N-Acetylcysteine and α-cyano-4-hydroxycinnamic acid alter protein kinase C (PKC)-δ and PKC-ζ and diminish dysmorphogenesis in rat embryos cultured with high glucose in vitro

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Abstract

Malformations and growth disturbances are two- to threefold more common in infants of diabetic mothers than in offspring of non-diabetic pregnancy. Several suggestions have emerged to explain the reasons for diabetic embryopathy, including enhanced mitochondrial production of reactive oxygen species leading to altered activation of protein kinase C. This study aimed to evaluate the effect of α-cyano-4-hydroxycinnamic acid (CHC) and N-acetylcysteine (NAC) addition on morphology and activity of protein kinase C-δ and protein kinase C-ζ in rat embryos exposed to a high glucose concentration in vitro. Day 9 embryos from normal rats were cultured in 10 or 30 mM glucose concentrations with or without supplementation of CHC, NAC, or protein kinase C inhibitors specific for protein kinase C-δ and protein kinase C-ζ. Embryos were evaluated for malformations, crown rump length, and somite number. Protein kinase C-δ and protein kinase C-ζ activities were estimated by western blot by separating membranous and cytosolic fractions of the embryo. We found increased malformations and growth retardation in embryos cultured in high versus low glucose concentrations. These abnormalities were diminished when CHC and NAC or specific protein kinase C-inhibitors were added to the culture medium. The activities of embryonic protein kinase C-δ and protein kinase C-ζ were increased in the high glucose environment after 24-h culture, but were normalized by the addition of CHC and NAC as well as respective inhibitor to the culture medium. These findings suggest that mitochondrial overproduction of reactive oxygen species is involved in diabetic embryopathy. Furthermore, such overproduction may affect embryonic development, at least partly, by enhancing the activities of protein kinase C-δ and protein kinase C-ζ.


Introduction


PKC signaling is associated with apoptosis, especially the isoforms PKC-δ and PKC-ζ (Leroy et al. 2005, Santiago-Walker et al. 2005). It has been suggested that PKC-δ is involved in stabilizing p53 proteins (Lee et al. 2006) and related to reactive oxygen species production (Domenicotti et al. 2003), both of which would ultimately lead to apoptotic cell death.

We recently found alterations in activation of PKC isoforms associated with a hyperglycemic environment (Gareskog & Wentzel 2004). Previous studies have suggested that a diabetic milieu causes mitochondrial overproduction of reactive oxygen species (ROS), which yields mitochondrial swelling and enhanced lipid peroxidation in the embryos. This is because excess pyruvate from glycolysis moves across the mitochondrial membrane, overloading the electron transport chain (Yang et al. 1998). Subsequently, more ROS are generated, which is suggested to give rise to apoptosis and inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The inhibition of GAPDH, the rate limiting enzyme in glycolysis, then leads to alterations in PKC-signaling pathways (Du et al. 2003). These metabolic abnormalities may be partly involved in the etiology of
embryonic dysmorphogenesis. A consequence of this notion in therapy is that dietary supplementation with antioxidants to the mother may prevent embryonic malformations in diabetic pregnancy by inhibition of enhanced mitochondrial production of ROS (Yang et al. 1998).

The mitochondrion has an important role in the apoptotic machinery and previous studies have suggested that an altered apoptotic rate may affect the maldevelopment of embryos subjected to a diabetic milieu (Moley et al. 1998, Eriksson et al. 2000, Moley 2001). In addition, we found that combined supplementation of two compounds with anti-oxidative features (folic acid and vitamin E) to pregnant diabetic rats diminished diabetes-induced dysmorphogenesis. The beneficial effects of this combined treatment were associated with normalization of apoptotic-associated protein levels (Gareskog et al. 2006).

In the present study, day 9 embryos of normal rats were cultured for 24 or 48 h with a low (10 mM) or high (30 mM) concentration of glucose with or without α-cyano-4-hydroxy cinnamic acid (CHC), a mitochondrial pyruvate transport inhibitor. CHC restricted the uptake of excess pyruvate into the mitochondria, thereby reducing the overproduction of ROS. In addition, we used a precursor to reduced glutathione and a powerful antioxidant, N-acetylcysteine (NAC), in the culture medium with high glucose concentration to reduce the effect of produced ROS. Furthermore, we used specific inhibitors against PKC isoforms δ and ζ in the culture medium with high glucose concentration to investigate the impact of reduced PKC activity on embryonic dysmorphogenesis. With these different experimental conditions, we aimed to analyze the relationship between compounds reducing ROS, embryonic dysmorphogenesis, and activation of PKC isoforms δ and ζ in a diabetic environment.

