IN UTERO EXPOSURE TO MATERNAL DIABETES IMPAIRS VASCULAR EXPRESSION OF PROSTACYCLIN RECEPTOR IN RATS OFFSPRING

Short running title: Maternal diabetes and abnormal aorta programming

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Submitted 3 March 2010 and accepted 3 July 2010.

This is an uncopyedited electronic version of an article accepted for publication in Diabetes. The American Diabetes Association, publisher of Diabetes, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes in print and online at http://diabetes.diabetesjournals.org.

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Epidemiologic studies have clearly identified modifications of fetal environment as a risk factor for the development of cardiovascular diseases in adulthood. In our experimental model, rats exposed in utero to maternal diabetes develop hypertension in adulthood.

Objective: to evaluate modifications of arterial structure, gene expression and function in our model of rats exposed to maternal diabetes.

Research Design and Methods: morphometric analysis of elastic vessels structure and determination of thoracic aortic gene expression profile with oligonucleotides chips (Agilent, G4130, 22k) were performed before the onset of established hypertension (3 months).

Results: arterial parameters of in situ fixed thoracic aorta were not significantly different between control mother offspring (CMO) and diabetic mother offspring (DMO). The aortic gene expression profile of DMO is characterized by modifications of several members of the arachidonic acid metabolism including a 2-fold under-expression of prostacyclin receptor (IP), which could contribute to decreased vasodilatation. This was confirmed by ex vivo experiments on isolated aortic rings. Pharmacological studies on conscious rats showed that SBP decline in response to a PGI2 analog was impaired in DMO rats.

Conclusion: these results suggest an abnormal vascular fetal programming of prostacyclin receptor in rats exposed in utero to maternal hyperglycemia that is associated with impaired vasodilatation and may be involved in the pathophysiology of HTA in this model.

Cardiovascular disease is one of the greatest health burdens worldwide. Cardiovascular risk is not only determined by conventional risk factors in adult life, but also by early life events resulting in re-setting of key physiological functions. Modifications of the intra-uterine environment during specific windows of fetal development are now recognized as important causes of fetal stress (1), leading to several responses such as loss of structure/function and pre-emptive adaptations to an adverse post-natal environment (2), and finally to adult diseases such as metabolic abnormalities and hypertension (1; 3).

Nutrition is one of the major intrauterine environmental factor that alters expression of the fetal genome and may have lifelong consequences leading to limited physiological function and disease (4; 5).

Particularly, the consequences of maternal diabetes in the adult offspring are gaining attention (6; 7). Interestingly, both metabolic disorders and raised blood pressure (BP) have increasingly been associated with in utero exposure to maternal diabetes (8).

Several animal models such as modification of maternal nutrition, reduction of uterine supply or glucocorticoids treatment, have contributed to the understanding of some of the mechanisms involved in fetal/perinatal programming by showing that kidney changes and alterations of hormones regulation are involved in the fetal programming of hypertension (3; 9; 10). More recently, alterations of vascular function in several models of fetal programming have been also implied (11; 12). In previous works (13; 14), we developed a rat streptozotocin-induced model of fetal exposure to maternal diabetes, characterized by moderate levels of maternal
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hyperglycemia, normal gestation and delivery with healthy pups without intrauterine growth retardation (IUGR). Offspring’s from diabetic mother (DMO) presented an established hypertension at 6 months of age (9). The exact mechanism leading to late onset of hypertension in this model is unknown. The present work was designed to evaluate changes in vascular properties through genetic profile and pharmacological studies in diabetic mother offspring compared with control mother offspring (CMO) at pre-hypertensive stage.

RESEARCH DESIGN AND METHODS

Animals. Diabetes was induced in Sprague–Dawley rats (250-300 g) at day 0 of gestation by using a single intraperitoneal injection of streptozotocin (Sigma, 35 mg/kg) as previously described (9; 13; 14). The diabetic state was checked in fasted rats by measuring the plasma glucose concentration (Accuchek, Roche) on tail blood. Only pregnant females whose plasma glucose ranged between 15 and 20 mmol/l were included in the study. This diabetic status was confirmed every two days until delivery. On the day of delivery, the newborn rats were weighted. Each litter was then reduced to 10 pups. All the animals were house kept at CEF (Centre d’Explorations Fonctionnelles). They were maintained in a temperature and light controlled room at 21°C with a 12 hours light cycle. They had free access to food (SAFE Laboratory) and tap water. All experiments were performed at 3 months of age corresponding to pre-hypertensive stage. Six to seven different litters of CMO and DMO were used for the entire study. Each experiment was performed on males obtained from a minimum of 3 different litters. All experiments were conducted in accordance with the institutional guidelines and the recommendations for the care and use of laboratory animals put forward by the French Ministry of Agriculture.

