Effect of treatment of diabetic rats with Dehydroepiandrosterone (DHEA) on vascular and neural function.

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Running title: DHEA treatment of diabetic rats

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Abstract

Nutritional supplementation with DHEA may be a candidate for treating diabetes-induced vascular and neural dysfunction. DHEA is a naturally occurring adrenal androgen that has antioxidant properties and is reportedly reduced in diabetes. Using a prevention protocol we found that dietary supplementation of streptozotocin-induced diabetic rats with 0.1, 0.25 or 0.5% DHEA caused a concentration-dependent prevention in the development of motor nerve conduction velocity and endoneurial blood flow impairment, which are decreased in diabetes. At 0.25%, DHEA significantly prevented the diabetes-induced increase in serum thiobarbituric acid reactive substances and sciatic nerve conjugated diene levels. This treatment also reduced the production of superoxide by epineurial arterioles of the sciatic nerve. DHEA treatment (0.25%) significantly improved vascular relaxation mediated by acetylcholine in epineurial vessels of diabetic rats. Sciatic nerve Na\(^+\)/K\(^+\) ATPase activity and myo-inositol content was also improved by DHEA treatment, whereas sorbitol and fructose content remained elevated. These studies suggest that DHEA, by preventing oxidative stress and perhaps improving sciatic nerve Na\(^+\)/K\(^+\) ATPase activity, may improve vascular and neural dysfunction in diabetes.
Introduction

Diabetic neuropathy is a multifactorial problem with a unique etiology. It has been described by some investigators to be a disease of the vasculature leading to nerve ischemia and altered nerve function (9,11,12,14,43,45,56,59). Other investigators have proposed that diabetic neuropathy is caused by metabolic defects associated with an increased flux of glucose through the aldose reductase pathway and depletion of intracellular myo-inositol levels leading to a defect in Na⁺/K⁺ ATPase activity and an alteration of signal transduction pathways in the nerve (26,31,36). Our studies suggest that vascular dysfunction of epineurial vessels and reduction in endoneurial blood flow (EBF) is responsible for the early stages of diabetic neuropathy (17). In addition, we have shown that oxidative stress, likely due to the generation of superoxide, causes vascular dysfunction in epineurial arterioles and accompanies the reduction in EBF, and these abnormalities precede the slowing of motor nerve conduction velocity (MNCV) (18,19).

Because of the multiple abnormalities contributing to the etiology of diabetic neuropathy it is impractical to attempt to treat this disorder by correcting each of these individually. Therefore, investigators are faced with the problem of determining the vascular or metabolic defects that have the greatest impact on the development of diabetic neuropathy and then design strategies to treat the more relevant defects. In this regard, we have found that treating diabetic rats with antioxidants corrects the vascular defects in epineurial vessels and improves neural function (18,19). We would now like to identify a safe and efficacious antioxidant therapy that will correct the vascular and neural dysfunctions induced by diabetes. One disadvantage of current antioxidant therapies is that a large concentration of antioxidant is usually required in order to achieve a significant benefit, which may result in undesired side effects. In addition, the method of delivery of some antioxidants is impractical for therapeutic use in patients (10).
However, dehydroepiandrosterone (DHEA) may be a compound that will circumvent these problems. DHEA is a naturally occurring adrenal steroid that can be provided as a nutritional supplement. Moreover, DHEA has been shown to have antioxidant properties and is reported by some investigators to be decreased in diabetes and aging (4,5,7,41,42,52). In these studies we demonstrate that treating diabetic rats with 0.25% DHEA in the diet for 4-5 weeks blocks the diabetes-induced increase in superoxide production in epineurial vessels and prevents the development of vascular and neural dysfunction.
Materials and/or Methods

