1 (PGC-1) and p68, transcription intermediary factors (TIFS), ubiquitin ligase E6-AP, multiprotein complexes termed TRAP/DRIP[153]. Proteins CBP and p300 are co-integrators that interact with a variety of transcription factors, bind directly to the nuclear receptors, and associate with the p160 family of coactivators [164]. Coactivators possess a nuclear receptor interacting domain (NR box) for association with ligand-bound receptors, which contains three highly conserved LXXLL motifs, where L is leucine and X any amino acid [165].

Similar to coactivators, corepressors are recruited by NRs in the absence of a ligand or in the presence of NR antagonists such as Tamoxifen and RU486 [166-167]. They contain two LXXLL motifs, named CoRNR boxes, which mediate recognition by transcriptionally inert NRs [168]. The latter are conserved in both position and sequence between the silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) and nuclear corepressor (NCoR) [153]. Corepressors lack intrinsic deacetylation domains and require recruitment of Sin3 and histone deacetylases (HDACs) to achieve this [166].

Hepatocyte nuclear factor 4

The adult liver executes several functions essential for metabolic homeostasis including plasma protein synthesis, carbohydrate, lipid and amino

acid metabolism and xenobiotic metabolism. The majority of these functions are mediated by hepatocytes. Studies on the transcriptional regulatory elements of genes expressed in hepatocytes identified hepatocyte nuclear factor 1 (HNF-1), HNF-3 (FOXA), HNF-6, CCAAT/enhancer binding protein (CEBP) families and HNF-4. Gene knockout studies of HNF-4 have been found to disrupt expression of a large number of genes, indicating that this factor is important in functions such as the control of energy metabolism, xenobiotic detoxification, bile acid synthesis, and serum protein production.

HNF-4 was first discovered by Sladek et al., as a liver-enriched nuclear orphan receptor that regulates the transcription of the genes encoding transthyretin, an important transporter of vitamin A and thyroid hormones in the blood, and apolipoprotein CIII, a constituent of chylomicrons and very-low-density lipoproteins and a regulator of lipoprotein lipase activity [169]. HNF-4 generally acts as a positive transcription regulator of many hepatocyte genes and can activate gene transcription in the absence of exogenous ligands. Initially, HNF-4, was believed to be an orphan receptor [169], but now it is known that HNF-4 binding activity may be modulated by fatty acyl-CoA thioesters [170] and also by protein kinase A-mediated phosphorylation [171]. HNF-4 is acetylated by CBP coactivator, a modification inducing HNF-4 binding to DNA, its nuclear localization and the recruitment of other coactivators [172]. HNF-4 binds DNA as a homodimer.

HNF-4 expression in the developing fetus is detected in the liver bud and gut and subsequently in the developing pancreas and kidney [173-174]. In the

adult, HNF-4 is highly expressed in the liver, kidney, intestine and pancreas [169]. Disruption of HNF-4 gene leads to a lethal phenotype due to impairment of endodermal differentiation and gastrulation [175]. In humans heterozygous mutations in the HNF-4 gene are associated with an early-onset form of type II diabetes called maturity onset diabetes of the young 1 (MODY) [176].

Hayhurst et al. used the Cre-loxP system to produce mice lacking HNF-4 in the adult liver [177]. These mice exhibited increased lipid levels in hepatocytes and greatly reduced serum cholesterol and triglyceride levels. Of great importance was the observation that the expression of SR-BI gene was markedly increased in HNF-4 null mice. Moreover, HNF-4 null mice present defects in bile acid transport with increased bile acid levels. These phenotypes can be explained by a) a defect in VLDL secretion as apoB and MTP, essential for VLDL secretion, show decreased expression, b) an increase in hepatic cholesterol uptake due to increased expression of SR-BI (**Figure 7**), and c) a decrease in bile acid uptake to the liver due to bile acid transporter down-regulation. Overall, this study provided strong evidence that HNF-4α is central to the maintenance of hepatic function and is a major *in vivo* regulator of genes involved in the control of lipid homeostasis.

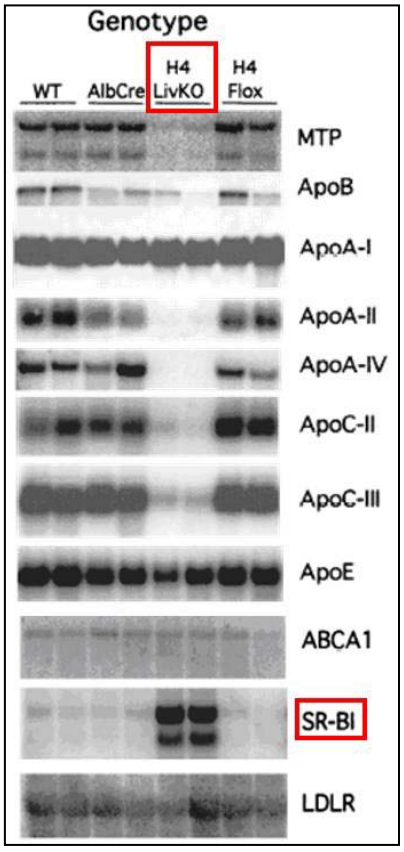


Figure 7| ***Northern blot analysis of liver RNA from H4LivKO and control animals [177].***

GLUCOCORTICOIDS

Functions of glucocorticoids

Glucocorticoids (GC) are steroid hormones that control various physiological actions and are essential for homeostatic regulation of a variety of organ functions. Glucocorticoids have outstanding therapeutic effects and are among the most important drugs in clinical use today. They are used in the treatment of a wide range of diseases such as rheumatoid arthritis, asthma, and other inflammatory diseases. These steroids influence intermediary metabolism (glucose homeostasis, protein, lipid and carbohydrate metabolism), development and programmed cell death. Also, they act as immunosupressors and anti-inflammatory agents, participate in the timing of circadian rhythms in peripheral cells and organs and have effects on the central nervous system (arousal, cognition, mood and sleep) [178-180].

Glucocorticoids are produced in the adrenal cortex. They are highly lipophilic and can easily pass through the plasma membrane. These hormones act as ligands of the glucocorticoid receptor (GR). The GR consists of three different domains with distinct functions: an N-terminal domain with transactivation function, a DNA-binding domain of the zinc finger type and a ligand-binding domain involved in the formation of the hydrophobic ligand-binding pocket. GR has two isoforms termed hGRα and hGRβ. hGRα is expressed in almost all human tissues and cells whereas hGRβ, although it is expressed in a variety of human tissues, it is present in lower concentrations than hGRα.

Glucocorticoid mechanism of action

hGRα exists in the cytosol as an inactive multiprotein complex with chaperones and co-chaperone molecules such as immunophilins p23 and Src. Upon hormone binding, the GR undergoes conformational changes leading to dissociation from chaperones and exposure of its nuclear localization signals. The activated GC/GR complex translocates to the nucleus within 20minutes. In the nucleus, it binds as a homodimer to positive or negative glucocorticoid responsive elements (GRE or nGRE respectively) within promoter or enhancer regions, and regulate the transcription of several genes by either enhancing (transactivation) or inducing (transrepression) their expression [181]. Transactivation of anti-inflammatory genes, such as interleukin-10 (IL-10), annexin-1, mitogen-activated protein kinase (MAPK) and NF-kB is mediated through activated GR binding to positive GREs. Transrepression is mediated by interaction between the activated GR and transcription factors, resulting in reduced expression of pro-inflammatory genes such as activator protein-1 (AP-1), nuclear factor-kB (NF-kB) or signal transducers and activator of transcription (STAT) [182] [183-184].

Glucocorticoids exert their effects mainly by genomic mechanisms through cytosolic receptors. These effects are exerted over a course of hours or days because they require: a) transrepression of genes, b) RNA and protein degradation, or c) transactivation of genes followed by protein synthesis (**Figure 8a, b, c**). However, some effects of glucocorticoids are very rapid and cannot be explained by genomic actions [185-186]. The GR has been shown to exert a "hit-

and run" mechanism of action. A study by McNally et al. showed that the GR resides on DNA for less that 10 s before being ejected and replaced by another GR [187]. The non-genomic effects include: a) non specific, caused by membrane interactions at high concentrations[188], and b) specific, either by interactions with intracellular receptors [189] or by binding to membrane bound glucocorticoid receptors (**Figure 8**) [185-186]. Glucocorticoids have been shown to activate eNOS in a non-genomic manner via PI3K and Akt phosphorylation. This leads to vasorelaxation, which might explain the cardioprotective role of glucocorticoids [189].

Binding of GRα to a GRE results in a conformational change in hGRα that promotes the recruitment of co-activators or co-repressors on the GR-DNA complex, such as CBP/p300, P/CAF, and SRC-1 [180, 190-191]. Repression of genes by the ligand-bound hGRα can be accomplished by directly binding to negative GREs. This is done by prevention of activating transcription factor binding to their cognitive binding sites by competing with GR. However, repression of most inflammatory genes by hGRα is independent of DNA binding. Activated hGRα physically interacts with the nuclear factor-k B (NF-kB), the activator protein-1 (AP-1), Smad protein, signal transduction and activator of transcription (STAT) and cAMP response element (CREB) [180, 190].

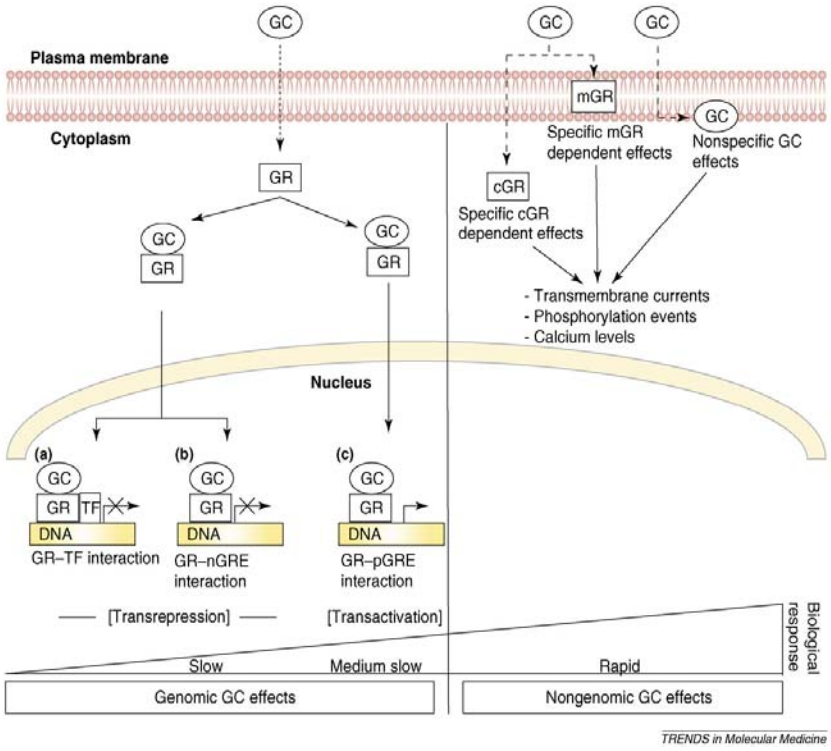


Figure 8| ***Glucocorticoids mechanism of action. Left***: *a,* Ligated GR directly inhibits pro-inflammatory transcription factors (i.e. AP-1, NFAT, NF-kB and STAT), or *b,* actively suppresses transcription (transrepression) of inflammatory genes (i.e. IL-1b and IL-2) through binding to negative GREs (nGRE). *c,* Activated GR induces transcription (transactivation) of immunosuppressive genes (i.e. IkB, annexin-1, IL-10, MAPK phosphatase-1, lipocortin-1, and annexin-1) via positive GREs (pGRE). ***Right:*** GCs induce rapid effects on transmembrane currents, signal transduction (such as TCR and MAPK signaling pathways), second-messenger cascades or intracellular Ca2+ mobilization. It is currently assumed that nongenomic GC effects are mediated by cytosolic or membrane-bound GRs, or via nonspecific interactions with cell membranes. cGR, cytosolic glucocorticoid receptor; GC, glucocorticoids; GRE, glucocorticoid receptor responsive element; MAPK, mitogen activated protein kinase; mGR, membrane-bound glucocorticoid receptor; TCR, T-cell receptor; TF, transcription factor. [184]

Glucocorticoid regulation

Glucocorticoids are under the control of the hypothalamic-pituitary-adrenal (HPA) axis. In response to stress factors, inputs from various brain regions, including the suprachiasmatic nucleus harboring the central circadian timekeeper, elicit the secretion of corticotrophin releasing factor (CRH) from specialized neurons in the hypothalamus. CRH stimulates the release of the adrenocorticotrophic hormone (ACTH) from the pituitary gland into the blood, which in turn triggers the synthesis and secretion of glucocorticoids in the adrenal cortex. Glucocorticoids repress their own synthesis through two negative feedback loops: 1) by inhibiting the synthesis of CRH in hypothalamic neurons and 2) by inhibiting the synthesis of ACTH in pituitary gland corticotrophs [192]. However, glucocorticoids synthesis has been documented in the absence of high levels of ACTH in a wide variety of cases such as depression, Alzheimer's disease, and ischemic injury and abdominal or brain injury [193].

Glucocorticoids and SR-BI

It has been shown that in steroidogenic tissues SR-BI expression is upregulated among others by trophic hormones (LH/hCG, FSH, ACTH), insulin and cAMP analogs [61, 69, 194-195]. Also, both adrenal SR-BI mRNA and protein levels are regulated in response to the synthetic corticosteroid, dexamethasone,

which inhibits the hypothalamic-pituitary axis and decreases ACTH secretion [69]. This observation supports the hypothesis that SR-BI expression in adrenal gland is regulated in response to ACTH. However, Cai et al. reported lack of glucocorticoid stimulation in response to ACTH in SR-BI null mice which indicates that glucocorticoid insufficiency is not due to impaired signalling in the hypothalamic-pituitary-adrenal axis upstream of the adrenals, but it is a result of primary adrenal deficiency due to an inadequate cholesterol supply [196]. Until now little is known about the mechanism of action of glucocorticoids on the hSR-BI gene and more studies are needed in order to elucidate the regulation of SR-BI by glucocorticoids.

CORTICOTROPIN RELEASING FACTOR

Corticotropin releasing hormone (CRH), also termed corticotropin releasing factor (CRF) or corticoliberin, is a 41-amino acid peptide and is the major regulator of the stress response. In the anterior pituitary, CRH stimulates the expression of the propiomelanocortin (POMC) gene in corticotroph cells, which is then cleaved to ACTH. CRH gene is widely expressed throughout the body. The adrenal gland is comprised of the cortex and medulla. The cortex is responsible for steroidogenesis, surrounds the medulla and consists of the zona glomerulosa, the zona fasciculata and the zona reticularis. ACTH stimulates the synthesis and secretion of cortisol from the zona fasciculata, the middle cortical zone.

CRH binds to the CRH-binding protein (CRH-BP), which controls its bioavailability by preventing its binding to the CRH receptors. CRH binds to two distinct CRH receptors, CRH receptor type 1 (CRH1) and 2 (CRH2), which belong to the superfamily of G protein-coupled receptors of brain-gut neuropeptides [197]. CRH1 and CRH2 share a 70% sequence homology, but CRH2 shows no affinity to CRH peptide, whereas CRH1 exhibits high affinity towards CRH. CRH1 is a 415 amino acid protein and is expressed in CNS and the anterior pituitary.

CRH peptides directly affect adrenal steroidogenesis and not via ACTH. This is supported by a study of Jones et al., where it was reported that exogenous CRH enhances adrenal steroidogenesis in hypophysectomized calves [198]. This

direct stimulatory effect of CRH peptides on cortisol synthesis is mediated by the CRH1 receptor [199].

Crh-/- mice born to heterozygous matings have normal viability, are fertile with normal longevity. However, the adrenal size is small in *Crh*-/- fetuses compared to their *Crh +/+* littermates. Also, the expression of StAR protein, which is a rate limiting enzyme in the biosynthesis of glucocorticoids and blood corticosterone levels are significantly lower compared to *Crh*+/+ fetuses [200]. Fetal CRH deficiency causes poor adrenal growth, diminished corticosterone secretions and impaired pulmonary development [200]. Overall, fetal CRH is necessary for the normal development and function of the fetal adrenal gland.

Purpose of study

The purpose of the present study was two-fold:

1. To study the transcriptional regulation of human SR-BI gene in steroidogenic tissues

- The identification and characterization of regulatory elements and transcription factors that regulate the hSR-BI gene in steroidogenic tissues
- The elucidation of the mechanism of feedback inhibition of SR-BI gene expression by glucocorticoids
- The regulation of SR-BI gene expression by Corticotropin releasing factor in adrenal cells

2. To study the transcriptional regulation of human SR-BI gene in the liver

- The identification and characterization of regulatory elements and transcription factors that regulate the hSR-BI gene in the liver
- The elucidation of the mechanism of regulation of the human SR-BI gene by Hepatocyte Nuclear Factor 4α

Significance of study

Coronary artery disease (CAD) accounts for approximately fifty per cent of deaths in the European population. Numerous epidemiological and clinical studies have shown that low high-density lipoprotein cholesterol (HDL-C) levels are associated with increased risk of fatal and non-fatal myocardial infarction independent of other risk factors or pre-existing CAD (primary or secondary prevention). These studies suggest that increasing HDL-C levels will reduce CAD risk. However, currently used drugs such as statins, niacin or fibrates or life-style changes (quit smoking, exercise, diet) did not have the anticipated therapeutic benefit. In addition, promising drugs such as the CETP inhibitor torcetrapid were removed from clinical trials despite the tremendous rise in HDL-C levels because they failed to reduce mortality in CAD patients. These findings suggested that the functionality rather than the concentration of HDL is the important parameter to consider during drug design.

The discovery of SR-BI as the physiologically relevant receptor for HDL offered new therapeutic opportunities for the prevention or treatment of atherosclerosis. Numerous studies in animal models unequivocally showed that SR-BI is a key factor in HDL metabolism and a potential drug target. SR-BI is mainly expressed in the liver, where it participates in the reverse cholesterol transport and facilitates cholesteryl ester uptake for biliary secretion and in steroidogenic tissues, where it supplies cholesterol for steroid hormone synthesis. Understanding the mechanisms of regulation of this important gene in

these cell types will provide novel insights into lipoprotein and glucocorticoid metabolism and is expected to open new avenues for the design of novel SR-BI based drugs for the treatment of CAD or adrenal disorders. Finally, the present study will lead to the generation of new knowledge concerning the mechanisms of transcriptional regulation of genes in adrenal cells as well as the mechanisms of action of the medically important glucocorticoid receptor.

Materials & Method

MATERIALS

Restriction enzymes and their modification buffers for the construction of deletion mutant plasmids were purchased from Minotech and New England Biolabs. Dulbecco's Eagle's medium (DMEM), DMEM F-12, OPTI-MEM, the antibiotic penicillin/streptomycin and Lipofectamine 2000 used for cell cultures were from Invitrogen/Life Technologies. Insulin transferring-sodium selenite supplement was from Roche Diagnostics. Fetal Bovine Serum was from BioChrom Labs. Trizol and Superscript Rnase H-reverse transcriptase for RNA extraction were from Invitrogen/Life Technologies. Dynabeads M-280 streptavidin magnetic beads used for Dnap and SYBR^{R} GreenER™ qPCR SuperMix for Real Time PCR were purchased from Invitrogen/Life Technologies. GoTaq, dNTPs, 5xBuffer and $MgCI_2$ used for PCRs were purchased from Sigma-Aldrich as were the SHIELDS&SANG INSECT m3 MEDIUM, ONPG and the ligands for nuclear receptors 22 (R)-hydroxycholesterol and 9-cis retinoic acid. The luciferase assay system, cell lysis buffer, Wizard SV gel, PCR cleanup system were all from Promega. The ECL substrate for development of Western blots was from Pierce. Corticosterone 125IRIA kit for measurement of corticosterone in mice was purchased from ICN Biomedicals. Infinity™ cholesterol liquid stable reagent and Data-Cal™ calibrator reference serum for measurement of serum cholesterol in mice were from Thermo Fisher Scientific. Metyrapone, dexamethasone, mifepristone and mithramycin A were purchased from Sigma-Aldrich. Silencer siRNA for human Glucocorticoid receptor was purchased from Ambion. CRF peptide was a generous gift from Dr. Tsatsanis and hCRF-1/pin4 plasmid was a

generous gift from Dr. Liapakis. pcDNA3, pcDNA3hGRa and GRE-luc were obtained from Pr. Russher.

Rabbit polyclonal anti-SR-BI was from Novus Biologicals. Glucocorticoid receptor antibody was from Santa Cruz Biotechnology. Anti-goat HRP and anti-rabbit HRP were from Sigma-Aldrich. Anti-mouse peroxidase-conjugated secondary antibody was purchased from Chemicon international Inc. (Temecula, CA). Anti-GFP antibody was from Minotech.

METHODS

Agarose gel electrophoresis

Depending on the size of DNA agarose gels of 0,5%, 1%, 1,5% and 2% agarose were used and prepared in 1xTAE (50xTAE: 2M tris HCI pH 7.5, 2mM EDTA and acetic acid). The mixture of 1xTAE and agarose is boiled and after cooling it, 7µl of ethidium bromide is added and poured in a tray. After stabilization the mixture is placed in an electrophoresis tank which contains 1xTAE and DNA is loaded in wells and electrophoresis takes place at 100-120Volt.

Ligation reaction

Cut DNA and plasmid vector individually to yield complementary ends. Combine in a ligation reaction in total volume of 10-15µl. Use 0.03pmol of vector DNA and 0.1 pmol of insert. Add T4 DNA ligase enzyme and 10x T4 DNA ligase buffer and adjust volume up to 10 or 15µl with ddH_2O. Incubate overnight at 4^oC or for 30minutes at 16^oC.

Transformation

- Add 100-150µl DH10β competent cells to the ligation reaction
- Leave 30min on ice
- Heat shock for 45sec at 42^oC
- Cool on ice for 2min and add 900µl growth medium
- Mix and IB for 1hr at 37^oC

- CF at 3.500rpm for 5min
- Discard using a pipette 900μl of the supernatant-medium and mix by pipetting up and down
- Spread all the cells on an LM agar plate
- Keep in the incubator O/N (37^{o}C)

Miniprep procedure-Micro screening

Following incubation of colonies picked in a 2ml LB-ampicillin (kanamycin etc) for 16-18hours at 37oC with vigorous shaking:

- Transfer bacteria from mini-cultures to 1.5ml eppendorfs
- CF at 13.000rpm for 1min, aspirate

Bacterial lysis with lysozyme

- Resuspend pellet in 600μl *E.Coli* lysis buffer, vortex
- Add 30μl lysozyme (10mg/ml in H_2O), vortex to dissolve pellet
- IB for 5min at RT
- Boil for 90sec at 100^{o}C
- Cool on ice for 2min
- CF at 13.000rpm for 15min and remove pellet with a toothpick
- Add 600μl isopropanol (cold), mix
- IB for 30min at -20^{o}C
- CF at 13.000rpm for 15min
- Aspirate supernatant and add 500μl 75%EtOH
- CF at 13.000rpm for 5min and dry pellet (either in speed vac or RT)

- Dissolve in 30/40µl TE with added RNase (10mg/ml)
- Flash spin 3x, mix

Bacterial alkaline lysis

- Add 150µl of P1 (with added RNAse out) and dissolve pellet by pipetting
- Add 150µl of P2 and mix
- Add 150µl of P3 and mix
- CF at 13.000rpm for 15min
- Transfer filtered supernatant to a clean eppendorf
- Add 2/3 of volume isopropanol
- IB for 15min at -20^{o}C
- CF at 13.000rpm for 15min
- Aspirate and add 250µl 75% EtOH
- Dry pellet and dissolve in water

✓ Load mini-preps in an agarose gel to check for positives

✓ If positives are obtained digest with the appropriate enzyme(s)

✓ IB for 1hr at 37^{o}C (depends on the enzyme using) and run the digestions in an agarose gel

✓ If positives are obtained pick one to use for streaking on an LM agar plate and incubate overnight at 37^{o}C

Isolation of plasmid DNA from large scale bacterial cultures

Grow 200ml cultures with antibiotic selection shaking overnight at 37^{o}C. For the isolation of plasmid DNA the Qiagen midi-maxi prep kit was used from Qiagen. The concentration of the DNA plasmid is measured at 260nm.

Ethanol precipitation

- Add to the sample 2.5 volumes of 100% EtOH and 1/10 volume 3M CH_3COONa, pH 5.5
- Vortex and keep at -80^{o}C for 10min
- CF at 13.000rpm for 10min
- Aspirate and add 1ml 75% EtOH
- CF at 13.000rpm for 5min
- Aspirate and resuspend in H_2O

Constructs

The hSR-BI wt and deletion promoter plasmids were constructed by PCR amplification and subsequent cloning into the pGL3Basic vector. The hSR-BI promoter plasmid -2,787/+212 was generated using as template genomic DNA from HepG2 cells and cloned into pGL3Basic vector with enzymes KpnI and XhoI. The hSR-BI promoter -408/+212 was cloned into pGL3Basic vector using the enzymes KpnI and XhoI. The hSR-BI promoter -1363/+78 was cloned into the Topo vector which was digested with HindIII to give the hSR-BI promoter -1,288/+78. The latter was cloned to pGL3Basic digested with HindIII. The hSR-BI promoters -944/+78, -673/+78, -290/+78, -201/+78, -88/+78 were all

constructed using the enzymes KpnI and HindIII. The SR-BI promoter -62/+78 was generated by digestion of the SR-BI promoter plasmid -1,288/+78 with SmaI. The sequences of all oligonucleotides used as primers in PCR amplification are shown in Table 1. We also used the expression vectors pcDNA3-6myc-SREBP-1a, pcDNA3-6myc-LXR, pcDNA3-6myc-RXR, that were already in the lab.

Cell lines

The cell lines used in this thesis are the following: the human embryonic kidney cell line HEK293T, the human hepatocelular carcinoma cell line HepG2, the human adrenocorticocarcinoma cell line H295R, the human ovarian cell line SKOV-3, the drosophila S2 cell line (Schneider's) and the 911 cell line. Cells lines HEK293T, HepG2, SKOV-3 and 911 were grown at 37^{o}C under a humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle medium (DMEM) which contains 4.5g/L D-glucose and with added 10% FBS and 1% P/S (penicillin/streptomycin). The human adrenocortical cells were grown at 37^{o}C under a humidified 5% CO_2 atmosphere in a 1:1 mixture of Dulbecco's Modified Eagle medium and Ham's F12 medium containing 15mM HEPES, 10μg/ml ITS (insulin, transferring, selenium), 10nmol/L hydrocortisone, 10nmol/l β-estradiol, 2% FBS and 1% P/S. For experiments with hormones, hydrocortisone, β-estradiol and FBS were excluded from the medium. Drosophila SL2 cells were cultured in Schneider's insect medium supplemented with 10% insect culture-tested fetal bovine serum and penicillin/streptomycin at 25^{o}C.

Transcient transfections

Transient transfections were carried out with calcium phosphate method and Lipofectamine 2000 method. In $CaCI_2$ transfection there is combination of $CaCI_2$ (2M), DNA and WFI. Then equal volume of 2x Hepes Buffered Saline (HBS) is added, the mixture is mixed by vortexing and incubated at RT for 10minutes before addition to cells. Cells are incubated and after 16-18 hours the medium is changed to fresh one (except S2). For lipofectamine transfection in 6-well plates, DNA (4μg) plus OPTI-MEM (192μl) is prepared in one tube and lipofectamine 2000 (6μl) plus OPTI-MEM (194μl) in a separate tube and incubated for 5min before addition of DNA mixture. Following 20minutes incubation at RT, 1.6ml of the desired medium is added to the mixture in order to have 2ml in total. The medium in the cultured cells is removed and 1ml of the mixture is added to cells in each well. Cells are incubated and after 16-18hrs medium is changed to fresh one. Ligands and repressors (dexamethasone, 9-*cis*-retinoic acid, 22-(R)-hydroxycholesterol, mifepristone) are added after the renewal of the medium and are incubated for 2hrs for RNA expression experiments and for 24 hours for transactivation experiments before harvest.

Charcoal stripped FBS (Foetal bovine serum)

When very low concentration of hormones is needed then CSS is used instead of FBS. This procedure removes lipophilic material such as the concentration of cholesterol and hormones (cortisol, estradiol, progesterone, testosterone, T3 and T4).

- Add to 50ml FBS 0.125gr ***charcoal*** and 0.0125gr ***dextran or glucose***

- Mix and IB at 56^{o}C for 30min (shake every 5min)
- CF at 3.000rpm for 15min
- Transfer the supernatant to new falcon and add again 0.125gr ***charcoal*** and 0.0125gr ***dextran or glucose***
- Mix and IB at 37^{o}C for 30min (shake every 5min)
- CF at 3.000rpm for 15min and transfer supernatant to a clean falcon
- Filter the supernatant using a 0.20μm filter and keep at -20^{o}C

Preparation of bio-oligonucleotides

Reaction mix: 400μM of Bio-primer

400μM of primer

2μl Neb2 buffer

Adjust with H_2O up to 20μl

- Boil at 92 ^{o}C for 2min
- Cool slowly at 4^{o}C
- Add water for injection to adjust the concentration to 0.58μM

DNA affinity precipitation assay (DNAp)

Nuclear extract isolation

- 1x wash with PBS
- Scrape cells with 1xPBS
- CF at 4.000rpm for 10min at 4^{o}C
- Measure pellet

- Resuspend quickly in 5x (cell volume) ***Hypotonic buffer*** + protein inhibitors
- CF at 4.000rpm for 5min at 4^{o}C
- Resuspend quickly in 3x (cell volume) ***Hypotonic buffer*** + protein inhibitors
- Leave for 10min on ice
- Homogenize with insulin syringe (27g) 20 times
- Pellet the nuclei at 6.000rpm for 15 at 4^{o}C
- Measure volume and resuspend in ½ (pellet volume) ***Low salt buffer + protein inhibitors,*** Vortex
- Add ½ (pellet volume) ***High salt buffer + protein inhibitors*** drop wise while vortexing
- Rotate for 30min at 4^{o}C
- CF at 13.000rpm for 40min at 4^{o}C
- Collect supernatant
- Perform Bradford/Lawry
- Can keep supernatant at -80^{o}C

Beads preparation

- Use 5µl ***Dynabeads M-280 Streptavidin*** (50µg) and wash once with 500µl 1x ***B&W***
- Add 7µl 2x ***B&W*** and 7µl ***Biotinylated oligo*** (0.58µM)
- IB for 15min RT (for control add to the beads 14µl of 1x B&W)
- Wash twice with 1x B&W
- Wash once with D buffer

Nuclear extract preparation

- Add:
    - 6µl of carrier mix [1µl dI-dC (3µg/µl) + 5µl 10x Binding buffer]
    - 30µg of nuclear protein extract (depends on nuclear extract)
    - D buffer up to 50µl
    - IB on ice for 15min

➢ Add the prepared nuclear extract to the beads

➢ IB on ice for 30min

➢ Wash three times with 500µl D buffer

➢ Add loading buffer and boil for 10min

Hypotonic buffer	Low salt buffer	High salt buffer	10xBinding buffer
10mM Hepes pH7.9	20mM Hepes pH7.9	20mM Hepes pH7.9	100mM Hepes pH7.9
1.5mM $MgCl_2$	1.5mM $MgCl_2$	1.5mM $MgCl_2$	20mM $MgCl_2$
10mM KCl	0,02M KCl	1,2M KCl	0,5M KCl
0,2mM EDTA	0,2mM EDTA	0,2mM EDTA	40mM Spermidine
PMSF (10µl/ml)	25% glycerol	25% glycerol	1mg/ml BSA
DTT (2µl/ml)	PMSF (10µl/ml)	PMSF (10µl/ml)	0.2mM Zn acetate
Benzam.(1µl/ml)	DTT (2µl/ml) Benzam. (1µl/ml)	DTT (2µl/ml) Benzam. (1µl/ml)	0.5% NP-40 (10%)

D buffer	2x B&W	BBRC
20mM Hepes pH7.9	10mM Tris-HCl pH 7.5	10mM Tris-HCl pH 7.5
10% glycerol	1mM EDTA	10% glycerol
40mM KCl	2mM NaCl	50mM KCl
0.5mM DTT		4mM $MgCl_2$, 0,2mM EDTA

Table 2 | *Buffers for DNap*. Adjust the volume of buffers with ddH_2O and always add inhibitors (PMSF, DTT, benzamidine) just before use.

