SREBP-1 Mediates Activation of the Low Density Lipoprotein Receptor Promoter by Insulin and Insulin-like Growth Factor-I*

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Transcription of the low density lipoprotein (LDL) receptor gene is regulated by intracellular cholesterol concentration, hormones, and growth factors. We studied the mechanisms by which insulin and estradiol stimulate promoter activity of the LDL receptor gene. Hormonal effects were analyzed in HepG2 cells after transient transfection with reporter gene constructs. Successive 5' deletions of the LDL receptor promoter fragment from -537 to +88 revealed the sterol regulatory element 1 (SRE-1) between -65 and -56 as an insulin- and estradiol-sensitive cis-element. If the SRE-1 is point mutated at position -59 (C to G), which abolishes the binding of the SRE binding proteins (SREBP-1 and SREBP-2), no insulin or estradiol stimulatory effect on reporter gene expression was observed, indicating a role of SRE binding proteins in this regulatory mechanism. The concentration of the 125-kDa membrane-integrated SREBP-1 precursor protein in LDL repressed HepG2 cells is not altered by hormone treatment. Concentrations of SREBP-1 mRNA and precursor protein are reduced significantly by high and stable expression of an SREBP-1 antisense cDNA fragment in HepG2 cells (SREBP1(-) cells). Transfection of SREBP1(-) cells with promoter construct pHDL4 (-105 to +88) reduces induction of reporter gene activity by insulin and insulin-like growth factor-I to 35 and 17% respectively, compared with HepG2 cells. The stimulatory effect of estradiol remains unchanged, and the inductions by pravastatin are enlarged. We conclude that different regulatory effects converge at SRE-1, but that SREBP-1 is selectively involved in the signal transduction pathway of insulin and insulin-like growth factor-I leading to LDL receptor gene activation.

Expression of the LDL receptor is mainly regulated at the transcriptional level by cellular cholesterol concentration (1). The regulatory cellular cholesterol pool is represented by the concentration of oxysterols, which are regulatory active substances repressing the transcription rate of the LDL receptor. The regulatory cis-element in the promoter of these genes is called SRE-1 (sterol regulatory element 1) (2, 3).

Recently, two SRE binding proteins (SREBP-1 and -2) have been characterized (4–7). They are activated by a novel proteolytic mechanism that controls the concentration of the SRE binding proteins and the transcription rate of the LDL receptor gene. SREBP-1 is synthesized as a 125-kDa precursor protein that is embedded in the membranes of the endoplasmic reticulum and nuclear envelope. The amino-terminal segment of SREBP-1 contains an acidic transcriptional activation domain and a basic helix-loop-helix leucine zipper (bHLH-Zip) region that mediates protein dimerization and DNA binding. In the absence of oxysterols a protease cleaves the precursor protein, and the amino-terminal fragment enters the nucleus and activates transcription. When sterols accumulate within the cells the activity of the protease diminishes, and the active transcription factor is no longer generated.

LDL activity is regulated by different hormones and growth factors in different cells. Insulin (8, 9) and estradiol (10, 11) increase LDL receptor activity at the cell surface. This seems to be a result of enhanced gene transcription (12, 13). We have focused on the characterization of the still unknown mechanisms by which these hormones stimulate the transcription of the LDL receptor gene.

EXPERIMENTAL PROCEDURES

Promoter Reporter Gene Constructs—A promoter fragment of the human LDL receptor gene 5'-untranslated region from -537 to +88 relative to the transcription start point was amplified from human DNA by PCR using oligonucleotides pr1 (attctct tgtacagttcattag) and pr2 (caggctctggaccagcttgccga). The fragment was subcloned into the Smal site of pUC 13 to generate pHDLR, in which the S' end of the promoter fragment is in proximity to the SaeI restriction site within the polylinker. Several 5' restricted and mutated constructs were obtained from this parental clone by PCR amplification using the universal primer pr3 (gggggaggctcaactactgcccc) and different promoter-specific primers with a normal or mutated sequence. After religation and propagation in bacteria the promoter fragments of all constructs were subcloned in sense orientation into the SmaI and PvuII sites of the pGlBasic vector (Promega).

