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Sex-dependent effect of liver growth factor on atherosclerotic lesions and fatty liver disease in apolipoprotein E knockout mice

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Summary. Objective: Since the hepatic mitogen, liver growth factor (LGF), improves vascular structure and function in a hypertensive rat model and exhibits antioxidant activity, it may play a role in the development of atherosclerosis. Methods: To test this hypothesis, 14 male and 11 female apolipoprotein E (apoE)-deficient mice with a C57BL/6J genetic background were injected intraperitoneally twice a week with 1.7 μg of LGF per mouse for ten weeks. Plasma carbohydrates, inflammatory and lipid parameters, apolipoproteins A-I and A-II and paraoxonase activity were assessed at the end of the experimental period. Histological and chemical analyses of the livers and quantification of aortic atherosclerotic lesions were also carried out. Results: LGF administration changed neither plasma lipid nor inflammatory parameters. ApoA-I and arylesterase activity were not affected by LGF either, while apoA-II decreased significantly in males but not in females. Plasma apoA-II correlated positively with liver fat in males but negatively in females. Atherosclerotic area lesions in males receiving LGF were 25% lower than in control mice. Likewise, a significant reduction of fatty liver disease was also observed in males in association with decreased levels of insulin, leptin and resistin. Conclusion: These results indicate that administration of LGF modulates atherosclerotic lesions in a sex-dependent manner. This effect is independent of plasma cholesterol, triglycerides, IL-6, MCP-1 and TNF-α and is related to a remodelling of HDL particles characterised by a decrease in apoA-II induced by changes in hepatic mRNA expression. Hence, LGF administration could be used as a safe alternative to control fatty liver disease and atherosclerosis in males.

Key words: Cholesterol, Apolipoprotein, Fatty liver disease, Atherosclerosis, LGF

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Introduction

Nowadays, atherosclerosis is recognised as an inflammatory process, in which the initial lesion is produced by the response of the injured endothelium to physical stress or to exposure to oxidised cholesterol, homocysteine or infectious agents, among other causes. This process involves humoral (cytokines, growth factors) and cellular (increased chemotaxis, adherence and infiltration of inflammatory cells) mechanisms that promote the recruitment of circulating cells into the subendothelial space (Libby and Aikawa, 2002). Interestingly, the initial cellular mechanisms of atherogenesis are similar to those observed in chronic inflammatory fibroproliferative diseases such as liver cirrhosis, pulmonary fibrosis, etc. (Cusa and Mazzone, 2002). In spite of this phenomenon, the prevalence of cardiovascular disease in cirrhotic patients is low (Kawakami et al., 2007), pointing to differential

Abbreviations. apo, apolipoprotein; HDL, high-density lipoproteins; HDL-c, high-density lipoprotein cholesterol; IL-6, interleukin 6; LDL, low-density lipoproteins; LDL-c, low-density lipoprotein cholesterol; LGF, liver growth factor; MCP-1, monocyte chemotactic protein-1; NEFA, nonesterified fatty acids; NO, nitric oxide; TG, triglycerides; TNF-α, tumour necrosis factor α; VLDL, very low-density lipoproteins

etiological factors among these pathological entities that should be explored.

Liver growth factor (LGF), an albumin-bilirubin complex with a covalent bond, is a hepatic mitogen (Diaz-Gil et al., 1986) that has shown both *in vivo* and *in vitro* activity, and its concentration in humans and in rats is significantly increased in the presence of hepatobiliary disorders or liver injury (Weiss et al., 1983; Somoza et al., 2006). In addition, Díaz-Gil et al. (2003) have observed that the first targets of LGF in liver are the endothelial cells around the portal vein. When endothelial cells were incubated with this compound, the mitogenic pathway was initiated by TNF-α secretion, but without inducing adhesion molecules. In addition, LGF has shown an antifibrotic effect associated with decreased hepatic levels of metalloproteinases 2 and 9 and transforming growth factor-beta 1 (Diaz-Gil et al., 2008), and a modulation of the activation state of fibrogenic liver cells (hepatic stellate cells and myofibroblasts) in fibrotic rats (Díaz-Gil et al., 2009). Biological actions have also been reported in other tissues. In fact, a short-term treatment of spontaneously hypertensive rats with LGF reduced blood pressure, improved nitric oxide-dependent vasodilation, and exerted vascular antifibrotic actions (Somoza et al., 2006). LGF was also able to protect endothelial NO from superoxide anion degradation (Condezo-Hoyos et al., 2009). These effects of LGF treatment on vascular tissues led us to hypothesize that LGF might also exert beneficial effects in other vascular diseases such as atherosclerosis. To address this question, we employed apoE-deficient mice, a well-characterized and widely used animal model that spontaneously develops atherosclerosis with features similar to those observed in humans (Sarría et al., 2006). Besides, this animal model displays hepatic steatosis that can be modulated by dietary interventions (Arbones-Mainar et al., 2006a; Acín et al., 2007). In the present study, apoE- deficient mice were subjected to long-term intraperitoneal administration of LGF, and both pathological entities, as well as carbohydrates, hormones, lipid and apolipoproteins, were monitored at the end of treatment to ascertain their involvement.

