

Dehydroepiandrosterone prevents lipid peroxidation and cell growth inhibition induced by high glucose concentration in cultured rat mesangial cells

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Abstract

The oxidative stress induced by high glucose concentration contributes to tissue damage associated with diabetes, including renal injury. Dehydroepiandrosterone (DHEA), the major secretory product of the human adrenal gland, has been shown to possess a multi-targeted antioxidant activity which is also effective against lipid peroxidation induced by high glucose. In this study we evaluated the effect of DHEA on the growth impairment which high glucose concentration induces in cultured rat mesangial cells. Primary cultures of rat mesangial cells were grown for 10 days in media containing either normal (i.e. 5.6 mmol/l) or high (i.e. 30 mmol/l) concentrations of glucose, without or with DHEA at different concentrations. The impairment of cell growth induced by high glucose was reversed by 100 nmol/l and 500 nmol/l

DHEA, which had no effect on mesangial cells cultured in media containing glucose at the normal physiological concentration (5.6 mmol/l). In high-glucose cultured mesangial cells, DHEA also attenuated the lipid peroxidation, as measured by thiobarbituric acid reactive substances (TBARS) generation and 4-hydroxynonenal (HNE) concentration, and preserved the cellular content of reduced glutathione as well as the membrane Na⁺/K⁺ ATPase activity. The data further support the protective effect of DHEA against oxidative damage induced by high glucose concentrations, and bring into focus its possible effectiveness in preventing chronic complications of diabetes.

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Introduction

Nephropathy is a very common complication of diabetes, accounting for nearly 50% of all patients in haemodialysis in Western countries (Del Prete *et al.* 1998). Chronic hyperglycaemia is thought to play a central role in the development of diabetic nephropathy, as in other complications of diabetes (Lorenzi 1992, Diabetes Control and Complications Trial Research Group 1993, UK Prospective Diabetes Study 1998). Mesangial cells are involved in the pathogenesis of glucose-induced kidney damage. It has been well documented that exposure to high glucose concentrations inhibits the proliferation of mesangial cells in a dose-dependent manner (Nahman *et al.* 1992, Trachtman 1994, Trachtman *et al.* 1994, Cosio *et al.* 1995). The mechanism behind this effect is not fully understood, but considerable evidence suggests that the oxidative stress induced by high glucose concentration contributes to tissue damage associated with diabetes, including renal injury (Shah 1989, Baynes 1991, Larkins &

Dunlop 1992, Trachtman *et al.* 1992, Ha & Kim 1995). We recently reported that dehydroepiandrosterone (DHEA), the major secretory product of the human adrenal gland, possesses a multi-targeted antioxidant activity (Boccuzzi *et al.* 1997) and protects bovine retinal capillary pericytes against glucose-induced toxicity *in vitro* (Brignardello *et al.* 1998).

Since the mesangial cell is a specialized pericyte in the glomerular microvasculature (Schlondorff 1987), we decided to investigate the effect of DHEA on the growth abnormalities induced by high glucose concentrations in primary culture of rat mesangial cells. In the same experimental model we also studied the effect of DHEA on the oxidative stress induced by high glucose concentrations.

Materials and Methods

DHEA, D-glucose, D-mannitol, collagenase and reagents for cell cultures were purchased from Sigma Chemical Co.

(St Louis, MO, USA). Culture flasks and dishes were from Corning (New York, NY, USA). Male Wistar rats (Harlan-Nossan, Correzzana, Italy) ranging from 200 to 220 g body weight were housed and given human care in compliance with the Italian Ministry for Health Guidelines and with the Principles of Laboratory Animal Care (NIH no. 85–23, revised 1985). Animals were provided with Piccioni pellet diet (no. 48, Gessate Milanese, Italy) and water and allowed to feed *ad libitum*.

