Intrauterine growth restriction promotes vascular remodelling following carotid artery ligation in rats

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ABSTRACT

Epidemiological studies revealed an association between IUGR (intrauterine growth restriction) and an increased risk of developing CVDs (cardiovascular diseases), such as atherosclerosis or hypertension, in later life. Whether or not IUGR contributes to the development of atherosclerotic lesions, however, is unclear. We tested the hypothesis that IUGR aggravates experimentally induced vascular remodelling. IUGR was induced in rats by maternal protein restriction during pregnancy (8% protein diet). To detect possible differences in the development of vascular injury, a model of carotid artery ligation to induce vascular remodelling was applied in 8-week-old intrauterine-growth-restricted and control rat offspring. Histological and immunohistochemical analyses were performed in the ligated and non-ligated carotid arteries 8 weeks after ligation. IUGR alone neither caused overt histological changes nor significant dedifferentiation of VSMCs (vascular smooth muscle cells). After carotid artery ligation, however, neointima formation, media thickness and media/lumen ratio were significantly increased in rats after IUGR compared with controls. Moreover, dedifferentiation of VSMCs and collagen deposition in the media were more prominent in ligated carotids from rats after IUGR compared with ligated carotids from control rats. We conclude that IUGR aggravates atherosclerotic vascular remodelling induced by a second injury later in life.

INTRODUCTION

Numerous epidemiological and animal studies suggest that besides the well-known candidates such as arterial hypertension, diabetes mellitus and hypercholesterolemia, IUGR (intrauterine growth restriction) is a major risk factor of atherosclerosis [1,2]. The incidence of diseases of the metabolic syndrome is significantly higher in IUGR patients and IUGR therefore promotes the development of atherosclerotic lesions at least in an indirect manner [3]. On the other hand, findings from human and animal studies propose an association between an early detectable alteration of vascular structure and composition and low birth weight [4–7]. These observations suggest that IUGR may also directly increase the vascular susceptibility to atherosclerosis, independent of its effect on classical risk factors.

The quantity of animal models capable of inducing IUGR reflects the numerous underlying pathomechanisms [8]. In this study, the maternal LP (low-protein)

Key words: atherosclerotic lesion, carotid artery ligation, intrauterine growth restriction, maternal protein restriction, vascular remodelling.

Abbreviations: BP, blood pressure; CTGF, connective tissue growth factor; IUGR, intrauterine growth restriction; LP, low-protein; NP, normal-protein; PAS, periodate–Schiff; PCNA, proliferating-cell nuclear antigen; VSMC, vascular smooth muscle cell.

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diet model in the rat was used because it is easy to handle and highly reproducible. Using this model, we were able to show that maternal protein restriction during pregnancy leads to aortic overexpression of profibrotic cytokines in the newborn offspring and an increased vascular deposition of collagens later in adolescence [9]. Skilton et al. [10] reported that after maternal LP diet reduced aortic wall thickness and elastin content could be detected in 12 week old IUGR animals.

The alteration of arterial blood flow induces vascular remodelling and fosters the development of subsequent atherosclerotic vascular lesions [11]. In the present study, we used an animal model of vascular injury originally developed by Kumar and Lindner [12] in which the ligation of the left common carotid artery leads to proximal vascular remodelling with neointimal formation and reduced luminal area. Recent animal studies provided evidence that intrauterine hypoxia, one of the main pathogenetic factors of IUGR, leads to manifest cellular and structural changes of the aortic tunica intima [2]. Against this background we tested the hypothesis that in our rat model of IUGR the ligation of the carotid artery leads to aggravated vascular remodelling and neointimal formation.

MATERIALS AND METHODS

Animal procedures

All procedures performed on animals were carried out in accordance with guidelines of the American Physiological Society, conform to the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the local government authorities (Regierung von Mittelfranken, AZ No. 54-2531.31-12/06).

