

Chapter 1

Oxidative Stress and Diabetes-Induced Vascular Dysfunction: Role in Diabetic Neuropathy

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1.1 Introduction

Oxidative stress is an important component of diabetes and its complications [1–15]. Studies showing that treatment with antioxidants prevents diabetes- and hyperglycemia-induced impairment of endothelium-dependent relaxation suggest that oxidative stress is a major factor in the development of diabetic vascular disease [7, 16–20]. In addition, treatment of streptozotocin-induced diabetic rats with antioxidants has demonstrated that oxidative stress and vascular dysfunction may be a major factor in the development of diabetic neuropathy [5, 6, 8, 21–23]. In this chapter I will present past studies from my laboratory that have focused on the effect of streptozotocin-diabetes-induced oxidative stress on vascular reactivity of epineurial arterioles and neural function.

1.2 Diabetes-Induced Oxidative Stress and Vascular Dysfunction in Epineurial Arterioles

My laboratory has for many years focused on the effect of diabetes on vascular and neural dysfunction. Our studies first demonstrated that vascular impairment of epineurial arterioles, blood vessels that provide circulation to the sciatic nerve, and reduced endoneurial blood flow precede neural dysfunction as determined by slowing of motor nerve conduction velocity (see Fig. 1.1 [24]). Our studies demonstrated that one week after the induction of diabetes using streptozotocin, that

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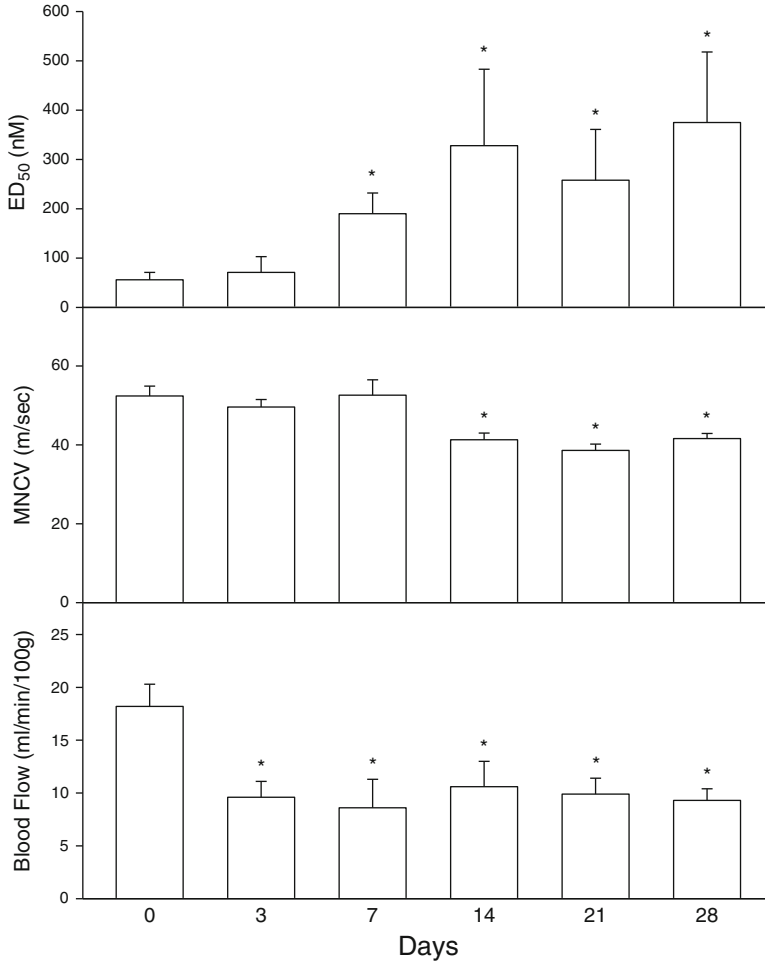


Fig. 1.1 The effect of streptozotocin-diabetes in the development of vascular and neural dysfunction. Vascular reactivity to acetylcholine by epineurial arterioles (section A ED₅₀), endoneurial blood flow (section B), and motor nerve conduction velocity (section C) was examined in control (0) and streptozotocin-induced diabetic rats following 6–30 days of diabetes. Data is presented as the mean \pm SEM. * $P < 0.05$ compared to control (0)

vasodilation in response to a low dose of acetylcholine was significantly impaired and after two weeks of diabetes, maximum impairment in acetylcholine-mediated vascular relaxation was observed [24]. During this time period endoneurial blood flow of the sciatic nerve was also reduced. Impairment in motor nerve conduction velocity was not observed until after two weeks of diabetes suggesting that vascular dysfunction may be an early development in diabetes and a major factor contributing to diabetic neuropathy.