Chromatin immunoprecipitation assay (Chip)

Chromatin purification

- Aspirate medium
- Wash with 7ml DMEM (RT) and aspirate
- Add 9ml DMEM + 1ml ***Formaldehyde*** (slowly)
- IB 10min at 37^{o}C (yeast incubator)
- Add 1ml ***Glycine*** (1.375m, 4^{o}C), dropwise, quickly and shake
- Aspirate and do 3 washes with 10ml ***1xPBS/0.5mM PMSF***
- Add 7ml ***1x PBS/0.5% NP-40/0.5mM PMSF***
- Scrape on ice and collect in 15ml falcon
- CF at 1.000rpm for 5min
- Aspirate, redissolve in 5ml ***Swelling buffer*** (firstly in 1ml, then add the rest 4ml)
- Keep on ice for 10min
- Break cells with Dounce (adjust to 2.5-3) ~30 times, keep on ice every 10 times
- Add 5μl of sample to 5μl 0.4% Trypan blue and check cells under microscope if they are broken and if nuclei are free and blue
- Transfer sample to a new 15ml falcon
- CF at 2.000rpm for 5min, 4^{o}C
- Redissolve pellet in 2ml ***Sonication buffer*** (1ml 1st + 1ml)
- Transfer to a 5ml plastic tube for sonication (no bubbles)
- Adjust sonicator to pulse 50-65% for 30'' (keep sample on ice for 2-3min between intervals)

- Transfer to a 2ml the eppendorf
- CF at 14.000rpm for 15min , 4°C
- Transfer again to a 2ml eppendorf
- Transfer 50μl from sample to a 1.5ml eppendorf and add 150μl ***H_2O*** and 10.5μl ***4M NaCl***
- IB at 65°C O/N and keep the rest of the sample at -80°C

Checking the quality of chromatin

- Add 2μl ***RNAseA*** 10mg/ml (1/10 in H_2O) to the sample that was IB at 65°C
- IB for 1hr at 37°C and add 2μL EDTA (0.5M)
- Add 210μl ***phenol/chlorophorm/isoamyl***, vortex
- CF at 13.000rpm for 5min RT
- Transfer the upper phase to a clean eppendorf and add 210μl ***chlorophorm*** and 4μl (5μg/Ml) ***glycogen,*** vortex
- CF at 13.000rpm for 5min, RT
- Tramsfer the upper phase (~200μl) to a clean eppendorf
- Add 1/10 of volume (20μl) 3M ***CH_3COONa*** and 2 ½ volumes (500μl) ***100% EtOH***
- Keep at -80°C for 30min and CF at 13.000rpm for 10min RT
- Discard supernatant and add 300μl ***75% EtOH***
- CF at 13.000rpm for 10min RT
- Discard supernatant, let pellet to dry in the hood
- Dissolve in 20μl ***WFI*** and leave on ice
- Run on a 1.5% agarose gel (7μl DNA + 2μl dye w/o Bromophenol blue)
- Chromatin must run low in the gel below 500bp

Preparation of G beads -Preclearing

NEED: 20µl G beads for w/o Ab sample
20µl G beads for + Ab
40µl G beads for preclearing
} 80µl per p100plate

- Take 170-180µl G protein beads and CF at 5.000rpm for 2min at 4°C
- Pipette remove the upper phase
- CF at 5.000rpm for 2min
- Pipette remove the upper phase (if not enough add more beads)
- Add 1.5ml ***Sonication buffer***
- Rotate for 10min at 4°C } x3
- CF at 5.000rpm for 2min at 4°C
- Pipette remove the upper phase
- Add 494µl ***Sonication buffer*** and 5µl (100mg/ml) ***BSA*** and 1µl (0.5µg/µl) ***λDNA***
- 2hr rotation at 4°C (defroze chromatin and measure OD if differ parameters)
- Transfer 180µl beads to 2 clean 1.5ml eppendorfs and store at 4°C (mix well before transfer in order to have equal volumes)
- The rest of the beads are for preclearing
- CF at 5.000rpm for 2min at 4°C
- Pipette remove upper phase
- Add 1500µl chromatin, 15µl ***BSA***, 3µl ***λDNA***
- 2hr rotation at 4°C
- CF at 5.000rpm for 2min at 4°C and transfer supernatant to new tubes
- Take: 65µl for INPUT (store at -20°C)

650µl for sample w/o Ab
650µl for sample + Ab
} 2hr rotation at 4°C

- CF at 5.000rpm for 3min at 4°C the beads that were stored at 4°C
- Pipette remove supernatant and keep the clean beads
- Transfer 650µl (w/o Ab) and 650µl (+Ab) to the clean beads
- Rotate at 4°C O/N

Washes

- CF at 5.000rpm for 2min at 4°C
- Pipette remove supernatant
- Add 1ml ***Wash buffer A***
- Rotate for 10min at 4°C
} x2
- CF at 5.000rpm for 2min at 4°C
- Pipette remove supernatant
- 2x washes as above with ***Wash buffer B***
- 2x washes as above with ***Wash buffer C***
- 2x washes with 1ml ***TE*** (remove supernatant completely)
- Add 150µl ***Elution buffer*** (warmed at 37°C)
- Vortex
- IB for 10min at 65°C
- Vortex
} x2
- CF 13.000rpm for 1 min at RT
- Transfer supernatant to new eppendorf
- Total supernatant from the 2 washes with elution buffer is ~300µl

- Add 100µl ***H_2O*** and 21µl 4M ***NaCl***
- Add to INPUT sample up to 300µl Elution buffer (235µl), 100µl H_2O and 21µl ***4M NaCl***
- Shake and flash spin **and** IB at 65°C O/N

DNA crosslinking

- Add 1µl ***RNase A*** to the samples kept at 65°C O/N
- IB at 37°C for 1hour
- Add 2µl ***EDTA*** (0.5M) and 2µl ***Proteinase K*** (10mg/ml)
- IB at 42°C for 2hours
- Add 200µl H_2O, 1/10 of volume ***CH_3COONa*** (60µl) and equal volume of ***phenol:chlorophorm:isoamyl*** (600µl)
- Vortex for 30sec
- CF at 10.000rpm for 5min
- Transfer supernatant to clean 2 ml tubes
- Clean with equal volume of ***chlorophorm*** (600µl)
- Vortex 30sec and CF at 10.000rpm for 5min
- Transfer supernatant to clean 2ml tubes
- Add 4µl ***glycogen*** and 2.5volumes EtOH 100% (1500µl)
- Vortex and keep at -20°C O/N

DNA purification

- CF at 13.000rpm for 30min at 4°C
- Aspirate and add 500µl 75% ***EtOH***
- CF at 13.000rpm for 10min at 4°C

- Remove supernatant and let pellet to dry in the hood
- Redissolve pellet of input in 100µl 10mM ***Tris*** (1/100) (pH 7.5) and of samples in 50µl 10mM ***Tris*** (pH 7.5)
- Perform PCR

Swelling buffer	Sonication buffer	Wash buffer A	Wash buffer B	Wash buffer C
25mM Hepes pH7.9	50mM Hepes pH7.9	50mM Hepes pH7.9	50mM Hepes pH7.9	20mM Tris-CI pH 8
1.5mM $MgCI_2$	140mM $MgCI_2$	140mM NaCI	500mM NaCI	250mM LiCi
10mM KCI	1% Triton X-100	1% Triton X-100	1% Triton X-100	0.5% NP-40
0.5% NP-40	1mM EDTA	1mM EDTA	1mM EDTA	1mM EDTA
	0.1% Na Deoxycholate	0.1% Na Deoxycholate	0.1% Na Deoxycholate	0.1% Na Deoxycholate
	0.1% SDS	0.1% SDS	0.1% SDS	
0.5mM PMSF, 2µg/ml Aprotinin, 1mMDTT	0.5mM PMSF, 2µg/ml Aprotinin	0.5mM PMSF, 2µg/ml Aprotinin	0.5mM PMSF, 2µg/ml Aprotinin	0.5mM PMSF, 2µg/ml Aprotinin

Elution buffer	TE+inhibitors
50mM Tris-CI pH 8	10mM Tris-CI pH 8
1mM EDTA	1mM EDTA
1% SDS	0.5mM PMSF, 2µg/ml Aprotinin
50mM $NaHCO_3$	

Table 3| ***Buffers for Chromatin immunoprecipitation assay.*** Adjust the volume of buffers with ddH_2O and always add inhibitors (PMSF, DTT, benzamidine) just before use.

RNA extraction from cells

- Aspirate medium
- Add 1ml PBS (ice cold)
- Aspirate and add ***1ml trizol*** and harvest cells in a 2ml tube
- IB at RT for no more than 5min
- Add ***200µl chlorophorm*** and vortex until a homogenized mixture is formed
- IB 2-3min at RT
- CF at 12.000rpm for 15min at 4^{o}C
- Transfer supernatant to a 1.5ml eppendorf
- Add ***500µl isopropanol*** at RT and shake mildly
- IB for 10min at RT
- CF at 12.000rpm for 15min at 4^{o}C
- Add 1ml 75% EtOH
- Vortex but pellet won't be dissolved
- CF at 12.000rpm for 15min at 4^{o}C
- Aspirate supernatant and dry pellet
- Redissolve in 30-50µl (depends on the pellet), mild shake and leave on ice
- Mix with pipette and leave on ice
- Measure OD at 260nm and calculate RNA concentration by the formula

  OD*200/1000*40 = µg/µl

RNA extraction from tissue

Homogenization if extraction from tissue

Homogenize sample in ***1ml trizol*** per 50-100mg of tissue in a 2ml tube (optional: CF and remove supernatant)

Phase separation

- IB homogenized sample for 5min at RT to permit complete dissociation of nucleoprotein complexes
- Add ***200µl chlorophorm*** per 1ml of trizol
- Shake tubes for 15sec by hand and IB for 2-3min at RT
- CF at 12.000rpm for 15min at 4^{o}C (2-8^{o}C)
- Following CF RNA remains in the colorless upper aqueous phase

RNA precipitation

- Transfer aqueous phase to a fresh tube (1.5ml) and save the organic phase if isolation of DNA or protein is desired
- Precipitate the RNA by mixing with ***500µl isopropanol***
- IB samples at RT for 10min and CF at 12.000rpm for 10min at 4^{o}C

RNA wash

- Remove supernatant and wash RNA pellet once with ***1ml 75% EtOH***
- Mix by vortexing and CF at no more than 7.500rpm for 5min at 4^{o}C

Redissolving RNA

- Dry pellet briefly and don't let the RNA pellet to dry complete
- Partially dissolved RNA samples have an $A_{260/280} < 1.6$

- Dissolve RNA in ***30-50µl RNase-free H_2O*** or ***0.5% SDS*** by passing solution few times through pipette tip
- Measure absorbance at 260nm on a nanodrop

Reverse transciption

Dilute RNA to a working concentration of 250ng-500ng in RNase-free H_2O.

Reaction mix: 1µg RNA

10mM dNTs

300pmol Random primers

Adjust with RNase-free H_2O up to 33.5µl

PCR reaction: 65°C for 5min→chill on ice and add 10µl 5xFS buffer and 5µl DTT

25°C for 2min→ add 1µl reverse transciptase and 0.5µl RNase out

25°C for 10min

42°C for 50min

70°C for 15min

Quantitative Real-Time PCR analysis

Real time PCR is a sensitive and reliable quantitative method for gene expression analysis were both genomic and reverse transcribed cDNA can be used as templates. In this thesis real-time PCR data were analyzed by relative quantification which relies on the comparison between expression of a target gene versus a reference gene and the expression of the same gene in target sample versus reference samples. Quantitative real-time RT-PCR was performed on a Stratagene MX3000 machine. For the detection and quantitation of gene

transcripts, SYBR GreenER was used which is a dye that fluoresces when bound non-specifically to double-stranded DNA. During denaturation step as DNA is single-stranded, SYBR GreeER is free in solution and produces little fluorescence. Glyceraldehyde-3-phosphate dehydrogenase gene was used as a reference gene as it is expressed in all cells and has the same copy number in all cells. Total RNA from mouse adrenals was reverse transcribed as described above and twenty five nanograms of resultant cDNA were used per 25µl reaction. The concentrations of primers used were 0.4µm for SR-BI gene and 0.1µM for GAPDH gene. Each cycle consisted of 30sec at 95^{o}C, 45sec at 58^{o}C and 30sec at 72^{o}C for a maximum of 35cycles. The primers used for the amplification of the mouse SR-BI and GAPDH genes are shown in Table 5.

Quantitation of the amount of cDNA in the sample is done when the amplification is logarithmic, at the beginning of the upturn of the curve while it is still linear. As the concentration of the product is rising the curve reaches a plateau phase. For data analysis the ΔΔCt model was used. Ct is the point at which the fluorescence crosses the threshold. The amount of target, normalized to the endogenous reference and relative to a calibrator, is given by the formula:

$$\textbf{Ratio} = 2^{-\Delta\Delta Ct}$$

$$\textit{where}\ \Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{target}$$

$$\textit{whereas}\ \Delta Ct_{reference} = \text{average } Ct_{control} - \text{average } Ct_{treatment}$$

$$\Delta Ct_{target} = \text{average } Ct_{control} - \text{average } Ct_{treatment}$$

A validation experiment was performed before using the ΔΔCt method in order to check if the efficiencies of target and reference genes are equal. This was

verified by plotting the absolute value of the slope of log input amount vs. ΔCt of serial dilutions of the template and it was found to be <0.1.

Collection of cells

Collection with Promega lx lysis buffer

- Wash cells with 1ml 1x PBS
- Scrape on ice and collect in 500µ-1ml 1x PBS
- CF at 5.000rpm for 5min
- Dissolve pellet in 50µl 1x lysis buffer (promega)
- Vortex and freeze at -80°C for 10min
- Thaw on ice
- CF at 13.000rpm for 5min
- Collect supernatant

Collection with Co-IP lysis buffer

- Wash cells with 1ml 1x PBS
- Scrape on ice and collect in 500µl -1ml 1x PBS
- CF at 5.000rpm for 5min at 4°C
- Aspirate and dissolve pellet in 100µl (depends on pellet) Co-IP lysis buffer (with PMSF and benzamidine)
- Rotate for 30min at 4°C
- CF at 13.000rpm for 5min
- Collect supernatant

Bradford/Lawry protein quantitation

- Duplicates
- Mix: 195μl H_2O + 5μl extract + 100μl ***A´***

A´ = 1ml A + 20μl S

or

190μl H_2O + 10μl extract + 100μl ***A´***

+

800μl ***B***

- IB for 15min RT
- Measure absorbance at 750nm (visual)
- Average absorbance of duplicates
- Use for calculations: $y = \alpha + \beta x$ =>

=> x = μgr of protein /5 or 10μl of extract

where $\alpha = 0.02348$

$\beta = 0.00745$

y = OD

- Then calculate the μgr needed and equilibrate the samples in order to have the same amount of protein in every sample

Whole protein determination in tissues

- Cut the tissue in small pieces (for liver ½ of lobe)
- Add RIPA buffer (for liver 1ml) and homogenize with polytron homogenizer
- Rotate for 20min at 4°C
- CF at 14.000 rpm for 30min at 4°C and keep the supernatant

- Perform Bradford/Lawry to estimate the quantity of protein obtained

RIPA buffer
50mM, pH 7.4 Tris –HCI
1% NP-40
0.25% Na Deoxycholate
150mM NaCI
1mM EDTA
1mM PMSF, 2μg/ml Aprotinin, 1mM NaF,1mM Na_3VO_4

Table 4| ***RIPA buffer for whole protein determination in tissues.*** Adjust the volume with ddH_2O and always add inhibitors just before use

Western blotting

The desired amount of cells lysates was boiled with 4x sample buffer for 15min, subjected to SDS-PAGE and transferred to nitrocellulose membranes, with a Bio-Rad Protean electroblot apparatus. Electrophoresis was performed on 10.5% polyacrylamide gel electrophoresis in 500 ml 1x TGS (1L 10x TGS: 30.3 gr Tris, 144.2 gr Glycine, 10 gr SDS, pH 8.3). Nitrocellulose membranes were washed with TBS-T (TBS + 0.1% Tween-20) for 10 min, at RT. Non-specific sites were blocked by soaking the membrane in TBB buffer (1x TBS + 5% non-fat milk, 0.1% Tween-20) for 1hr at 4^{o}C. Western blotting was performed with a 1:200 dilution of the anti-hGRα, a 1:2,500 dilution of the anti-SR-BI, a 1:1,000 anti-GFP and a 1:5,000 anti-actin antibodies in TBB overnight at 4^{o}C. The membranes were washed 3 times with TBS-T, for 10 min, at room temperature. As a secondary antibody anti-mouse or anti-rabbit horseradish peroxidase-conjugated (HRP) were used, in a 1:10,000 dilution in TBS-T, for 1 h at RT. After

3 washes of 10 min with TBS-T at RT, bands were visualized by enhanced chemiluminescent detection on Fuji medical X-Ray film (Super RX).

Animal housing and treatment

Male *Crh*+/+ and *Crh*−/− mice of 129×C57BL/6 genetic background were generated as previously described [201]. All mice were housed with *ad libitum* access to rodent chow on a 12:12 light/dark cycle (lights on at 7:00 a.m.). All experiments were approved by the Animal Committee of Medical of University of Crete. All experiments were performed in mice of 2-4 months of age. Animals were housed individually at least 16hours before each experiment. Crh-/- and Crh+/+ mice were administered with 10μg/ml corticosterone or ethanol respectively in drinking water for 3 days. The pharmacological adrenalectomy expreriment was carried out as previously described [202]. In brief, WT mice were injected intraperitoneally twice a day for 4 days with 200mg/Kg of metyrapone or 100μl 10% DMSO and on the fourth day 100mg/Kg aminoglutethimide was injected twice in combination with metyrapone.

Blood and tissue collection, plasma hormone and cytokine assays

Blood samples were collected by retroorbital eyebleeding of conscious mice. Mice were sacrificed by decapitation and adrenal tissues well collected and frozen rapidly on dry ice. Blood samples were centrifuged at 3.000 rpm for 10min at 4^{o}C, and plasma was separated, aliquoted, and stored at -80^{o}C until further use. Plasma corticosterone levels were measured using the ^{125}I Radioimmunoassay kit (RIA).

Determination of corticosterone in mouse plasma (^{125}I RIA KIT)

This kit utilizes the combination of a highly specific antiserum for corticosterone and a proprietary blocking function incorporated in the assay system. In this assay a limited amount of specific antibody (Ab) is reacted with the corresponding hormone (*H) labeled with a radioisotope. Upon addition of an increasing amount of the hormone (H), a correspondingly decreasing fraction of *H added is bound to the antibody. After separation of the bound from the free *H by various means, the amount of radioactivity in one or both of these two fractions is evaluated and used to construct a standard curve against which the unknown samples are measured. The assay was carried out according to the manufacturer's instructions.

Adenovirus contruction

Specifically, AdhGRα was constructed by extracting the cDNA of hGRα from the pcDNA3.1hGRα vector by enzymes KpnI and XbaI and following ligation to the KpnI and XbaI restriction sites of the pAd-Track CMV vector.

- Cloning the gene of interest into a pAD-Track CMV vector
- Ligation of the gene of interest to pAD-Track digested with the same enzymes
- Transformation in DH10β cells and selection for kanamycin resistance
- Screen for positive clones by digesting with restriction enzymes
- Mini-preparation and streaking of the positive clone
- Amplification and purification of the positive clone

- Linearize construct by digesting with enzyme P*me*I
- Extract and purify the resulting band by gel extraction
- Recombination of the linear band in 100µl E.coli BJ5183-AD1 cells by transformation (E.coli BJ5183-AD1 cells contain the adenoviral backbone plasmid, pAdEasy)
- Mini-preparation by alkaline lysis purification and transformation in E.coli DH10β cells by plating all cells and using DNA of the mini-preparation
- Select for kanamycin resistance and screen for positive clones by digesting with enzyme *Pac*I
- Amplification and purification of the positive recombinants
- Cleave the recombinant plasmid (~15µg) with enzyme *Pac*I
- Purify the upper band by EtOH precipitation

Infection and amplification of adenovirus into the packaging cell line 911

- Lipofectamine 2000 was used for infection of 10µg purified DNA into $1.5*10^6$ 911 cells in a p100 plate (Change medium to DMEM 2% HIHS 1%P/S before transfection)
- Incubate in a 37°C incubator under a humidified 5% CO_2 for 10-14 days (Next day after transfection check for fluorescence)
- Harvest lysed cells, freeze (-80°C) and thaw (37°C) 3 times and CF at 3.500rpm for 10min at 4°C
- Collect supernatant and infect a T175 flask with 911 cells with 2.5ml of adenovirus supernatant
- Incubate in a 37°C incubator under a humidified 5% CO_2 for 2-3 days

- Collect lysed cells and infect 5 T175 flasks with 911 cells with the virus collected
- Harvest cells before lysis occurs (when cells burst) and CF at 1.800rpm for 10min at 4°C
- Discard supernatant and add 1ml of DMEM 2% HIHS 1%P/S to dissolve one pellet
- Combine the 5 dissolved pellets to one (resulting virus: 1ml) and keep in cryovials at -80 °C
- Perform either plaque assay or FFU assay for calculating the MOI of the virus

Plaque assay

- Prepare 1.5% agarose solution (100ml): 1.5gr agarose, 4ml Hepes 1m, pH 7.4 and 96ml H_2O. Dissolve agarose by autoclaving.
- Prepare 10x mix: 250μl $MgCI_2$, 400μl HIHS and 9.35ml 2xMEM
- Prepare adenovirus dilutions in DMEM 2% HIHS as follows:

Tube 1: 50μl virus + 950μl medium

Tube 2: 10μl virus + 990μl medium

Tube 3: 10μl virus + 990μl medium

Falcon #4: 300μl virus + 2700μl medium

Falcon #5: 300μl virus + 2700μl medium

- Remove DMEM 10%FBS 1%P/S medium from 6well plate with 911 cells and add 1ml/well from the virus dilutions in falcons #4 and #5 i.e.

#4	#4	Blank
#5	#5	Blank

- IB for 15min at 37 ^{o}C (in the meantime add the 10x mix to 10ml agarose solution and place in a 55 ^{o}C waterbath)
- Remove virus from 6 well plate starting from blank→#5→#4
- Add 3ml of agarose mix kept at 55 ^{o}C
- IB in a 37^{o}C incubator under a humidified 5% CO_2 and wait for 10-12days

Calculating PFU and MOI

For PFU (Plaque forming units):

- ✓ if counting plaques in falcon #4: average plaques * 10^5/µl of virus

  i.e: 3*10^5/50µl of virus
- ✓ if counting plaques in falcon #5: average plaques * 10^6/µl of virus

  i.e: 3*10^6/50µl of virus

For MOI (Multiplicity of infection):

Use the formula: **$\alpha = (y*x)/\beta$**

where α = MOI

y = µl of virus

β = number of cells used in the experiment

x = virus concentration (PFU)

Fluorescence forming assay

- Split a confluent 75 cm^2 flask with 911 cells and use 1ml to plate three 35mm plates
- Next day coat plates with 1 ml (25µg/ml) collagen/ well. Keep the plates in the hood.(Stock solution: 1 mg/ml in 0.5 M acetic acid stored in -20º C. Dilute in PBS 25 µg/ml and keep at 4º C.)
- Let sit for 30 min or more
- When dry, recover the plates or reseal in plastic bags and store at RT
- Prepare 10 fold virus dilutions from 10^2 to 10^8 in DMEM 10% FBS 1% P/S
- Use dilutions 10^6, 10^7, 10^8 and add 1ml of virus dilution to a plate coated with collagen
- IB for 2hrs in a 37°C incubator under a humidified 5% CO_2
- Aspirate the virus solution off and wash with 1ml fresh growth medium
- Aspirate the medium off and add 2ml of fresh growth medium/plate and incubate in a 37°C incubator under a humidified 5% CO_2 for 48hrs
- Remove medium from the plates
- Wash once with PBS and dry the plates in the hood for 15-20 minutes
- Add carefully 1 ml 4 % Paraformaldehyde (PFA) to each plate to fix the cells
- Leave the plates for 2min in the hood
- Wash once with 1x PBS
- Aspirate and add fresh 1x PBS
- View cells under an inverted microscope to count the fluorescence forming units

Calculating FFU

- ✓ Count 20 grids and take the average of cells counted

  For example if you have an average of 5 cells in the 10^5 dilution plate, then

  $$FFU = 5*200*10^5 = 10*10^7/ml$$

- ✓ Tips: Grid area: 0.04 cm^2. Total grids: 200. So, total area 0.04 cm^2 x 200 = 8 cm^2

Table 5| ***Primers used for PCR cloning, RT-PCR, Real-Time PCR, DNAP and Chip***

Name of primer	Sequence 5′- 3′	Purpose
hSR-BI - 2787 F	CGGGGTACC CTG GAG ATA CAT CGA TAA GCA AAA C	PCR Cloning
hSR-BI - 1362 F	ATAGGTACC TCA TTC ATT TGC TCACGT GCT CAT CAT TGG	PCR Cloning
hSR-BI - 944 F	CGGGGTACC CCA CCT CCC AGT TTT CTT ATT GTA	PCR Cloning
hSR-BI - 673 F	CGGGGTACC CAC CAG CGT TAC GAA GGA TAC	PCR Cloning
hSR-BI - 408 F	CGGGGTACC GGA GGA GGG AAA AGG AAG GGT GAG	PCR Cloning
hSR-BI - 290 F	CGGGGTACC CCC ATA GAC GTT TTG GCT CAG	PCR Cloning
hSR-BI - 201 F	CGGGGTACC CGG GGC TTG TCT TGG CGG C	PCR Cloning
hSR-BI - 88 F	CGGGGTACC AAT CCT GAA GCC CAA GGC TG	PCR Cloning
hSR-BI +78 R	CCCAAGCTT CGG CGA CAG AGA CGA CAC AGG CGG GG	PCR Cloning
hSR-BI +212 R	CCG CTC GAG CCA GCA CAG CGC ACA GTA GCC CCG	PCR Cloning
GAPDH F	ACC ACA GTC CAT GCC ATC AC	RT-PCR

GAPDH R	TCC ACC ACC CTG TTG CTG TA	RT-PCR
hSR-BI -172 F	CTC ATC AAG CAG CAG GTC CTT A	RT-PCR
hSR-BI -478 R	GCA TGA CGA TGT AGT CGC TCT	RT-PCR
mSR-BI - 313F	CTC AAG AAT GTC CGC ATA GAC C	Real-Time PCR
mSR-BI - 615R	GAC CAA GAT GTT AGG CAG TAC AAT G	Real-Time PCR
Bio-GR F	GAC CCT AGA GGA TCT GTA CAG GAT GTT CTA GAT	DNAP
Bio-GR mut F	GAC CCT AGA GGA TCT CAA CAG GAT CAT CTA GAT	DNAP
GR R	ATC TAG AAC ATC CTG TAC AGA TCC TCT AGG GTC	DNAP
GRmut R	ATC TAG ATG ATC CTG TTG AGA TCC TCT AGG GTC	DNAP
Bio-hSR-BI-2787 F	BIOTIN - CTG GAG ATA CAT CGA TAA GCA AAA C	DNAP
Bio-hSR-BI-1491 F	BIOTIN - CTG GAG ATA CAT CGA TAA GCA AAA C	DNAP
Bio-hSR-BI-944 F	BIOTIN - CCA CCT CCC AGT TTT CTT ATT GTA	DNAP
Bio-hSR-BI-620 F	BIOTIN - CTG TGC AGG GTG TAT GGA GGC CC	DNAP
Bio-hSR-BI-290 F	BIOTIN - CCC ATA GAC GTT TTG GCT CAG	DNAP

hSR-BI- 2301 R	CTT TCC AAG GGC TAC CAT GA	DNAP
hSR-BI- 949 R	CGC CTG TAA TCC CAG CAC TTT GG	DNAP
hSR-BI-620 R	GCA ATT CTG TTC TCC CAG GTA TCC	DNAP
hSR-BI- 369 R	AGT CTC CCC TCT CGA CCC TCC T	DNAP
hSR-BI-292 R	CCT GGT GCC CCT CAC CCT CTT G	DNAP - ChIP
hSR-BI+78 R	CGG CGA CAG AGA CGA CAC AGG CGG GG	DNAP - ChIP
hSR-BI-673 F	CAC CAG CGT TAC GAA GGA TAC	ChIP
hSR-BI-290 F	CCC ATA GAC GTT TTG GCT CAG	ChIP
SLC19A2-252 F	GCA TTC CCA ACA GAT GAG C	ChIP
SLC19A2-135 R	GGA GGA CAT GTG GAA CTC C	ChIP
GEM F	CAG ATG CCT TCT CGA AGC TC	RT-PCR
GEM R	TGA ATG GCT CCA TGA CCA C	RT-PCR

Results

PART I. REGULATION OF SR-BI IN STEROIDOGENIC TISSUES

Functional analysis of the human SR-BI promoter in H295R cells

As a first step towards a better understanding of SR-BI gene regulation in adrenal cells, we performed a structure-function analysis of the human SR-BI promoter in the human adrenocortical H295R cell line. For this purpose, we cloned the promoter of the human SR-BI gene between nucleotides -2,787 and +78 upstream of the firefly luciferase reporter gene and generated a series of 5' deletions which are shown schematically in **Figure 9**. All these reporter plasmids were transiently transfected into H295R cells and their relative activity was scored by luciferase assays. This analysis revealed that the activity of the human SR-BI promoter in adrenal cells is determined by interplay between positive and negative regulatory elements. Specifically, we identified three positive regulatory regions between nucleotides -1288/ -944, -673/-408 and -290/-88 which are separated by two strongly negative regulatory regions defined by nucleotides -944/-673 and -408/-290 (**Figure 9)**. Also, the region upstream of the nucleotide -1,288 up to -2,787 shows strong negative activity, indicating the presence of negatively acting regulatory elements. Interestingly, the negative region between nucleotides -408/-290 coincides with the presence of a hormone response element that binds heterodimers of retinoid X receptor (RXR) with peroxisome proliferator activated receptor γ (PPARγ) identified previously to bind and mediate induction of the SR-BI promoter by a thiazolidinedione (BRL49653), in Hepa1c1c-7 cells [173] (**Figure 9**). The positive region between nucleotides -1288/-944 contains a previously described binding site for SREBP-1a and a hormone response element (HRE) that binds heterodimers of Retinoid X

Receptor (RXR) with Liver X Receptor (LXR) and the Liver Receptor Homolog 1 (LRH-1) [124].

