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Teratogenicity Involved in Experimental Diabetic Pregnancy

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Abstract
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Maternal diabetes is associated with increased risk of growth disturbances and congenital malformations. The malformations rate in the offspring of diabetic mothers is 2-3 fold higher compared to infants of nondiabetic mothers. In this thesis we have investigated the role of the protein kinase C (PKC) pathway and the apoptotic machinery in embryopathy.

We investigated the involvement of PKC isoforms in the embryopathy of diabetic rat pregnancy. Embryos of diabetic rats showed altered activity and protein distribution of several PKC isoforms compared with embryos of normal rats. Using whole embryo culture we found increased activity of PKC-delta and PKC-zeta after 24h of culture and increased rate of malformations and growth retardation in embryos cultured in high glucose concentration compared to embryos cultured in low glucose concentration. Addition of α-cyano-4-cinnamic acid and N-acetylcysteine to the culture medium normalized malformations and growth retardations whereas specific PKC-inhibitors abolished malformations and partly restored the growth retardations. All treatment normalized glucose-induced increase of PKC activity.

Estimated occurrence of apoptosis in embryos of diabetic rats and in embryonic cells exposed to high glucose concentration showed increased rate of pro-apoptotic markers. The increased apoptosis in the high glucose exposed embryonic cells was normalized by supplementation of N-acetylcysteine or apoptosis inhibitor. Treatment with vitamin E and folic acid to diabetic pregnant rats decreased diabetes-induced malformations and resorptions, concomitant with normalization of apoptotic protein levels.

These results suggest that oxidative stress is augmented in embryos of diabetic rats and that it also plays a role in the activation of PKC and apoptosis. We used antioxidative treatment with beneficial effect although we could not completely abolish the embryonic demise; this may indicate that other mechanisms are involved in diabetic embryopathy. Further studies are needed to develop multi-nutrient dietary supplement to eliminate embryonic abnormalities induced by maternal diabetes.

Keywords: Diabetes, Pregnancy, PKC, Apoptosis, Rat, Embryopathy, Vitamin E, Folic acid, CHC, NAC

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”Perfekt!” sa jag. ”Jag visste att du kunde!”
”Har vi kommit till slutet?” frågade Nasse.
”Ja”, svarade jag, ”jag antar det.”
”Det *verkar* vara slutet”, sa Puh.
”Det gör det. Och ändå-”
”Ja, vadå, Nasse?”
”På sätt och vis verkar det också som en början.”

Benjamin Hoff, Te enligt Nasse, 1992

Pappa fosskar förstår du, ja just de!

Agust, 2.5 år

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List of Papers

This thesis is based on the following papers:

I Mattias Gäreskog, Parri Wentzel
Altered Protein Kinase C Activation Associated with Rat Embryonic Dysmorphogenesis *Pediatric Research* 56: 849-857, 2004.

II Mattias Gäreskog, Jonas Cederberg, Ulf J. Eriksson, Parri Wentzel
Maternal diabetes *in vivo* and high glucose concentration *in vitro* increases apoptosis in rat embryos *Reproductive Toxicology, in press 2006.*

III Mattias Gäreskog, Ulf J. Eriksson, Parri Wentzel
Combined Supplementation of Folic Acid and Vitamin E Diminishes Diabetes-Induced Embryotoxicity in Rats *Birth Defects Research A,* 76: 483-490, 2006.

IV Mattias Gäreskog, Parri Wentzel
N-acetylcysteine and CHC alter PKC-δ and PKC-ζ and diminish dysmorphogenesis in rat embryos cultured with high glucose *in vitro (Manuscript)*.

The papers will be referred to by their Roman numerals. Reproductions were made with permission from the publishers.

Contents

Introduction....11
Diabetes mellitus....11

Background....13
Diabetic pregnancy....13
Protein kinase C....15
PKC-delta....16
PKC-zeta....16
Apoptosis....16
p53....18
Bcl-2....18
Caspases....19
Oxidative stress....19
Treatment....20
Vitamin E....20
Folic acid....21
N-acetylcysteine....21
α-cyano-4-cinnamic acid....22
Embryonic development....22
Streptozotocin....23
Animal model....24

Aims....25

Material & Methods....26
Animals....26
Treatment (Paper III)....26
Preparation of total RNA (Paper I & II)....27
Preparation of cDNA (Paper I & II)....27
Analysis of mRNA levels (Paper I & II)....27
Estimation of PKC activity markers (Paper I and IV)....28
Fractionation of embryonic cells....28
Immunoblot analysis....29
Measurement of protein....29
Western blot analysis (Paper II & III)....29
Immunostaining of embryos (Paper I & II)....30

Whole embryo culture (Paper II and IV)....30
Culture of embryonic cells (Paper II)....30
Analysis of Nuclear factor-κ B activation (Paper III)....31
Extraction of nuclear proteins....31
Electromobility shift assay....31
Detection of cell death and apoptosis (Paper II)....31
Activated Caspase 3 detection in living cells....31
Propidium iodide flow cytometry....32
Vital staining with propidium iodide and Hoechst 33342....32
Statistical considerations....32

Results and discussion....33
Paper I....33
Paper II....34
Paper III....36
Paper IV....38

Conclusions....41

Summary in Swedish....43

Acknowledgements....45

References....47

Abbreviations

ANOVA	Analysis of variance
AU	Arbitrary unit
CHC	α-cyano-4-hydroxycinnamic acid
Cp	Crossing point
CuZnSOD	Copper-Zinc superoxid dismutase
ECL	Enhanced chemiluminescent
EMSA	Electromobility shift assay
FACS	Fluorescence activated cell sorter
GAPDH	Glyceraldehyd-6-phosphate dehydrogenase
GSH	Glutathione
G6PDH	Glucose-6-phosphate dehydrogenase
IDDM	Insulin dependent diabetes mellitus
MD	Manifestly diabetic
MnSOD	Manganese superoxid dismutas
N	Non-diabetic
NAC	N-acetylcysteine
NF-κB	Nuclear factor-κ B
NIDDM	Non insulin dependent diabetes mellitus
PARP	Poly ADP-ribose polymerase
PCR	Polymerase chain reaction
PIP_2	Phosphatidylinositol bisphosphate
PKC	Protein kinase C
PTP	Permeability transition pore
ROS	Reactive oxygen species
STZ	Streptozotocin
TCA	Tricarboxylic acid cycle
$\Delta\Psi m$	Mitochondrial membrane potential

Introduction

Diabetes mellitus

Diabetes mellitus is a combination of several different diseases with various etiologies. All these diseases have one aspect in common, high glucose level in the blood due to insufficient secretion of insulin from the beta cells of the Langerhans islets in the pancreas. This condition has been known for over 3000 years, and previously was called “honey urine” because of the large amounts of sweet urine excreted by the patients. The two most common forms of diabetes are insulin dependent diabetes mellitus (IDDM), also called type 1 diabetes, and non insulin dependent diabetes mellitus (NIDDM), also called type 2 diabetes.

Type 1 diabetes represents approximately 5-10% of all cases of diabetes and is often called juvenile-onset diabetes because the onset of the disease is most common in younger age groups. This type of diabetes is characterized by an autoimmune destruction of the beta cells, which leads to an inability to produce insulin, and to a lifelong need for insulin treatment.

Type 2 diabetes is estimated to represent 90-95% of all cases of diabetes. It is a metabolic disease that develops due to a deteriorated function of the beta cells, in combination with a substantial insulin resistance in the body. It has previously been considered as an adult-onset form of diabetes. However, this is not always the case today since some young individuals have been diagnosed with type 2 diabetes and obesity is considered as a risk factor for this form of diabetes.

Today, more than 230 million people have diabetes worldwide and the number is increasing (1). If nothing is done to slow the epidemic, the number of sufferers will exceed 350 million by 2025 (1). Today there is extensive research on transplantation of beta cells to type 1 patients, but also considerable efforts are made to change dietary and exercise habits of people with increased risk of developing the diseases.

A life with high glucose concentrations in the blood does not only imply a life with injections or efforts in controlling diet and weight but also several secondary complications associated with the diabetic state. These complications are the major cause of morbidity and mortality in persons with type 1 and type 2 diabetes (2). After some years with the disease patients may ex-

perience problems with eyesight, peripheral nerve sensitivity, kidney function and circulation. Another risk for complications occurs when the diabetic women get pregnant. The risk of having a malformed child in a diabetic pregnancy is 2-3 fold compared to a normal pregnancy, in addition to increased risk for preeclampsia, miscarriage and stillbirths (3-6).

In recent years, advances in monitoring of blood glucose levels have decreased the incidence and severity of these complications (7-9). However, it is still difficult to keep normal blood glucose levels constantly. For certain complications a good metabolic control is insufficient to diminish the problems, it can merely mitigate and detain them. With regard to complications in diabetic pregnancy, the intense clinical monitoring of the pregnant diabetic woman during the last decade has decreased the rate of congenital malformations. However the incidence of fetal dysmorphogenesis is still increased in the diabetic pregnancy (5, 10-15).

Background

Diabetic pregnancy

Before the introduction of insulin, successful pregnancies among women with IDDM were few. There was a concern in the medical community that diabetes was inconsistent with pregnancy (16). In 1882 Van Noorden observed that only 5 % of 427 diabetic women became pregnant (17) and only 10 pregnancies occurred in 1300 diabetic women between 1898 and 1917 (18). Indeed, before the discovery and use of insulin in 1922 the risk of complications during pregnancy was extremely high. The maternal mortality was 45% and the perinatal mortality as high as 70% (19). When it also became clear that a pregnant diabetic woman, with intensive insulin treatment should try to achieve normoglycemia, several studies reported a dramatic decrease of the risk for fetal and neonatal complications (20-22). These experiences, i.e., that strict metabolic control in the periconceptional and early pregnancy lowered frequency of malformation and abortion, gave rise to further studies highlighting good glucose control throughout full gestation and even better outcome.