Acetylcholine-induced vasodilation of epineurial arterioles is mediated by two mechanisms involving the production of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) [25]. This is important since one mechanism by which superoxide/oxidative stress can cause vascular dysfunction is by quenching the bioactivity of NO (see below). A primary factor contributing to diabetes-/hyperglycemia-induced impairment in vascular relaxation in epineurial arterioles is increased oxidative stress [26, 27]. Oxidative stress occurs when the balance between the production of oxidation products and the ability of antioxidant mechanisms to neutralize these products is shifted in the favor of formation/accumulation of oxidative stress products.

It is widely known that diabetes causes an increase in the production of reactive oxygen species [18, 28–30]. The most common forms are superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and peroxynitrite ($ONOO^-$) [31]. There are many potential sources for production of these compounds. Superoxide can be produced by the electron transport chain of the mitochondria, NADH oxidase, NAD(P)H oxidase, xanthine oxidase, nitric oxide synthases, cyclooxygenase, lipoxygenase, and cytochrome P-450 [31]. Superoxide can spontaneously acquire an electron to form hydrogen peroxide. Hydrogen peroxide can also be formed from superoxide via superoxide dismutase (SOD), of which there are three isoforms: manganese (Mn)-SOD, which is located in the mitochondria, and two isoforms of copper and zinc (Cu, Zn)-SOD, which are located in the cytosol or extracellularly, respectively [31]. Hydrogen peroxide can be converted to water by the action of catalase or by glutathione peroxidase in the presence of reduced glutathione [31]. However, in the presence of trace metals such as iron (Fe), hydrogen peroxide can form OH^- via a process known as the Fenton reaction [31]. The formation of peroxynitrite is also important pathologically and occurs by the reaction of O_2^- and NO [30, 31]. We have demonstrated that superoxide and peroxynitrite, as indicated by the presence of nitrotyrosine staining, formation is increased in epineurial arterioles from diabetic rats (see Fig. 1.2 [26, 27]).

In a hallmark study Brownlee et al. [32, 33] presented a unifying hypothesis that increased production of superoxide by the mitochondrial chain is a causal link between elevated glucose and three of the main biochemical pathways (glucose-induced activation of protein kinase C, increased formation of glucose-derived advanced glycation end products, and increased glucose flux through the aldose reductase pathway) responsible for diabetes/hyperglycemia complications [32, 33]. Our studies have indicated that in epineurial arterioles from diabetic rats, the increased formation of superoxide seems to be primarily derived from the mitochondria [34]. We had previously demonstrated that reducing superoxide formation and oxidative stress in diabetic rats by treatment with several different types of antioxidants improved vasodilation by acetylcholine in epineurial arterioles of the sciatic nerve [26, 27]. In studies designed to investigate the source of superoxide formation in epineurial arterioles of the sciatic nerve from diabetic rats, we demonstrated that antioxidants were capable of preventing superoxide formation and

