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An aldose reductase inhibitor reverses early diabetes-induced changes in peripheral nerve function, metabolism, and antioxidative defense

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ABSTRACT

Aldose reductase inhibitors (ARIs) prevent peripheral nerve dysfunction and morphological abnormalities in diabetic animal models. However, some experimental intervention studies and clinical trials of ARIs on diabetic neuropathy appeared disappointing because of either 1) their inadequate design and, in particular, insufficient correction of the sorbitol pathway activity or 2) the inability to *reverse* established functional and metabolic deficits of diabetic neuropathy by AR inhibition in general. We evaluated whether diabetes-induced changes in nerve function, metabolism, and antioxidative defense are corrected by the dose of ARI (sorbinil, 65 mg/kg/d in the diet), resulting in complete inhibition of increased sorbitol pathway activity. The groups included control rats and streptozotocin-diabetic rats treated with/without ARI for 2 weeks after 4 weeks of untreated diabetes. ARI treatment corrected diabetes-induced nerve functional changes; that is, decrease in endoneurial nutritive blood flow, motor and sensory nerve conduction velocities, and metabolic abnormalities (i.e., mitochondrial and cytosolic NAD^+/NADH redox imbalances and energy deficiency). ARI restored nerve concentrations of two major non-enzymatic antioxidants, reduced glutathione (GSH) and ascorbate, and completely arrested diabetes-induced lipid peroxidation. In conclusion, treatment with *adequate* doses of ARIs (that is, doses that completely inhibit increased sorbitol pathway activity) is an effective approach for reversal of, at least, early diabetic neuropathy.

Key words: diabetic neuropathy • NAD^+/NADH ratio • nerve blood flow • nerve conduction • oxidative stress • reversal

Numerous findings from leading experimental groups demonstrate that diabetes-induced peripheral nerve conduction deficits (1–5), metabolic imbalances (6–8), neurotrophic changes (9–11), and morphological abnormalities (12–14), as well as indices of autonomic neuropathy (4, 15–17), are prevented by structurally diverse aldose reductase

inhibitors (ARIs), thus implicating increased activity of the sorbitol pathway of glucose metabolism in the pathogenesis of diabetic neuropathy (DN). However, the past clinical trials of ARIs in DN appeared disappointing (18, 19), and it is unclear whether this failure resulted from the problems with trials *per se*; that is, inadequate design, including insufficient correction of increased sorbitol pathway activity, the lack of acceptable systemic toleration of the agents tested, or the inability to *reverse* DN by AR inhibition in general. The latter concern is strengthened by growing evidence that diabetes-associated increase in AR activity initiates within weeks numerous aberrations in signal transduction and metabolic pathways; that is, oxidative stress (20), NAD(P)-redox imbalances (21), changes in phosphoinositide (6) and arachidonic acid (7) signaling, activation of protein kinase C (PKC, 22), and mitogen-activated protein kinases (23), which, in turn, contribute to complex pathological changes of DN.

Despite a clear need to address the reversibility of diabetes-induced nerve functional and metabolic changes with AR inhibition for potential future development of well-tolerated potent ARIs, less than 5% (1, 3, 11, 24–30) out of more than 300 studies of ARIs in experimental diabetic neuropathy used the intervention approach; that is, the treatment was introduced after development of functional and metabolic abnormalities of early DN. Most of these studies revealed the reversal of conduction deficits of early DN (1, 3, 24–29) by different ARIs. However, two groups (11, 30) failed to demonstrate any increase of motor nerve conduction velocity (MNCV) deficit, despite restoration of normal sorbitol pathway activity, with ARI treatment. One group (3, 28, 29) has reported that structurally diverse ARIs correct diabetes-induced decrease in endoneurial nutritive blood flow (NBF). The ability of ARIs to correct established metabolic abnormalities other than sorbitol accumulation, *myo*-inositol depletion, and decrease in Na, K-ATP-ase activity and to reverse oxidative injury in the diabetic peripheral nerve has never been assessed. The present study was designed to evaluate whether diabetes-induced neurovascular dysfunction, nerve conduction deficits, metabolic changes, and vulnerability to oxidative stress can be reversed by treatment with an adequate dose of ARI; that is, the dose that inhibits completely the increased sorbitol pathway activity.

MATERIALS AND METHODS

We performed experiments in accordance with regulations specified by the National Institutes of Health “Principles of Laboratory Animal Care, 1985 Revised Version” and the University of Michigan Protocol for Animal Studies.

Animals

Barrier-sustained, cesarean-delivered male Wistar rats (Charles River, Wilmington, MA), body weight 250–300 g, were fed a standard rat chow diet (ICN Biomedicals, Cleveland, OH) and had access to water *ad libitum*. We induced diabetes by a single intraperitoneal (i.p.) injection of streptozotocin (Upjohn, Kalamazoo, MI, 55 mg/kg body weight, i.p., in 0.2 ml of 10 mM citrate buffer, pH 5.5) to animals that had been fasted overnight. Blood samples for measurements of glucose were taken from the tail vein ~48 h after the streptozotocin injection and the day before the animals were killed. The rats with blood glucose $\geq$13.8 mM were considered diabetic. The experimental groups comprised control rats and diabetic rats treated with or without the ARI sorbinil (Pfizer, 65 mg$\bullet$ kg body weight$^{-1}\bullet$ day $^{-1}$, in the diet). The sorbinil-containing diet was made commercially (ICN Biomedical, Aurora, OH) based on the average food intake of 40 g

food/day for 250–300 g diabetic rat. The average food intake was calculated from the results of several studies performed in our laboratory. The treatment of diabetic rats started one month after streptozotocin injection. The duration of treatment was 2 wk.

Reagents

Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from Sigma Chemical (St. Louis, MO). Methanol (HPLC grade), perchloric acid, hydrochloric acid, and sodium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). Ethyl alcohol (200 proof dehydrated alcohol, U.S.P. punctilious) was purchased from Quantum Chemical Co. (Tiscola, IL). β-D-glucose, sorbitol, N.F., and D-fructose, U.S.P. were purchased from Pfanstiehl Laboratories, Inc. (Waukegan, IL). Kits for malondialdehyde and 4-hydroxyalkenals assay were purchased from Oxis International (Portland, OR).