However, it does not seem that the blood glucose concentration is the only reason for the increased risk for preeclampsia, miscarriage, stillbirths, macrosomia and malformations in a diabetic pregnancy compared to a non-diabetic pregnancy (11, 23). Despite insulin treatment complications still occur. Besides increased glucose levels (24), which are considered to be the major diabetic teratogen, other suggestions for the induction of diabetic embryopathy have emerged during recent years.

Studies have shown, in a high glucose concentration milieu *in vitro*, decreased inositol levels due to impaired uptake (25) yielding an embryonic deficiency of inositol and subsequent embryonic dysmorphogenesis (26, 27). These findings have been confirmed by addition of inositol to high glucose cultured embryos (28, 29) and by dietary addition to diabetic pregnant rodents (30, 31) which yielded less embryonic maldevelopment. Furthermore studies have shown that treatment with the competitive inhibitor scyllo-inositol to culture medium induces both inositol deficiency and embryonic dysmorphogenesis of similar type as the damage caused by high glucose

alone (27, 32). These effects can be diminished by addition of inositol. These findings show that inositol deficiency plays a role in diabetic teratogenesis.

Disturbed metabolism of arachidonic acid and prostaglandins has been found in previous studies of experimental diabetic pregnancy. Addition of arachidonic acid to embryonic high glucose culture medium, intraperitoneal injections, and diet enrichment of arachidonic acid to pregnant diabetic rats have all been found to block embryonic dysmorphogenesis (33-36). These findings indicate a disturbance in the arachidonic acid cascade as a consequence of a diabetic milieu.

In addition, measurements of PGE_2 have indicated that this prostaglandin is decreased in embryos of diabetic rodents during neural tube closure in high glucose cultured embryos (37, 38) compared to that embryos of non-diabetic rats. In high glucose cultured embryos the expression of PGE_2 decreases after neural tube fusion to the same level as embryos from diabetic rats indicating that PGE_2 has an important role in the process of neural tube fusion. A non-fused neural tube is a common malformation in diabetic pregnancy. Adding PGE_2 to culture medium blocks glucose-induced teratogenicity *in vitro*, as well as maldevelopment of embryos cultured in diabetic serum (28, 36).

The notion that diabetes is associated with oxidative stress has been suggested (39-41). Increases of several indicators of oxidative stress have been found in diabetic rats, such as serum F_2-isoprostanes and protein carbonyls in tissues (38, 42, 43). Studies have suggested that the diabetes-induced generation of oxidative stress could be implicated in diabetic complications (44, 45). Findings that strengthen these ideas are the anti-teratogenic interventions with antioxidants. Dietary addition of butylated hydroxytoluene (46), vitamin E (47-49) and vitamin C (50) diminished perturbed embryonic development *in vivo,* whereas addition of SOD (41, 51), catalase (41) and NAC (52, 53) diminished embryonic dysmorphogenesis *in vitro*. Thus, oxidative stress plays an important role in the pathogenesis of diabetes.

When different animals and different strains are exposed to hyperglycemia the outcome of the insult can result in various effects. One reason for this is probably genetic predisposition. Cederberg and Eriksson found an impaired expression of the scavenging enzyme catalase in the malformation-prone U rat strain. There was a decreased activity of catalase in the U strain compared to the malformation-resistant H rat strain (54). Studies of the catalase gene revealed two differences between the H and U rat, a heterozygosity in the promoter region of the malformation-resistant strain and a base substitution in the 3'UTR region of the mRNA of the malformation-prone strain. Indeed, genetics is involved in the development of malformations in diabetic pregnancy.

In recent years several studies have been undertaken to elucidate the risk of having a malformed child in a diabetic pregnancy. The hypothesis is that the growing awareness and tight monitoring of blood glucose today would

yield a risk for malformations in pregnant diabetic women that does not differ from that in nondiabetic pregnancy. A Swedish study between 1987-97 found a malformation rate of 9.5% in mothers with pre-existing diabetes (14) while a Finnish study found a rate of 4.2% during the same period (13). The difference in these values is probably because of different definitions of malformations. Today, it is generally agreed that the malformation rate in a diabetic pregnancy is increased 2-3 times compared to that in a nondiabetic pregnancy (3).

Some anomalies are associated with diabetes, mainly cardiovascular, central nervous system, and musculoskeletal malformations (4, 55). These anomalies are 5- to 10-fold more frequent in infants of diabetic mothers than in those of nondiabetic mothers (11). The malformation that is considered to be the most strongly connected to diabetes is the caudal regression syndrome, which is 200- to 400-fold more frequent than in nondiabetic pregnancies (3). Nonetheless, the caudal regression syndrome is still rare. To avoid these malformations it is important for the pregnant diabetic women to carefully adjust blood glucose levels during pregnancy and especially during the first trimester when organogenesis take place. Still, there are diabetic women that do not know that they are pregnant and also pregnant women that do not know that they have diabetes. Moreover, even good control of blood glucose levels does not completely reduce the risk of having a malformed child.

Protein kinase C

Protein kinase C (PKC) isoforms have been suggested to be involved in diabetic complications (56). PKC is a family of structurally and functionally related proteins and belong to the serine/threonine kinases. The PKC family participates in various signal transduction pathways, as a response to specific hormonal, neuronal and growth factor signals (57). Today, 12 isoforms of PKC have been cloned and characterized (58). These isoforms have been divided into three different subclasses based on structure and ability to bind co-factors. The conventional isoforms alfa, beta1, beta2 and gamma require diacylglycerol and Ca^{2+} -ions to be activated. The novel isoforms delta, epsilon, eta, theta and mu require diacylglycerol but not Ca^{2+} -ions. The atypical isoforms zeta and iota/ lambda need neither Ca^{2+} -ions nor diacylglycerol. All these isoforms have varied distributions in different tissues. Each PKC isoform is a separate gene product except PKC-beta1 and beta2, which are alternatively spliced variants of the same gene product. The PKC isoforms consists of a single polypeptide chain with an amino-terminal regulatory domain and a carboxy-terminal catalytic domain. The regulatory domain possesses motifs involved in binding phospholipids, co-factors and Ca^{2+}, and participates in protein interactions regulating PKC activity and localization.

The catalytic domain is a kinase region involved in ATP and substrate binding.

PKC-delta

PKC-delta was the first novel isoform to be identified and is expressed ubiquitously among cells and tissues suggesting that PKC-delta has universal rather than cell-type-specific roles in mammals. It is activated by diacylglycerol, produced by hydrolysis of membrane inositol phospholipids, as well as by tumour-promoting phorbol esters through the binding of these compounds to the C1 region in its regulatory domain. It is also cleaved by Caspase to generate catalytically active fragments, and it is converted to an active form without proteolysis through the tyrosine phosphorylation reaction.

Studies have shown that PKC signaling is associated with apoptosis, especially the isoform PKC-delta (59). It has been suggested that PKC-delta is involved in stabilizing p53 proteins (60), or related to reactive oxygen species production (61) or both. Both suggestions would ultimately lead to apoptotic cell death. This pro-apoptotic role has been recognized in various cells (62-65). Another finding of worth is that PKC-delta translocates to mitochondria to alter its function (66, 67).

PKC-zeta

PKC-zeta was originally discovered as a unique PKC isotype (68). Today it has been classified into the atypical subfamily of PKC. It is expressed in several tissues such as brain, kidney, heart and aorta. The mechanism of activation mainly consists of two events: release of the pseudosubstrate sequence from the substrate-binding cavity and phosphorylation of the kinase domain (69). The molecule responsible for the phosphorylation of PKC-zeta is the 3'-PI-dependent protein kinase 1 (PDK1) (70). PKC-zeta may also be activated by lipid components such as phosphatidylinositols (71) and arachidonic acid (72). PIP_2 contributes to the activation of PKC-zeta in two ways: by direct modulation of the inhibiting pseudosubstrate, and indirect modulation by phosphorylation of the kinase domain through PDK1.

PKC-zeta has been suggested to regulate nuclear events essential for the initiation of the apoptotic pathway (73). PKC-zeta regulates the NF-κB signalling system and the intrinsic mitochondrial apoptotic route (74) through influencing Bcl-2 family protein expression (75-77).

Apoptosis

Apoptosis is a programmed cell death occurring at predictable locations and times during development (78). Apoptotic death signals are transduced by

biochemical pathways to activate Caspases that cleave specific proteins. The proteolysis of these critical proteins then initiates cellular events of cell death morphologically characterized by membrane budding, nuclear and cytoplasmic shrinkage and chromatin condensation (79). These steps prepare apoptotic cells for phagocytosis and result in the efficient recycling of biochemical components (80).

Apoptosis is divided into two pathways: the extrinsic and the intrinsic pathway. The extrinsic pathway is also called the death-receptor pathway and is triggered by members of the death-receptor family such as CD95 and tumour necrosis factor receptor I. The intrinsic pathway is mediated by mitochondria. This pathway is often triggered by external insults through the activation of pro-apoptotic members of the Bcl-2 family.

Cell division and cell migration are essential in a developing organism, but apoptosis or programmed cell death is just as important in regulating cell numbers to create the right proportion of organs and to remove cells that could be harmful. Most of the cells produced during mammalian embryonic development undergo physiological cell death before the end of the perinatal period (81). A teratogenic insult is often followed by distortions in the pattern of apoptosis in embryonic organs destined to be malformed (82).