Materials and Methods

Animals

Embryos were obtained from females of a local outbred Sprague–Dawley rat strain with an increased incidence of congenital malformations in diabetic pregnancy (Eriksson et al. 1982). The rats were fed food (AB Analycen, Lidköping, Sweden) and tap water available ad libitum and were maintained at an ambient temperature of 22 °C with 12 h light:12 h darkness cycle. Female and male rats were caged overnight and the morning with a positive vaginal smear (containing sperm) was designated gestational day 0. On gestational day 9, the pregnant rats were killed by cervical dislocation after light ether anesthesia. Each embryo with intact surrounding membranes was carefully dissected free from the uterine and decidual tissue.

Whole embryo culture

On gestational day 9, embryos from 5 to 6 non-diabetic rats were collected as described above and prepared for in vitro embryo culture by using New's (1978) method.

The freed embryos, within their intact yolk sacs, were transferred to a 50 ml culture tube (Falcon 2070, Becton Dickinson, Lincoln Park, NJ, USA) with 4 ml rat serum and 1 ml saline with appropriate addition of glucose (10 or 30 mM), and CHC (0.4 mM) or NAC (0.5 mM; Sigma-Aldrich Sweden AB) to the respective experimental groups. Male retarded breeders served as serum donors. Control experiments with the addition of CHC and NAC to embryos cultured with 10 mM glucose were performed. Furthermore, we added ethanol to embryos cultured in low glucose concentration since CHC was dissolved in ethanol. None of these supplementations negatively affected the embryonic development (data not shown).

In addition, we added isoform-specific inhibitors directed towards PKC-δ (Rottlerin, Biaffin GmbH & Co KG, Kassel, Germany; Gschwendt et al. 1994) and PKC-ζ (Pseudosubstrate, Myristoylated, Nordic Biosite AB, Täby, Sweden; Standaert et al. 1997) to embryos cultured in high glucose (30 mM) concentration 24 and 48 h. Pilot experiments of CHC and NAC addition were performed to determine the optimal concentration. Four to five embryos from different rat mothers were carefully mixed in each culture tube. After 24 or 48 h of culture, embryos were dissected out of their yolk sacs and examined under a stereomicroscope. The embryos were assessed for crown rump length, somite number, and occurrence of malformations. The observed malformations consisted of open neural tube and rotational defects.

Estimation of PKC activity markers

Fractionation of embryonic cells Embryos were collected and washed in PBS before they were lysed with a Kontes Pellet Pestle motor 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-mercaptoethanol, 1 mM phenyl methyl sulfonyl fluoride). The homogenized sample was allowed to lyse for 5–10 min. Next, a 10 μl lyse sample was stored for protein determination before centrifuging cell debris and nuclei. A cold Beckman rotor was loaded with the supernatant transferred to centrifuge tubes. The samples were centrifuged at 160 000g for 20 min in a Beckman Airfuge (Beckman Instruments Inc., Palo Alto, CA, USA). The supernatant (cytosolic fraction) was transferred to clean tubes and stored on ice. The pellet (membrane fraction) was resuspended in 30 μl loading buffer, and the cytosolic fraction was precipitated with three volumes of acetone. The precipitated proteins were centrifuged for 10 min at 5°C. The acetone was poured out and the pellet was suspended in 30 μl loading buffer.

Immunoblot analysis Prepared samples were lysed and fractionated by SDS-PAGE at 14 mA for 1 h. Proteins were transferred to nitrocellulose membrane (Hybond-P, Amersham Biotech) overnight at 30 V.
The membranes were blocked overnight with 5% non-fat dried milk and incubated subsequently with the primary antibody (PKC-δ, sc-213, PKC-ζ, sc 216, rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The unbound antibody was removed by washing with PBS-Tween (0.010 M phosphate buffer (pH 7.4), 0.0027 M KCl, 0.140 M NaCl and 0.05% Tween 20). The membranes were then incubated with the secondary antibody (antirabbit Ig, Amersham Life Sciences) diluted in the ratio of 1:1000 in 25 ml of PBS-Tween + 2.5% BSA (Miles Scientific, Naperville, IL, USA 60566) for 30 min.