Experimental procedure

We have previously reported (21, 31) that urethane anesthesia distorts the profile of peripheral nerve metabolites, whereas rat sedation by a short (~15–20 s) exposure to carbon dioxide with immediate cervical dislocation preserves reduced metabolite and high-energy phosphate levels in the range of those obtained after decapitation without any narcosis (21, 31). For this reason, two different sets of animals were used for functional and metabolic studies. In the first set, the rats were anesthetized by urethane (1–1.2 g/kg, i.p.). We measured sciatic motor nerve conduction velocity (MNCV) before assessing nerve blood flow (NBF) on the contralateral nerve. In all measurements, body temperature was monitored by a rectal probe and maintained at 37°C with a warming pad. Hindlimb skin temperature was also monitored by a thermistor and maintained between 36–38°C by radiant heat. In the second set, the rats were sedated by CO_2 in a specially designed chamber (21) and were killed immediately by cervical dislocation. The femoral segments of the left sciatic nerve from each rat were dissected rapidly (~30 s), blotted carefully with fine filter paper to remove any accompanying blood, and frozen in liquid nitrogen for subsequent measurements of β-hydroxybutyrate, acetoacetate, pyruvate, lactate, phosphocreatine, creatine, and ATP. The remaining part of the left nerve and the right nerve were used for measuring total malondialdehyde plus 4-hydroxyalkenals, GSH, total ascorbate, and sorbitol pathway intermediates.

Functional studies

Sciatic endoneurial nutritive NBF

NBF was assessed by microelectrode polarography and hydrogen clearance (32). We cannulated the left carotid artery with polyethylene tubing and maintained patency with heparinized saline (50 U/ml normal saline). We connected the catheter to a transducer and monitored the blood pressure by a MacLab data acquisition system. A tracheostomy was performed, and the animal was respired artificially with O_2:N_2 (20%:80%) by using a small animal ventilator (Harvard Apparatus, South Natick, MA). The right sciatic nerve was exposed and dissected gently away from the surrounding tissue. The skin around the incision was positioned to create a reservoir. We inserted a ground electrode subcutaneously into the flank of the rat. Using a micromanipulator, we inserted a H_2-sensitive platinum electrode (tip diameter 0.2 μm; World

Precision Instruments, Sarasota, FL) into the nerve above the trifurcation. Mineral oil at 37°C was used to fill the reservoir and prevent diffusion of gases out of the nerve. The nerve was polarized with 0.25V and, when a stable baseline was achieved, the animal received a gas mixture containing 10% H_2, which continued until the current change stabilized (10–30 min), at which time H_2 flow was terminated. Current recordings were made every 30 s until baseline levels were achieved (30–60 min). After the experiment, mono- or bi-exponential clearance curves were fitted to the data (Graphpad Software, La Jolla, CA). Nutritive NBF was taken as the slow component of the curve. An average of two determinations at different sites was used to determine nutritive NBF.

Sciatic MNCV

The left sciatic nerve was stimulated proximally at the sciatic notch and distally at the ankle via bipolar electrodes with supramaximal stimuli (8V) at 20 Hz. The latencies of the compound muscle action potentials were recorded via bipolar electrodes from the first interosseous muscle of the hind paw and measured from the stimulus artifact to the onset of the negative M-wave deflection. We calculated MNCV by subtracting the distal latency from the proximal latency and divided the result into the distance between the stimulating and recording electrode.

Digital SNCV

We recorded hindlimb SNCV in the digital nerve of the second toe by stimulating it with a square-wave pulse of 0.05 msec duration with the smallest intensity current, which resulted in a maximal amplitude response. The sensory nerve action potential was recorded behind the medial malleolus. The distance between recording and stimulating electrodes was approximately 25 mm and that was between indifferent and active recording needles maintained at 10 mm. Approximately 10 responses were averaged to obtain the position of the negative peak. The maximal SNCV was calculated by measuring the latency to the onset/peak of the initial negative deflection and the distance between stimulating and recording electrodes.

Metabolic studies

Preparation of perchloric extract

Femoral segments (~20 mg) of the left nerve were weighed, homogenized in 1.5 ml of ice-cold 6% $HClO_4$, and centrifuged at 4,000 g for 10 min. After centrifugation, the samples were neutralized immediately with 5 M K_2CO_3 to pH 6–7 and were centrifuged again at 4,000 g for 5 min, to precipitate insoluble $KClO_4$.

Biochemical measurements

Glycolytic intermediates, ketone bodies, phosphocreatine, creatine, and ATP

The steady-state concentrations of glucose, acetoacetate, β-hydroxybutyrate, pyruvate, lactate, phosphocreatine, creatine, and ATP were assayed in perchloric extracts of femoral segments of the left sciatic nerve spectrofluorometrically (Perkin-Elmer LS-5B, Norwalk, CT) by enzymatic

procedures as described by Lowry and Passonneau (33). The lower limit for all spectrofluorometric procedures in our study, including sorbitol, fructose, and GSH, was 0.1×10^{-9} M.

GSH

We modified the method (34) and mixed 0.1 ml of neutralized nerve perchloric extract with 0.89 ml of 0.02 M EDTA in 1.0 M tris-HCI buffer, pH 8.1. The reaction was initiated by addition of 0.01 ml of 0-phthaldialdehyde (10 mg–1 ml methanol). Initial and final readings were taken at λ excitation: 345 nm, λ emission: 425 nm, slits: 5 and 5. The differences in initial and final readings were compared with those in corresponding GSH standards ($1–10\times 10^{-9}$ M) processed in the same run.

Total ascorbate

Approximately 20-mg segments of the sciatic nerves were weighed, homogenized in 1 ml of ice-cold 5% metaphosphoric acid, and centrifuged at 4,000 *g* for 10 min. Aliquots (0.1 ml) of the supernatant were mixed with 0.9 ml 2 M Na-acetate buffer, pH 6.2, and 10 U of ascorbate oxidase to convert free AA to DHAA. The reaction is completed in 30 min. Then, 0.02 ml 92.5 mM O-phenyl-enediamine is added (35), and the fluorophore formation is completed after 30 min. The initial and final readings are taken at λ excitation: 350 nm, λ emission: 430 nm.