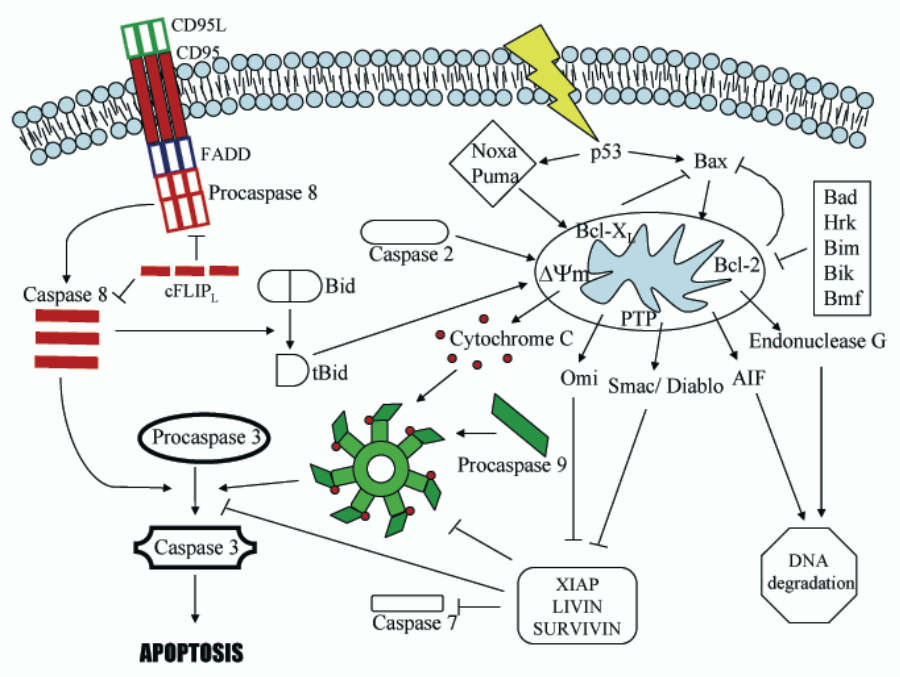

Fig 1. Apoptotic pathways

p53

p53 is a transcription factor and a tumor suppressor that regulates the cell cycle and functions as a tumor suppressor. It has been described as “the guardian of the genome” due to its role in conserving stability by preventing genome mutation (83). p53 is central to many of the cell’s anti-cancer mechanisms. It can activate DNA repair proteins when DNA has sustained damage, it can hold the cell cycle at the G_1/ S phase on DNA damage recognition and it can initiate apoptosis if the damage proves to be irreparable. p53-induced apoptosis is mediated by activation of genes involved in both the intrinsic pathway (Bax, Noxa, Puma, p53AIP1, PIGs, and APAF-1) and the extrinsic pathway (Killer/DR5, FAS, and PIDD). p53 also transcriptionally represses cell survival genes, such as IGFR, Bcl-2, or survivin, leading to apoptosis induction (84, 85). p53 is activated by many environmental stimuli, including DNA-damaging agents such as irradiation and chemicals, physiological effects such as depletion of growth factors and certain conditions such as diabetes. Besides its role in cancer and genetic stability, p53 is involved in apoptosis, inhibition of angiogenesis and embryonic development (86, 87).

Bcl-2

Bcl-2 family plays an important role as regulator of programmed cell death and apoptosis. Several of the proteins in this family were originally identified at the chromosomal breakpoint of the translocation between chromosome 14 and 18 in B-cell lymphomas. The Bcl-2 family comprises members that induce death (Bax, Bak, Bcl-X_S, Bad, Bid, Bik and Hrk) and inhibit death (Bcl-2, Bcl-X_L, Bcl-w, Bfl-1, Brag-1, Mcl-1 and A1), which differ in their tissue localization and activation pattern. These members also differ structurally; most proteins in the Bcl-2 family possess a carboxy-terminal transmembrane region, except Bad and Bid. Additionally, they possess variable amounts of Bcl-2 homology regions that determine their capacity to interact with each other and with other unrelated proteins. The ability to form heterodimers and homodimers between anti-apoptotic and pro-apoptotic members is suggested to play a neutralizing role in the rheostat theory, which suggests that the balance between death inducers and death inhibitors decides the outcome of the specific cell. Most studies suggest that Bcl-2 related proteins have to localize to mitochondria to regulate apoptosis by triggering or protecting the mitochondrial membrane potential.

Caspases

Caspases are a family of cysteine proteases that is activated specifically in apoptotic cells. They are homologous to each other and highly conserved through evolution from insects and nematods to humans. At least 14 Caspases have been identified in human and about two-thirds of these have been suggested to function in apoptosis. Caspases have been divided into subfamilies based on their substrate preference, extent of sequence identity and structural similarities. These are cytokine processing Caspases, initiator Caspases and executioner Caspases (88). Caspases are synthesized as inactive pro-Caspases that are proteolytically processed, at critical aspartate residues, to their active form. All pro-Caspases contain a protease domain and a NH_2 terminal prodomain. The protease domain contains two subunits that associate to form a heterodimer after proteolytic processing. Two heterodimers then associate to form a tetramer, which is the active form of Caspase. The NH_2 terminal domain varies in length depending on the functional aspects of the Caspase. A recent article by Boatright and Salvesen (89) proposes different activation mechanisms for initiator and executioner Caspases. Initiator Caspases are activated by dimerization and executioner Caspases that exist as preformed dimers are activated by cleavage.

Oxidative stress

Oxidative stress is a condition when the balance between reactive oxygen species (ROS) and antioxidant defence has tipped in favour to the former. The reason for this could be a depletion of antioxidants due to malnutrition or an excess production of ROS via exposure to elevated O_2 concentrations, the presence of toxins that are metabolized to produce free radicals or excessive activation of natural radical-producing systems.

These excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids and eventually cell death. These disturbances may lead to several conditions complicating the human life quality.

The group of ROS consists of the free radicals, hydroxyl (OH•) and superoxide (O_2•). The hydroxyl radical is the most potent oxidant known; it has a very short half-life and reacts at a diffusion-controlled rate with almost all molecules in living cells. The superoxide radical is produced by phagocytic cells and helps them to inactivate viruses and bacteria. These molecules are extremely reactive due to the possession of unpaired electrons. Some non-radical derivates of O_2 exist, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3).

The body has a major antioxidant defence mechanism to protect itself from free radical attack. The superoxide dismutase (SOD) enzymes are pow-

erful molecules in the defence system against ROS and exist mainly in two isoforms: MnSOD and CuZnSOD. MnSOD contains manganese at its active site and is situated mainly in the mitochondria. CuZnSOD contains copper and zinc at its active site and is situated mainly in the cytosol. These enzymes dismutate O_2 to generate H_2O_2 and work in collaboration with other antioxidants such as catalase and glutathione peroxidase to further convert H_2O_2 to water and ground state oxygen. Antioxidants such as vitamin E, β-carotene, and coenzyme Q are present within the cell membranes and are also important factors against ROS.

The role of ROS in diabetic pregnancy has been investigated during many years; the first evidence of the detrimental impact of ROS on diabetic pregnancy was established in 1991 by Eriksson and Borg (41). They found that glucose-induced embryonic dysmorphogenesis could be diminished by addition of free oxygen radical scavengers. Since, others have confirmed the involvement of ROS in diabetic pregnancy. Furthermore, recently Brownlee and associates have proposed a model for a unifying mechanism for all complications in diabetes based on an overproduction of ROS from the mitochondrial electron transport chain (90). Thus, the ROS production seems to have a major role in the developing complications of diabetes.

Treatment

Vitamin E

Vitamin E is absorbed from the intestine together with dietary fats and is released into the circulation with chylomicrons (91). The α-tocopherol concentration in a human fetal liver is approximately 20-40% of that in adult livers (92). The vitamin E concentration in human fetal cord serum is approximately one quarter of that in maternal serum and the concentration in fetal serum correlated with that in maternal serum (93).

The α-tocopherol transfer protein (a-TTP) exists in the liver and facilitates the incorporation of α-tocopherol (94). The presence of α-tocopherol in uterus has recently been demonstrated in mice (95) playing an important role in supplying the placenta and the fetus with α-tocopherol throughout pregnancy. Otherwise, little is known about the transfer of lipid-soluble vitamins across the placenta.

Dietary supplementation with α-tocopherol to pregnant rats increased the concentration of this vitamin in a dose-dependent manner in maternal plasma and liver. Fetal liver concentration of α-tocopherol increased in a dose-dependent manner by maternal vitamin supplementation (96). However, studies suggest that the vitamin E transfer through the placental barrier is low (97, 98).

Conclusively, oral supplementation of the mother should be a good means of increasing fetal vitamin E concentration.

Folic acid

The cellular uptake of folate involves two distinct pathways: 1) The reduced folate carrier (RFC) pathway, via an integral transmembrane protein present in all cells (99) and 2) the membrane-associated folate receptors (FRs) pathway (100). The FRs are variably expressed in tissues as three different isoforms: alfa, beta and gamma (101). The alfa and beta isoforms of the FRs are expressed in human adult and fetal tissues (102) and are abundant in human placental tissue, where they play a major role in maternal-fetal folate transport (103).

Transfer of folate across the human placenta from mother to fetus involves participation of a folate receptor expressed in the syncytiotrophoblasts (104). FRs are also strongly expressed on the endometrium and deciduas of the pregnant uterus. Embryos express FRs even on early-cleavage cells. The egg cylinder, all three germ layers of the embryo, the ectoplacental cone, extraembryonic membranes, maternal giant cells and deciduas all express FRs, which indicates that these receptors have a function in folate transport at all stages of embryogenensis (105).

It is now well established that administration of folic acid before conception and during pregnancy reduces the occurrence of congenital abnormalities (106-108).

