Immediate Postnatal Overfeeding in Rats Programs Aortic Wall Structure Alterations and Metalloproteinases Dysregulation in Adulthood

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BACKGROUND
Alterations in the nutritional perinatal environment, such as intrauterine growth retardation with subsequent postnatal catch-up growth, program cardiovascular disease in adulthood, possibly through alterations in matrix metalloproteinase (MMP)-2 and -9. However, experimental evidences demonstrating that changes in the nutritional perinatal environment can program MMP-2 and -9 with subsequent alterations of vessel wall are lacking.

AIM
The current study evaluated whether immediate postnatal overfeeding is able to alter vascular morphological indexes and circulating and/or vascular MMP-2 and -9 status.

METHODS
Aortic morphology (wall thickness and percentage of incomplete elastin lamellae) and circulating and aortic MMP-2 and -9 activity (measured by gelatin zymography) and aortic MMP-2 and -9 mRNA (measured by reverse transcription polymerase chain reaction (RT-PCR)) were studied in adult male rats overfed (OF) or normofed (NF) during the immediate postnatal period.

Clinical evidences show that changes in nutritional factors both during fetal life and in infancy and childhood can lead to a programming of the metabolic syndrome and its associated cardiovascular diseases, the major cause of premature death in developed countries. Intrauterine growth retardation has been related to mortality from coronary disease and the development of hypertension and diabetes.1 Leeson et al.2 have demonstrated that low birth weight is negatively associated with impaired endothelial function in childhood. Such relationship was still present in early adult life, especially in those with lower cardiovascular risk profile,3 indicating that the above-mentioned phenomenon can directly result from programming of the vascular wall structure and/or function. Indeed, low birth weight babies have stiffer conduit arteries and higher blood pressure in adulthood.4 Several mechanisms could account for the above-mentioned phenomenon. Although the molecular basis is not yet established, it has been suggested that glucocorticoid overexposure, impaired nephrogenesis, epigenetic changes occurring during gestation, or rapid postnatal weight gain (the catch-up phenomenon) may play a causal role.5 In addition, Sesso and Franco6 have suggested that perinatal programming of vascular dysfunction can be related to alterations in matrix metalloproteinase (MMP) pathways. Indeed, they demonstrated that children born small-for-gestational-age exhibited elevated amounts of circulating MMP-2, MMP-9, MMP-2/tissue inhibitor of metalloproteinase-2 (TIMP-2) ratio, and MMP-9/TIMP2 ratio. MMP-2 and -9 are endopeptidases with capacity to cleave several components of the extracellular matrix such as collagen and elastin.7,8 MMP-2 is constitutively expressed in endothelial and smooth muscle cells and highly regulated at a posttranscriptional level, whereas MMP-9 activity is regulated by its expression and

RESULTS
Postnatal overfeeding induced early onset obesity. Adult OF rats presented with increased blood pressure and circulating MMP-2 and -9 activity. In the thoracic aorta, postnatal overfeeding increased wall thickness and decreased elastin integrity (as demonstrated by an increased percentage of incomplete elastin lamellae). OF rats showed enhanced aortic MMP-2 activity and MMP-9 mRNA levels. Circulating and aortic MMP-2 activity correlated positively with the percentage of incomplete elastin lamellae and aortic wall thickness, respectively.

CONCLUSION
Our data demonstrate for the first time that immediate postnatal nutritional programming induces increases in circulating and aortic MMP-2 activity with parallel aortic wall alterations, such as decreased elastin integrity and enhanced thickening, showing that this experimental model is suitable for the study of perinatal nutritional programming of vascular functions.

Keywords: aortic wall; blood pressure; elastin lamellae; gelatin zymography; hypertension; MMP-2; MMP-9; postnatal programming.

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Original Article

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secretion. MMP-2 and -9 have been related to physiologic and pathologic vascular conditions involving remodeling of the vascular wall including atherosclerosis, development of restenotic lesions, atherosclerotic plaque vulnerability, and rupture, as well as arterial aneurisms.9 It has been recently reported that transient high oxygen exposure in newborn rats, which programs hypertension, leads to decreased elastin content and increased MMP-2 staining in the aorta of 4-week-old animals.10 However, experimental evidences demonstrating that changes in the nutritional perinatal environment are able to program changes in MMP-2 and -9 activities with subsequent alterations of vessel wall morphology are lacking.

