Liver growth factor induces testicular regeneration in EDS-treated rats and increases protein levels of class B scavenger receptors

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Liver growth factor induces testicular regeneration in EDS-treated rats and increases protein levels of class B scavenger receptors. Am J Physiol Endocrinol Metab 308: E111–E121, 2015. First published November 11, 2014; doi:10.1152/ajpendo.00329.2014.—The aim of the present work was to determine the effects of liver growth factor (LGF) on the regeneration process of rat testes after chemical castration induced by ethane dimethanesulfonate (EDS) by analyzing some of the most relevant proteins involved in cholesterol metabolism, such as hormone sensitive lipase (HSL), 3β-hydroxysteroid dehydrogenase (3β-HSD), scavenger receptor SR-BI, and other components of the SR family that could contribute to the recovery of steroidogenesis and spermatogenesis in the testis. Sixty male rats were randomized to nontreated (controls) and LGF-treated, EDS-treated, and EDS + LGF-treated groups. Testes were obtained on days 10 (T1), 21 (T2), and 35 (T3) after EDS treatment, embedded in paraffin, and analyzed by immunohistochemistry and Western blot. LGF improved the recovery of the seminiferous epithelium, the appearance of the mature pattern of Leydig cell interstitial distribution, and the expression of mature SR-BI. Moreover, LGF treatment resulted in partial recovery of HSL expression in Leydig cells and spermatogonia. No changes in serum testosterone were observed in control or LGF-treated rats, but in EDS-castrated animals LGF treatment induced a progressive increase in serum testosterone levels and 3β-HSD expression. Based on the pivotal role of SR-BI in the uptake of cholesteryl esters from HDL, it is suggested that the observed effects of LGF would facilitate the provision of cholesterol for sperm cell growth and Leydig cell recovery.

class B scavenger receptor; scavenger receptor-BI; scavenger receptor-BI; lysosomal integral membrane protein II; hormone-sensitive lipase; tests; epididymis; cholesterol esters; steroidogenesis; spermatogenesis; liver growth factor; ethane 1,2-dimethane sulphonate; fertility; sterility

There is a tight relationship between fertility and changes in cholesterol metabolism during spermatogenesis (33). A constant supply of cholesterol is required within Leydig cells for the synthesis of steroid hormones (steroidogenesis) (21, 39), whereas in the seminiferous tubules cholesterol is involved in germinal cell proliferation and differentiation to spermatozooids (24). Early experiments examining the role of de novo cholesterol synthesis revealed elevated rates of [14C]acetate incorporation into cholesterol during the leptotene, zygotene, and pachytene spermatocyte stages of development (35).

The majority of nutrients needed for spermatogenesis, including lipids, are provided by Sertoli cells, which have the capacity to synthesize cholesterol from acetate in vitro (49). Because the amount of cholesterol required to support spermatogenesis far exceeds the biosynthetic capacity of Sertoli cells, specialized cholesterol transporters are likely to facilitate the influx of cholesterol from the circulation (2). The major source of cholesterol for Sertoli cells is high-density lipoprotein (HDL), as demonstrated in the rat (17, 18).

There are multiple systems for cellular cholesterol delivery, but the vast majority of lipoprotein-derived cholesterol utilized for steroidogenesis in murine testis is obtained via class B scavenger receptors (SR-Bs). SR-Bs are responsible for the “selective uptake of cholesteryl esters (CEs)” from HDL by steroidogenic cells (5, 36, 40, 44). In the testis, SR-BI is expressed most abundantly in steroidogenic Leydig cells (27, 39), with lesser amounts in Sertoli cells (42).

Experimental deficiency of SR-BI causes abnormalities in the development of female gametes. A significant proportion of ovulated oocytes die soon after ovulation, all resulting in sterility in SR-BI gene-knockout (KO) female mice (47). The CEs that are taken up and stored in lipid droplets must be hydrolyzed by nonlysosomal CE hydrolases such as hormone-sensitive lipase (HSL) before the unesterified cholesterol can be transferred to mitochondria for steroid production (26, 41).