Regulation of SR-BI promoter activity by Sp1 transcription factors

The positive region between nucleotides -201 and -88 contains five putative binding sites for members of the Sp1 family transcription factors that were identified previously by in silico analysis [203]. We wanted to examine the involvement of Sp1 family transcription factors (Sp1, Sp2 and Sp3) on the activity of the human SR-BI promoter and for this reason we used the *Drosophila* Schneider's SL2 cells which lack endogenous Sp1 expression. SL2 cells were transiently cotransfected with the hSR-BI-1288/+78-luc reporter or the hSR-BI-290/+78-luc reporter along with the Drosophila-specific expression vectors for Sp1, Sp2 and Sp3. As shown in **Figure 10**, the activity of both promoters was strongly induced by Sp1 and Sp3 but there was only a minor induction by Sp2. Furthermore, we transiently transfected HepG2 cells with the hSR-BI-1288/+78-luc or the hSR-BI-290/+78-luc reporter, in the absence or in the presence of mithramycin A (**Figure 11**). Mithramycin A (also known as aureolic acid, mithracin or plicamycin) is an aureolic acid-type polyketide produced by the soil bacteria *Streptomyces argillaceus* [204]. Mithramycin A is a drug that binds to GC-rich regions in chromatin and interferes with the transcription of genes that bear GC-rich motifs in their promoters which include binding sites for Sp1 transcription factors [205-206]. As shown in **Figure 11**, Mithramycin A

treatment repressed the activity of both hSR-BI-1288/+78 and hSR-BI-290/+78 promoters.

The combined findings of **Figures 10 and 11** indicate that Sp1 and Sp3 transcription factors bind to the GC-rich regions present in the proximal SR-BI promoter and regulate the expression of the SR-BI gene in a positive manner.

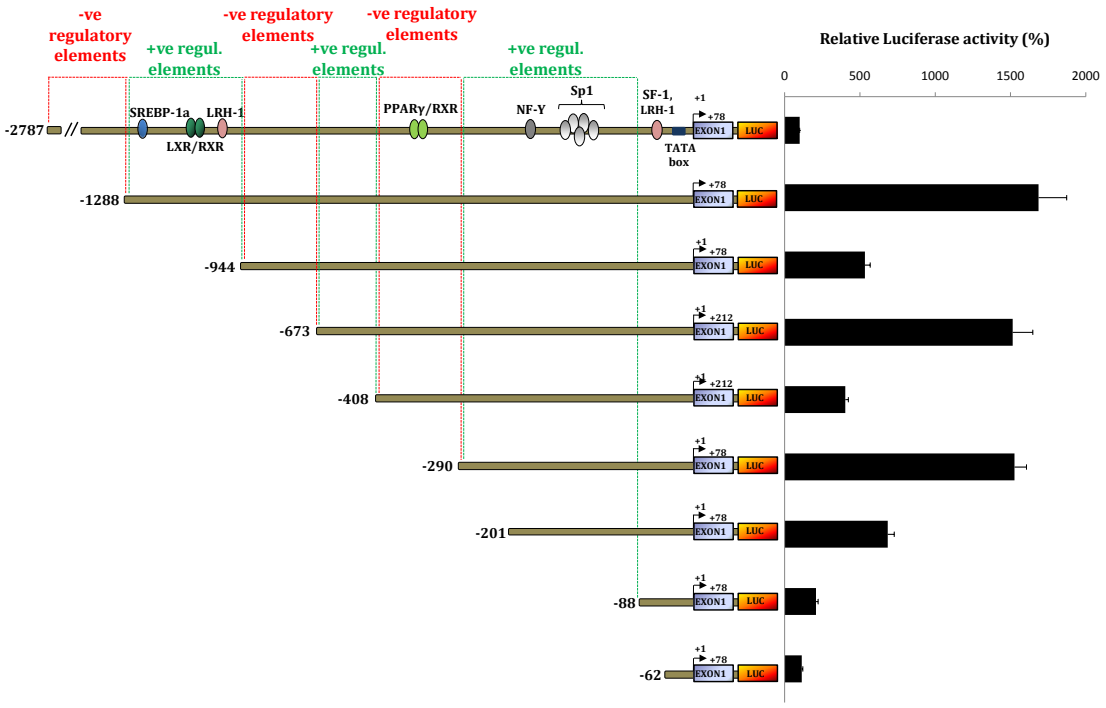


Figure 9| ***Structure-function analysis of the hSR-BI promoter in H295R adrenal cells.*** H295R cells were transiently transfected with the indicated reporter plasmids bearing the luciferase gene under the control of different SR-BI promoter fragments (0.5μg each) along with the CMV-β-gal plasmid expressing β-galactosidase (1μg) for normalization of transfection. Relative luciferase activity (±SEM) was calculated and the experiment was performed in duplicate. Transcription factors that have been shown to bind to the hSR-BI promoter are indicated, as well as the different positive (in green) and negative (in red) regulatory regions.

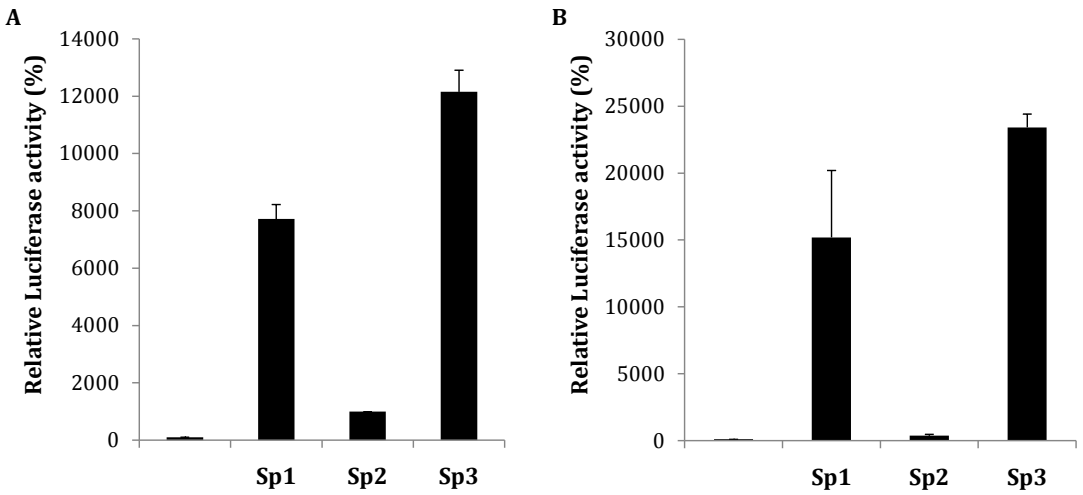


Figure 10| ***Human SR-BI promoter is induced by Sp1 family transcription factors. A, B,*** *Drosophila* Schneider's SL2 cells were transiently cotransfected with the hSR-BI-1288/+78-luc reporter (A) or the hSR-BI-290/+78-luc reporter (B) (0.5µg each) along with the Drosophila-specific expression vector s for Sp1, Sp2, and Sp3 (25ng). The hsp-lacZ plasmid expressing b-galactosidase (1µg) was included in each sample for normalization of transfection variability. Relative luciferase activity (±SEM) was calculated and the experiments were performed in duplicate.

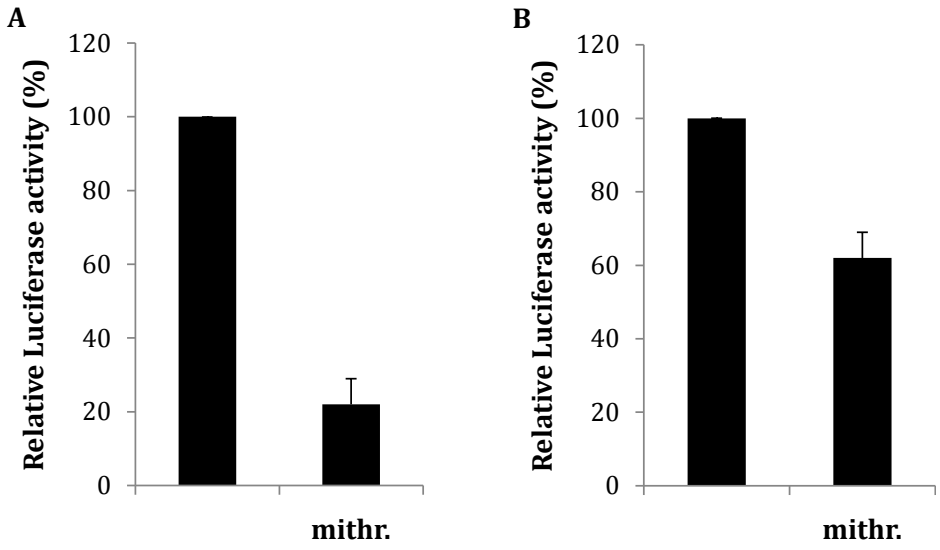


Figure 11| ***Human SR-BI promoter is repressed by Sp1 inhibitor, mithramycin A. A, B,*** HepG2 cells were transiently transfected with the hSR-BI-1288/+78-luc reporter (A) or the hSR-BI-290/+78-luc reporter (B) (0.5μg each) and treated with 200ng/ml mithramycin A. Mithramycin A was added 16hours post-transfection and 24 hours prior to cell collection. Relative luciferase activity (±SEM) was calculated and the experiments were performed in duplicate.

Glucocorticoids inhibit SR-BI gene expression in adrenal and ovarian cells

We investigated the role of glucocorticoids and their receptor in SR-BI gene expression in adrenal cells and to achieve this goal we utilized the H295R adrenal cell line. As shown in **Figure 12**, administration of dexamethasone (Dex) to H295R cells for 2 hours caused a 37% reduction in SR-BI mRNA levels. This reduction was fully blocked by cycloheximide (CHX), a protein synthesis inhibitor suggesting that inhibition of SR-BI gene expression by glucocorticoids requires *de novo* protein synthesis.

Next, we examined the effect of glucocorticoids on the activity of the human SR-BI promoter. For this purpose, H295R cells were cotransfected with the hSR-BI-1288/+78-luc reporter vector along with an expression vector for hGRα in the presence of dexamethasone. As shown in **Figure 13A**, hGRα in the presence of dexamethasone inhibited the activity of the hSR-BI -1288/+78 promoter to 45% relative to the untreated H295R cells. In contrast, the activity of the SR-BI promoter was not repressed in the presence of mifepristone (RU486) a non-selective glucocorticoid receptor antagonist. As a control, a reporter plasmid bearing the luciferase gene under the control of a single glucocorticoid response element (GRE-luc) [207] was used and showed that hGRα in the presence of dexamethasone induced the activity of this promoter 29-fold in H295R cells (**Figure 13B**).

To confirm the above findings, we used a reporter plasmid bearing a different deletion mutant of the SR-BI promoter (hSR-BI-944/+78). As shown in

Figure 13C, mifepristone abolished the repression of the hSR-BI-944/+78 promoter by hGRα.

To verify the data of **Figure 13**, we performed a similar experiment in hepatic HepG2 cells. For this purpose we transiently transfected a reporter plasmid bearing the luciferase gene under the control of a single glucocorticoid response element (GRE-Luc) along with hGRα in the presence of dexamethasone (Dex), mifepristone (RU486) or the vehicle only (ethanol). As shown in **Figure 14**, the GRE-luc reporter plasmid was unaffected in the presence of dexamethasone, mifepristone or vehicle only. As expected, ligand-activated GRα strongly induced GRE-luc reporter activity, reaching a 20 fold induction level. On the contrary, the induction of the GRE-luc reporter by the ligand-activated GRa was repressed in the presence of mifepristone by almost 60%. This experiment confirmed the activation of GRα in the presence of its ligand dexamethasone and its nonselective antagonist, mifepristone, on the GRE-luc reporter in hepatic cells.

The effect of glucocorticoids on SR-BI gene expression and promoter activity was also examined in a different steroidogenic cell type, the ovarian adenocarcinoma SKOV-3 cells [208]. First, we showed that administration of dexamethasone to SKOV-3 cells reduced the SR-BI mRNA levels by approximately 60% relative to untreated cells (**Figure 15A**). Second, we showed that cotransfection of SKOV-3 cells with the hSR-BI-1288/+78-luc reporter vector along with an expression vector for hGRα in the presence of dexamethasone caused a 60% reduction in SR-BI promoter activity (**Figure 15B**). In a control experiment, ligand-activated hGRα strongly induced the activity of the GRE-luc reporter in SKOV-3 cells (**Figure 15C**). A similar

transactivation experiment was carried out in the human embryonic kidney HEK293T cells. These cells were transiently cotransfected with the hSR-BI-1288/+78 promoter or the GRE-luc reporter along with the hGRα expression vector in the presence of dexamethasone. As shown in **Figure 16**, ligand-activated GRα repressed the activity of the hSR-BI-1288/+78 promoter by 70% (panel A), and strongly induced the activity of the GRE-luc reporter (panel B).

The combined findings of **Figures 12-16** indicate that glucocorticoids are negative regulators of the SR-BI gene in two steroidogenic cell lines, the adrenal H295R and the ovarian SKOV-3 cells. Glucocorticoids activate endogenous GR and repress the transcriptional activity of the hSR-BI promoter.

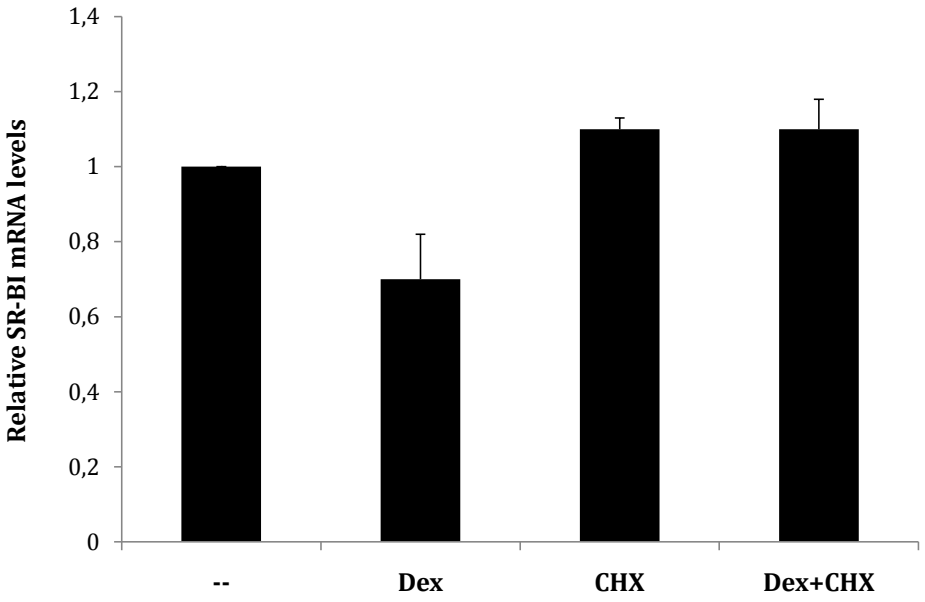


Figure 12| ***Inhibition of SR-BI gene expression by glucocorticoids requires de novo protein synthesis.*** H295R cells were treated with 1μM dexamethasone and/or 10mg/ml cycloheximide for 2 hours. Total RNA was isolated and analyzed by RT-PCR. Values were normalized to those obtained for the housekeeping GAPDH gene.

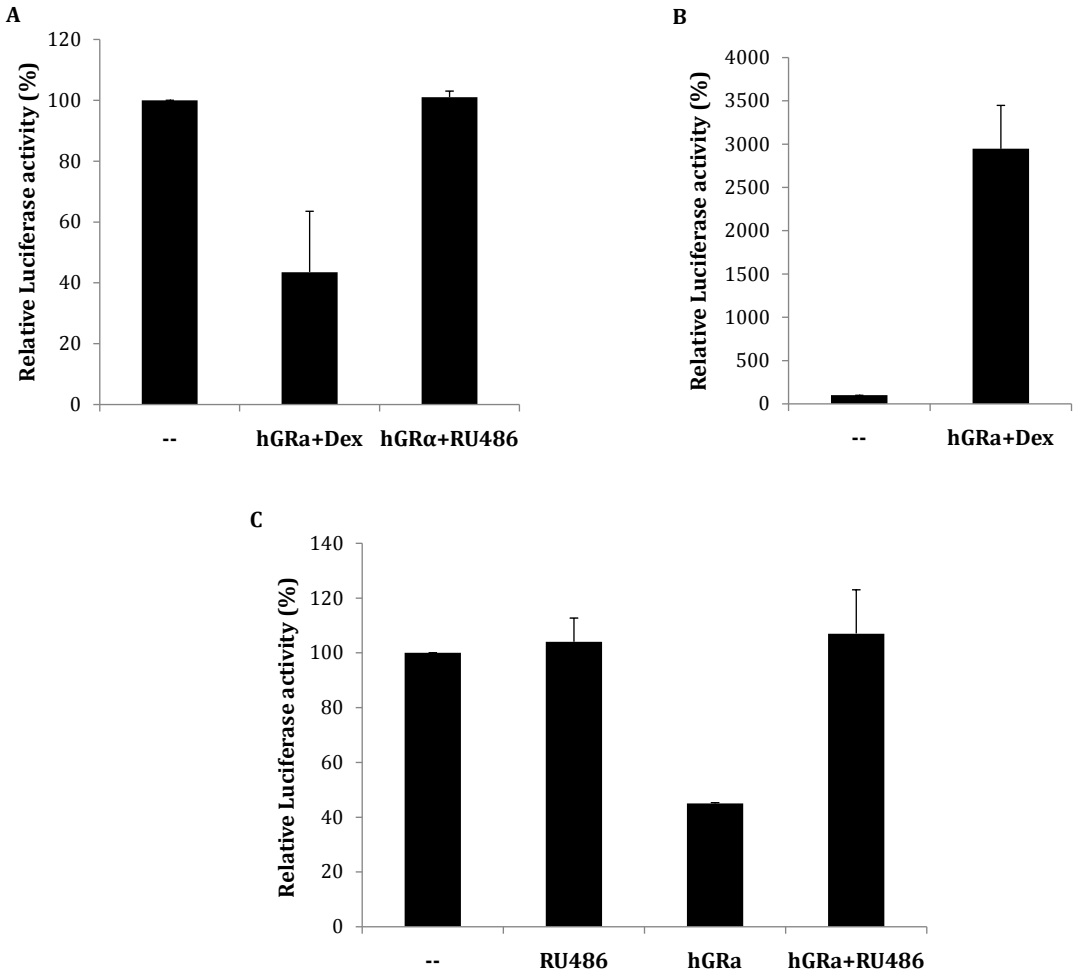


Figure 13|***Ligand activated GR inhibits the transcriptional activity of the SR-BI promoter in adrenocortical H295R cells. A, C,*** H295R cells were transiently transfected with the hSR-BI-1288/+78-Luc reporter (A), or the hSR-BI-944/+78-luc reporter (C) along with an expression vector for hGRα (1µg each) in the presence of dexamethasone (Dex, 1µM), mifepristone (RU486, 1µM), or the vehicle only (--). ***B,*** H295R cells were transiently transfected with the GRE-Luc reporter (1µg) along with an expression vector for the hGRα (1µg) in the presence of dexamethasone (Dex, 1µM) or the vehicle only (--). Ligands were added for 24hours before cell collection. Relative luciferase activity (±SEM) was calculated from at least three independent experiments performed in duplicate.

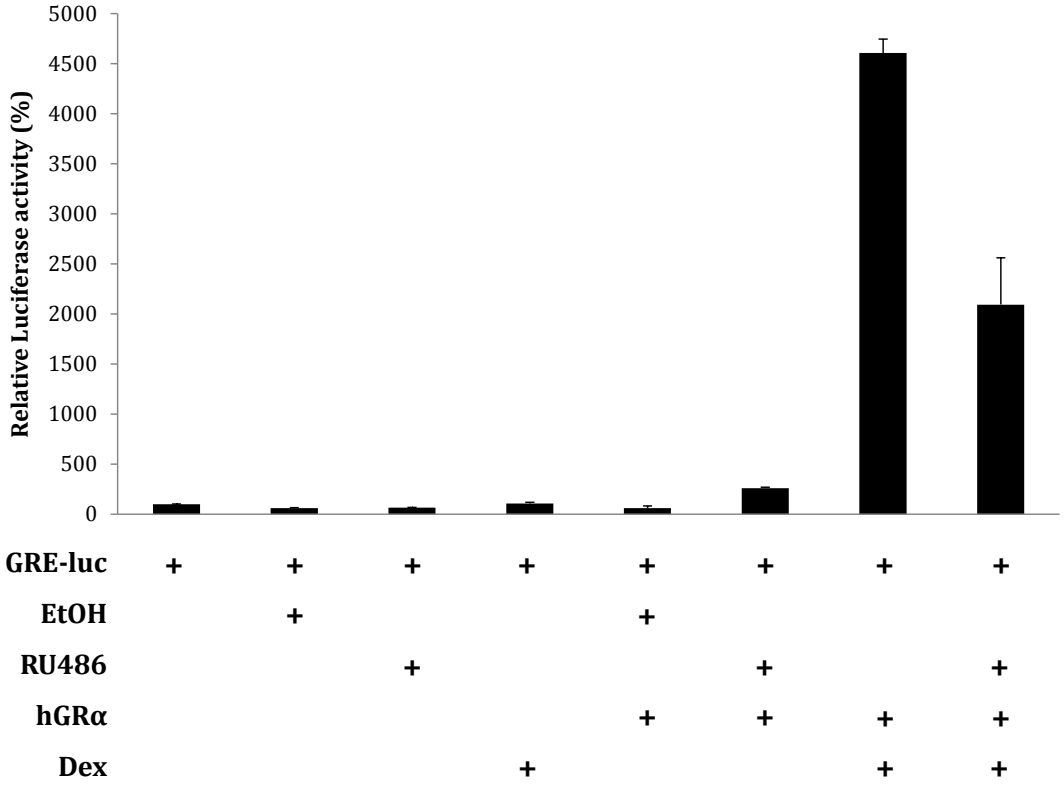


Figure 14| ***Glucocorticoid induction of GRE-luc is repressed by mifepristone in hepatic HepG2 cells.*** HepG2 cells were transiently transfected with reporter plasmid bearing the luciferase gene under the control of a single glucocorticoid response element (GRE-Luc) along with hGRα expression vector (0,5μg each) in the presence of dexamethasone (Dex, 1μM), mifepristone (RU486, 1μM), or vehicle only (ethanol, EtOH). Ligands were added for 24h before cell collection. Relative luciferase activity (%) (±SEM) was calculated and the experiment was performed in duplicate.

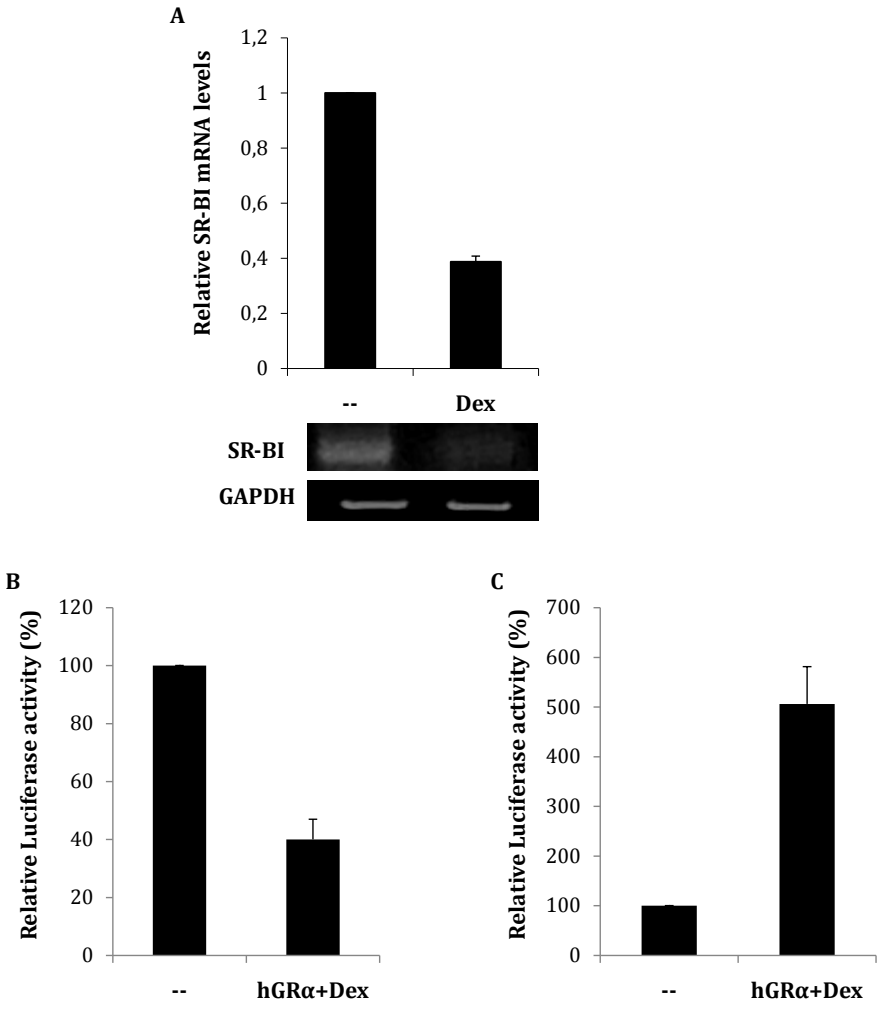


Figure 15| ***Ligand activated GR inhibits SR-BI expression in ovarian SKOV-3 cells. A,*** SKOV-3 cells were treated with 10μM dexamethasone for 2 hours. Total RNA was isolated and analyzed by RT-PCR. Values were normalized to those obtained for the housekeeping GAPDH gene. **B, C,** SKOV-3 cells were transiently transfected with the full-length hSR-BI-1288/+78-luc reporter (B) or the GRE-luc reporter (C) (1μg each) along with the expression vector for hGRα in the presence of dexamethasone (1μM). Dexamethasone was added 24hours before cell collection. Relative luciferase activity (±SEM) was calculated and the experiments were performed in duplicate.

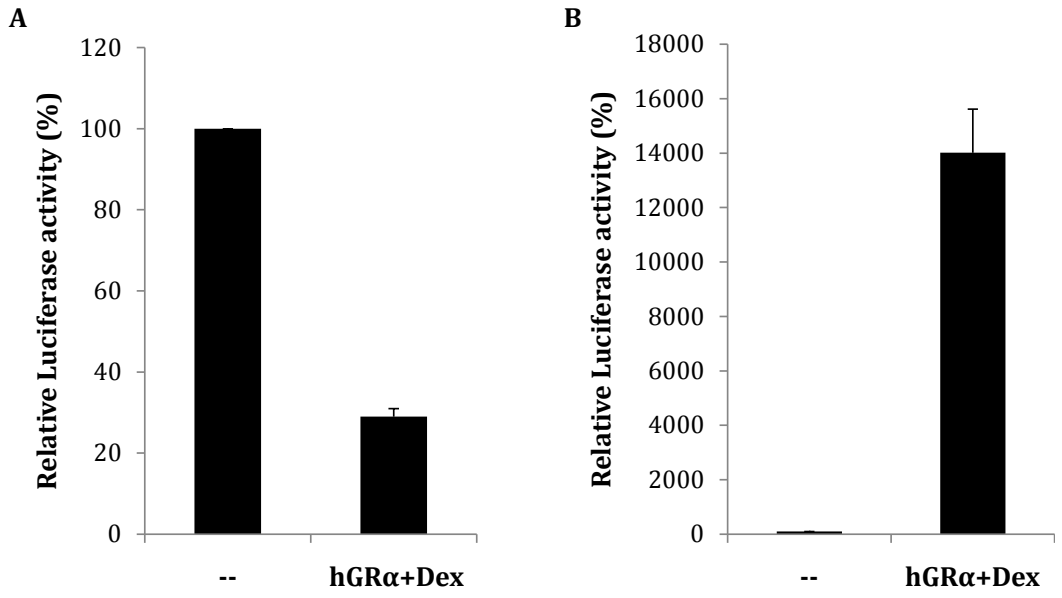


Figure 16| ***Ligand activated GR inhibits the transcriptional activity of the SR-BI promoter in human embryonic kidney HEK293T cells. A, B,*** HEK293T cells were transiently transfected with the full-length hSR-BI-1288/+78-luc reporter (A) or the GRE-luc reporter (B) along with the expression vector for hGRα (1μg each) in the presence of dexamethasone (1μM). Dexamethasone was added 24hours before cell collection. Relative luciferase activity (±SEM) was calculated and the experiments were performed in duplicate.

A recombinant adenovirus expressing human GRα suppressed SR-BI gene expression in H295R cells

Adenovirus-mediated gene transfer technology was used to investigate further the role of GR in SR-BI gene expression. In order to achieve this goal, we generated and titrated a recombinant adenovirus expressing human GRα (**Figure 17A**) (see Materials and Methods). Using antibodies against human GRα and GFP in immunoblotting experiments we showed that H295R cells were productively infected by the AdGRα adenovirus in a dose-dependent manner (multiplicity of infection 0-25) (**Figure 17B**). Using direct immunofluorescence we monitored the infection of H295R cells by the recombinant hGRα adenovirus as well as the control adenovirus AdGFP (**Figure 18**)

Next, we overexpressed the human GRα in H295R cells via AdhGRα and we observed that this overexpression was associated with a significant reduction in the SR-BI mRNA levels compared to a control adenovirus expressing only the green fluorescent protein (AdGFP) (**Figure 19A**). A microarray analysis in dexamethasone treated A549 adenocarcinoma cells showed that GEM (gon extragenic modifier) was one of 73 genes that were down-regulated by GRα [209]. We utilized this information and used GEM as a positive control in our experiments. As shown in **Figure 19B**, overexpression the human GRα in H295R cells via AdhGRα was associated with a significant reduction in the GEM mRNA levels compared to the control adenovirus expressing only the green fluorescent protein (AdGFP). Moreover, overexpression of the hGRα in H295R cells caused a

significant reduction in SR-BI protein levels (**Figure 19C**) levels compared to the control adenovirus expressing only the green fluorescent protein (AdGFP).