Cell culture

Primary cultures and subcultures were obtained as described by Striker & Striker (1985). In brief, kidneys were obtained from normal untreated rats. The separated cortex was minced and forced through progressively smaller wire mesh sieves to isolate uncapsulated glomeruli. After exposure to a collagenase solution (300 U/ml), the glomeruli were placed in 25 cm² plastic culture flasks and cultured in RPMI supplemented with 20% FCS, 2 mmol/l L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin, to obtain mesangial cells. Culture flasks were kept in a humidified atmosphere containing 5% (v/v) CO₂ at 37 °C. The identity of mesangial cells was confirmed by their morphological appearance: stellate cells growing in interwoven bundles. Moreover, immunofluorescence studies were performed as described by Biancone *et al.* (1992), with modifications. Briefly, immunofluorescence staining of mesangial cells was negative for factor VIII and cytokeratin antigens, whereas uniform staining was obtained with anti smooth-muscle myosin antibodies.

After 3 weeks in primary culture, mesangial cells were trypsinized, viable cells counted and 2.5×10^4 cells/well were plated on 24-well culture plates. Mesangial cells up to the sixth passage were used for the experiments. Cells were allowed to attach overnight after which the seeding medium was completely removed and replaced with one of the experimental media.

Mesangial cell growth

In order to explore the effects of high glucose and DHEA on mesangial cell growth, cells were cultured in one of the following phenol red free Dulbecco's modified Eagle's medium (DMEM) experimental media: (a) 5.6 mmol/l glucose (control); (b) 30 mmol/l glucose; (c) 5.6 mmol/l glucose + 50 nmol/l DHEA; (d) 5.6 mmol/l glucose + 100 nmol/l DHEA; (e) 5.6 mmol/l glucose + 500 nmol/l DHEA; (f) 30 mmol/l glucose + 50 nmol/l DHEA; (g) 30 mmol/l glucose + 100 nmol/l DHEA; (h) 30 mmol/l glucose + 500 nmol/l DHEA. These concentrations were chosen on the basis of our previous report on bovine retinal capillary pericytes (Brignardello *et al.* 1998). DHEA was diluted in ethanol, its final concentration not exceeding 0.1%. This concentration had no detectable effects on

mesangial cell growth; however, the same concentration of ethanol was also added to the medium of control cultures. The experimental media were renewed every 3 days. After 10 days of culture, cells were trypsinized, stained with trypan blue to select vital cells and counted using a Bürker chamber.

The effect of D-mannitol on the proliferation of mesangial cells was also studied. As in experiments with D-glucose, cells were grown in phenol red free DMEM medium containing 5.6 or 30 mmol/l D-mannitol and counted after 10 days' exposure.

All the experiments were carried out in triplicate. Results are expressed as percentages of cells in the control condition (i.e. 5.6 mmol/l glucose).

Oxidative state in mesangial cells

The effect of DHEA and glucose on the oxidative state of mesangial cells was evaluated after 10 days' exposure to a medium containing 5.6 or 30 mmol/l glucose, without or with 500 nmol/l DHEA. Medium was renewed every 3 days. This DHEA concentration was chosen since it showed the maximum protective effect against glucose-induced toxicity on bovine retinal capillary pericytes (Brignardello *et al.* 1998) and against cumene-induced lipid peroxidation on Chang liver cells (Gallo *et al.* 1999).

Lipid peroxidation was evaluated by measuring both thiobarbituric acid reactive substances (TBARS) generation and 4-hydroxynonenal (HNE) concentration. TBARS generation was measured spectrophotometrically at 543 nm. Mesangial cells (2.5×10^6 cells/ml) were incubated for up to 4 h at 37 °C in a shaking bath. For the standard curve, commercially available malonildialdehyde bis-(dimethylacetal), followed by acid hydrolysis, was used. The HNE concentration was evaluated by the method described by Esterbauer *et al.* (1991), with modifications. An aliquot of each experimental media was added to an equal volume of an acetonitrile–acetic acid mixture (96:4, v:v). Samples were then centrifuged at 250 g for 20 min at 4 °C and the supernatant was directly injected to HPLC (Waters, Milford, MA, USA) with a RP-18 column (Merck, Darmstadt, Germany). The mobile phase used was 42% acetonitrile:biodistilled water (v:v). Commercially available HNE (Calbiochem, La Jolla, CA, USA) was used to prepare a standard curve.