However, folic acid is essential for embryogenesis because this vitamin is the source of the reduced folate cofactors that transfer the one-carbon units required for the synthesis of nucleic acids and some amino acids in these highly proliferative embryonic cells (109). This could be a primary effect on the developing embryo.

N-acetylcysteine

N-acetylcysteine (NAC) is an amino acid with a molecular weight of 163.2 which was introduced as a mucolytic agent for chronic pulmonary diseases about 50 years ago (110).

It acts as an antioxidant, both directly as a glutathione (GSH) substitute by providing SH groups and scavenge ROS, and indirectly as a precursor to GSH (111).

GSH is the most abundant low-molecular-weight thiol in animal cells and plays a central role in the antioxidant defence against ROS. For GSH synthesis, the availability of cysteine is generally the limiting factor, and one of the effective precursors of cysteine is its synthetic derivative, NAC (112).

NAC may also have other actions due to its precursor actions of GSH. GSH may act as a co-factor in some enzyme reactions. Most relevant to hy-

perglycemia is the glyoxalase pathway, which is responsible for metabolism of reactive triose phosphate-derived 3-carbon intermediates such as methylglyoxal (113). Because methylglyoxal has been implicated in the formation of advanced glycation end products and diabetic complications (114, 115), the reduction of this dicarbonyl intermediate by NAC could be an alternate possibility to reduce diabetes-induced complications (116).

α-cyano-4-cinnamic acid

The transport of pyruvate into the mitochondria is maintained via a specific mitochondrial pyruvate carrier (MPC) discovered in 1974 with the use of the inhibitor α-cyano-4-cinnamic acid (CHC) (117). It was shown that CHC inhibited mitochondrial pyruvate transport by specifically modifying a thiol group on the MPC (118).

Pyruvate uptake into the mitochondria during hyperglycaemic conditions seems to be increased, which in the long term could lead to an increased generation of ROS. Our hypothesis is that CHC restricts the uptake of excess pyruvate into the mitochondria, thereby reducing the harmful overproduction of ROS in the cells.

Embryonic development

This work has focused on rat embryonic development at gestational day 10 and 11, which corresponds to human development during the 4^{th} and 5^{th} week postconception. During these two days the embryo is growing rapidly and develops several important organs such as the heart and connecting vessels, the neural tube and facial structures e.g., the mandible. A disturbance of the development at this time may result in congenital malformations. In diabetic pregnancy we find malformations affecting specifically the heart and the mandible, and the neural tube is often not completely fused. These malformations can be reversed by supplementation of antioxidants and folic acid (96). Day 10 and day 11 can be considered as two separate embryonic periods where antiteratogenic treatments may exert completely different effects on the embryo, mainly because the shift from a yolk sac placenta to the permanent chorioallantoic placenta occurs during these two days.

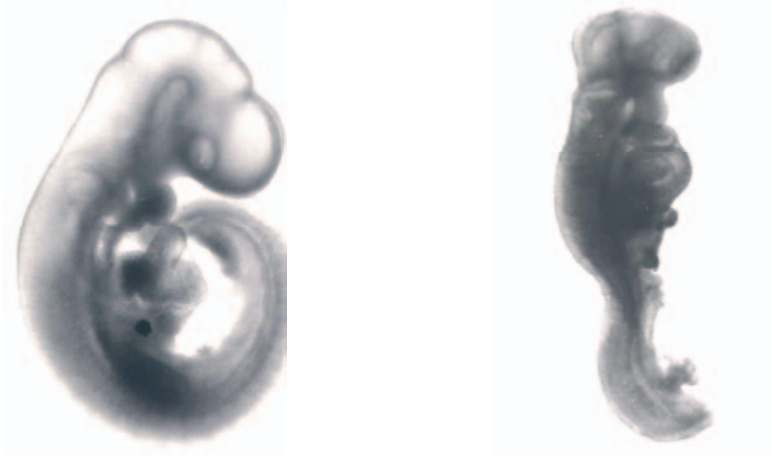

Fig 2. Normal embryo at gestational day 11. Malformed embryo at gestational day 11.

Streptozotocin

Streptozotocin (STZ) is derived from the soil microorganism Streptomyces achromogenes and was developed in the 1960s as an antibiotic (119). In 1963, STZ was found to be specifically toxic to beta cells in the islets of Langerhans and it is now widely used to induce diabetes in experimental animal models (120, 121).

STZ consists of 1-methyl-1-nitrosourea connected to position C2 of a D-glucose molecule (122). The glucose moiety of STZ accounts for its specificity and penetration into beta cells, a passage that is mediated via the transmembrane glucose transporter GLUT 2 (123). The toxicity of STZ within the beta cells is due to the methylnitrosurea which decompose and lead to DNA damage (124, 125). Streptozotocin is extremely labile in solution at physiological pH and body temperature and its half-life in blood is about 5-10 min (126). Previous studies have shown that it is eliminated from the body within 6 h after intravenous administration (127).

The beta–cell toxicity of STZ is likely to be related to its capacity to produce methyl ions ($CH_3^{\cdot}$) that cause DNA damage, which in turn activates the nuclear DNA repair enzyme poly ADP-ribose synthetase (128). Activation of this enzyme results in a marked decrease in cellular levels of NAD^+, the substrate of the poly ADP-ribose synthetase. NAD^+ depletion is critical for the pro-insulin synthesis (128) and ATP production (129) which is important for cellular metabolism and survival leading to decreases in beta cell performance and viability.

STZ has been shown to directly affect the developing rat embryo both in vivo and in vitro (130, 131). On the other hand, insulin treatment of either mice or rats made diabetic with STZ significantly decreased the occurrence

of malformed offspring. This suggests that diabetes was the major cause of the defective fetal development and not a direct effect of STZ (131, 132).

Animal model

In this thesis we have used rats from a local outbred strain of Sprague-Dawley with an increased incidence of congenital malformations in diabetic pregnancy, called the Uppsala-strain (U). The U strain developed out of the Hanover (H) strain during 20 years (1962-1982) when it was kept in a commercial breeding facility in Sweden. During that time it was discovered that the two strains differed in their toxicological susceptibility. Since 1982, the U strain has been kept under outbreeding conditions in a colony at the Laboratory Animal Resources of the BioMedical Centre in Uppsala. The H strain has been outbred in a colony by a commercial breeder in Sweden (B&K Universal AB, Sollentuna, Sweden) since 1982.

Observed malformations in this strain due to diabetes affect most commonly the CNS, heart, kidney and the facial skeleton. These malformations are also commonly found in human diabetic pregnancy. These observations support the view that in the rat, as in man, the offspring of a diabetic mother exhibits a wide range of malformations which are not characteristic of the diabetic state as such (133). Efforts have been made to identify the reason for the increased rate of malformations in the U rat compared to the malformation-resistant H rat. These efforts resulted in a difference in the activity of the scavenging enzyme catalase, probably due to differences in the catalase gene (54, 134).

Aims

The aims of this study were:

- To characterize a possible association between altered PKC activity and disturbed embryonic development in a diabetic environment.
- To evaluate the role of apoptosis in the embryonic development of diabetic rat pregnancy.
- To evaluate the effect of folic acid and vitamin E treated pregnant rats for embryonic morphology and apoptosis.
- To evaluate the effect of α-cyano-4-hydroxycinnamic acid and N-acetylcysteine addition on morphology and activity of protein kinase C-delta and protein kinase C-zeta in rat embryos exposed to high glucose concentration *in vitro*.

Material & Methods

Animals

Embryos were obtained from normal and diabetic female rats of a local outbred Sprague-Dawley strain with an increased incidence of congenital malformations in diabetic pregnancy (135).

Diabetes was induced with a single injection of 40 mg/kg STZ into a tail vein. Blood glucose was measured after one week. Rats with a glucose concentration exceeding 20 mM were considered to be manifestly diabetic (MD). After establishing the diabetic status, overnight mating of the MD females commenced: a positive vaginal smear the following morning designated gestational day 0. Non-STZ injected females served as non-diabetic controls (N).

On gestational day 9, 10 and 11 the normal and diabetic rats were killed by cervical dislocation after light ether anaesthesia. Each collected embryo was carefully freed of the surrounding tissues.

The Animal Ethical Committee of the Medical Faculty of Uppsala University approved the research protocol including all experimental procedures involving animals.

Treatment (Paper III)

N and MD rats were given 15 mg/kg folic acid by daily subcutaneous injections in the neck pouch of 0.5 ml of 10 mg/ml folic acid (dissolved in redistilled water with pH adjustment to 7.8-7.9). The injections commenced on gestational day 0 and continued until termination of pregnancy on gestational day 10 or 11. A second group of N and MD rats were supplemented with 5% of vitamin E mixed in the food during the same period of time. A third group received a combined treatment with folic acid and vitamin E. Untreated N and MD pregnant rats served as controls.

On gestational day 10 and 11 the N and MD rats were killed by cervical dislocation after light ether anesthesia. Each embryo was carefully dissected out and examined in a stereomicroscope for malformations, crown rump length and somite numbers. In particular, the occurrence of disturbed embryonic development was noted, such as open neural tube, tail twist and somatic malrotation.

Preparation of total RNA (Paper I & II)

Total RNA from each embryo was isolated with QiaGEN RNeasy mini kit according to the manufacturer's instructions. One microliter of RNase inhibitor was added to each sample.

Preparation of cDNA (Paper I & II)

One microgram of total RNA was used for reverse transcription according to manufacturer's instructions. First strand cDNA synthesis used first strand beads.

