Specific Local Cardiovascular Changes of \(\text{N}^\varepsilon\text{-}(\text{Carboxymethyl)lysine, Vascular Endothelial Growth Factor, and Smad2 in the Developing Embryos Coincide With Maternal Diabetes–Induced Congenital Heart Defects\) 

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OBJECTIVE—Embryos exposed to a diabetic environment in utero have an increased risk to develop congenital heart malformations. The mechanism behind the teratogenicity of diabetes still remains enigmatic. Detrimental effects of glycation products in diabetic patients have been well documented. We therefore studied a possible link between glycation products and the development of congenital cardiovascular malformations. Furthermore, we investigated other possible mechanisms involved in this pathogenesis: alterations in the levels of vascular endothelial growth factor (VEGF) or phosphorylated Smad2 (the latter can be induced by both glycation products and VEGF).

RESEARCH DESIGN AND METHODS—We examined the temporal spatial patterning of the glycation products \(\text{N}^\varepsilon\text{(carboxymethyl)lysine (CML) and methylglyoxal (MG) adducts, VEGF expression, and phosphorylated Smad2 during cardiovascular development in embryos from normal and diabetic rats.\)

RESULTS—Maternal diabetes increased the CML accumulation in the areas susceptible to diabetes-induced congenital heart disease, including the outflow tract of the heart and the aortic arch. No MG adducts could be detected, suggesting that CML is more likely to be indicative for increased oxidative stress than for glycation. An increase of CML in the outflow tract of the heart was accompanied by an increase in phosphorylated Smad2, unrelated to VEGF. VEGF showed a time-specific decrease in the outflow tract of embryos from diabetic dams.

CONCLUSIONS—From our results, we can conclude that maternal diabetes results in transient and localized alterations in CML, VEGF expression, and Smad2 phosphorylation overlapping with those regions of the developing heart that are most sensitive to diabetes-induced congenital heart disease. Diabetes 58: 1222–1228, 2009

Diabetes increases a women’s risk to give birth to a child with a congenital malformation. Newborns of mothers suffering from type 1 diabetes have a two- to sixfold higher risk of developing congenital malformations (1–3), while that of children from women with type 2 diabetes is raised with a factor of 3–11 (4–6). The majority of these malformations concern the cardiovascular system. Epidemiological studies indicate that maternal diabetes is one of the most important risk factors for the development of congenital heart disease (CHD) (7). Considering the rapid increase of pregnant women with diabetes, the occurrence of maternal diabetes–induced CHD is a serious and increasing health problem.

How maternal diabetes induces CHD still remains to be elucidated. We have studied the involvement of two mechanisms causing complications in adult diabetic patients, being glycation and disturbance of vascular endothelial growth factor (VEGF) levels, assuming that these might also harm embryonic tissues and could thereby disturb embryonic cardiovascular development.

The formation and accumulation of advanced glycation end products (AGEs) such as \(\text{N}^\varepsilon\text{(carboxymethyl)lysine (CML) and methylglyoxal (MG) adducts are important pathophysiological mechanisms in the development of cardiovascular complications in diabetic patients (8). Accumulation of AGEs has been reported in diabetic patients suffering from micro- and macrovascular complications and delayed wound healing (9,10) as well as in diabetic rat models (11,12). In patients, the occurrence of the complications could be prevented using pharmacological inhibitors of AGE formation such as aminoguanidine (13,14), emphasizing the importance of this mechanism in the development of diabetes complications. In rat embryos cultured in high-glucose medium, an increase in a major precursor in the formation of AGEs, i.e., 3-deoxyglucosone, was observed (10), indicating a role for glycation in the development of congenital malformations. Indeed, high values of A1C during pregnancy do not only indicate suboptimal glycemic control, they are also related to the incidence of congenital malformations (15). If their levels in pregnant women become high (A1C >14.4), malformations are identified in 40% of the neonates (16).

To study the impact of glycation on cardiovascular development, we investigated the presence of the AGEs...
CML and MG adducts in embryos derived from diabetic and nondiabetic rats. We used Sprague-Dawley–derived outbred U (Uppsala) rats, previously noted for a high rate of congenital malformations in response to maternal diabetes (17,18). In addition, we used embryos of the L and B inbred rat strains derived from U and H rats, respectively. In the latter strain, diabetic pregnancy did not increase congenital malformations in the offspring.