In this study we used the experimental paradigm of immediate postnatal overfeeding in rats. We and others previously demonstrated that postnatal overfeeding in rats, obtained by reducing the size of the litter in the immediate postnatal period, results in early onset obesity with glucose intolerance and increased circulating free fatty acid levels. Such experimental paradigm is followed in adulthood by disturbances comparable to those described in the metabolic syndrome, upregulation of basal and stress-induced circulating cytokines and local glucocorticoid metabolism (overexpression of glucocorticoid receptors and 11β-hydroxysteroid dehydrogenase type 1) in mesenteric adipose tissue.11 However, experimental evidences are lacking.

METHODS

Animals

All experimental procedures were approved by the Local Animal Care and Use Committee. Wistar rats (Janvier, Le Genest St Isle, France) were housed under standard conditions of light (12-hour light/dark cycle; lights on at 0800) and temperature (22–24 °C), with free access to tap water and standard pellet diet. Virgin females were time-mated. At postnatal day 3, male pups were randomly distributed to the mothers. The litters were culled to 10 newborns for normofed (NF) rats or 3 pups for overfed (OF) rats.12,13 Animals were subsequently left undisturbed, except for weighing and cage cleaning at postnatal days 8 and 21. Rats were weaned at P21.

Blood pressure measurements were performed by the tail cuff methodology (Biosbe, Chaville, France) in 5-month-old rats (n = 7 in each group, originating from 3 litters for NF or OF rats (2–3 rats per litter), a group size comparable to those used by Plagemann et al.11 to measure blood pressure in NF or OF rats using the same methodology). The animals were lightly sedated with isoflurane (2% in air), the body temperature was maintained at 38.5 °C using a heating blanket, and blood pressure and heart rate were recorded (3 measurements within 6 minutes). Averaged values for each rat were used for the subsequent statistical analysis.

Five-month-old rats (n = 9 in each group, originating from 3 or 4 litters for NF (2–4 rats per litter) or OF rats (2–3 rats per litter), respectively) were anesthetized with an intramuscular injection (1 ml/kg b.w.) of a mixture containing 40 mg/ml ketamine (Panpharma, Fougeres, France) and 1 mg/ml largactyl (Sanofi-Aventis, Paris, France). Body weight was recorded, the chest was opened, and blood was collected by cardiac puncture into citrated tubes which were centrifuged at 4,000 rpm for 15 minutes at 15°C and the resulting plasma was stored at −80°C until analysis. The aorta was flushed with 10 ml of 5 IU/ml heparin in saline and collected. The middle portion of the descending thoracic aorta was dissected free from the surrounding adipose tissue and either snap frozen or fixed in 4% formaldehyde.

Histological analysis

After paraffin embedding, 5-μm-thick sections were cut with a Leica microtome (Leica Microsystems, Wetzlar, Germany), apposed onto slides (Superfrost Plus; CML, Nemours, France), then subsequently dewaxed, rehydrated, colored using Masson’s trichrome technique and mounted. For each slide 5 crosswise-cut media sections were digitalized using a ProgRes charge-coupled device camera (Jenoptik GmbH, Jena, Germany) coupled to a DM-RB microscope (Leica) at ×40 magnification. Aortic wall thickness was quantified and vascular smooth muscle cells (VSMCs) nuclei, total and incomplete elastin lamellae in the media were manually counted. The investigator was blinded to treatment allocation. Averaged values from 5 images were used for the subsequent statistical analysis. For elastin and collagen staining slides were dewaxed, rehydrated, mounted with Fluoprep and photomicrographed using fluorescent light and a 480 nm excitation/521–558 emission filter (relying on elastin autofluorescence). Then slides were unmounted, colored using picrosiris red, and the same regions were captured under polarized light on a Leica DM-RB microscope.

Immunohistochemistry for MMP-2 was performed as previously described15 using a monoclonal antibody raised against MMP-2 (Abcam #37150, Cambridge, UK). Control sections were incubated without the primary antibody.

Factor VIII (FVIII) and fibrinogen determination

Circulating FVIII and fibrinogen were assayed using the STA-R device and commercially available kits and reagents from Diagnostica Stago (Asnières, France) including the corresponding normal and pathologic control plasmas. Fibrinogen plasma levels were assayed using the STA-Fibrinogen kit (Clauss’ method) and FVIII procoagulant activity was determined using the corresponding STA-deficient plasmas.

