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Effects of antioxidants on nerve and vascular dysfunction in experimental diabetes

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Abstract

Reactive oxygen species (ROS) are elevated by metabolic changes in diabetes, including autoxidation and increased advanced glycation. Endogenous protection by the glutathione redox cycle is also compromised by the competing NADPH requirement of elevated polyol pathway flux. Antioxidant treatment strategies prevent or reverse nerve conduction velocity (NCV) deficits in diabetic rats. These include lipophilic scavengers such as butylated hydroxytoluene, probucol and vitamin E, more hydrophilic agents like α-lipoic acid and acetyl cysteine, and transition metal chelators that inhibit autoxidation. In the long-term, elevated ROS cause cumulative damage to neurons and Schwann cells, however, they also have a deleterious effect on nerve blood flow in the short term. This causes endoneurial hypoxia, which is responsible for early NCV deficits. Antioxidant treatment corrects the blood flow deficit and promotes normal endoneurial oxygenation. ROS cause antioxidant-preventable vascular endothelium abnormalities, neutralizing nitric oxide mediated vasodilation and increasing reactivity to vasoconstrictors. Unsaturated fatty acids are a major target for ROS and essential fatty acid metabolism is impaired by diabetes. γ-Linolenic acid stimulates vasodilator prostanoid production, and there are marked synergistic interactions between γ-linolenic acid and antioxidants. This has encouraged the development of novel drugs such as ascorbyl-γ-linolenic acid and γ-linolenic acid-lipoic acid with enhanced therapeutic potential.

Keywords: Diabetes; Neuropathy; Nerve conduction velocity; Blood flow; Ischemia; Antioxidant; Free radicals; Nitric oxide; Vascular endothelium; Vitamin E; α-Lipoic acid

1. Introduction

Reduced peripheral nerve perfusion causes endoneurial hypoxia, which is a major factor in the etiology of diabetic neuropathy in patients and animal models [1–4]. In streptozotocin-induced diabetic rats, sciatic nerve blood flow is reduced by 40–50% within a week or two of diabetes induction [5,6] and impaired perfusion has also been noted in the BB-Wistar spontaneously diabetic model [7]. Prevention or correction of the blood flow deficit, by a variety of means, improves measures of nerve function such as conduction velocity (NCV) and resistance to hypoxic conduction failure. Successful treatments include chronic electrical nerve stimulation, vasodilators,

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and drugs that compensate for some of the metabolic changes in diabetes such as *n*-6 essential fatty acids, aldose reductase and protein kinase C inhibitors, anti-advanced glycation agents and antioxidants [3,8]. Fig. 1 shows the relationship between sciatic motor NCV and nutritive (capillary) endoneurial blood flow for groups of diabetic rats pooled from a large number of experiments in which various levels of these treatments were used (reviewed in 4), including antioxidants which are the main topic of this paper. These diverse treatments all fit the same relationship, as blood flow increases so does NCV, up to an asymptote which is within the normal range.

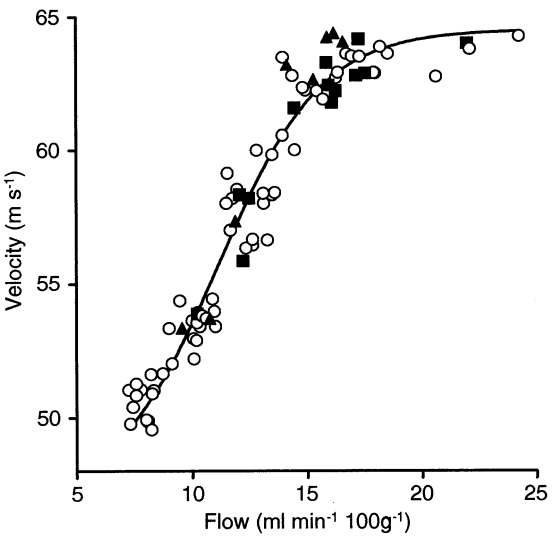

Fig. 1. Relationship between sciatic nutritive endoneurial blood flow and motor conduction velocity in groups of streptozotocin-diabetic rats given different drug treatments in our laboratory. The open circles correspond to groups treated with various vasodilators, essential fatty acids, and miscellaneous metabolically active compounds such as carnitine derivatives, aminoguanidine, sorbitol dehydrogenase inhibitors, protein kinase C inhibitors and myoinositol. Diabetes duration was 1–3 months and treatment was preventive or corrective. The filled squares represent groups treated with different antioxidants and the filled triangles are from aldose reductase inhibitor studies. All treatment effects appear to follow the same relationship; conduction velocity is low at low flow rates and reaches an asymptote which corresponds to the nondiabetic level as flow increases.