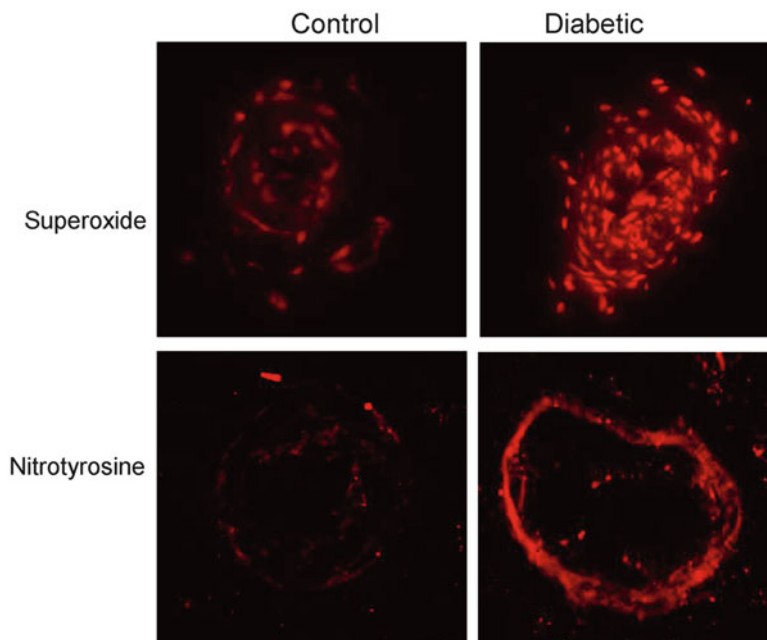


Fig. 1.2 Representative images for superoxide and nitrotyrosine staining in epineurial arterioles from control and streptozotocin-induced diabetic rats. Duration of diabetes was 8 weeks

reversing diabetes-induced vascular impairment *in vitro*. Dihydrolipoic acid and to a lesser extent α -lipoic acid were effective in decreasing superoxide formation and restoring acetylcholine-mediated vasodilation to arterioles from diabetic rats. α -Lipoic acid is capable of scavenging hydroxyl radicals, hypochlorous acid, and singlet oxygen, but not superoxide or peroxy radicals [35, 36]. α -Lipoic acid is also effective at chelating transition metals. In contrast, in its reduced form as dihydrolipoic acid, it is a good scavenger of superoxide and prevents initiation of lipid peroxidation [35, 36].

In vivo α -lipoic acid can be converted into dihydrolipoic acid [35]. In addition, both α -lipoic acid and dihydrolipoic acid can regenerate other cellular antioxidants including dehydroascorbate, ubiquinol, oxidized glutathione, and, indirectly, the tocopherols [35]. The combination of these properties was likely responsible for the effectiveness of α -lipoic acid and dihydrolipoic acid in decreasing superoxide formation [34]. Tempol, a superoxide dismutase mimetic, also reversed the diabetes-induced impairment of acetylcholine-mediated vasodilation and increased superoxide formation in epineurial arterioles [37]. This is in agreement with other studies, which demonstrated that tempol or M40403, another superoxide dismutase mimetic, restores diabetes-induced endothelial dysfunction [27, 37, 38]. The decrease in superoxide formation by α -lipoic acid, dihydrolipoic acid, or tempol

and the reversal of the diabetes-induced impairment in vasodilation suggest that the increased formation of superoxide and perhaps scavenging of nitric oxide is responsible in part for the reduced vascular response to acetylcholine in epineurial arterioles from diabetic rats. This is supported by our previous studies demonstrating the formation of superoxide and/or peroxynitrite by epineurial arterioles of the sciatic nerve from diabetic rats causes vascular dysfunction that is prevented with treatment by antioxidants in vivo [26, 27]. This was further supported by studies demonstrating that pretreatment with L-arginine in vitro improved acetylcholine-mediated vasodilation in epineurial arterioles from diabetic rats without decreasing the formation of superoxide by these vessels. Acute pretreatment with L-arginine of vessels from diabetic rats as well as L-arginine treatment of diabetic animal models and humans has led to the suggestion that reduced availability of nitric oxide during periods of hyperglycemia may be responsible for impaired vascular relaxation [39–43]. This may be due to a limitation in arginine as a substrate for nitric oxide synthase in diabetes or an increase in scavenging of nitric oxide by superoxide [26, 27, 44]. Our studies would support the latter conclusion. We have demonstrated increased superoxide and peroxynitrite formation in epineurial arterioles of diabetic rats and impairment in endothelium-dependent vascular relaxation that is prevented by antioxidant treatment [26, 27].