After extensive washing with PBS-Tween, the membranes were covered with ECL Plus western blotting detection system fluid (RPN 2132, Amersham Biosciences). After 5 min, excess fluid was removed with Whatman filter paper. The membranes were confined in a plastic film and placed in an exposure box. Film (Hyperfilm MP, 18×24 cm, Amersham Pharmacia Biotech) 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 densitometric measurement of the membrane fractions and the cytosolic fraction of each sample was normalized by division of the protein content of the whole sample. The ratio of the membrane-bound fraction and the cytosolic fraction was subsequently used as a marker of isoenzyme activity (Alcazar et al. 1997, Ceolotto et al. 1999, Lehtihet et al. 2003).

The total protein content was estimated in each sample from an aliquot of 10 µl lysate by the method of Lowry and collaborators using BSA as standard (Lowry et al. 1951).

The risks for loading/pipetting errors or differences in transfer were controlled by blotting with total extracellular regulated kinase (ERK) that is shown to be constant in diabetic milieu (Longuet et al. 2005).

Ethical and statistical considerations

The Animal Ethical Committee of the Medical Faculty of Uppsala University approved the research protocol including all experimental procedures involving animals. Statistical significance was determined by ANOVA. In the case of significance ($P<0.05$), individual groups were compared according to Fisher’s protected least significant difference post hoc test. Differences between fractions of malformed and viable embryos were analyzed with two-tailed $\chi^2$-statistics. Analyses were performed using the program StatView for Macintosh (SAS Institute, Cary, NC, USA).

Results

Morphological evaluation

Malformations We found more malformations in embryos cultured in 30 mM (high) glucose concentration for 24 h when compared with embryos cultured in 10 mM (low) glucose. The addition of CHC and NAC to the culture medium with high glucose normalized the malformations in embryos cultured for 24 h. The addition of PKC-δ- and PKC-ζ-specific inhibitors to culture medium with high glucose also normalized the malformations (Fig. 1).

![Figure 1 Malformations. Embryos cultured in low and high glucose for 24 h (10 mM G 24 h, 30 mM G 24 h) and 48 h (10 mM G 48 h, 30 mM G 48 h), supplemented with CHC, NAC, and specific PKC-δ (delta inhib) and PKC-ζ (zeta inhib) inhibitors were evaluated for malformations, calculated in percentage (%) of total implantations. Sample size= 4–16. Significances *$P<0.05$ versus 10 mM G embryos and †$P<0.05$ versus 30 mM G embryos ($\chi^2$-test).](https://www.endocrinology-journals.org)
In embryos cultured for 48 h in high glucose medium, we found substantially more malformations when compared with those cultured in low glucose medium. Fewer malformations were observed with the addition of CHC to culture medium with high glucose concentration. The addition of NAC to culture medium with high glucose concentration normalized the malformations of embryos cultured for 48 h. Furthermore, the addition of PKC-δ- and PKC-ζ-specific inhibitors to high glucose culture medium also normalized malformations of embryos cultured for 48 h (Fig. 1). The addition of CHC or NAC to embryos cultured in low glucose concentration for 24 or 48 h yielded no malformations (data not shown). The observed malformations consisted of open neural tube and rotational defects.

**Crown rump length** Crown rump length was lower in embryos cultured in high glucose concentration when compared with those cultured in low glucose concentration for 24 h. The addition of CHC or NAC to culture medium with high glucose concentration normalized crown rump length, whereas the addition of PKC-δ- and PKC-ζ-specific inhibitors had no effect (Fig. 2).

In embryos cultured for 48 h in medium with a high glucose concentration, we found decreased crown rump length when compared with embryos cultured in low glucose concentration. The addition of CHC and NAC to a medium with high glucose concentration normalized crown rump length, whereas the addition of PKC-δ- and PKC-ζ-specific inhibitors to the culture medium with high glucose concentration had no effect on the crown rump length of embryos cultured for 48 h (Fig. 2).