Reactive oxygen species (ROS) are increased in diabetes; the main sources are metabolic including autoxidation of glucose and its metabolises, advanced glycation, altered prostanoid production and abnormal or inefficient mitochondrial function [9,10]. In patients, levels of ROS rise with poor metabolic control [11]. ROS are also produced by macrophages during their respiratory burst, to destroy infectious organisms and in inflammatory diseases. For peripheral nerve, ROS can directly damage neurons and Schwann cells and in combination with diabetes antioxidant protection mechanisms are compromised. Thus, in diabetic rats, sciatic nerve lipid peroxidation was increased and levels of superoxide dismutase (SOD) and the reduced form of glutathione (GSH) were decreased, although there were no changes in glutathione peroxidase and reductase [12–15]. In the longer term, this could lead to cumulative neurodegenerative changes such as axonopathy and demyelination, as well as deleterious effects on cell bodies and their mitochondria as has recently been observed in dorsal root ganglion [15,16]. However, ROS also have effects on blood vessel function, which compromise perfusion of several organs including peripheral nerve. This is responsible for the earliest defects in nerve function in experimental models and will exacerbate nerve damage by causing further ROS-dependent ischemia-reperfusion effects [4,5].

Nitric oxide (NO) is an important vascular target for ROS. Superoxide neutralizes NO [17] and the peroxynitrite formed is a source of hydroxyl radicals that can cause endothelial damage [18]. Oxidative stress therefore diminishes vessel endothelium-dependent relaxation, which is apparent in some experimental preparations even after acute exposure to hyperglycemia [19,20]. Defective endothelium-dependent relaxation has been observed in chronic diabetic animals [21–26], and also in type 1 and type 2 diabetic subjects [27–31] and is an important potential target for antioxidant treatment.

Table 1
Antioxidant and related strategies used against neurovascular dysfunction in diabetic rats

Lipophilic scavengers	
Butylated hydroxytoluene	*Transition metal chelators*
Probucol and analogues	Deferoxamine
Vitamin E	Trientine
β-Carotene	α-Lipoic acid
Hydrophilic scavengers	*Indirect antioxidant actions*
Glutathione	Aldose reductase inhibitors
Vitamin C (also a prooxidant)	Aminoguanidine
Acetyl cysteine	
α-Lipoic acid	

2. Antioxidant effects on neural and vascular dysfunction in experimental diabetes

The various antioxidant strategies that have proved effective against neural and vascular complications in diabetic rats are summarized in Table 1. These include lipophilic free radical scavengers such as vitamin E, β-carotene, butylated hydroxytoluene and probucol as well as hydrophilic scavengers such as acetyl cysteine and vitamin C [21–23,32–38]. Currently, there is a substantial interest in α-lipoic acid which is both lipid and water soluble [14,39,40]. An alternative approach to scavenging ROS is to prevent their formation by autoxidation, the Fenton reaction and the advanced glycation process, all of which are catalyzed by free transition metal ions. This can be accomplished using transition metal chelators, including α-lipoic acid which acts as a chelator as well as a scavenger [41,42]. Some non antioxidant treatments are also included in Table 1 as they reduce oxidative stress. Aminoguanidine prevents AGE formation [43], and aldose reductase inhibitors (ARIs) can improve endogenous antioxidant defences by increasing tissue GSH levels [44,45].

A substantial number of studies have shown that antioxidant treatment can prevent or correct reduced motor and sensory NCV in diabetic rats. Where measured, there was accompanying improvement in nerve perfusion [32,34–36,38,40,41]. This is illustrated in Fig. 2 for probucol [34]. Diabetes causes a decrease in sciatic endoneurial nutritive (capillary) blood flow (Fig. 2A), which is prevented by probucol treatment. There are corresponding decreases in mean endoneurial oxygen tension (Fig. 2B) and motor NCV (Fig. 2C). Furthermore, diabetes causes an increase in plasma angiotensin converting enzyme activity (Fig. 2D), a marker of endothelial damage, which is attenuated by probucol treatment. Also shown in Fig. 2 is the effect of treating nondiabetic rats with the prooxidant and antimalarial agent, primaquine, which reproduced these effects of diabetes without any elevation of plasma glucose concentration, further supporting the notion that oxidative stress causes neurovascular dysfunction. Effects on plasma angiotensin converting enzyme levels suggest that there is increased activation of the vasoconstrictor renin–angiotensin system in diabetes, and studies with angiotensin converting enzyme inhibitors and angiotensin AT_1 receptor antagonists have shown that this is important for vasa nervorum [46]. Oxidative stress also stimulates endothelial endothelin-1 synthesis and this interacts with the angiotensin II system to increase vasa nervorum vasoconstriction [47].

