ORIGINAL RESEARCH

Down regulation of the Proliferation and Apoptotic Pathways in the Embryonic Brain of Diabetic Rats

María Sol Kruse · Joaquín Barutta · María Cristina Vega · Héctor Coirini

Abstract Compelling evidence shows that the offspring subjected to uncontrolled hyperlycemia during gestation display behavioral, neurochemical, and cellular abnormalities during adulthood. However, the molecular mechanisms underlying these defects remain elusive. Previous studies have shown an increased rate of apoptosis and a decreased index of neuronal proliferation associated with diabetic embryopathy. The aim of the present study was to determine whether impairments in apoptotic related proteins also occur in the developing central nervous system from non-malformed embryos exposed to uncontrolled gestational hyperglycemia. Pregnant rats injected with either streptozotocin or vehicle were killed on gestational day 19. Offspring brains were quickly removed to evaluate protein expression by Western blotting. Embryonic brains from diabetic rats exhibited a decrease in the cell survival p-Akt expression ($52.83 \pm 24.35\%$) and in the pro-apoptotic protein Bax ($56.16 \pm 6.47\%$). Moreover, the anti-apoptotic protein Bcl-2 showed a non-significant increase while there were no changes in Procaspase 3 or cleaved Caspase 3 proteins. The cytoskeleton proteins NF-200 and GFAP were also examined. Neither NF-200 nor GFAP showed differences in embryonic brains from diabetic rats compared to controls. Altogether, these results indicate that both proliferation and apoptotic pathways are decreased in the brain from the developing offspring of diabetic rats. Since selective neuronal apoptosis, as well as selective cell proliferation, are specifically involved in brain organogenesis, it is possible that simultaneous impairments during the perinatal period contribute to the long lasting alterations observed in the adult brain.

Keywords Akt · Bax · Bcl-2 · Caspase 3 · GFAP · NF-200

Introduction

Normal development of the vertebrate nervous system requires the formation of synapses, which are regulated by neurotropins. A second process removes unnecessary neurons by apoptosis leaving a more efficient synaptic configuration. Several agents are able to disturb the developing process resulting in altered cell number and neural function (White and Barone 2001). For example, prolonged hyperglycemia during critical periods of development is known to elicit malformations in the fetal brain (Dheen et al. 2009; Van Lieshout and Voruganti 2008). Similarly, a high incidence of major congenital anomalies and neonatal deaths have been described in infants of diabetic mothers (Casson et al. 1997; Greene et al. 1989; Hawthorne et al. 1997). Although optimal control of glucose levels during pregnancy is desirable, the occurrence of malformation remains 3- to 5-fold higher than that for non-diabetic pregnancy (Reece 1999).

Multiple factors have been associated with hyperglycemia-induced malformations. These include the imbalance of maternal fuels early in gestation, the deficiency in essential

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fatty acid and proteins as well as the excess of embryonic oxygen-free radicals production (Goldman et al. 1985; Homko et al. 1999; Reece 1999; Reece and Wu 1997; Reece et al. 1996; Sivan et al. 1997). Embryos cultured with high levels of glucose or with diabetic serum showed cell death in the neural tube (Sadler 1980a, b). Similarly, embryos from diabetic mice exhibited increased apoptosis on the surface of neural folds during organogenesis (Fine et al. 1999; Phelan et al. 1997). Damage to yolk sac cell membranes was also found during a critical period of organ formation by hyperglycemia (Reece et al. 2005), an effect associated with hyperglycemia-induced apoptosis (Moley 2001; Pampfer et al. 1997; Phelan et al. 1997).