Material and methods

Animals and treatments

Fourteen male and 11 female 4-month-old apoE-deficient mice with a C57BL/6J genetic background were used. During the experimental period, the animals were fed with a standard chow diet (2014 Global Rodent Maintenance, Harlan Teklad) and had free access to chow and water. To avoid the potential confounding effects of variation among batches of chow, 25 kg from a single batch were reserved and used throughout the experiment. Food intake was recorded during the first two weeks of the experimental period. Animals were randomly distributed into two groups according to their initial plasma cholesterol concentration. One of the groups received an intraperitoneal injection, twice a week for ten weeks, of 150 μl of a solution containing 1.7 μg of the Liver Growth Factor (Diaz-Gil et al., 1986), and the other (control), the same volume of saline solution. The mice were housed in a temperature-controlled facility and handled observing criteria from the European Union for the care and use of laboratory animals in research, and the protocol was approved by the Ethics Committee for Animal Research of the University of Zaragoza, Spain.

Sample collection

Following an overnight fast, blood samples were taken from the retro-orbital plexus, using capillary tubes under light anaesthesia with isofluorane, at the beginning of the experimental period to quantify initial plasma cholesterol and paraoxonase activity. After ten weeks of treatment, the anaesthetized animals were sacrificed by exsanguination and the plasma obtained was frozen and stored until analysis. Immediately after sacrifice, the liver was removed and frozen in liquid nitrogen and an aliquot was stored in a 10% buffered formaldehyde solution for histological analysis.

The heart was perfused with phosphate-buffered saline (pH 7.4) and dissected. Once covered with OCT (Bayer Diagnostic, Germany), it was frozen in dry ice-chilled isopentane (Panreac, Barcelona, Spain) and then stored at -80°C until histological analysis.

Analytical procedure

Plasma total cholesterol (TC) and triglycerides (TG) were quantified by enzymatic methods (Thermo, Madrid, Spain). Plasma high density lipoprotein cholesterol (HDL-c) was quantified by a fluorescence method (Amplex Red, Molecular Probes, USA) in the supernatant obtained after precipitation with phosphotungstic acid-$MnCl_2$ (Roche, Barcelona, Spain) of apolipoprotein B-containing particles. Paraoxonase was assayed as arylesterase activity according to the rate of hydrolysis of phenylacetate, as previously described (Acín et al., 2005), and the results were expressed as μmol phenylacetate hydrolysed$\cdot$min$^{-1}\cdot$L^{-1} (IU$\cdot$L^{-1}). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assayed at 37 ºC using kits from Alfawassermann (Woerden, Holland) and expressed as IU$\cdot$L^{-1}. Glucose concentrations were measured by a fluorescence method (Amplex Red, Molecular Probes, USA). Insulin, interleukin 6, leptin, monocyte chemotactic protein-1, resistin and TNFα were determined using a Milliplex kit from Millipore (Millipore, Madrid, Spain). Apolipoproteins A-I and A-II were quantified by ELISA using specific rabbit and goat polyclonal antibodies (Biodesign, Saco, ME, USA) respectively, as previously described (Navarro et al., 2005). All assays were carried out in triplicate and processed on the same day.

Histological analysis of aorta and liver

Several sections of frozen proximal aorta and aortic sinus were cut with a cryostat (Microm HM505E, Barcelona, Spain) and the 6 μm-cryosections were stained with Sudan IV for lipids and counterstained with haematoxylin (Harris solution, Sigma Chemical Company). Histological images were taken with a Canon digital camera fitted to a Nikon microscope. The lesion sizes were quantified from an average of four sections from each animal using Scion Image software (Scion Corporation, Frederick, Maryland, USA).

Liver samples stored in formaldehyde were embedded in paraffin. Sections (4-μm) were stained with haematoxylin and eosin and observed with a Nikon microscope. Hepatic fat content was evaluated by quantifying the extent of fat droplets in each liver section with Adobe Photoshop 7.0 and expressed as percentage of the entire liver section (Guillén et al., 2008).

Isolation of RNA and quantification of mRNA

Total RNA was isolated using Trigent reagent (Sigma). DNA contamination was removed by treatment with TURBO DNAse using the DNA removal kit from AMBION (Austin, TX, USA). RNA was quantified by absorbance at $A_{260/280}$ (the $A_{260/280}$ ratio was greater than 1.75). The integrity of the 28 S and 18 S ribosomal RNAs was verified by formaldehyde- agarose gel electrophoresis followed by ethidium bromide staining, and the 28S/18S ratio was greater than 2. Equal amounts of DNA-free RNA from each sample of each animal were used in reverse transcription- polymerise chain reaction (qRT-PCR) analyses to quantitate mRNA expression. First-strand cDNA synthesis and the PCR was performed using the SuperScript II Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen), according to the manufacturer's instructions and as previously described (Arbones-Mainar et al., 2006a). The primers used were designed by Primer Express® (Applied Biosystems, Foster City, CA) and checked by BLAST analysis (NCBI) to verify gene specificity. The primers used for the *Apoa2* and for *Pipb* genes have been previously described (Arbones-Mainar et al., 2006a; Guillen et al., 2009). The specificity of the PCR was confirmed by the temperature dissociation curves. Real time PCR was performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) following the standard procedure. The relative amount of all mRNAs was calculated using the comparative 2-ΔΔCt method and normalised to the invariant cyclophilin B (*Pipb*) mRNA expression.

Hepatic lipid analysis

Tissues (10 mg) were homogenised in 1 ml of PBS. An aliquot was saved to determine protein concentration by the BioRad dye-binding assay (BioRad, Madrid, Spain). One volume of homogenate was extracted with two volumes of chloroform: methanol (2:1) twice. The separated organic phases were combined and evaporated under N_2 stream. Extracts were dissolved in 100 μL of isopropanol to estimate cholesterol and TG concentrations using commercial kits as described above.