Sorbitol pathway intermediates and total MDA plus 4-hydroxyalkenals

Nerve segments (~40 mg) of the remaining part of the left nerve or the right nerve were weighed and homogenized in 1 ml 0.1 M Na-phosphate buffer, pH 6.5. We then added 100 µl of 0.3 M zinc sulfate, followed by an equivalent of barium hydroxide, to 0.4 ml of the homogenate for protein precipitation. The samples were centrifuged at 4,000 g for 10 min and 100 µl aliquots of the supernatant were taken for spectrofluorometric measurements of sorbitol and fructose by enzymatic procedures as we described previously (36, 37). The rest of the homogenate was centrifuged at 3,000 g for 5 min. Aliquots (200 µl) of the supernatant were used for measurements of malondialdehyde plus 4-hydroxyalkenals (4-HA) with the kits LPO-586 from Oxis International (Portland, OR). The method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and 4-HA after their extraction with methanesulfonic acid at 45°C (38). The absorbance of chromogenic product was measured at 586 nm by using a spectrophotometer Beckman DU 640 (Fullerton, CA) and was compared with the absorbance in corresponding standards.

Calculations of free mitochondrial and cytosolic NAD^+:NADH ratios

According to classical publications of Krebs' laboratory (39, 40) and other studies (41), direct measurements of NAD, NADH, NADP, and NADPH do not provide information on compartmentalization of nicotinamide adenine nucleotides between cytosol and mitochondria and do not separate free from protein-bound forms (only free forms determine direction and free-energy changes of dehydrogenase reactions). The same studies proposed an alternative approach for assessment of free $NAD(P)^+$/NAD(P)H ratios in the cytoplasm and mitochondria from ratios

of the concentrations of oxidized and reduced metabolites of suitable NAD(P)-linked dehydrogenase systems. Using this approach, we calculated free $NAD^+/NADH$ ratios for mitochondria and cytoplasm from the steady-state metabolite concentrations and the equilibrium constants of β-hydroxybutyrate and lactate dehydrogenase systems as described (39, 41):

$$\frac{[NAD^+]}{[NADH]}\text{mit} = \frac{[\text{Acetoacetate}]}{[\beta\text{-Hydroxybutyrate}]} \times \frac{1}{K_1},$$

where K_1 is the equilibrium constant of β-hydroxybutyrate dehydrogenase.

$$\frac{[NAD^+]}{[NADH]}\text{cyt} = \frac{[\text{Pyruvate}]}{[\text{Lactate}]} \times \frac{1}{K_2},$$

where K_2 is the equilibrium constant of lactate dehydrogenase.

Statistical analysis

The results are expressed as mean ± SD. Data were subjected to equality of variance F test and then to log transformation, if necessary, before one-way analysis of variance. Where overall significance ($P<0.05$) was attained, we made individual, between-group comparisons using the Student-Newman-Keuls multiple-range test. We defined significance at $P \leq 0.05$. When between-group variance differences could not be normalized by log transformation (datasets for body weights, plasma glucose, and some metabolic parameters), we analyzed the data by the nonparametric Kruskal-Wallis one-way analysis of variance, followed by the Fisher's protected least significant difference test for multiple comparisons.

RESULTS

Final body weights were lower in diabetic rats than in control rats (Table 1). The initial body weights were similar in control and diabetic groups. No statistically significant difference was found between final body weights in diabetic rats treated with ARI and the corresponding untreated group.
Blood glucose concentration was increased by 561% in diabetic rats compared with control group. ARI treatment had no effect on blood glucose concentration in diabetic rats.

Nerve glucose, sorbitol, and fructose concentrations were 275%, 963%, and 440% higher in diabetic rats compared with those in control rats (Table 2). Nerve glucose concentration was not affected by ARI treatment. Diabetes-induced increase in both sorbitol and fructose concentrations was normalized completely with 65 mg/kg/day-dose of sorbinil.

Sciatic NBF, mean systemic blood pressure (BP) and endoneurial vascular conductance (VC) were 47%, 20%, and 34% lower in diabetic rats compared with those in control rats ($P<0.01$ for NBF and VC and <0.05 for BP, Fig. 1A–C). NBF was 47% higher in ARI-treated diabetic rats compared with untreated diabetic group ($P<0.01$) but still remained 22% lower than in nondiabetic controls ($P<0.01$). BP was slightly increased because ARI treatment was not

statistically different from either control or diabetic groups. Diabetes-induced decrease in VC was essentially corrected by ARI treatment (a 38% increase vs. untreated diabetic group, $P<0.01$); no statistically significant difference in VC was found between ARI-treated diabetic group and non-diabetic control group.

Sciatic MNCV was decreased by 22% in diabetic rats compared with controls ($P<0.01$, Fig. 1D). MNCV was 21% higher in ARI-treated diabetic group compared with untreated diabetic group ($P<0.01$). No statistically significant difference was found between MNCV in ARI-treated diabetic group and nondiabetic controls. SNCV was 16% lower in diabetic rats compared with controls ($P<0.01$, Fig. 1E), and this decrease was essentially corrected by ARI treatment (a 13% increase vs. untreated diabetic group, $P<0.01$).

Concentrations of acetoacetate and β-hydroxybutyrate were increased by 258% and 693% in diabetic rats compared with those in control rats (Table 3). Acetoacetate concentration tended to decrease with ARI treatment, but the difference with untreated diabetic group did not achieve statistical significance. β-hydroxybutyrate concentration was 49% lower in ARI-treated diabetic rats compared with untreated diabetic group. Pyruvate concentration was decreased by 25% in diabetic rats vs. controls, and this decrease was not corrected by ARI treatment. Lactate concentration was increased by 56% in diabetic rats compared with controls. This increase was corrected completely in diabetic rats treated with ARI. Nerve ATP concentrations were similar in control rats and diabetic rats treated with or without ARI. PCr concentration was reduced by 40% in diabetic rats vs. control group, and PCr/Cr ratios were reduced in diabetic rats (by 40% and 43% vs. control group). PCr concentration tended to increase because ARI treatment was not significantly different from either control or diabetic groups. Cr concentrations were not different among control rats and diabetic rats treated with or without ARI.