Reverse transcription polymerase chain reaction analysis

Total RNA was extracted using TriReagent (Molecular Research Center, Cincinnati, OH), according to the
MMPs zymography

Thoracic aorta were homogenized in RIPA buffer and centrifuged at 20,000× g for 30 min at 4 °C. Aorta supernatant (corresponding to 10 μg of total proteins) or 5 or 10 μl (for MMP-2 or -9, respectively) of plasma diluted 1/5 were added to non-reducing sample buffer (v/v) and loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels containing copolymerized gelatin (0.1% w/v, Sigma, Saint-Quentin Fallavier, France). Separate gels were used for MMP-2 or -9 zymograms and recombinant MMP-2 or -9 proteins were used as standards. Electrophoresis was performed at 75 V for 30 minutes and then at 100 V for 2 hours at 4 °C. Gels were rinsed twice with 2.5% Triton X-100 to remove SDS and to renature the MMPs and incubated overnight at 37 °C in 50 mM Tris-HCl buffer pH 7.6 containing 5 mM CaCl2, 200 mM NaCl and 0.02% Brij 35 with gentle agitation. Gels were stained with coomassie blue, photographed and normalized against 18S. Primers were as follows: MMP-2: forward 5'-GACCACCCGAGATTACGAC-3’, reverse 5'-GCACCCCGAGATTACGAC-3’; MMP-9: forward 5'-GTACGGCGTCAAGCAAGC-3’, reverse 5'-GTACGGCGTCAAGCAAGC-3’; TIMP-1: forward 5'-CATGGAGAGCCTCTGTGGAT-3’, reverse 5'-GATGGTCAATTGCCGTTC-3’; TIMP-2: forward 5'-CGTGGGTTGCAATTGCAGC-3’, reverse 5'-GATGGGTTCGACCAGT-3’.

Statistical analysis

Data are presented as means ± SE. Statistical analysis was performed with the Statview analysis program using Student’s t-test or the Mann–Whitney U-test. The Kolmogorov–Smirnov test was used to compare aortic wall thickness. The association between variables was assessed by the Spearman rank correlation test followed by the r to z Fisher’s test.

RESULTS

Body parameters and blood pressure

Table 1 shows the effects of early postnatal overfeeding on various body parameters. Body weight was not statistically different between groups at P3. An increase in body weight was found at postnatal day 3 and disappeared at 5 months. Postnatal overfeeding induced a significant (P = 0.0074 and P = 0.0080, respectively) increase in diastolic (118 ± 5% of NF) and systolic (113 ± 3% of NF) blood pressure whereas heart rate was not affected.

Morphological parameters of the aortic wall

Figure 1A,B shows photomicrographs of representative sections of the thoracic aorta obtained in NF or OF rats and counterstained using Masson’s trichrome technique. Eosinstained elastin lamellae appear as thick bundles between each lamellae unit. Some of the lamellae are incomplete, either because they stop at some point in the observed microscopic field (Figure 1B “S”) or because they are partially discontinuous (Figure 1B “P”). VSMC nuclei (“N”) appear slightly colored by hematoxylin in the lamellar units which are comprised between the elastin lamellae and are characterized by a typical spindle shape. No other kind of nuclei, particularly with a rounded shape, were found, strongly demonstrating that aorta was not infiltrated by other cell types such as leukocytes. In order to evaluate the impact of postnatal overfeeding on vascular morphological alterations we measured aortic wall thickness and the percentage of incomplete elastin lamellae. The density of VSMCs nuclei as well as the total number of elastin lamellae were unaffected by the experimental manipulation (40.6 ± 6.2 vs. 33.3 ± 3.5 nuclei per field and 8.4 ± 0.2 vs. 8.6 ± 0.2 elastin lamellae per media in NF or OF rats, P > 0.05). OF rats showed an increase in aortic wall thickness and in the percentage of incomplete elastin lamellae, as illustrated in Figure 2. Thinn collagen depots were observed in the media, mainly in its outer half, in both NF and OF rats (Figure 1C: NF, D: OF, yellow and red staining of type I collagen by picrosirius red revealed under polarized light). Collagen staining was found to outline the elastin thick bundles revealed by autofluorescence (Figure 1E,F merged captions of picrosirius and autofluorescence in the same fields than C and D, respectively). Immunohistochemistry for MMP-2 in the aortic wall of a OF rat revealed a strong expression in the VSMCs (Figure 1G). Control experiment, that is incubation without the primary antibody, showed a lack of signal (Figure 1H), demonstrating the specificity of the experimental procedure.
MMPs status