**Materials:** Unless stated otherwise all chemicals used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO).

**Animals:** Male Sprague-Dawley (Harlan Sprague Dawley, Indianapolis, IN) rats 8-9 weeks of age were used for these studies. The animals were housed in a certified animal care facility and food (Harlan Teklad, #7001 (meal form), Madison, WI) and water were provided ad libitum. All institutional and NIH guidelines for use of animals were followed. Diabetes was induced by intravenously injecting streptozotocin (60 mg/kg in 0.9% NaCl, adjusted to a pH 4.0 with 0.2 M sodium citrate). Control rats were injected with vehicle alone. The rats were anesthetized with methoxyflurane before injection. Diabetes was verified 48h later by evaluating blood glucose levels with the use of glucose-oxidase reagent strips (Lifescan Inc., Milpitas, CA). Rats having blood glucose level of 300 mg dl⁻¹ (16.7 mM) or greater were considered to be diabetic. At this time the diabetic rats were randomly divided into four groups, three to receive 0.1%, 0.25% or 0.5% DHEA (5-androsten-3β-ol-17-one, dehydroepiandrosterone, Steraloids, Inc., Newport, RI) in the diet (based upon the average amount of food consumed per day by control and diabetic rats the amount of DHEA received from the three diets was 0.05, 0.125, and 0.25 g/day/kg rat and 0.075, 0.1875 and 0.375 g/day/kg rat for control and diabetic rats, respectively), the fourth group received an unmodified diet. Control rats were also fed an unmodified diet. Additional control rats were fed a diet containing 0.25 or 0.5% DHEA. Dietary treatments with DHEA were started on the day hyperglycemia was verified. All studies were conducted approximately 4-5 weeks after the verification of diabetes.
Motor nerve conduction velocity: MNCV was determined as previously described using a noninvasive procedure in the sciatic-posterior tibial conducting system in a temperature controlled environment (14,15,16,50).

Endoneurial blood flow: Immediately after determination of MNCV, sciatic nerve endoneurial nutritive blood flow was determined using the hydrogen clearance method as described by Cameron et al. (11) and adapted by our laboratory (54). The hydrogen clearance data was fitted by computer to a mono- or bi-exponential curve using commercial software (Prism, GraphPad, San Diego, CA). Nutritive blood flow (ml min^{-1} 100g^{-1}) was calculated using the equation described by Young (62) and vascular conductance, (ml min^{-1} 100g^{-1} mm Hg^{-1}) determined by dividing nutritive blood flow by the average mean arterial blood pressure.

Vascular reactivity: Videomicroscopy was used to investigate in vitro vasodilatory responsiveness of epineurial arterioles supplying the region of the sciatic nerve (branches of the superior gluteal and internal pudendal arteries) to acetylcholine (10^{-4} to 10^{-8} mol l^{-1}) or sodium nitroprusside (10^{-4} mol l^{-1}) as previously described (54). In separate studies the direct effect of DHEA (10^{-6} to 10^{-10} mol l^{-1}) and 17β-estradiol (10^{-6} to 10^{-10} mol l^{-1}) on vasodilation in vitro was examined using videomicroscopy. DHEA or 17β-estradiol were dissolved in ethanol and diluted in Krebs Henseleit physiological saline solution (54).

Detection of superoxide: Hydroethidine (Molecular Probes Inc., Eugene, OR), an oxidative fluorescent dye, was used to evaluate in situ levels of superoxide (O2^{-}) in epineurial vessels as described previously (17,18,19). Hydroethidine is permeable to cells and in the presence of O2^{-} is oxidized to fluorescent ethidium bromide, where it is trapped by intercalating with DNA. This method provides sensitive detection of O2^{-} in situ. Superoxide levels were also
measured in the aorta by lucigenin-enhanced chemiluminescence as described previously (17,18,19,38).

*Sciatic nerve Na\(^+\)/K\(^+\) ATPase activity and sorbitol, fructose and myo-inositol content:* The left sciatic nerve was removed, desheathed, and divided into four samples for determination of Na\(^+\)/K\(^+\) ATPase activity, conjugated diene level, glutathione level and sorbitol, fructose and myo-inositol content as previously described (17,18,19).

*Additional biological parameters:* Sciatic nerve glutathione (GSH), serum TBARS and sciatic nerve conjugated diene levels were determined as additional markers of oxidative stress. Sciatic nerve glutathione levels were determined according to Lou et al. (34). Tissue was weighed and homogenized in 1 ml of cold 10% trichloroacetic acid and centrifuged for 15 min at 1000g. The supernatant (100 µl) was mixed with 0.89 ml of 1.0 M Tris, pH 8.2 and 0.02 M EDTA. Afterwards, 10 µl of dithionitrobenzene (DTNB) was added and change in absorbance measured at 412 nm. A glutathione standard curve (100-500 ng) was performed for each assay. The data were recorded as µg mg\(^{-1}\) wet weight. TBARS level in serum was determined by the method of Mihara et al. (37) as modified by Siman and Eriksson (51). Briefly, 200 µl of serum was boiled in 0.75 ml of phosphoric acid (0.19 M), 0.25 ml thiobarbituric acid (0.42 mM) and 0.3 ml water for 60 min. Afterwards, the samples were precipitated with methanol/NaOH and centrifuged for 5 min. The supernatant was measured fluorometrically at excitation wavelength 532 nm and emission wavelength 553 nm. Standards were prepared by the acid hydrolysis of 1,1,3,3-tetraethoxypropane. The data were reported as µg ml\(^{-1}\) serum. Sciatic nerve conjugated diene levels were determined according to the method of Recknagel and Ghoshal (46) and Low and Nickander (34). Briefly, a segment of the sciatic nerve was extracted with chloroform and methanol. The lipid extract was evaporated and redissolved in 1 ml cyclohexane. Conjugated
diene levels were determined by measuring the absorbance at 233 nm with extraction blanks used as references. An extinction coefficient of 2.52 x 10⁴ M was used to determine the amount of conjugated diene present. The data was reported as µmol mg⁻¹ wet weight. Serum free fatty acid and triglyceride levels were determined using commercial kits from Roche Diagnostics, Mannheim, Germany and Sigma Chemical Co., St. Louis, MO, respectively.