Compelling evidence indicates that a dysregulation of the normal programmed cell death pathways is implicated in the malformations observed in diabetes. Bax, a death-promoting member of the Bcl-2 family of proteins is expressed at high levels in embryos from hyperglycemic diabetic mice (Lei et al. 2002). These embryos also demonstrated increased apoptosis, suggesting that hyperglycemia may induce an apoptotic response via Bax (Moley et al. 1998; Reece et al. 2005; Yang et al. 2008a, b). Alterations of cell survival and proliferation have also been observed in embryos from diabetic mothers. Akt, a key mediator protein of cell survival and cell growth (Brazil and Hemmings 2001; Datta et al. 1999; Hanada et al. 2004; Scheid and Woodgett 2001) is down regulated in yolk sack and umbilical vein tissue from embryonic rats subjected to hyperglycemia (Reece et al. 2005; Varma et al. 2005). However, it remains elusive whether Akt is affected in the fetal brain under prolonged hyperglycemia.

The present study is designed to determine the impact of gestational hyperglycemia induced by streptozotocin (STZ) on non-malformed embryonic brain signaling proteins related to apoptosis during the late neonatal period. Here, we tested the hypothesis that high circulating levels of glucose are linked to impaired insulin signaling, leading to an alteration in the levels of the proapoptotic factor Bax, and the antiapoptotic factor Akt.

Methods

Experimental animals

Animal procedures have been approved by the Animal Care and Use Ethical Committee of the School of Medicine, University of Buenos Aires, Argentina, in accordance to guidelines defined by the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals procedures. Animals were kept under standard laboratory conditions at 24°C, with light/dark cycles of 12/12 h

and food and water ad libitum. Sixty days-old female Sprague–Dawley rats weighing 210–260 g ($n = 8$) were placed overnight in cages with males of the same strain. Vaginal smears were examined the next morning and the presence of spermatozoa was considered as day 1 of gestation. Diabetes was induced on gestational day (GD) 3 by a single femoral i.v. injection of 45 mg/Kg streptozotocin (STZ, Sigma-Aldrich) dissolved in saline 0.9% acidified to pH 4.5 using citric acid ($n = 4$) (Coirini et al. 1980). Vehicle-injected rats served as control ($n = 4$). Forty-eight hours after STZ administration, a pronounced glucosuria (>2 g/100 mL, Diastix; Bayer) and elevation of blood sugar levels >180 mg/dL were detected in all rats. Both control and STZ-treated dams were killed on GD 19 by decapitation. The embryos were removed and their brains were rapidly dissected, frozen on dry ice and stored at −80°C. Glucose levels were determined in pregnant rats using blood from the tail 1 h before killing. The offspring glycemia was determined from troncal blood immediately after decapitation. In all cases, a commercial strip and glucometer were used (OneTouch Ultra, Johnson & Johnson).

Western blotting

Homogenates from frozen brains were obtained by sonication in ice-cold lysis buffer (40 mM Tris–HCl, 120 mM NaCl, 1 mM EDTA, 10 mM NaF, 50 mM glycerol phosphate, 2 mM Na_3VO_4 and 1% Triton 100, pH 7.5) containing a protease inhibitor cocktail (Roche Diagnostics). Then samples were sonicated and centrifuged for 5 min at $5000 \times g$ at 4°C. The supernatants were collected and protein concentrations were determined by Bradford (Bradford 1976) using BSA as standard. 20 μg of protein was separated on 10% SDS-PAGE in Tris–glycine electrophoresis buffer at 100 V for 90 min. PageRuler™ Prestained Protein Ladder (Fermentas) was used as protein molecular weight marker. Proteins from gels were transferred onto PVDF membranes (Bio-Rad, Argentina) and membranes were blocked with TBS-T (20 mmol/L Tris, pH 7.5; 150 mmol/L NaCl and 0.1% Tween-20) containing 5% of fat-free milk for 1 h. Blocked membranes were incubated with primary antibody in TBS-T containing 5% fat-free milk at 4°C overnight. The following primary antibodies were used: phospho-Akt kinase (1:800, Cell Signaling Technologies Inc), Bax (1:1,000, Santa Cruz Biotech.), Caspase 3 (1:1,000, Santa Cruz Biotech.), Bcl-2 (1:300, Santa Cruz Biotech.) Glial Fibrillary Acid Protein (GFAP) (1:600, Sigma-Aldrich), NF-200 (1:800, Sigma-Aldrich), and F-Actine (1:1,000, Santa Cruz Biotech.). Immunoblots were then washed with TBS-T three times and incubated at RT for 1 h with the respective HRP-conjugated secondary antibodies (1:5,000, GE Healthcare Life Sciences, Argentina). Chemiluminescence was detected with the ECL system (GE Healthcare Life Sciences, Argentina) and