The cellular content of the endogenous antioxidant reduced glutathione (GSH) was evaluated. Cells were diluted in PBS to 5×10^6 cells/ml and lysed by sonication. GSH content was measured by the Owens and Belcher method (Owens & Belcher 1965). Aliquots of lysed cells were prepared in trichloroacetic acid (TCA)–EDTA (10%:10 mmol/l, v:v), then centrifuged and the supernatant used. A mixture was directly prepared in the cuvette: 0.05 mol/l sodium phosphate buffer, pH 7.0; 1 mmol/l EDTA, pH 7.0; 10 mmol/l DTNB plus an

aliquot of the sample, which was monitored at 412 nm for 2 min.

To evaluate cell membrane integrity, Na/K ATPase (Na^+ , K^+ -ATPase Mg^{2+} dependent ATP phosphohydrolyase, EC 3.6.1.3) activity was assayed in a medium containing imidazolo-HCl buffer pH 7.4, 120 mmol/l NaCl, 10 mmol/l KCl, 5 mmol/l MgCl_2 and 4 mmol/l ATP. The reaction was started by adding 25 μg of cell membrane protein and carried out as described by Shallom & Katayare (1985).

Statistical methods

All results are presented as means \pm standard deviation. Statistical comparison between groups was carried out by two-tailed paired data Student's *t*-test.

Results

Effects of DHEA on the growth of mesangial cells cultured in normal or high glucose concentrations

DHEA had no effect on the growth of mesangial cells cultured at a normal glucose concentration (5.6 mmol/l) (Fig. 1). As expected, increasing the glucose concentration in the culture medium from 5.6 to 30 mmol/l reduced the number of mesangial cells (72% \pm 10 of the control condition, i.e. 5.6 mmol/l glucose) ($P < 0.001$; Fig. 1). The addition of 100 or 500 nmol/l DHEA shielded the mesangial cells against glucose toxicity: compared with the control condition, the number of mesangial cells grown in high glucose concentration (30 mmol/l) was 84% \pm 15 in the presence of 100 nmol/l DHEA ($P < 0.05$ vs 30 mmol/l glucose; Fig. 1) and 88% \pm 15 in the presence of 500 nmol/l DHEA ($P < 0.02$ vs 30 mmol/l glucose; Fig. 1).

In order to exclude the possibility of the effect of high glucose concentration being due to hyperosmolality, we also studied the effect of D-mannitol on the proliferation of mesangial cells. As has been reported for bovine retinal capillary pericytes (Chibber *et al.* 1994), high concentrations of D-mannitol (30 mmol/l) did not affect the growth of mesangial cells (95% \pm 10 compared with 5.6 mmol/l glucose).

Effects of high glucose and DHEA on lipid peroxidation in mesangial cells

Lipid peroxidation in mesangial cells, as evaluated by determination of TBARS generation, was higher in 30 mmol/l glucose (211 \pm 35 pmol/mg protein (prot.)) than in the control condition (168 \pm 16 pmol/mg prot.; $P < 0.02$; Fig. 2). The presence of 500 nmol/l DHEA in the culture medium, which completely reversed the antiproliferative effect of high glucose concentrations, also