Analysis of mRNA levels (Paper I & II)

One microgram of the cDNA purified from embryos containing 10 ng of converted total RNA was amplified and measured with Real Time PCR using the Roche LightCycler. Specific primers were designed and manufactured by Cybergene AB for PKC and by TIB Molbiol for Caspase 3 and p53 (Table 1). According to the Light Cycler protocol, 1 µl of the cDNA was amplified in a final volume of 10 µl containing 6.2 µl RNase-free water, 1 µl FastStart DNA Master SYBR Green I, 2 mM $MgCl_2$, 0.5 µmol/l of the sense and antisense primers. For relative quantification, G6PDH were used as control (Table 1).

Controls were included in each run of the Real Time PCR assay; for each primer pair one sample with no cDNA (containing only RNAse free water) was included. Furthermore, to exclude the possibility of remaining DNA fragments in the samples, 10 ng of the total RNA of each sample was amplified in the Light Cycler. We found no PCR product in the water or in the total RNA samples. Furthermore, we excluded the essential AMV-RT enzyme in the cDNA preparation and found that no PCR product could be amplified.

In a separate pilot study, we compared the expression of Glucose-6-phosphate dehydrogenase (G6PDH), actin, and ribosomal protein S-28 in embryos of normal and diabetic rats, and found G6PDH to yield the most stable expression (data not shown).

Results were analysed for each sample with relative quantification comparing the difference between sample and control crossing point (Cp) values. To render a true value for mRNA levels, the calculated difference were transformed according to the expression.

$$2^{-(Cpsample-CpG6\ PDH)}$$

to yield the ratio sample/G6PDH.

Table 1. PCR primer sequences

Primer		Sequences	Annealing temperature (°C)
G6PDH	Forward	5'-ATTGACCACTACCTGGGCAA-3'	60
	Reverse	5'-GAGATACACTTCAACACTTTGACCT-3'	
PKC-beta1	Forward	5'-CACAAGTTTAAGATCCACACCTACTCC-3'	60
	Reverse	5'-ATGTGGGCCTGGATGTAGATGCGGCCA-3'	
PKC-beta2	Forward	5'-TTTGGCAGAGAGACAAGAGA-3'	60
	Reverse	5'-GACATACTCTGGGTTAGT-3'	
PKC-delta	Forward	5'-TATAACTACATGAGCCCCACC-3'	60
	Reverse	5'-CCAGAGACAGCTGTCTTCTTC-3'	
PKC-epsilon	Forward	5'-CAAGTTCATGGCCACCTA-3'	60
	Reverse	5'-ACCTCGTCAGGGGTTTCCTG-3'	
PKC-gamma	Forward	5'-AAAACCGCAAAGACTGAACAG-3'	60
	Reverse	5'-AGCTGTGGCGCCCAGAGGCTGTCAA-3'	
PKC-zeta	Forward	5'-CAGCTCCTCCGTATCCATGCCGC-3'	60
	Reverse	5'-ACAATGGGCTGGGTGGGTCTCCG-3'	
p53	Forward	5'-TGAAGAAATTATGGAATTGATGGAT-3'	52
	Reverse	5'-ACCGCAGTCCAGCTCTGTA-3'	

Estimation of PKC activity markers (Paper I and IV)

Fractionation of embryonic cells

The embryos were washed in PBS before they were lysed with hand homogenizer in buffer (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 5 mM EDTA, 0.2% Triton, 10 mM Benzamidine, 50 mM β-mercaptoetanol, 1mM Phenyl Methyl Sulfonyl Fluoride). The homogenized sample was allowed to lysate for 5-10 min. A 20 µl volume of the lysated sample was drawn and stored for protein determination before spinning down cell debris and nuclei. A cold Beckman rotor was loaded with the supernatant transferred to centrifuge tubes. The samples were spun at 160 000G for 20 min. The supernatant (cytosolic fraction) was transferred to clean tubes and stored on ice. The pellet (membrane fraction) was resuspended in 30 µl loading buffer. The cytosolic fraction was precipitated with acetone. The precipitated proteins were centrifuged for 10 min in cold environment and the pellet was suspended in 30 µl loading buffer. The samples were heated to 100 °C for 2-3 min before they were applied at a 5% stacking gel with a 7% running gel. After electrophoresis the gel was put in Western blot-buffer for 10-15 min. A protran nitrocellulose membrane was used for the transfer which proceeded overnight.

Immunoblot analysis

The membranes were pretreated with 5% non-fat dry milk to block non-specific binding. Primary antibody was applied and incubation occurred. Membranes were rinsed for 1.5 h on a shake board and washed with PBS-Tween. They were then incubated with the secondary antibody.

ECL+plus western blotting detection system was applied drop-wise on the membrane. The membranes were confined in plastic film and placed in an exposure box. Hyperfilm was applied in a darkroom and was exposed for 1 min. The film was developed with Agfa Curix 60.

The developed film was scanned to a computer and evaluated densitometrically. The densitometric measurement of the membrane and cytosolic fractions of each sample were normalized by dividing by the protein content of the whole sample and the resulting ratio, expressed as AU/µg, was used as a measure of total PKC isoenzyme content. The membrane-bound fraction was subsequently used as a marker of isoenzyme activity.

Measurement of protein

Total protein content was estimated in each sample from an aliquot of 20 µl lysate by the method of Lowry and collaborators (136) using bovine serum albumin as standard.

Western blot analysis (Paper II & III)

Tissue homogenates were lysed and fractionated by SDS-PAGE (12%) at 14 mA for 1 h. Proteins were transferred to nitrocellulose membrane overnight at 30V.

The membranes were blocked overnight with 5% non-fat dried milk and subsequently incubated with the primary antibody. Unbound antibody was removed by washing with PBS-Tween. Membranes were then incubated with the secondary antibody diluted 1:1000 in 25 ml of PBS-Tween + 2.5% BSA for 30 min.

After extensive washing with PBS-Tween, membranes were covered with ECL+plus Western blotting detection system fluid. After 5 min excess fluid was removed with a Whatman paper. Membranes were confined in plastic film, with the proteins upward, and placed in an exposure-box. Film was applied in darkroom and developed with Agfa Curix 60.

The developed film was scanned to a computer and densitometrically evaluated with Kodak Digital Science 1D, the protein density was expressed in AU.

Immunostaining of embryos (Paper I & II)

Embryos were fixated in 4% paraformaldehyde and stored in 70% ethanol before they were embedded in paraffin. The embedded embryos were sectioned in 5-µm thick sections.

Slides were deparaffinized, rehydrated, and rinsed in PBS. Sections were covered with trypsine diluted in 0.1% $CaCl_2$. Sections were then washed and subjected to H_2O_2. Slides were rinsed and subjected to normal goat serum. We removed the goat serum and added either primary antibody PKC-beta1/PKC-beta2/Bax/Bcl-2/Caspase 3 or blocking peptide + antibody PKC-beta1/PKC-beta2/Bax/Bcl-2/Caspase 3 before incubation overnight.

Slides were washed and incubated with secondary antibody. The slides were washed again and then developed with Sigma Fast™ 3,3'-Diaminobenzidine tablet sets. Finally the slides were mounted with cover slip.

Whole embryo culture (Paper II and IV)

Embryos used for whole embryo culture were collected on day 9 as described above and prepared for *in vitro* embryo culture using the method of New (137).

The freed embryos, within their intact yolk sacs, were transferred to a 50-ml culture tube with 4 ml rat serum and 1 ml saline with appropriate addition of glucose (10 or 30 mM). In addition, during whole embryo culture we added different compounds (N-acetylcysteine, α-cyano-4-hydroxycinnamic acid, specific PKC-delta and PKC-zeta inhibitor and ethanol) to different culture tubes.

Culture of embryonic cells (Paper II)

Embryos were transferred from PBS into Dulbecco's minimal essential medium (DMEM) containing 5.5 mM glucose. Whole embryos were minced, to generate tissue clumps and single cells. The cell suspension was placed into culture dishes precoated with 1% gelatin supplemented with DMEM containing 10% fetal calf serum (FCS). Dishes serving as high glucose cultures were supplemented with glucose to a final concentration of 30 mM glucose.

Analysis of Nuclear factor-κ B activation (Paper III)

Extraction of nuclear proteins

Embryos from control and diabetic rats, day 10 and day 11, were pooled and processed to extract the nuclear proteins. Briefly, the embryos were homogenized, pelleted and lysed in 50 µl of buffer A (10mM Tris pH 7.5, 1.5mM $MgCl_2$, 10mM KCl, 2mM dithioreithol, 0.4mM Pefabloc). After centrifugation the pellet was resuspended in buffer A. Nuclei were pelleted and nuclear proteins were extracted by addition of 50 µl buffer C (20mM Tris pH 7.5, 1.5mM $MgCl_2$, 0.42M KCl, 20% glycerol, 2mM dithioreitol, 0.4mM Pefabloc) and sonication. After sonication the samples were kept on ice for 20 min. Nuclear protein extracts were stored in –70°C.

Electromobility shift assay

For electromobility shift assays (EMSA) of Nuclear factor-κ B (NF-κB), we used the following double-stranded oligonucleotide: 5'-AGCTTCAGAGGGGACTTTCCGAGAGG-3'. The oligonucleotide was labelled with [^{33}P] ATP using T4 polynucleotide kinase and purified with Chroma spin columns. Nuclear protein extracts (4.5 µl) were denaturated with formamid and incubated in a 20-µl reaction mixture (0.1 ng DNA (14 000 cpm), 10mM Tris pH 7.5, 40mM NaCl, 1mM EDTA, 0.2% deoxycholic acid, 4% glycerol, 1mM β-mercaptoethanol, 2 µg polydeoxyinosinic-deoxycytidylic acid) for 30 min at room temperature. A 100-fold excess of non-labelled oligonucleotide was used as negative control. Samples were separated on a non-denaturing polyacrylamid gel and exposed to X-ray film.

