Dehydroepiandrosterone protects bovine retinal capillary pericytes against glucose toxicity

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Abstract

Pericyte loss is an early feature of diabetic retinopathy and represents a key step in the progression of this disease. This study aimed to evaluate the effect of dehydroepiandrosterone (DHEA) on glucose toxicity in retinal capillary pericytes. Bovine retinal pericytes (BRP) were cultured in a high glucose concentration, with or without DHEA. After 4 days of incubation the number of BRP was significantly reduced by the high glucose concentration. The addition of DHEA to the medium reversed the adverse effect of high glucose: BRP proliferation partially recovered in the presence of 10 nmol/l DHEA, and completely recovered in the presence of DHEA at

concentrations equal to or greater than 100 nmol/l. At physiological glucose concentrations, DHEA had no effect on BRP growth. Data show that DHEA, at concentrations similar to those found in human plasma, protects BRP against glucose toxicity. This effect seems specific for DHEA, since its metabolites, 5-en-androstene-3 β ,17 β -diol, dihydrotestosterone and estradiol did not alter BRP growth in normal or high glucose media. Various pieces of evidence link the antioxidant properties of DHEA to its protective effect on glucose-induced toxicity in BRP.

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Introduction

Dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) are the most abundant hormonal steroids circulating in humans, but their physiological roles are unknown. Besides its function as a precursor of androgens and estrogens, DHEA has been reported to possess beneficial effects on cancer (Schwartz 1979, Boccuzzi *et al.* 1994), atherosclerosis (Gordon *et al.* 1988), obesity (Nestler *et al.* 1988), autoimmune diseases (Van Vollenhoven *et al.* 1994), infections (Yang *et al.* 1994), aging (Morales *et al.* 1994) and diabetes. Focusing on diabetes, DHEA has been shown to reduce hyperglycemia and water consumption in genetically diabetic mice (Coleman *et al.* 1982), to increase insulin sensitivity of target organs (Buffington *et al.* 1991) and to improve insulin resistance in diabetic patients (Buffington *et al.* 1993). A variety of theoretical explanations have been offered as basic mechanisms for DHEA pleiotropic actions (Regelson & Kalimi 1994), but the process by which the clinical course of diabetes may be improved remains poorly defined. Moreover, it is still not known if DHEA can affect the progression of chronic diabetic complications.

The loss of retinal capillary pericytes is a characteristic change in the early phase of diabetic retinopathy (Cogan *et al.* 1961, Kefalides 1981) and is considered a key step in the progression of this disease. In rats made diabetic by streptozotocin, soon after the increase in glycemic levels the normal pericyte to endothelial cell ratio is remarkably reduced (Sharma *et al.* 1985). Furthermore, high glucose concentrations have been reported to suppress the cell multiplication and the mitotic rate of cultured bovine retinal capillary pericytes (BRP) (Li *et al.* 1984, Chibber *et al.* 1994, Porta *et al.* 1994). In this study, we used this *in vitro* model to investigate the effect of DHEA and its metabolites on the loss of BRP induced by high glucose. Data show that DHEA, at concentrations similar to those found in human plasma, prevents the suppression of BRP growth caused by high glucose. This effect is specific and might be related to DHEA antioxidant properties.

Materials and Methods

DHEA, D-glucose and reagents for cell cultures were purchased from Sigma Chemical Co. (St Louis, MO, USA). Tissue culture flasks and dishes were from Corning (New York, NY, USA).