Free nerve mitochondrial (Fig. 2A) and cytosolic (Fig. 2B) NAD^+:NADH ratios were decreased by 52% and 57% in diabetic rats compared with those in control rats ($P<0.01$ for all both ratios). Diabetes-induced mitochondrial and cytosolic NAD-redox changes were partially corrected by ARI treatment. Free mitochondrial NAD^+:NADH ratio was 48% higher in ARI-treated diabetic rats than in untreated diabetic group ($P<0.05$) but still remained 30% lower than in nondiabetic controls ($P<0.01$). Free cytosolic NAD^+: NADH ratio in ARI-treated diabetic rats was 60% higher than in untreated diabetic group ($P<0.01$) and 44% lower than in nondiabetic controls ($P<0.05$). PCr/Cr ratio (Fig. 2C) was reduced by 43% in diabetic rats vs. control group. PCr/Cr ratio was 54% higher in ARI-treated diabetic rats compared with the corresponding untreated group; no statistically significant difference in PCr/Cr ratios was found between ARI-treated diabetic group and nondiabetic controls.

Nerve MDA plus 4-HA concentration (Fig. 2D) was increased by 62% in diabetic rats vs. controls ($P<0.01$), and this increase was corrected completely by ARI treatment ($P<0.01$ vs. untreated diabetic group). Nerve GSH concentration (Fig. 2E) was decreased by 38% in diabetic rats vs. controls ($P<0.01$), and this decrease was essentially corrected in the ARI-treated diabetic group ($P<0.01$ vs. corresponding untreated group). Nerve total ascorbate concentration (Fig. 2F) was 33% lower in diabetic rats compared with controls ($P<0.01$). This concentration was 29% higher in diabetic rats treated with ARI compared with untreated diabetic group ($P< 0.05$). No statistically significant difference in total ascorbate concentrations was found between ARI-treated diabetic and nondiabetic control rats.

DISCUSSION

The involvement of AR in the pathogenesis of peripheral DN is supported by at least five lines of evidence: 1) similarity of a number of functional, metabolic, and morphological abnormalities in animal models of diabetes and galactose feeding (13, 42–44); 2) demonstration of preventive effects of structurally diverse ARIs on functional, biochemical, and structural changes of DN (1–14, 18, 28, 29); 3) potentiation of galactose-induced neuropathy in the transgenic mice expressing human AR (45) and the absence of functional deficits of DN in the AR knockout (AR-/-)mice (46); 4) identification of high AR protein level as an independent risk factor for DN in patients with both Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetes mellitus (47); and 5) finding of a 30.2% increase in the frequency of the Z-2 allele of the AR gene, known to be associated with two- to threefold AR expression (48), in patients with DN compared with uncomplicated group. Despite such strong support of the AR concept of DN from both experimental and human studies, the contradictory results of intervention animal studies (1, 3, 24–29 and 11, 30) and the inefficacy of a number of ARIs in clinical trials (18, 49) raise the question of reversibility of existing abnormalities of peripheral DN with AR inhibition. To address this question, we performed experiments with the well-studied ARI sorbinil administered at a dose known to inhibit entirely diabetes-induced increase in the sorbitol pathway activity (1, 6, 20, 50, 51) for 2 wk after 4 wk of untreated diabetes. We have demonstrated previously (52) that the rat model with 3-wk duration of streptozotocin diabetes already displays clearly manifested peripheral nerve blood flow and conduction deficits, NAD^+/NADH redox imbalances, energy deficiency, and evidence of enhanced oxidative stress.

As expected from previous studies (1, 6, 20, 50, 51, 52, 54), nerve glucose concentrations remained unaffected, whereas both sorbitol and fructose were normalized completely with 65 mg/kg/d of sorbinil. Thus, we achieved a complete inhibition of diabetes-induced sorbitol pathway hyperactivity (that is, met an obligatory criterion for addressing a principal question of this study).

Diabetes-induced decrease in NBF in our experiments is in agreement with other reports for both exposed (2–4, 12, 28–32, 55–59) and unexposed (60, 61) diabetic nerve. Correction of sciatic endoneurial NBF and vascular conductance deficits in sorbinil-treated diabetic rats is consistent with the results reported previously for structurally diverse ARIs, ZD5522 (3), WAY-121509 (28), tolrestat (29), and NZ-314 (62). Evaluation of ARIs on NBF assessed by laser Doppler flowmetry resulted in contradictory findings. Prevention of laser Doppler flow deficit in streptozotocin-diabetic rats has been found with the ARIs ponalrestat (2, 3), ZD5522(3), and ONO-2235 (12), whereas sorbinil and imirestat (51) appeared ineffective despite prevention of the sorbitol pathway hyperactivity. All the ARIs in the aforementioned studies prevented diabetes-induced nerve conduction slowing. Thus, the laser Doppler flow [reflecting the composite of epi-, peri-, and endoneurial blood flow and dominated by epi/perineurium as demonstrated in perineurial window experiments (63)] does not show as good a correlation with MNCV and SNCV as sciatic endoneurial nutritive BF assessed by microelectrode polarography and hydrogen clearance. Total (non-nutritive and nutritive) endoneurial BF measured by hydrogen clearance is not prevented or restored by a number of pharmacological agents; that is, the ARIs ZD5522, ponalrestat, tolrestat, and WAY-121509, as well as N-acetyl-L-cysteine and aminoguanidine (3, 64 and Norman E. Cameron, unpublished observations) all of which

effectively correct both diabetes-induced endoneurial nutritive BF and conduction deficits in early DN (3, 28, 29, 65). Endoneurial nutritive BF appears to provide the best assessment of diabetes-induced neurovascular dysfunction for studies of the pathogenesis of DN and development of new therapies compared with either laser Doppler or total (non-nutritive and nutritive) endoneurial BF.