Detection of cell death and apoptosis (Paper II)

Activated Caspase 3 detection in living cells

For analysis of apoptosis in cells we followed the manufacturer's description using carboxyfluorescein (FAM) labelled peptide fluoromethyl ketone (FMK) Caspase inhibitor (FAM-Peptide-FMK) to detect activated Caspase 3 in embryonic cells cultured in low glucose (5.5 mmol) and high glucose (30 mmol), from normal and diabetic rats. Embryos were prepared according to culture of embryonic cells.

The same procedure was performed with embryonic cells from whole embryo culture. In this experiment we treated some groups with 0.5 mM N-acetylcycstein (NAC), an antioxidant, to evaluate a possible influence of NAC on Caspase 3 activation.

Furthermore embryonic cells from N rats (day 10 and 11) were cultured for 48 h in 5.5 mM or 30 mM together with apoptosis inhibitor II (NS3694). The same procedure to detect activated Caspase 3 was then performed.

Propidium iodide flow cytometry

Embryonic cells were prepared as above and incubated with propidium iodide added to the culture medium. The cells were then washed with PBS, trypsinized for 5 min, centrifuged at 500 g for 1 min and resuspended in PBS twice before FACS analysis.

After resuspension in PBS the cells were analyzed using a FACSCalibur fluorescence activated flow cytometer for forward scatter and FL3. Data analysis was performed using CellQuest software.

Vital staining with propidium iodide and Hoechst 33342

Embryonic cell culture were incubated with propidium iodide (10μg/ml) and Hoechst 33342 (5μg/ml), for 10 min at 37°C, added to culture medium. The cells were then washed with PBS, trypsinized, centrifuged and resuspended in PBS before they were placed on a slide for examination with fluorescence microscopy using a UV-2A filter.

Statistical considerations

Statistical significance was determined by analysis of variance (ANOVA). In the case of significance ($P<0.05$), individual groups were compared according to Fisher's protected least significant difference post-hoc test in Paper I, III and IV. Students t-test and Mann-Whitney's test were used in Paper II. We have also used χ^2-test in Paper II, III and IV. Analyses were performed using the program Statview® for Macintosh.

Results and discussion

Paper I

We found that the activity of several isoforms of PKC was altered in offspring from diabetic rats compared to offspring from non-diabetic rats. The embryos from diabetic rats showed an increased activity of PKC-alfa, PKC-beta1, PKC-gamma, PKC-delta and PKC-zeta compared to embryos from non-diabetic rats and, in addition the malformed embryos showed a superimposed increase in activity of PKC-gamma and PKC-delta. All of these changes were found on gestational day 10. The same experiments conducted on gestational day 11 showed a decreased activity of PKC-alfa and PKC-zeta in embryos of diabetic rats compared to embryos from normal rats. These results support the idea of increased activation of PKC in diabetes-exposed tissues but this effect seems to be confined to the period up to day 10 in embryogenesis.

However, when we investigated distribution and abundance of the PKC isoforms in embryos from normal and diabetic rats on gestational day 10 and 11, we could not find any differences between the groups with regard to PKC-alfa, PKC-gamma, PKC-delta, PKC-epsilon and PKC-zeta. In contrast, we found enhanced accumulation of PKC-beta1 and PKC-beta2 proteins in the neural tube, heart and facial tissues. We found no differences between embryos from normal rats and normal formed embryos from diabetic rats; however, in malformed embryos from diabetic rats we found intensified staining for both PKC-beta1 and PKC-beta2 compared to the embryos from normal rats and normal formed embryos from diabetic rats. These findings suggest an association between embryonic maldevelopment and enhanced protein distribution of these two PKC isoforms.

Furthermore, we assessed gene expression of PKC-alfa, PKC-beta1, PKC-beta2 PKC-gamma, PKC-delta, PKC-epsilon and PKC-zeta, and detected three changes: an increased gene expression of both PKC-beta1 and PKC-zeta on gestational day 10 and a decreased gene expression of PKC-gamma on gestational day 11 in embryos from diabetic rats. The changes in mRNA levels correspond approximately to the changes in activity recorded for these three PKC isoforms; however, most alterations in isoenzyme activity were not reflected in changes of gene expression. Evidently, the regulation of gene expression and activity of the different PKC proteins are not identical.

These results resemble findings of another research group that recently reported an association between exposure to a diabetic environment and enhanced embryonic PKC activity in a mouse model of diabetic embryopathy (138). On the other hand, inhibition of PKC has been shown to cause malformations in rodent embryos and furthermore, administration of a PKC inhibitor to embryos subjected to teratogenic glucose concentrations *in vitro* failed to diminish the disturbed embryonic development, indicating a need for a basal level of PKC activity for normal embryogenesis (139, 140). Diabetic embryopathy has therefore been associated with both low and high activity of PKC. In the present study it seems that structural defects in the embryo and enhanced activity of PKC are associated. The question is whether embryos develop a set of active PKC isoenzymes due to the diabetes-induced damage or whether PKC activation precedes and contributes to embryonic maldevelopment. Activation of PKC may therefore either be a consequence of embryonic dysmorphogenesis or be involved in the induction of the developmental disturbance.

Paper II

The most important finding in the present study was decreased levels of the anti-apoptotic protein Bcl-2 and increased levels of the pro-apoptotic proteins Bax and Caspase 3 in embryos of diabetic rats compared to embryos from nondiabetic rats, indicating increased apoptotic occurrence in embryos exposed to a diabetic milieu.

In addition, we found a tendency towards increased p53 protein levels at both day 10 and 11, as well as increased p53 gene expression at day 11 in embryos of diabetic rats compared to embryos from nondiabetic rats. Furthermore, we found increased activation of Caspase 3 in embryonic cells cultured in high glucose concentration compared to cells cultured in low glucose concentration. The same result was found in embryonic cells from whole embryo culture. Increased activation of Caspase 3 was normalized by NS3694, which inhibits the formation of the apoptosome by preventing the association of cytochrome c or dATP to their respective domain on Apaf-1 (141), and thereby blocks the intrinsic pathway of programmed cell death. Furthermore, the increased activation of Caspase 3 was normalized with the addition of NAC to the embryo, suggesting a role for increased oxidative stress.

Interestingly, cells from embryos of diabetic rats displayed increased activation of Caspase 3 compared to cells from embryos of nondiabetic rats even when cultured in low glucose concentration for 48 h. This may reflect hyperglycemic memory, i.e., when hyperglycemia-induced effects on cells persist or progress despite subsequent periods of normal glucose levels (142).

Furthermore, flow cytometry detected increased uptake of propidium iodide in embryonic cells from diabetic rats compared to embryonic cells from nondiabetic rats, indicating an increased apoptotic rate in embryonic cells from diabetic rats. These findings were confirmed by vital staining.

In a previous study, Forsberg et al (143) failed to demonstrate an increased apoptotic rate in embryos exposed to a diabetic environment with the TUNEL assay. However, other authors have detected increased apoptosis with TUNEL staining in different tissues of embryos of diabetic rodents during organogenesis (144, 145). Indeed, recent studies propose an association between high glucose milieu and apoptosis in embryos (146-148). Together with the result from the present study we therefore suggest that a diabetic environment may increase the occurrence of apoptosis in embryos.

Several studies have proposed the hyperglycemic condition to be responsible for both microvascular and macrovascular complications in diabetes. The toxicity and teratogenicity of increased glucose concentration are also established in human diabetic pregnancy, where increased maternal HbA1c concentration in early gestation has been found to be correlated with increased risk for fetal malformation (13). Studies clearly indicate that glucose is utilized (149, 150) by embryonic cells, in particular by cardiac cells (151). The diabetes-induced malformations often occur in the central nervous system, as well as in the heart and great vessels (4). In previous experimental work, we have found maldevelopment of these tissues in rat offspring exposed to hyperglycemia *in vivo* and *in vitro* (41, 52, 152-154). These tissues balance proliferation and apoptosis during their development and could be vulnerable to changes in apoptotic rate.

Maternal diabetes is proposed to affect the mitochondrial morphology (155), and hyperglycemia is suggested to force the mitochondrial electron transport chain to generate increased amounts of reactive oxygen species (150, 156). These actions alter the mitochondrial transmembrane potential and the permeability transition pore (PTP) opens. This leads to leakage of proteins involved in apoptosis, e.g., Bcl-2, Bax, and cytochrome C. Cytochrome C induces the formation of the multisubunit apoptosome composed of apoptotic protease activating factor-1 (Apaf-1), pro-Caspase 9, and ATP. The apoptosome is responsible for the activation of Caspase 3 (157), leading to apoptosis by activation of Caspase 6, DNA fragmentation factor (DFF) and poly ADP-ribose polymerase (PARP). Previous studies have shown that PARP ribosylates and inactivates the rate limiting enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) in the glycolysis and thereby may cause increased PKC activation, enhanced hexosamine flux, increased AGE accumulation and increased amount of NF-κB (45). These pathways would in turn be responsible for several diabetic complications and, possibly, embryonic dysmorphogenesis. In addition, increased oxidative stress has been suggested to activate PARP via DNA strand breaks (45) and studies have demonstrated that different antioxidants positively affect mitochondrial mor-

phology (158) as well as embryonic maldevelopment (41, 52, 152). Furthermore, we have recently found decreased GAPDH activity in rat embryos exposed to a diabetic milieu *in vivo* and *in vitro* (159). This study shows that the antioxidant NAC suppresses Caspase 3 activation in cells from embryos exposed to low and high glucose concentrations. These findings, which link disturbed mitochondrial morphology and function with developmental disturbances via oxidative stress in the embryo, may support the therapeutic use of antioxidants in diabetic pregnancy.