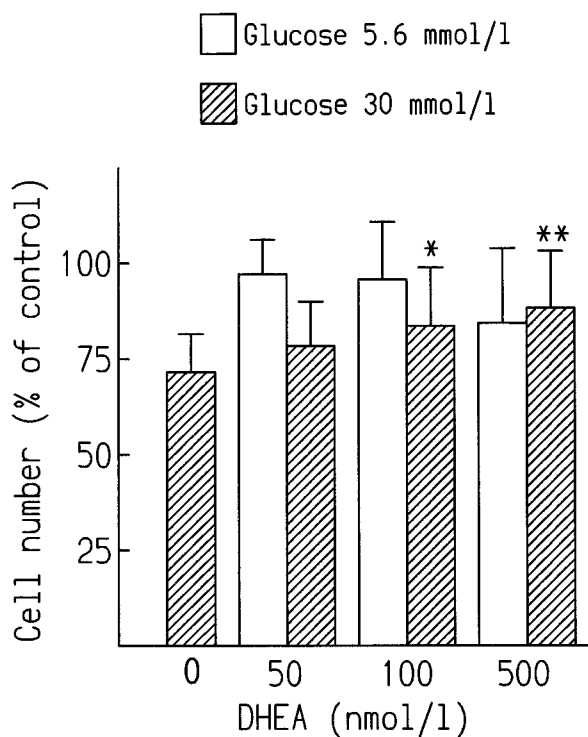


Figure 1 Effect of increasing DHEA concentration on the growth of rat mesangial cells cultured in 5.6 or 30 mmol/l glucose. The bars represent means \pm s.d. ($n = 7$). * $P < 0.05$, ** $P < 0.02$ vs 30 mmol/l glucose alone.

reduced TBARS generation in high-glucose cultured mesangial cells (135 \pm 24 pmol/mg prot.; $P < 0.02$ vs 30 mmol/l glucose; NS vs control; Fig. 2). When cells were cultured in 5.6 mmol/l glucose, the presence of 500 nmol/l DHEA had no effect on TBARS generation (Fig. 2).

HNE levels were also much higher in the medium of mesangial cells grown in a high glucose concentration (1.29 \pm 0.23 mmol/ 10^6 cells) than in the control condition (0.36 \pm 0.11 mmol/ 10^6 cells; $P < 0.005$; Fig. 2). The presence of 500 nmol/l DHEA in the culture medium, which had no effect on HNE concentration in 5.6 mmol/l glucose, dramatically reduced HNE levels in the media of mesangial cells grown in 30 mmol/l glucose (0.53 \pm 0.15 mmol/ 10^6 cells; $P < 0.01$ vs 30 mmol/l glucose; NS vs control; Fig. 2).

Effects of high glucose and DHEA on non-enzymatic defences and cell membrane integrity

The cellular content of GSH in the different experimental conditions is summarised in Table 1. GSH levels were lower in the high glucose concentration media (0.33 \pm 0.03 $\mu\text{g}/10^6$ cells) than in the control condition (0.49 \pm 0.04 $\mu\text{g}/10^6$ cells) ($P < 0.01$). The presence of