A second strong candidate for a role in maternal diabetes–induced CHD is VEGF. For normal embryonic and cardiovascular development, it is essential that VEGF is maintained between critical threshold levels. Mice missing only one allele die at E10.5, displaying abnormal vessels (19), while also a two- to threefold overexpression of VEGF results in severe cardiovascular malformations (20). Malformations of the cardiac outflow tract, such as Tetralogy of Fallot, that are increased in the offspring of diabetic women can be induced by selective knockout of both the VEGF188 and VEGF164 isoforms in murine embryos and are related to a local increase of the VEGF120 isoform and VEGF signaling in the heart (21,22).

Culture of young mouse embryos (E7.5 until E9.5) in elevated glucose levels resulted in abnormal development of the vitelline circulation. This phenomenon could be prevented by the addition of VEGF (23), suggesting that the effect of elevated glucose on vascular development is mediated by a downregulation of VEGF. Furthermore, VEGF plays a role in vascular complications in adults with diabetes (24), underscoring the importance of fine balanced VEGF levels for vascular tissue.

We studied VEGF expression locally in the developing heart using in situ hybridization on sections of rat embryos from dams with or without diabetes.

Disturbance of Smad2 signaling in the fourth pharyngeal arch artery (PAA) has been linked to arch malformations in the transforming growth factor (TGF)-β knockout mice model (25). Because both glycation and VEGF influence localization of CML and MG adducts in E13 and E14 embryos of the malformation-prone L and U rat strains and the resistant B rats with (MD) and without (ND) diabetes.

At E13, CML was identified in all the MD offspring of the Sprague-Dawley rat strain more resistant to diabetes-induced congenital malformations, respectively. All rats were fed a commercial pellet diet (AB Analycen, Lidköping, Sweden) and had free access to food and tap water. They were maintained at an ambient temperature of 22°C with a 12-h light-dark cycle. Introduction of diabetes in female rats was performed by selective knockout of both the VEGF188 and VEGF164 isoforms in murine embryos and are related to a local increase of the VEGF120 isoform and VEGF signaling in the heart (21,22). Because both glycation and VEGF influence localization of CML and MG adducts in E13 and E14 embryos of the malformation-prone L and U rat strains and the resistant B rats with (MD) and without (ND) diabetes.

To identify the potential of AGEs in the development of diabetes-induced CHD, we analyzed the presence and localization of CML and MG adducts in E13 and E14 embryos from the malformation-prone L and U rat strains and the resistant B rats with (MD) and without (ND) diabetes. At E13, CML was identified in all the MD offspring of the malformation-susceptible rats L-MD and U-MD (Fig. 1). The CML+ area was identified in the PAAAs (Fig. 1B and D). More precisely, it overlapped with segments derived from the fourth and sixth PAA and extended just proximal to the connection to the subclavian arteries. This we have shown in the three-dimensional reconstruction of the developing cardiovascular system of an E13 L-MD embryo (Fig. 2B–D) and its schematic representation indicating the various PAA segments (Fig. 2A). Formation of CML also occurred in the vessel wall of the ascending aorta and pulmonary trunk, where staining was more intense in the aortic wall than in the pulmonary trunk (compare Fig. 2C to Fig. 2D). Furthermore, CML+ cells were identified in the outflow tract myocardium bordering the cushions of the outflow tract (Fig. 2B, blue). CML accumulated in the cytoplasm rather than in the extracellular matrix. No CML could be detected in any of the E13 offspring of the L-ND or in MD or ND embryos derived from the malformation-resistant B strain (Fig. 3). In embryos of U-ND rats, we observed a weak CML staining (Fig. 1). Using immunohis-
tochemical analysis, no MG adducts could be detected in any of the embryos at this stage.

At E14, CML was detected in all embryos in the proximal outflow tract myocardium, in the ascending aorta, all PAAs, the subclavian arteries, the dorsal aorta, the pulmonary trunk, and the pulmonary arteries. CML was also present in the cushions of the outflow tract, where it was increased in the MD offspring of the U and L rats (Fig. 4B, D, and F), compared with that in ND embryos or MD embryos of the resistant B rats (Fig. 4A, C, and E). Again no MG adducts could be detected using immunohistochemical analysis.