The present study shows increased apoptosis in cells from embryos of diabetic rats compared to embryos of nondiabetic rats. This increased apoptotic rate may be an indicator of the general embryonic demise (leading to delayed maturation and fetal resorptions), but may also be partly involved in some of the developmental disturbances occurring in the embryo exposed to a diabetic milieu, such as non closure of the neural tube (160) and heart malformations (4, 161).

Paper III

The most important findings in this study were the positive effects of combined administration of folic acid and vitamin E on diabetes-induced embryonic malformations and resorptions as well as the findings supporting a role for NF-κB in rat organogenesis. Together with the effect of the combined treatment to reduce malformations and resorptions we found normalizing on Bcl-2 protein levels on day 10 but not day 11. This complex pattern of effects on apoptosis-related proteins not only indicates a possible role for apoptosis in the demise occurring in the embryo due to a hyperglycemic environment, but also suggests that other mechanisms are involved in diabetic embryopathy.

This study focused on rat embryonic development at gestational day 10 and 11. These two days were chosen because they are critical in neural tube closure, early heart development and other somatic malformations found in diabetic pregnancy. Day 10 and day 11 can be seen as representative of two separate embryonic periods where anti-teratogenic treatments may exert completely different effects on the embryo, mainly because of the shift from a yolk sac placenta to the permanent chorioallantoic placenta, which occurs during these two days.

It has been proposed that NF-κB is responsible for pro-apoptotic events in cells with NF-κB-regulated genes such as Fas, FasL and p53 (162-164). However, several recent studies have demonstrated anti-apoptotic products from NF-κB-regulated genes such as Bcl-2, Bcl-X_L and MnSOD (165-167). Bcl-2, Bcl-X_L and Bax are important during embryonic development since they inhibit (Bcl-2 and Bcl-X_L) and promote (Bax) apoptosis in cell populations (168). When the ratio of Bcl-2 to Bax changes to the advantage of Bax,

the downstream effector in the apoptotic pathway, Caspase-3, is activated. This activation is important in fetal development (169). The present study showed a strong activation of NF-κB in embryos of normal rats and a decreased activity in embryos of diabetic rats, whereas the normalizing effect with both folic acid administration and combined supplementation of folic acid and vitamin E may indicate a connection between ROS and NF-κB. Indeed, a recent study shows that NF-κB inhibits ROS in part by up regulating the Ferritin heavy chain (FHC), one of two subunits of Ferritin and the primary iron-storage protein complex of the cell (170). Moreover NF-κB inhibits MKK7 in the JNK-signalling pathway by Gadd45β preventing cell death (171).

In the present study, we found, in general, an attenuating effect on markers of apoptosis by treatment with vitamin E and folic acid. However, we were unable to demonstrate a clear additive or synergistic effect by the treatments. Furthermore, we noted inappropriate effects of the treatments: for instance the combined administration of the two treatments was not able to normalize embryonic development on gestational day 10 although the Bcl-2 levels were normalized. Likewise, on gestational day 11, the combined treatment differed from folic acid administration with regard to Bcl-2 and Bax protein levels in the embryos. These findings may be due to stochastic coincidence, or even the negative interaction between folic acid and vitamin E, at least in specific dosage intervals. The possible biochemical background of such negative interaction is not clear, however, and the main result of this study remains the positive effects on diabetes-induced developmental disturbances and apoptotic markers exerted by vitamin E and folic acid. The absence of an additive or synergistic effect suggests that these agents affect the same teratological pathway(s).

The fact that diabetic women still have an increased risk of giving birth to malformed children, despite intensive insulin therapy, obliged us to do further research in this field. Several suggestions have emerged to explain the reasons for the increased malformation rate, such as oxidative stress, abnormal activation of protein kinase C, apoptosis, disturbed arachidonic acid pathway and prostaglandin synthesis, and hypoxia (18, 33, 41, 144, 172, 173). It is likely that all these suggestions are involved in the etiology of diabetic embryopathy (174).

The question is whether we can affect these disturbed pathways with one universal treatment. Based on the results from the present study we may need combined treatments to normalize the increased risk of fetal dysmorphogenesis associated with maternal diabetes.

In a previous study we found that folic acid administration to pregnant diabetic rats, both in vivo and in vitro, clearly affected malformations and growth retardation (173). Other studies using folic acid in clinical trials have shown beneficial effects preferably for neural tube defects (106, 175). Follow-up studies on the folic acid fortification of the US food supply have

found similar results (176, 177). Several experimental studies have used supplementation of vitamin E alone and found protective effects against diabetic embryopathy for malformations and resorptions (47-49). A recent study reported decreased malformation rate in embryos exposed to a diabetes-like milieu treated with vitamin E *in vivo* and folic acid *in vitro*, concomitant with a reverse of diabetes-induced diminished prostaglandin E_2 levels (173).

These results in combination with the present results may indicate that the doses of each individual antioxidant, rather than a combined synergistic effect of folic acid and vitamin E, determine the protective effect against diabetic embryopathy. A study reported decreased fetal malformations and diminished oxygen radical-related tissue damage in diabetic rats treated with a combination of vitamin E and vitamin C (96). However, the authors could find no synergistic effect between the two antioxidants.

In conclusion, folic acid and vitamin E, two compounds with antioxidative features (178, 179) diminish the increased oxidative stress in the diabetic milieu. This reduction may be exerted by either folic acid, or vitamin E or both combined. Since we could not demonstrate additive or synergistic beneficial effects of the combined treatment, we conclude that both compounds block the same teratological processes. There are probably mechanisms involved in diabetic embryopathy separated from oxidative stress that these antioxidative properties cannot normalize. As a consequence, attempts to completely normalize fetal outcome in diabetic pregnancy may have to include some other combination and antiteratogenic substances.

Paper IV

The most important finding in the present study was the close association between glucose-induced dysmorphogenesis and increased activity of the isoenzymes PKC-delta and PKC-zeta in the embryos. In addition, we found that CHC and NAC treatment normalized both glucose-induced maldevelopment and increased enzymatic activity of PKC-delta and PKC-zeta. Furthermore, specific inhibitors against PKC-delta and PKC-zeta also normalized the glucose-induced dysmorphogenesis as well as increased PKC activity. These results suggest that disturbed PKC-delta and PKC-zeta activity play a role in diabetic embryopathy.

In the present study we found an increase in malformations and growth disturbances in embryos cultured in high glucose concentrations for 24 h or 48 h *in vitro*, corresponding to gestational day 10 and day 11 *in vivo*. Addition of CHC or NAC to medium with high glucose concentration prevented malformations in embryos cultured for 24 h. Embryos cultured for 48 h in high glucose concentration had a higher rate of malformations, which could be reduced with addition of CHC and NAC to the culture medium. Growth

disturbances (crown rump length and somite numbers) in high glucose concentrations were normalized in embryos cultured for 24 h and 48 h when CHC and NAC were added to the culture medium. Both compounds were anti-teratogenic; however, NAC appeared to be slightly more effective in reducing glucose-induced embryonic dysmorphogenesis.

Recent studies have suggested that PKC has a role in embryonic development; disturbed levels were found to induce dysmorphogenesis both in rat and mouse models (138-140). In the present study we found an increased activation of PKC-delta and PKC-zeta in embryos exposed to a teratogenic hyperglycemic environment. Addition of CHC and NAC normalized PKC-delta and PKC-zeta activity and corrected embryonic development. Addition of PKC-delta specific inhibitor normalized PKC-delta activity, and both PKC-delta and PKC-zeta specific inhibitors normalized embryonic dysmorphogenesis. The increased activation of both PKC isoforms was confined to embryos cultured for 24 h *in vitro*, corresponding to day 10 *in vivo*. No consistent effects due to CHC, NAC or specific inhibitors of PKC-delta and PKC-zeta were found on embryos cultured for 48 h *in vitro*, corresponding to day 11 *in vivo,* except for normalized PKC-delta activity due to the PKC-delta inhibitor.

In a high glucose environment, increased pyruvate is transported into the mitochondria where it is oxidized by the tricarboxylic acid cycle (TCA) to CO_2 and yields increased levels of NADH and $FADH_2$. These products serve as electron donors and generate a high mitochondrial membrane potential by pumping protons across the mitochondrial inner membrane in the mitochondrial electron transport chain and, ultimately, an excess of superoxide is produced (90).

The superoxide overproduction of the mitochondria is suggested to cause DNA strand breaks which in turn may activate PARP. PARP ribosylates GAPDH, the rate-limiting enzyme in the glycolysis, thereby leading to decreased GAPDH activity. This phenomenon diverts excess of glycolytic metabolites into different pathways upstream of GAPDH, among those the PKC pathway which later leads to an increased activation of PKC (45).

The result of the present study agrees with the notion of ROS-mediated GAPDH inhibition since the high glucose environment increased the activity of PKC-delta and PKC-zeta, and addition of CHC and NAC normalized this activation. Enhanced PKC activities are suggested to affect blood-flow, angiogenesis, capillary and vascular occlusion, and pro-inflammatory gene expression, leading to diabetic complications (90). This could also be true for embryonic dysmorphogenesis. In addition, an abnormal activation of PKC could initiate increased apoptosis, especially PKC-isoforms delta and zeta which have been associated with apoptosis (59, 61, 180). A recent study suggested that the increased production of free radicals of the mitochondrial electron transport chain damages proteins and lipids and leads to dysfunction of mitochondria. This in turn releases pro-apoptotic factors that activate the

cysteine protease family of Caspases, which then propagate a death cascade (181).