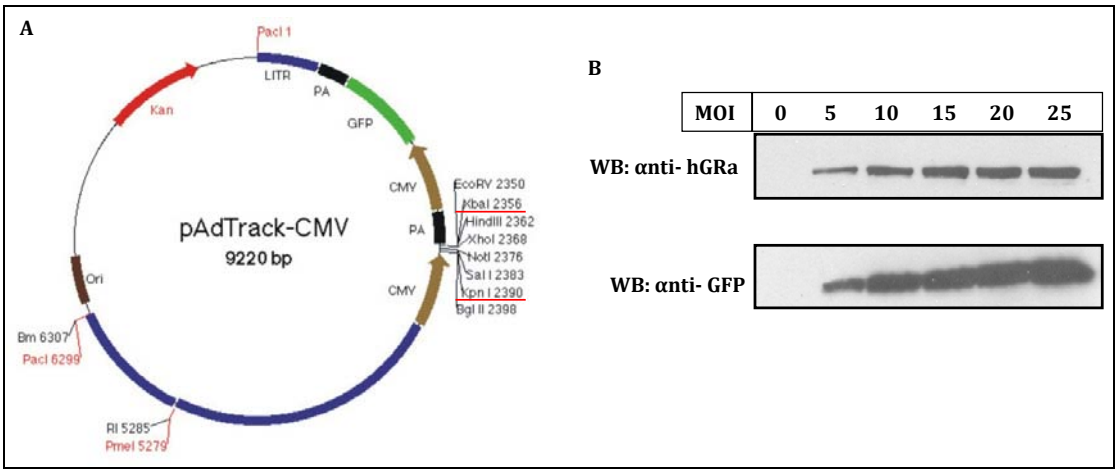


Figure 17| ***A recombinant adenovirus expressing human GRα productively infected H295R cells. A,*** AdhGRα recombinant adenovirus was generated as described in Materials & Methods by ligation of the hGRα cDNA to the KpnI and XbaI restriction sites of the pAd-Track CMV vector. ***B,*** H295R cells were infected with a recombinant adenovirus expressing the human GR (AdhGRα) with increasing multiplicity of infection (MOI) values. Equal amounts of whole cell extracts from the infected cells were subjected to SDS-page and immunoblotting using antibodies against hGRα and GFP.

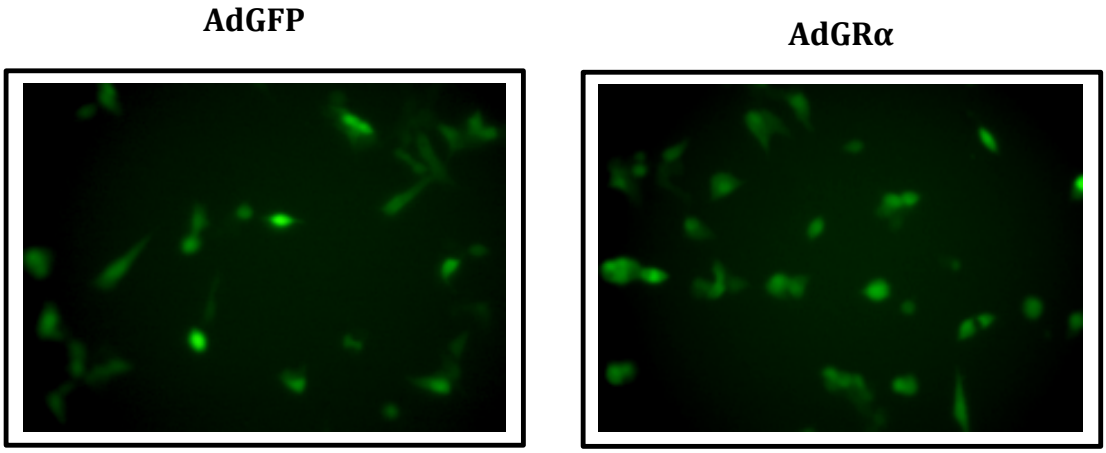


Figure 18| ***Monitoring the infection of H295R cells by the recombinant adenoviruses expressing GFP and human GRα*** H295R cells were infected with a control adenovirus expressing the green fluorescent protein (AdGFP) or with an adenovirus expressing AdhGRα (MOI=10). Direct immunofluorescence was utilized for the detection of the efficacy of adenovirus infection with MOI=10.

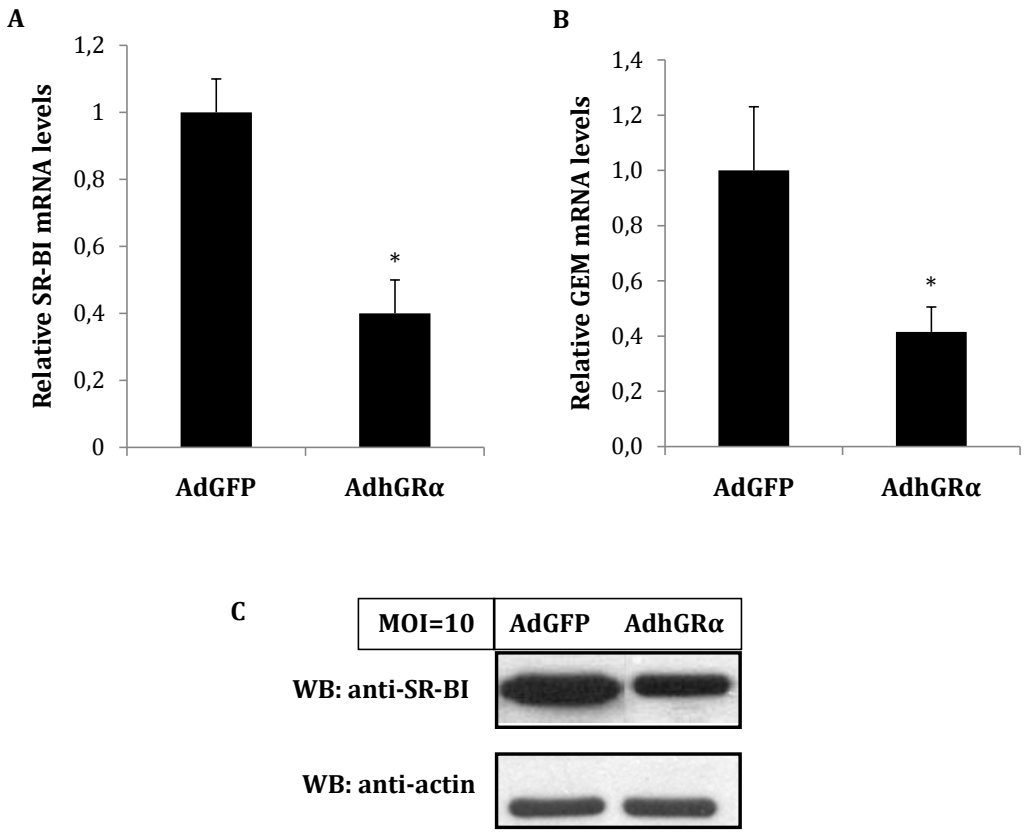


Figure 19| ***A recombinant adenovirus expressing human GRα suppressed SR-BI gene expression in H295R cells. A, B, C,*** H295R cells were infected with a control adenovirus expressing the green fluorescent protein (AdGFP) or with an adenovirus expressing AdhGRα (MOI=10). The mRNA levels of SR-BI (A) or GEM genes (B) were quantitated by RT-PCR, normalized relative to the housekeeping gene GAPDH and plotted. ***C,*** Protein SR-BI levels were detected by immunoblotting using a polyclonal anti-SR-BI antibody. The endogenous levels of β actin were used for normalization of the protein extracts. *, $P<0.05$ by one-way ANOVA.

Glucocorticoid receptor is required for the inhibition of the SR-BI promoter by glucocorticoids

To investigate further the role of the glucocorticoid receptor in the inhibition of SR-BI promoter activity by glucocorticoids in adrenal cells, we employed siRNA-mediated gene silencing technology. For this purpose, we transfected H295R cells with the hSR-BI-1288/+78-luc reporter in the presence of a scrambled siRNA or a GR-specific siRNA and treated with dexamethasone (**Figure 20A**). We found that silencing of GRα was associated with a 2.5-fold increase in the activity of the SR-BI promoter suggesting that endogenous GR plays an inhibitory role in SR-BI gene expression in adrenal cells (**Figure 20A**).

The efficiency of GRα gene silencing by siRNA was monitored by immunoblotting in HEK293T cells that had been transiently cotransfected with an expression vector for hGRα (pcDNA3hGRα). As shown in **Figure 20B**, the SR-BI protein levels were reduced by more than 50% compared to the SR-BI protein levels in the presence of the control siRNA. It must be pointed out that siRNA-mediated silencing was not very efficient because of the high levels of expression of endogenous GRα in these cells.

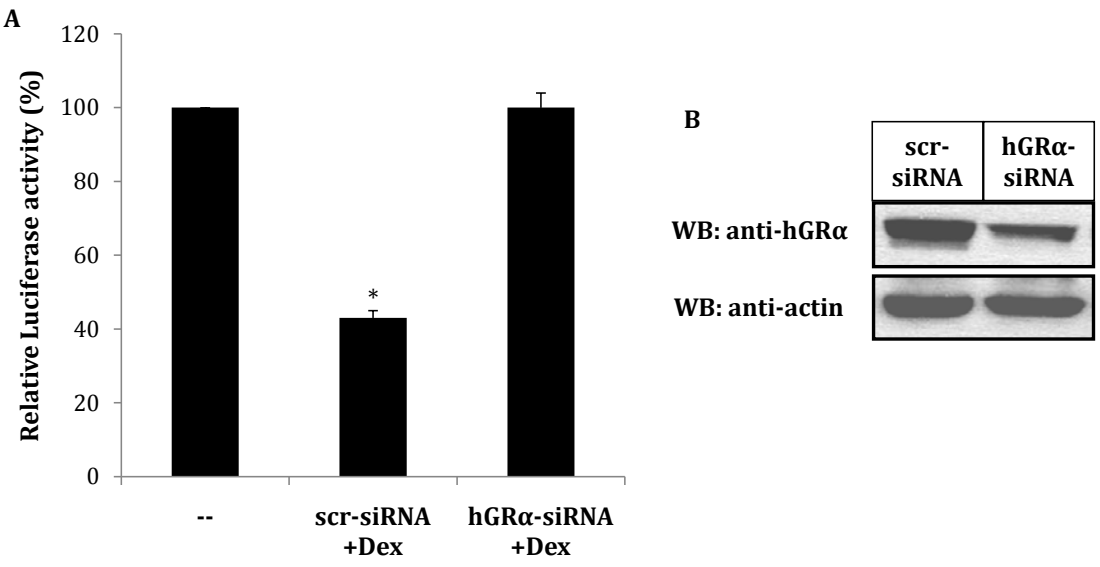


Figure 20| ***The GR is required for the inhibition of the SR-BI promoter by glucocorticoids.*** ***A,*** H295R cells were transiently transfected with the hSR-BI-1288/+78-luc reporter (1μg) in the presence of a scrambled siRNA or a GR-specific siRNA (200pmol) and treated with 1μM dexamethasone for 24 hours prior to cell harvesting. Relative luciferase activity (±SEM) was calculated and plotted. Each experiment was performed in duplicate. ***B,*** Human embryonic kidney HEK293T cells were transiently transfected with pcDNA3hGRα vector in the presence of scrambled siRNA or a GR-specific siRNA (200pmol). Whole cell extracts from the transfected cells were subjected to SDS-PAGE and immunoblotting using antibodies for hGRα and actin. *, $P<0.05$ by one-way ANOVA.

The proximal region of human SR-BI promoter is sufficient for repression by glucocorticoids in H295R adrenal cells

To understand better the mechanism of SR-BI gene regulation by glucocorticoids in adrenal cells, we transiently transfected selected 5' deletion reporter plasmids of the SR-BI promoter (-944, -408, -290, -201 and -62) into H295R cells in the presence or absence of dexamethasone and their relative activity was scored by luciferase assays. It was observed that the transcriptional activity of all except the most proximal reporter plasmid hSR-BI-62/+78 was repressed by dexamethasone (**Figure 21**). This finding indicates that the minimal region required for repression of the hSR-BI promoter by dexamethasone is the region between nucleotides -201 and -62. A closer examination of the nucleotide sequence of this region failed to identify any putative glucocorticoid responsive element (GRE) suggesting that inhibition of SR-BI promoter by glucocorticoids is exerted via an indirect mechanism (see below). This hypothesis is also supported by the findings of **Figure 12** which showed that new protein synthesis is required for dexamethasone-mediate repression of SR-BI gene expression in adrenal cells.

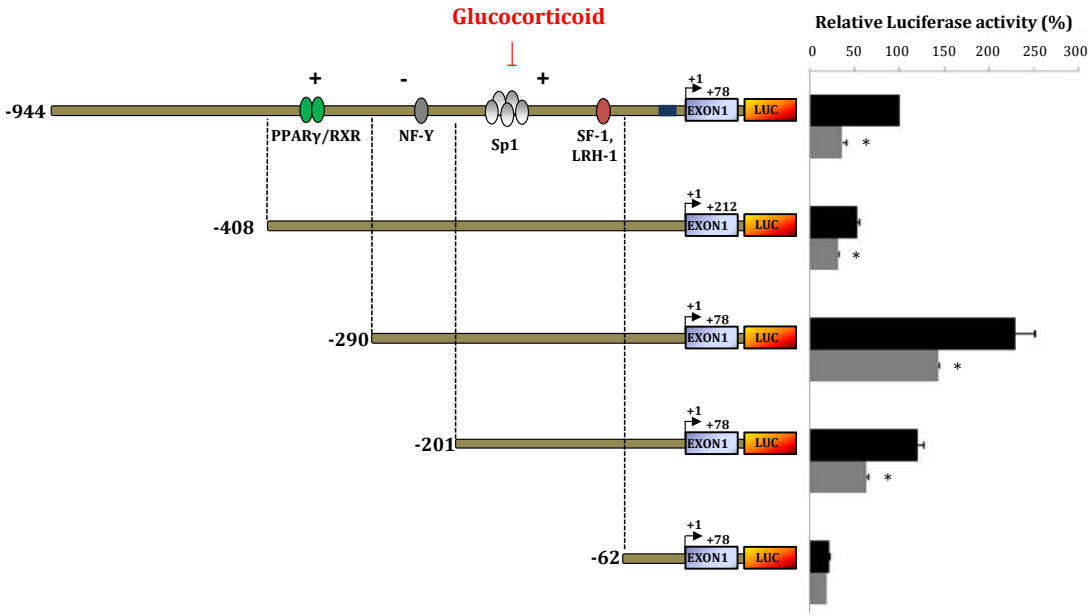


Figure 21| ***The proximal region -201/-62 of the hSR-BI promoter is sufficient to mediate repression by glucocorticoids in H295R cells.*** H295R cells were transiently transfected with the indicated SR-BI reporter plasmids in the absence (black bars) or in the presence (gray bars) of dexamethasone (1μM). The CMV-β-gal plasmid expressing β-galactosidase (1μg) was included in each sample for normalization of transfection variability. Relative Luciferase activity (±SEM) was calculated and the experiment was performed in duplicate. Positive (+) and negative (-) regulatory regions (data taken from **Figure 9**) and the region of the SR-BI promoter that mediates inhibition by glucocorticoids are indicated. *, $P<0.05$ by one-way ANOVA.

The proximal region of the SR-BI promoter downstream of -201 is required for inhibition by the glucocorticoid receptor in adrenal cells

Following this initial characterization of glucocorticoid-responsive elements in the SR-BI promoter in adrenal cells, we examined the effect of the glucocorticoid receptor on the activity of different SR-BI promoter fragments. For this purpose, we cotransfected H295R cells with various deletion mutants of the hSR-BI promoter along with an expression vector for the human glucocorticoid receptor (hGRα). In agreement with the findings of **Figure 21**, this deletion analysis showed the minimal region of the SR-BI promoter that is required for inhibition by GR is the region between nucleotides -201/-88 (**Figure 22).**

In conclusion, the combined findings of **Figures 21 and 22** indicate that the proximal region of the SR-BI promoter between nucleotides -201 and -88 should contain regulatory elements that control the activity of the hSR-BI promoter in the presence of the glucocorticoid receptor. A similar deletion analysis was carried out in human embryonic kidney HEK293T cells. HEK293T cells were cotransfected with the deletion mutants -2787, -944, -673, -408, -290, -88 and -62 of the hSR-BI promoter along with an expression vector for the human glucocorticoid receptor (hGRα) (**Figure 23**). The findings of this experiment are in agreement with the results from the analysis in adrenal H295R cells and confirm that the minimal region of the hSR-BI promoter that is necessary for repression by the glucocorticoid receptor is the proximal region -201/-88.

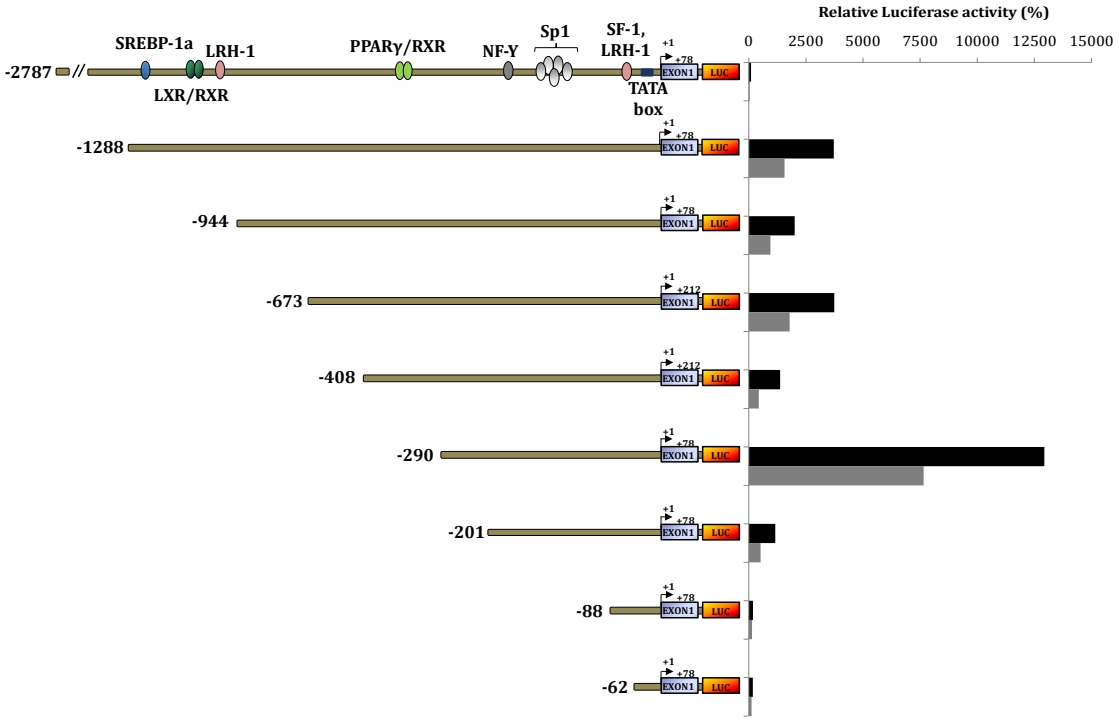


Figure 22| ***The glucocorticoid receptor inhibits the hSR-BI promoter in H295R cells.*** H295R cells were transiently cotransfected with the indicated reporter plasmids (black bars) and an expression vector for the glucocorticoid receptor (hGRα) (gray bars). The reporter plasmids for hSR-BI bear the luciferase gene under the control of different SR-BI promoter fragments (0.5μg each) along with the CMV-β-gal plasmid expressing β-galactosidase (1μg) for normalization of transfection. Relative luciferase activity (%) (±SEM) was calculated and the experiment was performed in duplicate.

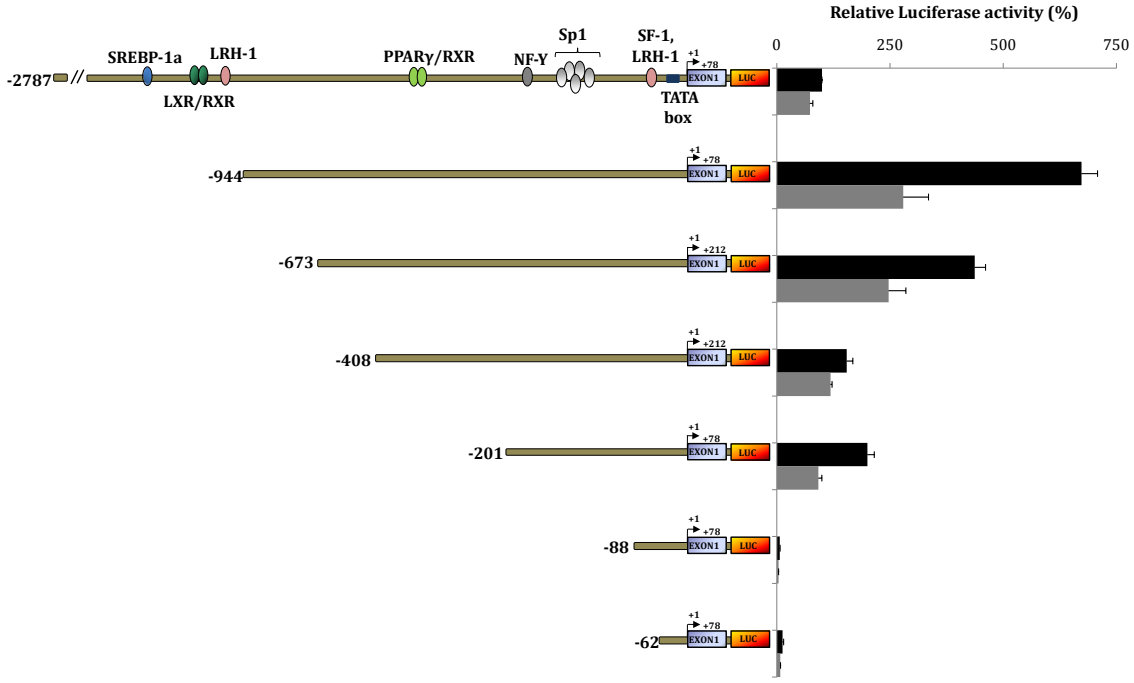


Figure 23| ***The glucocorticoid receptor inhibits the hSR-BI promoter in HEK293T cells.*** HEK293T cells were transiently cotransfected with the indicated reporter plasmids (0.5µg each) in the absence (black bars) or in the presence (gray bars) of an expression vector for the glucocorticoid receptor (hGRα). The CMV-β-gal plasmid expressing β-galactosidase (1µg) was also included for normalization of transfection. Relative luciferase activity (±SEM) was calculated and plotted. The experiment was performed in duplicate.

The GR does not bind to the SR-BI promoter

To understand better the mechanism of inhibition of SR-BI gene transcription by GRα in adrenal cells, we performed protein-DNA interaction assays in order to identify potential GRα binding sites in the SR-BI promoter. First we used the DNA affinity precipitation assay which allows the detection of DNA bound proteins *in vitro* by immunoblotting and for this purpose we generated a series of non-overlapping biotinylated PCR fragments covering the entire SR-BI promoter between nucleotides -1491 to +78. More specifically, the amplified regions, which are shown schematically in **Figure 24A**, were: -1491/-949, -944/-620, -620/-292 and -290/+78. These biotinylated PCR fragments were allowed to interact with extracts from HEK293T cells that had been transiently transfected with the expression vector for hGRα. DNA-bound GR could be detected by immunoblotting using the anti-GR antibody. As shown in **Figure 24B**, no interaction was detected between GR and the biotinylated proximal SR-BI promoter fragment -290/+78 shown to be sufficient to mediate repression by glucocorticoids (**Figure 21**). Binding of GRα to the other three biotinylated PCR fragments could be observed, but this binding was very weak compared with the binding of GRα to a control biotinylated double stranded oligonucleotide containing a well-characterized GRE (BioGR) [210]. No binding of GRα was observed to negative control oligonucleotides bearing a mutated GRE (BioGRmut) (**Figure 24B**).

We then performed chromatin immunoprecipitation assays in dexamethasone-treated H295R cells and found no recruitment of endogenous

GRα on the proximal -290/+78 or the more distal -673/-292 SR-BI promoter (**Figure 25B**). As a control, we used primers that amplify the promoter of the solute carrier family member 2 (SLC19A2) shown previously to bind GR *in vivo* (**Figure 25B**) [209].

Overall, the findings of the protein-DNA interaction assays of Figures 24 and 25 combined with the findings of Figure 12 (cycloheximide experiment) support an indirect mechanism of suppression of SR-BI gene transcription by glucocorticoids in adrenal cells.

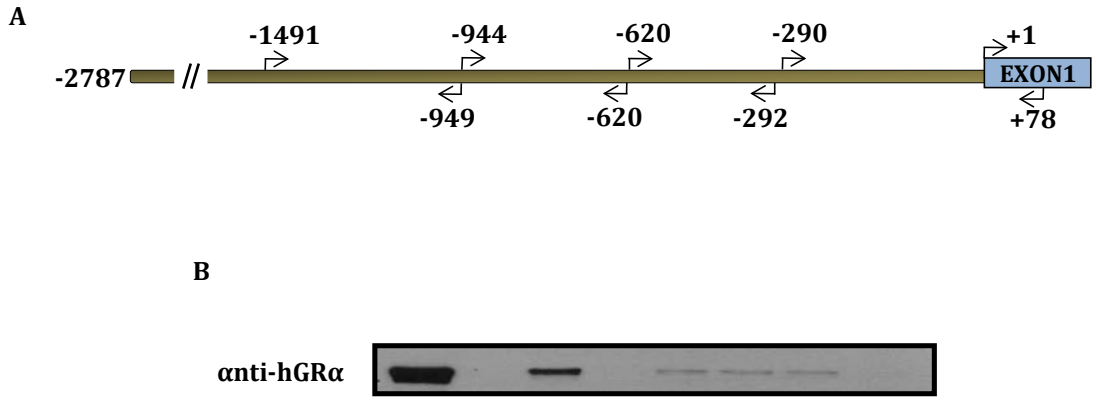


Figure 24| ***The GR does not bind to the SR-BI promoter in vitro. A,*** Schematic representation of the SR-BI 5′ upstream region showing the non-overlapping biotinylated PCR fragments that were used for the *in vitro* DNA affinity precipitation assays of B. ***B,*** DNA affinity precipitation experiment was carried out using extracts from HEK293T cells transiently transfected with the expression vector for hGRα. As controls, a Biotinylated double stranded oligonucleotides bearing one copy of a consensus glucocorticoids response element (BioGR) and one bearing a mutated element (BioGRmut) were used. Streptavidin agarose beads were coupled to Biotinylated control oligonucleotides or the Biotinylated PCR fragments and incubated with the cell lysates as described in Materials and Methods. DNA-bound GR was detected by immunoblotting using the anti-hGR antibody.

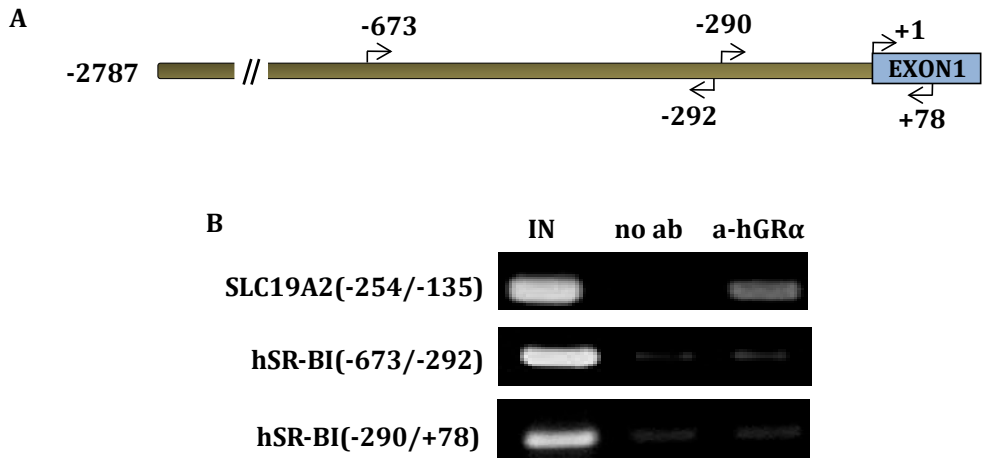


Figure 25| ***The GR does not bind to the SR-BI promoter in vivo. A*****,** Schematic representation of the SR-BI 5′ upstream region showing the pairs of primers used for the chromatin immunoprecipitation analysis of B. *B,* Chromatin immunoprecipitation analysis in H295R cells treated with 1μM dexamethasone for 2 hours. Immunoprecipitations were performed using the anti-hGRα antibody or no antibody as a control (no ab). Primers that amplify the distal -673/-292 or the proximal -290/+78 SR-BI promoter region or the -254/-135 promoter region of the solute carrier family member 2, SLC19A2 (positive control) were used for PCR analysis and are shown in Table 5.

Is KLF15 the SR-BI repressor?

A closer examination of the sequence of the -201/-88 region of the human SR-BI promoter revealed the presence of previously characterized or putative regulatory elements that could serve as binding sites for transcriptional activators or inhibitors (**Figure 26**). More specifically, this region contains five binding sites for the ubiquitous zinc finger transcription factor Sp1 characterized previously in the rat promoter [211] as well as a putative binding site for Nuclear factor Y (NF-Y) both of which are activators of gene transcription. In fact, deletion the SR-BI promoter region between nucleotides -201 and -62 was associated with a significant reduction in basal SR-BI promoter activity in adrenal H295R cells, suggesting that these factors may play an essential role on SR-BI gene expression in adrenal tissue (**Figure 9**). Interestingly, the same region contains several putative binding sites for transcriptional repressors including the zinc finger factor 219 (ZNF219), zinc finger 9 (ZF9), and the kruppel-like factor 15 (KLF15) (**Figure 26**). Among these factors, KLF15 deserves special attention because the gene encoding for this transcriptional repressor was recently identified as a glucocorticoid-responsive gene in cardiac cells in a microarray study [212]. In another study, it was shown that the KLF15 gene was induced by dexamethasone in cultured rat hepatocytes [213]. Thus we hypothesized that enhanced glucocorticoid biosynthesis in adrenal cells in response to increase cholesteryl ester uptake leads to activation of GR that binds to the promoter of the KLF15 gene thus activating its transcription. KLF15 that is

produced by this glucocorticoid response could bind to the proximal region of the human SR-BI promoter thus repressing its activity.

To test experimentally our hypothesis that KLF-15 is a repressor of the hSR-BI promoter, we cotransfected HEK293T cells with the hSR-BI-201/+78-luc reporter along with an expression vector for the human KLF-15. As is shown in **Figure 27A**, hKLF-15 repressed the hSR-BI-201/+78 promoter by 50%. In a control experiment we cotransfected HEK293T cells with the hSR-BI-88/+78-luc reporter, which lacks the putative site for KLF-15, along with an expression vector for the human KLF-15. As shown in Figure **27B,** KLF-15 did not affect the activity of the hSR-BI-88/+78 promoter suggesting that the SR-BI promoter region between -201 and -88 contains a KLF15 responsive element. This needs to be investigated further by additional experimentation.

