Short-term treatment of spontaneously hypertensive rats with liver growth factor reduces carotid artery fibrosis, improves vascular function, and lowers blood pressure

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Abstract

Objective: Liver growth factor (LGF), a mitogen for liver cells, reduces fibrosis in a rat model of cirrhosis. The present study assesses the possible vascular antifibrotic and antihypertensive effects of LGF treatment on spontaneously hypertensive rats (SHR).

Methods: Six-month-old male SHR and normotensive Wistar Kyoto rats (WKY) were treated with LGF (4.5 μg LGF/rat i.p. twice a week for 2 weeks). Haemodynamic parameters were measured in anaesthetized rats. Vascular structure and function were studied in carotid arteries using optical and confocal microscopy, radioimmunoassay for desmosine, and isometric tension recording.

Results: LGF reduced systolic and diastolic blood pressure only in SHR. When compared to those of untreated SHR, carotid arteries from LGF-treated SHR showed: 1) a 50% reduction in collagen area and an increase in vascular smooth muscle cell number in the media, 2) no difference in total elastin content, but an increase in size of fenestrae in the internal elastic lamina, and 3) enhanced relaxation to acetylcholine, sodium nitroprusside, and forskolin. These effects were specific for SHR, since no changes were observed in LGF-treated WKY.

Conclusion: Short-term treatment with a low dose of LGF induced a large improvement in vascular structure and function and significantly reduced blood pressure in a rat model of essential hypertension. The present results could open future research to explore the vascular effects of this endogenous factor in order to determine its potential as an antifibrotic and antihypertensive agent in humans.

Keywords: Arterial fibrosis; Liver growth factor; Hypertension; Vasodilatation; Extracellular matrix

1. Introduction

Conduit arteries play a key role in the cardiovascular system, buffering pressure pulsations during the cardiac cycle. Increasing attention is being focused on changes of large arteries in hypertension which increase in stiffness, thereby loosing this buffering function [1,2]. Thus, large artery compliance and distensibility have been shown to be reduced in human hypertension and these alterations are known to influence the development of complications in hypertensive vascular disease [1–5].

The fibrous components of the extracellular matrix (ECM), collagen and elastin, are the main elements

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Liver growth factor (LGF), a hepatic mitogen with both in vivo and in vitro activity, was purified by Diaz-Gil and co-workers some years ago [12]. Following an in-depth chemical and immunological study, this group demonstrated that LGF is an albumin–bilirubin complex [13,14]. LGF is nearly undetectable in sera from healthy humans or rats, but its concentration is dramatically increased in the presence of hepatobiliary disorders or liver injury [15,16]. In a model of CCl4-induced cirrhosis induced in rats, LGF injection decreased total liver collagen, restored partial architectural integrity, necrotic tissue and serum enzymes, reducing ascites and improving hemodynamics [17]. Diaz-Gil has recently demonstrated that the first targets of LGF in liver are the endothelial cells around portal spaces, which secrete TNF-α, initiating the mitogenic pathway [18]. It is worth noting that, in endothelial cells in vitro, LGF acts at a very low concentration (5 pg/ml) [18], much lower than that required for the hepatocyte growth factor (HGF) to act on the same cells. HGF, another growth factor, the chemical structure of which is different from that of LGF, is active in the ng/ml range [19].

The reduction of liver fibrosis by LGF treatment leads us to hypothesize that LGF might also exert antifibrotic actions in other diseases, such as hypertension. Therefore, we have investigated the vascular effects of LGF in spontaneously hypertensive rats (SHR), a model of hypertension that involves vascular fibrosis, abnormal wall architecture and endothelial dysfunction [1].

2. Methods

LGF was purified from rat serum following the procedure previously reported by Diaz-Gil and co-workers. Purity of the LGF preparation was assessed according to standard criteria [12,20]. LGF preparations were lyophilized and stored at 4°C until used, at which time, aliquots were dissolved in saline for i.p. injection. Before using LGF in these experiments, we checked its activity in vivo at several doses, injecting it 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, Dreiech, Germany) into DNA [12]. The optimal dose of LGF used was 4.5 μg/rat.