Virgin female Wistar rats (220–300 g) were obtained from Charles River and were housed in a room maintained at 22 ± 2 °C, exposed to a 12 h dark/12 h light cycle. The animals were allowed unlimited access to standard chow (No. 1320; Altromin) and tap water. A total of eight dams were time-mated by the appearance of sperm plugs and then fed on a diet containing 17.2 % protein (casein) or an isocaloric diet containing 8.4 % protein (casein) throughout pregnancy. Diets were obtained from Altromin (#C1000 and C1003). Sodium content (0.25 %) of both diets was equal. Rats delivered spontaneously with comparable litter sizes in both diet groups and the litters were reduced to six male pups per dam as described previously [13] to achieve equal lactation conditions. During lactation, rat mothers were fed standard chow. The offspring was nursed by their mothers until weaned at day 21 to standard chow. The animals used for experiments were derived from four litters in each group. At 8 weeks of age, vascular remodelling was induced by dissecting the left common carotid artery and ligating it with 6.0 silk (Ethicon) near the carotid bifurcation as described for mice by Kumar and Lindner [12] in xylazine/ketamine anaesthesia. All ligated rats recovered without any symptoms of stroke. For evaluation of vascular lesions, we chose a time point 8 weeks after ligation, because in a pilot study vascular changes after 4 weeks of ligation were still very weak. At 8 weeks after ligation of the carotid artery the animals [12 offspring from the LP-treated group and 12 offspring from the NP (normal protein)-treated group] were killed for histological evaluation. Carotids were perfused with physiological saline solution and subsequently perfusion fixed with methyl carnoy solution via the left ventricle similar as described by Kumar and Lindner [12]. The unligated right common carotid arteries served as controls, because Kumar and Lindner [12] did not detect any differences between the unligated right carotid arteries and carotid arteries from unmanipulated animals regarding morphometric parameters. The left and right common carotid arteries were excised. To avoid the analysis of unspecific changes caused by the ligature itself, care was taken to exclude the most proximal part of the carotid to the ligation from histological evaluation. From the proximal part of the vessels near the bifurcation 2 mm and from the distal part adjacent to the aortic arch 5 mm were discarded. Then, they were immersed in methyl carnoy solution and embedded in paraffin. Serial sections were cut for morphological and immunohistochemical analyses.

Serum measurements

For determination of serum cholesterol, blood samples were obtained when animals were killed by heart puncture and were analysed with the automatic analyser Integra 800 (Roche Diagnostics).

Histological analysis

Paraffin-embedded sections were PAS (periodate–Schiff) stained. All histological analyses were done using a Leica DMR microscope (Leica Instruments) and a Nikon Digital Sight DS-U1 Camera. The cross-sectional areas of the media, the lumen and the neointima were measured by computer-assisted area integration (Metaview software; Visitron Systems). The inner and outer perimeter of the media was traced, and media thickness and media area as well as lumen diameter and lumen area was calculated from the perimeter data (based on a cycle perimeter formula). Media/lumen ratios and the size of neointimal area were calculated from these results.

Immunohistochemistry

Paraffin-embedded vascular sections were layered with the primary antibody, and incubated at 4 °C overnight. After addition of the secondary antibody [dilution 1:500; biotin-conjugated goat anti-(rabbit IgG) or rabbit

Table 1 Body weights and serum cholesterol in offspring after maternal LP and NP diet

Values are means (95 % CI); $n = 12$ litters from four NP dams and 12 litters from four LP dams. LP, offspring of dams with low-protein diet; NP, offspring of dams with normal-protein diet.