Blood pressure measurements. Blood pressure measurements were performed on CMO and DMO animals at 3 and 12 months of age respectively to confirm the development of hypertension. Rats were instrumented under sodium pentobarbital anaesthesia (60 mg/kg, i.p.; Sanofi) with an arterial catheter (PE-50 fused to PE-10: internal diameter – 0.28 mm and outside diameter, 0.61 mm) inserted via the femoral artery to record BP. The catheter was led subcutaneously to exit between the scapulae. Upon regaining consciousness, animals were housed in individual cages. After 3 days of recovery, BP was recorded on conscious unrestrained animals during 30 minutes. The arterial catheter was connected to a pressure transducer (P10EZ, Becton Dickinson) linked with a Gould RS 3400 polygraph in order to measure continuously pulsatile BP.

Histomorphometry analysis. Morphological studies were performed on the thoracic aorta of 3 months old CMO and DMO animals. Arterial samples were fixed in 10% buffered formalin and embedded in paraffin. Sections (6 µm) were stained with orcein for elastic fibers and sirius red for collagen in order to measure internal and external media perimeters, intima-media thickness, medial cross-sectional area, elastic fiber and collagen densities of the media by computer-directed color analysis (NIS-Elements AR2.3, Nikon). Briefly, collagen and elastin densities are the ratio of the area of collagen and elastin (stained with sirius red and orcein, respectively) detected and measured with the camera and the program-driven computer on the area of the tissue in a given microscopic field.

Microarray analysis. Gene expression profile was performed on thoracic aorta in 3 months old CMO and DMO animals. RNA extraction of total RNA and preparation of cDNA: total RNA was extracted from thoracic aortic samples by TRizol reagent (Invitrogen). The quality and concentration
were checked by the Agilent technology. Good quality RNA was amplified by the Amino Allyl MessageAmp™ aRNA kit (Applied Biosystems), according to the manufacturer’s instructions. Ten samples (5 CMO and 5 DMO) were isolated with sufficient yield and integrity for complete microarray analysis. 

**Hybridization on microarrays:** Transcript profiling was performed using rat Agilent G4130A arrays. These microarrays contained ~22,000 distinct oligonucleotide probes (60 mers). The list of the 22,575 probes is available online (http://www.chem.agilent.com). Antisense RNA were coupled with Cy3 and Cy5 post-labeling reactive dyes (Amersham) by a reverse transcription step. After a post-processing step, microarrays were hybridized with labeled-RNA as previously described (15). Slides were scanned with a ScanArray 5000 (GSI Lumonics) and images were quantified with the GenePix Pro 5.0 program (Axon Instruments,). This technique offers the possibility to hybridize two samples on a same slide: a control and a pathological sample. Values of pathological samples were normalized to values of control samples in order to compare pathological samples between them. Moreover, because incorporations of Cy3 and Cy5 dyes can differ significantly, data were further normalized using a dye-swap method (16); this method required duplication of experiments. For that purpose, a first microarray was hybridized with pathological and control samples labeled with Cy3 and Cy5, respectively. A second “swap” microarray was hybridized with pathological and control samples labeled with inverted dyes. Statistical analysis was performed using SAM software (Significance Analysis of Microarray) as described in Figure 1. Microarray result has been deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under GEO Series Accession Number GSE16750.

**Confirmation of modifications observed in arachidonic acid metabolism.** *Real-time RT-PCR:* reverse transcription from 2 µg of total RNA was carried out to generate cDNA. Real-time PCR was then performed for each cDNA preparation from thoracic aorta of 3-month-old rats, using the Applied Biosystem together with the SYBRGreen master mixture for prostacyclin receptor (IP) or with the TaqMan technology for Cyp4f2. Expressions were normalized to the expression level of β-actin. *Western blot:* western blot analysis of proteins of interest was performed following previously described techniques (17). Briefly, proteins were extracted from arterial biopsies of CMO and DMO. Total protein content was determined by the Bradford technique (18) and equal amounts (50 µg) of the denatured proteins were used. Membranes were incubated with polyclonal antibodies directed against IP and Cyp4f2. The detection was performed by chemiluminescence emitted from luminol oxidized by peroxidase (ECL system, Amersham).