Cell culture

Primary cultures and subcultures (up to the second passage) of BRP were established as previously described (Wong *et al.* 1987). In brief, retinas were obtained from freshly slaughtered cattle, homogenized in Dulbecco's modified Eagle's medium (DMEM, 5.6 mmol/l glucose, Sigma) and passed through an 85 µm nylon mesh. A 1 mg/ml collagenase-dispase solution in phosphate buffered saline Dulbecco 'A' (PBS) (Oxoid, Basingstoke, Hants, UK) was used to digest the trapped microvessels for 30 min at 37 °C. The digested microvessels were plated in 75 cm² tissue culture flasks after filtration through a 53 µm nylon mesh. Cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 UI/ml penicillin and 100 µg/ml streptomycin. Pericytes in culture were identified by morphological criteria and by immunocytochemical staining with 3G5 monoclonal antibody (Nayak *et al.* 1988). Cultures of BRP were trypsinized, viable cells counted and 2×10^4 cells/well were plated in 6-well (9.6 cm²) culture plates. BRP of the first and second passage were used for the experiments. Cells were allowed to attach overnight, then the medium was completely removed and replaced with an experimental one.

Experimental conditions

To test the effect of DHEA on the adverse consequence of high glucose concentration, BRP were cultured in either 5.6 or 30 mmol/l glucose, with or without DHEA at four different concentrations (10 nmol/l; 100 nmol/l; 500 nmol/l; 1000 nmol/l).

Since DHEA is a precursor of both androgens and estrogens, and 5-en-androstene-3β,17β-diol (ADIOL) is one of its major metabolites, we also tested the following experimental media: (a) 5.6 or 30 mmol/l glucose, with or without estradiol (E₂) at two different concentrations (1 and 500 nmol/l); (b) 5.6 or 30 mmol/l glucose, with or without dihydrotestosterone (DHT) at two different concentrations (1 and 500 nmol/l); (c) 5.6 or 30 mmol/l glucose, with or without ADIOL at two different concentrations (1 and 500 nmol/l). Furthermore, the effect of a steroid without hormonal properties was tested by growing BRP in a medium containing 5.6 or 30 mmol/l glucose, with or without 500 nmol/l cholesterol.

The compounds were diluted in ethanol; the final concentration of ethanol in the medium did not exceed 0.1%, a concentration which had no detectable effect on BRP growth. However, the same concentration of ethanol was also added to the medium of control cultures.

After 4 days of culture, cells were trypsinized and counted in a Bürker chamber using trypan blue. All the experiments were carried out using two replicate dishes per treatment, and repeated on 4 to 6 separate occasions. Results are expressed as percentage of cell number in the control condition (i.e. 5.6 mmol/l glucose).

Lipid peroxidation in BRP

In additional experiments, the effects of DHEA and glucose on lipid peroxidation were tested by growing BRP in a medium containing glucose at either 5.6 or 30 mmol/l, with or without DHEA (100 or 500 nmol/l). After four days of culture, lipid peroxidation in BRP was determined spectrophotometrically by measuring the formation of thiobarbituric acid reactive substances (TBARS) at 543 nm. BRP (2.5×10^6 cells/ml) with or without DHEA were incubated for up to four hours at 37 °C in a shaking bath (Slater & Sawyer 1971). For the standard curve, commercially available malonyldialdehyde bis-(dimethylacetal), followed by acid hydrolysis, was used.

Statistical methods

All results are presented as means ± s.d. Statistical comparison between groups was carried out by analysis of variance (ANOVA). For each comparison, the threshold of significance was calculated according to Bonferroni's method.

Results

Effects of DHEA on BRP grown in normal or high glucose concentration

The growth of BRP cultured at normal glucose levels (5.6 mmol/l) was unaffected by DHEA (Fig. 1). As expected, increasing the glucose concentration in the culture medium from 5.6 mmol/l (control) to 30 mmol/l significantly reduced BRP number ($P < 0.001$; Fig. 2). The addition of DHEA, at concentrations ranging from 10 to 1000 nmol/l, shielded pericytes from the high glucose effect (Fig. 2): when cultured in 30 mmol/l glucose, BRP numbers were lower in the absence of DHEA ($65.3 \pm 6.0\%$) than in the presence of varying doses of DHEA (10 nmol/l: $83.5 \pm 12.4\%$, $P < 0.05$; 100 nmol/l: $89.0 \pm 11.0\%$, $P < 0.01$; 500 nmol/l: $93.8 \pm 14.3\%$, $P < 0.01$; 1000 nmol/l: $88.3 \pm 8.1\%$, $P < 0.05$) (Fig. 2). A DHEA concentration of 100 nmol/l completely reversed the adverse effect of 30 mmol/l glucose on BRP growth (Fig. 2).