2.1. LGF treatment of WKY and SHR rats

Six month old male rats were randomly divided in two groups: LGF-treated (LGF-SHR, n=20; LGF-WKY, n=13) and untreated (SHR, n=21; WKY, n=18). LGF treatment was carried out as follows: LGF (4.5 μg/rat) was injected i.p. at 3–4 day intervals four times (days 0, 3, 6 and 9). At day 12, haemodynamic parameters were measured in all rats under anesthesia (50 mg/kg sodium pentobarbital i.p.), through a cannula inserted in the right iliac artery connected to a pressure transducer (Statham, Harvard Apparatus GmbH, Germany). Systolic and diastolic blood pressures (SBP, DBP, respectively) and heart rate (HR) were measured. All procedures conformed to the Guidelines for Care and Use of Laboratory Animals (NIH publication No. 85-23, revised in 1996; RD 223/1988).

2.2. Rat carotid artery structure

After haemodynamic parameter measurements, a group of rats (WKY n=8, LGF-WKY n=6, SHR n=8 and LGF-SHR n=8) was perfused with a 4% solution of paraformaldehyde (PFA) at a flow rate of 1 ml/min per 100 g of rat weight for 3 h [21]. At the end of the perfusion both common carotid arteries were dissected and kept in 4% PFA. One of the arteries was embedded in paraffin. Transversally cut sections were stained with Massons trichromic method for determination of the collagen area. The contralateral carotid artery was kept in 4% PFA and was used to determine structural parameters (internal diameter and media thickness), the number of VSMC in the media, elastin content and organization of the internal elastic lamina (IEL) by confocal microscopy. From this second artery one ring and one longitudinal section were obtained. Rings were stained with the nuclear dye Hoechst 33342 (10 μM) for 10 min at room temperature and washed for 15 min. They were then visualized with a Leica TCS SP2 confocal system (Leica Microsystems, Germany, Ex 364nm) and 10 serial optical images of the ring (1 μm thick) were obtained with a 10× objective (for determination of the internal diameter) and 40× (for determination of the media thickness and VSMC number). From these serial images, VSMC were counted with Metamorph Image Analysis software (Universal Imaging Corporation, Downingtown, PA, USA) and the total number was calculated in a volume, taking into account media thickness of the ring. To visualize the IEL, longitudinal sections from the carotid arteries were mounted on a slide with the endothelial side facing up and visualized with the 488/515 nm line of the microscope to detect elastin (autofluorescent at this wavelength). Stacks of serial optical sections (0.5 μm thick) were captured from each artery with a 63× oil-immersion objective under identical conditions of laser intensity, brightness and contrast and a maximal projection was obtained. Quantification of the relative area occupied by fenestrae was performed in binary images with Metamorph.
software [22]. In the remaining segment of carotid artery the elastin content was estimated by radioimmunoassay for desmosine, a crosslinked residue specific unique to elastin [23], and total protein content assessed by ninhydrin-based assay, as previously described [24,25].

2.3. Rat carotid artery function

Vascular function was studied in carotid arteries (WKY n = 7, LGF-WKY n = 6, SHR, n = 9; LGF-SHR, n = 7) by isometric tension recording [26]. Concentration-response curves to Ach (1 nM to 100 μM), forskolin (0.1 nM to 1 μM) and sodium nitroprusside (SNP, 1 pM to 1 μM) were then generated for endothelial-intact segments pre-contracted with 0.1 μM noradrenaline (NA). All experiments were performed simultaneously in segments from untreated and LGF-treated SHR and WKY.

2.4. Statistical study

Comparisons between groups were made by performing a one-way or two way analysis of variance (ANOVA), using Graph Pad Prism 04. Post-hoc comparisons were carried out with the Newman–Keuls test. pD2 values were calculated by log-probit analysis according to the standard method. Statistical significance was set at p<0.05.

3. Results

3.1. LGF treatment reduced blood pressure only in SHR.

Body weight was similar between strains, and was not modified by treatment in either SHR or WKY rats. SBP, DBP and HR were significantly higher in SHR when compared to WKY. LGF treatment significantly reduced SBP and DBP, but not HR, in SHR. However, LGF treatment did not modify any of the haemodynamic parameters in WKY rats (Table 1).