Restoration of normal MNCV and SNCV in sorbinil-treated diabetic rats is consistent with findings (1, 3, 24–29) obtained in the intervention studies with other ARIs. Our results do not confirm the failure to reverse diabetes-induced MNCV slowing by AR inhibition (11, 30). It is important that beneficial effects on nerve conduction have also been observed with suboptimal doses of ARIs (51, 54, 66, 67) and, in particular, with 1 mg/kg SNK-860, the dose equipotent to 20 mg/kg sorbinil (67). A close negative correlation (r=–0.95) was found between MNCV and sciatic nerve sorbitol concentrations in the aforementioned study.

The decrease in free mitochondrial NAD^+/NADH ratio is a metabolic indicator of tissue hypoxia. Changes in nerve-free mitochondrial NAD^+/NADH ratios in diabetic rats treated with or without vasoactive compounds; that is, vasodilators, antioxidants, and ARIs parallel those of NBF (31, 52, 55) and peripheral nerve oxygen tensions (68–70). Consistent with other studies of our group (27, 52), nerve free cytosolic NAD^+/NADH ratio was also decreased in diabetic rats. We have reported previously that nerve-free cytosolic NAD^+/NADH redox changes in diabetic rats are preventable by 1) the vasodilator prazosin despite the absence of any effect on nerve sorbitol pathway intermediates (52) and 2) the antioxidant DL-α-lipoic acid, regardless of further increase in nerve sorbitol pathway activity (31), and are not preventable by a sorbitol dehydrogenase (SDH) inhibitor despite a 91% inhibition of the increased flux through SDH (21). Therefore, our findings do not support the concept of "diabetic pseudohypoxia," which suggests that diabetes-induced cytosolic NAD-redox imbalances are due to increased oxidation of sorbitol to fructose, coupled to reduction of NAD to NADH, by SDH (71). Considering that mitochondrial and cytosolic pools of nicotinamide adenine dinucleotides are linked through dicarboxylate carriers and that peripheral nerve strongly depends on oxidative (aerobic) metabolism (72), it is reasonable to suggest that diabetes-associated cytosolic NAD-redox imbalances have a mitochondrial origin; that is, reflect a metabolic response of the cytoplasm to endoneurial hypoxia developing due to decreased NBF. Activation of anaerobic glycolysis and accumulation of its product, lactate, result from the shift from normal (transport into mitochondria) to abnormal (NADH-dependent pyruvate reduction by lactate dehydrogenase) disposition of cytosolic NADH under hypoxic conditions. Partial correction of both mitochondrial and cytosolic NAD^+/NADH redox states in ARI-treated diabetic rats is consistent with corresponding changes of NBF.

The presence of endoneurial hypoxia in diabetic rats is manifested by a decrease in PCr concentration and PCr/Cr ratio, which are the most sensitive variables of peripheral nerve energy state. Nerve PCr concentration rapidly reacts to changes in perfusion/oxygenation (72, 73), and both PCr concentration and PCr/Cr ratio in the diabetic nerve increase in response to vasodilator treatment (52). The correction of nerve energy state in ARI-treated diabetic rats in the present experiments is in line with restoration of NBF, MNCV, and SNCV. The aforementioned studies in animal models of ischemia and hyperbaric oxygenation (72, 73) and our findings with the α_1-adrenoceptor antagonist prazosin (52) and now ARI in the diabetic rat model suggest that out of a variety of metabolic parameters, nerve energy state correlates best with nerve conduction. The

latter is not surprising given that nerve plasma membrane potential is maintained by energy-dependent processes (74); that is, it depends on cytosolic ATP/ADP ratio. The decrease in PCr/Cr ratio [an index of free *cytosolic* ATP/ADP ratio (42)] may account for chronic depolarization and Na^+ channel inactivation with resulting reduced excitability and nerve conduction deficit in diabetic nerves. Membrane depolarization has been found in human glial cells, treated with metabolic inhibitors (75), and rat peripheral nerve fibers exposed to anoxia (76).

The effects of ARIs on indices of DN are unidirectional with those of inhibitors of non-enzymatic glycation (57, 65, 77) and PKC (62, 78) as well as antioxidants (31, 55, 64, 70,79–83). Over past several years, the continuing debate about a "primary mechanism" of diabetic complications has centered on oxidative stress and its relationship with other hyperglycemia-initiated factors (84). Recently, it has been suggested that three pathways leading to diabetic complications; that is, increased sorbitol pathway activity, nonenzymatic glycation/glycoxidation, and PKC activation, originate from oxidative stress, and, in particular, production of superoxide anion radicals in mitochondria (85). However, this concept, at least, the part related to the sorbitol pathway, is not supported by experimental studies demonstrating the absence of any suppression of tissue sorbitol pathway activity by antioxidants; that is, those neutralizing superoxide anion radicals [DL-α-lipoic acid (86), taurine (87), and probucol (88)] in diabetic animal models [31, 64, 89, 90]. Furthermore, it does not explain why interventions with totally different pharmacological agents; that is, inhibitors of AR, glycation, and PKC effectively prevent or reverse diabetic complications, including neuropathy. Based on evidence of contribution of increased AR activity (20, 91–93), the Maillard reaction (94), the interaction of advanced glycation end-products (AGE) with their receptors (95), and, recently, PKC activation (96) to hyperglycemia-induced oxidative injury, it would be logical to assume that the common component for the pathways leading to diabetic complications; oxidative stress is localized downstream from primary hyperglycemia-initiated mechanism(s) (Fig. 3). The input of the aforementioned mechanism(s) to free radical damage varies for different tissue sites and stages of diabetic complications. The absence of AGE accumulation in the peripheral nerve (97) and the failure of aminoguanidine to counteract nerve oxidative stress in short-term diabetes (78) suggest that advanced glycation plays no role in nerve oxidative injury in early DN. The contribution of PKC is unclear considering that the enzyme activity has been reported down-regulated (96), unchanged (62,78), and up-regulated (89) in the peripheral nerve in early diabetes and no measurements of oxidative stress other than GSH (78) has been performed in PKC inhibitor-treated rats. Based on endothelial cell culture studies (96, 99), however, one could assume that the inhibitors of both nonenzymatic glycation and PKC counteract diabetes-associated oxidative stress in *vas nervorum*. A number of findings (20, 100, 101), including those from our group (101) suggest that increased AR activity has a key role in peripheral nerve oxidative injury in, at least, short-term diabetes. AR inhibition has been reported to prevent or correct nerve lipid peroxidation product accumulation (20), GSH depletion (100, 101), increase in GSSG/GSH ratio (100) and down-regulation of superoxide dismutase (100) in diabetic animals. On the contrary, sorbitol accumulation above the "diabetic threshold" in SDH inhibitor-treated diabetic rats exacerbates nerve malondialdehyde and 4-hydroxyalkenal accumulation and GSH depletion (21). The present study demonstrates that even short (two-week) treatment with an adequate dose of ARI appears sufficient to reverse depletion of key nonenzymatic antioxidants, GSH and ascorbate, and to bring about normal lipid peroxidation in early DN.