Determination of Serum DHEA and Estrogen: Assays for serum level of DHEA and estrogen were performed using RIA assay kits from Diagnostic Systems Laboratories, Inc., Webster, TX according to the manufactures instructions.

Data Analysis: The results are presented as mean ± SE. Comparisons between the groups for MNCV, EBF, sciatic nerve Na⁺/K⁺ ATPase activity, sciatic nerve sorbitol, fructose and myo-inositol content, serum TBARS, sciatic nerve conjugated diene, serum free fatty acid and triglyceride level, and lens and sciatic nerve glutathione levels were conducted (following data transformation one-way ANOVA analysis was applied with Newman-Keuls test for multiple comparisons (Prism software, GraphPad, San Diego, CA). Dose response curves for acetylcholine-induced relaxation were compared using a two way repeated measures analysis of variance with autoregressive covariance structure using proc mixed program of SAS (17,18,19,54). Whenever significant interactions were noted specific treatment-dose-effects were analyzed using a Bonferroni adjustment. A p value of less 0.05 was considered significant.
Results

Change in body weight, blood glucose level and serum DHEA and estrogen level. Data in Table 1 show the change in body weight for control and diabetic rats treated with or without DHEA for the period of the study. At the time of experimentation blood glucose was significantly increased in diabetic rats treated with or without DHEA. There was no difference in blood glucose level between diabetic rats and diabetic rats treated with DHEA. Data in Figure 1 demonstrate that feeding diabetic rats 0.1-0.5% DHEA caused a concentration-dependent increase in DHEA level in the serum. At 0.25% and 0.5% the increase in DHEA in the serum was significant compared to control rats. The level of estrogen in the serum was also increased but the change from control rats was not significant. Basal serum DHEA and estrogen level in control and non-treated diabetic rats were similar. Measurement of serum free fatty acid and triglyceride levels revealed that treating diabetic rats with DHEA does not change the diabetes-induced increase in free fatty acid or triglyceride level (data not shown).

Sciatic nerve Na\(^+\)/K\(^+\) ATPase activity and sorbitol, fructose and myo-inositol content. Data in Table 2 demonstrate that diabetes caused a 30% decrease in sciatic nerve Na\(^+\)/K\(^+\) ATPase activity. Treating diabetic rats with 0.25% or 0.5% DHEA caused a significant increase in Na\(^+\)/K\(^+\) ATPase activity compared to control or untreated diabetic rats. Data in Table 2 also demonstrate that diabetes caused a significant increase in the fructose content and a decrease in myo-inositol level in the sciatic nerve. Treating diabetic rats with 0.25% or 0.5% DHEA prevented the decrease in myo-inositol the content in the sciatic nerve.

Evaluation of oxidative stress. Data in Table 3 demonstrate that glutathione level in the sciatic nerve was decreased by diabetes but this difference was not statistically significant. Treating diabetic rats with 0.25% or 0.5% DHEA tended to prevent the decrease in sciatic nerve
glutathione level. Diabetes caused a significant increase in sciatic nerve conjugated diene level, which was prevented by treating diabetic rats with 0.25% or 0.5% DHEA. Diabetes also caused a significant increase in serum thiobarbituric acid reactive substances (TBARS), which was prevented by treating diabetic rats with 0.25% DHEA.

Data in Figure 2 demonstrate that treating streptozotocin-induced diabetic rats with 0.25% DHEA markedly decreased the diabetes-induced increase in the level of superoxide in epineurial vessels as measured by hydroethidine fluorescence compared to paired analysis of untreated diabetic rats in two separate studies. We also measured the superoxide level in the aorta by lucigenin-enhanced chemiluminescence. These studies demonstrated that the superoxide level is increased in the aorta of diabetic rats by about 50% compared to control rats (2.43 ± 0.14 vs. 1.64 ± 0.13 relative light units (RLU) min⁻¹ mm⁻¹, respectively, n = 6 for both sets of animals) and treating diabetic rats with 0.25% DHEA decreased the diabetes-induced increase of superoxide (1.97 ± 0.09 RLU min⁻¹ mm⁻¹, n = 11). These changes were not statistically significant although the trend was similar to the data reported in Figure 2 for superoxide formation by epineurial arterioles of the sciatic nerve. Treating diabetic rats with 0.1% or 0.5% DHEA was less effective than treatment with 0.25% DHEA in preventing superoxide production by the aorta (2.07 ± 0.29 and 2.31 ± 0.38 RLU min⁻¹ mm⁻¹, respectively, n = 6 for both sets of animals) or epineurial arterioles of the sciatic nerve (Figure 2).