**Pharmacological studies.** To validate functional effect of decrease in IP in DMO, we studied the effect of an analog of prostacyclin (PGI2) on vascular dilatory function in isolated thoracic aorta and on blood pressure in conscious CMO and DMO at 3 months of age. *Ex vivo study:* vascular dilatory function was assessed in response to beraprost (Cayman) in isolated thoracic aorta obtained from 7 CMO and 10 DMO animals. Arterial segments (2 mm long) were dissected and mounted on a wire-myograph (Danish MyoTechnology, DMT) as previously described (19). Briefly, 2 tungsten wires (25 µm in diameter) were inserted into the lumen of the arteries and fixed to a force transducer and a micrometer, respectively. Arteries were bathed in a 5 ml organ bath containing a physiological salt solution (PSS) maintained at a pH of 7.4, a PO2 of 160 mmHg and a PCO2 of 37 mmHg. After wall tension normalization, arteries
were allowed to stabilize for one hour. Endothelial integrity was assessed by evaluating the vasodilator effect of $10^{-6}$ mol/l acetylcholine (Ach, Sigma-Aldrich), after preconstriction treatment with $10^{-6}$ mol/l phenylephrine (Phe, Sigma-Aldrich). Cumulative concentration-response curve (CRC) to beraprost (0.001 to 30µmol/L) was performed after phenylephrine-induced preconstriction (1 µmol/L). Endothelium-independent relaxation to sodium-nitroprusside was obtained at the end of the protocol.

**In vivo study:** 8 CMO and 6 DMO were instrumented as described above with a supplementary venous catheter inserted into the right jugular vein to allow the injection of the pharmacological product and the two catheters were led subcutaneously to exit between the scapulae. Systolic BP was extracted from the whole BP signal and heart rate was calculated. Recordings were performed at rest during 30 minutes. The experiment started when cardiovascular parameters were stable. The effect of another analog of PGI$_2$, iloprost (Schering, i.v) was determined on arterial BP and heart rate.

**Statistical analysis.** For microarray analysis, significance analysis of microarrays (SAM, Stanford University CA) was applied to identify genes differentially expressed between DMO and CMO. Briefly, SAM computes a score for each gene that measures the strength of transcript correlation with survival. This score (d) is the maximum-likelihood score statistic from Cox's proportional hazards model (Cox score). A threshold value was chosen to give a reasonably low false positive rate (less than 10%), as estimated by repeatedly permuting the survival times and counting the number of genes that were significant at each threshold. Missing data were handled using the K-nearest neighbors imputer (k = 10) of the SAM imputation engine.

For others experiments, all results are expressed as the mean ± SEM values. Statistical analysis was performed by non parametric Mann Whitney test or by analysis of variance (Anova) when appropriate. Statistical significance was defined as p < 0.05.

**RESULTS**

**Development of hypertension.** DMO and CMO had similar SBP at 3 months of age (141.9 ± 3.2 mmHg, n=17 vs. 136.9 ± 3.6 mmHg, n=16). SBP was significantly higher in DMO than in CMO at 12–months of age (166.9 ± 8.4 mmHg, n=8 vs. 136.6 ± 4.5 mmHg, n=10, p<0.01). SBP increased significantly in DMO between 3 and 12-months of age (p<0.01).

**Vascular structure.** Table 1 shows that arterial parameters of in situ fixed thoracic aorta were not significantly different between CMO and DMO at 3-months of age. Under light microscopy, orcein stain did not reveal morphological changes of the elastic bundles in DMO compared to CMO (data not shown).

**Microarrays analysis.** RNA samples of 3 months of age aorta were analyzed. We isolated 119 genes differentially expressed between DMO and CMO (Fig 1A); the full list of these 119 genes is presented in Data Supplement Table 2. Transcriptome analysis is often criticized for a lack of reproducibility of quantification. However the requirement of duplication of experiments (use of swap) has improved this reproducibility of quantification. The specific signal associated with a spot (representative of a gene) was defined by the geometrical average of the specific intensities of this spot in direct and swapped experiments; We found a very good correlation between the intensity of a spot in direct experiment and that in swap experiment (Fig 1B).

We classified the 119 transcripts differentially expressed between groups, according to the 7
functional categories described by Hwang et al. (20). As a group, transcripts encoding proteins potentially involved in the mechanical regulation of vascular function (‘signaling/communication’ and ‘structure/motility’) constituted the most important group with 35% of the annotated transcripts (Fig 2A). Examples of the former groups are genes related to extracellular matrix, cytoskeleton or cellular junctions (Fig 2B). Interestingly, most differentially expressed genes were implicated in the arachidonic acid metabolism. Among these genes, IP (i.e. D28966) expression was strongly reduced (d=-1.990, fold-change=0.5). Genes implicated in production of 20-hydroxyeicosatetraenoic acid, a potent vasoconstrictor (i.e. Cyp4f2, Cyp4f4) were over-expressed (Fig 2B). These genes have the most significant “d” value correlated to the better fold-change and are then considered as major targets. Modifications induced by modulation of expression of these genes involved in vascular biology could contribute to hypertension development. Thus, the following study focused on IP and Cyp4f2.