As hyperglycemia influences VEGF expression and alterations in the amount of VEGF can disturb cardiovascular development, we performed VEGF in situ hybridizations in embryos from ND and MD dams. At E13, a high expression of VEGF was seen in all myocardial cells aligning the endocardial cushions of both the inflow tract and the outflow tract of the heart, the myocardial cells in the ventricular septum, and the fibrous tissue protruding into the base of the atrial septum, the so-called spina vestibuli. At this stage of development, VEGF expression was similar in both ND and MD embryos of L, U, or B rats. E14 ND embryos and the MD embryos of the resistant B rats.

**FIG. 1.** CML detection in E13 embryos of the susceptible L and U strain. Immunohistochemical detection of CML is shown in sections derived from the offspring of rats of the susceptible L and U strain being either nondiabetic (ND) or diabetic (MD). A–D are at the level of the PAA. In E–H, we can see the presence of CML in the outflow tract (which is not yet septated at this stage of development). Ao, aorta; dAo, descending aorta; EC, endocardial cushions; LVOT, left ventricular outflow tract; RVOT, right ventricular outflow tract. Scale bars in A–F, 60 μm; in G and H, 200 μm. (A high-quality digital representation of this figure is available in the online issue.)

**FIG. 2.** Indication of the localization of CML in a three-dimensional reconstruction of an E13 embryo from a diabetic L rat. In this Amira reconstruction (B–D), the position of CML is indicated in purple (when present in the vessel wall) or blue (when present in the myocardium of the outflow tract). The aorta is indicated in red, the pulmonary trunk and ductus arteriosus in green, and the myocardium in gray. The frontal plane (B) illustrates that the CML* area is partly located in the myocardium, the outflow tract (attached to the endocardial cushion) indicated in blue, and partly in the vessel wall of the ascending aorta indicated in purple. The view from the left (C) shows the CML* area on the aortic side of the outflow tract (arrow) as well as in the PAA (arrowhead). The view from the right side (D) shows the presence of CML on the pulmonary side of the outflow tract (arrow) and in the right-sided PAA (arrowhead). In the artist’s impression (A), the location of the third PAA (future carotic artery), the fourth PAA (the left-sided fourth will become part of the aortic arch and the right-sided fourth will be part of the brachial cephalic artery), and the sixth PAA (ductus arteriosus) are indicated. The transparent purple indicates the CML* area. (A high-quality digital representation of this figure is available in the online issue.)

**FIG. 3.** CML detection in the resistant B strain embryos at E13. Immunohistochemical shows that there is no CML accumulation in sections derived from the offspring of rats from the resistant B strain being either nondiabetic (ND) or diabetic (MD). Sections A and B are at the level of the PAA and in C and D at the outflow tract (which is not yet septated at this stage of development). Ao, aorta; dAo, descending aorta; EC, endocardial cushions; LVOT, left ventricular outflow tract, RVOT, right ventricular outflow tract. Scale bar = 60 μm. (A high-quality digital representation of this figure is available in the online issue.)
strain showed a VEGF mRNA expression pattern similar to that seen on E13. However, a diabetes-induced decrease in VEGF expression was observed in five of six MD embryos derived from the malformation-prone L and U strain. This decrease in VEGF could be observed in all areas that normally have high VEGF expression level (Fig. 5).

Because both glycation products and VEGF influence the Smad2 signaling, we analyzed active Smad2 using an antibody against phosphorylated Smad2. At E13, phosphorylated Smad2 was increased in the CML+ positive vessel wall of the ascending aorta and pulmonary trunk as well as in the myocardium of the outflow tract (Fig. 6D) in L and U MD embryos, compared with either the surrounding CML− cells or to the same area from MD-B embryos (Fig. 6B) or ND offspring. In the PAAs of all embryos, the level of phosphorylated Smad2 was relatively high compared with other arteries without a clear distinction for CML+ or CML− areas.

At E14, phosphorylation of Smad2 overlapped with the increased CML patterning, which in this stage of development was present in the cushions of the outflow tract of the heart (Fig. 6F–H). In addition, phosphorylated Smad2 was higher in the cushions of the E14 MD offspring (Fig. 6F) than in the ND offspring (Fig. 6F).