*Endoneurial blood flow and motor nerve conduction velocity.* Data in Figure 3 demonstrate that treating diabetic rats with 0.25% or 0.5% DHEA prevented the decrease in EBF compared to untreated diabetic rats. Data in Figure 4 demonstrate that treating diabetic rats with 0.25% DHEA significantly prevented the development of the diabetes-induced slowing in MNCV.
Arteriolar vascular reactivity. As demonstrated in Figure 5, diabetes caused a significant decrease (p < 0.05) in acetylcholine (10⁻⁴ to 10⁻⁸ mol l⁻¹) mediated vascular relaxation in epineurial arterioles of the sciatic nerve. Treating diabetic rats with 0.25% DHEA significantly prevented the development of the diabetes-induced impairment in acetylcholine mediated vascular relaxation (p < 0.05). In contrast, treating diabetic rats with 0.1% or 0.5% DHEA was less effective. Treating control rats with 0.25% or 0.5% DHEA had no effect on acetylcholine-mediated vasodilation (data not shown). Maximal endothelium-independent vasodilation induced by sodium nitroprusside (10⁻⁴ M) was not affected by diabetes or treatment of diabetic rats with 0.25% DHEA (100.1 ± 3.3, 90.0 ± 4.8 and 91.1 ± 2.8 in control (n = 16), untreated diabetic (n = 14) and diabetic rats treated with 0.25% DHEA (n = 36), respectively. Baseline diameters of the vessels used in these studies were not different for control, untreated diabetic or diabetic rats treated with DHEA (data not shown).

Since DHEA is a precursor for the synthesis of estradiol we were interested in determining whether DHEA or 17β-estradiol alone can cause vasodilation in vitro in epineurial arterioles of the sciatic nerve. At 10⁻⁶ M concentration 17β-Estradiol or DHEA caused only a 9.9 ± 6.5% and 19.8 ± 7.3% vasodilation, respectively (n = 6).
Discussion

Previously we had demonstrated that the diabetes induced decrease in EBF and impairment of acetylcholine-induced vasodilation of epineurial arterioles of the sciatic nerve, precedes slowing of motor nerve conduction and decrease in Na\(^+\)/K\(^+\) ATPase activity in the sciatic nerve (54). In addition we showed that the generation of superoxide in epineurial arterioles of the sciatic nerve accompanies the diabetes-induced impairment in vasodilation (17). In these studies we have demonstrated that treating streptozotocin-induced diabetic rats with 0.25% DHEA partially prevented the diabetes-induced production of superoxide in epineurial vessels of the sciatic nerve, slowing of MNCV, reduction in EBF, and impairment of acetylcholine-mediated vascular relaxation in epineurial arterioles of the sciatic nerve.

DHEA and its sulfated metabolite, DHEAS, are the most abundant circulating adrenal steroids in humans. Plasma concentrations of DHEA peak in the third decade and fall gradually thereafter to levels of 20% of peak by the eighth decade (58). Circulating levels of DHEA are significantly lower in rodents than humans (61). In these studies, feeding rats a diet containing 0.25% DHEA resulted in a large increase in DHEA in serum (~ 300 nM). Baseline level of DHEA in humans is about 10 nM (57). However, when taking DHEA supplements ranging from 50-2250 mg/day the DHEA level in serum has been shown to increase to as high as 230 nM (54). Even though the amount of DHEA required in the diet of diabetic rats was high in order to achieve an effect on vascular and neural function, the amount of DHEA in the serum of diabetic rats fed a diet containing 0.25% DHEA is achievable in humans when taking oral supplements (57). Apart from its role as a precursor to androgens and estrogens, other biological effects of DHEA have been described (21). DHEA has well characterized effects on satiety, obesity and diabetes in rodents (47). It may also affect satiety, obesity and cardiovascular disease in humans,
though these reports remain controversial and the mechanisms of these effects remain unclear (6,13,30). The reciprocal relationship of plasma DHEA concentration in age-related disorders and diabetes mellitus, has prompted exploration of a possible role for DHEA in these disorders. In this regard, it has been shown that DHEA and its metabolites can prevent/reduce hyperglycemia in the ZDF/Gmi-fa/fa rat and C57BL/KsJ-db/db mouse (8,15,16). In these studies treatment with DHEA and/or metabolites began before the onset of hyperglycemia and in the db/db mouse was proposed to maintain islet integrity (8,15,16). Moreover, Dillon et al., have demonstrated that DHEAS acts on β cells to increase glucose stimulated insulin secretion through a transcriptional mechanism (20). However, in streptozotocin-induced diabetic DHEA treatment did not cause a decrease in plasma glucose levels (2). In the latter study as well as ours treatment with DHEA was not initiated until after the onset of hyperglycemia (2). In contrast, in the studies using the ZDF rats and db/db mouse, genetic diabetic animal models, DHEA treatment was begun prior to the development of hyperglycemia and β cell dysfunction (8,15,16). Therefore, it is unlikely that in the streptozotocin-induced diabetic rat, which is a model of β cell injury and death, that DHEA is capable of improving hyperglycemia by modifying glucose uptake.