The following is a list of generated constructs, subcloned promoter regions, and used primers: pHDL1 (-537 to +88), subcloned from pHDLR; pHDL2 (-222 to +88), pr4 (ctctctctctctctctctctc)/pr3; pHDL3 (-367 to +88), pr5 (aacaactctctctctctctc)/pr3; pHDL4 (-105 to +88), pr6 (gacaactctctctctctc)/pr3; pHDL5 (-69 to +88), pr7 (gagaactctctctctctctctctc)/pr3; pHDL6 (-35 to +88), pr8 (gagaactctctctctctctc)/pr3; pHDL7 (-105 to +88, C → G at position -59), pr9 (gagaactctctctctctctctctc)/pr10 (aatgcgcttcactactgcccc)/pr10. The constructs were subcloned in sense orientation into the SaeI and HindIII sites of the pGlBasic vector (Promega).

The following is a list of generated constructs, subcloned promoter regions, and used primers: pHDL1 (-537 to +88), subcloned from pHDLR; pHDL2 (-222 to +88), pr4 (ctctctctctctctctctctc)/pr3; pHDL3 (-367 to +88), pr5 (aacaactctctctctctctc)/pr3; pHDL4 (-105 to +88), pr6 (gacaactctctctctctc)/pr3; pHDL5 (-69 to +88), pr7 (gagaactctctctctctctctctc)/pr3; pHDL6 (-35 to +88), pr8 (gagaactctctctctctctc)/pr3; pHDL7 (-105 to +88, C → G at position -59), pr9 (gagaactctctctctctctctctc)/pr10 (aatgcgcttcactactgcccc)/pr10. The constructs were subcloned in sense orientation into the SaeI and HindIII sites of the pGlBasic vector (Promega).
Stable Transfection of HepG2 Cells with pcSREBP1—HepG2 cells were transiently transfected with pcSREBP1 DNA labeling kit (Boehringer Mannheim) using 50 μCi of [α-32P]dCTP (3000 Ci/mmol) and 20 ng of fragment. Specific mRNA levels of the LDL receptor and glyceraldehyde-3-phosphate dehydrogenase as an internal standard were determined by a reverse transcription polymerase chain reaction titration assay (17). Standard RNA was prepared by in vitro transcription using T7 RNA polymerase (AmpliScribe), Biozyme Laboratories, Ltd.) of a human LDL receptor cDNA (18) fragment (base pairs 1055–1424) containing a HindIII cutting site generated by in vitro mutagenesis (19) of C/A (base pair 1376).

Antibodies and Immunoblot Analysis of Nuclear Extracts—Nuclear protein extracts were prepared from HepG2 cells following the method described by Digman et al. (20) according to the modifications of Wang et al. (21).

A polyclonal antibody against SREBP-1 (K10, 1,500, Santa Cruz Biotechnology, Inc.) directed against a peptide sequence from amino acids 470 to 479 and an alkaline phosphatase-coupled anti-rabbit IgG (Boehringer Mannheim), or alternatively the BM chemiluminescence Western blotting kit (Boehringer Mannheim), was used for Western blot analysis of SREBP-1 in the nuclear membrane fractions according to the manufacturer’s instructions. Protein extracts were separated on a 7.5% SDS-PAGE gel containing prestained SDS-PAGE standards high range (Bio-Rad). Protein concentrations were determined by the method of Bradford (22).

RESULTS

Localization of an Insulin- and Estradiol-sensitive Promoter Element—HepG2 cells were transiently transfected with promoter reporter gene constructs, which contain normal and mutated successive 5′-deleted fragments of the LDL receptor untranslated region from −537 to +88 relative to the transcription start point (Fig. 1). Maximal luciferase activity was obtained with the construct phLDL2 (100%). The decrease of promoter activity obtained with the longer constructs (phLDL1, 72% and phLDL3, 39%) and the next shortest one (phLDL4, 46%) was not investigated. Construct phLDL4 contains the promoter region from all known transcriptional active cis-elements. Deletion of the distal SP1 recognition site and further deletion of SRE-1 and the proximal SP1 recognition site, respectively, reduces promoter activity to 35% (phLDL5) and 1% (phLDL6). SRE-1 is essential for maximal promoter activity. A point mutation at −59 (C to G) within the SRE-1, which prevents the binding of SRE binding proteins, and a deletion from −68 to −58 reduces the promoter activity to 12 and 23%, respectively.