Statistical analysis

Data were analysed by the Kolmogorov-Smirnov test to check the normal distribution of variables and by Bartlett's test to assess the homogeneity of variances. One-way ANOVA was used and *post hoc* tests were performed using Fisher's least significant difference. When the variables did not exhibit a Gaussian distribution, groups were compared using a Kruskal-Wallis test, and differences between pairs were tested using the Mann-Whitney U-test. Unless otherwise stated, results are expressed as mean±SD. Correlations between variables were tested by calculating the Pearson or Spearman's correlation coefficient using SPSS software, version 15.0 (SPSS Inc, Chicago, IL, USA). Differences were considered significant when $P<0.05$.

Results

Effect of LGF on somatometric parameters

The treatment was well tolerated in both female and male mice, as shown in Table 1. In fact, after 10 weeks of LGF treatment, body weight did not experience any significant change in either sex despite the obvious, sex-related difference in this parameter. Likewise, LGF administration did not induce any significant change in liver weight either. The sex-related differences observed in the latter were corrected when referred to body weight. Using this ratio, no significant variation was observed after administration of LGF either.

Effect of LGF on liver histology and hepatic lipid content

Figure 1 shows representative histological images of livers from control and treated mice of both sexes.

Table 1. Somatometric parameters of ApoE-deficient mice receiving Liver Growth Factor (LGF).

	Female		Male	
	Control	LGF	Control	LGF
Body weight (g)	22.3±2.1	22.8±1.7	29.7±6.3	32.1±3.0
Liver weight (g)	1.02±0.16	0.92±0.10	1.24±0.40	1.35±0.16
Liver/body weight ratio (x100)	4.5±0.4	4.1±0.2	4.0±0.7	4.2±0.2

Data are expressed as mean ± standard deviation. Control and LGF mice received intraperitoneal injections of saline or LGF, respectively, twice a week for ten weeks. Statistical analysis was carried out by one-way ANOVA and post-hoc test was Fisher LSD. ns, $P>0.05$.

ApoE-deficient mice, in agreement with the reported accumulation of lipids in this model (Arbones-Mainar et al., 2006a; Acín et al., 2007), showed macrovesicular steatosis in liver lobule zone 2 and microvesicular in hepatocytes outside this region, a phenomenon that was more evident in males (Fig. 1C,D). LGF administration clearly alleviated the lesion pattern in both sexes, reducing the surface occupied by lipids (Fig. 1B,D). Quantitative evaluation of the percentage of the area occupied by lipid droplets in all animals is shown in Figure 1E and corroborates the above pattern in the sense that male mice receiving LGF showed a significant decrease in the hepatic fat content. When chemical analysis was carried out (Fig. 1F,G), the decrease observed in treated male mice was due to reduced amounts of both cholesterol and TG. On the other hand, a significant increase in hepatic cholesterol was observed in the treated female group. These results suggest that LGF is involved in the control of hepatic fat in a sex-dependent way.

LGF and plasma carbohydrate and lipid parameters

The plasma carbohydrate and lipid parameters assayed are shown in Table 2. Plasma glucose levels were significantly more elevated in females receiving LGF, but not in treated males. However, the levels of

Table 2. Sex-related effects of liver growth factor (LGF) administration on plasma parameters in ApoE-deficient mice.

	Female		Male	
	Control	LGF	Control	LGF
Glucose (mmol/L)	12±4	19±3[a]	10±5	13±3
Insulin (pg/mL)	424±103	201±144[a]	173±107	79±48[a]
Cholesterol (mmol/L)	9.7±2.0	8.8±1.6	13.6±4.5	11.7±1.6
HDL cholesterol (mmol/L)	0.12±0.04	0.09±0.01	0.15±0.06	0.22±0.10
Triglycerides (mmol/L)	1.7±0.5	2.3±1.3	1.7±0.6	2.0±0.9
NEFA (mg/dL)	1.1±0.3	1.1±0.3	1.4±0.1	1.1±0.3
Arylesterase activity (IU/mL)	16.4±1.2	15.5±1.9	13.8±3.1	13.6±1.7
ApoA-I (AU)	37±7	34±2	46±8	51±12
ApoA-II (AU)	5.8±2.2	7.1±1.2	16.3±3.2	12.8±2.5[a]
ApoA-II/ g liver (x100)	5.8±2.4	7.8±1.4	14.3±6.1	9.6±2.4[a]

Data are expressed as mean ± standard deviation. Control and LGF mice received intraperitoneal injections of saline or LGF, respectively, twice a week for ten weeks. Statistical analysis was carried out by one-way ANOVA with post-hoc analysis using Fisher LSD test. [a]: P<0.05 vs control.