**RT-PCR and protein analysis.** RT-PCR and western blot analysis were performed on CMO and DMO thoracic aorta in another independent experimental set of rats to confirm the modulation of expression of IP and Cyp4f2 genes. A significant down-regulation was detected in IP gene in DMO compared to CMO. Relative gene expression levels were 0.46 ± 0.22 in DMO and 1.10 ± 0.24 in CMO (respectively n=7 and n=10, each from 4 different litters, p<0.05, Fig 3A). The decreased expression of IP transcript was confirmed at the protein level (n=5 from 3 different litters, Fig 3B), with an approximately 2.5-fold decrease in DMO compared to CMO (p=0.048), consistent with the 0.5 fold under-expression of the corresponding gene observed by microarray and RT-PCR. We did not detect any significant increase of Cyp4f2 at gene and protein levels (data not shown).

**Pharmacological studies.** We examined whether the decrease in IP protein level was associated with functional modifications. Ex vivo experiments on isolated thoracic aorta rings showed a significantly decreased vasodilatory function in DMO compared to CMO (Fig 4, p<0.01) in response to beraprost.

During in vivo experiments, we observed a blunted decrease in SBP in response to 4 ng/kg/ml iloprost in DMO (-10.7%, from 139 to 124 mmHg) compared to CMO (-24.1%, from 134.4 to 105.8 mmHg, p=0.040 for difference, Fig 5), whereas SBP was identical at lower dosage. Heart rate was similar in the 2 groups (377.0 ± 10.9 bpm in DMO vs. 371.5 ± 12.6 bpm in CMO) at any dose.

**DISCUSSION**

The major result of the present study is that exposure to maternal diabetes is associated with profound changes in aortic gene expression and function in the adult offspring. Indeed, we identified a specific gene expression profile of the thoracic aorta in DMO rats, including genes related to extracellular matrix, cytoskeleton or cellular junctions and arachidonic acid metabolism. We focused on members of the arachidonic acid metabolism and found that IP expression was reduced by 50 percent at the messenger and protein levels in aorta of the DMO rats. Functional implication of IP down-expression was demonstrated in aortic ring experiments with an impaired vasodilatation response to PGI\(_2\) analog of vessels issued from DMO rats. Concurrently, in vivo experiments showed that PGI\(_2\) analog-dependant effect on systolic blood pressure was blunted in DMO.

Our experimental study showing a reduced vascular expression of IP in animals exposed in utero to maternal diabetes reinforce the evidence of a re-programming of vascular function by modifications of the
intrauterine environment. Indeed, vascular functions in the rat offspring has been recently investigated in models of impairment of maternal diet during pregnancy i.e. high fat, hypocaloric or low protein diets. These studies have evidenced a vascular dysfunction mainly related to impaired endothelial functions through the assessment of NO pathway (11; 12; 21; 22). One must note that, in contrast to our experimental protocol, several of these works were conducted in established hypertension and thus did not address early abnormalities of the vessel wall. Thus, it is still unclear whether these abnormalities i.e. impaired endothelium dependant relaxation occur first or develop as a consequence of hypertension. However, endothelial dysfunction may occur without hypertension, Rodford et al. recently identified reduced endothelial responsiveness to acetylcholine, unexpected increased eNOS vascular expression and, systemic reduction of antioxidant protection in a protein-restricted rat model with normal blood pressure (23). Concerning exposure to maternal diabetes, Rocha et al. showed a decreased endothelium-dependent vasodilatation in mesenteric arteries of 12-month-old rats (after several months of hypertension) issued from diabetic mothers (24).

Vascular fetal programming of PGI\textsubscript{2}/IP pathway has never been reported until now. Since prostacyclin plays a key role in several vascular functions in particular in the control of vascular tone by promoting vasodilatation and differentiation of vascular smooth muscle cell (25), our results raise the question of the consequences of an early down-expression of vascular IP (3 month-old animals) in view of the hypertension that will be established at 6 months in our model. Firstly, prostacyclin analog was less effective in reducing blood pressure in DMO rats as compare to CMO. This indicates that IP down-expression is involved in the abnormal regulation of blood pressure in DMO. Secondly, at the vascular level, although vasodilatation properties of resistance arteries i.e. mesenteric arteries are not evaluated here, impaired vasodilatation response to PGI\textsubscript{2} analog is evidenced in DMO aortic rings. Together, these results suggest that IP decreased expression may participate to abnormal regulation of arterial pressure in DMO through impaired vasodilatation responses. Interestingly, vascular expression of IP has been studied in spontaneous hypertensive rats (SHR). In this strain, Numaguchi et al. showed a diminished expression of IP mRNA in thoracic aorta before the onset of hypertension (1 month of age) (26). However, in established hypertension (9 months of age), Tang et al. showed similar levels of IP transcripts in vascular smooth muscle cells of SHR compared to Wistar Kyoto (27). At a functional level, relaxation obtained in aortic rings in response to PGI\textsubscript{2} or IP agonist, was impaired in 3 months-old SHR and was further decreased in older animals (28).