In studies to investigate the possible sources of superoxide formation in epineurial arterioles of diabetic rats, we found that increased formation of superoxide by epineurial arterioles was attenuated by preincubation with rotenone but not *m-chlorophenylhydrazone* (CCCP) or thenoyltrifluoroacetone (TTFA) [34]. Rotenone is an inhibitor of complex I of the mitochondrial electron transport chain, TTFA is an inhibitor of complex II, and CCCP is an uncoupler of oxidative phosphorylation. We are unsure why CCCP was less effective than rotenone in reducing superoxide formation by epineurial arterioles of the sciatic nerve of diabetic rats. It is possible that CCCP did not penetrate the vascular wall under the incubation conditions. Nonetheless, this study implicated complex I of the mitochondrial electron transport chain in the production of superoxide by epineurial arterioles of the sciatic nerve of the diabetic rat. In our studies increased formation of superoxide by epineurial arterioles from diabetic rats was also partially decreased by diphenylene iodonium (DPI). DPI has been used for many years as a NAD(P)H oxidase inhibitor [45]. Therefore, our studies at first would suggest that NAD(P)H oxidase may also be a source for the production of superoxide by epineurial arterioles of the diabetic rat. However, Li and Trush have reported in studies with monocytes that DPI at concentrations that inhibit NAD(P)H oxidase diminished the production of superoxide by mitochondrial respiration [46]. They found that DPI was as potent as rotenone in inhibiting the production of superoxide by the mitochondria, likely by complex I. If the studies by Li and Trush are correct, we cannot unequivocally state that NAD(P)H oxidase is a source of superoxide formation by epineurial arterioles of the sciatic nerve.

1.3 Neural Dysfunction

In two separate studies we examined the effect of treating streptozotocin-diabetic rats with α -lipoic acid or M40403 on vascular dysfunction, endoneurial blood flow, and nerve activity, as determined by measuring motor nerve conduction velocity [26, 27]. These studies demonstrated that treating diabetic rats using a prevention protocol with α -lipoic acid or M40403 prevented the diabetes-induced decrease in motor nerve conduction velocity and endoneurial blood flow (Fig. 1.3) and impairment of acetylcholine-mediated vascular relaxation by epineurial arterioles (Fig. 1.4). These treatments generally improved markers of oxidative stress including serum thiobarbituric acid reactive substance and superoxide and nitrotyrosine staining of epineurial arterioles [26, 27]. These studies imply that diabetes causes the increased production of superoxide and peroxynitrite in neural microvascular tissue and this is responsible for impaired vascular function. Moreover, improving vascular function in diabetes by use of antioxidants also restores endoneurial blood flow and neural activity.

Diabetes has been shown to cause an increase flux of glucose through the aldose reductase pathway that leads to the accumulation of sorbitol by nerve and other tissues [33]. Numerous investigators have demonstrated that treating diabetic rats with an aldose reductase inhibitor improves nerve function, and we have shown that treatment with an aldose reductase inhibitor also improves vascular dysfunction in epineurial arterioles [47]. The mechanism responsible for improving diabetes impaired vascular and nerve function by aldose reductase inhibitor treatment is

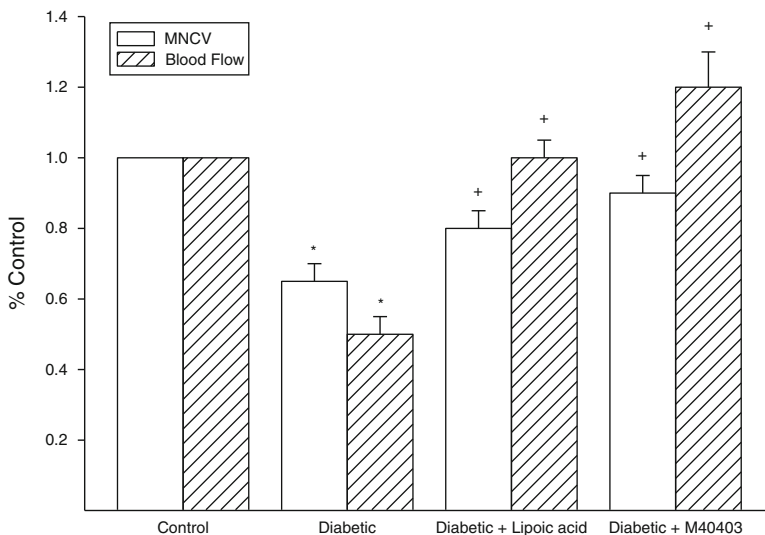


Fig. 1.3 Effect of treatment of streptozotocin-induced diabetic rats with 0.5 % α -lipoic acid or M40403 on motor nerve conduction velocity and endoneurial blood flow. Data is presented as the mean \pm SEM % of control. * $P < 0.05$ compared to control; * $P < 0.05$ compared to diabetic