	Offspring of dams fed on an		
Parameter	NP diet	LP diet	*P* value
Birth weight (g)	6.48 (6.32–6.65)	5.3 (5.15–5.45)	<0.001
Body weight at 16 weeks (g)	495.1 (477.11–513.09)	465.54 (446.75–484.32)	0.043
Total serum cholesterol (mg/dl)	58.97 (46.59–71.35)	63.45 (50.48–76.43)	0.624

anti-(mouse IgG); all from Dianova], the staining procedures were carried out by a peroxidase detection method as described previously [14]. Each slide was counterstained with haematoxylin/eosin. As a negative control, we used equimolar concentrations of pre-immune rabbit or mouse IgG. To detect macrophages, sections were stained with a monoclonal antibody against ED-1 at a dilution of 1:250 (Serotec, Biozol), proliferating cells were stained with a monoclonal antibody against PCNA (proliferating-cell nuclear antigen) at a dilution of 1:50 (Dako). Counting of proliferating cells was performed after staining. PCNA-positive cells per cross-section were counted. To relate PCNA-positive cells to the total number of VSMCs (vascular smooth muscle cells), cell nuclei were counted in the media of carotid cross sections. Smooth muscle actin was detected with a monoclonal antibody (1:50 dilution) from Serotec. The primary rabbit antibody against collagen I was from Biogenesis and used at a dilution of 1:1000. The rabbit polyclonal antibody against collagen IV was used at a 1:500 dilution (Southern Biotechnology Associates). Expansion of smooth muscle actin, and collagen I and IV staining in the media was evaluated using Metaview software (Visitron Systems). The stained area was expressed as a percentage of the total medial area.

Statistical analysis

Quantile–quantile plots were used to assess the normality of the data. After having verified that the outcome variables of interest were normally distributed, we used linear mixed model analysis to analyse the data [15]. To account for the fact that measurements obtained from the same dam were not independent, we included a normally distributed random 'dam' effect into the mixed model equation. Likelihood ratio tests were applied to detect significant effects of diet and ligature on the respective outcome variables. All *P* values obtained from statistical hypothesis tests were adjusted for multiple comparisons [16]. Results were considered significant when the *P* value was less than 0.05. All statistical analyses were performed using statistical software package R, version 2.13 [17].

RESULTS

Average birthweights of the offspring born to dams fed on the LP diet were significantly lower than the birthweights of the offspring of dams fed on the NP diet throughout pregnancy (Table 1; $P < 0.001$). LP offspring somewhat caught up with their body weights during the first weeks of life, but at the age of 16 weeks, average body weights of the LP offspring were still significantly different from NP offspring (Table 1; $P = 0.043$). Total serum cholesterol levels were comparable in NP and LP offspring at 16 weeks of age (Table 1).

Ligation of the left carotid artery for 8 weeks led to vascular remodelling, similar to the one described for mice [12] and shown in Figure 1. Neointima formation was observed in the ligated carotid arteries in two out of 12 NP animals and in the ligated carotid arteries of ten out of 12 LP animals. In neointimal areas, macrophage infiltration was commonly observed (Figure 1). In control carotid arteries, no neointima formation was detected (Figure 1). The size of the area of neointima was measured in serial sections of ligated carotid arteries in NP and LP offspring, i.e. between 2 and 9 mm from the ligature neointimal area was assessed every 1 mm. Neointimal size was smaller on average in NP as compared with LP until 9 mm distal from the ligature (Figure 2). Differences between NP and LP offspring were significant until 6 mm distal from the ligature (Figure 2). Further histological and immunohistological analyses were performed in sections from the middle part of the embedded carotid arteries.

The area of the carotid artery media was not significantly different between groups (Table 2). However, the average media was significantly thicker in ligated carotid arteries of LP than in ligated carotid arteries of NP rats ($P = 0.005$, Figure 3a). There was a significant increase in average media thickness in carotid arteries after ligation in LP animals ($P = 0.002$, Figure 3a), while in NP animals, no significant differences were observed between ligated and control carotid arteries (Figure 3a). Lumen diameters are reduced most prominently in LP after ligation (mean difference, 254.2 μm; $P < 0.001$ (Table 2). In NP, also a significant reduction in lumen diameter