To conclude, our experimental study shows that exposure to maternal diabetes is associated with a decreased IP vascular expression in the adult offspring. Although it is not currently known if fetal programming of IP may be relevant in human, a recent study on a natural mutation of IP (R212C variant, defective in adenyl cyclase activation) has shown that impaired PGI\textsubscript{2} signaling appears to contribute to cardiovascular phenotype in a risk factor-dependent manner (29). If applicable in human, the concept of prenatal resetting of vascular functions by maternal nutrition and particularly by maternal diabetes would certainly open new epidemiologic and therapeutic perspectives.

**Author Contributions.** Duong Van Huyen JP researched data, contributed to discussion and wrote manuscript; Vessières E researched data; Perret C researched data;
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Troise A researched data; Prince S researched data; Guihot AL researched data; Barbry P researched data and contributed to discussion; Henrion D researched data and contributed to discussion; Bruneval P researched data and contributed to discussion; Laurent S contributed to discussion; Lelièvre-Pégorier M contributed to discussion; Fassot C researched data, contributed to discussion and wrote manuscript.

ACKNOWLEDGMENTS
We thank Dr Didier Heudes for his help in image analysis and computing. This research was supported by Fondation de France, ALFEDIAM and INSERM Institutes.

REFERENCES


Table 1: Histomorphological parameters of the thoracic aorta in 3 month old control mother offspring (CMO) and diabetic mother offspring (DMO). MCSA: medial cross-sectional area, IMT: intima-media thickness. Values are mean ± SEM.

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<th>DMO (n=6)</th>
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<td>483 ± 22</td>
<td>461 ± 11</td>
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<tr>
<td>IMT, µm</td>
<td>97.1 ± 3.6</td>
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<td>4921.1 ± 134.0</td>
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<td>Collagen density, %</td>
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<td>15.2 ± 0.02</td>
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**Figure Legends**

**Figure 1. Analysis of profiling data**
Scheme for (a) analysis of profiling data at 3 months of age and (b) demonstration of the robustness of microarray technology with the swap method: correlation between Cy3 values and Cy5 values obtained with the corresponding swap slide for the 22,575 transcripts. DMO: diabetic mother offspring and CMO: control mother offspring.

**Figure 2. Microarray analysis**
(a) Classification of the differentially expressed genes between DMO and CMO according to the 7 functional categories described by Hwang et al. (20) at 3 months of age and (b) genes of interest implicated in extracellular matrix, cytoskeleton, cellular junctions and metabolism of arachidonic acid (d= statistical score).

**Figure 3. Confirmation of modifications observed in arachidonic acid metabolism**
(a) Relative gene expression of IP receptor and in DMO (open bars, n=7 from 4 litters) and CMO (solid bars, n=10 from 4 litters). (b) Western blot analysis of IP receptor levels in the thoracic aorta of 3 months of age DMO and CMO. Each line corresponds to one animal from different litters. Values are mean ± SEM. ** p<0.01 vs. CMO. DMO: diabetic mother offspring and CMO: control mother offspring.

**Figure 4**
Changes in diameter in response to beraprost mediating vasodilatation in thoracic aortic rings of CMO (open circle, n=7) and DMO (solid circle, n=10) at 3 months of age. Values are mean ± SEM. ** p<0.01 vs. CMO. DMO: diabetic mother offspring and CMO: control mother offspring.

**Figure 5. Pharmacological studies**
Dose-response curve to iloprost in CMO (open circle) and DMO (solid circle). Values are mean ± SEM. * p<0.05 vs. CMO. DMO: diabetic mother offspring and CMO: control mother offspring

**Figure 1**
Figure 2

A

Gene expression 27%
Cell communication 23%
Cell structure/motility 12%
Cell/organism defense 6%
Metabolism 8%
Cell division 3%
Unclassified 21%

B

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Figure 3

A

Arbitrary gene expression level normalized to β-actin

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B

IP/β-actin (AU)

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* indicates significant difference.
Figure 4

Figure 5