One possible mechanism for the action of DHEA is through its conversion to estradiol. This is supported by studies of hypertensive rabbits treated with 0.3% DHEA in the diet. About 50% of the DHEA effect in reducing atherosclerosis in this animal model was due to the increased production of estradiol (29). Estrogens have a wide array of biological effects, targeting both genomic and nongenomic mechanisms (39). Recent studies have shown that estrogen can increase endothelial nitric oxide synthase (eNOS) expression and/or activity leading to increased vascular relaxation (22,48,53,55,63). We have recently shown that DHEA can also
stimulate eNOS activity in vascular endothelial cells by a specific G protein coupled plasma membrane receptor (33). This effect of DHEA on endothelial cell nitric oxide production was independent of the action of estrogen. In the present studies, feeding rats a diet containing 0.1% to 0.5% DHEA caused a concentration-dependent increase in estrogen in circulation. However, the increase in estrogen level in the serum was not statistically significant compared to control rats. It is unlikely that the effect of DHEA in improving diabetes-induced impairment in vascular relaxation were due to an increase in estrogen levels. We demonstrated that estrogen, at a concentration similar to estrogen levels found in rats fed 0.25% DHEA (~0.5 nM), did not induce vasodilation in epineurial vessels in vitro. 17β-Estradiol or DHEA at 1 nM caused only a 2% or 11% vasodilation, respectively. In another study estrogen has been shown to down-regulate mRNA expression of Na⁺/K⁺ ATPase α1 subunit in renal tissue (32). This finding is inconsistent with our studies and those by Aragno et al. (2), which demonstrated that DHEA treatment of diabetic rats increased Na⁺/K⁺ ATPase activity in the sciatic nerve and renal tissue, respectively. These studies argue against a role for increased estrogen production from DHEA in the regulation of Na⁺/K⁺ ATPase activity in these studies. However, we cannot rule out a role for chronic increased levels of estrogen in DHEA fed rats on vascular and neural function. Additional studies will be necessary using an aromatase inhibitor such as fadrozole and perhaps estradiol supplementation to address this issue (24).

DHEA has been shown to have antioxidant properties (4,5,7,41,42,52). Our previous studies have demonstrated that increased oxidative stress contributes to neural dysfunction in diabetes (18,19). In streptozotocin-treated diabetic rats, treatment with DHEA was shown to reduce serum and tissue thiobarbituric acid reactive substances and fluorescent chromolipids (5). DHEA treatment also restored glutathione levels in the liver and kidney as well as the enzymatic
activity of catalase, superoxide dismutase and glutathione peroxidase (5). DHEA has been demonstrated to prevent damage induced by acute and chronic hyperglycemia and restore the redox state in ganglion synaptosomes isolated from diabetic rats (1,3,4,5). In a recent study, Aragno et al. reported that treatment of streptozotocin-induced diabetic rats with DHEA might delay the progression of diabetic kidney disease by reducing the inflammatory response to oxidative stress (2). In the present studies, 0.25% DHEA treatment of diabetic rats clearly prevented oxidative stress. Compared to our previous studies, 0.25% DHEA treatment was almost as effective as α-lipoic acid in preventing superoxide formation in vascular tissue and other markers of oxidative stress in streptozotocin-induced diabetic rats, as well as preventing impairment in EBF, vascular function and MNCV (18). Potential sources for increased superoxide production in diabetes include the mitochondria, NAD(P)H oxidase, xanthine oxidase as well as nitric oxide synthase. Schwartz and Pashko have demonstrated that administering DHEA to laboratory mice and rats inhibits development of experimental tumors (49,50). They suggested that the antiproliferative and tumor preventive effects of DHEA were due to inhibition of glucose-6-phosphate dehydrogenase and the pentose phosphate pathway. This pathway is an important source of NADPH, a critical reductant for many biochemical reactions that generate oxygen free radicals (49,50). Therefore, one possible mechanism for the DHEA-mediated decrease in oxidative stress is by reducing NADPH levels, and thus generation of superoxide by NAD(P)H oxidase.