3.2. LGF treatment improved carotid artery structure in SHR

LGF treatment did not change the internal diameter or media thickness in rat carotid arteries from either WKY or SHR (Table 2). On the other hand, in SHR LGF significantly altered carotid artery composition, modifying both extracellular and cellular components in the media (Fig. 1B, C and D). Carotid collagen content was reduced by half after LGF treatment (Fig. 1B and C). This was paralleled by an increase in VSMC number and a reduction in VSMC nuclear size in LGF-SHR, as compared to untreated SHR (Fig. 1B and D). This was confirmed by the inverse correlation observed between medial collagen content and cell number ($R^2 = -0.71$, p < 0.05). There was also a positive correlation between collagen content and SBP ($R^2 = 0.83$, p < 0.05), as well as an inverse correlation between VSMC number and SBP ($R^2 = -0.6$, p < 0.05). In WKY rats, collagen content was much lower when compared to SHR, and was not modified by LGF treatment (Fig. 1A and C). The only change induced by LGF in carotid arteries from WKY rats was a significant increase in cell number in the media (Fig. 1D).

LGF significantly enlarged the area occupied by fenestrae in the IEL of carotid arteries from SHR, but not from WKY (Fig. 2A and B). The amount of elastin in carotid arteries, measured by desmosine content, was significantly greater in SHR than in WKY, although it was not significantly modified by LGF treatment (Fig. 2C).

3.3. LGF treatment improved carotid artery vasodilator capacity in SHR

In carotid arteries from SHR, LGF-treatment significantly increased relaxation to the endothelium-dependent vasodilator acetylcholine (Fig. 3A) and to the endothelium-independent vasodilators forskolin and sodium nitroprusside (SNP), which stimulate cAMP and cGMP formation, respectively (Fig. 3B and C). Concentration-response curves to all the vasodilator agents tested were significantly shifted to the left with a significant reduction in pD2 values and an increase in maximal response. This effect was specific for

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>WKY (n=18)</th>
<th>SHR (n=21)</th>
<th>LGF-WKY (n=13)</th>
<th>LGF-SHR (n=20)</th>
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<tr>
<td>Body weight (g)</td>
<td>378 ± 9.0</td>
<td>389 ± 7.3</td>
<td>384 ± 13.0</td>
<td>405 ± 12.9</td>
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<tr>
<td>SBP (mm Hg)</td>
<td>122 ± 5.7</td>
<td>192 ± 7.0*</td>
<td>125 ± 6.2</td>
<td>163 ± 5.1*</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>89 ± 6.7</td>
<td>148 ± 5.7**</td>
<td>90 ± 5.4</td>
<td>122 ± 4.5*</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>251 ± 12.1</td>
<td>332 ± 14.0*</td>
<td>269 ± 9.5</td>
<td>316 ± 10.2</td>
</tr>
</tbody>
</table>

SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto rats. Rat number is indicated in parentheses. * p < 0.01 LGF-treated SHR or WKY versus the respective untreated rats; ** p < 0.01 untreated SHR versus untreated WKY.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>WKY (n=8)</th>
<th>SHR (n=8)</th>
<th>LGF-WKY (n=6)</th>
<th>LGF-SHR (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal diameter (μm)</td>
<td>696 ± 14.2</td>
<td>657 ± 69</td>
<td>703 ± 19</td>
<td>726 ± 40</td>
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<tr>
<td>Media thickness (μm)</td>
<td>68 ± 1.6</td>
<td>73.5 ± 12</td>
<td>71 ± 2.8</td>
<td>65 ± 12</td>
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</tbody>
</table>

SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto rats. Rat number is indicated in parentheses.
4. Discussion

The main finding of this work is the demonstration for the first time that the antifibrotic mitogen LGF induces striking changes in vascular composition and function, together with a substantial blood pressure reduction in SHR. These effects are achieved at a low dose of LGF within a short period of treatment.