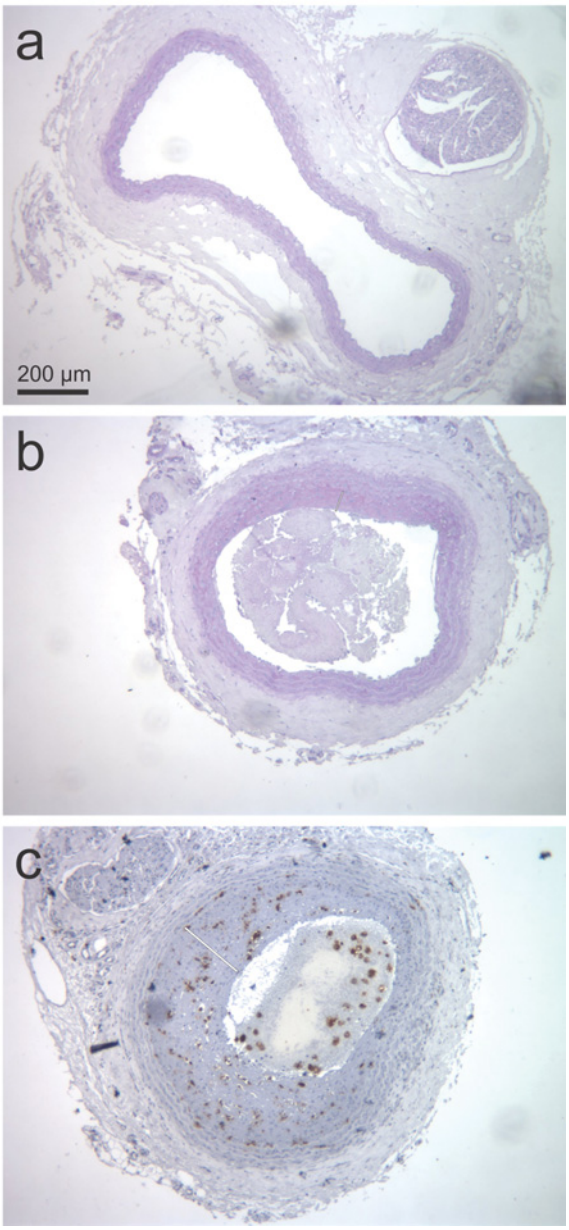

Figure 1 Neointima formation after carotid artery ligation

Representative photomicrographs showing (a) a control right carotid artery (PAS stain) and (b) a carotid artery after 8 weeks of ligation (PAS stain). (c) Immunohistochemical detection of macrophages (ED-1; brown stain) in a ligated carotid artery. The white bar marks the thickness of the neointima.

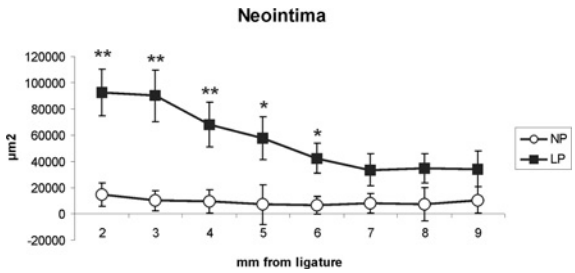

Figure 2 Neointima formation in ligated carotid arteries of LP-treated IUGR and NP-treated rats

Values are means ± S.E.M. ($n = 12$ litters from four NP dams and 12 litters from four LP dams.), as obtained from linear mixed effects models. Mean differences between LP-ligated carotid artery and NP-ligated carotid artery were significant until 6 mm from the ligature. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the respective NP-ligated carotid artery.

was shown after ligation (mean difference, 105.6 μm; $P < 0.001$ (Table 2). Comparing the effect of carotid artery ligation on lumen diameters in LP and NP animals, a more severe reduction in LP animals could be detected. ($P = 0.041$; Table 2). Media/lumen ratios, both with regard to area and diameter, were significantly increased in LP after ligation ($P < 0.001$; Figure 3b and Table 2). In contrast, media/lumen ratios in NP animals were only significantly increased with regard to area after ligation ($P = 0.016$; Figure 3b) but not with regard to diameter ($P = 0.104$; Table 2). Ligation resulted in higher media/lumen ratios in LP than in NP animals, both with regard to area and diameter ($P \leqslant 0.013$; Figure 3b and Table 2). Dedifferentiation of smooth muscle cells was assessed after staining with α-smooth muscle actin. Ligation of the carotid arteries led to dedifferentiation of smooth muscle cells in the media, both in NP and LP offsrping ($P < 0.001$; Figure 3c). Carotid arteries of LP offspring were more strongly affected by smooth muscle cell dedifferentiation in the media due to ligation than carotid arteries of NP animals (Figure 3c).