exposed to hyperfilm (GE Healthcare Life Sciences, Argentina). The antibodies used for Western blot analysis revealed in each case single bands at the expected molecular masses. All membranes were then stripped and reprobed for F-Actin as a loading control. Signals in the immunoblots were scanned and analyzed by Scion Image software. The amount of target protein was indexed to F-Actin in all cases to ensure correction for the amount of total protein on the membrane. Statistical analyses were performed using the StatView software. Values are expressed as mean $\pm$ SD and comparisons were analyzed using Student's t-test.

Results

Maternal hyperglycemia decreases the viability of the offspring

In the present study, all malformed embryos were excluded. Only embryos exhibiting correct body shape flexure, both anterior and posterior neural pole closure, and no evidences of mandible or other somatic malformations (Gareskog et al. 2007; Reece et al. 2005; Wentzel et al. 2008) were studied. All non-malformed embryos examined were from STZ-treated dams whose glucose levels were between 300 and 500 mg/dL. The offspring blood glucose levels were over 100 mg/dL, which were twice the values of control embryos (CE). As shown in Table 1, 53 embryos were obtained from 4 control dams while 42 embryos were recovered from 4 STZ-treated dams (diabetic offspring, DO). In accordance to previous studies, a significant reduction in the viability of the embryos was observed in the STZ group (Gareskog et al. 2007; Wentzel et al. 2008). Overall, the incidence of malformations and fetal loss found in our STZ cohort (Table 1) was similar to that described by others (Wentzel et al. 2008).

Decreased p-Akt in the embryonic brains from offspring of diabetic rats

The effect of hyperglycemia on the neuronal survival-signaling pathway was analyzed by assessing the anti-apoptotic

protein p-Akt. Akt serine/threonine kinase activity is primarily activated by phosphorylation through PI3K (Zdychova and Komers 2005). Here, we measured the activity of p-Akt by means of Western blotting. We found a significant decrease in p-Akt levels ($52.83 \pm 24.35\%$; $p < 0.05$) in DO brains relative to the control group (Fig. 1).

Altered apoptosis in the embryonic brains from offspring of diabetic rats

It is well known that during the organogenesis period, there is an excessive apoptosis in embryos exposed to hyperglycemia, an effect that is typically associated with increased expression of pro-apoptotic proteins (Gareskog et al. 2007; Reece et al. 2005; Sun et al. 2005; Yang et al. 2008a, b; Zabihi et al. 2007; Zhao et al. 2009). Here, we determined the levels of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 in CE and DO brains by Western blot. A significant decrease of Bax ($56.16 \pm 16.47\%$; $p < 0.001$) was observed in the DO brains compared to controls (Fig. 2a). In contrast, a non-significant increase in Bcl-2 levels was found in the DO (Fig. 2b). The Bax/Bcl-2 balance was lower in DO than CE (CE $= 0.62 \pm 0.10$ vs. DO $= 0.45 \pm 0.24$; $p > 0.05$). The expression of the downstream effectors in the apoptotic pathway, the
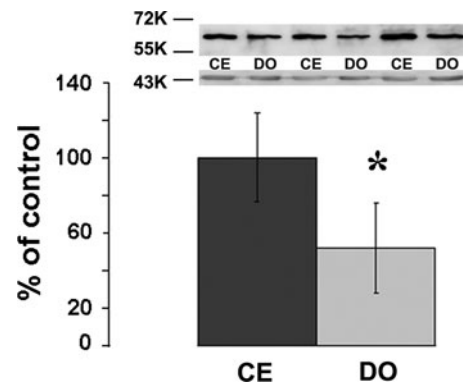