The activity and the genomic expression of MMP-2 and -9 and their inhibitors were assessed in both plasma and aortic extracts using gelatin zymography and reverse transcription polymerase chain reaction, respectively. Results are presented in Figure 3. In plasma postnatal overfeeding induced a large (about 260% of control) increase in the activity of both MMP-2 and -9. In order to search for a possible programming-induced inflammatory syndrome we measured plasma FVIII and fibrinogen. Both circulating FVIII and fibrinogen concentrations were unaffected by the postnatal manipulation (FVIII: 100.0 ± 6.8 vs. 109.2 ± 8.6% and fibrinogen: 2.73 ± 0.1 vs. 2.94 ± 0.9 g/l in NF or OF rats, respectively; \( P > 0.05 \)).

In aortic extracts, MMP-2 mRNA concentration was unaffected by the experimental manipulation, whereas MMP-2 activity rose to 136% of controls. Aortic MMP-9 mRNA levels increased 3 times in OF compared with NF rats (Figure 3). MMP-9 activity was not detectable by gelatin zymography in aortic extracts. Aortic TIMP-1 and 2 mRNA levels did not change in neonatally OF rats compared with
MMP-2 and -9 activity and aortic MMP-2 activity and demonstrate that adult OF rats had increased circulating the aortic wall and increased blood pressure. Indeed we and/or activity with parallel morphological alterations of environmental changes during the perinatal period are able to program upregulation of MMPs gene expression and/or activity was not changed, allowing circulating MMPs to be fully active. Our present data are consistent with the lack of TATA or CAAT box in the gene coding for MMP-2, which implies that it is constitutively expressed.16

Our data show that OF rats had increased blood pressure compared with NF animals, consistent with the report of Plagemann et al.11 We choose to measure blood pressure in rats lightly sedated with isoflurane but not in immobilized conscious animals to avoid artifactual increases related to the exacerbated response to emotional stress characteristic of OF rats.12 One can assume that such experimental procedure did not affect our results since it has been demonstrated that a single isoflurane anesthesia did not change blood pressure in adult male Wistar rats.17

OF rats showed changes in aortic wall morphologic indexes, that is increase in the percentage of incomplete elastic lamellae of the aortic wall. Several mechanisms could account for the fragmentation of elastin lamellae. When submitted to repeated cyclic loading, arterial elastin fractures, a phenomenon called fatigue failure. Because the rate of elastin synthesis in adulthood is thought to be negligible, in both human and rodents, it is believed that damaged elastin is not replaced.4 Since we found that OF rats have high blood pressure, it could be hypothesized that part of the increased percentage of incomplete elastin lamellae found in programmed animals could be due to fatigue-induced fracturing.4 Alternatively, because elastin lamellae fragmentation leads to a gradual transfer of mechanical load to collagen, which is much stiffer than elastin, the increased percentage of incomplete elastin lamellae found OF animals aorta could be one of the pathophysiological mechanisms responsible for the higher blood pressure found in this experimental group. In addition, we found that circulating MMP-2 activity correlated positively with the percentage of incomplete elastin lamellae, suggesting that enzymatically induced mechanisms could participate in the phenomenon of premature failure of elastin in OF rats. Our findings showing that aortic TIMP-1 and -2 mRNA levels were unaffected by the experimental manipulation suggest that aortic elastase inhibitors activity was not changed, allowing circulating MMPs to be fully active. Our present data are consistent with the report of Yasmin et al.18 who demonstrated, in patients with isolated systolic hypertension, that circulating MMP-2 immunoreactivity correlated with aortic pulse wave velocity, a measure of distensibility. They also support the findings of Sesso and Franco19 who found increased circulating MMP-2 /TIMP-2 ratio in children born small-for-gestational age.

We found that OF rats had increased aortic wall thickness as compared with NF animals, suggesting the existence of a remodeling of the extracellular matrix. Such data are consistent with clinical findings. Fetuses, neonates,

NF animals (104.7 ± 5.9 vs. 100.0 ± 6.2 and 102.7 ± 6.4 vs. 100.0 ± 6.7 %, respectively, P > 0.05).