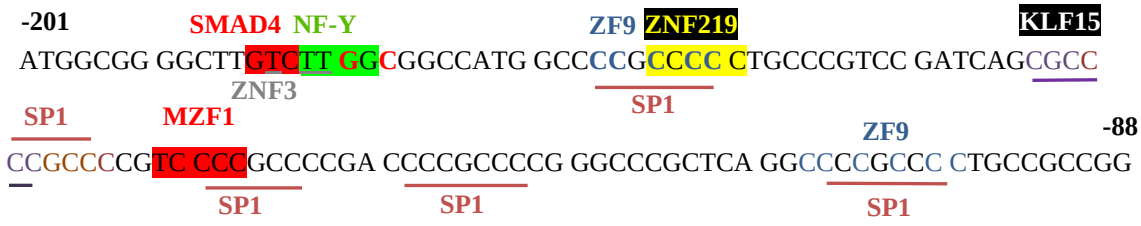


Figure 26| ***Binding sites for activators or inhibitors in the -201/-88 region of the human SR-BI promoter.***

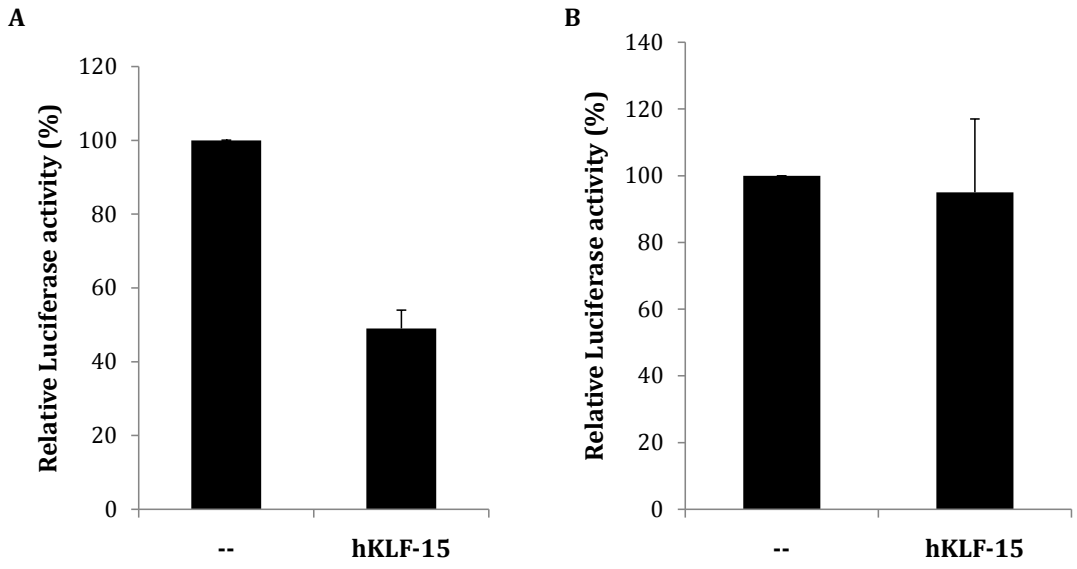
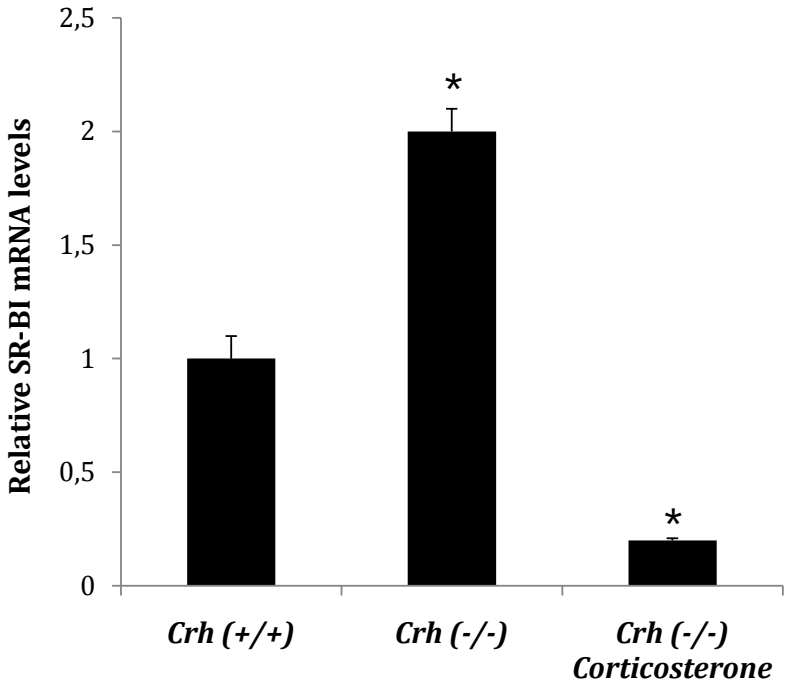


Figure 27| ***KLF-15 represses the activity of the hSR-BI proximal promoter region -201/+78. A, B,*** HEK293T cells were transiently cotransfected with the hSR-BI-201/+78-luc reporter (1μg; A) or the hSR-BI-88/+78-luc reporter (1μg; B) along with an expression vector for the human KLF-15 (2μg). Relative luciferase activity (±SEM) was calculated and plotted. The experiment was performed in duplicate.

Glucocorticoids down-regulate SR-BI gene expression in mouse adrenal

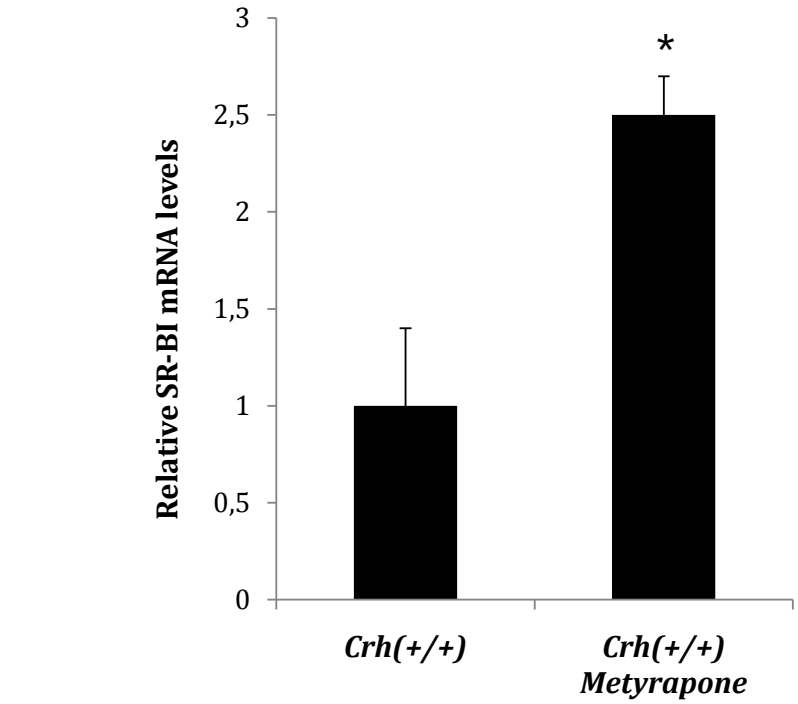
In order to study the effect of glucocorticoids on SR-BI gene expression *in vivo* we used a mouse model of corticosterone insufficiency (*Crh-/-* mice). Previous studies have shown that corticosterone production in *Crh-/-* mice was markedly disturbed [199, 201, 214]. As shown in **Figure 28**, corticosterone depletion was associated with a 2-fold increase in the mRNA levels of the SR-BI gene in the adrenals. On the other hand, administration of exogenous corticosterone in these mice decreased adrenal SR-BI mRNA levels by 80% compared to untreated mice suggesting that glucocorticoids play an inhibitory role on SR-BI gene expression in adrenals (**Figure 28**).

To corroborate our findings regarding the negative effect of corticosteroids on SR-BI gene regulation, we treated wild type mice with metyrapone. Metyrapone is a drug used for diagnosis of adrenal insufficiency, and used for the treatment of patients with Cushing's syndrome which is characterized by elevated cortisol levels [215]. Metyrapone blocks cortisol synthesis by inhibiting the activity of the enzyme steroid 11β hydroxylase, a rate limiting enzyme in the production of cortisol. As shown in **Figure 29**, metyrapone administration to mice caused a 4-fold decrease in blood corticosterone levels and this was associated with a 2.5-fold upregulation of SR-BI gene expression in adrenal cells in agreement with the findings of **Figure 28**.



Corticost. (µg/dl)	2.2	0,9	18

Figure 28| ***Inhibition of human SR-BI gene expression by glucocorticoids in mouse adrenals.*** Corticosterone (10µg/ml) or vehicle was administered to *Crh (-/-)* mice in drinking water for 3 days. Total RNA from these mice as well as from *Crh (+/+)* mice were isolated and reverse transcribed. Quantitative real-time PCR was carried out as described in Materials and Methods. Plasma corticosterone levels were measured using the ^{125}I RIA kit (RIA). Each value is the mean of three different mice of a representative experiment.*,P<0.05.



Corticost. (µg/dl)	8.0	2.0

Figure 29| ***Upregulation of human SR-BI gene expression by metyrapone in mouse adrenals.*** *Crh (+/+)* mice were injected intraperitoneally with 200mg/Kg of metyrapone or 10% dimethylsulfoxide twice a day for 4 days. On the fourth day, mice were injected twice with a combination of metyrapone with 100 mg/Kg aminoglutethimide. Total RNA from these mice as well as from *Crh (+/+)* mice were isolated and reverse transcribed. Quantitative real-time PCR was carried out as described in Materials and Methods. Plasma corticosterone levels were measured using the ^{125}I RIA kit (RIA). Each value is the mean of three different mice of a representative experiment. *, $P<0.05$.

Corticotropin releasing factor does not induce glucocorticoid signaling

Following the activation of the hSR-BI gene in *Crh -/-* mice (**Figure 28**), we examined the effect of corticotropin releasing factor (CRF) on the activity of the SR-BI promoter. For this purpose, we cotransfected HEK293T cells with the deletion mutants -1288/+78 and -290/+78 of the hSR-BI promoter along with an expression vector for the human corticotropin releasing factor receptor 1 (CRF-1) in the presence of its ligand, CRF, or vehicle. As shown in **Figure 30**, CRF inhibited the activity of both SR-BI promoter deletion mutants. Also, overexpression of the CRF-1 receptor strongly repressed the activity of both SR-BI promoters. More specifically, CRF repressed the activity of the hSR-BI-1288/+78 promoter by 25% and of the hSR-BI-290/+78 promoter by 20% compared to the untreated promoters. The CRF-1 receptor repressed the activity of the -hSR-BI-1288/+78 promoter by 60% (**Figure 30A**) and of the hSR-BI -290/+78 promoter by 70% (**Figure 30B**). Thus, these findings provided additional evidence for the repression of the human SR-BI promoter by CRF.

In parallel, we investigated the effect of CRF on the activation of endogenous GR in adrenal cells. For this purpose, we cotransfected adrenal H295R cells with a reporter plasmid bearing the luciferase gene under the control of a single glucocorticoid response element (GRE-luc) in the presence of CRF or vehicle. As shown in **Figure 31**, the activity of the GRE-luc reporter was unaffected by CRF. This finding indicates that CRF does not induce glucocorticoid signaling in adrenal cells and that the mechanism of repression of SR-BI gene expression by CRF is not mediated by glucocorticoids or GR.

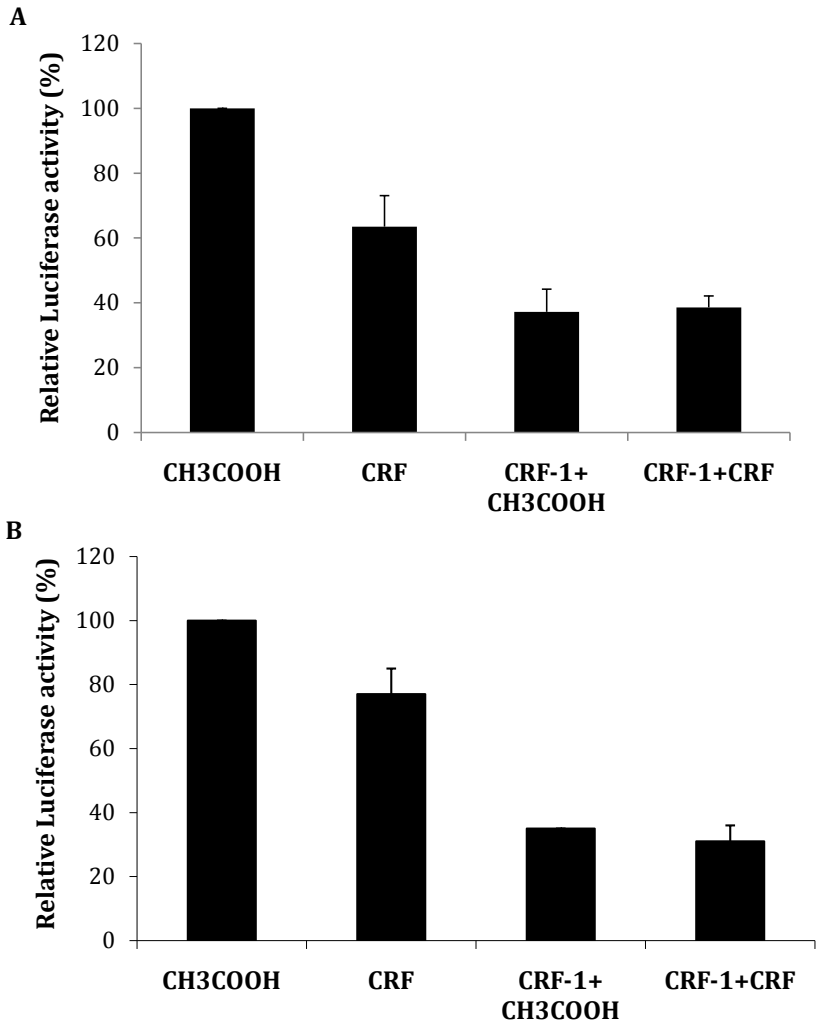


Figure 30| ***CRF and its receptor CRF-1 down-regulate the human SR-BI promoter. A, B,*** H295R cells were transiently cotransfected with the full-length hSR-BI-1288/+78–luc reporter (A) or the hSR-BI-290/+78-luc reporter (B) (1μg each) along with the expression vector for CRF-1 (0,5μg) in the presence of CRF or vehicle. CRF or CH_3COOH were added 24 hours prior to cell collection. Relative luciferase activity (±SEM) was calculated from at least three independent experiments performed in duplicate.

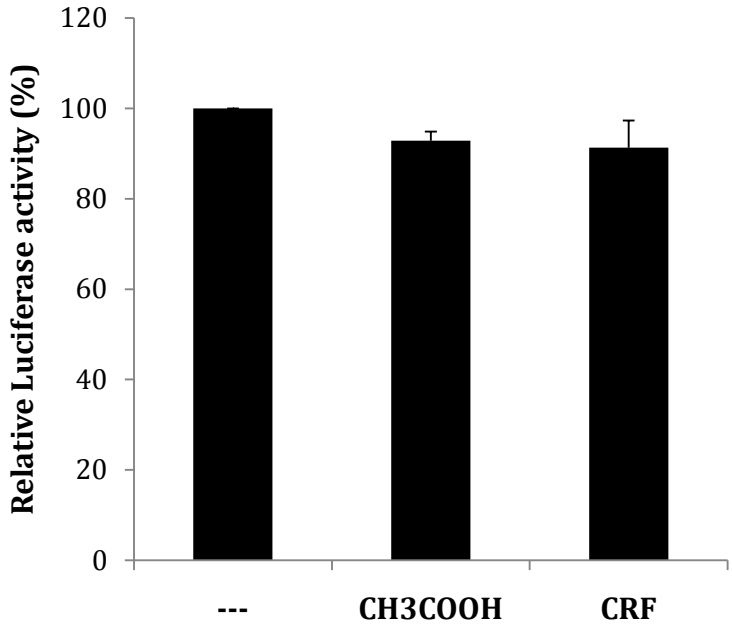


Figure 31| ***CRF does not induce the activity of the GRE-luc reporter.*** HEK293T cells were transiently transfected with the GRE-luc reporter (1μg) in the presence of CRF or vehicle. CRF or CH_3COOH were added 24 hours prior to cell collection. Relative luciferase activity (±SEM) was calculated from at least three independent experiments performed in duplicate.

PART II. REGULATION OF SR-BI IN THE LIVER

Functional analysis of the human SR-BI promoter in hepatic HepG2 cells

As discussed in the introduction, the liver is one of the major sites of high SR-BI gene expression and the tissue in which this receptor plays a crucial role in HDL cholesteryl ester uptake. As a first step towards a better understanding of SR-BI gene regulation in hepatic cells, we performed a structure-function analysis of the human SR-BI promoter in the human hepatoblastoma cell line HepG2. For this purpose, a series of SR-BI reporter plasmids were transfected into HepG2 cells and their relative activity was scored by luciferase assays. This analysis revealed that the activity of the human SR-BI promoter in hepatic cells, similar to adrenal cells, is determined by a complex interplay between positive and negative regulatory elements (**Figure 32**). Specifically, we identified two positive regulatory regions between nucleotides -1288/-408, and -290/-88 which are separated by a strong negative regulatory region defined by nucleotides -408/-290. Also, the distal region -2,787/-1288 shows strong negative activity, indicating the presence of negatively acting regulatory elements.

As discussed in the previous section, the region of the SR-BI promoter between nucleotides -1288/-944 contains previously described binding sites for SREBP-1a and a hormone response element that binds heterodimers of RXR with liver X receptor (LXR) and the liver receptor homolog 1 (LRH-1). To investigate whether these elements are functional in hepatic cells, HepG2 cells were transiently cotransfected with the hSR-BI-1288/+78-luc reporter along with expression vectors for LXR and RXR in the absence or presence of the LXR ligand

22-*R*-OH-cholesterol (**Figure 33**). It was observed that ligand-activated LXR/RXR heterodimers increased four-fold the activity of the human SR-BI promoter. Moreover, transient cotransfection of HepG2 cells with the hSR-BI-1288/+78-luc reporter (**Figure 34A**) or the hSR-BI-290/+78-luc reporter (**Figure 34B**) along with an expression vector for SREBP-1a revealed that the activity of the human SR-BI promoter is induced by SREBP-1a. More specifically, the hSR-BI-1288 promoter was induced 2,5 ford whereas the hSR-BI-290/+78 promoter was induced almost 3-fold by SREBP-1a. The rat SR-BI promoter proximal region -290/+78 contains a characterized binding site for SREBP-1a at position between nucleotides -117 and -105 which bears a 75% homology with the human SR-BI promoter sequence. Thus it is possible that SREBP-1a binds to the proximal region and induces the activity of the human SR-BI promoter.

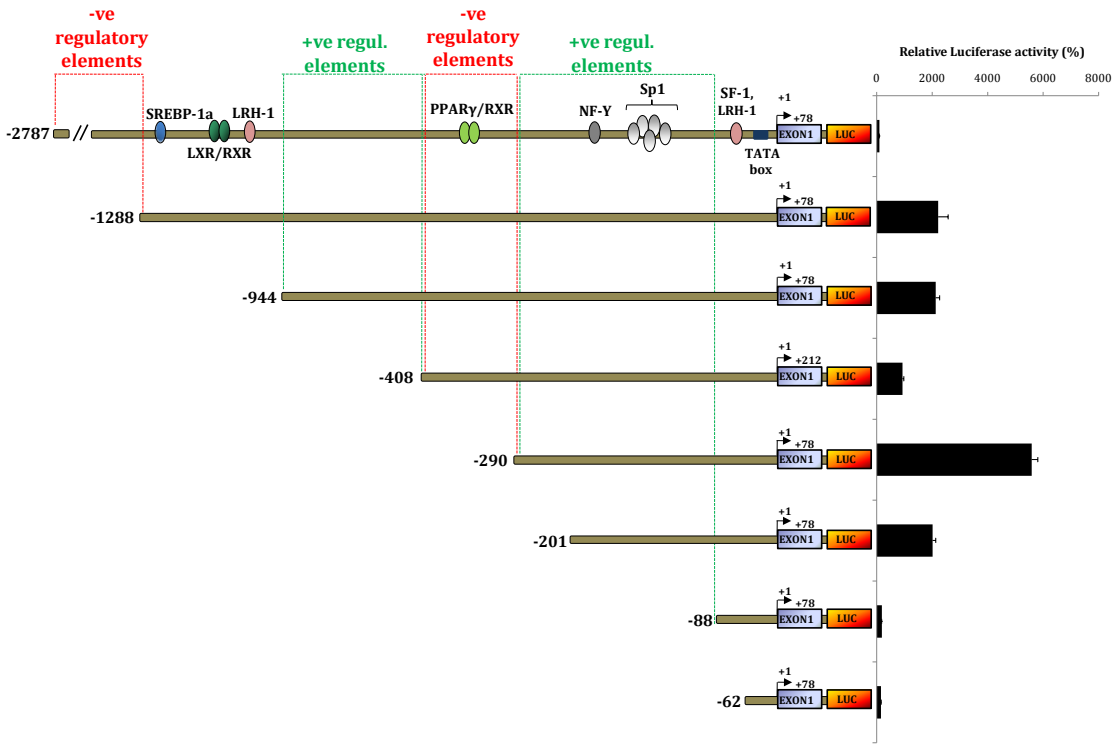


Figure 32 | *Structure-function analysis of the hSR-BI promoter in hepatic HepG2 cells.* HepG2 cells were transiently transfected with the indicated reporter plasmids bearing the luciferase gene under the control of different SR-BI promoter fragments (0.5µg each) along with the CMV-β-gal plasmid expressing β-galactosidase (1µg) for normalization of transfection. Relative luciferase activity (±SEM) was calculated and the experiment was performed in duplicate. Transcription factors that have been shown to bind to the hSR-BI promoter are indicated, as well as the different positive (in green) and negative (in red) regulatory regions.

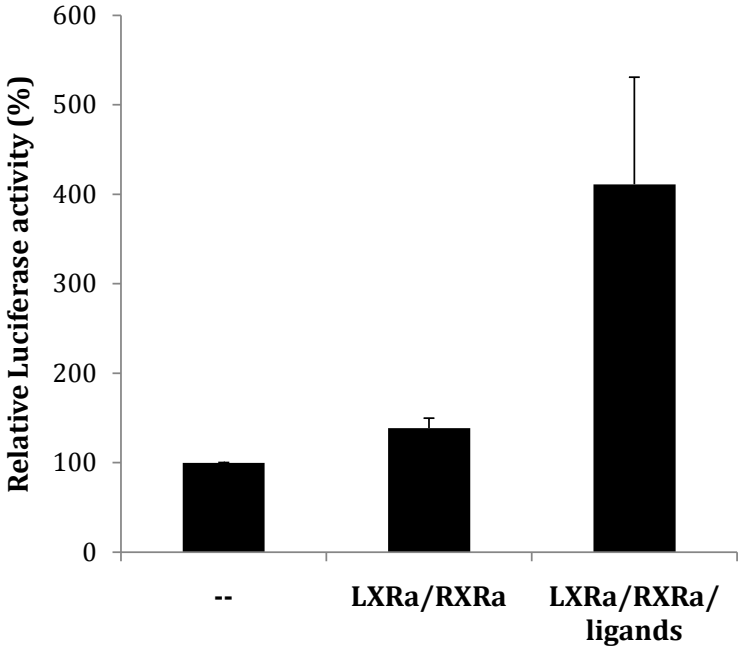


Figure 33|*The transcriptional activity of the human SR-BI promoter is induced by ligand activated heterodimer LXR/RXR.* HepG2 cells were transiently cotransfected with the hSR-BI-1288/+78-luc reporter along with expression vectors pcDNA3-6myc-LXR and pcDNA3-6myc-RXR (0,5µg each). Sixteen hours post-transfection, cells were treated with 1µM 22-*R*-OH-cholesterol and incubated for additional 24 hours in lipoprotein-depleted medium. Relative luciferase activity (±SEM) was calculated and the experiments were performed in duplicate.

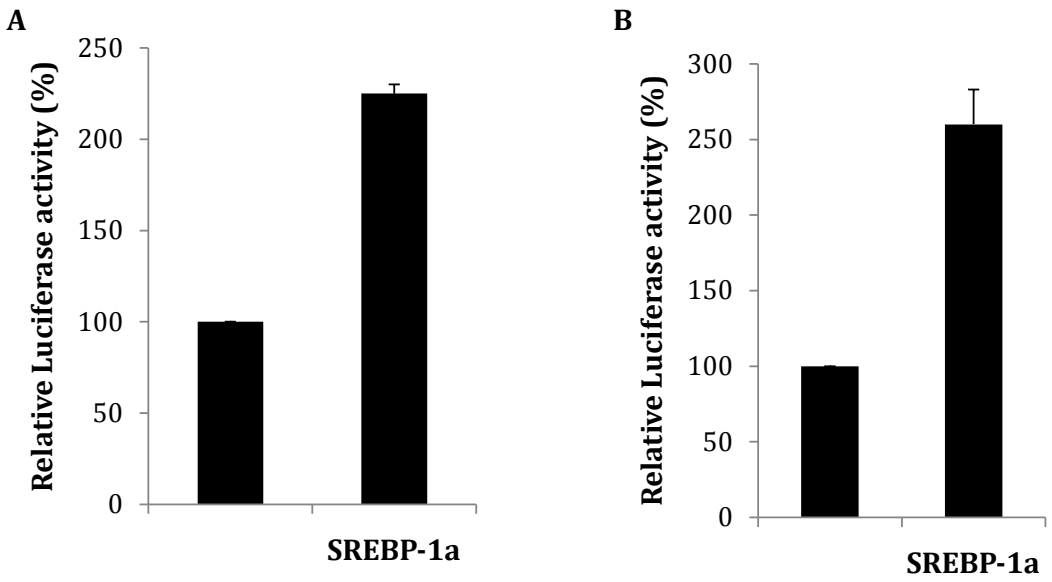


Figure 34| ***The transcriptional activity of the human SR-BI promoter is induced by SREBP-1a.*** HepG2 cells transiently cotransfected with the hSR-BI-1288/+78-luc reporter (A) or hSR-BI-290/+78-luc reporter (B) (0,5μg each) along with the expression vector pcDNA3-6myc-SREBP-1a (1μg). Relative luciferase activity (±SEM) was calculated and the experiments were performed in duplicate.

FOXA2 (HNF-3β) induces the activity of the SR-BI promoter

In the present study, we investigated the role of FOXA2/ Hepatocyte Nuclear Factor 3β (HNF-3β), one of the major liver-specific transcription factors, in SR-BI gene regulation in hepatocytes. FOXA proteins are expressed in endoderm-derived tissues such as the liver, gut and endocrine pancreas [216] and play a central role in maintaining normal glucose homeostasis by regulating, among others, the expression of genes encoding the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in the liver and the insulin secretion-regulating ion-channel in β-cells of the pancreatic islets [216-217]. FOXA2 ablation in hepatocytes demonstrated that FOXA2 is required for the execution of the hepatic gluconeogenic program and for bile acid homeostasis [218]. Analysis of *cis*-regulatory elements of genes encoding gluconeogenic enzyme has led to the finding that binding sites for CREB and GR are often located in close proximity to those of nuclear factors expressed predominantly in hepatocytes, including hepatocyte nuclear factor 4α (HNF-4α) and FOXA proteins.

HepG2 cells were transiently cotransfected with the deletion mutants of the hSR-BI promoter along with an expression vector for FOXA2. As shown in **Figure 35**, FOXA2 activated the transcription of all SR-BI promoter deletion mutants. Specifically, the proximal region -201/+78 showed the highest level of activation (4-fold) and activation was also evident in the minimal deletion mutant -62/+78, which contains only the TATA box. Thus, FOXA2 seems to be a regulator of SR-BI gene transcription.

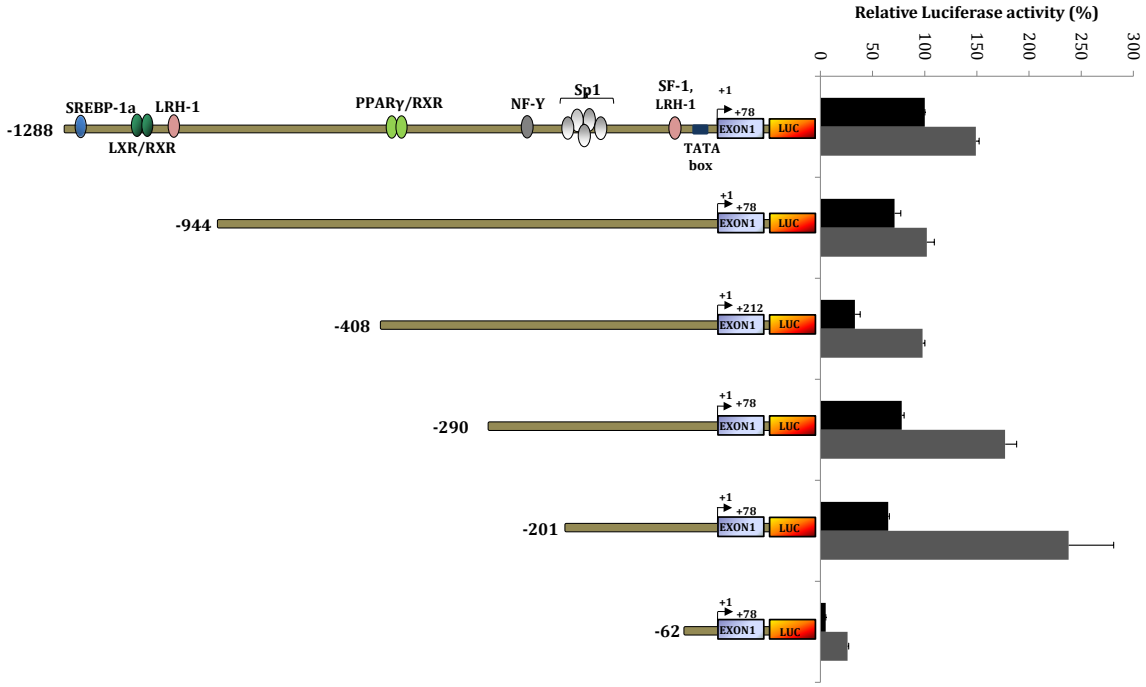


Figure 35| ***FOXA2 induces the transcriptional activity of the human SR-BI promoter.*** HepG2 cells were transiently cotransfected with the deletion mutants of the hSR-BI promoter (0.5µg) indicated in the figure along with an expression vector for FOXA2 (CMV-FOXA2, 1µg). Relative luciferase activity (±SEM) was calculated and the experiment was performed in duplicate.

Effect of HNF-4 on SR-BI gene expression

As discussed in the Introduction, the expression of SR-BI gene was markedly increased in the liver of HNF-4α null mice suggesting that this transcription factor may play a negative role in the regulation of SR-BI in this tissue (**Figure 7**) [177]. HNF-4 is a transcriptional activator that plays key roles in the maintenance of hepatic function and is a major *in vivo* regulator of genes involved in the control of lipid homeostasis [219].

In the present study, we investigated the role of HNF-4 in the regulation of SR-BI gene expression in hepatocytes by various experimental approaches. As a first approach we transiently transfected HEK293T cells, which lack endogenous HNF-4α, with an expression vector for HNF-4 and measured the endogenous SR-BI mRNA levels. As shown in **Figure 36A**, endogenous SR-BI mRNA levels were reduced by 60% compared to untransfected HEK293T cells. In a second approach, we transiently cotransfected hepatic HepG2 cells with the hSR-BI-1288/+78-luc reporter along with a vector expressing a short hairpin RNA targeting HNF-4 (sh-HNF4) or a control vector (**Figure 36B**). We observed that the silencing of HNF-4 by shRNA in HepG2 cells was associated with an induction in hSR-BI promoter activity confirming that HNF-4 is a negative regulator of SR-BI gene transcription in hepatic cells.