To localize the cis-elements in the promoter of the LDL receptor gene responsible for the insulin and estradiol action, HepG2 cells were transfected with the different promoter reporter gene constructs. One day after transfection the normal culture medium was changed to medium containing 0.5% LPDS for 24 h, leading to a high basal expression of the reporter gene. 5 h before harvesting the hormones were added to
the medium. Fig. 2 shows the inducibility of luciferase activity for the different constructs by insulin (100 nM) and estradiol (10 µg/ml) relative to the basal reporter gene expression determined in medium containing 0.5% LPDS alone. Insulin increases luciferase activity about 2-fold in cells transfected with construct phLDL4 or a longer one. In all constructs with a deleted or mutated SRE-1 (phLDL6, phLDL7, and phLDL10) luciferase activity is not stimulated by insulin. Deletion of the distal SP1 recognition site reduces the inducibility only slightly, from 2.0-to 1.6-fold. The results are similar for induction with estradiol, but there is already a stepwise reduction of the inducibility from about 2-fold with construct phLDL2 to 1.6-fold with phLDL4 and to 1.35-fold with phLDL5. A complete and functional SRE-1 is necessary for the stimulation of the LDL receptor gene promoter by insulin and estradiol.

The Role of SREBP-1 in the Stimulation of LDL Receptor Promoter Activity by Hormones—We and others (12) have found that insulin increases the specific mRNA concentration of the LDL receptor gene in the presence of repressing concentrations of LDL cholesterol (Fig. 3). One proposed mechanism is that insulin increases the activity of the oxysterol-repressed protease, which liberates the mature SREBP-1. Enhanced transcription of the LDL receptor gene would be expected as a consequence of this mechanism. To measure the amount of SREBP-1 precursor protein and hormonal effects on its concentration the nuclear membrane fraction of HepG2 cells was extracted by high salt and detergent. Fig. 4 shows a Western analysis of membrane extracts from HepG2 cells. When cells were cultured in 0.5% LPDS there was much less SREBP-1 precursor protein present than after the repression of the proteolytic mechanism by LDL. The addition of insulin and estradiol for 2 h did not alter this level significantly. A Northern analysis of HepG2 cells cultured under the same conditions revealed no hormone-induced changes of SREBP-1 and SREBP-2 mRNA levels (data not shown).

Characterization of SREBP1(−) Cells—To find out if SREBP-1 is involved in the transcriptional activation by hormones and growth factors we reduced the cellular SREBP-1 concentration by expression of an SREBP-1-specific antisense mRNA. HepG2 cells were transfected with construct pcSREBP1−. It contains an SREBP-1 cDNA fragment coding for amino acids 34–178 inserted in antisense orientation in the polylinker of pcDNA3 where it is expressed constitutively by the cytomegalovirus promoter at a high level. After selection in G418-containing medium and propagation of single clones, one clone SREBP1(−) was further characterized. First we compared the specific mRNA concentrations for SREBP-1 and SREBP-2 in SREBP1(−) cells and HepG2 cells by Northern analysis (Fig. 5). Approximately 2 µg of poly(A) mRNA of each cell line were hybridized with specific probes against SREBP-1, SREBP-2, and glyceraldehyde-3-phosphate dehydrogenase to normalize mRNA concentrations. Densitometrical analysis revealed that in SREBP1(−) cells the concentration of SREBP-1 mRNA is reduced to 40% compared with HepG2 cells, whereas
HepG2 cells and SREBP1(2) cells. The expression of SREBP-2 seems not to be the concentration of SREBP-2 remains unaltered in SREBP1(−) cells. The expression of SREBP-2 seems not to be up-regulated as a consequence of reduced SREBP-1 expression. The signals at 4.2 and 5.2 kilobases are proposed to result from different polyadenylation signals of the same gene (7).