We next sought to estimate the impact of the changes in the MMPs status on aortic wall. Results are depicted in Figure 4. There was a positive linear relationship (r = 0.641, P = 0.0055) between the activity of circulating MMP-2 and the percentage of incomplete elastin lamellae. Aortic wall MMP-2 activity correlated positively (r = 0.591, P = 0.0077) with the aortic wall thickness. There was no significant relationship between plasma MMP-9 activity or aortic MMP-9 mRNA levels and the percentage of incomplete elastin lamellae or the aortic wall thickness (data not shown).

DISCUSSION

Our data present the first experimental evidence that environmental changes during the perinatal period are able to program upregulation of MMPs gene expression and/or activity with parallel morphological alterations of the aortic wall and increased blood pressure. Indeed we demonstrate that adult OF rats had increased circulating MMP-2 and -9 activity and aortic MMP-2 activity and MMP-9 gene expression and thickening of the aortic wall with decreased elastin integrity. We found in OF rats an increase in aortic MMP-2 activity whereas aortic MMP-2 mRNA levels were unaffected, suggesting that, under our experimental conditions, aortic MMP-2 is regulated at a posttranscriptional level. This observation is consistent with the lack of TATA or CAAT box in the gene coding for MMP-2, which implies that it is constitutively expressed.16

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We found that OF rats had increased aortic wall thickness as compared with NF animals, suggesting the existence of a remodeling of the extracellular matrix. Such data are consistent with clinical findings. Fetuses, neonates,
and children with intrauterine growth retardation have increased aortic intima-media thickness compared with those born appropriate for gestational age. However, when studied at the adulthood, the above-mentioned phenomenon was dependent upon the rate of postnatal growth. In the atherosclerosis risk in young adults (ARYA) study, carotid intima-media thickness was inversely associated with birth weight in subjects who showed exaggerated catch-up growth during infancy whereas it was positively associated with birth weight in patients with minimal growth during infancy. Under our experimental conditions, aortic wall thickness was positively correlated with aortic MMP-2 activity and MMP-2 protein was strongly expressed in VSMCs. Although one cannot exclude that common factors could program aortic wall growth and the regulation of aortic MMP-2 activity, it could be suggested that enhanced MMP-2 activity is responsible, at least in part, for the increase in aortic wall thickness found in OF animals. In hemodialyzed patients serum MMP-2 concentrations are an independent predictor of elevated intima-media thickness. Although plasma MMP-9 activity and aortic MMP-9 mRNA levels were increased in OF rat, we did not find any significant correlation between these parameters and the percentage of incomplete elastin lamellae or aortic wall thickness, suggesting that, in our experimental model, the observed changes in circulating or aortic MMP-9 are not involved in the alterations of the aortic wall in OF animals.

The mechanisms responsible for early postnatal overfeeding-induced MMP-2 and -9 increases in adulthood are not clear. Because circulating FVIII and fibrinogen levels were unaffected by the postnatal manipulation one can exclude that programming-induced increases in MMP-2 and -9 were subsequent to an inflammatory syndrome. Therefore, hormonal or metabolic alterations are more likely to be involved in the programming-induced regulation of MMP-2 and -9 expression and/or activity. It has been reported that, during the perinatal period, transient glucocorticoids administration can have long-lasting effects on the regulation of MMPs activity. We already demonstrated that postnatal overfeeding accelerates the maturation of the hypothalamo-pituitary-adrenal axis with increased circulating glucocorticoids concentrations detected as soon as postnatal day 14. Therefore, the above-mentioned observations suggest that the increase in plasma and aortic MMPs found in the present study could be subsequent, at least in part, to a glucocorticoid-induced programming phenomenon occurring during the immediate postnatal period. Alternatively, alterations in programming-induced glucose homeostasis could be responsible for the observed alterations in circulating and/or vascular MMP-2 and -9 activities. It has been demonstrated that patients with the metabolic syndrome have increased circulating MMP-2 activity and MMP-9 immunoreactivities. In vivo in rats, pharmacologic hyperinsulinemia increased aortic MMP-2 and MMP-9 protein, a phenomenon which was further augmented by free fatty acids infusion. Interestingly, adult OF rats show insulin resistance and increased circulating free fatty acids.

In conclusion our data demonstrate for the first time that immediate postnatal nutritional programming induces increases in circulating and aortic MMP-2 activity with parallel aortic wall alterations, such as decreased elastin integrity and enhanced thickening, showing that this experimental
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DISCLOSURE

The authors declared no conflict of interest.

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