Fig. 1 Expression of pAkt in embryonic brains. Western blot of p-Akt in brain tissue from offspring of control non-diabetic (CE) and diabetic (DO) dams. Data were quantified by densitometric analysis and corrected for F-Actin as loading control. Representative pictures of p-Akt expression and F-Actin as loading control are shown above the bars. Data are presented as mean $\pm$ SD from at least three independent experiments. *$p < 0.05$ Student's t-test; $n = 11$/group

Table 1 Maternal and fetal glucose blood levels and viability

Group	Maternal glycemia (mg/dL)	Fetal glycemia (mg/dL)	Total nonmalformed embryos	Total malformed embryos	Average number of embryos per mother
Control	91 ± 4	45 ± 5	53	0	13.3 ± 1.7
Diabetic	380 ± 94*	120 ± 15*	36	6*	10.5 ± 0.6

Blood obtained from the tail vein was used to determine glucose levels of pregnant rats 1 h prior cesarean and the offspring glycemia was determined from troncal blood obtained immediately after decapitation. Data are presented as mean $\pm$ SD from 4 animals per group * $p < 0.05$ Student's t-test
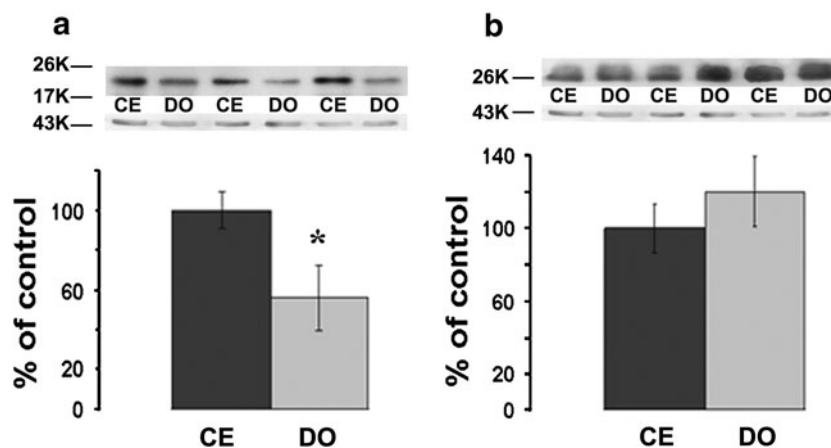
Fig. 2 Expression of apoptotic and anti-apoptotic proteins in embryonic brains. Data were quantified by densitometric analysis and corrected for F-Actin as loading control. **a** A significant decrease of the pro-apoptotic protein Bax was observed in diabetic offspring (DO) brains when compared to non-diabetic offspring. **b** A non-significant

caspase executioner Caspase 3, was also studied. Neither Procaspase 3 nor the cleaved Caspase 3 showed significant differences between DO and CE brains (Fig. 3).

No alterations in the cytoskeletal organization in embryonic brains from offspring of diabetic rats

Hyperglycemia produces a variety of structural alterations in the nervous system (Biessels et al. 1994; Gispen and Biessels 2000; Reagan 2012). Among them, abnormalities of the neurofilament have been suggested to account for

elevation in the anti-apoptotic protein Bcl-2 levels were observed between CE and the DO group. Representative pictures of Bax, Bcl-2, and F-Actin expression are shown above the bars. Data are presented as mean ± SD from at least three independent experiments. *$p < 0.05$ Student's t-test; $n = 7$/group