To compare the amount of SREBP-1 precursor protein in HepG2 cells and SREBP1(−) cells before and after repression with LDL cholesterol, nuclear protein extracts were analyzed by Western blot (Fig. 6). Cells were harvested after culturing in medium containing 0.5% LPDS for 36 h. The SREBP-1-liberating proteolytic mechanism was repressed by the addition of LDL (250 μg/ml) and 25-hydroxycholesterol (10 μg/ml) 12 and 24 h before harvesting. The accumulation of SREBP-1 precursor protein is several-fold stronger in HepG2 cells than in SREBP1(−) cells after 12 and 24 h of incubation (Fig. 6).

Hormonal Effects on Reporter Gene Expression in SREBP1(−) Cells—The concentration and biosynthesis of SREBP-1 is efficiently reduced in SREBP1(−) cells by the antisense construct. Therefore these cells were used as a system to study the role of SREBP-1 in the transcriptional activation of the LDL receptor gene by hormones. HepG2 cells and SREBP1(−) cells were transiently transfected with constructs pHDL4 and pSVβgal. The normal culture medium was changed to medium containing 0.5% LPDS 1 day after transfection for an additional 24 h, and thereafter the cells were harvested. To study hormonal effects on reporter gene expression the hormones were added 16 h before harvesting to the 0.5% LPDS-containing medium. Basal luciferase activity was more than 60% decreased in SREBP1(−) cells compared with HepG2 cells. This activity was always defined as 1 to compare the effects of added substances. In both cell lines luciferase activity was repressed by LDL to the same degree, whereas the induction by pravastatin was more effective in SREBP1(−) cells than in HepG2 cells, increasing from 1.9-fold to nearly 3-fold. In contrast, the stimulatory effect of insulin (2-fold induction) and IGF-I (3.2-fold induction) on reporter gene activity in HepG2 cells was reduced to 35 and 17%, respectively, in SREBP1(−) cells. The action of estradiol was not affected by the diminished SREBP-1 concentration. Forskolin, an activator of protein kinase A, stimulated luciferase activity 1.7-fold in HepG2 cells and 2.1-fold in SREBP1(−) cells (Fig. 7).

**FIG. 4.** Hormonal effects on the proteolytic mechanism cleaving SREBP-1 precursor protein. Nuclei of HepG2 cells were extracted with detergent and salt as described under “Experimental Procedures,” and proteins were separated on a 7.5% SDS-PAGE. The concentration of SREBP-1 precursor protein was determined in a Western blot. HepG2 cells were cultured in medium containing 0.5% LPDS alone (lane 1), with LDL (250 μg/ml) 12 h after the addition of LDL (250 μg/ml) and 25-hydroxycholesterol (10 μg/ml) 12 and 24 h before harvesting. The accumulation of SREBP-1 precursor protein is severalfold stronger in HepG2 cells than in SREBP1(−) cells after 12 and 24 h of incubation (Fig. 6).

**FIG. 5.** Reduction of SREBP-1 mRNA concentration by antisense mRNA in SREBP1(−) cells. Two μg of poly(A) mRNA from SREBP1(−) cells and from HepG2 cells were hybridized successively with specific cDNA probes against SREBP-1 (4 kilobases) and SREBP-2 (4.2 and 5.2 kilobases). Hybridization with a cDNA probe against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed for normalization. The marker lane is indicated by M. Autoradiographs were quantified densitometrically.

**DISCUSSION**

We showed that reporter gene constructs of the LDL receptor promoter transiently transfected into HepG2 cells are stimulated approximately 2-fold by both insulin and estradiol. Although several insulin-responsive elements have been identified recently (23) there seems to be no unique sequence mediating the insulin effect. The molecular mechanisms that show how steroid hormones activate transcription are understood in more detail (24), but the DNA consensus sequence (25) recognized by estrogen receptors is not present in the promoter region of the LDL receptor gene. It is possible that the estrogen receptor does not bind directly to the DNA.