The role of HSL in testis was revealed by the phenotype of HSL-deficient mice (11, 32). Male mice homozygous for the mutant allele (HSL−/−) are sterile and show abnormalities in spermatogenesis, resulting in profound alterations in spermatic maturation and oligosperma (32). Recently, we have shown that the lack of HSL in mouse testis induces augmented protein levels of SR-BI, SR-BII, and lysosomal integral membrane protein (LIMP-II), alters lipid raft microdomains, and induces activation of key proteins (p-ERK, p-Akt, and p-SRC) in cell signaling pathways involved in differentiation, proliferation, and other cellular processes during spermatogenesis (9).
LIMP-II belongs to the CD36 family. However, it lacks the glycine dimerization motif present in the other members of this family. This motif is involved in homodimerization and lipid uptake activity (18a); thus, LIMP-II is not expected to participate in the selective uptake of HDL CEs. The physiological role of LIMP-II is not well known, and studies on the expression of LIMP-II at the cellular level are scarce.

Treatment with ethane 1,2-dimethane sulphonate (EDS) has been used to destroy mature Leydig cells and to study the spontaneous recovery of spermatogenesis that occurs thereafter from stem cells (6, 15, 30, 45, 46, 50). This temporary chemical castration produces an initial fall in serum testosterone, one with subsequent loss of differentiating germ cells (7, 8). However, massive germ cell loss after EDS administration is not a general phenomenon. The degree to which germ cell loss occurs after EDS administration appears to be rat strain specific (25, 30).

Liver growth factor (LGF) is an albumin-bilirubin complex purified from rat serum and patients with hepatobiliary disorders (13) that stimulates cell proliferation, with no signs of toxicity or tissue degeneration (15), and has anti-fibrotic, anti-hypertensive, and neuroregenerative activities (19, 37, 43). In preliminary studies, we showed that LGF prevents germ cell sloughing and Sertoli cell damage and promotes germinal cell growth after EDS-induced Leydig cell depletion (29). In mice treated with busulfan, which damages the testicular germinal epithelium, it has been shown recently that LGF partially restores spermatogenesis as well as sperm production and motility (34).

In the present work, we studied the effects of LGF administration on testicular regeneration in rats previously treated with EDS, analyzing the protein levels of class B scavenger receptors (SR-BI, SR-BII, and LIMP-II) and HSL in the testis to gain insight on the contribution of these proteins involved in cholesterol metabolism to the recovery of steroidogenesis and spermatogenesis.

MATERIALS AND METHODS

Animal care and experiment design. We used sexually mature male Wistar rats weighing 300 g. The rats were maintained at 22–24°C under standard conditions of illumination (0800–2000) and feeding (Purina Chow diet; Panlab, Barcelona, Spain). The animals were fed ad libitum and had continuous access to tap water. The experiments were performed at the Center for Animal Experimentation of the University of Alcalá de Henares (Register no. ES28005001165), following the laws of the European Community (867609/EEC) and Spain (R. D. 1201/2005). The experimental protocol was approved by the Animal Research Ethics Committee of the Hospital Universitario Ramón y Cajal (Madrid, Spain).

The animals were allocated to the following groups according to the procedures used in treatment. In the EDS group, 15 rats received a single intraperitoneal (ip) injection of EDS (75 mg/kg) dissolved in dimethylsulfoxide and water (23). The animals were euthanized 10 (T1), 21 (T2), and 35 (T3) days after treatment with EDS at a rate of 5 animals/time period. In the LGF group, 15 rats received LGF (4.5 µg/animal) ip twice/wk starting on day 7 and ending on day 32 (4 wk total). The rats were euthanized on the same days as the rats in the EDS group (always 3 days after the last injection of LGF for a stable situation). In the EDS + LGF group, 15 rats received a single injection of EDS and LGF twice/wk, as in the LGF group. In the control group, 15 rats were injected ip twice/wk with the vehicle in which LGF (saline) was dissolved, as in LGF group, and were euthanized at the same times as the rats in the previous groups.

The rats were anesthetized by inhalation of halothane. Animals were euthanized by decapitation between 1000 and 1100 and after normal overnight access to food. The blood was obtained by intra-cardiac puncture and collected into chilled heparinized receptacles; the serum was separated by centrifugation and kept at −80°C until processing. The testes and the epididymis were removed and weighed. The right testis was frozen in liquid nitrogen and stored at −80°C until processing for protein extraction and expression (see Western blotting below). The left testis was divided in two pieces, one-half of which was fixed in formalin for 24 h and the other half in Bouin’s fluid. The fixed tissues were embedded in paraffin following conventional methods (28) and sectioned at 5 µm either to be stained with hematoxylin and eosin or to be used for immunohistochemistry.