In this study, the diminishing effect exerted by NAC on the glucose-induced embryonic maldevelopment supports the notion of an excess of free radical production in the embryos (41, 182). NAC increases the levels of intracellular antioxidant reduced glutathione by providing the rate-limiting amino acid, cysteine, for the synthesis of glutathione. Addition of NAC initiates the ability to take care of excess of reactive oxygen species. CHC, the pyruvate transport inhibitor, reduces the uptake of pyruvate into the mitochondria and TCA by specifically modifying a thiol group on the mitochondrial pyruvate carrier. The reduced substrate to the TCA leads to reduced production of superoxide, leading to less pronounced embryonic dysmorphogenesis.

Still, there is a question why this phenomenon seems to be restricted to day 10. Perhaps a longer exposure to high glucose concentrations is more difficult to reverse. Both PKC-delta and PKC-zeta could have specific roles in the development of embryonic organogenesis on day 10, which are terminated on day 11.

Nonetheless, these results support the notion that diabetic embryopathy depends at least partly on overproduction of reactive oxygen species in embryonic mitochondria and may be associated with enhanced activity of the PKC isoforms delta and zeta.

Conclusions

During the recent years we have tried to identify some of the pathways responsible for the increased risks of miscarriage, malformations, stillbirth and pre-eclampsia to which a pregnant woman with diabetes is exposed. An important gain from identifying the molecular and cellular mechanism behind these increased risks would be the design of a treatment to decrease them. We have tested different treatments *in vivo* and *in vitro* to try to normalize or reduce embryonic dysmorphogenesis. Our main conclusions are as follows:

- Diabetic rat embryopathy is associated with altered activity and tissue distribution of several PKC isoforms in early organogenesis.

- Exposure to a diabetic milieu during organogenesis increases apoptosis in embryonic cells and embryos. This enhanced apoptotic rate may have a role in diabetic embryopathy by inducing disturbed embryonic maturation, increased rates of resorptions and congenital malformations.

- Combined supplementation of folic acid and vitamin E to pregnant rats diminishes diabetes-induced malformations and resorptions, concomitant with normalization of apoptotic protein levels.

- CHC and NAC as well as PKC-delta and PKC-zeta specific inhibitors have a protective effect on high glucose-induced embryonic dysmorphogenesis with a concomitant normalizing effect on the increased activation of PKC-delta and PKC-zeta in rat embryos.

We have found a disturbed pattern of protein kinase C activity in diabetic rat pregnancy correlated to embryonic dysmorphogenesis. The disturbance consisted mainly of an increased activation of several isoforms. This notion is consistent with several studies using different cells and tissues. However, other studies suggest that some isoforms of PKC may be decreased in a diabetic milieu and that the use and dose of inhibitors should be carefully considered since a decreased activation below normal may also be deleterious. The pattern of PKC differs widely depending on species, tissues and cells.

We treated high glucose-induced dysmorphogenesis and increased PKC-delta and PKC-zeta activity in rat embryos with NAC and CHC. Both com-

pounds had a beneficial effect and since NAC is a powerful antioxidant we may suggest that oxidative stress is involved. CHC limits the uptake of pyruvate into the mitochondria which generates oxidative stress. These findings imply that high glucose concentration generates an increased uptake of pyruvate into the mitochondria and subsequently an increased generation of oxidative stress. One of the actions exerted by the ROS could be activation of PKC which in turn also participates in the dysmorphogenesis of the embryo. The notion that PKC is partly involved is verified by treatment with PKC-delta and PKC-zeta specific inhibitors which positively affect glucose-induced dysmorphogenesis.

Our findings suggest that apoptosis is markedly increased in rat embryos during organogenesis exposed to a diabetic environment and in embryonic cells exposed to high glucose concentration. This increased cell death may contribute to embryonic maldevelopment. Indeed, treatment of diabetic rats with folic acid and vitamin E normalized several apoptotic markers concomitant with diminished diabetes-induced embryonic dysmorphogenesis. Both folic acid and vitamin E have been ascribed antioxidative features although folic acid has an important role as one-carbon donator. The fact that this treatment beneficially affects abnormal apoptosis and embryonic dysmorphogenesis again implicates a role for oxidative stress, this time in abnormal activation of apoptosis during organogenesis.

One conclusion of this work is that a diabetes-induced overproduction of ROS during rat organogenesis exerts detrimental effects on the embryo partly by activating different isoforms of PKC and apoptotic pathways. These alterations can be blocked by antioxidants, but not completely. Further studies are needed to develop a multi-nutrient dietary supplement to eliminate embryonic abnormalities induced by maternal diabetes.

Summary in Swedish

Diabetes mellitus är något som plågat mänskligheten sedan urminnes tider. Tillståndet beskrevs för flera tusen år sedan i den fornegyptiska skriften Papyrus Ebers. År 130 beskrev en grekisk läkare sjukdomen och fann att det som patienten dricker rinner fort igenom kroppen och att urinen var mycket söt. Därav namnet diabetes, det grekiska ordet för ”rinna igenom” och mellitus det latinska namnet för ”söt som honung”.

Nästa milstolpe inom diabetes spikades 1921 när Banting och Best lyckades extrahera hormonet insulin som sedan administrerades till en sockersjuk pojke som var nära döden. Pojken överlevde och Banting fick nobelpris!

Idag finns över 230 miljoner diabetiker i världen. Många lever med dagliga insulininjektioner, dessvärre utvecklas ibland komplikationer till diabetes. Dessa består av problem med synen, försämrad känslighet i armar och ben, njur och cirkulationsproblem. Dessa komplikationer är den största orsaken till sjuklighet och dödlighet bland diabetiker.

Diabetes leder även till en ökad risk för problem i samband med graviditet. Innan insulinets upptäckt var det få diabetiska graviditeter som lyckades föda friska barn. Det var mycket svårt att bli gravid, Elliot Joslin fann vid tidigt 1900-tal att bara några få procent av de diabetiska kvinnorna blev gravida. När en kvinna väl blev gravid var risken för maternell dödlighet 45% och risken för att barnet dog nära 75%. Användandet av insulin bidrog till en avsevärd förbättring men idag har den diabetiska och gravida kvinnan fortfarande en förhöjd risk för missfall, havandeskapsförgiftning och missbildade barn. Risken för att få barn med missbildningar vid diabetes är ungefär tre gånger högre jämfört med en normal graviditet. Man har sedan en lång tid tillbaka försökt att förstå den molekylära mekanismen bakom dessa problem utan att lyckats komma fram till någon fullständig lösning. En mängd förslag har framkommit och bidragit till ökad kunskap, bland annat inositolbrist, störd arakidonsyrametabolism och ökad oxidativ stress. Det har föreslagits att oxidativ stress påverkar olika enzym system, inklusive signalproteiner som protein kinas C (PKC).

Under de fyra år som jag har forskat har vi undersökt hur aktiveringen av det viktiga signalproteinet protein kinas C blir påverkad av en diabetisk miljö och hur mängden av apoptos (celldöd) påverkas av en hög socker miljö.

Vi fann en ökad aktivering hos ett flertal av de 7 undersökta isoformerna av embryonal PKC från diabetiska råttor jämfört med embryon från normala råttor. När vi i en senare studie använde oss av embryoodling fann vi en

förhöjd aktivering av PKC i hög glukoskoncentration, denna gång av PKC-delta och PKC-zeta samtidigt som vi fann fler missbildningar och tillväxthämning hos dessa embryon. För att försöka normalisera dessa effekter använde vi oss av fyra substanser som tillsattes till embryoodlingen i närvaro av hög sockerkoncentration: α-cyano-4-hydroxycinnamic acid (CHC) som är en hämmare av pyruvat-transport in i mitokondrien, N-acetylcysteine (NAC) som är en antioxidant och specifika inhibitorer mot PKC-delta och PKC-zeta. Samtliga av dessa tillsatser normaliserade den förhöjda PKCaktiviteten och hade en mycket god effekt på den embryonala utvecklingen, även om CHC och NAC var effektivare än PKCinhibitorerna. Dessa resultat tyder på en ökad produktion av fria radikaler från mitokondrien som dels leder till störd utveckling och dels leder till en förhöjd aktivering av vissa PKCisoformer som även i sin tur påverkar embryot på ett negativt sätt.

När vi undersökte embryon utsatta för en diabetisk miljö och celler utsatta för hög glukoskoncentration fann vi en ökad mängd av celldöd jämfört med embryon och celler som varit utsatta för en normal sockerkoncentration. Samtidigt fann vi ökat antal missbildningar och tillväxthämning hos embryon från diabetiska råttor. Vid behandling av de diabetiska råttorna med vitamin E och folsyra under dräktighetsperioden kunde vi normalisera diabetesframkallade missbildningar och förbättra tillväxthämningen samtidigt som vi normaliserade de markörer för apoptos som tyder på en förhöjd nivå av celldöd i en diabetisk miljö. Resultaten från dessa studier tyder på en ökad produktion av fria radikaler som leder till en störd utveckling möjligtvis via en onormal stimulering av celldöd. Vid behandling med vitamin E som är en antioxidant, och folsyra som förutom antioxidativa egenskaper också har en viktig roll som enkols-donator, kunde den oxidativa stressen minskas, embryoutvecklingen normaliseras och celldöd reduceras.

Sammantaget tyder resultaten på att en diabetisk miljö leder till en ökad aktivering av ett flertal former av PKC samt en ökad förekomst av celldöd hos embryot. Dessa mekanismer kan möjligtvis framkallas av en diabetesrelatrerad ökning av fria radikaler som ger oxidativ stress i vävnaderna. Förändringarna i PKCaktivering och celldöd leder vidare till en ökad förekomst av missbildningar och tillväxthämningar hos embryon. Den behandling vi har använt oss av har visat på en god effekt men inte tillräckligt god, mer forskning behövs för att utveckla ett supplement av ett flertal kombinerade näringsämnen för att eliminera embryonala abnormaliteter framkallade av maternell diabetes.

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