Another mechanism by which DHEA treatment may be improving neural function is through preventing the decrease in sciatic nerve Na⁺/K⁺ ATPase activity and myo-inositol content. Greene and co-investigators have demonstrated that the diabetes-induced decrease in sciatic nerve Na⁺/K⁺ ATPase activity and the reciprocal increase in sorbitol levels and decrease
in myo-inositol content are linked to nerve dysfunction (24,27,31). It should be noted that in contrast to previous studies the diabetes-induced decrease in Na\(^+\)/K\(^+\) ATPase activity in these studies did not reach statistical significance. Nonetheless, our studies demonstrated that 0.25% and 0.5% DHEA treatment of diabetic rats prevented the 30% diabetes-induced decrease in sciatic nerve Na\(^+\)/K\(^+\) ATPase activity and myo-inositol content. DHEA treatment was more effective than α-lipoic acid, and as effective as treatment with aldose reductase inhibitors, in preventing the decrease in sciatic nerve Na\(^+\)/K\(^+\) ATPase activity (18,23,25). Aragno et al. have demonstrated that treating diabetic rats with DHEA significantly prevented the diabetes-induced decrease in renal Na\(^+\)/K\(^+\) ATPase activity (2). These investigators concluded that the reduction in 5- and 15-hydroperoxy-eicosatetraenoic (HPETE) acid and 4-hydroxynonenal levels in the kidney of diabetic rats treated with DHEA contributed to the improvement of Na\(^+\)/K\(^+\) ATPase activity. Both 5- and 15-HPETE are potent inhibitors of Na\(^+\)/K\(^+\) ATPase activity (2). In our studies 0.25% and 0.5% DHEA treatment of diabetic rats resulted in an increase in sciatic nerve Na\(^+\)/K\(^+\) ATPase activity. The mechanism responsible for this effect of DHEA is not clear. It is unlikely to be due to preventing the redox imbalance induced by diabetes, since sciatic nerve sorbitol and fructose levels are still elevated in DHEA-treated diabetic rats. It is possible that improvement in oxidative stress caused by DHEA treatment or an increase in expression of Na\(^+\)/K\(^+\) ATPase in diabetic rats could have contributed to an increase in Na\(^+\)/K\(^+\) ATPase activity. Liu and Dillon (33) have demonstrated, using cultured bovine aorta endothelial cells, that DHEA through binding to a G-protein coupled receptor activates mitogen-activated protein kinase and tyrosine kinase activity. It has been shown by several laboratories that activation of tyrosine kinase and/or mitogen-activated protein kinase pathway increases Na\(^+\)/K\(^+\) ATPase activity likely by increasing protein expression (28,40,44). Therefore, activation of these kinases may be
responsible for the increase in Na⁺/K⁺ ATPase activity in sciatic nerve of diabetic rats treated with DHEA.

Treating diabetic rats with 0.1% DHEA generally had little effect in preventing oxidative stress and vascular and neural dysfunction, whereas treatment with 0.5% DHEA, with exception to its effect on endoneurial blood flow and sciatic nerve Na⁺/K⁺ ATPase activity, appeared to exacerbate oxidative stress and some of the diabetes-induced vascular and neural defects compared to 0.25% DHEA treatment suggesting that, at higher doses, DHEA may be pathogenic. As demonstrated in Table 1, diabetic rats fed a diet containing 0.5% DHEA had a tendency to lose more weight than diabetic rats fed a diet containing 0.25% DHEA. However, treating non-diabetic rats with 0.25% or 0.5% DHEA appeared to have no adverse effects on vascular or neural function or metabolic measurements (data not shown). We do not have a straightforward explanation why treatment of diabetic rats with 0.5% DHEA appeared to be beneficial in some aspects but less so in regard to other vascular and neural functions. Clearly 0.5% DHEA improved sciatic nerve endoneurial blood flow and Na⁺/K⁺ ATPase activity. In contrast, vascular dysfunction, motor nerve conduction velocity and markers of oxidative stress were not improved to the same extent with 0.5% DHEA as seen with dietary supplementation of diabetic rats with 0.25% DHEA. One possible interpretation is that at higher doses DHEA, or perhaps one of its metabolites, may have direct deleterious effects on neural and/or vascular function. Some of the ambiguity may also be due to the increased formation of estrogen that occurs with supplementation of the diet with 0.5% DHEA. It is possible that estrogen may be improving endoneurial blood flow by affecting vascular function of the endoneurial blood vessels in the sciatic nerve. Another possible explanation for the increase in endoneurial blood flow in diabetic rats fed a diet containing 0.5% DHEA, is that DHEA treatment could be causing an increase in
NO production by the endothelium leading to improved vascular relaxation. This is supported by studies by Liu and Dillon (33) that have demonstrated that DHEA activates endothelial cell nitric oxide synthase by a specific plasma membrane receptor coupled to G-proteins in cultured bovine aorta endothelial cells. Nonetheless, our studies suggest that the proper dosage of DHEA is important for treatment of diabetes-induced vascular and neural dysfunction. This may be one reason for some of the inconsistent results obtained with DHEA treatment in some studies (60).