axonal atrophy and axonal loss in diabetes (Fernyhough et al. 1999; McLean 1997; Medori et al. 1985; Scott et al. 1999). We first examined whether hyperglycemia during gestation affects the NF-200 expression, a critical component of the axonal cytoskeleton. No differences were observed between DO and CE brains (Fig. 4a). We next investigated the expression of glial fibrillary acidic protein (GFAP), since astrocytes are reduced in different brain regions of diabetic rats (Dennis et al. 2005; Garcia-Caceres et al. 2008; Lechuga-Sancho et al. 2006). We found no apparent differences in GFAP levels between CE and DO brains (Fig. 4b).
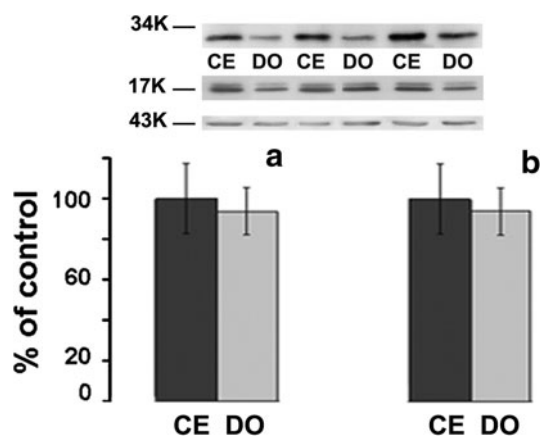
Discussion

Periconceptional hyperglycemia is teratogenic and can lead to congenital malformations and miscarriage (Gareskog et al. 2007; Reece et al. 2005; 2006; Yang et al. 2008a, b). The human brain is particularly vulnerable to the effects of hyperglycemia, and the relative risk of central nervous system malformations is 15.5 times higher than in normal pregnancies (Cannon et al. 2002). Previous studies have shown an increased rate of apoptosis and a decreased index of neuronal proliferation during organogenesis associated with disturbed embryonic maturation, increased embryonic resorption and congenital malformations (Gao and Gao 2007; Sun et al. 2005; Zhao et al. 2009). The results of the present study expand upon these findings by showing that exposure to uncontrolled gestational hyperglycemia can disrupt both neuronal proliferation and neuronal survival in non-malformed embryos from STZ-induced diabetic rats. These alterations may contribute to develop future alterations in the adult brain. It is now known that the offspring

Fig. 3 Expression of Procaspase 3 and Caspase 3 in embryonic brains. Densitometric analysis of the Western blot bands from the Procaspase 3 (**a**) and active cleaved Caspase 3 (**b**) showed no differences between control non-diabetic offspring (CE) and diabetic offspring (DO) groups. Data were corrected for F-Actin as loading control. Representative pictures are shown above the bars. Data are presented as mean ± SD from at least three independent experiments. $p > 0.05$ Student's t-test; $n = 7$/group