LGF treatment significantly reduced both SBP and DBP in SHR, but not in WKY rats, suggesting that its effects are specific for the hypertensive state. The fact that blood pressure reduction was not accompanied by changes in HR suggests that this factor might exert its effects by modifying vascular structure and/or function in SHR, both of which are known to be altered in hypertension [7,11,27]. This vascular effect does not exclude other possible actions on the central nervous system, including the baroreceptor reflex. LGF treatment did not change gross carotid artery geometry, but it significantly altered wall composition, modifying both extracellular and cellular components of the vascular media in SHR. Thus, medial collagen content was reduced by nearly 50% by treatment, while there was an increase in VSMC number. This inverse correlation between collagen and cell number suggests that collagen in carotid arteries from SHR was replaced by VSMC during LGF treatment. One of the abnormalities of VSMC in hypertension is the increase in polyplody [10]. This is most likely related to a defect in mitotic spindle cell cycle check point [28], so that cells replicate their DNA but do not undergo cell division [29,30]. The increase in VSMC number, together with the reduction in nuclear size induced by treatment, would suggest that LGF reduces ploidy by stimulation of mitosis. This finding is not surprising due to the reported mitogenic activity of LGF for other cell types [12]. In WKY rats, LGF also increased VSMC number but it did not modify collagen content. It is therefore possible that LGF stimulates the proliferation of the mature VSMC phenotype. This is of particular importance since there is strong evidence that the switch of VSMC to an immature phenotype plays a major role in a number of vascular diseases such as atherosclerosis and hypertension [31]. The striking antifibrotic and mitotic effects of LGF on elastic arteries of hypertensive animals would suggest that the action of LGF is not restricted to the liver, and that LGF might in fact be a pleiotropic agent with wider scope.

The present study demonstrates that LGF also has an effect on elastin, the resilient protein of the vascular wall. Elastin is a major component of conduit arteries [32] and, together with collagen, is a key protein for vascular
mechanical properties [33]. It has been previously documented that the elastin content of large arteries is increased by hypertension [34]. This was confirmed in our study, where the desmosine content was significantly higher in SHR carotid arteries compared to those of WKY. However, newly synthesized elastin under pathological conditions may not be as physiologically effective as that produced during development [33]. In fact, we have previously reported that in the IEL from SHR carotid [9] and resistance arteries [22], elastin is abnormally organized, with a reduced fenestra area. The present study demonstrates that total elastin content was not significantly altered by LGF, but that treatment enlarged fenestra area in SHR carotid arteries. This is of special interest, since we have recently reported that, in small arteries, the IEL fenestra area plays a more prominent role in intrinsic vascular mechanical properties than total elastin content [35]. Therefore, the increase in fenestra size, together with the reduced collagen content, induced by LGF treatment in SHR carotid arteries, is likely to improve vascular mechanical properties in these vessels.

The above mentioned structural modifications were accompanied by a significant increase in relaxation only in SHR. The fact that this enhancement was observed in both endothelium-dependent and independent agents, acting via different pathways (cGMP, cAMP), suggests that LGF exerts these effects through a general improvement of wall architecture. It is known that fibrosis induced by excessive collagen deposition influences arterial responses to vasodilators by...
limiting the expansion of the vessel wall [11]. Moreover, the fact that the first target for LGF action in liver is the endothelium [18], does not exclude the possibility that the positive effect of LGF on vasodilatation might be partially due to an enhancement of vasoactive endothelial factors. In studies related to this concept, other authors have detected two specific receptors for “modified albumin” (albumin with some ligand bonds) in endothelial cells [36,37], that resemble LGF, an albumin–bilirubin complex [13]. Whether these receptors bind LGF and initiate the regeneration cascade remains to be determined. In addition, LGF could also improve vasodilatation through an antioxidant effect, as previously reported in hepatocytes, erythrocytes and myocytes [38–40]. Safar and co-workers have highlighted the importance of both the ECM and the endothelium for large artery compliance [41]. Therefore, it is likely that LGF-treatment might improve vascular mechanics through normalization of both cellular function and the ECM.

We would like to stress the short period and low dose of LGF treatment needed to reduce fibrosis, to increase vasodilatory responses and to reduce blood pressure. This is especially relevant if we compare it with classical pharmacological treatments that usually require several months to improve vascular structure and function [42–44].
In summary, two-week LGF-treatment induces striking changes in elastic artery structure, function and blood pressure in SHR. These results could open future research to explore the vascular effects and mechanism of this endogenous factor in order to determine its potential as an anti-inflammatory and antihypertensive agent.

Acknowledgements

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