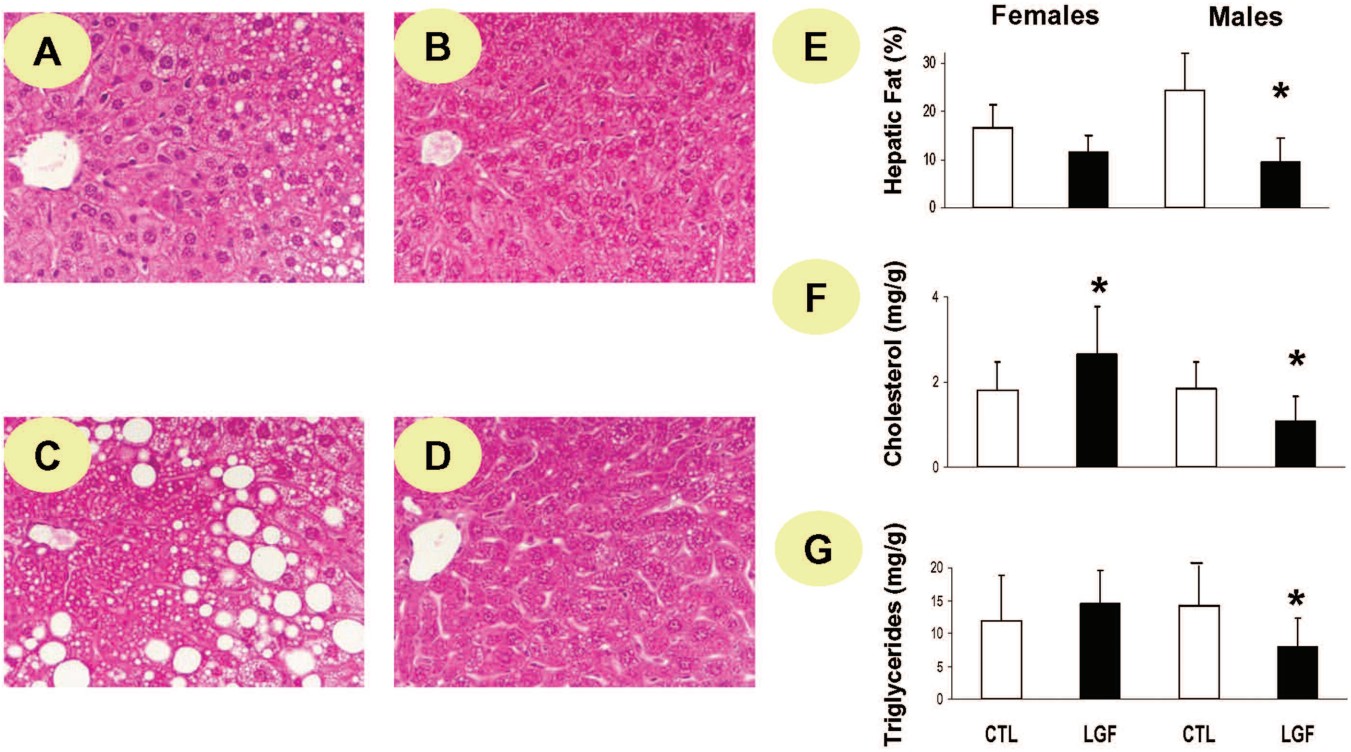

Fig. 1. Sex-related effect of LGF administration on hepatic fat in ApoE-deficient mice. Representative micrographs of liver at x400 magnification from C57BL/6J control female **(A)**, LGF-treated female **(B)**, male control **(C)** and male LGF-treated mice **(D)**. Liver sections (4 μm) from each mouse were stained with haematoxylin and eosin and evaluated blindly. Changes in hepatic fat content expressed as percentage of surface occupied by lipids **(E)**, analysis of cholesterol **(F)** and triglyceride content **(G)** in different experimental groups. Data are mean values with SD. Control (CTL) and LGF mice received intraperitoneal injections of saline or LGF, respectively, twice a week for ten weeks. Statistical analysis was carried out by one-way ANOVA with post-hoc analysis using Fisher LSD test. *: P<0.05 vs CTL.

plasma insulin were found to be significantly decreased in treated mice of both sexes. Although total and HDL cholesterol were higher in males than in females, LGF administration did not induce any significant change in any of these parameters in either sex. No significant differences were observed in the values of TG and nonesterified fatty acids (NEFA) following LFG treatment either. To characterise the influence on HDL particles, we measured the concentrations of plasma apolipoproteins A-I and A-II and paraoxonase. These parameters showed statistical differences between sexes. Thus, apoA-I and apoA-II levels were significantly higher ($P<0.01$ and $P<0.001$, respectively), while arylesterase activity was lower ($P<0.05$) in males than in females. LGF treatment modified neither the apoA-I concentration nor arylesterase activity in either sex. However, there was a sex-related difference in the apoA-II response to this treatment: while no significant change was observed in females, male mice receiving LGF showed a significant decrease in plasma apoA-II. When this plasma apoA-II concentration was referred to the liver mass due to the predominant role in its biosynthesis (Tailleux et al., 2002), a further decrease was observed in the group of males receiving LGF (Table 2). To better know the characteristics of HDL after treatment, apoA-II/HDL-c and apoA-II/apoA-I ratios were calculated and are shown in Figure 2, panels A and B, respectively. In control animals, both ratios displayed significantly lower values in females than in males, and LGF administration induced a significant reduction of both ratios only in males. To verify whether this decrease in apoA-II resulting from the effect of LGF treatment was due to a variation in its hepatic mRNA, the steady-state levels of this message were determined by quantitative PCR. As shown in Fig 3, males receiving LGF showed a significant decrease in the levels of this transcript. Overall, these results suggest that LFG exerts a sex-specific action in the remodelling of HDL particles by decreasing the plasma concentration of apoA-II mediated by a decrease in its hepatic mRNA.