Fig. 4 NF-200: and GFAP expression in embryonic brains. Densitometric analysis of NF-200 (**a**) and GFAP (**b**) Western blot bands showed no differences between the offspring of control non-diabetic (CE) and diabetic (DO) dams. Data were corrected for F-Actin as loading control. Representative pictures are shown above the bars. Data are presented as mean ± SD from at least three independent experiments. $p > 0.05$ Student's t-test; $n = 4$/group
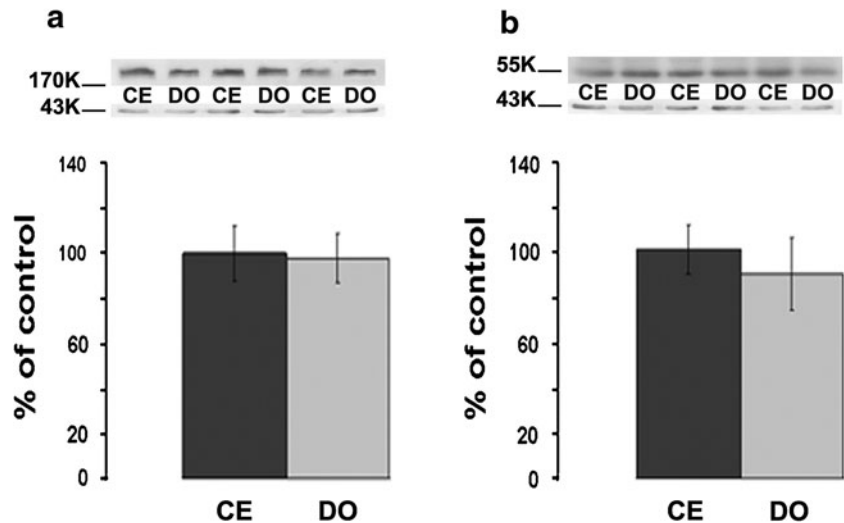
of diabetic mothers typically exhibit a wide range of behavioral, neurochemical, and cellular abnormalities during adulthood. Rats born from STZ-induced diabetic rats exhibited decreased brain weight and dendritic spine density (Yamano et al. 1986), increased anxiety-like behaviors and locomotors hyperactivity (Ramanathan et al. 2000) and neurotransmitter alterations in the hypothalamus (Foglia et al. 1987), midbrain, and caudate putamen (Bhattacharya and Saraswati 1991). However, the mechanisms underlying these alterations are not fully understood. Here, we found that the activity of Akt was significantly reduced in the DO brains from non-malformed embryos. Since Akt plays a crucial role in insulin signaling as well as in cell proliferation and cell survival, we hypothesized that the Akt decrease observed in our experiments is likely to be associated with alterations in that kind of processes as it was described in malformed embryos (Reece et al. 2005) or in embryonic endothelial cells (Varma et al. 2005) following uncontrolled gestational hyperglycemia. Moreover, uncoupling of downstream insulin signaling at Akt has been implicated in the development of insulin resistance and Type II diabetes (Vosseller et al. 2002), a pathology often developed by the offspring of diabetic mothers (Fetita et al. 2006; Han et al. 2007).

Apoptosis activation is related to dysmorphogenesis in embryos exposed to diabetes during organogenesis (Gareskog et al. 2007; Reece et al. 2005; Yang et al. 2008a, b; Zabihi et al. 2007; Zhao et al. 2009). In contrast, we found a reduction of the expression of the pro-apoptotic protein Bax, and a non-significant increase in the anti-apoptotic protein Bcl-2, indicating that the apoptotic pathway is decreased in the brain of DO embryos. Moreover, the lack of changes in the level of Caspase 3 and the altered Bax/Bcl-2 balance indicates that hyperglycemia during gestation is not triggering apoptosis in DO brains. It is therefore very likely that a developmental disruption of the natural

cell death programming (apoptosis) is implicated. In fact, the process of synaptic pruning is especially important during this stage of gestation when approximately 50% of overproduced neurons in the brain are eliminated (Jacobson 1973). Altogether, the reduction of Bax and p-Akt could implicate an alteration of the natural apoptosis and neuronal proliferation, which in turn may lead to the formation of abnormal neuronal networks in the adulthood and increase liability to develop neurological and psychiatric disorders.

We found no changes in GFAP levels and heavy neurofilament expression (e.g., NF-200) in the brain from offspring of diabetic rats. As embryos from diabetic pregnancies are typically hyperinsulinemic (Han et al. 2007), we speculate that the concurrent high levels of insulin may confer some degree of neuroprotection and prevent the expected decrease in GFAP and NF-200. This interpretation is consistent with previous studies showing that insulin treatment prevents or attenuates the GFAP and heavy neurofilament changes induced by hyperglycemia in adult diabetic animals (Cannon et al. 2002; Christianson et al. 2007).

In summary, our study demonstrates that the several signaling processes that mediate the normal brain developmental is also compromised in non-malformed offspring embryos from STZ-induced diabetes rats. Surprisingly, we found that both the proliferation and apoptotic pathways were down regulated in the DO brains, indicating that uncontrolled hyperglycemia during pregnancy may cause changes in cell proliferation and natural cell death programming in the neuronal progenitors that may result in abnormal neurodevelopment. Since selective cell proliferation, combined with selective apoptosis, sculpts the developing central nervous system, we hypothesized that several enduring neurobiological consequences are expected to occur in the adult brain from non-malformed offsprings exposed to uncontrolled prenatal hyperglycemia.

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