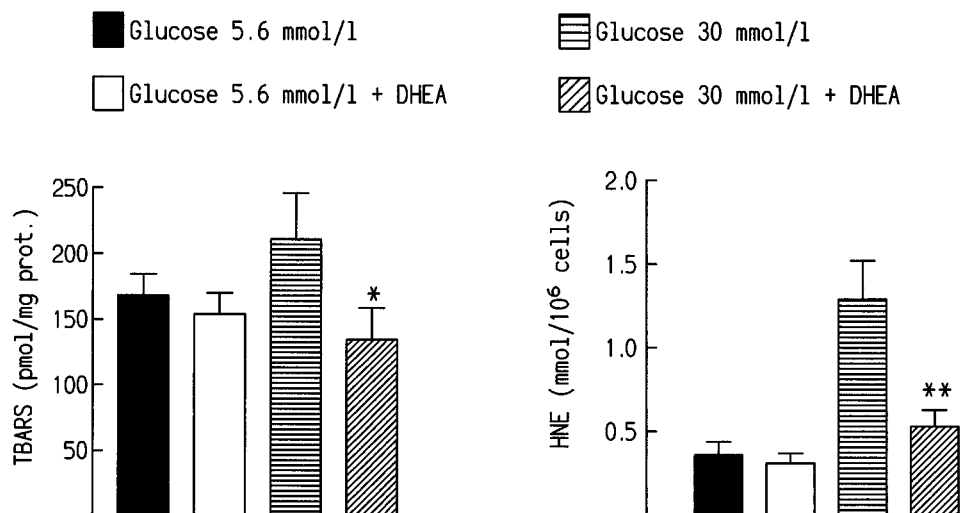


Figure 2 Effect of DHEA 500 nmol/l on lipid peroxidation induced by 30 mmol/l glucose in cultured rat mesangial cells. The bars represent means \pm s.d. of TBARS generation (left panel, $n = 5$) and HNE concentration (right panel, $n = 3$). * $P < 0.02$, ** $P < 0.01$ vs 30 mmol/l glucose.

500 nmol/l DHEA in high glucose medium restored GSH levels to control values ($0.47 \pm 0.05 \mu\text{g}/10^6$ cells).

The integrity of mesangial cell membrane, evaluated by determination of Na/K ATPase activity, was also markedly reduced in 30 mmol/l glucose ($0.53 \pm 0.09 \mu\text{mol}$ inorganic phosphorus (Pi)/mg prot./1 h) versus the control condition ($1.19 \pm 0.18 \mu\text{mol}$ Pi/mg prot./1 h) ($P < 0.01$, Table 1). The presence of 500 nmol/l DHEA protected Na/K ATPase activity ($0.79 \pm 0.11 \mu\text{mol}$ Pi/mg prot./1 h) against the effect of high glucose ($P < 0.05$ vs 30 mmol/l glucose; Table 1).

Discussion

Our data show that, *in vitro*, DHEA protects rat mesangial cells against injury caused by exposure to high glucose concentrations, reducing oxidative stress and preserving cell growth. This effect resembles that exerted by DHEA on bovine retinal capillary pericytes (Brignardello *et al.* 1998). Moreover, it occurs at the same concentrations of

the steroid, which are similar to those found in human tissues and not too different from that of human plasma (Van Landeghem *et al.* 1985, Brignardello *et al.* 1990). Indeed, a number of similarities exist between diabetic retinopathy and nephropathy: the loss of smooth muscle-like cells (i.e. pericytes in the retina and mesangial cells in the glomerulus) has been reported in both retinopathy and nephropathy (Lorenzi & Cagliero 1991, Saito *et al.* 1988). Furthermore, mesangiolysis in diabetic glomeruli appears to play a role in the formation of microaneurisms (Saito *et al.* 1988).

Mesangial cells are highly susceptible to glucose toxicity and the inhibition of their proliferation induced by high glucose concentrations has been well documented (Nahman *et al.* 1992, Trachtman *et al.* 1994, Yao & Li 1994, Cosio 1995). In mammals, most types of cells down-regulate their glucose transporters' expression after exposure to high glucose concentrations (Kahn 1992). In contrast, mesangial cells increase the glucose uptake via the heightened expression of the glucose transporter GLUT1 (Heilig *et al.* 1997), which is the only GLUT

Table 1 GSH content and cell membrane Na/K ATPase activity in rat mesangial cells (mean \pm s.d.)

	5.6 mmol/l glucose	30 mmol/l glucose	30 mmol/l glucose + 500 mmol/l DHEA
Reduced glutathione ($\mu\text{g}/10^6$ cells)	0.49 ± 0.04 ($n=3$)	$0.33 \pm 0.03^*$ ($n=3$)	0.47 ± 0.05 ($n=3$)
Na/K ATPase (mmol Pi/mg prot./1 h)	1.19 ± 0.18 ($n=3$)	0.53 ± 0.09 ($n=3$)	$0.79 \pm 0.11^\dagger$ ($n=3$)

* $P < 0.01$ vs control; $^\dagger P < 0.05$ vs 30 mmol/l glucose.

isoform expressed in cultured mesangial cells (Heilig *et al.* 1997) and is not regulated by insulin.