**Somite numbers** We found fewer somites in embryos cultured in high glucose concentration for 24 h when compared with embryos cultured in low glucose concentration. The addition of CHC or NAC to culture medium with high glucose concentration-normalized somite numbers, whereas the addition of PKC-δ- and PKC-ζ-specific inhibitors partly restored somite numbers (Fig. 3).

In embryos cultured for 48 h in high glucose concentration, we found fewer somites when compared with embryos cultured in low glucose concentration. The addition of CHC and NAC to culture medium with high glucose concentration normalized somite numbers. The addition of PKC-δ- and PKC-ζ-specific inhibitors to the culture medium with high glucose concentration also normalized somite numbers of embryos cultured for 48 h (Fig. 3).

**Estimation of PKC-δ and PKC-ζ activities**

**PKC-δ** PKC-δ activity was higher in embryos cultured in medium with high glucose for 24 h when compared with embryos cultured in low glucose concentration. The addition of CHC and NAC to high glucose medium normalized PKC-δ activity. Furthermore, the addition of PKC-δ-specific inhibitor to high glucose medium also normalized PKC-δ activity (Fig. 4A).

There was no difference between embryos cultured for 48 h in medium with high glucose when compared with those cultured in medium with low glucose concentration. The addition of CHC and NAC to a medium with high glucose concentration did not affect the PKC-δ activity in embryos cultured for 48 h (Fig. 4B). However, the addition of PKC-δ-specific inhibitor partly normalized the PKC-δ activity. The addition of CHC or NAC to culture medium...
with low glucose concentration did not affect the PKC-δ activity during 24 or 48 h culture (data not shown). Figure 4C shows representative western blot bands with the normalization control ERK.

PKC-ζ PKC-ζ activity was higher in embryos cultured in high glucose concentration for 24 h when compared with embryos cultured in low glucose concentration. The addition of CHC and NAC to high glucose medium normalized PKC-ζ activity. The addition of PKC-ζ-specific inhibitor also normalized PKC-ζ activity (Fig. 5).

PKC-ζ did not differ in embryos cultured for 48 h in high and low glucose concentrations. The addition of CHC, NAC, or PKC-ζ-specific inhibitor to high glucose medium did not affect the PKC-ζ activity in embryos cultured for 48 h (Fig. 5B). The addition of CHC or NAC to low glucose concentration medium did not affect the PKC-ζ activity during 24 or 48 h culture (data not shown). Figure 5C shows representative western blot bands with the normalization control ERK.

**Discussion**

The most important finding in the present study was the close association between glucose-induced dysmorphogenesis and increased activity of the isoenzymes PKC-δ and PKC-ζ in the embryos. In addition, we found that CHC and NAC treatment normalized both glucose-induced maldevelopment and increased enzymatic activities of PKC-δ and PKC-ζ. Furthermore, specific inhibitors against PKC-δ and PKC-ζ also normalized the glucose-induced dysmorphogenesis as well as increased PKC activity. These results support a role for disturbed PKC-δ and PKC-ζ activities 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 or 48 h *in vitro*, corresponding to gestational days 10 and 11 *in vivo*. The addition of CHC or NAC to a 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 the 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 when CHC and NAC were added to the culture medium. However, in embryos cultured for 48 h in high glucose concentration, CHC diminished the increased growth disturbances, whereas NAC completely normalized these parameters, at the dose levels chosen. 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 lead to dysmorphogenesis both in rat and in mouse models (Ward *et al.* 1998, Wentzel *et al.* 2001, Hiramatsu *et al.* 2002). In the present study, we found an increased activation of both PKC-δ and PKC-ζ in embryos exposed to a teratogenic hyperglycemic environment. The addition of CHC and NAC normalized PKC-δ and PKC-ζ activities and corrected embryonic development. The addition of PKC-δ-specific inhibitor normalized the PKC-δ activity, and both PKC-δ and PKC-ζ-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-δ and PKC-ζ were found on embryos cultured for 48 h in vitro, corresponding to day 11 in vivo, except for diminished PKC-δ activity due to the PKC-δ inhibitor.