The L and B strains are inbred rat strains of which the L strain is known to present with extra-cardiac malformations such as micrognatia, cleft palate, or skeletal malformations, whereas the B strain is more resistant to these malformations. Cardiovascular anomalies have not been described in these strains. It was not known if the diabetes-induced facial anomalies were linked to cardiovascular malformations. We have therefore studied cardiac morphology of E16 L-MD and B-MD embryos and compared the identified malformations with those we have previously characterized in the U strain embryos of E16 and E18 (18). In the inbred L rats, all MD fetuses had a CHD compared with 67% in the outbred U-MD offspring. Outflow tract anomalies were identified in 74% of the L-MD offspring compared with 63% in the U-MD offspring. Defects of the fourth PAA were seen in 47% of the L-MD and 11% of the U-MD embryos (Table 1). In the B-MD offspring resistant to the maternal diabetes–induced extra-cardiac malformations, the number of cardiovascular malformations is also considerably lower (Table 1). From these data, we can conclude that the L rat strain is susceptible to diabetes-induced CHD comparable to the U rat strain.

**DISCUSSION**

At E13, CML accumulation appeared in a small area of the developing outflow tract and in the PAAs in MD embryos of the susceptible L and U strains. At this developmental stage, the PAAs are still paired and their remodeling has not yet started (18). The addition of myocardium from the secondary heart field to the primary heart tube is almost
FIG. 6. (Co)localization of CML and phosphorylated Smad2. In this figure, we show the (co)localization of CML and phosphorylated Smad2 in the developing cardiovascular system. At E13, no CML (A) and no increase in phosphorylated Smad2 (B) were observed in the outflow tract of the B-MD offspring. In the MD offspring of the susceptible L strain, both CML (C) and phosphorylated Smad2 (D) were increased in the outflow tract of the heart. At E14, CML is identified in the endocardial cushions, although more CML is identified in the U-MD offspring (G) compared with the U-ND offspring (E). At the same time, phosphorylated Smad2 is increased in the cushions of the outflow tract in embryos of the MD offspring (H) compared with those from nondiabetic dams (F). Scale bar in A–D = 60 μm and in E–H = 200 μm. (A high-quality digital representation of this figure is available in the online issue.)

The accumulation of CML and the increase in phosphorylated Smad2 at E13 is asymmetric, being higher on the aortic side than on the side of the pulmonary trunk. This is exactly opposite to the lacZ expression identified in the y96-Myf5-nLacZ-16 transgenic mice, which is predominantly seen on the side of the pulmonary trunk (39). From this, Bajolle et al. (39) hypothesize that deviations from the left/right asymmetry inhibits outflow tract rotation and result in anomalies such as a double outlet right ventricle. Analogous and asymmetric increase in CML accumulation might disturb the left/right signaling in MD embryos and subsequently the rotation of the outflow tract, also resulting in CHD, such as a double outlet right ventricle that is frequently seen in our MD offspring (18). Furthermore, embryos that are heterozygous for both a Smad2 and nodal mutation (40) show defects in left-right patterning, resulting in abnormal cardiac looping. This indicates that also the asymmetric increase in phosphorylated Smad2 (co-localizing with the asymmetric CML increase) in the E13 MD embryos is likely to play a role in diabetes-induced congenital heart malformations.

At E14, septation is almost completed and the process of PAA remodeling is still in progress. Although CML could be detected in the embryonic vasculature in both MD and ND offspring of all strains, increased CML was found in the cushions of the outflow tract in the MD offspring of the susceptible U and L dams. Abnormal cushions as seen on E14 could lead to congenital heart malformations such as Tetralogy of Fallot or double outlet right ventricle. We have previously identified these malformations in combination with hypoplastic cushions in the MD offspring of these rats (18), in which we now show an increase in CML.

CML is an AGE that can be formed on lysine residues in proteins by a glyoxidation reaction (41). In the embryos of our study, we failed to detect MG adducts, which are produced from a glycation reaction. This raises the question whether the CML accumulation indicates an increase in the formation of AGEs or an increase in oxidative stress. MG adducts–mediated formation of nonenzymatic glycation did not take place in sufficient amounts. It cannot be ruled out that other AGEs, which are not detected by the antibodies used in this study, may play a role in the formation of CHD. However, we favor the explanation that, in our model, the increase in CML can be used as a biomarker for oxidative damage, as has been suggested before (41,42). We base this opinion on the fact that antioxidant supplementation reduces the number and severity of the diabetes-induced CHD in the offspring of these rats (17). In addition, increasing the oxidative stress by injection of antimycin A in pregnant mice results in mycardium from the secondary heart field and the function of neural crest cells are hampered in diabetic pregnancies (17,18,36–38).