LGF preparations. LGF preparations were lyophilized and stored in aliquots at 4°C until being used for ip injection dissolved in saline. Before LGF was used in these experiments, we checked the activity in vivo at several doses, injecting LGF into normal rats to establish the dose that produced the greatest stimulation of liver DNA synthesis, as determined by incorporation of [3H]thymidine (New England Nuclear, Dreieich, Germany) into DNA (15).

Sperm class analyzer for determination of concentration, motility, and viability of spermatozoa. After the animals were euthanized, the cauda epididymis and vas deferentia were retrieved and placed in M2 medium (Sigma-Aldrich, St. Louis, MO) after all excess adipose tissue and blood vessels were removed to minimize the risk of contamination (22). Spermatozoa were released from the epididymis by slicing with a 30-gauge needle and left in the medium for 5 min to recover motility.

The sperm concentration was determined with a Bürker haemocytometer. The number of spermatozoa was counted under a light microscope. The percentage of motile spermatozoa was assessed by a Sperm Class Analyzer (SCA 2002; Micropptic, Barcelona, Spain). The proportions of living and dead spermatozoa were assessed by a live/dead sperm viability kit (Molecular Probes, Eugene, OR). At least 500 spermatozoa were counted for each treatment.

Radioimmunoassay of testosterone. Serum and testis testosterone was measured by radioimmunoassay with 125I-testosterone (Spectria; Orion Diagnostica, Espoo, Finland), following the manufacturer’s instructions. All measurements were performed three times. The sensibility of the method was 0.1 nmol/ml. The coefficients of variation intra- and interassay were <10%.

Total number of Leydig cells in rat testis. The stereological analyses were performed according to the methods described previously (3), using the ImageJ image analysis software (National Institutes of Health free software) and analyzing the 3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase (3β-HSD)-positive interstitial cells. A total of 12 consecutive, independently selected sections were used for each testis and experimental condition at T3. The volume density of the nucleus (Vn) was calculated by considering the Leydig cell nuclei as ellipsoids. Length and width were measured in the longitudinal sections, and the volume was calculated (VT = 4πVn²/3).

The different parameters were obtained by analyzing a total of 30 hits recorded per section, using 12 nonconsecutive, randomly selected sections for each testis. The following parameters were calculated: 1) volume density of each nucleus (Vn), that is, the surface area occupied by the nuclei divided by the total surface area of the field; and 2) the numerical density (ND) of the Leydig cells, calculated by the average nuclear volume, considering the Leydig cell nuclei as ellipsoids (Vn = 4πVn²/3) with minor (b) and major (a) semiaxes. The total number of Leydig cells in the testis (ND) was calculated according to the following formula: (Vn/Vn) × VT.

Immunohistochemistry. Streptavidin peroxidase immunostaining was performed as described previously (4, 28). Briefly, the sections
were incubated for 12 h at 4°C with a primary antibody, 3β-HSD P-18 (sc-30820; Santa Cruz Biotechnology), (1/300), anti-scavenger receptor (SR)-BI (NB-400-102, lot P1), SR-BII (NB-400-102, lot D5) (Novus Biologicals, Littleton, CO) (1/1,000), anti-LIMP-II (AF 1966, lot KK-401) (R & D Systems, Minneapolis, MN) (1/100), and anti-HSL (AB3525, lot LV871084) (Chemicon, Billerica, MA) (1/800), and chicken polyclonal antiserum directed against HSL from white rat adipose tissue diluted in 0.3% normal goat serum, 0.001% Triton X-100, and 0.01% glycine in TBS (pH 7.6). The sections were washed twice in TBS to remove unbound primary antibody and then incubated with the secondary antibody for 1 h at room temperature. The secondary biotin-conjugated antibodies were anti-rabbit IgG (1/500; sc-2004, lot E1710) (Santa Cruz Biotechnology) for SR-BI and SR-BII (Novus Biologicals, Littleton, CO), anti-goat IgG (1/400; sc-2768, lot C0408) for LIMP-II (R & D Systems), and goat anti-chicken IgY (1/200; RDI-703035155, lot 51570) for HSL (Vector Laboratories, Burlingame, CA) diluted in 0.3% normal rabbit (539845; Calbiochem) or goat serum (50007A; Labclincins-Serolab), 0.001% Triton X-100, and 0.01% glycine in TBS (pH 7.6). The sections were rinsed in TBS and incubated with the streptavidin-peroxidase complex (Zymed Laboratories, San Francisco CA) for 30 min and washed in TBS followed by Tris-HCl buffer (pH 7.6). Peroxidase activity was developed using 3–3′-diaminobenzidine tetrahydrochloride as the chromogen (plus substrate cromogen system, lot 10072031, Dako K3468; Dako and Agilent Technologies, Barcelona, Spain). The sections were counterstained with Carazzi’s hematoxylin. Thereafter, the sections were dehydrated in ethanol, cleared with xylene, mounted in DePeX, and observed under a light microscope.