Effect of LGF on atherosclerotic lesion areas

The individual cross-sectional atherosclerotic areas after 10 weeks of LGF administration are reflected in Fig. 4, panel A. This clearly indicated that females receiving LGF did not experience any significant variation. In contrast, the atherosclerotic lesion in males receiving LGF was 25% smaller than in the mice of the control group, although this difference did not reach statistical significance ($P=0.06$), probably due to an insufficient number of animals. When data from male mice from this experiment were combined with those obtained in a similar experiment carried out one year earlier (n=12 per group), and expressed as percentage of change to correct for the influence of different chow batches (panel B), a significant decrease was recorded in males receiving LGF, while no change was observed for females (data not shown), indicating the importance of employing an adequate sample size of 12 animals to obtain a significant value. This result suggests a sex-specific action of LGF with preference for males.

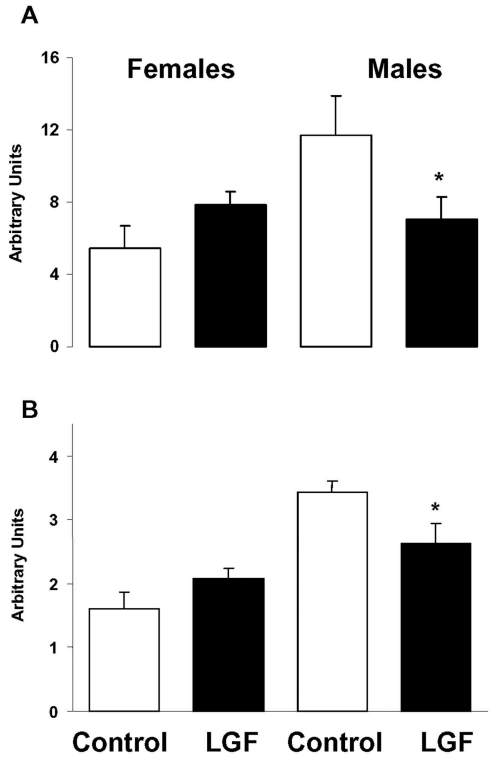

Fig. 2. Effect of LGF administration on ApoA-II-related parameters in ApoE-deficient mice. Relationship of plasma ApoA-II to HDL cholesterol concentrations in panel **A.** Ratio of ApoA-II to ApoA-I plasma concentrations in **B.** Data are mean values with SEM. Control and LGF conditions as described in previous figure. Statistical analysis was carried out by one-way ANOVA with post-hoc analysis using Fisher LSD test. *: $P<0.05$ vs control.

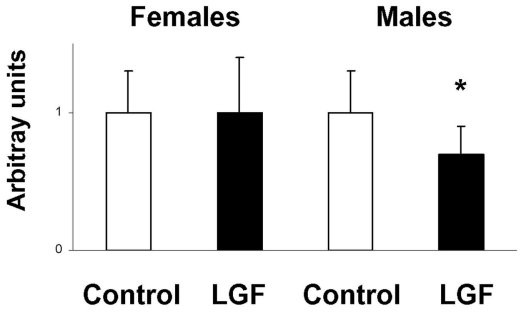

Fig. 3. Effect of LGF on hepatic *Apoa2* mRNA levels in ApoE-deficient mice. Data represent arbitrary units normalised to the cyclophilin B (*Ppib*) expression for each condition with the qRT-PCR. Values are mean values with SD. *: $P<0.05$ vs control.

Effect of LGF on necrosis, inflammatory and adipocyte markers

Data summarising these outcomes are shown in Table 3. Catalytic plasma concentrations of ALT and AST did not show any significant change after LGF treatment in females, in contrast with the significant decrease in treated male mice, a finding in agreement with the decrease in fatty liver disease in this sex.

Table 3. Effect of LGF administration on necrosis, inflammatory and adipocyte markers in ApoE-deficient mice according to sex

	Female		Male	
	Control	LGF	Control	LGF
ALT (IU/L)	27±14	20±3	44±14	27±9[a]
AST (IU/L)	58±22	73±22	146±80	51±24[a]
IL-6 (pg/mL)	7±8	8±9	16±11	23±20
MCP-1 (pg/mL)	44±22	41±13	57±19	56±20
TNFα (pg/mL)	6±2	5±1	7±2	7±1
Leptin (pg/mL)	852±740	558±112	3316±2546	669±863[a]
Resistin (pg/mL)	1502±289	1563±205	1554±434	947±261[a]

Data are expressed as mean ± standard deviation. Control and LGF mice received intraperitoneal injections of saline or LGF, respectively, twice a week for ten weeks. Statistical analysis was carried out by one-way ANOVA with post-hoc analysis using Fisher LSD test. [a]: P<0.05 vs control.

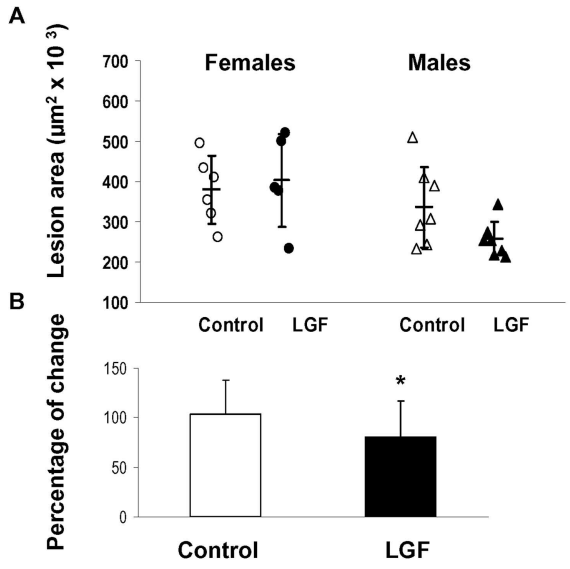

Fig. 4. Sex-dependent effect of LGF administration on atherosclerotic lesion areas in ApoE-deficient mice. **Panel A** shows individual mean values and SD of cross-sectional areas of atherosclerotic lesions. **Panel B**, comparison of male LGF-treated and male control mice from two independent experiments carried out with an interval of one year with diets of different batches of chow. Due to the difference in lesion areas between the two experiments, the effects observed are expressed as percentage of change. *: P<0.05 vs control.