Glucose-induced oxidative damage has been suggested to play a role in the development of diabetic nephropathy (Shah 1989, Larkins & Dunlop 1992, Trachtman *et al.* 1992, Ha & Kim 1995). Hyperglycaemia not only generates more oxygen free radicals, it also attenuates antioxidative mechanisms through glycation of scavenger enzymes (Ha & Kim 1995). The resulting imbalance between oxygen free radical production and cellular defence mechanisms could cause membrane lipid peroxidation and cell damage. Here we show that in high glucose concentrations DHEA reduces lipid peroxidation and preserves both the cellular content of GSH and the membrane Na^+/K^+ ATPase activity, suggesting that its protective effect on mesangial cell growth might be due, at least in part, to its antioxidant properties. These results agree with previous reports showing that DHEA possesses antioxidant activity (Aragno *et al.* 1997, Boccuzzi *et al.* 1997, Aragno *et al.* 1999) and that antioxidants reverse the antiproliferative effect of high glucose on cultured mesangial cells (Trachtman 1994, Trachtman *et al.* 1994).

Apart from oxidative stress, other mechanisms have been hypothesized to explain the glucose-induced inhibition of mesangial cell growth. Some evidence suggests a role for the glucose-induced increase of protein kinase C levels (Williams 1989, Cosio 1995). A mechanism involving transforming growth factor- β (TGF- β), whose production is increased by high glucose, has also been hypothesized (Yao & Li 1994, Del Prete *et al.* 1998). However, it is unlikely that the protective effect of DHEA on glucose toxicity in mesangial cells is linked to these mechanisms, since DHEA itself has been shown to act as a positive stimulator for both TGF- β and protein kinase C production in human and animal experimental models (Bodine *et al.* 1995, Wu *et al.* 1997, Ishizuka *et al.* 1999).

The mechanism underlying the protective effect of DHEA against oxygen free radical damage is still undefined and might implicate DHEA metabolism. Experiments performed by adding DHEA directly to either isolated microsomes or lipoproteins failed to exert any protective effect, throwing doubt on whether DHEA itself acts as a direct antioxidant (Boccuzzi *et al.* 1997). In rats, DHEA given i.p. 3 h before dextrose administration protects tissues against lipid peroxidation induced by hyperglycaemia, whereas a 1 h pre-treatment is ineffective (Aragno *et al.* 1997). Thus, DHEA does appear to need a 'lag-phase' before becoming active, and it could be speculated that during this time it is converted into an active metabolite. Indeed, most effects of DHEA are mediated by one or other of its metabolites, such as 5-en-androsten-3 β ,17 β -diol for the hormonal effect (Boccuzzi *et al.* 1994) or 5-en-androsten-3 β ,7 β ,17 β -triol for the action on the immune system (Padgett & Loria 1994). The active compound (i.e. DHEA or one of its metabolites) might modify the structure or lipid composition of the cell

membrane. A similar mechanism has been reported for another steroid hormone, i.e. oestradiol, which induces stable modifications of lipoproteins making them more resistant to attack by free radicals (Shwaery *et al.* 1997). Indeed, DHEA protects plasma low density lipoprotein (LDL) against lipid peroxidation (Boccuzzi *et al.* 1997, Khalil *et al.* 1998) and is able to change the fatty acid composition of the phospholipid membrane of mitochondria in rats (Mohan & Cleary 1991).

Whatever the mechanism, these results further confirm the multi-targeted antioxidant properties of DHEA (Aragno *et al.* 1997, Brignardello *et al.* 1998, Aragno *et al.* 1999) and, together with our previous study on bovine retinal capillary pericytes (Brignardello *et al.* 1998), focus on the possible effectiveness of DHEA in the prevention of chronic complications of diabetes.

Moreover, since the DHEA(S) plasma level is a highly specific individual marker (Thomas *et al.* 1994) with a wide variability within the normal population (Orentreich *et al.* 1992), a relationship between DHEA plasma levels and individual susceptibility to developing diabetic nephropathy might be hypothesized.

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