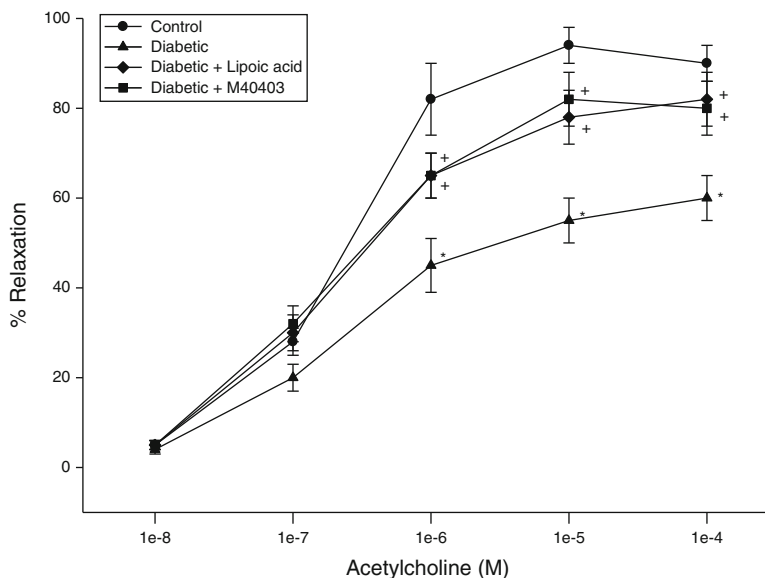


Fig. 1.4 Effect of treatment of streptozotocin-induced diabetic rats with 0.5 % α -lipoic acid or M40403 on acetylcholine-mediated vascular relaxation by epineurial arterioles. Data is presented as the mean \pm SEM % of control. * $P < 0.05$ compared to control; + $P < 0.05$ compared to diabetic

unclear but in part may be due to reducing oxidative stress [47]. Previously we had reported that treating streptozotocin-induced diabetic rats with 0.5 % α -lipoic acid (see above) provides maximum protection against diabetes-induced oxidative stress and the development of vascular and neural dysfunction [26]. We have also reported that sorbinil, an aldose reductase inhibitor, partially prevented the development of diabetes-induced vascular and neural defects but were not as efficacious as antioxidant therapies [26, 47]. We next sought to determine whether combining these therapies at lower doses may be synergistic [48]. We found that the combination of 0.25 % α -lipoic acid and fidarestat (3 mg/kg B.W.), an aldose reductase inhibitor, completely prevented the diabetes-induced impairment of acetylcholine-mediated vascular relaxation in epineurial arterioles of the sciatic nerve (Fig. 1.5) and that this combination was more effective in preventing diabetes-induced vascular dysfunction than monotherapy of either compound. Our explanation for these results was that treatment of diabetic rats with fidarestat in combination with α -lipoic acid favored the formation of dihydrolipoic acid. α -Lipoic acid is a good metal chelator and is capable of scavenging hydroxyl radicals, hypochlorous acid, and singlet oxygen, but not superoxide or peroxy radicals [35, 36, 49, 50]. However, in its reduced form, as dihydrolipoic acid, it is a good scavenger of superoxide and prevents initiation of lipid peroxidation [35, 36, 49, 50]. In vivo, the conversion of α -lipoic acid to dihydrolipoic acid requires either NADH or NADPH [49, 51]. In the mitochondria, preferentially R(+)- α -lipoic acid is converted to dihydrolipoic acid by the action of dihydrolipoamide dehydrogenase which requires NADH [50, 51].