Effects of E₂, DHT and ADIOL on BRP grown in high glucose concentration

E₂ and DHT, at concentrations of 1 nmol/l which have maximal effects on hormone-responsive cells (Lippman *et al.* 1977, Bélanger *et al.* 1990), had no effect on BRP numbers: cell numbers of BRP grown in 30 nmol/l glucose were not different from those of BRP grown in 30 nmol/l glucose plus 1 nmol/l E₂ ($70.0 \pm 9.9\%$) or 1 nmol/l DHT ($69 \pm 1.4\%$). Even at 500 nmol/l, a

Figure 1 Effect of increasing DHEA concentrations on the growth of BRP cultured in 5.6 mmol/l glucose. Bars represent the mean $\pm$ S.D. of two replicate cultures repeated on 6 separate occasions.

Figure 2 Effect of increasing DHEA concentrations on the growth of BRP cultured in 30 mmol/l glucose. Bars represent the mean $\pm$ S.D. of two replicate cultures repeated on 6 separate occasions. * $P < 0.05$, ** $P < 0.01$ vs 30 mmol/l glucose alone.

concentration at which DHEA shows its maximal protective effect, E_2 and DHT were ineffective (Fig. 3). ADIOL at concentrations of 1 nmol/l (data not shown) and 500 nmol/l (Fig. 3) also had no effect. Furthermore,

cholesterol, a steroid without hormonal properties, when added to the culture medium at a concentration of 500 nmol/l, did not influence the inhibitory effect of high glucose on BRP growth (Fig. 3).

Inhibition of glucose-induced lipid peroxidation in BRP by DHEA

Lipid peroxidation in BRP, determined by measuring TBARS generation, was higher in 30 mmol/l glucose than in the control condition (i.e. 5.6 mmol/l glucose; Fig. 4). The addition of DHEA to the culture medium, at a concentration (100 nmol/l) that prevents the inhibitory effect of high glucose on BRP growth, also protected BRP against glucose-induced lipid peroxidation: TBARS generation was significantly lower at 30, 60, 120 and 180 min (Fig. 4). Similar results were obtained with 500 nmol/l DHEA (data not shown). The addition of either 100 or 500 nmol/l DHEA to the control medium had no effect on the time course of lipid peroxidation (data not shown).

Discussion

Our data show that DHEA, which has no effect on the number of BRP grown in normal glucose, completely abolishes the inhibitory effect of high glucose on BRP multiplication at concentrations equal to or higher than 100 nmol/l. To our knowledge, this is the first report of a protective effect exerted by an endogenous steroid on glucose toxicity in retinal pericytes.

The link between high glucose concentration and pericytes loss in early diabetic retinopathy is debatable. High glucose concentration has been reported to suppress the cell growth and the mitotic rate of cultured BRP (Li *et al.* 1984, Chibber *et al.* 1994, Porta *et al.* 1994). However, retinal pericytes appear to be a highly stable cell population and their decrease in early streptozotocin diabetes is viewed as the result of increased cell death which cannot be replaced (Sharma *et al.* 1985). Moreover, the fall in BRP numbers after fluctuations of glucose concentration in the culture medium has recently been related to apoptosis (Li *et al.* 1996). Thus, it is not clear if the reduction in BRP numbers in high glucose culture medium is due to impaired cell growth, increased death, or both.

The mechanism underlying the protective effect of DHEA on glucose toxicity in BRP is unknown. Our *in vitro* model excludes any action through the increase in insulin sensitivity due to DHEA and supports a direct effect on pericytes. In this perspective, it has recently been shown that DHEA directly affects glucose uptake by cultured cells *in vitro* (Nakashima *et al.* 1995) and modifies many enzymatic activities involved in fuel metabolism (Pascale *et al.* 1995).