The specificity of the immunohistochemical procedures was assessed by means of negative controls that were performed as follows: omitting the primary antibodies, using nonimmune serum (0.3% donkey normal serum 017-000-121; Jackson ImmunoResearch) instead of the primary antibodies, and incubating with an inappropriate secondary antibody (anti-goat biotin 1/200 from Millipore for SR-BI, SR-BII, and HSL; and anti-rabbit biotin 1/500 from Dako for LIMP-II) after the incubation with the primary antibodies at optimal titers.

Western blotting. Western blot analysis was performed as described previously (4, 9). The samples were homogenized in 10 mM Tris-HCl buffer [1% (w/v) containing 1 mM EDTA, 12 mM 2-mercaptoethanol, 1 mM benzamidine, and 1 mM phenylmethylsulphonyl fluoride with the addition of a cocktail of protease inhibitors (10 µg/ml leupeptin and 1 µg/ml aprotinin) and phosphatase inhibitors (10 mM sodium fluoride and 1 mM sodium orthovanadate) in the presence of 0.5% Triton X-100. An equal amount of protein from whole cell lysate (20–50 µg) was subjected to 8–12% SDS-PAGE and transferred to polyvinylidene difluoride or nitrocellulose membranes (GE Millipore, Bedford, MA). After blocking, the blots were incubated for 2 h at room temperature with the primary antibody as follows: 3β-HSD (sc-30820, 1/1,000; Santa Cruz Biotechnology), SR-BI (NB-400-102, lot P1), SR-BII (NB-400-102, lot D5, 1/1,000; Novus Biologicals), and HSL (AB3525, lot LV871084, 1/18,000; Chemicon, Billerica, MA) in 5% nonfat dried milk-TBS-0.1% Tween. The incubation was followed by a 1-h incubation with the secondary peroxidase-conjugated antibody as follows: anti-rabbit IgG (1/2,000, sc-2004, lot E1710; Santa Cruz Biotechnology) for SR-BI and SR-BII and donkey anti-chicken IgY (1/20,000, RDI-703035155, lot 51570) for HSL (RDI Research Diagnostics, Flanders, NJ) in 5% nonfat dried milk-TBS-0.1% Tween. All steps were performed at room temperature, and the blots were washed between incubation steps with TBS-0.1% Tween. The immunoreactive bands were visualized using the enhanced chemiluminescence detection method according to the manufacturer’s instructions (ECL system; Amersham, Dübendorf, Switzerland) and subsequent exposure of the membrane to X-ray film. To correct for loading, glyceraldehyde-3-phosphate dehydrogenase (sc-25778, lot L3113; Santa Cruz Biotechnology) was used as the internal control housekeeping protein in Western blot analysis. Quantity 217 One software was used for the analysis and quantification of Western blot image. The specificity of the procedure was assessed by means of negative controls that were performed, omitting the primary antibody and incubating with the secondary antibody alone at optimal titers.

Statistical analysis. Data are presented as means ± SE unless otherwise stated. All statistical analyses were performed with GraphPad Prism software (version 4; GraphPad Software, San Diego, CA). For statistical comparisons between the groups, two-way ANOVA was conducted. Testosterone data were analyzed using one-way ANOVA, and the statistical comparison between groups was performed by Neuman-Keuls multiple comparison test (P < 0.05). These data indicate that LGF treatment after EDS damage induces the recovery of testosterone levels on T2 and T3, which is statistically significant. EDS treatment produced a decrease in epididymis weight on T1 and T2 and spermatozoa concentration at the three times studied (Fig. 1, A and B). As the time progressed, the epididymis weight in EDS and EDS + LGF rats increased faster than in the other groups, resulting in differences with the control no longer being statistically significant on T3.