Analysis of the hormonal effects with 5′-deleted constructs revealed the region between −69 and −36, where the SRE-1 and the proximal SP1 site are located, as the hormone-sensitive promoter sequence. The point mutation (C to G at position −59) in construct pHDL7 within the SRE-1 destroys the recognition...
sequence for SRE binding proteins (3, 4). Insulin and estradiol have no promoter-stimulating effect on this mutated construct. Similar results were obtained with construct phLDL10 in which the SRE-1 is deleted. This construct has a higher promoter activity than phLDL7, which can be explained by the reduced distance of the two SP1 binding sites. We conclude that SRE-1 might be the hormone sensitive cis-element and that binding of the transcription factors SREBP-1 or -2 is involved in the promoter activation by insulin and estradiol.

Insulin increases the LDL receptor mRNA concentration in the presence of repressing concentrations of LDL (12) (see Fig. 3). One possible explanation for such action is that insulin increases the nuclear concentration of SRE binding proteins by activating the proteolytic mechanism that liberates the native SRE binding proteins from their membrane-bound precursor form. We studied the influence of insulin and estradiol on the liberation mechanism of SREBP-1 in LDL-repressed HepG2 cells and found no hormone-induced changes of the SREBP-1 precursor protein pool (Fig. 4) and no change of the mRNA concentrations of SREBP-1 and -2 under these conditions (data not shown). Therefore, it appears that insulin and estradiol do not affect the liberation mechanism of SREBP-1; it is unlikely that a higher SREBP-1 gene expression compensates for this mechanism.

Reduction of SREBP-1 concentration in HepG2 cells enabled us to differentiate between stimulatory effects on LDL receptor promoter activity. High expression of SREBP-1 antisense mRNA in SREBP1(−) cells resulted in a 60% reduction of SREBP-1 mRNA concentration and an even greater decrease in specific protein concentration in the nuclear membrane fraction of LDL cholesterol-repressed HepG2 cells. In transfected SREBP-1-deficient cells only the stimulatory effects of insulin and IGF-I were reduced to 35 and 17%, respectively, whereas the stimulatory effect of estradiol was unchanged and the induction by the cholesterol synthesis inhibitor pravastatin was even increased. However, the reduced SREBP-1 mRNA level in SREBP1(−) cells was not compensated by a higher expression of SREBP-2. The role of SREBP-2 was not determined in greater detail in this study, but one possible explanation is that SREBP-2 plays a more dominant role in SREBP-1-deficient HepG2 cells, e.g. mediating the effect of the cholesterol synthesis inhibitor. In accordance with that explanation, the depletion of sterols in hamster liver by treating animals with a cholesterol synthesis inhibitor and a bile acid-binding resin led to an increase in nuclear SREBP-2 (26).

Although expression and processing of SRE binding proteins might not be similar between cultured cells, including HepG2 and intact liver, these data indicate that SREBP-1 and SREBP-2 couple different intracellular signal transduction pathways to the sterol response promoter element. After our paper was submitted, a report by Lloyd and Thompson (27) appeared in which the in vivo pattern of protein-DNA interactions in the LDL receptor promoter element was investigated. Interestingly, they show that incubation of HepG2 cells with insulin associated with a hypermethylation at position −59 in the SRE of the LDL receptor promoter, indicating insulin-induced alterations in protein binding to the SRE region. We conclude from our results that different regulatory effects converge at SRE-1 but that SREBP-1 is selectively involved in the signal transduction pathway of insulin and IGF-I leading to LDL receptor gene activation.

Fig. 7. Differential role of SREBP-1 in the induction of LDL receptor promoter activity. SREBP1(−) (gray bar) and HepG2 cells (black bar) were transiently transfected with construct phLDL4 and incubated with LDL (250 μg/ml), pravastatin (10−4 M), insulin (10−7 M), IGF-I (10−9 M), estradiol (10−9 M), and forskolin (10−5 M) before harvesting as described under "Experimental Procedures." Bars represent the induction of luciferase activity relative to the values measured in 0.5% LPDS-containing medium alone (No). Values are means of 4–8 separate experiments ± S.D., each done in triplicate. Statistical significances (Student's t test) were calculated for the difference between the effects observed in HepG2 versus SREBP(−) cells for insulin and IGF-I (p < 0.0001) and pravastatin (p < 0.002).
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