Ligation of the carotid arteries resulted in a significant reduction of total cell number in the media. This effect was observed for both LP and NP animals ($P \leqslant 0.012$; Table 2). The percentage of proliferating cells tended to be lower in LP animals than in NP, regardless of whether the carotid artery was ligated or not (Table 2). The difference in media thickness between ligated carotids from NP and LP animals (Figure 3a) was reflected by an increased deposition of collagens I and IV in the media of ligated carotids from LP animals compared with ligated carotids NP animals ($P \leqslant 0.012$, Figure 4).

DISCUSSION

The main finding of the present study is that in the animal model of maternal protein restriction IUGR aggravates the course of vascular remodelling and neointimal formation after acute arterial injury. Altered arterial flow and endothelial shear stress are both relevant pathomechanisms in the development of atherosclerosis [11]. The model of left carotid artery ligation was chosen because, in contrast with other experimental models of acute arterial injury, e.g. balloon angioplasty, the integrity of the endothelium is not affected. Furthermore, defining the paired carotid as target-vessel provides the

Table 2 Morphometric parameters, total cell number in the media and media proliferation of carotid arteries in offspring after maternal LP and NP diet.

Values are means (95% CI); $n = 12$ litters from four NP dams and 12 litters from four LP dams.

	Control carotid artery			Ligated carotid artery			*P* value†	
	Offspring of dams fed on an			Offspring of dams fed on an			Offspring of dams fed on an	
Parameter	NP diet	LP diet	*P* value*	NP diet	LP diet	*P* value*	NP diet	LP diet
Area of media (mm^2)	0.15 (0.12–0.18)	0.16 (0.13–0.19)	0.844	0.15 (0.13–0.17)	0.17 (0.15–0.18)	0.267	0.966	0.966
Lumen diameter (μm)	821.8 (740.6–903.0)	873.8 (794.1–953.4)	0.463	716.2 (660.4–772.1)	619.6 (563.7–675.4)	0.041	<0.001	<0.001
Media/lumen ratio (medial diameter/lumen diameter)	15.37 (13.59–17.14)	14.06 (12.36–15.76)	0.463	18.51 (14.9–22.12)	26.35 (22.74–29.96)	0.013	0.104	<0.001
Cell number in the media/carotid cross section	204.1 (162.2, 245.9)	242.8 (200.9, 284.6)	0.463	158.45 (136.8, 180.0)	146.5 (123.8, 169.1)	0.454	0.012	0.001
Media proliferation (% PCNA-positive nuclei)	1.47 (0.32, 2.61)	0.16 (0.00, 1.31)	0.463	1.67 (0.76, 2.58)	0.59 (0.00, 1.53)	0.176	0.626	0.083

*Statistical significance between each group in either the control or ligated carotid artery.

†Statistical significance for results in the control compared with the ligated carotid artery within each group.

Figure 3 Changes of the carotid media in LP-treated IUGR and NP-treated rats

(a) Media thickness of ligated (lig) and control (co) carotid arteries. (b) Media/lumen ratio of ligated (lig) and control (co) carotid arteries. (c) Evaluation of smooth muscle actin staining as a marker of smooth muscle differentiation in the media of ligated (lig) and control (co) carotid arteries. Values are means $\pm$ S.E.M. ($n = 12$ litters from four NP dams and 12 litters from four LP dams), as obtained from linear mixed model analysis. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

opportunity to examine the untreated counterpart as controls [12]. Although the technique of carotid artery ligation is well established in mice, only few animal studies exist that have investigated the effects of carotid artery ligation in rats [18,19].