RESULTS

Epididymis weights, number of spermatozoa, and viable spermatozoa per rat epididymis. No significant differences were observed between the LGF and control groups in terms of body and epididymis weight (Fig. 1A) or viable spermatozoa and concentration on T1, T2, and T3 (Fig. 1, B and C), indicating that LGF treatment did not induce an increase in epididymis weights or an improvement in sperm quality in normal testis. The amount of spermatozoa of LGF group on T2 was significantly higher than the amount of the same group on T1, and this difference is statistically significant. EDS treatment produced a decrease in epididymis weight on T1 and T2 and spermatozoa concentration at the three times studied (Fig. 1, A and B).

Serum testosterone concentration in rats. Serum testosterone levels were decreased significantly in EDS and EDS + LGF-treated rats on T1, T2, and T3 compared with control and LGF-treated rats (Fig. 2A). On T2 and T3, serum testosterone levels in EDS + LGF-treated rats were significantly elevated compared with EDS group at those times. These data indicate that LGF treatment after EDS damage induces the recovery of testosterone levels at T2 and T3 and improves Leydig cell recovery.

Effects of LGF in 3β-HSD localization, protein levels, and total number of Leydig cells in testis from rats treated previously with EDS. Adult Leydig cells capable of testosterone synthesis express high levels of 3β-HSD, which catalyzes the oxidative conversion of Δ5-3-β-hydroxysteroids to the Δ4-3-keto configuration and is therefore essential for the biosynthesis of all classes of hormonal steroids, including androgens. In testes from control and LGF-treated rats, 3β-HSD-positive staining was found in Leydig cells located at the center of the intertubular spaces and in some stromal perivascular cells (Fig. 2B1). In rats treated with EDS, no 3β-HSD staining in Leydig cells was observed on T1, consistent with the depletion of this cell type by the effect of EDS. On T2, we observed a small amount of 3β-HSD-positive Leydig cells and stromal perivascular and peritubular cells. These Leydig cells were located mainly at the peritubular region. On T3, EDS-treated rats showed 3β-HSD-positive cells with predominant peritubular distribution and some immunopositive perivascular cells. In EDS + LGF-treated rats, just scarce peritubular and perivascular 3β-HSD positive cells were detected on T1. However, abundant peritubular 3β-HSD-positive cells were observed on T2, along with some perivascular immunopositive cells. On
Effects of LGF on the protein levels and localization of class B scavenger receptor (SR-BI, SR-BII) in testis from rats treated previously with EDS. We analyzed the protein levels of the different SR-Bs on the different testicular cell types because of the relevance for cholesterol utilization in testicular cell proliferation, steroidogenesis, and spermatogenesis. We comparatively studied the immunolocalization of two SR-Bs in rodents (SR-BI and SR-BII) to ascertain whether changes in their localization and protein levels by effect of EDS and LGF treatments were parallel or not to evaluate their potential as biomarkers of the testis function.
Fig. 2. Effects of LGF on serum testosterone levels (A), immunolocalization (B1) and protein levels of 3β-hydroxysteroid dehydrogenase (3β-HSD; B2), Leydig cell no. (B3), and changes in the histology of the testis (C) in EDS-treated rats. Rats were C, treated with LGF, and treated with a single intraperitoneal injection of EDS or with EDS + LGF. The animals were euthanized 10 (T1), 21 (T2), and 35 (T3) days after treatment with EDS at the rate of 5 animals per time period. Data in A are expressed as the mean ± SE of 5 rats/time in each group. Statistical comparisons are shown vs. C (**P < 0.01; ***P < 0.001) and EDS + LGF vs. EDS (+ + P < 0.001) by Neuman-Keuls multiple comparison test. B1 and C show the results of 1 representative testis micrographs taken on 5-μm-thick paraffin-embedded sections stained with 3β-HSD (B1) or hematoxylin and eosin (C), respectively. The diameter of seminiferous tubules (STs) and the localization of Leydig cells (LC) are indicated in the testis micrographs (C). Bars, 12.5 μm (B1) and 25 μm (C).
Table 1. Effects of LGF on seminiferous tubule diameter in EDS-treated rat testes