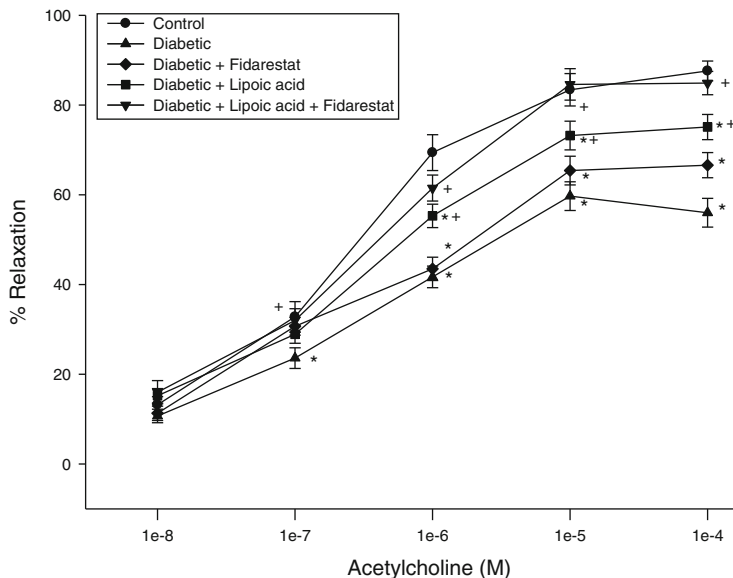


Fig. 1.5 Effect of treatment of streptozotocin-induced diabetic rats with 3 mg/kg fidarestat and/or 0.25 % α -lipoic acid on acetylcholine-mediated vascular relaxation by epineurial arterioles. Data is presented as the mean \pm SEM % of control. * P <0.05 compared to control; + P <0.05 compared to diabetic

Both stereo-isomers of α -lipoic acid can be reduced in the cytosol by glutathione reductase or thioredoxin reductase, both require NADPH [50–52]. In neutrophils, as well as rat heart, kidney, and brain, NADH-dependent reduction of α -lipoic acid is prominent, whereas with rat liver, NADH- and NADPH-dependent pathways were about equally active [50, 52]. In erythrocytes and endothelial cells, NADPH is the primary reducing cofactor for α -lipoic acid [50, 53]. In diabetes, NADPH levels are reduced due to the increased flux of glucose through the aldose reductase pathway [40, 41]. Therefore, blocking the aldose reductase pathway with an aldose reductase inhibitor such as fidarestat likely protects cellular NADPH levels permitting the formation of dihydrolipoic acid. This explanation is supported by our studies demonstrating that serum dihydrolipoic acid levels are increased in diabetic rats treated with α -lipoic acid and fidarestat [48]. These studies suggest that in addition to protecting glutathione production, treatment of diabetic rats with an aldose reductase inhibitor may promote the formation of dihydrolipoic acid. This result may explain the antioxidant properties of aldose reductase inhibitors [54]. In these studies there was a synergistic effect on improving lens glutathione levels when treating diabetic rats with the combination of α -lipoic acid and fidarestat. Treatment of diabetic rats with fidarestat alone independently improved endoneurial blood flow and motor nerve conduction velocity, by 50 and 60 %, respectively, and reduced superoxide formation in the aorta. Furthermore, treating diabetic rats with 3 or 15 mg/kg body weight of fidarestat had a concentration-dependent effect on improving endoneurial

blood flow, motor nerve conduction velocity, and acetylcholine-mediated vasodilation in epineurial arterioles. Taken together our results imply that some markers of oxidative stress and neural function are significantly improved by monotherapy using α -lipoic acid; however, the greatest beneficial effects were observed on all markers of oxidative stress and vascular function when the combination treatment consisting of α -lipoic acid and fidaostat was used.

In summary, diabetic neuropathy is a multifactorial disorder and vascular dysfunction in part due to an increase in oxidative stress is a contributing factor. Since diabetic vascular and neural disease is multifactorial, combination therapy may be the best approach for an effective treatment. The studies presented above suggest that an effective combination therapy should include an antioxidant such as α -lipoic acid and an aldose reductase inhibitor.

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