Plasma levels of IL-6, MCP-1 and TNFα were not modified by LGF administration in either sex. Interestingly, plasma levels of leptin and resistin were significantly decreased in male mice after LGF administration, suggesting a sex-related action of this factor on these adipokines.

Association among parameters

In this study, plasma apoA-II levels were negatively associated with hepatic fat content in female mice (Fig. 5A), whereas this association was positive in male mice (Fig. 5B). In the latter sex, the association was influenced by the treatment whose values were in the lower range. Likewise, in males hepatic fat was found to be associated with plasma levels of leptin (Fig. 5C) and resistin (Fig. 5D), and with hepatic *Apoa2* mRNA levels (Fig. 5E), and a direct association between resistin and plasma ApoA-II was also found (Fig. 5F). In addition, significant positive associations were observed among the aortic atherosclerotic lesion and the plasma apoA-II/ apoA-I ratio (Fig. 5G) and very low-density lipoprotein plus low-density lipoprotein cholesterol (VLDL-c+LDL-c) (Fig. 5H), although only in males. The clearly lower values observed for the apoA-II/ apoA-I ratio in LGF-treated mice are indicative of an important role in the male-specific development of atherosclerosis.

Discussion

The long-term intraperitoneal administration of LGF at the dose used here (1.7 µg/animal) in apoE-deficient mice was well tolerated, with no toxicity in terms of body weight gain and no liver damage. Remarkably, LGF had a sex-dependent effect. Thus, in males, it led to a significantly smaller atherosclerotic lesion area and to a lesser degree of hepatic steatosis and circulating aminotransferases. The changes took place in presence of no variation in plasma TG, NEFA or total or HDL cholesterol, and no changes in apolipoprotein A-I or paraoxonase. Insulinaemia was found to be significantly decreased in treated mice of both sexes, although only in females was this translated into hyperglycaemia. However, a decrease in the plasma levels of pro-atherogenic apoA-II was found in males, and the ratio of the main HDL apolipoproteins displayed a strong association with atherosclerosis development, suggesting that the agent may act by improving the anti-atherogenic properties of HDL in this sex. Likewise, decreased levels of adipokines such as leptin and resistin were also observed in males and were associated with the hepatic area occupied by lipid droplets. Based on these findings, this compound is a promising new agent for the control of atherosclerosis and fatty liver disease, with the limiting particularity that only male mice may be able to benefit from it.

This study showed a decrease in atherosclerotic lesion areas in LGF-treated males with no differences between females and males in the control group (Fig.