<table>
<thead>
<tr>
<th>Seminiferous Tubule Diameter, μm</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
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<tbody>
<tr>
<td>Control</td>
<td>266.1 ± 3.7a</td>
<td>292.0 ± 6.3a</td>
<td>294.4 ± 3.5a</td>
</tr>
<tr>
<td>LGF</td>
<td>270.3 ± 3.9a</td>
<td>281.0 ± 2.6a</td>
<td>271.3 ± 3.6b</td>
</tr>
<tr>
<td>EDS</td>
<td>189.2 ± 4.5b</td>
<td>199.4 ± 3.5b</td>
<td>230.5 ± 3.7c</td>
</tr>
<tr>
<td>EDS + LGF</td>
<td>188.4 ± 3.4b</td>
<td>207.4 ± 3.0b</td>
<td>233.3 ± 3.6c</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. LGF, liver growth factor; EDS, ethane 1,2-dimethane sulphonate; T1, day 10; T2, day 21; T3, day 35. Rats were untreated (control) or treated with LGF (4.5 μg/animal) injected ip twice/wk from day 7 to day 32 (4 wk), with a single ip injection of EDS (75 mg/kg), or with EDS + LGF. The animals were euthanized on T1, T2, and T3 after treatment with EDS at a rate of 5 animals/time period. We measured 20 fields/section of testis, 5 rats/group, and at 3 times (T1, T2, and T3). Statistical differences are shown by superscripted letters. Different letters express significant statistical differences between the groups.

In testes from control rats, SR-BI staining was found in the membranes of the Leydig cells, some spermatogonias, acrosomes of the spermatocytes, and elongated spermatids at the three times studied (T1, T2, and T3; Fig. 3A). In LGF-treated rats, a similar SR-BI staining pattern was found. The residual bodies of elongated spermatids showed positive SR-BI staining in LGF-treated testes on T3. In rats treated with EDS on T1, no staining for SR-BI staining was observed except on the acrosomes of spermatids. On T2, EDS-treated rats showed some positive SR-BI staining in Leydig cells and increased staining in spermatogonias compared with T1. On T3, SR-BI staining in EDS-treated rats was demonstrated in the membranes of the Leydig cells, acrosomes of the spermatids, and some spermatogonias, similar to what was found in the testes from control rats. Taken together, these observations show the partial recovery of testis morphology and SR-BI expression in EDS-treated rats.

Regarding the effect of LGF in EDS-treated rats, on T1, some SR-BI staining in interstitial cells, as well as spermatocytes and spermatogonias, was found in testes from EDS + LGF-treated rats, indicating a better condition compared with EDS-treated rats. Yet SR-BI staining in interstitial cells of EDS + LGF testes was much less than in controls and LGF-treated rats (Fig. 3A). Since this time, the recovery of SR-BI staining in EDS + LGF-treated rats was similar to the EDS group.

To get a more global insight on SR-BI expression, we then determined SR-BI protein levels by Western blotting in total lysates of rat testes (Fig. 3A). Rat testis expresses two SR-BI-immunoreactive proteins [82 (mature protein, light band) and 57 kDa (immature protein, intense band)]. This pattern was not altered by the effect of LGF. In testis from EDS-treated rats, protein levels of the 82-kDa mature form were markedly reduced at any time compared with the control (Fig. 3A). In contrast, the levels of the 57-kDa SR-BI isoform were highly increased, especially at T2. Administration of LGF to EDS-treated rats resulted in a remarkable increase in the levels of both SR-BI proteins, reaching levels on T2 and T3 that were even higher than the control. This regain of SR-BI protein levels in testes may be interpreted as an improvement of testes EDS damage by the effect of LGF treatment. Whether the increase in total SR-BI content is a reflection of the increased number of SR-BI-expressing cells or due specifically to increased expression by Leydig cells remains to be clarified.

Rat testis also expresses two SR-BII-immunoreactive proteins (82 and 57 kDa), like SR-BI. Globally, EDS treatment resulted in a profound decrease of SR-BII protein levels in the rat testis, as examined by Western blotting (Fig. 3B), which persisted on T3. Examination of the histological preparations shows that SR-BII is localized to the germinal cells (spermatocytes and spermatids) from the seminiferous tubulus, whereas both Leydig and Sertoli cells are immunonegative for SR-BII staining (Fig. 3B). In EDS-treated rats, the remaining spermatocytes and spermatids were also immunostained for SB-II, but the density of stained cells was much lower than in controls at any time studied (Fig. 3B). Compared with EDS-treated rats, in testis from EDS + LGF-treated rats, an increase in SR-BII staining in spermatogonia was observed on T1 (Fig. 3B), but thereafter, the immunostaining pattern was very similar in these two groups, and SR-BII protein level in total tissue was practically undetectable by Western blotting compared with control rats (Fig. 3B).