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similar CHD, as seen in the offspring of diabetic mice (43),
an effect that could be diminished by antioxidant supple-
mentation. Furthermore, CHD was reduced by the addi-
tion of antioxidant in chicken embryos in which neural
crest cells were exposed to elevated glucose (37). Next to
these experimental models, it has been shown that women
who gave birth to a child with a CHD have higher levels of
biomarkers for oxidative stress (44). On the basis of these
data, we are convinced that a local glucose-induced in-
crease in oxidative stress, as pointed out by CML, is an
important mechanism in the development of congenital
malformations in the offspring from diabetic pregnancies.

Elevated glucose levels can disturb the cardiovascular
development by influencing the expression of VEGF (45).
The addition of high glucose concentrations to cultured
mice embryos inhibits endothelial to mesenchymal trans-
formation in the endocardial cushions by a reduction of
VEGF levels (45). In the MD embryos, a clear overlap
between CML accumulation, reduced VEGF expression,
and hypoplastic endocardial outflow tract cushions was
identified. We assume that a decrease in VEGF causes
 cushion hypoplasia and the related maldevelopment of the
outflow tract (double outflow right ventricle and Tetralogy
of Fallot). In addition to our results, decreased levels of
VEGF were identified in cultured embryos and in vitro
endocardial cushions after exposure to elevated glucose
(23,45). This negative relation between diabetes and VEGF
expression in the embryo that we and others found is
opposite to data for adult endothelial cells (46,47).

At E14 in the endocardial cushions of the outflow tract
of the heart, CML and phosphorylated Smad2 were in-
creased, whereas VEGF expression was decreased. AGEs
can induce Smad2 phosphorylation in vitro and in vivo
(26), and therefore CML might be responsible for the raise
in phosphorylated Smad2. However, VEGF has been re-
ported to inhibit Smad2 phosphorylation (48); therefore, a
reduced level of VEGF in these structures could also relate
to the identified increase in Smad2 phosphorylation. Al-
though endothelial to mesenchymal transformation can be
increased by TGFβ/Smad2 signaling (49,50), the hypoplas-
ic appearance of the outflow cushions contradicts this
assumption. We assume that the lack of myocardial VEGF
expression is more detrimental for cushion development
than the stimulating effect one might expect from Smad2.
Unfortunately, the pathways for activation and action of
Smad2 are complex, involving interactions with multiple
players, and at this time, it is not possible to fully elucidate
them.

The incidence of full-blown CHD in fetuses (>E16)
between the L and U strain clearly reveals their genetic
predisposition for diabetes-induced congenital cardiovas-
cular malformations. The high percentage of CHD in the
MD offspring of the inbred L strain, for which rats were
selected on the basis of extra-cardiac malformations,
implies that there is a link between cardiovascular and
noncardiac congenital malformations. The similarity be-
 tween the number and type of malformations identified in
the L-MD and U-MD fetuses justifies the use of the L strain
next to the U strain for the identification of the pathways
involved in diabetes-induced CHD.

In this research, we have provided a novel proof for a
specific temporal-spatial pattern rather than a general
increase in CML accumulation, suggesting a raise in ox-
idative stress. The increase in CML and phosphorylated
Smad2 strikingly overlapped with those cardiovascular
regions that are at high risk during development. Further-
more, the hyperglycemia-related decrease in VEGF was
observed in the same regions. We postulate that local
rather than general changes in CML accumulation, Smad2
phosphorylation, and VEGF expression are detrimental for
the developing cardiovascular system in MD embryos.

ACKNOWLEDGMENTS
This work was funded by the Netherlands Heart Foun-
dation grants NHS2002B035 and NHS2006B074, the Ernfors
Family Fund, the Swedish Diabetes Association, and the
Swedish Research Council Grant 12X-7475.

This work was also funded by the Novo Nordisk Foun-
dation. No other potential conflicts of interest relevant to
this article were reported.

We would like to thank P ten Dijke for the gift of the
antibody raised against phosphorylated Smad2. We also
thank Jan Lens for graphics and layout.

Parts of this study were presented at the 4th Interna-
tional Symposium on Diabetes and Pregnancy, 29–31
March 2007, Istanbul, and at the 12th Annual Weinstein
Cardiovascular Development Conference, 19–22 May
2005, Tucson, Arizona.

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