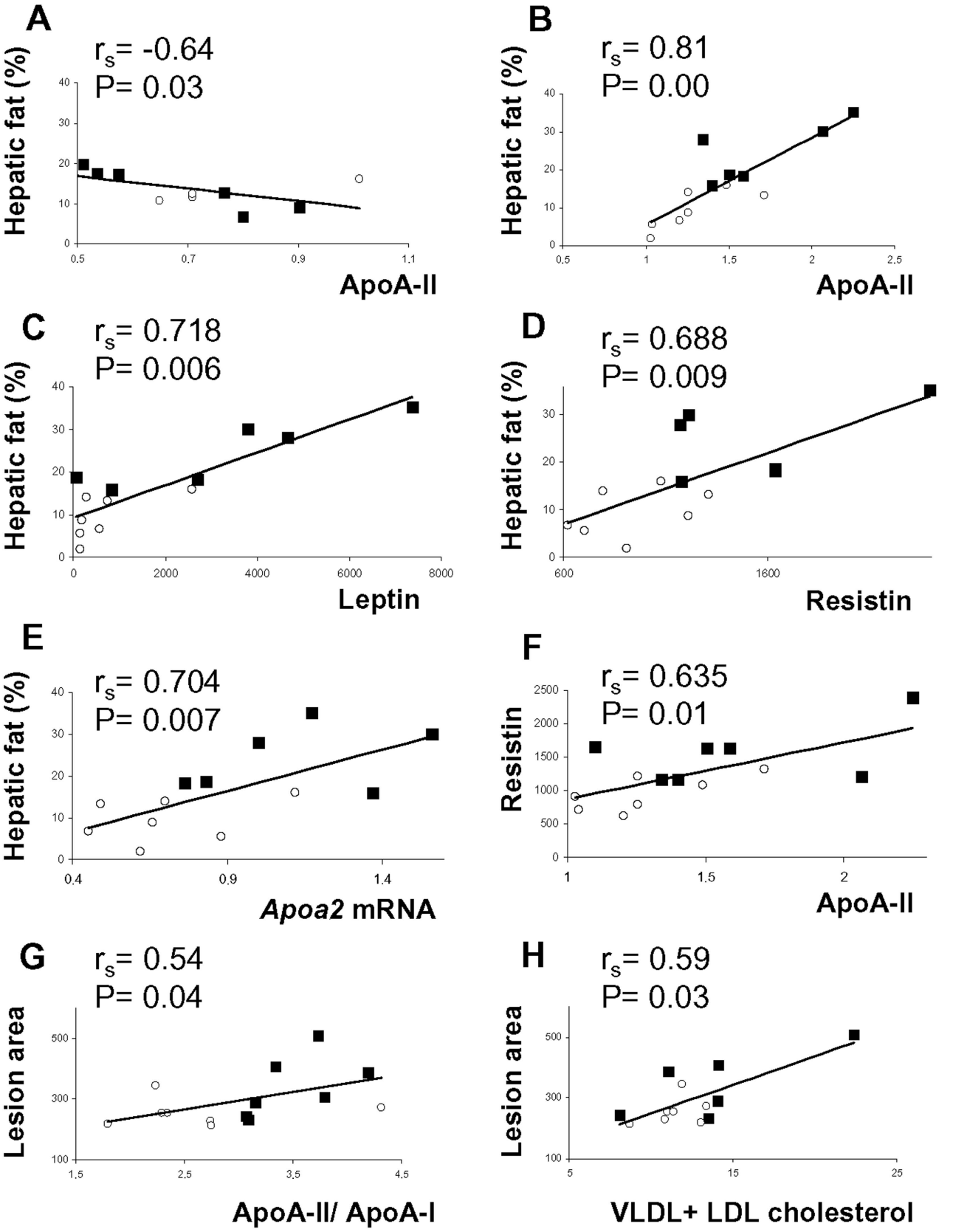

Fig. 5. Relationships among parameters. Association between plasma ApoA-II and hepatic fat content in females **(A)** and in males **(B)**. Associations, in males, between hepatic fat content and plasma leptin **(C)**, plasma resistin **(D)**, and hepatic *Apoa2* mRNA **(E)**. Association between plasma ApoA-II and resistin concentrations in males **(F)**. Also in males, associations between aortic atherosclerotic lesions and the ApoA-II/ApoA-I ratio **(G)** and the lesions and VLDL+LDL cholesterol **(H)**. Correlation analysis was done according to Spearman's test and values corresponding to all experimental groups have been included. Black squares and empty circles correspond to control and LGF- treated mice, respectively.

4A). The latter findings are in agreement with the results of our previous experiments (Acín et al., 2005) and with those of other authors (Caligiuri et al., 1999), despite the reported protective effect of estradiol administration on fatty streak formation in apoE- deficient mice (Elhage et al., 1997). This datum would suggest that only a pharmacological dose of estradiol would make a difference. In our case, the similarity in the atherosclerotic lesion is maintained in spite of the significant differences in plasma cholesterol levels between sexes (Table 2). Since the difference in this parameter between control and LGF-treated males was not statistically significant either, an independent cholesterol effect on male atherosclerotic lesion may be involved in LGF action. A fact that supports this conclusion may also be derived from the association between VLDL-c+LDL-c and the atherosclerotic area shown in Figure 5H, where LGF had no action either. Variations in inflammatory markers such as IL-6, TNF and MCP-1 were not associated with the atherosclerotic changes, either.

Epidemiological and clinical studies support the inverse relationship between HDL-c levels and atherosclerosis (Tall, 1990; Amarenco et al., 2008) and the risk for coronary heart disease (Wilson et al., 1998). Also, apoA-I, a major structural apolipoprotein on the HDL-c particles, protects in part against atherosclerosis, promoting efflux of cholesterol excess from macrophages in the arterial wall (Rader, 2002). Taking data from both sexes, HDL-c and apoA-I levels were inversely correlated with the area of the atherosclerotic lesion (-0.49 and -0.46, $P<0.05$, respectively; data not shown), although both plasma parameters were strongly related to liver weight. Thus, this fact may explain the differences observed between sexes both in HDL-c and in apoA-I levels (Table 2). Interestingly, the liver weight explained the variation of 54% in apoA-I levels. In this respect, when HDL-c and apoA-I levels were expressed in terms of grams of liver, no relationship was detected corroborating the observation that the liver is the main source of these lipoproteins and the origin of the sexual dimorphism regarding these analytes (data not shown). On the other hand, recent pharmacological studies in humans (Kastelein et al., 2007) and dietary experiments in apoE-deficient mice (Arbones-Mainar et al., 2006b) have shown that raised plasma HDL-c in some particular cases may be associated with progression of atherosclerosis. This apparent discrepancy concerning the anti-atherogenic properties of HDL-c may be explained by the heterogeneous class of HDL particles, reflecting the complex relationships that exist between HDL subfractions (Kontush and Chapman, 2006). Some evidence suggests that HDL particles may differ in their composition in apolipoproteins and enzymes that substantially modulate HDL functions. Therefore, not all subtypes of HDL particles are equally efficient in promoting cholesterol efflux as well as anti-inflammatory, pro-fibrinolytic and/or antioxidant activities (Assmann and Nofer, 2003). In this regard, apoA-II, the second most abundant protein in HDL, is predominantly synthesized in the liver (Tailleux et al., 2002; Aouizerat and Kane, 2003), and its physiological role has not been fully defined. ApoA-II has been shown to exert a pro-atherogenic effect in animal models (Warden et al., 1993; Meyers and Kashyap, 2004) that may antagonize the action of apoA-I, impairing the anti-atherosclerotic action of HDL particles (Castellani et al., 2008). This apolipoprotein has been observed to be increased and associated with atherosclerosis in a dietary intervention with linoleic acid isomers (Arbones-Mainar et al., 2006a). The present results provide new evidence to support this interpretation. Thus, in our experimental setting, the significantly smaller atherosclerotic lesions observed in LGF-treated males (Fig. 4B) corresponded with lower apoA-II values in animals receiving the agent than in controls (Table 2). It has been shown that an increase in the apoA-II contained in HDL-c may promote a remodelling of HDL-c particles by displacing apoA-I from the lipoprotein (Aouizerat and Kane, 2003), rendering the particle less efficient (Castellani et al., 1997). The opposite may have a beneficial effect, as our results indicate. Indeed, a significant and positive association was observed between the ratio of apoA-II/apoA-I and the atherosclerotic lesion (Fig. 5G), where male animals receiving the agent showed more favourable results. The difference in the starting plasma levels of apoA-II between females and males may play a role in this peculiar sex-dependent benefit (Table 2).