In a high glucose environment, increased pyruvate is transported into the mitochondria where it is oxidized by the

**Figure 4** (A) Estimated activity of PKC-δ, day 10. Embryos cultured in low and high glucose for 24 h (10 mM G, 30 mM G), supplemented with CHC, NAC and PKC-δ-specific inhibitor were used for western blots to estimate PKC activity by the separation of membranous and cytosolic fractions. Sample size = 4–9. Data are given as means ± S.E.M. Significances: *$P < 0.05$ versus 10 mM G embryos and **$P < 0.05$ versus 30 mM G embryos (ANOVA). (B) Estimated activity of PKC-δ, day 11. Embryos cultured in low and high glucose for 48 h (10 mM G, 30 mM G), supplemented with CHC, NAC, and PKC-δ-specific inhibitor were used for western blots to estimate the PKC activity by separation of membranous and cytosolic fractions. Sample size = 4–10. Data are given as means ± S.E.M. Significances: *$P < 0.05$ versus 10 mM G embryos and **$P < 0.05$ versus 30 mM G embryos (ANOVA). (C) Representative western blot bands with the normalization control ERK.

**Figure 5** (A) Estimated activity of PKC-ζ, day 10. Embryos cultured in low and high glucose for 24 h (10 mM G, 30 mM G), supplemented with CHC, NAC, and PKC-ζ-specific inhibitor were used for western blots to estimate PKC activity by separation of membranous and cytosolic fractions. Sample size = 4–9. Data are given as means ± S.E.M. Significances: *$P < 0.05$ versus 10 mM G embryos and **$P < 0.05$ versus 30 mM G embryos (ANOVA). (B) Estimated activity of PKC-ζ, day 11. Embryos cultured in low and high glucose for 48 h (10 mM G, 30 mM G), supplemented with CHC, NAC, and PKC-ζ-specific inhibitor were used for western blots to estimate the PKC activity by separation of membranous and cytosolic fractions. Sample size = 4–10. Data are given as means ± S.E.M. Significances: *$P < 0.05$ versus 10 mM G embryos and **$P < 0.05$ versus 30 mM G embryos (ANOVA). (C) Representative western blot bands with the normalization control ERK.
tricarboxylic acid cycle (TCA) to CO₂ and yields increased levels of NADH and reduced flavin adenine dinucleotide (FADH₂). These products serve as electron donors and, ultimately, an excess of superoxide is produced (Brownlee 2001).

The superoxide overproduction of the mitochondria is suggested to cause DNA strand breaks that in turn may activate poly ADP-ribose polymerase (PARP). PARP ribosylates GAPDH, the rate-limiting enzyme in the glycolysis, thereby leading to a decreased GAPDH activity. This phenomenon diverts excess glycolytic metabolites into different pathways upstream of GAPDH, among those the PKC pathway which later in the long term leads to an increased activation of PKC (Du et al. 2003).

The result of the present study is in concert with the notion of ROS-mediated GAPDH inhibition since the high glucose environment increased the activities of PKC-δ and PKC-ζ, and the 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 (Brownlee 2001). This could also be true for embryonic dysmorphogenesis. In addition, an abnormal activation of PKC could initiate increased apoptosis, especially PKC isoforms δ and ζ which have been associated with apoptosis (Domenicotti et al. 2003, Leroy et al. 2005, Santiago-Walker et al. 2005). 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 proapoptotic factors that activate the cysteine protease family of caspases, which then propagate a death cascade (Vincent et al. 2004).

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 (Eriksson & Borg 1991, 1993). NAC increases the levels of intracellular antioxidant reduced glutathione by providing the rate-limiting amino acid, cysteine, for the synthesis of glutathione. The addition of NAC initiates the ability to take care of excess 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-δ and PKC-ζ could have specific roles in the development of embryonic organogenesis on day 10, which are terminated on day 11. In conclusion, we found that CHC and NAC as well as PKC-δ- and PKC-ζ-specific inhibitors have a protective effect on high glucose-induced embryonic dysmorphogenesis in vitro, and concomitantly we found a normalizing effect of NAC and CHC on increased activation of PKC-δ and PKC-ζ in rat embryos. These effects were confined to gestational day 10. The 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 δ and ζ.

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