In conclusion, diabetes-induced neurovascular dysfunction, nerve conduction deficits, key metabolic changes and oxidative injury in, at least, early DN are reversed by treatment with an adequate dose of ARI; that is, the dose that completely inhibits the increased sorbitol pathway activity. These data support the need for continued development of new potent and well-tolerated ARIs. The analysis of the past ARI clinical trials indicates that their failure resulted from either inadequate doses or insufficient inhibition of the sorbitol pathway activity, unacceptably high systemic toxicity of the inhibitors rather than inapplicability of the AR concept for DN in humans (18). Robust inhibition of AR with Zenarestat in diabetic human nerve has yielded dose-dependent efficacy on nerve structure and function; at least, 80% lowering of nerve sorbitol concentration appeared required for improvement in nerve electrophysiology and fiber density (102). We would like also to point out that, whereas experimental studies in animal models indicate the reversibility of early DN, all the clinical trials in the United States have been performed in patients with nerve structural changes; that is, far more advanced DN, a stage of the disease that may be highly resistant to any drug therapy. An approach more likely to be successful may be a prevention or early intervention, which was modeled by the experiments reported herein. The ARIs are used widely for treatment of patients with DN in Japan, and are prescribed to patients with newly diagnosed diabetes mellitus, together with insulin or hypoglycemic agents, or with early small fiber neuropathy. This approach is justified considering that tight blood glucose control with the complete lack of intermittent hyperglycemia is difficult, often impossible, to achieve and has not been shown to reverse established neuropathy (103).

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Table 1

Final body weight and blood glucose concentrations in control rats and diabetic rats treated with or without ARI (Mean ± SEM, *n*=12–15)

	Control	Diabetic	Diabetic+ ARI
Final body weight, g	428 ± 7	300 ± 14**	308 ± 21**
Blood glucose, mmol/l	3.23 ± 0.09	18.12±0.75**	18.46 ± 0.68**

**Significantly different compared with controls ($P<0.01$).

Table 2

Nerve glucose, sorbitol, and fructose concentrations[a] in control rats and diabetic rats treated with or without ARI (Mean ± SEM, *n*=7–10).

	Control	Diabetic	Diabetic + ARI
Glucose	3.57 ± 0.16	13.4 ± 1.0**	13.5 ± 0.63**
Sorbitol	0.128 ± 0.019	1.36 ± 0.133**	0.062 ± 0.016##
Fructose	1.21 ± 0.13	6.54± 0.42**	1.21 ± 0.13##

[a] μmol/g wet weight ;

**Significantly different compared with controls (P<0.01); ##significantly different compared with untreated diabetic group (P<0.01).

Table 3

Steady-state metabolite concentrations and variables of energy state[a] in control rats and diabetic rats treated with or without ARI (Mean ± SEM, *n*=7–10).

	Control	Diabetic	Diabetic + ARI
Acetoacetate	0.119 ± 0.007	0.427 ± 0.045**	0.357 ± 0.051**
β-Hydroxybutyrate	0.067 ± 0.005	0.531 ± 0.080**	0.273 ± 0.018$^{**,\#\#}$
Pyruvate	0.156 ± 0.011	0.117 ± 0.015*	0.119 ± 0.005*
Lactate	1.74 ± 0.14	2.71 ± 0.37*	1.83 ± 0.13$^{\#}$
ATP	0.716 ± 0.103	0.711 ± 0.113	0.881 ± 0.089
PCr	3.78 ± 0.42	2.28 ± 0.29**	2.93 ± 0.09
Cr	3.80 ± 0.44	4.27 ± 0.32	4.61 ± 0.42

[a]In μmol/g wet weight.

*,**Significantly different compared with controls (*P*<0.05 and < 0.01, respectively);$^{\#,\ \#\#}$- significantly different compared with untreated diabetic group (*P*<0.05 and < 0.01, respectively).