On the other hand, Castellani et al. (2008) have proposed that one function of apoA-II is to regulate the metabolism of TG-rich lipoproteins, with HDL serving as a plasma reservoir of apoA-II that is transferred to the TG-rich lipoproteins in much the same way as VLDL and chylomicrons acquire most of their apoC from HDL. Based on the maintenance of plasma TG and cholesterol (Table 2), and the change observed in apoA-II after LGF treatment, this possibility is not likely to explain our results either.

It is a well-documented fact that apoE-deficient mice fed a chow diet develop hepatic steatosis (Mensenkamp et al., 2001; Arbones-Mainar et al., 2006a; Guillen et al., 2008), attributed to an impairment of VLDL-TG secretion and consequent accumulation of these particles (Kuipers et al., 1997). Also, our present results, in agreement with previous findings by Guillen et al. (2008), point out that male apoE-deficient mice consuming the chow diet had a higher hepatic fat content and smaller atherosclerotic lesion area than females, indicating a sex-dependent difference in the outcome and its involvement in atherosclerosis development. In fact, in the present report (data not shown), the hepatic fat was also associated positively with the lesion area (0.59, $P<0.05$) in males. The significantly lowered hepatic fat content due to LGF administration in males (Fig. 1) suggests a sex-specific regulation of intracellular lipid metabolism, because no significant changes were observed either in plasma TG as the outcome product or in plasma NEFA as the incoming substrate (Table 2). In

this respect, it is interesting to highlight that there was an opposite trend between sexes in the significant correlations between plasma apoA-II and liver fat (Fig. 5a,b): positive for males (rs=0.81, P<0.00) and negative for females (rs=-0.64, P<0.03). Overall, these results suggest a sex-dependent connexion between hepatic lipids and apoA-II, and would agree with the data indicating a sexual regulation by estrogens (Jeong and Yoon, 2007). However, our data provide a new endocrine paradigm of regulation: both leptin and resistin were found to be associated with morphometrically measured hepatic fat (Fig. 5C,D) in males, and the latter parameter was associated with hepatic expression of *Apoa2* mRNA (Fig. 5E). Furthermore, plasma resistin levels were also significantly associated with those of apoA-II (Fig. 5F). Contradictory results concerning the actions of these adipokines have been observed. Thus, the role of leptin in insulin resistance remains unclear, as does that of resistin (Antuna-Puente et al., 2008), and the hepatic lipid content in this setting has not been assessed. The present experimental approach provides several interesting outcomes; first, insulin levels are quite sensitive to LGF administration irrespective of sex. Second, low levels of insulin are not translated into hyperglycaemia, or an increased triglyceridaemia when accompanied by decreased levels of resistin and leptin in males. The opposite takes place in females only for glycaemia. Finally, the target organ for the consequences of insulin, resistin and leptin variations due to LGF administration is clearly the liver, where the TG and cholesterol content were decreased in males, as were the number and size of lipid droplets (Fig. 1). It has recently been proposed that the smaller the size of the latter, the more active they become in delivering the fatty acids of their TG to the different metabolic fates (Puri and Czech, 2008). In this respect, the finding of a significant association between morphometrically evaluated hepatic fat and the hepatic expression of Apoa2 mRNA is suggestive (Fig. 5E). Why this gene is so particularly sensitive to these changes and whether this is exerted through the release of lipid mediators or other indirect actions, such as variations in hypoxia factors, are questions for further research. In this line, the anti-steatosis activity of LGF provides an additional regenerative/preventive property, due to the decrease in physical impairment that large lipid droplets pose to a correct oxygenation and nutrition of hepatocytes, and the precursor role of fatty liver in the development of more serious diseases, such as cirrhosis (Jou et al., 2008; Turkish 2008).

In conclusion, long-term LGF administration was a relatively safe alternative to reduce the atherosclerotic lesion and hepatic steatosis in a sex-dependent manner. The first effect was independent of plasma cholesterol, TG and inflammatory markers, and related to a remodelling of HDL particles through loss of apoA-II. The second effect was in close connection with a particular hormonal milieu of low insulin, leptin and resistin levels, the latter levels being significantly associated with apoA-II. Overall, these results point to a difference in the regulation of this apolipoprotein between sexes in response to this agent, and introduce sex as an important variable to take into consideration to predict outcomes in situations in which there are elevated levels of circulating LGF, such as cirrhosis, with absence of atherosclerosis.

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