**DISCUSSION**

In the present work, we studied the effects of LGF, an albumin-bilirubin complex with mitogenic activity (14), in animals subjected to testicular damage induced by EDS. Hist-
Fig. 3. Effects of LGF in the immunolocalization and protein levels of class B scavenger receptors SR-BI and SR-BII in EDS-treated rat testis. Rats were control (C), negative control (C−), treated with LGF, and treated with a single ip injection of EDS or with EDS + LGF. The animals were euthanized 10 (T1), 21 (T2), and 35 (T3) days after treatment with EDS at the rate of 5 animals/time. Testes were removed from rat, embedded in paraffin, sectioned, incubated with the primary antibody followed by a secondary antibody conjugated with biotin, and counterstained with Carazzi’s hematoxylin. A: immunohistochemistry with SR-BI antibody shows positive staining in the cytoplasm of LC and elongated spermatids (Sp) of the STs of rat testes. Western blotting analysis of SR-BI protein rat testis shows the expression of the 82-kDa mature isoform and 57-kDa immature isoform. B: immunohistochemistry with SR-BII shows positive staining in the membrane of the germ cells [spermatocytes (Sc) and round spermatids (rSp)] and negative staining in elongated Sp, Sertoli cells, and LC. Western blotting analysis of SR-BII protein rat testis shows the expression of the 82-kDa mature isoform and 57-kDa immature isoform. Bar graphs show the densitometric analysis of SR-BI and SR-BII individual band Western blots (arbitrary units) (n = 5; means ± SE).
Fig. 4. Effects of LGF in the immunolocalization and protein levels of LIMP-II and hormone-sensitive lipase (HSL) in EDS-treated rat testis. Rats were C, treated with LGF, and treated with a single ip injection of EDS or with EDS + LGF. The animals were euthanized 10 (T1), 21 (T2), and 35 (T3) days after treatment with EDS at the rate of 5 animals/time. Testes were removed from rat, embedded in paraffin, sectioned, incubated with the primary antibody followed by a secondary antibody conjugated with biotin, and counterstained with Carazzi’s hematoxylin. A: immunohistochemistry with LIMP-II shows positive staining in the membranes of the Sertoli cells (S) and LC. B: immunohistochemistry with HSL antibody shows positive staining in the cytoplasm of LC and S, spermatogonia (Sg), Sc, and elongated Sp of the STs of rat testis. Western blotting analysis of HSL protein rat testis shows the expression of the 130-, 82-, and 67-kDa bands. The results of 1 representative immunohistochemistry staining and 1 immunoblot of 5 individual experiments/group are shown. Bars, 25 μm.
tolerological studies showed profound changes in the testis in adult rats by the effect of EDS as a result of Leydig cell destruction, confirming previous results by others (31, 50). Herein, we show that LGF treatment in EDS-treated animals induces a rapid recovery of serum testosterone levels, 3β-HSD protein levels, and testis morphology. Treatment with LGF also accelerated the recovery of Leydig cell numbers, as well as germ cells, in most of the seminiferous tubules, although at the times studied mature spermatozoa inside the lumen of the epididymal ducts were still absent. These results are in agreement with previous findings communicated by us showing that LGF increases the proliferation index in Leydig and germ cells measured by BrdU incorporation and diminishes apoptosis in the STs and interstitial tissue in rats treated with EDS (29).

Pérez-Crespo et al. (34) reported recently that ip administration of LGF was able to restore spermatogenesis in mice sterilized with busulfan. This compound induces a complete depletion of germ cells, but only partial depletion of testicular stem cells, which allows spontaneous recovery to occur (16). Busulfan treatment produces some morphological abnormalities of the seminiferous tubules but apparently does not affect Sertoli or spermatogonial stem cells (51). The recovery of the normal histological structure after LGF administration to animals treated previously with busulfan showed not only a reactivation of the spermatogenic process but also the regeneration of the microenvironment necessary for successful spermatogenesis (34). Thus, this study suggested that LGF collaborates in the mobilization of testicular stem cells to restore spermatogenesis after germinal epithelium damage (34). It cannot be overlooked that LGF may have a role in normal development of spermatogenesis, as reported for hepatocyte growth factor, which modulates germ cell proliferation and survival in vitro (10). Nevertheless, to date, the mechanism of action of LGF in testicular tissue and the possible receptors involved in the signal transduction of the LGF effects have not been elucidated.