In summary, the beneficial effects of DHEA we observed on vascular and neural function are dose dependent. The administration of 0.25% DHEA in the diet of diabetic rats, likely through reducing oxidative stress, prevented vascular and neural dysfunction, suggesting that further study of the potential benefits of DHEA in preventing diabetic complications is warranted.
Acknowledgments

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References


31. **Kim J, Kyriazi H, and Greene DA.** Normalization of Na\(^+\) -K\(^+\) -ATPase activity in isolated membrane fraction from sciatic nerves of streptozotocin-induced diabetic rats by


Figure Legends

Figure 1: Determination of DHEA and estrogen levels in serum from control, diabetic and diabetic rats treated with 0.1%, 0.25% or 0.5% DHEA. The number of animals in each group is indicated in Table 1. The * denotes a significant difference (p < 0.05) compared to control rats, + denotes a significant difference (p < 0.05) compared to untreated diabetic rats.

Figure 2: Detection of superoxide level in arterioles from control, diabetic rats and diabetic rats treated with 0.1%, 0.25% or 0.5% DHEA. The duration of diabetes and treatments for these studies was 4-5 weeks. Fluorescent photomicrographs of confocal microscopic sections of epineurial arterioles of the sciatic nerve from the two different sets of animals were examined on different days. Arterioles were labeled with the hydroethidium. Recordings of fluorescence were taken at identical laser and photomultiplier settings for both control and untreated and DHEA-treated diabetic rats.

Figure 3: Determination of endoneurial blood flow from control rats, diabetic rats and diabetic rats treated with 0.1, 0.25 and 0.5% DHEA. Endoneurial blood flow, reported as nutritive flow or vascular conductance was determined for the same number of rats described in Table 1. Data is presented as the mean ± SEM. The * denotes a significant difference (p < 0.05) compared to control rats, + denotes a significant difference (p < 0.05) compared to untreated diabetic rats.

Figure 4: Determination of motor nerve conduction velocity from control rats, diabetic rats and diabetic rats treated with 0.1, 0.25 and 0.5% DHEA. Motor nerve conduction velocity was
determined for the same number of rats described in Table 1. Data is presented as the mean ± SEM. The * denotes a significant difference (p < 0.05) compared to control rats, + denotes a significant difference (p < 0.05) compared to untreated diabetic rats.

Figure 5: Determination of the effect of treatment of diabetic rats with 0.1%, 0.25% or 0.5% DHEA on acetylcholine-mediated vascular relaxation in epineurial arterioles of the sciatic nerve. Pressurized arterioles were constricted with U46619 (30-50%) and incremental doses of acetylcholine were added to the bathing solution while recording steady state vessel diameter. The number of experimental animals used in these studies was the same as noted in Table 1. The * denotes that the response to acetylcholine was significantly attenuated (p < 0.05) in diabetic rats. The + denotes that the response to acetylcholine was significantly different (p < 0.05) compared to untreated diabetic rats.
TABLE 1
Change in Body Weight and Blood Glucose Levels