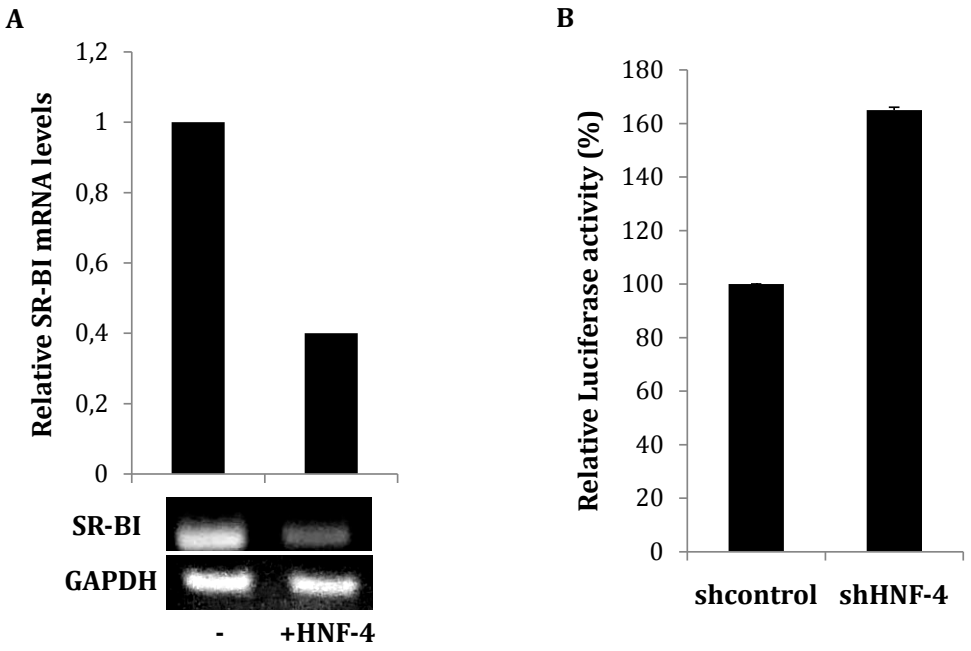


Figure 36| ***Effect of HNF-4 on SR-BI gene expression.*** ***A,*** HEK293T cells were transiently transfected with an expression vector for HNF-4. SR-BI mRNA levels were quantitated by RT-PCR and normalized relative to the housekeeping gene GAPDH and plotted. **B,** HepG2 cells were transiently cotransfected with the hSR-BI-1288/+78-luc reporter and a sh control vector or a shHNF-4 vector. Relative luciferase activity (±SEM) was calculated and the experiment was performed in duplicate.

HNF-4 represses the transcriptional activity of the hSR-BI promoter in hepatic cells

In order to elucidate the role of HNF-4 on the activity of the human SR-BI promoter we cotransfected hepatic HepG2 cells with the hSR-BI-1288/+78-luc reporter and increasing amounts of an expression vector for HNF-4. As shown in **Figure 37B**, HNF-4 inhibited the transcriptional activity of the human SR-BI -1288/+78 promoter in a dose dependent manner. We also cotransfected HepG2 cells with the hSR-BI-1288/+78-luc reporter along with increasing amounts of a constitutively active form of HNF-4, HNF-4 (1-370) which lacks the inhibitory domain (**Figure 37A**) [220]. Again, we observed a dose depended reduction of the transcriptional activity of the hSR-BI-1288/+78 promoter by the constitutively active HNF-4 mutant. In contrast, the activity of the hSR-BI -1288/+78 promoter was induced in a dose dependent manner by a dominant negative form of HNF-4, HNF-4 (1-360) DN that lacks the Activation Function-2 (**Figure 38A**) [220].

Next, we wanted to define the minimal region of the hSR-BI promoter required for repression by HNF-4. For this reason we transiently cotransfected HepG2 cells with the hSR-BI-408/+212-luc, hSR-BI-290/+78-luc or the hSR-BI-88/+78-luc reporter plasmids along with an expression vector for the constitutively active HNF-4 (1-370) (Fig. 39). As shown in **Figure 39A**, HNF-4 (1-370) reduced the transcriptional activity of the hSR-BI -408/+212 promoter by ~ 45% and the activity of the hSR-BI -201/+78 promoter by ~ 70%. The most

proximal region -88/+78 of the hSR-BI promoter was not repressed by HNF-4 (1-370), but it was induced by 60%.

In summary, the findings of **Figures 37-39** indicate that HNF-4 is a negative regulator of the human SR-BI expression and that the minimal region required for the repression by HNF-4 is the proximal region -201/-88 of the hSR-BI promoter.

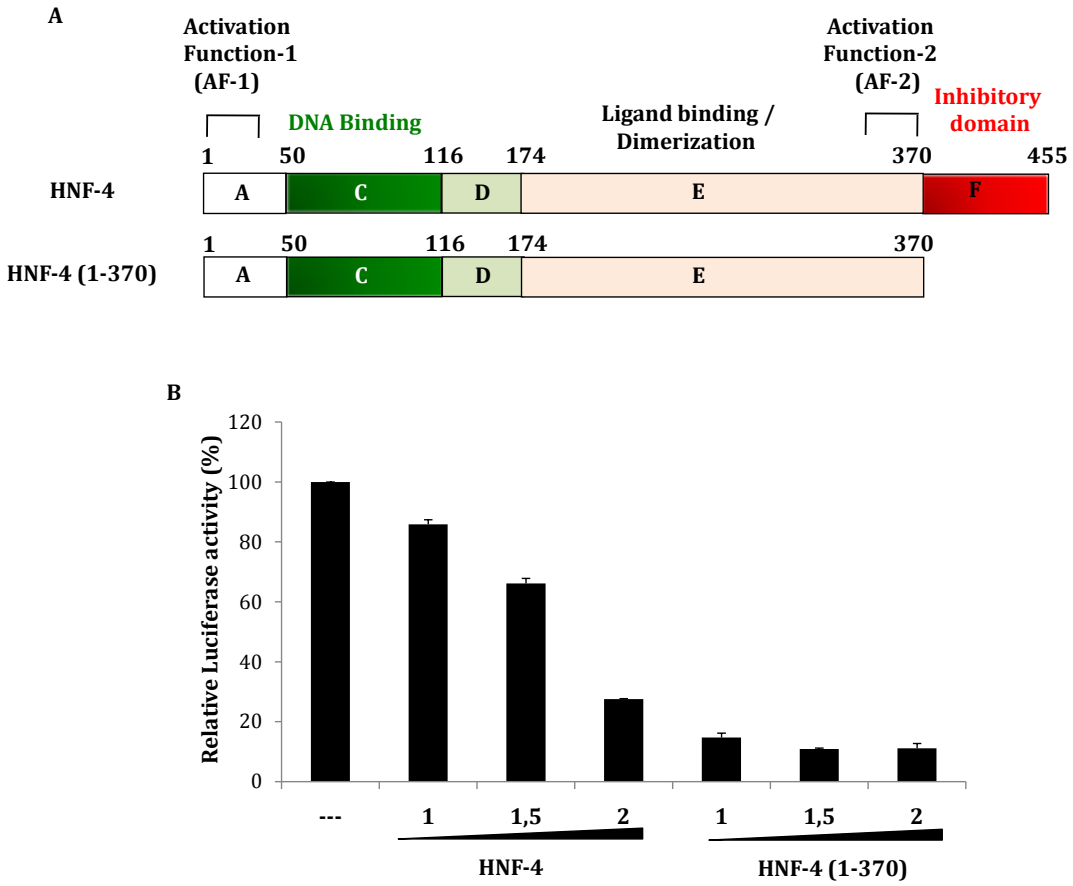


Figure 37| ***HNF-4 reduces the activity of the human SR-BI promoter in hepatic cells.*** A, Schematic representation of HNF-4 and it's constitutively active form, HNF-4 (1-370). B, HepG2 cells were transiently cotransfected with the hSR-BI-1288/+78-luc reporter (0.5µg) and increasing amounts of the expression vector for HNF-4 (1-2µg) or the expression vector for the constitutively active HNF-4 (1-370) (1-2µg). Relative luciferase activity (±SEM) was calculated and the experiment was performed in duplicate.

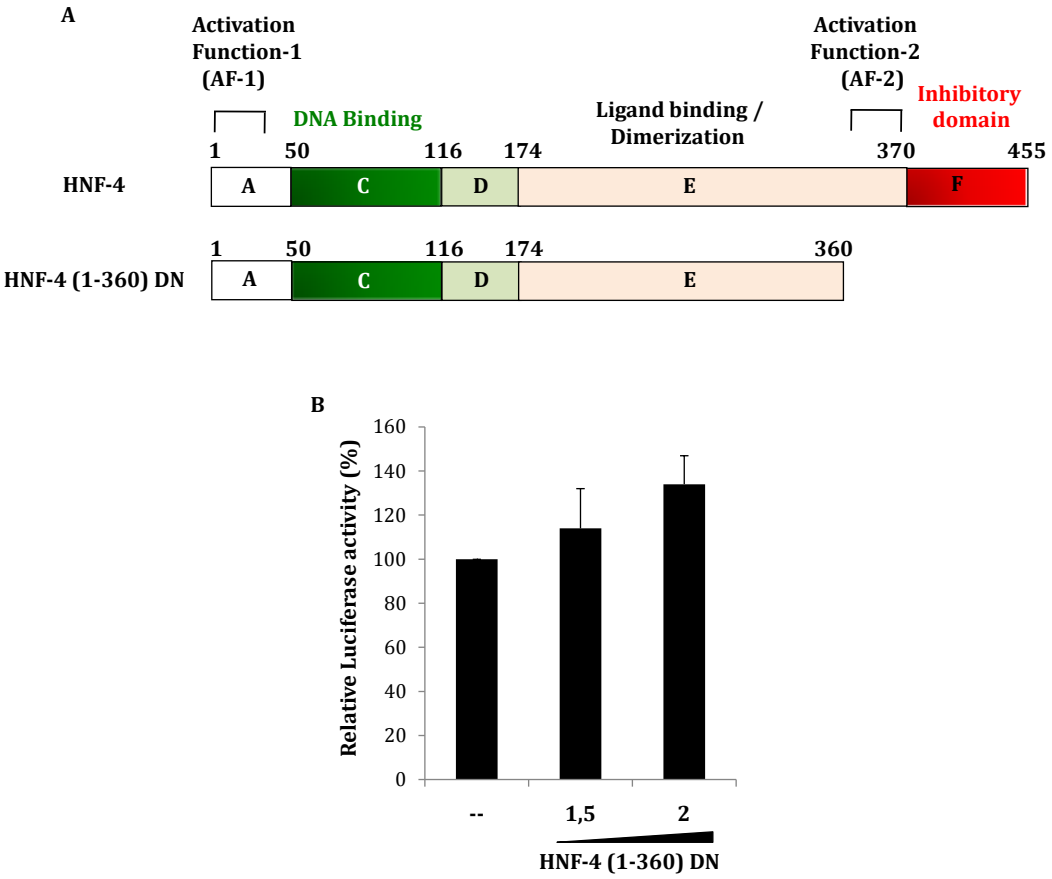


Figure 38| ***A dominant negative form of HNF-4 induces the activity of the hSR-BI promoter in hepatic cells. A,*** Schematic representation of HNF-4 and it's dominant negative form, HNF-4 (1-360)DN. ***B,*** HepG2 cells were transiently cotransfected with the hSR-BI-1288/+78-luc reporter (0.5µg) and increasing amounts of the expression vector for HNF-4 (1-360)DN (1-2µg). Relative luciferase activity (±SEM) was calculated and the experiment was performed in duplicate.

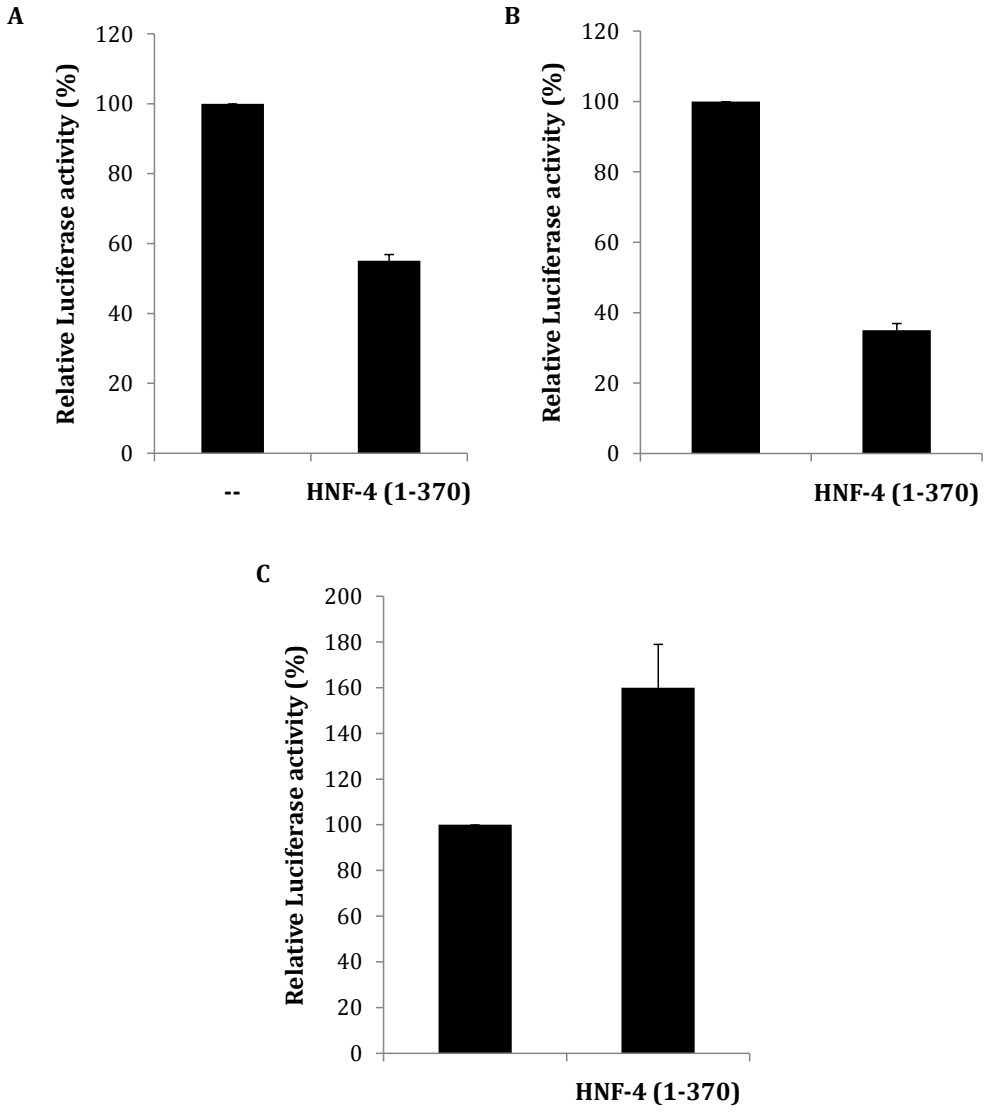


Figure 39| ***Constitutively active HNF-4(1-370) reduces the transcriptional activity of the proximal human SR-BI promoter. A, B, C,*** HepG2 cells were transiently transfected with the hSR-BI-408/+212-luc reporter (A) or the hSR-BI-201/+78-luc reporter (B) or the hSR-BI-88/+78-luc reporter (C) (0.5μg each) along with an expression vector for the constitutively active form, HNF-4 (1-370) (2μg). Relative luciferase activity (±SEM) was calculated and the experiments were performed in duplicate.

Putative HNF-4 elements on the hSR-BI promoter

We performed a sequence analysis of the SR-BI promoter in order to identify putative responsive elements for HNF-4. We used NUBIScan, a fast algoritm for the prediction of nuclear receptor DNA binding sites. The analysis revealed three putative responsive elements (DR1 sites) for HNF-4 similar to the published consensus response element for HNF-4. The positions of the DR1s identified are located between nucleotides -2536/-2524, -898/-885 and -276/-264 along the SR-BI promoter (**Figure 40**).

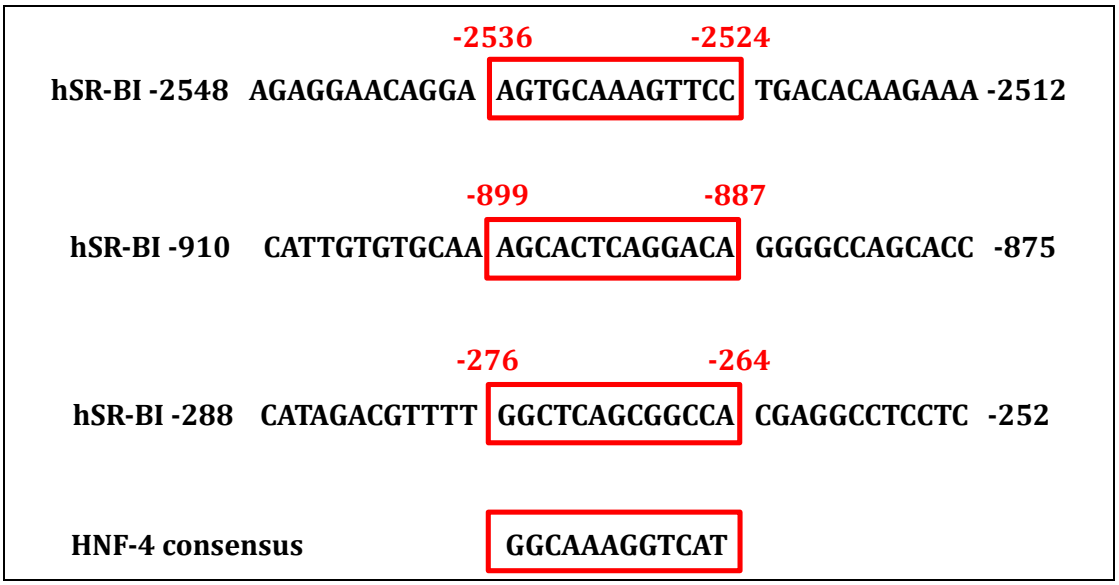


Figure 40| ***Putative HNF-4 binding sites on the hSR-BI promoter.*** DR1s were identified by NUBIScan and are indicated within the red boxes. HNF-4 consensus is adapted from [169].

Recruitment of HNF-4 to the hSR-BI promoter

The next step was to understand the mechanism of inhibition of SR-BI gene expression by HNF-4. For this reason we performed protein-DNA interaction assays in order to investigate whether HNF-4 binds to the putative binding sites mentioned above in the SR-BI promoter. First we used the DNA affinity precipitation assay which allows the detection of DNA bound proteins *in vitro* by immunoblotting and for this purpose we generated a series of non-overlapping biotinylated PCR fragments covering the entire SR-BI promoter between nucleotides -2787 to +78. The amplified regions, which are shown schematically in **Figure 41A**, are: -2787/-2301, -944/-620, -620/-369, -620/-292 and -290/+78. We found that HNF-4α interacted with all the biotinylated hSR-BI promoter fragments examined. More specifically, strong binding was observed to the biotinylated PCR fragments -2787/-2301, -944/-620, -620/-369, and -290/+78 of the hSR-BI promoter. Very weak binding was observed to the biotinylated PCR fragment -620/-292 compared to the binding of HNF-4 to the other PCR fragments of the hSR-BI promoter (**Figure 41B**).

We then performed chromatin immunoprecipitation assays in HepG2 cells and found recruitment of endogenous HNF-4 to the distal -2787/-2301 and the -944/-292 or the more proximal -290/+78 SR-BI promoter (**Figure 42B**). The regions -944/-620, -620/-369, -620/-292 used in the DNA affinity precipitation assay are included in the region -944/-292 used in the chromatin immunoprecipitation assay.

In conclusion, our findings support a direct mechanism of suppression of SR-BI gene by HNF-4 in hepatic cells which is achieved through multiple binding sites along the SR-BI promoter.

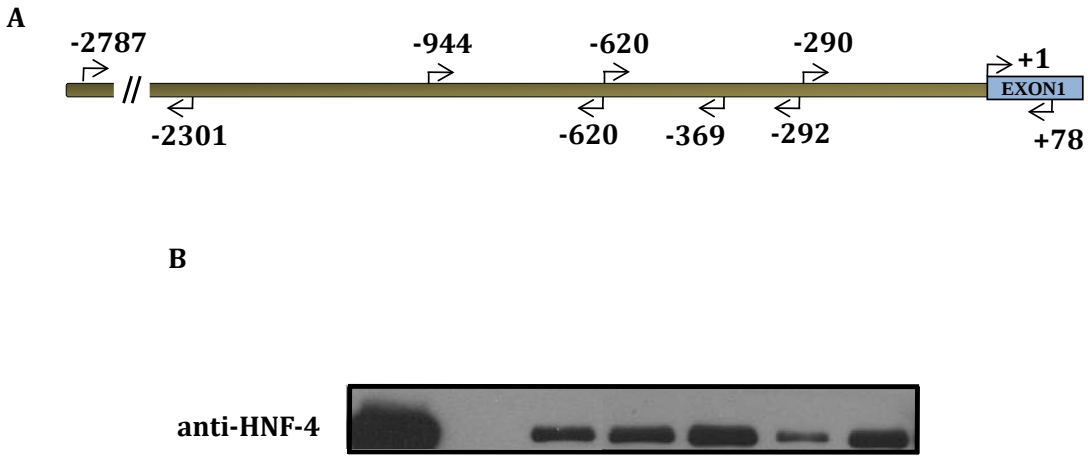


Figure 41| ***HNF-4 is recruited to multiple sites on the SR-BI promoter in vitro.*** ***A,*** Schematic representation of the SR-BI 5′ upstream region showing the non-overlapping Biotinylated PCR fragments that were used for the in vitro DNA affinity precipitation assays of B. ***B,*** DNA affinity precipitation experiment was carried out using extracts from HEK293T cells that had been transiently transfected with an expression vector for HNF-4. Streptavidin agarose beads were coupled to Biotinylated control oligonucleotides or the Biotinylated PCR fragments and incubated with the cells lysates as described in Materials and Methods. Primers used for the experiment are shown in Table 5.

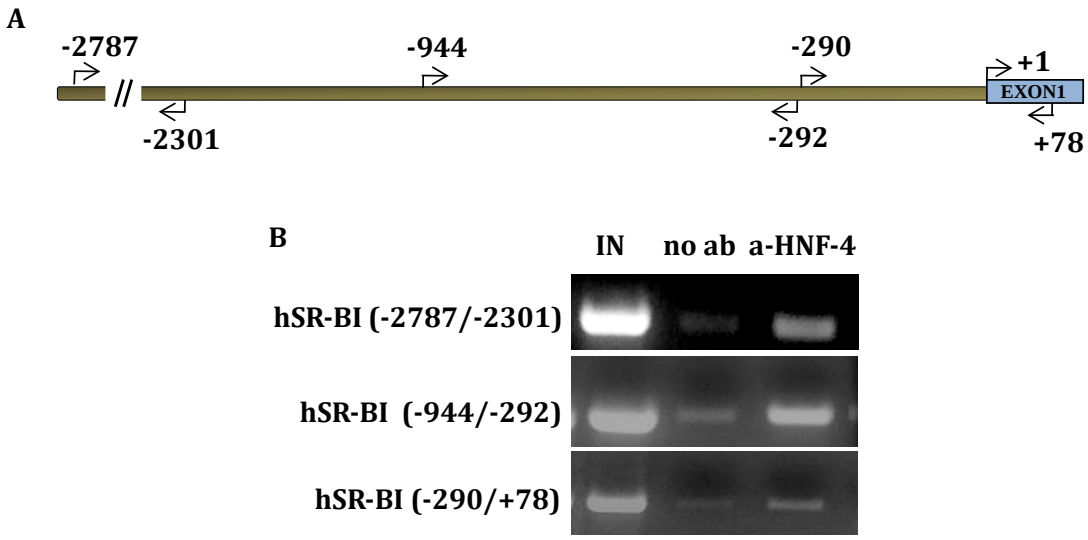


Figure 42| ***HNF-4 is recruited to the SR-BI promoter in vivo. A,*** Schematic representation of the SR-BI 5′ upstream region showing the pairs of primers used for the chromatin immunoprecipitation analysis of B. ***B,*** Chromatin immunoprecipitation analysis in HepG2 cells. Immunoprecipitations were performed using the anti-HNF-4 antibody or no antibody as a control (no ab). Primers that amplify the distal -2787/-2301, -944/-292 or the proximal -290/+78 SR-BI promoter region were used for PCR analysis and are shown in Table 5.

Discussion

Physiological significance of SR-BI gene regulation by glucocorticoids in steroidogenic tissues

Work from several laboratories during the last two decades has established that plasma lipoproteins are the major source of cholesterol for the production of steroid hormones in steroidogenic tissues such as adrenal, ovary and testis. Lipoprotein-derived cholesterol can be delivered through the use of a specialized pathway known as the selective cholesteryl ester uptake. A key player in this pathway is SR-BI, which interacts with apolipoprotein A-I present in HDL to mediate the uptake process. Analysis of mice with targeted disruption of the SR-BI gene established the important role of this membrane receptor in adrenocortical cholesterol metabolism. The SR-BI KO mice are characterized by depletion of cholesteryl ester stores in the adrenals and the ovaries. Similar disturbances in adrenal cholesterol metabolism were observed in mice lacking the *apolipoprotein A-I* and the *LCAT* genes, which are important players in plasma HDL biogenesis and remodeling. Importantly, these mice exhibit elevated levels of SR-BI gene expression in the adrenals, possibly as a compensatory mechanism for the reduced plasma HDL and the diminished HDL/SR-BI-mediated cholesteryl ester uptake. Despite the mechanisms for storing cholesteryl ester in lipid droplets and synthesis of endogenous cholesterol in steroidogenic tissues, the SR-BI-mediated cholesteryl ester uptake pathway provides a major supply of cholesterol for steroidogenesis.

In rat adrenals, ACTH induces cholesteryl ester uptake. Adrenals from mice lacking proopiomelanocortin are characterized by deficiency in CE and attenuated levels of key proteins involved in selective CE uptake and steroidogenesis such as SR-BI, whereas adrenal CE levels and SR-BI gene expression were restored with ACTH treatment. Thus, there seems to be a good correlation between the induction of cholesteryl ester uptake, SR-BI gene expression, and steroidogenesis in adrenal cells. Additional studies suggest that ACTH can stimulate SR-BI gene expression by interacting directly with adrenal cells. Also, the normal ACTH response in patients with familial hypercholesterolemia, lacking a functional LDL receptor, is consistent with the involvement of SR-BI [221].

During a stress response there is increased glucocorticoid synthesis and secretion. It has been shown that SR-BI protects against endotoxemia by facilitating adrenal glucocorticoids production in response to LPS and stress. Adrenocortical cells store relatively small amounts of glucocorticoids, thus primary adrenal failure in endotoxic shock is usually due to insufficient glucocorticoid synthesis. In SR-BI KO mice observations such as glucocorticoid insufficiency, reduced clearance of LPS, increased inflammatory response to LPS, low corticosterone levels led to the conclusion that the adrenal SR-BI is required to provide sufficient cholesterol for glucocorticoids production under conditions of high adrenal stimulation by ACTH, as occurs during LPS-induced inflammation [196].

A question that arises is whether or not SR-BI-mediated delivery of cholesterol to the adrenal gland is of equal importance in humans as in mice.

Early studies in human cells in culture showed that the LDL receptor may be more important in cholesterol delivery than SR-BI. However, this requires further investigation as little attention has been paid to the function of SR-BI in humans. The data of the present thesis support for the first time an indirect mechanism of inhibition of human SR-BI gene transcription by glucocorticoids in adrenal and ovarian cells involving endogenous GR. More experimentation on the function of SR-BI in steroidogenic tissues will provide answers for the better understanding of patients with adrenal insufficiency during a stress response or sepsis and in addition shed some light on the causes of female infertility in SR-BI KO mice.

GR acting as a transcriptional repressor

The GR regulates gene transcription in response to glucocorticoids by associating with GREs present on the promoters of target genes [222]. GREs can be a) imperfect palindromes to which GR is the unique-specific DNA-binding protein, or b) composite GREs, that specify binding sites for GR and for one or more non-receptor factors. Alternatively, GR may also regulate transcription through physical interactions with other transcription factors by influencing their ability to stimulate or inhibit the transcription rates of respective target genes that do not contain GREs. Examples are the inhibitory interactions of GR with nuclear factor kB, activator protein 1, and STATs, which facilitate the suppression of immune function and inflammation by glucocorticoids [223-225].

The ability of GR to repress gene transcription in response to glucocorticoids was also revealed by gene expression profiling experiments. It was shown by microarray analysis in dexamethasone treated A549 adenocarcinoma cells that 108 genes were activated and 73 genes were repressed by GRα [209].

Proposed mechanism of inhibition of human SR-BI gene expression by glucocorticoids via GR in adrenal cells

In the present study, we showed that inhibition of SR-BI gene transcription by glucocorticoids requires GR but is dependent of *de novo* protein synthesis. This was demonstrated by treating the cells with the cell cycle inhibitor cycloheximide. Using a similar approach, Rogatsky et al [226], had identified novel genes that are direct targets of glucocorticoids in osteosarcoma and epithelial lung carcinoma cells. This finding combined with the observation that GR does not bind to the SR-BI promoter *in vitro* and *in vivo* prompted us to postulate an indirect mechanism of SR-BI gene repression by GR/glucocorticoids (**Figure 43**). According to our hypothesis, cholesteryl ester uptake by adrenal or ovarian cells via SR-BI leads to cholesterol accumulation that serves as a substrate for steroid biosynthetic enzymes leading to the increase in intracellular glucocorticoids concentration. The latter can be reversed by metyrapone, which blocks cortisol synthesis by inhibiting the activity of the enzyme steroid 11β hydroxylase, a rate limiting enzyme in corticosterone production. GR activation

by glucocorticoids is associated with an indirect inhibition of SR-BI gene transcription presumably via activation of a transcriptional repressor. This repressor could subsequently bind to the proximal region of the human SR-BI promoter between nucleotides -201 and -88 and repress its activity. KLF15 is a possible candidate for the repression of the SR-BI gene expression in response to increase cholesteryl ester uptake, leading to activation of GR that binds to the promoter of the KLF15 gene, and activating its transcription. KLF15 that is produced by this glucocorticoid response could bind to the proximal region -201/-88 of the human SR-BI promoter thus repressing its activity. Our pleminary data using KLF15 in transactivation assays support this model which needs to be validated by additional experimentation.