In our animal model of IUGR, we could detect a significantly higher occurrence of neointimal formation after carotid artery ligation than in control animals with normal birth weights. The fact that we did not observe neointima formation in all ligated control animals, while in other studies neointimal formation was described consistently in all ligated animals, is likely due to histological analysis performed closer to the ligation

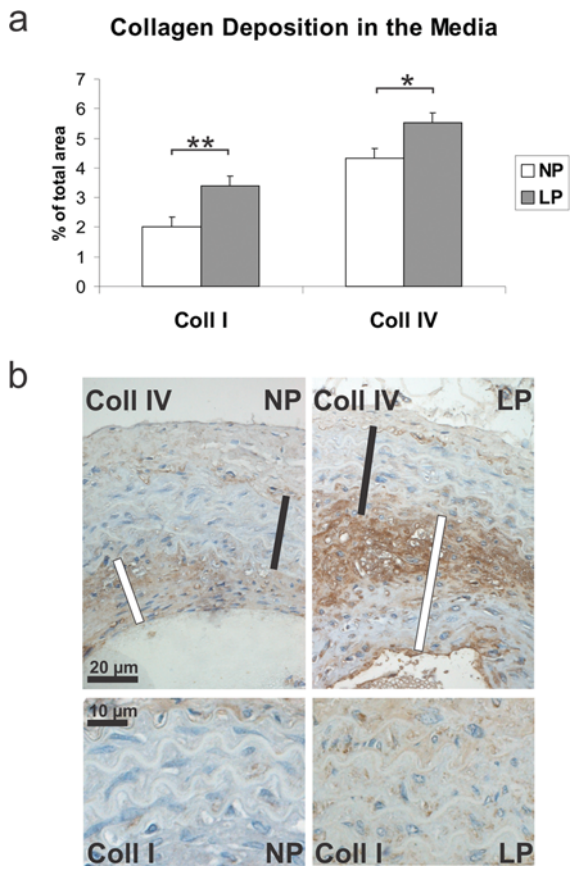

Figure 4 Deposition of collagens in the media of ligated carotids of LP-treated IUGR and NP-treated rats

(a) Evaluation of medial collagen I and IV staining. Values are means ± S.E.M. ($n = 12$ litters from four NP dams and 12 litters from four LP dams), as obtained from linear mixed model analysis. $^{*}P < 0.05$ and $^{**}P < 0.01$. (b) Representative photomicrographs of ligated carotid arteries from IUGR (LP) and control (NP) rats stained for collagen I (Coll I) or collagen IV (Coll IV). The black bars indicate the thickness of the media, whereas white bars indicate the neointima.

site in these studies. In this context Lowe et al. [19] showed that in the rat the degree of neointimal thickening diminished directly proportional to the distance between the ligation site and the location of histological analysis. Therefore in our study the absence of neointimal formation in some control animals is due to the fact that vascular remodelling was confined to the closer proximity of the ligation site, not reaching the location of histological analysis. On the other hand, the larger distance between the ligation site and the location of histological analysis minimizes the risk of detecting artificial local effects directly induced by the ligature itself.

Neointimal thickening is known to be triggered by dedifferentiation and subsequent proliferation and migration of VSMCs [20,21]. This is in line with our observations of a much higher percentage of dedifferentiated VSMCs in the tunica media of ligated carotids in IUGR animals than in the control group. Dedifferentiation of VSMCs is followed by the loss of their contractile and adhesive properties in the tunica media and migration of these cells into the intima. This migratory process is assumed to be the corner stone of neointimal formation. Neointimal thickening is thought to be the result of VSMC proliferation and secretion of components of the extracellular matrix [22].