<table>
<thead>
<tr>
<th>Animal</th>
<th>Change in Body Weight (g)</th>
<th>Blood glucose mg dl⁻¹</th>
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<tbody>
<tr>
<td>Control (n = 6)</td>
<td>153 ± 19</td>
<td>85 ± 2</td>
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<tr>
<td>Diabetic (n = 6)</td>
<td>31 ± 13*</td>
<td>457 ± 36*</td>
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<tr>
<td>Diabetic + 0.1% DHEA</td>
<td>-18 ± 11*</td>
<td>508 ± 20*</td>
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<td>(n = 6)</td>
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<tr>
<td>Diabetic + 0.25% DHEA</td>
<td>25 ± 14*</td>
<td>481 ± 17*</td>
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<tr>
<td>(n = 13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic + 0.5% DHEA</td>
<td>-36 ± 11*⁺Ψ</td>
<td>428 ± 26*</td>
</tr>
<tr>
<td>(n = 6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The average change in body weight over the 4-5 week experimental period and blood glucose measurements on the day of experimentation is shown. The data are means ± S.E.M. The number of rats used in each group is indicated in parentheses. The * denotes a significant difference (p < 0.05) compared to control rats, + denotes a significant difference (p < 0.05) compared to untreated diabetic rats, Ψ denotes a significant difference (p < 0.05) compared to diabetic + 0.25% DHEA treated rats.
TABLE 2

Effect of Treatment of Streptozotocin-induced Diabetic Rats with DHEA on Sciatic Nerve Na⁺/K⁺ ATPase activity and Sorbitol, Fructose and myo-Inositol Levels

<table>
<thead>
<tr>
<th>Animal</th>
<th>Na⁺/K⁺ ATPase activity (µmol ADP mg⁻¹ wet wt h⁻¹)</th>
<th>Intracellular content (nmol mg⁻¹ wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sorbitol</td>
</tr>
<tr>
<td>Control (n = 6)</td>
<td>311 ± 46</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Diabetic (n = 6)</td>
<td>212 ± 21</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Diabetic + 0.1% DHEA (n = 6)</td>
<td>237 ± 54</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Diabetic + 0.25% DHEA (n = 13)</td>
<td>466 ± 52</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Diabetic + 0.5% DHEA (n = 6)</td>
<td>582 ± 43</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

The data are means ± S.E.M. The * denotes a significant difference (p < 0.05) compared to control rats, + denotes a significant difference (p < 0.05) compared to untreated diabetic rats, Ψ denotes a significant difference (p < 0.05) compared to diabetic + 0.1% DHEA treated rats.
TABLE 3
Effect of Treatment of Streptozotocin-induced Diabetic Rats with DHEA on Sciatic Nerve Glutathione and Conjugated Diene Level, Lens Glutathione Level and Serum Thiobarbituric Acid Reactive Substances (TBARS)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Sciatic Nerve Glutathione (µg mg⁻¹ wet wt)</th>
<th>Sciatic Nerve Conjugated Dienes (µmol mg⁻¹ wet wt)</th>
<th>Serum TBARS (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>0.23 ± 0.04</td>
<td>2.7 ± 0.3</td>
<td>8.2 ± 1.7</td>
</tr>
<tr>
<td>Diabetic (n = 6)</td>
<td>0.10 ± 0.03</td>
<td>5.2 ± 0.3*</td>
<td>15.7 ± 1.6*</td>
</tr>
<tr>
<td>Diabetic + 0.1% DHEA (n = 6)</td>
<td>0.09 ± 0.01</td>
<td>6.4 ± 1.0*</td>
<td>11.6 ± 2.0</td>
</tr>
<tr>
<td>Diabetic + 0.25% DHEA (n = 13)</td>
<td>0.18 ± 0.03</td>
<td>3.9 ± 0.5</td>
<td>8.4 ± 0.8+</td>
</tr>
<tr>
<td>Diabetic + 0.5% DHEA (n = 6)</td>
<td>0.20 ± 0.03</td>
<td>3.2 ± 0.3</td>
<td>14.9 ± 1.4*</td>
</tr>
</tbody>
</table>

The data are means ± S.E.M. The * denotes a significant difference (p < 0.05) compared to control rats, + denotes a significant difference (p < 0.05) compared to untreated diabetic rats, Ψ denotes a significant difference (p < 0.05) compared to diabetic + 0.25% DHEA treated rats.
Figure 1
Figure 2
Control Diabetic 0.1% 0.25% 0.5%

Nutritive Blood Flow (ml/min/100g)

5 10 15 20 25 30 35

0.05 0.10 0.15 0.20 0.25 0.30

Conductance (ml/min/100g/mmHg)

Conditions

Diabetic + DHEA

Figure 3
Figure 4

Conditions

Control  Diabetic  0.1%  0.25%  0.5%  Diabetic + DHEA

Motor Nerve Conduction Velocity (m/sec)
Figure 5

Graph showing the effect of acetylcholine on relaxation in different groups: Control, Diabetic, Diabetic + 0.1% DHEA, Diabetic + 0.25% DHEA, and Diabetic + 0.5% DHEA. The x-axis represents acetylcholine concentration in [-log M], and the y-axis represents the percentage of relaxation.