One of the aims of the present work was to determine the protein levels of relevant proteins involved in cholesterol metabolism in the testis. Spermatogenesis occurs in a series of proliferative and differentiation stages that can be subdivided into mitotic, meiotic, and spermatogenic phases. A constant supply of cholesterol is required within Leydig cells to serve as the precursor for the synthesis of steroid hormones (steroidogenesis) (21, 39), whereas in the seminiferous tubules, cholesterol is involved in germinal cell differentiation to spermatozoa (spermatogenesis or gametogenesis) (24). There is also considerable evidence indicating that cholesterol is required for the development of gametes and fertility in both sexes. Because of the extensive production of germ cells throughout spermatogenesis, the requirement for cholesterol is significant. In the testis, the main pathway to obtain cholesterol is the selective uptake of CEs from HDL-mediated by the SR-BI (1, 38, 39). Moreover, it has been demonstrated directly that HDL is the main source of cholesterol in Sertoli cells (17, 18). In the present work, we showed that treatment with LGF accelerates the expression of mature SR-BI in the whole testis, and particularly in the Leydig cells. Based on the pivotal role of SR-BI in the uptake of CEs from HDL, this effect of LGF would augment cholesterol accretion, which may potentially be used for cell division of the remaining stem cells of the testis to favor testicular regeneration and to restore steroidogenesis and spermatogenesis. Because of the rapid reappearance of SR-BI in EDS-treated rats upon LGF administration, it may be suggested that SR-BI expression is an early marker of spermatogenesis recovery.

Despite these favorable changes induced by LGF administration, testicular function was not fully recovered in the time span studied. One of the causes that may have contributed to this is the slow recovery of the expression of HSL. Thus, although EDS treatment caused a severe decrease in the protein levels of HSL in rat testis, LGF treatment allowed only partial recovery of HSL protein levels in Leydig cells and spermatogonias, reaching relatively low levels. HSL is required for the hydrolysis of CEs (26), and in conjunction with SR-BI it contributes to the provision of free cholesterol in testis cells (5, 26, 40). Remarkably, HSL-deficient mice exhibit male sterility (11, 32). Therefore, it is likely that the observed HSL deficiency is contributing to the slow recovery of testicular function. Nevertheless, the detection of HSL staining in spermatocytes on T3 in rats treated with EDS plus LGF, but not in those treated with EDS alone, may indicate adequate progression along spermatogenesis by the effect of LGF. It would be interesting to study the effects of LGF in testosterone levels as well as in SR-BI and HSL expression in other rat strains less sensitive to EDS (20).

EDS treatment caused the destruction of Leydig cells, and during the first 2 wk after EDS treatment there was a stage-dependent loss of germ cells within seminiferous tubules that led to a profound disturbance of spermatogenesis. This was associated with the sequential highly depressed levels of SR-BI, the major isoform expressed in the germ cells, after EDS treatment. The restoration of the seminiferous epithelium also followed in a stage-specific manner in relation to development of a new Leydig cell population (6). In correlation with this, the expression of SR-BI, expressed mainly by Leydig cells, took place shortly after application of LGF. The lack of effect of LGF on SR-BI during the observation period is thus attributed to the gap between the reappearance of Leydig cells (early responders) and the recovery of germ cells (long depending on Leydig cell function).

In summary, we showed that ip administration of LGF accelerates the recovery of spermatogenesis in rats treated previously with EDS. In parallel, treatment with LGF intensely increased the protein levels of SR-BI, which is involved in the uptake of cholesterol from HDL required for cell division and spermatogenesis, all of which suggests that regained SR-BI in EDS-treated rats facilitated the expansion of the remaining cells of the testis to favor testicular regeneration and to restore steroidogenesis and spermatogenesis. In this sense, SR-BI expression in the rat testis could be considered as an early marker of spermatogenesis recovery after LGF treatment in rats, with Leydig cell and germinal epithelium damage induced by EDS.

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DISCLOSURES

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.
AUTHOR CONTRIBUTIONS


REFERENCES