In the media of ligated carotids, a significant reduction of the total cell number was detected only in IUGR animals, not in control rats. We presume that this reduced cell number might be due to an augmented media-to-intima migration of VSMCs [23]. Besides, we could observe an increase of media thickness in IUGR animals after carotid ligation, which might reflect concomitant structural alterations in this vascular layer. This is supported by the fact that the deposition of collagens I and IV was more prominent in ligated carotid arteries of IUGR rats, compared with ligated carotid arteries of control rats. Dysregulated VSMC proliferation rates in the tunica media are thought to play a crucial role in vascular remodelling after vascular injury [24]. In our study, however, we did not detect statistically significant differences in cellular proliferation in the tunica media of IUGR rats after carotid artery ligation when compared with ligated control rats. Only a trend towards lower proliferation rates in ligated IUGR rats compared with ligated control rats were observed. This might be due to the relatively high variance of the values from animals of the same group and a rather low value of statistical power for this endpoint. Hence we cannot rule out that we might well miss potential differences in proliferation rates. On the other hand, the relatively late time point we chose for read-out might be suboptimal to evaluate early events in the development of atherosclerotic lesions.

Vascular remodelling is able to induce the enlargement but also a significant reduction of the vessel lumen. Enlargement is observed mainly in the early stages of remodelling after arterial injury due to altered haemodynamics and the structural lesions in the tunica intima and media. Later on in the development of neointimal formation vascular remodelling turns into a constrictive process [25]. On the basis of the investigations in other experimental models of arterial injury there is some evidence that after migration towards the neointima VSMCs recover in terms of contractility and adhesivity [26]. This is regarded as the main pathomechanism of constrictive vascular remodelling followed by lumen reduction and an increased media to lumen ratio, as observed in our study.

The pathomechanism leading to an increased susceptibility of IUGR animals to the development of atherosclerotic lesions is not completely understood. Early endothelial dysfunction is frequently detected in IUGR individuals [27] and could be one of the initial steps leading to vascular remodelling. On the other

hand, an early imbalance of structural components of the vessel wall was detected in a study by Skilton et al. [10] without altered endothelial function. Using the model of maternal LP diet we were able to show that the expression of extracellular matrix proteins and their regulators was dysregulated in IUGR rats very early in life [9]. In juvenile IUGR animals we could detect a significant overexpression of aortic collagen, which is thought to be an essential component for constrictive vascular remodelling and consecutive lumen shrinkage after arterial injury [28]. This is consistent with the findings of our present study, showing that the deposition of collagens in vascular remodelling is more prominent in animals after IUGR. Furthermore, our recent study revealed that maternal protein restriction induces a significant increase in the vascular expression of CTGF (connective tissue growth factor) in the offspring already at the time of birth. CTGF, which is produced by VSMCs and considered a key player in the profibrotic TGFβ (transforming growth factor β) signalling pathway, is capable of controlling and mediating vascular remodelling after arterial injury [29].

IUGR in humans and animal models is frequently associated with the appearance of features of the metabolic syndrome, such as increased blood pressure (smooth muscle cell) [30] or increased serum cholesterol levels [31]. It is, however, unlikely that the increased susceptibility for developing atherosclerotic lesions in our model is secondary to increased blood pressure or high cholesterol levels: serum cholesterol levels measured in our study were not different in IUGR and control rats. Moreover, BP recordings in our model of LP diet did not reveal increased BP in IUGR compared with control rats [9].

Taken together, in IUGR animals the ligation of the carotid artery induces more VSMC dedifferentiation, and leads to a higher incidence of neointima formation and vascular remodelling than in control rats. This might be due to an early compromised structural integrity of the vasculature after IUGR. Our study might imply that children born with IUGR are at a higher risk of developing more severe lesions after vascular injury.

AUTHOR CONTRIBUTION

Carlos Menendez-Castro, Andrea Hartner and Karl Hilgers designed the study and contributed to the paper. Kerstin Amann and Wolfgang Rascher contributed to the design of the study and critically revised the paper. Carlos Menendez-Castro, Fabian Fahlbusch and Nada Cordasic collected, analysed and interpreted the data. Carlos Menendez-Castro interpreted the data and drafted the paper. Matthias Schmid carried out the statistical analysis of the data.

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