Figure 3 Effect of 500 nmol/l DHEA, cholesterol (Chol.), ADIOL, DHT, and E₂ on BRP grown in 5.6 mmol/l glucose (open bars) or 30 mmol/l glucose (hatched bars). Bars represent the mean ± S.D. of two replicate cultures repeated on 4 separate occasions. *P < 0.001 vs 5.6 mmol/l glucose alone; NS, not significant.

Figure 4 Effect of DHEA on lipid peroxidation in BRP induced by high glucose concentration. TBARS generation after incubation of BRP for 30, 60, 120, 180, or 240 min in 5.6 mmol/l glucose (solid bars), 30 mmol/l glucose (hatched bars) or 30 mmol/l glucose plus 100 nmol/l DHEA (open bars) is shown. Bars represent the mean ± S.D. of two replicate cultures repeated on 4 separate occasions. *P < 0.05, **P < 0.02 vs 30 mmol/l glucose.

DHEA itself exerts a weak androgenic action (Boccuzzi *et al.* 1992) and is a precursor of both estrogens and androgens (Abul-Hajj 1975). However, the androgen or the estrogen receptor are not involved, since E₂ and DHT, at concentrations which have the maximal proliferative effect on hormone-responsive tissues (Lippman *et al.* 1977, Bélanger *et al.* 1990), do not protect BRP against glucose toxicity. In addition, ADIOL, the main DHEA metabolite showing both androgenic and estrogenic properties (Boccuzzi *et al.* 1994), is unable to maintain pericyte number in the presence of high glucose.

A non-specific mechanism, related to the common chemical structure of steroids, is also excluded: at concentrations equimolar to the effective one of DHEA, cholesterol, ADIOL, E₂, and DHT do not protect BRP against glucose toxicity.

Several effects of DHEA have been ascribed to the inhibition of glucose-6-phosphate-dehydrogenase (G6PD) activity, a key enzyme of the pentose phosphate shunt (Schwartz *et al.* 1988). The decrease in NADPH generation due to G6PD inhibition might impair aldose reductase activity, thus reducing the accumulation of sorbitol within BRP. However, this mechanism is unlikely, since the well known inhibitory effect of DHEA on G6PD is observed only at concentrations much higher than those used in this study (Boccuzzi *et al.* 1993, Butler *et al.* 1993, Di Monaco *et al.* 1997).

We have shown that high glucose concentration in the culture medium causes lipid peroxidation of BRP, as unveiled by an increased generation of TBARS. A link between high glucose concentration and oxidative stress has already been demonstrated (Hunt *et al.* 1990, Ceriello *et al.* 1996) and it has been suggested that oxidative stress contributes to tissue damage associated with diabetes (Baynes 1991). The addition of DHEA to the high glucose medium, at the same concentration that protects BRP against the growth inhibitory effect, counteracts the overproduction of TBARS. This result fits with the multi-targeted antioxidant activity of DHEA which we have recently reported (Boccuzzi *et al.* 1997) and suggests that the protective effect of DHEA on the number of BRP grown in high glucose medium is due to its antioxidant properties.

However, quite apart from the mechanism involved, it is notable that the protective effect of DHEA on the number of BRP exposed for four days to high glucose occurs even at concentrations comparable to those found in human plasma (19 nmol/l). The different susceptibility to diabetic retinopathy observed in patients with similar duration and degree of chronic hyperglycemia has been related to poorly characterized genetic factors (Dornan *et al.* 1982). The DHEA(S) plasma level is a highly specific individual marker (Thomas *et al.* 1994), and it shows a wide variability among individuals within the normal population (Orentreich *et al.* 1992). A relationship between DHEA plasma level and the individual suscepti-

bility to the development of diabetic retinopathy might be speculated, but it needs to be explored by clinical studies.

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