Fig. 1

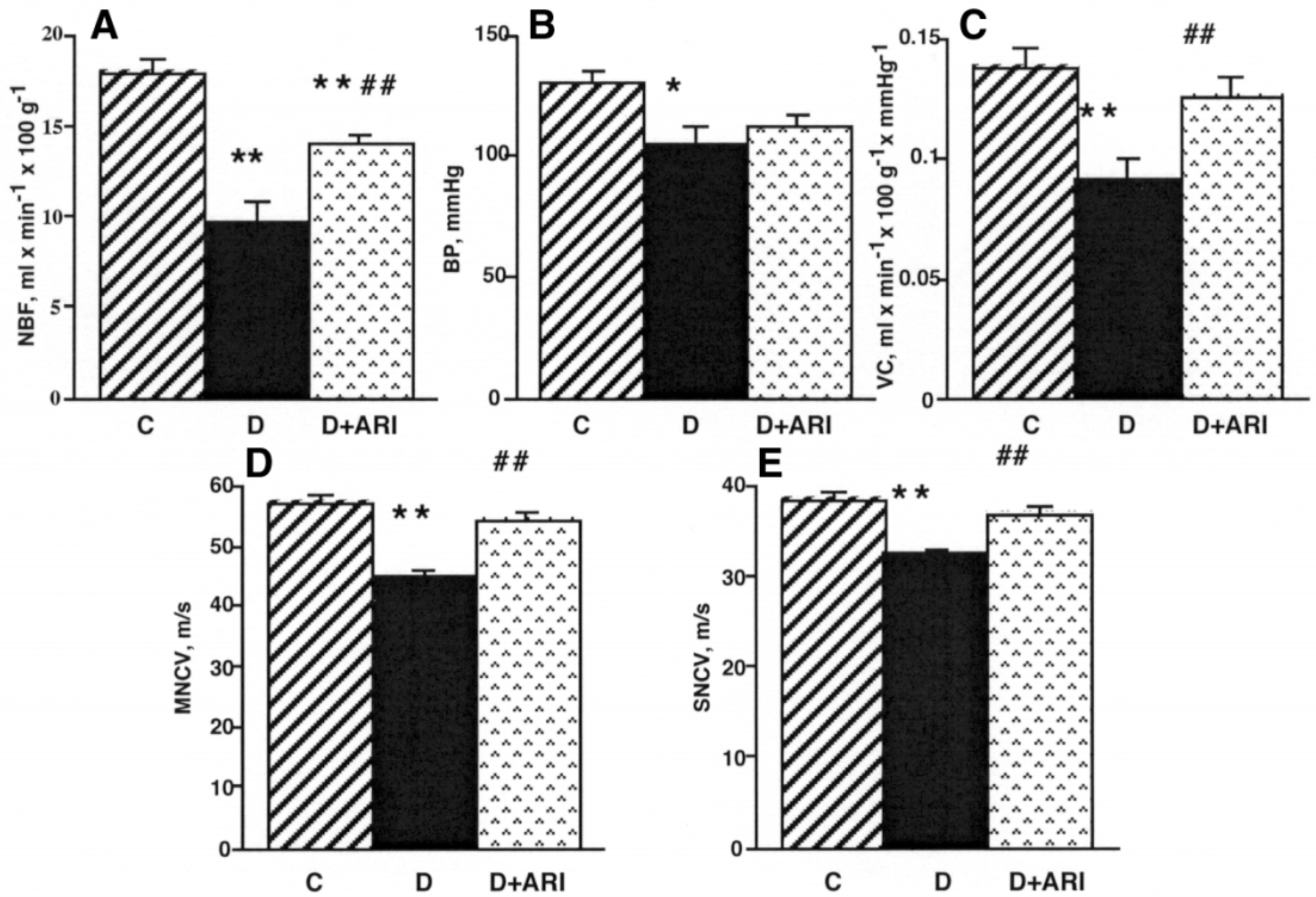

Figure 1. Evaluation of an ARI on diabetes-induced changes in peripheral nerve function. A) Sciatic endoneurial NBF; **(B)** blood pressure (BP); **(C)** endoneurial vascular conductance (VC); **(D)** MNCV; and **(E)** SNCV (mean ± SE, n=10–12). (C) Controls; (D) untreated diabetic group; (D+ARI) diabetic group treated with ARI. *,**Significantly different vs. controls (P<0.05 and <0.01, respectively); ##Significantly different vs. untreated diabetic group (P<0.01).