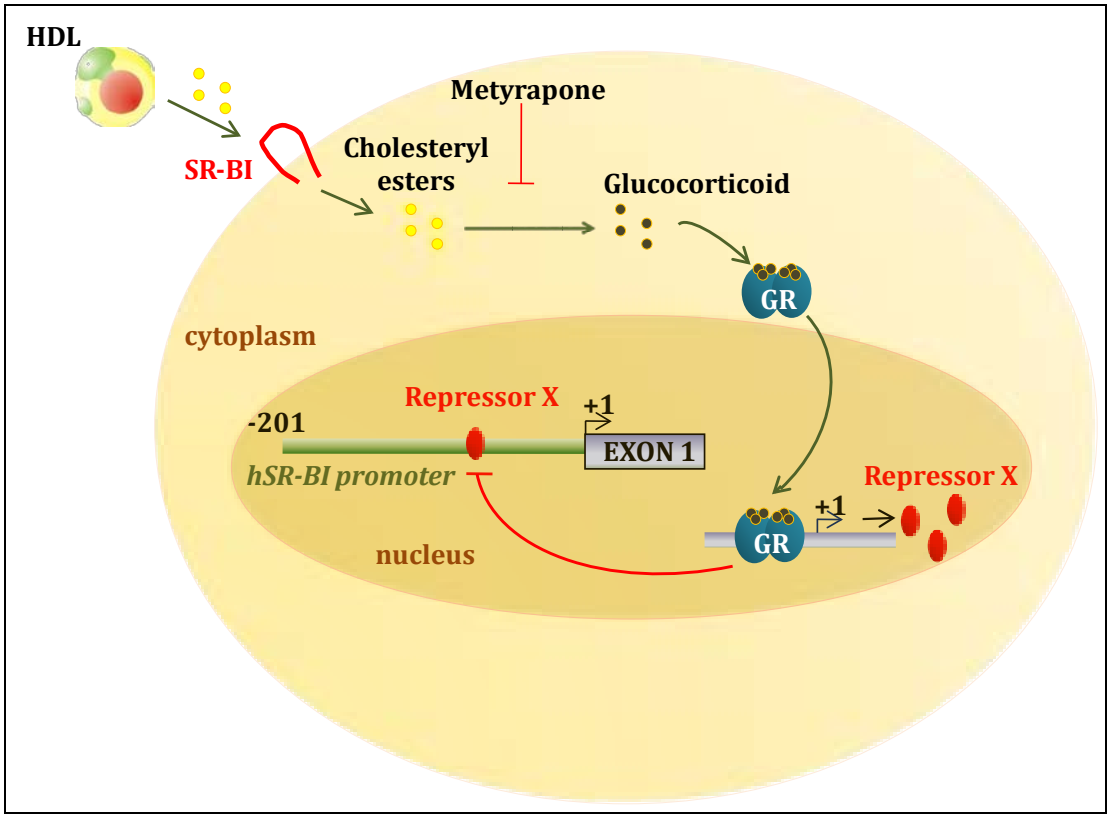


Figure 43| ***Proposed mechanism of inhibition of human SR-BI gene expression by glucocorticoids via GR in adrenal cells.*** See text for details.

Physiological significance of SR-BI gene regulation by HNF-4 in the liver

SR-BI receptor is a key player in HDL metabolism in the liver, as it facilitates cholesteryl ester uptake from HDL for subsequent secretion into the bile. In the liver the overall gene expression profile of hepatic cells is subject to continuous changes during organ development and in response to environmental stimuli. FOXA (HNF-3β) proteins are expressed in endodermal progenitor cells and play a role in the specification of the hepatic lineage [227]. HNF-4α gene is activated later and is required for fibroblast differentiation [228]. A recent genome-wide promoter occupancy study in adult human liver revealed that >40% of the promoters of active genes were bound by HNF-4α and that HNF-1α or HNF-6 bound promoters were also occupied by HNF-4α. It has been shown that disruption of the HNF-4 gene leads to a lethal phenotype due to impairment of endodermal differentiation and gastrulation, indicating that HNF-4 is essential for mammalian development [175]. Liver-specific HNF-4 null mice at their eight week of life show about 70% mortality and reveal lipid abnormalities due to impaired expression of HNF-4-regulated genes involved in lipid and bile acid metabolism and transport [177]. Also, mutations in the HNF-4α gene cause the disorder maturity onset diabetes of the young (MODY) [176]. Thus, HNF-4 seems to be a key switch in the control of lipid homeostasis and represent a promising target of new therapeutic agents for the prevention and treatment of metabolic diseases and atherosclerosis.

A recent study demonstrated that HNF-4 is involved in microsomal triglyceride transfer protein (MTP) gene expression, either directly or indirectly through elevated HNF-1 levels and that reduction of MTP mRNA levels were due to increased expression of the small heterodimer partner (SHP). The transcriptional activity of HNF-4 is regulated by interaction with SHP, an atypical negative nuclear receptor lacking a DNA-binding domain [229-230]. Moreover, it has been shown previously in our lab that the mRNA levels of SHP are highly induced by HNF-4 expression (V. Neokosmidou, PhD thesis, 2007). SHP can be induced by FXR and its ligands, the bile acids, to control the transcriptional activity of several other nuclear receptors including the constitutive androstane receptor, thyroid receptor, retinoid X receptor (RXR), the liver X receptor (LXR) and the liver receptor homolog-1(LRH-1) [231-236].

Global gene expression profiling combined with chromatin immunoprecipitation assays in transgenic mice constitutively expressing SHP in the liver, demonstrated that SHP affects genes involved in diverse biological pathways. More specifically, SHP affects genes involved in consecutive steps of cholesterol degradation, bile acid conjugation, transport and lipogenic pathways. It has been shown that among others, the SR-BI mRNA levels were significantly decreased in SHP-Tg livers [237]. This indicates that SHP is important for SR-BI expression. Overall, regulation of SR-BI gene in the liver is of great importance as it is a key factor in the reverse cholesterol transport, and its regulation by HNF-4α needs more analysis in order to elucidate the exact mechanism by which it suppresses SR-BI activity.

HNF-4 acting as a transcriptional repressor

Previous studies had shown that the gene encoding the HDL receptor SR-BI is strongly upregulated in the liver of HNF-4 KO mice suggesting that HNF-4 is a negative regulator of hSR-BI gene expression in hepatic cells. In this study we further investigated the role of HNF-4 on SR-BI regulation. First, deletion analysis of the hSR-BI promoter in hepatic cells revealed the presence of positive and negative regulatory regions. The finding that silencing of HNF-4 by shRNA was associated with an increase in the activity of the SR-BI promoter and that overexpression of wt HNF-4 or its constitutively active form, HNF-4 (1-370) led to a dramatic suppression of the activity of the promoter in HepG2 cells imply the importance of this transcription factor in the SR-BI gene expression. In agreement with these findings, overexpression of HNF-4 in fibroblasts was associated with a reduction in endogenous SR-BI mRNA levels. The above evidence indicate that HNF-4 acts as a transcriptional repressor of the SR-BI gene.

Possible mechanisms of inhibition of human SR-BI gene expression by HNF-4α in the liver

We propose two mechanisms that could account for the negative effect of HNF-4 on SR-BI gene expression, a direct mechanism and an indirect mechanism. The direct effect of HNF-4 on the SR-BI gene is based on our protein-DNA assays

which confirmed the recruitment of HNF-4 to three regions in the hSR-BI promoter. Thus, HNF-4α, bind to its responsive elements on the SR-BI promoter and facilitates direct suppression of SR-BI expression (**Figure 44**).

The binding of HNF-4α to the SR-BI promoter may be non functional i.e. HNF-4 may bind to the SR-BI promoter without any regulatory effect. For this reason we propose an indirect mechanism which involves the activation of a repressor. This is based on our findings that HNF-4 increased the expression of SHP, which in turn represses the activity of LRH-1. Liver SR-BI expression is decreased in *LRH-/-* mice, providing i*n vivo* evidence of SR-BI regulation by LRH-1[142]. SR-BI promoter contains two responsive elements for LRH-1, a distal and a proximal that can bind LRH-1 and induce the activity of the SR-BI promoter (Figure 44) [143] [142]. Thus we propose an indirect mechanism where HNF-4 activates SHP expression which in turn suppresses LRH-1 binding and subsequent induction of SR-BI activity. It must be pointed out that LRH-1 can be repressed through the SHP induction by FXR together with bile acids.

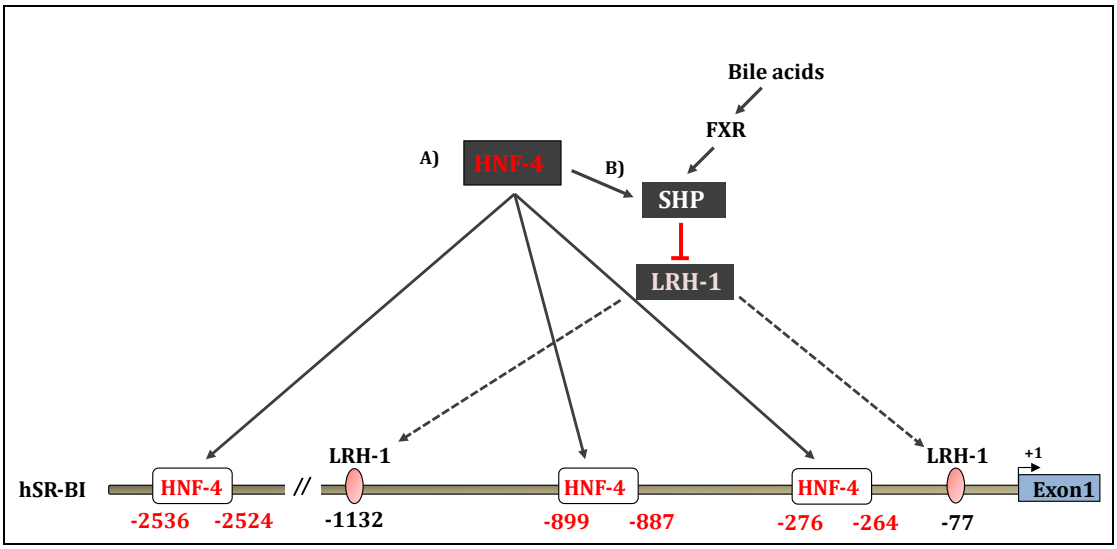


Figure 44| ***Proposed mechanisms of inhibition of human SR-BI gene expression by HNF-4α in the liver. A,*** Direct mechanism via binding of HNF-4α to its responsive elements identified by sequence analysis. ***B,*** Indirect mechanism through HNF-4α induction of SHP and subsequent LRH-1 repression, leading to inhibition of SR-BI transcription.

Clinical relevance of SR-BI with respect to HDL metabolism

A balance between plasma HDL levels and SR-BI expression is required in order for HDL to function as an anti-atherogenic molecule. When SR-BI expression is too high, HDL levels decrease, which result in alteration of reverse cholesterol transport. On the contrary, when SR-BI expression is very low, HDL-cholesterol turnover is impaired [99]. Overall, loss of SR-BI expression in rodents has a negative effect on cardiovascular physiology, whereas overexpression of the receptor suppresses atherosclerosis by increasing RCT.

Murine regulation of SR-BI gene expression, distribution, and activity are similar to those of human SR-BI [56, 67, 95, 203]. It was suggested that SR-BI may play a role in lipid metabolism in humans similar to its role in rodents. Polymorphisms in the human SR-BI gene were associated to a limited extent with variations in plasma lipid levels and body-mass index in a small, homogeneous population [238]. Furthermore, several studies demonstrated that plasma HDL concentration in humans is also determined by the levels of SR-BI expression [238-241]. In humans, SR-BI is involved in the metabolism of both HDL and LDL, as variations in the SR-BI locus are related to changes in LDL levels [238, 240-241]. Thus, SR-BI plays a dual role in the prevention of atherosclerosis in humans, by enhancing RCT through HDL and by reducing the levels of LDL which contain the atherogenic apoB apolipoprotein.

Great insights into the HDL metabolism were obtained by the studies of transgenic or knock animal models for the genes that participate in the biogenesis

and catabolism of HDL [43]. One apparent paradox that emerged from these studies was that mice that lack the HDL receptor SR-BI have high levels of abnormal HDL, but are more prone to atherosclerosis due to the formation of abnormal HDL particles [101-102]. In addition, few human subjects were identified with high HDL levels and documented coronary heart disease that was attributed to the atherogenic rather than atheroprotective properties of HDL that was formed in these patients [242].

This new knowledge suggests that what is important for protection from CHD is not necessarily a high level of HDL cholesterol, but more importantly the presence of functional HDL. The functionality of HDL can be influenced by the abundance of various bioactive proteins and lipids that exert anti-inflammatory, anti-oxidative, anti-coagulative and other atheroprotective functions, as well as by the acquisition of atypical proteins, for example serum amyloid A in the course of inflammation, which interfere with regular HDL function [243-244].

The modulation of HDL metabolism by dietary, pharmacological and gene-therapy approaches in order to reduce the risk of atherosclerosis is an appealing subject that needs more basic and clinical research.

In conclusion, the discovery of SR-BI as the receptor for HDL might offer new therapeutic avenues for the prevention or treatment of atherosclerosis and might be useful in assessments of risk factors and drug efficacy.

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Publications

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Feedback Inhibition of Human Scavenger Receptor Class B Type I Gene Expression by Glucocorticoid in Adrenal and Ovarian Cells

Sofia Mavridou, Maria Venihaki, Olga Rassouli, Christos Tsatsanis, and Dimitris Kardassis

Departments of Basic Sciences (S.M., D.K.) and Clinical Chemistry (M.V., O.R., C.T.), University of Crete Medical School, and Institute of Molecular Biology and Biotechnology (D.K.), Foundation of Research and Technology-Hellas, Heraklion 71003, Greece

Scavenger receptor class B type I (SR-BI) facilitates the reverse transport of excess cholesterol from peripheral tissues to the liver via high-density lipoproteins. In steroidogenic tissues, SR-BI supplies cholesterol for steroid hormone production. We show here that the transcription of the human *SR-BI* gene is subject to feedback inhibition by glucocorticoid in adrenal and ovarian cells. SR-BI mRNA levels were increased in adrenals from corticosterone-insufficient $Crh^{-/-}$ mice, whereas corticosterone replacement by oral administration inhibited *SR-BI* gene expression in these mice. SR-BI mRNA levels were increased in adrenals from wild-type mice treated with metyrapone, a drug that blocks corticosterone synthesis. Experiments in adrenocortical H295R and ovarian SKOV-3 cells using cycloheximide and siRNA-mediated gene silencing revealed that glucocorticoid-mediated inhibition of *SR-BI* gene transcription requires *de novo* protein synthesis and the glucocorticoid receptor (GR). No direct binding of GR to the SR-BI promoter could be demonstrated *in vitro* and *in vivo*, suggesting an indirect mechanism of repression of *SR-BI* gene transcription by GR in adrenal cells. Deletion analysis established that the region of the human SR-BI promoter between nucleotides −201 and −62 is sufficient to mediate repression by glucocorticoid. This region contains putative binding sites for transcriptional repressors that could play a role in *SR-BI* gene regulation in response to glucocorticoid. In summary, this is the first report showing that glucocorticoid suppress SR-BI expression suggesting that steroidogenic tissues maintain steroid hormone homeostasis by prohibiting SR-BI-mediated high-density lipoprotein cholesterol uptake when the endogenous levels of glucocorticoid are elevated. **(*Endocrinology* 151: 3214–3224, 2010)**

Scavenger receptor class B type I (SR-BI) is an 82-kDa membrane glycoprotein that serves as receptor of high-density lipoproteins (HDL) and plays an essential role in reverse cholesterol transport (1, 2). SR-BI facilitates the selective cholesteryl ester uptake from HDL and low-density lipoproteins as well as the bidirectional trafficking of unesterified cholesterol (2–11). SR-BI is a member of the cluster determinant 36 (CD36) superfamily of membrane proteins that includes, among others, CD36 and lysosomal integral protein II (LIMPII) (12, 13). SR-BI has a putative horseshoe-like structure with a large extracellular domain, two transmembrane domains as well as N-terminal and C-terminal cytoplasmic tails, and is localized in specialized plasma membrane domains called caveolae (14–17). An alternatively spliced form of SR-BI, designated SR-BII, has been described, which has a different C-terminal cytoplasmic tail (18). Major sites of SR-BI expression are the liver, adrenals, ovaries and testis (2, 19–21).

The regulation of *SR-BI* gene expression is exerted mainly at the level of transcription. The promoters of the human and rat *SR-BI* genes contain DNA sequences that bind several positively acting transcription factors in response to exogenous or endogenous stimuli. These factors

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Abbreviations: FBS, Fetal bovine serum; GADPH, glyceraldehyde3-phosphate dehydrogenase; GFP, green fluorescent protein; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HDL, high-density lipoprotein; ITS, insulin transferring-sodium selenite; SR-BI, scavenger receptor class B type I; SRE, sterol responsive element; SREBP, sterol-responsive element binding-protein.

include hormone nuclear receptors such as the liver X receptors α and β (LXRα, LXRβ) and the peroxisome proliferator activated receptor α (PPARα) that positively regulate the expression of the human *SR-BI* gene in response to oxysterols and fibrates respectively (22, 23); the liver receptor homolog 1 (LRH-1) (24); and the estrogen receptors α and β (ERα and β), which bind to three different estrogen-responsive elements on the rat SR-BI promoter and regulate its activity in response to estrogens (25). Other transcription factors include the steroidogenic factor-1 (SF-1), which activates the human and the rat SR-BI promoters and seems to be the key mediator of the cAMP-dependent regulation of the *SR-BI* gene (26), and SREBP-1a, which binds to two sterol-responsive elements (SREs) on the rat SR-BI promoter and regulates *SR-BI* gene expression in response to intracellular sterol levels (20, 27).

In addition to the positively acting transcription factors described above, the SR-BI promoter is also regulated by negatively-acting factors, including the nuclear receptor dorsal-sensitive sex adrenal hypoplasia congenital critical region on the X chromosome gene 1 (DAX-1), a protein that plays an important role in adrenal development (26); the Yin Yang-1 (YY-1) transcription factor, which represses the activity of the SR-BI promoter by inhibiting the binding of sterol-responsive element binding-protein (SREBP)-1a (28); and the pregnane X receptor, which represses the human SR-BI promoter activity in response to the pregnane X receptor agonists rifampicin and lithocholic acid (29).

In steroidogenic tissues, SR-BI supplies the cells with exogenous cholesterol for storage or for the synthesis of steroid hormones. In these tissues, SR-BI expression was shown to be upregulated by adrenorticotropic hormone (ACTH) (30–35). Suppression of ACTH by the synthetic corticosteroid dexamethasone (which inhibits the hypothalamic-pituitary axis and decreases ACTH secretion) decreased SR-BI levels (31). However, the mechanism by which ACTH and glucocorticoid regulate the expression of the *SR-BI* gene in stereoidogenic tissues is unclear.

Materials and Methods

Materials

DMEM, DMEM F-12 1:1, and penicillin/streptomycin for cell cultures were purchased from Invitrogen/Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from BioChrom Labs (Terre Haute, IN). Insulin transferring-sodium selenite (ITS) supplement was from Roche Diagnostics (Indianapolis, IN). Metyrapone, dexamethasone, mifepristone (RU-486), and poly(deoxyinosine/deoxycytosine) were purchased from Sigma-Aldrich (St. Louis, MO). Cycloheximide was a generous gift from Dr. Aristidis Iliopoulos (University of Crete). Restriction enzymes and modifying enzymes were purchased from Minotech (Heraklion, Greece), New England Biolabs (Beverly, MA), or Roche (Mannheim, Germany). GoTaq DNA polymerase, dNTPs, the luciferase assay system, and the Wizard SV gel and PCR cleanup system were purchased from Promega (Madison, WI). Trizol reagent for RNA extraction, SuperScript Rnase H-reverse transcriptase, SYBR GreenER qPCR SuperMix for real-time PCR, Lipofectamine 2000, and Dynabeads M-280 streptavidin magnetic beads were purchased from Invitrogen/Life Technologies (Carlsbad, CA). The Super Signal West Pico chemiluminescent substrate was purchased from Pierce (Rockford, IL). Corticosterone ^{125}I RIA kit for measurement of corticosterone was purchased from ICN Radiochemicals Inc (Irvine, CA). Silencer siRNA against human Glucocorticoid receptor (GR) was purchased from Ambion, Inc. and scrambled siRNA was synthesized at MWG (Ebersberg, Germany). pcDNA3hGRa and GRE-luc plasmids were obtained from Dr. H. Russcher (Erasmus MC, University Medical Center, Rotterdam). Anti-SR-BI antibody was purchased from Novus Biologicals (Littleton, CO), anti-hGRα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-green fluorescent protein (GFP) antibody was purchased from Minotech. The anti-rabbit and antimouse peroxidise conjugated secondary antibodies were purchased from Chemicon International Inc. (Temecula, CA). Biotinylated oligonucleotides were synthesized by VBC-Genomics (Vienna, Austria). All other oligonucleotides were synthesized at the microchemical facility of the Institute of Molecular Biology and Biotechnology (IMBB) (Heraklion, Greece).

Animal studies

Male $Crh^{+/+}$ and $Crh^{-/-}$ mice of 129×C57BL/6 genetic background were generated as previously described (36). All mice were housed with *ad libitum* access to rodent chow on a 12-h light, 12-h dark cycle (lights on at 0700 h). All experiments were approved by the Animal Committee of Medical School of the University of Crete. All experiments were performed in mice of 2–4 months of age. Animals were housed individually at least 16 h before each experiment. For the glucocorticoid replacement experiments, $Crh^{-/-}$ mice were administered with 10 μg/ml corticosterone or vehicle in their drinking water for at least 3 d before the onset of the experiment. The pharmacological adrenalectomy experiment was carried out as previously described (37). In brief, wild-type ($Crh^{+/+}$) mice were injected intraperitoneally twice a day for 4 d with 200 mg/Kg of metyrapone or 100 μl 10% dimethylsulfoxide, and on the fourth day 100 mg/Kg aminoglutethimide was injected twice in combination with metyrapone.

Blood and tissue collection and plasma corticosterone assay

Blood samples were collected by retroorbital eye bleeding of conscious mice. Mice were killed by decapitation, and adrenals well collected and frozen rapidly on dry ice. Blood samples were centrifuged at 3000 rpm for 10 min at 4 C, and plasma was separated, aliquoted, and stored at −80 C until further use. Plasma corticosterone levels were measured using the ^{125}I RIA kit (RIA) according to the manufacturer's instructions.

Plasmid construction

The SR-BI promoter (wild-type and deletion mutants) reporter plasmids were constructed by PCR amplification and subsequent cloning into the pGL3Basic vector. The SR-BI promoter

TABLE 1. Oligonucleotides used in PCR cloning, RT-PCR, real-time PCR, DNA affinity precipitation (DNAP), and chromatin immunoprecipitation (ChIP) experiments

Name of primer	Sequence (5′–3′)	Purpose
hSR-BI-2787 F	CGG GGT ACC CTG GAG ATA CAT CGA TAA GCA AAA C	PCR cloning
hSR-BI-1362 F	ATA GGT ACC TCA TTC ATT TGC TCA CGT GCT CAT CAT TGG	PCR cloning
hSR-BI-944 F	CGG GGT ACC CCA CCT CCC AGT TTT CTT ATT GTA	PCR cloning
hSR-BI-673 F	CGG GGT ACC CAC CAG CGT TAC GAA GGA TAC	PCR cloning
hSR-BI-408 F	CGG GGT ACC GGA GGA GGG AAA AGG AAG GGT GAG	PCR cloning
hSR-BI-290 F	CGG GGT ACC CCC ATA GAC GTT TTG GCT CAG	PCR cloning
hSR-BI-201 F	CGG GGT ACC CGG GGC TTG TCT TGG CGG C	PCR cloning
hSR-BI-88 F	CGG GGT ACC AAT CCT GAA GCC CAA GGC TG	PCR cloning
hSR-BI+78 R	CCC AAG CTT CGG CGA CAG AGA CGA CAC AGG CGG GG	PCR cloning
hSR-BI+212 R	CCG CTC GAG CCA GCA CAG CGC ACA GTA GCC CCG	PCR cloning
GAPDH F	ACC ACA GTC CAT GCC ATC AC	RT-PCR
GAPDH R	TCC ACC ACC CTG TTG CTG TA	RT-PCR
hSR-BI-172 F	CTC ATC AAG CAG CAG GTC CTT A	RT-PCR
hSR-BI-478 R	GCA TGA CGA TGT AGT CGC TCT	RT-PCR
hSR-BI-1027 F	TTC TGC CCG TGC CTG GAG TC	RT-PCR
hSR-BI-1680 R	GCT GTC TGC TGG GAG AGT C	RT-PCR
mSR-BI-313F	CTC AAG AAT GTC CGC ATA GAC C	Real-time PCR
mSR-BI-615R	GAC CAA GAT GTT AGG CAG TAC AAT G	Real-time PCR
Bio-GR F	Biotin - GAC CCT AGA GGA TCT GTA CAG GAT GTT CTA GAT	DNAP
Bio-GR mut F	Biotin - GAC CCT AGA GGA TCT CAA CAG GAT CAT CTA GAT	DNAP
GR R	ATC TAG AAC ATC CTG TAC AGA TCC TCT AGG GTC	DNAP
GRmut R	ATC TAG ATG ATC CTG TTG AGA TCC TCT AGG GTC	DNAP
Bio-hSR-BI-1491 F	Biotin - CTG GAG ATA CAT CGA TAA GCA AAA C	DNAP
Bio-hSR-BI-944 F	Biotin - CCA CCT CCC AGT TTT CTT ATT GTA	DNAP
Bio-hSR-BI-620 F	Biotin - CTG TGC AGG GTG TAT GGA GGC CC	DNAP
Bio-hSR-BI-290 F	Biotin - CCC ATA GAC GTT TTG GCT CAG	DNAP
hSR-BI-949 R	CGC CTG TAA TCC CAG CAC TTT GG	DNAP
hSR-BI-620 R	GCA ATT CTG TTC TCC CAG GTA TCC	DNAP
hSR-BI-292 R	CCT GGT GCC CCT CAC CCT CTT G	DNAP - ChIP
hSR-BI+78 R	CGG CGA CAG AGA CGA CAC AGG CGG GG	DNAP - ChIP
hSR-BI-673 F	CAC CAG CGT TAC GAA GGA TAC	ChIP
hSR-BI-290 F	CCC ATA GAC GTT TTG GCT CAG	ChIP
SLC19A2-252 F	GCA TTC CCA ACA GAT GAG C	ChIP
SLC19A2-135 R	GGA GGA CAT GTG GAA CTC C	ChIP

plasmid −62/+78 was generated by digestion of the SR-BI promoter plasmid −1288/+78 with *Sma*I. The sequences of all oligonucleotides used as primers in PCR amplification are shown in Table 1.

Cell culture and treatments

Human embryonic kidney cells HEK293T, human ovarian cells SKOV-3 and 911 cells were cultured in DMEM supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin at 37 C in a 5% CO_2 atmosphere. The human adrenocortical cells H295R were grown at 37 C under a humidified 5% CO_2 atmosphere in a 1:1 mixture of DMEM Ham's F12 medium containing 15 mM HEPES, 10 μg/ml ITS, 10 nmol/liter hydrocortisone, 10 nmol/liter β-estradiol, 2% FBS, and 1% P/S. For treatment of cells with dexamethasone and RU486, DMEM F-12 without phenol red and serum-free was used, and ligands were added for 24 h at a final concentration of 1 μM.

Transient cell transfections, siRNA silencing, and infections with recombinant adenoviruses

Transient transfections in HEK293T cells and SKOV-3 cells were performed using the $Ca_3(PO_4)_2$ coprecipitation method using 6 μg of DNA/well in six-well plates. Transient transfections in H295R cells were performed with the lipofectamine 2000 reagent according to the manufacturer's instructions using 2 μg of DNA/well in six-well plates. siRNA silencing experiments were carried out with the lipofectamine 2000 reagent according to the manufacturer's instructions using 200 pmol/ml or 250 pmol/ml in six-well plates. AdGFP and AdhGRα adenoviruses were amplified and titrated in 911 cells as described previously (38) and adenoviral infections of H295R cells using multiplicity of infection (MOI) = 10 were performed as previously described (39). Luciferase assays were performed using the luciferase assay kit from Promega Corp. according to the manufacturer's instructions. Normalization for transfection efficiency was performed by β-galactosidase assays.

Quantitative real-time RT-PCR analysis of RNA isolated from mouse adrenals

Total RNA from mouse adrenals was reverse transcribed, and 25 ng of resultant cDNA were used per 25 μl reaction. Quantitative real-time RT-PCR was performed on a Stratagene MX3000 and analyzed by using the $\Delta\Delta$Ct (cycle threshold) method. Each cycle consisted of 30 sec at 95 C, 45 sec at 58 C, and 30 sec at 72 C for a maximum of 35 cycles. Values were normalized to those obtained for the housekeeping glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene. Each PCR

was carried out in triplicates. The primers used for the amplification of the SR-BI and *GAPDH* genes are shown in Table 1.

RT-PCR analysis of RNA isolated from tissue culture cells

Total RNA extraction was carried out as described previously (40). Twenty-five nanograms of the resulting cDNA were used for PCR analysis. The program for PCR analysis for SR-BI was as follows: initial denaturation at 95 C for 3 min; 24 cycles at 95 C for 15 sec, 56 C for 30 sec, 72 C for 90 sec; a final extension step at 72 C for 5 min. For the normalization of the samples, the cDNA of the housekeeping *GAPDH* gene was also amplified by PCR. Quantitation of PCR products was carried out using TINA Scan program. The primers used for the amplification of the SR-BI and GAPDH cDNAs are shown in Table 1.

DNA affinity precipitation

Nuclei from HEK293T cells that had been transfected with pcDNA3-hGRα were washed with ice-cold 1× PBS and collected by centrifugation at 4000 rpm at 4 C for 10 min and resuspended in hypotonic buffer (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.2 mM EDTA) supplemented with protease inhibitors. After a 10-min incubation on ice nuclei were homogenized 20 times with a 27-gauge syringe and centrifuged at 6000 rpm at 4 C for 15 min. Nuclei were resuspended in a low-salt buffer (20 mM HEPES, pH 7.9; 25% glycerol; 1.5 mM $MgCl_2$; 0.02 M KCl; 0.2 mM EDTA) and a high-salt buffer (20 mM HEPES, pH 7.9; 25% glycerol; 1.5 mM $MgCl_2$; 1.2 M KCl; 0.2 mM EDTA) supplemented with protease inhibitors and rotated on a rotating platform at 4 C for 30 min. Nuclei were purified by centrifugation at 13,000 rpm at 4 C for 40 min. Dynabeads were washed once with 1× B&W buffer (5 mM Tris/HCl, pH 7.5; 0.5 mM EDTA; and 1 mM NaCl), mixed with 0.58 μM of biotinylated oligonucleotide and incubated at room temperature (25 C) for 15 min. The oligonucleotide-coupled beads were washed twice with 1× B&W buffer and once with D buffer (20 mM HEPES, pH 7.9; 10% glycerol; 40 mM KCl; 0.5 mM dithiothreitol). The protein-DNA binding reactions were allowed to proceed for 15 min on ice. Each reaction mixture included 50 μg of nuclear protein extracts, 3 μg of competitor poly(deoxyinosine/deoxycytosine) and the biotinylated oligonucleotide-coupled Dynabeads, or uncoupled Dynabeads as controls in a total reaction volume of 50 μl. All oligonucleotides used for DNA affinity precipitation are shown in Table 1. hGRα bound to the oligonucleotides was detected by SDS-PAGE and immunoblotting using anti-hGRα antibody.

Chromatin immunoprecipitation assay

H295R cells were grown in P-100 plates and treated with dexamethasone (1 μM) for 24 h before cell collection, and chromatin immunoprecipitation assay was performed as described previously (40) using anti-hGRα antibody. Immunoprecipitated chromatin was analyzed by PCR using primers shown in Table 1. PCR products were analyzed by agarose gel electrophoresis.

Western blotting

Cell lysates were collected, subjected to SDS-PAGE, and transferred to nitrocellulose membranes (Life Sciences and Schleichers & Schuell), with a Bio-Rad Protean electroblot apparatus. Electrophoresis was performed on 10.5% polyacrylamide gel electrophoresis in 500 ml 1× TGS (1 liter 10× TGS: 30.3 g Tris; 144.2 g glycine; 10 g sodium dodecyl sulfate, pH 8.3). Nitrocellulose membranes were washed with TBS-T (TBS + 0.1% Tween 20) for 10 min, at room temperature. Nonspecific sites were blocked by soaking the membrane in TBB buffer (1× TBS + 5% nonfat milk, 0.1% Tween 20) for 1 h at 4 C. Western blotting was performed with a 1:200 dilution of the anti-hGRα, a 1:2500 dilution of the anti-SR-BI, a 1:1000 anti-GFP, and a 1:5000 anti-actin antibodies in TBB overnight at 4 C. The membranes were washed three times with TBS-T, for 10 min, at room temperature. As a secondary antibody we used antimouse or antirabbit horseradish peroxidase-conjugated, in a 1:10,000 dilution in TBS-T, for 1 h at room temperature. After three washes of 10 min with TBS-T at room temperature, bands were visualized by enhanced chemiluminescent detection on Fuji medical x-ray film (Super RX).

Statistics

In all animal experiments, each group consisted of at least three mice, and each individual experiment was performed at least twice.

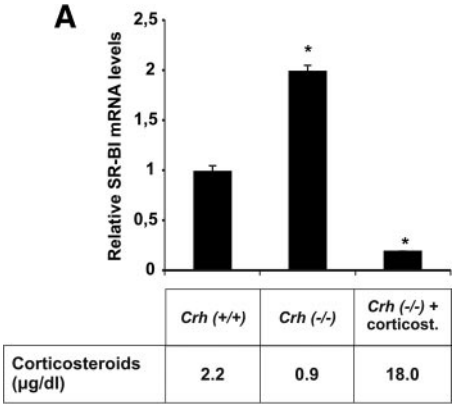

FIG. 1. Inhibition of human *SR-BI* gene expression by glucocorticoid in mouse adrenals. A, Corticosterone (10 μg/ml) or vehicle was administered to *Crh* (−/−) mice in drinking water for 3 d. Total RNA from these mice as well as from *Crh* (+/+) mice was isolated and reverse transcribed. B, *Crh* (+/+) mice were injected intraperitoneally with 200 mg/Kg of metyrapone or 10% dimethylsulfoxide twice a day for 4 d. On the fourth day, mice were injected twice with a combination of metyrapone with 100 mg/Kg aminoglutethimide. Quantitative real-time PCR was carried out as described in *Materials and Methods*. Plasma corticosterone levels were measured using the ^{125}I RIA kit (RIA). Each value is the mean of three different mice of a representative experiment. Statistics were performed as described in *Materials and Methods*. *, $P < 0.05$.

Data were analyzed by ANOVA, followed by Scheffé's and Fisher's least significant difference post hoc multiple comparison tests. For all analyses, $P < 0.05$ was considered significant.

Results

Glucocorticoid down-regulates *SR-BI* gene expression in adrenal cells

To examine the negative effect of glucocorticoid on the regulation of *SR-BI* gene expression in steroidogenic tissues *in vivo*, we used CRH (*Crh*) knockout mice ($Crh^{-/-}$ mice). Corticosterone production in $Crh^{-/-}$ mice was markedly disturbed as reported previously (Refs. 36 and 41–43 and Fig. 1A). Corticosterone insufficiency was associated with a 2-fold increase in the mRNA levels of the *SR-BI* gene in the adrenals of $Crh^{-/-}$ mice, whereas administration of exogenous corticosterone in these mice drastically decreased adrenal SR-BI mRNA levels suggesting that glucocorticoid plays an inhibitory role on *SR-BI* gene expression in adrenals (Fig. 1A).

To corroborate our findings regarding the negative effect of corticosteroids on *SR-BI* gene regulation, we treated wild-type mice with metyrapone. Metyrapone blocks corticosterone synthesis by inhibiting the activity of the enzyme steroid 11β-hydroxylase and is used for the treatment of patients with Cushing's syndrome, which is characterized by elevated cortisol levels (44). As shown in Fig. 1B, metyrapone administration to mice caused a 4-fold decrease in blood corticosterone levels, and this was associated with a 2.5-fold up-regulation of *SR-BI* gene expression in adrenal cells, in agreement with the findings of Fig. 1A.

Ligand-activated GR inhibited SR-BI promoter activity in adrenal and ovarian cells

To validate the negative role of glucocorticoid in *SR-BI* gene expression in adrenal cells, we performed experi-

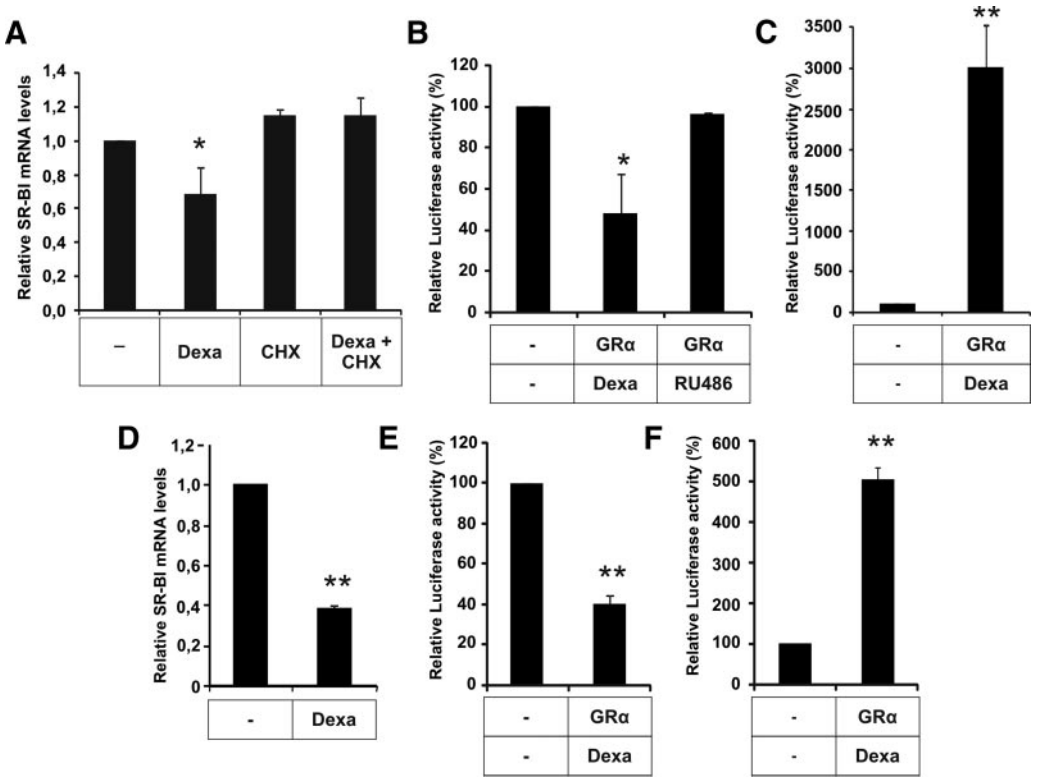

FIG. 2. Ligand-activated GR inhibits the transcriptional activity of the SR-BI promoter in adrenocortical H295R and ovarian adenocarcinoma SKOV-3 cells. A, Inhibition of human *SR-BI* gene expression by glucocorticoid in adrenal cells requires *de novo* protein synthesis. Human adrenocortical H295R cells were treated with 1 μM dexamethasone and/or 10 mg/ml cycloheximide for 2 h. Total RNA was isolated and analyzed by RT-PCR as described in *Materials and Methods*. Values were normalized to those obtained for the housekeeping *GAPDH* gene. The primers used for the amplification of the *SR-BI* and *GAPDH* genes are shown in Table 1. B, H295R cells were transiently transfected with the −1288/+78 SR-BI-Luc reporter (1 μg), along with an expression vector for the GR α (1 μg) in the presence of dexamethasone (Dexa, 1 μM), mifepristone (RU486, 1 μM), or the vehicle only (−). Ligands were added for 24 h before cell collection. C, H295R cells were transiently transfected with the GRE-Luc reporter (1 μg) along with an expression vector for the GR α (1 μg) in the presence of dexamethasone (Dexa, 1 μM) or the vehicle only (−). Ligands were added for 24 h before cell collection. D, SKOV-3 cells were treated with 10 μM dexamethasone for 2 h. Total RNA was isolated and analyzed by RT-PCR. Values were normalized to those obtained for the housekeeping *GAPDH* gene. E and F, SKOV-3 cells were transiently transfected with the full-length −1288/+78 SR-BI-Luc reporter (1 μg; E) or the GRE-Luc reporter (1 μg; F) along with the expression vector for hGRα (1 μg) in the presence of dexamethasone (1 μM). The relative luciferase activity (±SEM) calculated from at least three independent experiments performed in duplicate, is shown in the histogram. *, $P < 0.05$; **, $P < 0.001$ by one-way ANOVA.

ments in the human adrenocortical H295R cell line (45). As shown in Fig. 2A, administration of dexamethasone (dexa) to H295R cells for 2 h caused a 37% reduction in SR-BI mRNA levels. This reduction was fully blocked by cycloheximide, a protein synthesis inhibitor suggesting that inhibition of *SR-BI* gene expression by glucocorticoid requires *de novo* protein synthesis.

We next examined the effect of glucocorticoid on the activity of the human SR-BI promoter. For this purpose, we cotransfected H295R cells with the full-length −1288/+78- SR-BI luc reporter vector along with an expression vector for GRα in the presence of dexamethasone. As shown in Fig. 2B, GRα in the presence of dexamethasone strongly inhibited the activity of the −1288/+78 SR-BI promoter to 45% relative to the untreated promoter in H295R cells. In contrast, the activity of the SR-BI promoter was not repressed in the presence of mifepristone (RU486), a nonselective GR antagonist (Ref. 46 and Fig. 2B). As a control, we used a reporter plasmid bearing the luciferase gene under the control of a single glucocorticoid response element (GRE-Luc) (47) and showed that GRα in the presence of dexamethasone induced the activity of this promoter 29-fold in H295R cells (Fig. 2C).

The effect of glucocorticoid on *SR-BI* gene expression and promoter activity was also examined in a different steroidogenic cell type, the ovarian adenocarcinoma SKOV-3 cells (48). First, we showed that dexamethasone reduced the SR-BI mRNA levels in SKOV-3 cells by approximately 60% relative to untreated cells (Fig. 2D) and that GRα in the presence of dexamethasone inhibited the activity of the −1288/+78 SR-BI promoter by 60% (Fig. 2E). In a control experiment, ligand-activated GRα strongly induced fivefold the activity of the GRE-Luc reporter in SKOV-3 cells (Fig. 2F).

A recombinant adenovirus expressing human GRα suppressed *SR-BI* gene expression in H295R cells

We used adenovirus-mediated gene transfer technology to investigate further the role of the GR in *SR-BI* gene expression. To achieve this goal, we generated a recombinant adenovirus expressing human GRα. Using antibodies against human GRα and green fluorescent protein

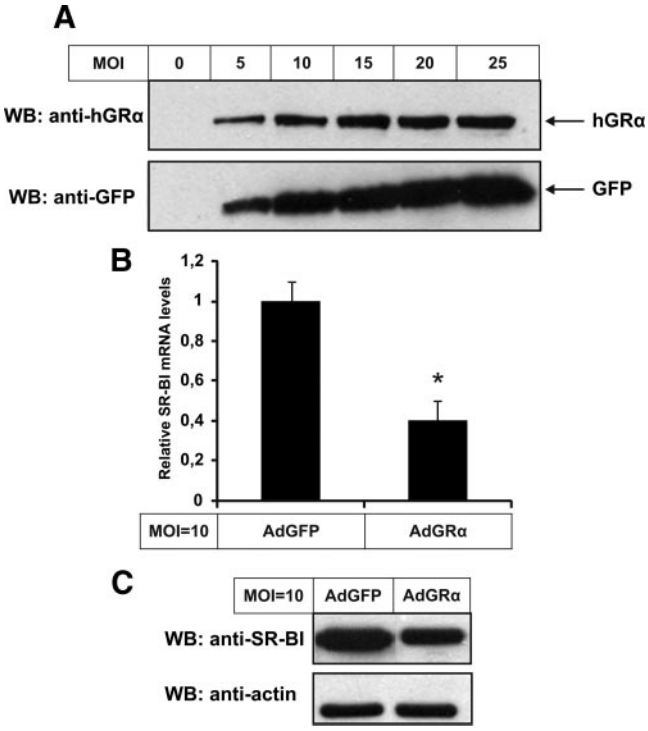

FIG. 3. A recombinant adenovirus expressing human GRα suppressed *SR-BI* gene expression in H295R cells. A, H295R cells were infected with a recombinant adenovirus expressing the human GR (AdGRa) with increasing multiplicity of infection (MOI) values. Equal amounts of whole cell extracts from the infected cells were subjected to SDS-PAGE and immunoblotting using antibodies against human GRα and GFP. B and C, H295R cells were infected with a control adenovirus expressing the green fluorescent protein (AdGFP) or with an adenovirus expressing AdGRα (MOI =10). SR-BI mRNA levels (B) were quantitated by RT-PCR, normalized relative to the housekeeping gene GAPDH and plotted. Protein SR-BI levels (C) were detected by immunoblotting using a polyclonal anti-SR-BI antibody. The endogenous levels of β actin were used for normalization of the protein extracts. *, $P < 0.05$ by one-way ANOVA.

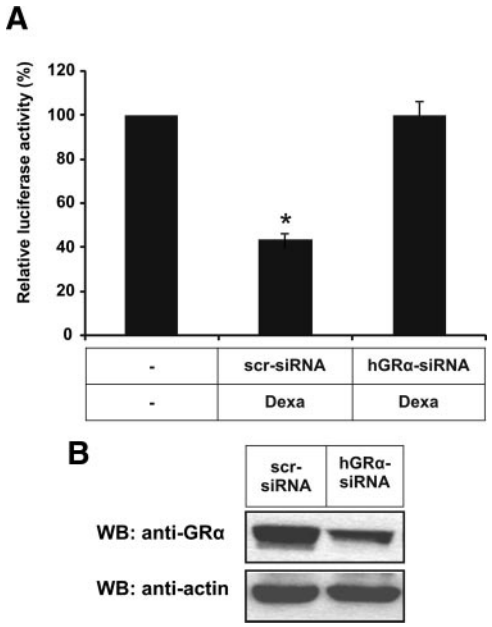

FIG. 4. The GR is required for the inhibition of the SR-BI promoter by glucocorticoid. A, H295R cells were transiently transfected with the −1288/+78 SR-BI-Luc reporter (1 μg) in the presence of a scrambled siRNA or a GR-specific siRNA (200 pmol) and treated with 1 μM dexamethasone for 24 h before cell harvesting. The relative luciferase activity (±SEM) calculated from at least three independent experiments performed in duplicates is shown in the histogram. B, Human embryonic kidney HEK293T cells were transiently transfected with pcDNA3hGRα vector in the presence of a scrambled siRNA or a GR-specific siRNA (200 pmol). Whole cell extracts from the transfected cells were subjected to SDS-PAGE and immunoblotting for the detection of human GR gene and β actin using the corresponding antisera. *, $P < 0.05$ by one-way ANOVA.

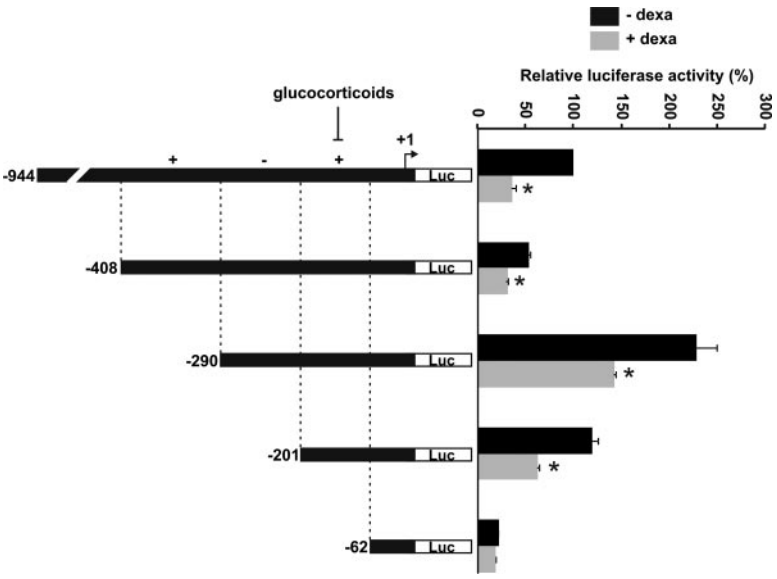

FIG. 5. The proximal region of the human SR-BI promoter is sufficient to mediate repression by glucocorticoid in H295R adrenal cells. H295R cells were transiently transfected with the indicated reporter plasmids bearing the luciferase gene under the control of different SR-BI promoter fragments along with the CMV-β-gal plasmid expressing β-galactosidase (1 μg) for normalization of transfection in the absence (black bars) or in the presence (gray bars) of dexamethasone (1 μM). The relative luciferase activity (±SEM) calculated from at least three independent experiments performed in duplicate is shown in the histogram. SR-BI promoter regions that affect the activity in a positive or a negative manner are indicated by (+) and (−), respectively. The region of the SR-BI promoter that mediates inhibition by glucocorticoid is indicated. *, $P < 0.05$ by one-way ANOVA.

in immunoblotting experiments we showed that H295R cells were productively infected by the AdGRα adenovirus in a dose-dependent manner (Fig. 3A). We then showed that overexpression of human GRα in H295R cells via AdGRα was associated with a significant reduction in the SR-BI mRNA (Fig. 3B) and protein (Fig. 3C) levels compared with a control adenovirus expressing only the green fluorescent protein (AdGFP). These data provided additional evidence for the negative role of GRα in *SR-BI* gene expression in adrenal cells.

The GR is required for the inhibition of the SR-BI promoter by glucocorticoid

To investigate further the role of the GR in the inhibition of SR-BI promoter activity by glucocorticoid in adrenal cells, we used siRNA-mediated gene silencing technology. For this purpose, we transfected dexamethasone-treated H295R cells with the −1288/+78 SR-BI-Luc reporter in the presence of a scrambled siRNA or a GRα-specific siRNA. We found that silencing of GRα by the siRNA was associated with a 2.5-fold increase in the activity of the SR-BI promoter, suggesting that endogenous GR plays an inhibitory role in *SR-BI* gene expression in adrenal cells (Fig. 4A). Figure 4B shows by immunoblotting the silencing of the *GRα* gene by the GRα-siRNA in HEK293T cells that had been transiently cotransfected with the pcDNA3hGRα vector. siRNA-mediated silencing was not so efficient because of the high levels of expression of exogenous GRα in these cells.

Functional analysis of the human SR-BI promoter in H295R cells

To understand better the mechanism of *SR-BI* gene regulation by glucocorticoid in adrenal cells, we performed a structure-function analysis of the human SR-BI promoter. For this purpose, we generated a series of 5′ deletions of the SR-BI promoter (−944, −408, −290, −201, and −62) and cloned them upstream of the luciferase reporter gene (Fig. 5). These reporter plasmids were transiently transfected into dexamethasone-treated or untreated H295R cells, and their relative activity was scored by luciferase assays. This analysis revealed that the activity of the human SR-BI promoter in adrenal cells is determined by an interplay between positive and negative regulatory regions. More specifically, we identified positive regulatory regions between nucleotides −944/−408, −290/−201, and −201/−62 and a strongly negative regulatory region defined by nucleotides −408/−290. We also showed that the minimal region of the SR-BI promoter that is required for repression by glucocorticoid is the region between nucleotides −201 and −62.

The GR does not bind to the SR-BI promoter

We then performed protein–DNA interaction assays to identify potential GRα binding sites in the SR-BI promoter. First, we used the DNA affinity precipitation assay and for this purpose we generated a series of nonoverlapping biotinylated PCR fragments covering the entire SR-BI promoter between nucleotides −1491 to +78. More specifically, the amplified regions, which are shown schematically in Fig. 6A, were as follows: −1491/−949, −944/−620, −620/−292, and −290/+78. As shown in Fig. 6B, no interaction of GRα could be detected with the biotinylated proximal SR-BI promoter fragment −290/+78 which was shown to be sufficient to mediate repression by glucocorticoid (Fig. 5). Binding of GRα to the other three biotinylated PCR fragments could be observed, but this binding was very weak compared with the binding of GRα to a control biotinylated double stranded oligonucleotide containing a well-characterized GRE (BioGR) (49). No binding of GRα was observed to a negative control oligonucleotide bearing a mutated GRE (BioGRmut).

We then performed chromatin immunoprecipitation assays in dexamethasone-treated H295R cells and found

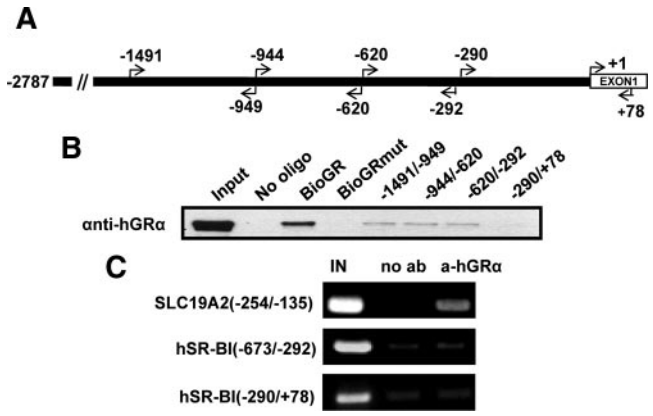

FIG. 6. The GR does not bind to the SR-BI promoter *in vitro* and *in vivo*. A, Schematic representation of the SR-BI 5′ upstream region showing the nonoverlapping biotinylated PCR fragments that were used for the *in vitro* DNA affinity precipitation assays of B. B, DNA affinity precipitation experiment was carried out in HEK293T cells transiently transfected with the expression vector for hGRα. As controls, a biotinylated double stranded oligonucleotide bearing one copy of a consensus glucocorticoid response element (BioGR) and one bearing a mutated element (BioGRmut) were used. Streptavidin agarose beads were coupled to biotinylated control oligonucleotides or the biotinylated PCR fragments and incubated with the cells lysates as described in *Materials and Methods*. C, Chromatin immunoprecipitation analysis in H295R cells treated with 1 μM dexamethasone for 2 h. Immunoprecipitations were performed using the anti-hGRα antibody or no antibody as a control (no ab). Primers that amplify the distal −673/−292 or the proximal −290/+78 SR-BI promoter region or the −254/−135 promoter region of the solute carrier family member 2, SLC19A2 (positive control) were used for PCR analysis. All primers used are shown in Table 1.

no recruitment of endogenous GRα to the proximal −290/+78 or the distal −673/−292 SR-BI promoter (Fig. 6C). As a control, we used primers that amplify the promoter of the solute carrier family member 2 (SLC19A2) shown previously to bind GR *in vivo* (Ref. 50 and Fig. 6C). Hence, these findings support an indirect mechanism of suppression of *SR-BI* gene transcription by glucocorticoid in adrenal cells.

Discussion

Physiological significance of *SR-BI* gene regulation by glucocorticoid in steroidogenic tissues

Work from several laboratories during the last two decades has established that plasma lipoproteins are the major source of cholesterol for the production of steroid hormones in steroidogenic tissues such as adrenal, ovary, and testis (51, 52). Lipoprotein-derived cholesterol can be delivered through the use of a specialized pathway known as the selective cholesteryl ester uptake (1). A key player in this pathway is SR-BI, which interacts with apolipoprotein A-I present in HDL to mediate the uptake process (32, 52–56). Analysis of mice with targeted disruption of the *SR-BI* gene established the important role of this membrane receptor in adrenocortical cholesterol metabolism. The SR-BI KO mice are characterized by depletion of cholesteryl ester stores in the adrenals and the ovaries (57). Similar disturbances in adrenal cholesterol metabolism were observed in mice lacking the *apolipoprotein A-I* and the *LCAT* genes, which are important players in plasma HDL biogenesis and remodeling (30, 58, 59). Importantly, these mice exhibit elevated levels of *SR-BI* gene expression in the adrenals, possibly as a compensatory mechanism for the reduced plasma HDL and the diminished HDL/SR-BI-mediated cholesteryl ester uptake (30, 58, 59).

In rat adrenals, ACTH induces cholesteryl ester uptake (60). Adrenals from mice lacking proopiomelanocortin are characterized by deficiency in CE and attenuated levels of key proteins involved in selective CE uptake and steroidogenesis such as SR-BI, whereas adrenal CE levels and *SR-BI* gene expression were restored with ACTH treatment (61). Thus, there seems to be a good correlation between the induction of cholesteryl ester uptake, *SR-BI* gene expression, and steroidogenesis in adrenal cells. Additional studies suggest that ACTH can stimulate *SR-BI* gene expression by interacting directly with adrenal cells (31). Although glucocorticoid may have an indirect effect on the expression of *SR-BI* gene in steroidogenic cells, our present data support for the first time a direct inhibition of *SR-BI* gene transcription by glucocorticoid in adrenal and ovarian cells and that this mechanism requires the endogenous GR.

GR acting as a transcriptional repressor

The GR regulates gene transcription in response to glucocorticoid by associating with GREs present on the promoters of target genes (62). Alternatively, GR may also regulate transcription through physical interactions with other transcription factors by influencing their ability to stimulate or inhibit the transcription rates of respective target genes that do not contain GREs. Examples are the inhibitory interactions of GR with nuclear factor κB, activator protein 1, and signal transducers and activators of transcription, which facilitate the suppression of immune function and inflammation by glucocorticoid (63–65). The ability of GR to repress gene transcription in response to glucocorticoid was also revealed by gene expression profiling experiments. Thus, it was shown by microarray analysis in dexamethasone-treated A549 adenocarcinoma cells that 108 genes were activated and 73 genes were repressed by GRα (50).

In the present study, we showed that inhibition of *SR-BI* gene transcription by glucocorticoid requires GR but is dependent on *de novo* protein synthesis. This was demonstrated by treating the cells with the cell cycle inhibitor cycloheximide. Using a similar approach, Rogatsky *et al.* (66) had identified novel genes that are

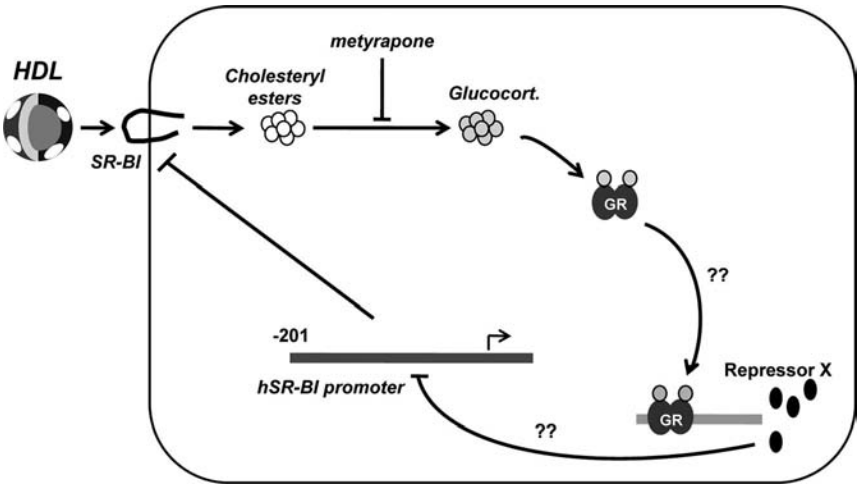

FIG. 7. Proposed mechanism of inhibition of human *SR-BI* gene expression by glucocorticoid via GR in adrenal cells. See text for details.

direct targets of glucocorticoids in osteosarcoma and epithelial lung carcinoma cells. This finding combined with the observation that GR does not bind to the SR-BI promoter *in vitro* and *in vivo* prompted us to postulate an indirect mechanism of *SR-BI* gene repression by GR/glucocorticoid (Fig. 7). According to our hypothesis, cholesteryl ester uptake by adrenal or ovarian cells via SR-BI leads to cholesterol accumulation that serves as a substrate for steroid biosynthetic enzymes leading to the increase in intracellular glucocorticoid concentration. GR activation by glucocorticoid is associated with an indirect inhibition of *SR-BI* gene transcription presumably via activation of a transcriptional repressor. This repressor could subsequently bind to the proximal region of the human SR-BI promoter between nucleotides −201 and −62 and repress its activity (Fig. 7).

A closer examination of the sequence of the −201/−88 region of the human SR-BI promoter revealed the presence of previously characterized or putative regulatory elements that could serve as binding sites for transcriptional activators or inhibitors (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org/). More specifically, this region contains five binding sites for the ubiquitous zinc finger transcription factor Sp1 characterized previously in the rat promoter (67) as well as a putative binding site for Nuclear factor Y (NF-Y) both of which are activators of gene transcription. In fact, deletion of the SR-BI promoter region between nucleotides −201 and −62 was associated with a significant reduction in basal SR-BI promoter activity in adrenal H295R cells (Fig. 6), suggesting that these factors may play an essential role on *SR-BI* gene expression in adrenal tissue. Interestingly, the same region contains several putative binding sites for transcriptional repressors including the zinc finger factor 219 (ZNF219), zinc finger 9 (ZF9), and the Kruppel-like factor 15 (KLF15) (Supplemental Fig. 1). Among these factors, KLF15 deserves special attention because the gene encoding for this transcriptional repressor was recently identified as a glucocorticoid-responsive gene in cardiac cells in a microarray study (68). In another study, it was shown that the *KLF15* gene was induced by dexamethasone in cultured rat hepatocytes (69). Thus, we are tempted to speculate that enhanced glucocorticoid biosynthesis in adrenal cells in response to increase cholesteryl ester uptake leads to activation of GR that binds to the promoter of the *KLF15* gene thus activating its transcription. KLF15 that is produced by this glucocorticoid response binds to the proximal region of the human SR-BI promoter thus repressing its activity but this model needs to be validated by additional experimentation.

In conclusion, the present study showed that gene encoding for the HDL receptor SR-BI is subject to feedback inhibition by glucocorticoid in adrenal and ovarian cells, which depend heavily on this receptor for their supply of cholesterol to be used for steroid hormone biosynthesis. This study establishes that the *SR-BI* gene is an indirect target of the GR and provides insight into a novel mechanism of transcriptional repression, which is different from the well-characterized tethering mechanism used by this receptor to inhibit the activity of inflammatory transcription factors such as nuclear factor-κB and activator protein 1.

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Address all correspondence and requests for reprints to: Dimitris Kardassis, Laboratory of Biochemistry, University of Crete Medical School, Vassilika Vouton, Heraklion 71003, Greece. E-mail: kardasis@imbb.forth.gr.

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Disclosure Summary: The authors have nothing to declare.

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