Fig. 2

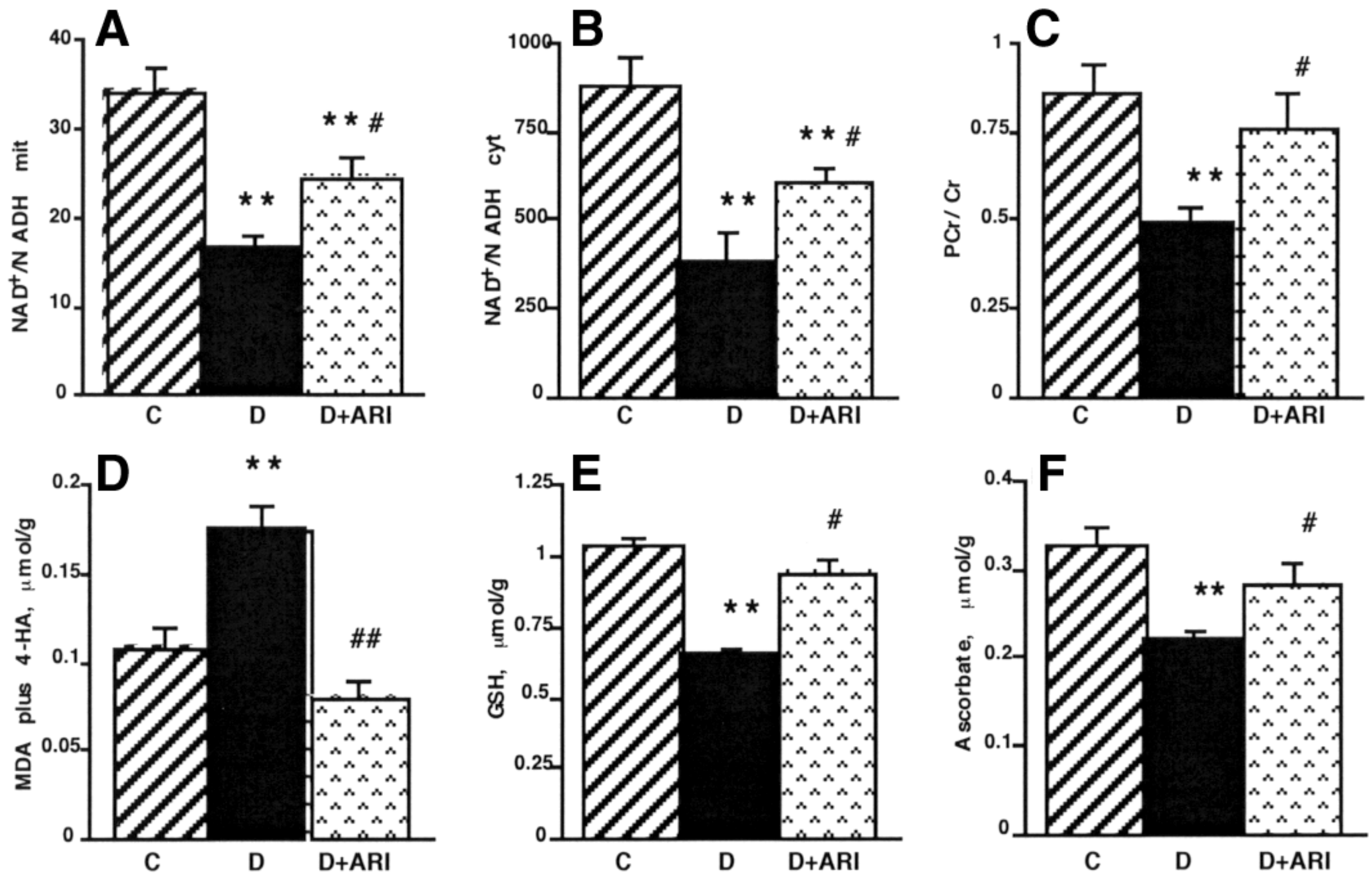

Figure 2. Evaluation of an ARI on diabetes-induced changes in the key metabolic variables and enhanced oxidative stress in peripheral nerve. A) Free mitochondrial NAD^+/NADH ratios; **(B)** Free cytosolic NAD^+/NADH ratios; **(C)** PCr/Cr ratios; **(D)** Lipid peroxidation product concentrations; **(E)** GSH concentrations; **(F)** Ascorbate concentrations (mean ± SE, n=6–10). (C) Controls; (D) untreated diabetic group; (D+ARI) diabetic group treated with ARI. **Significantly different vs. controls (*P*<0.01); #,##Significantly different vs. untreated diabetic group (*P*<0.05 and <0.01, respectively).

Fig. 3

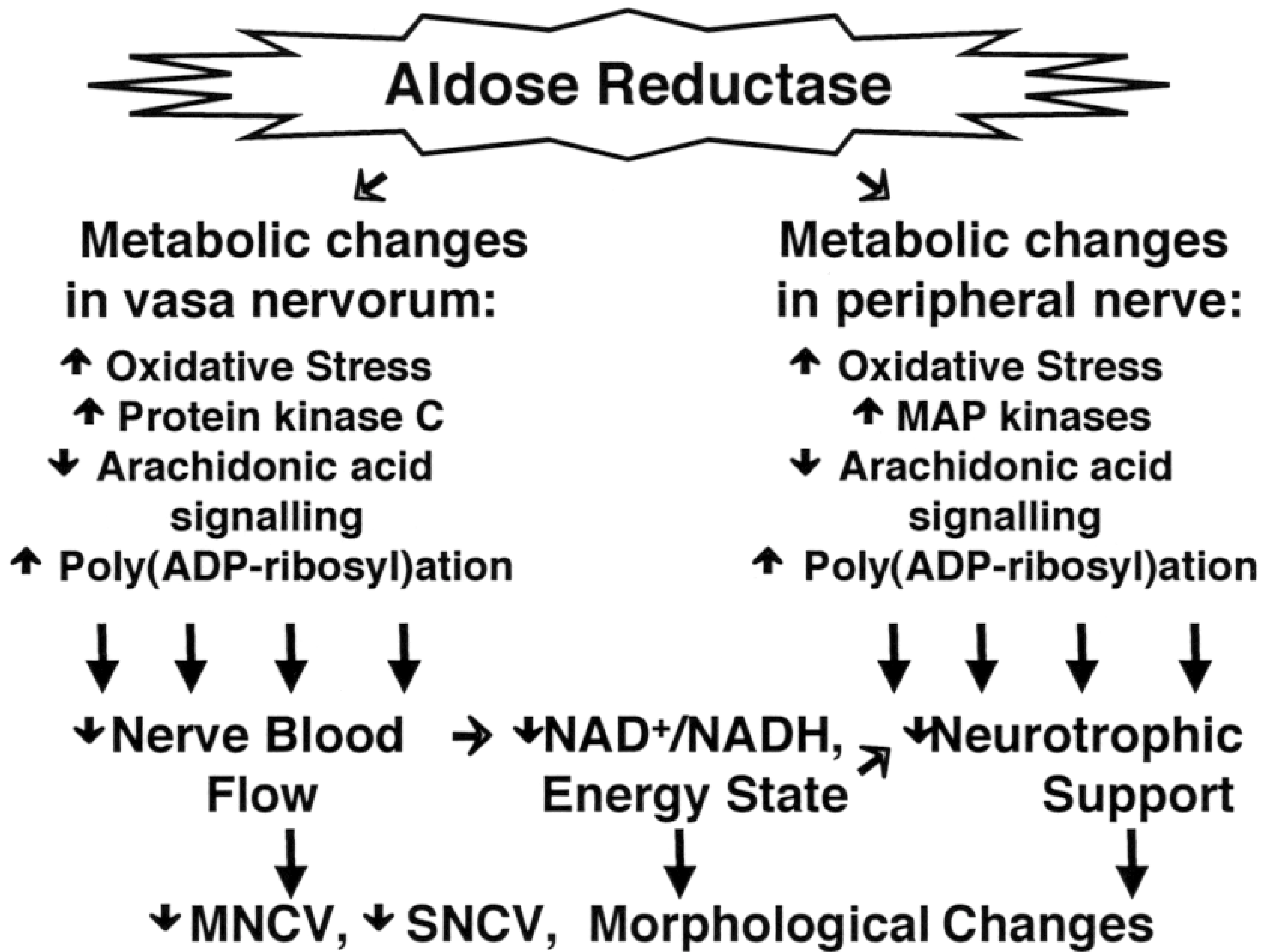

Fig.3. The key primary role for AR in functional, signal transduction, metabolic, neurotrophic, and morphological abnormalities characteristic for peripheral diabetic neuropathy.