The tendency towards increased vasoconstriction is exacerbated by reduced vasa nervorum NO synthesis or action [48,49]. This is reflected in other vessels and vascular beds and may be prevented by antioxidant treatments [21–23,42]. Fig. 3 shows an example of defective NO-mediated endothelium dependent relaxation from aortas of diabetic rats, which was prevented by treatment with the lipophilic scavenger, butylated hydroxytoluene [22]. Antioxidant effects to improve nerve blood flow and NCV in diabetic rats are blocked by co-treatment with a low dose of a NO synthase inhibitor [36], which further reinforces the argument that the effects on vasa nervorum endothelium are very important for antioxidant action and nerve function in experimental diabetes.

Hydrophilic scavengers such as acetyl cysteine also protect against NCV, NO and blood flow defects in diabetic rats [22,37,38]. Furthermore, acetyl cysteine allowed normal nerve maturation in young diabetic rats, preventing a reduction in mean nerve fiber size. It improved the regenerative response to nerve damage, inhibited an increase in plasma tumor necrosis factor activity,

and prevented red cell lipid peroxidation and a reduction in red cell GSH content [37,38]. Other antioxidants, such as vitamin E and butylated hydroxytoluene also prevent blunted nerve growth and regeneration in young diabetic rats, and in the galactosemic model of enhanced polyol pathway activation [50,51]. Similar phenomena were noted for vasodilator treatment [46] therefore it is likely that antioxidant-mediated improvements in nerve perfusion are largely responsible for growth and regeneration promoting actions.

Natural antioxidants present in the diet can influence NCV and blood flow in diabetic rats. However, very high doses which far exceed normal availability may be required; for example lg kg^{-1} day^{-1} of vitamin E was needed to give $\sim 80\%$ protection of sciatic motor NCV in diabetic rats [32]. For vitamin C, 150 mg kg^{-1} day^{-1} gave an optimal level of protection which was relatively modest at $\sim 35\%$. At high doses (500 mg kg^{-1} day^{-1}) protection was even less, perhaps because vitamin C can also act as a

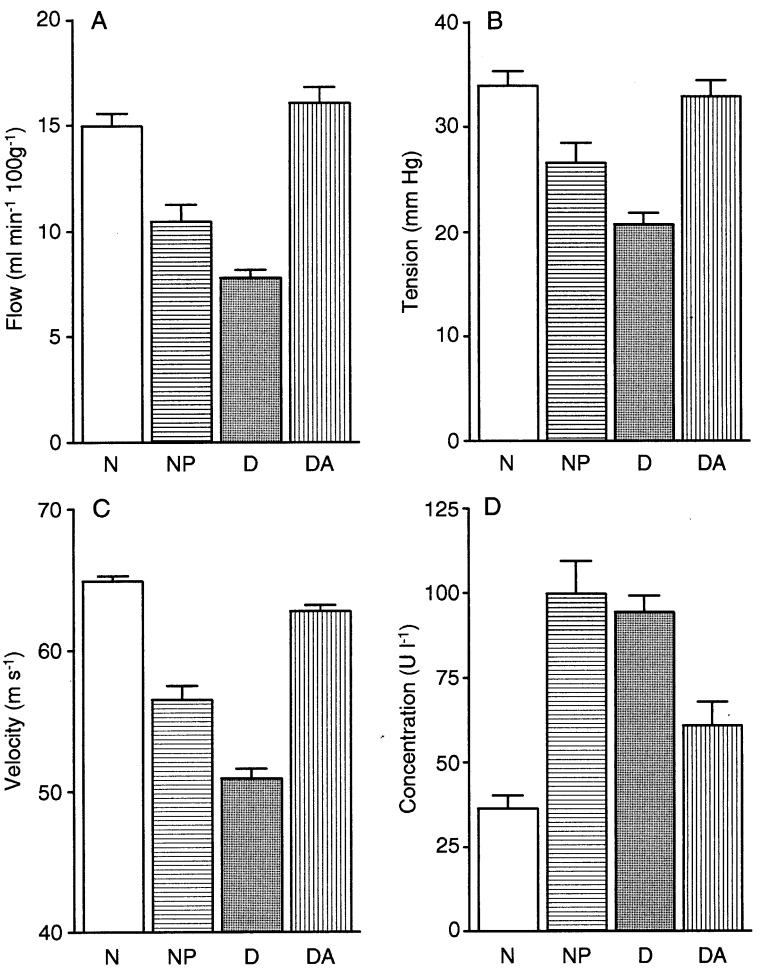

Fig. 2. Effects of antioxidant treatment with probucol in streptozotocin-diabetic rats and prooxidant treatment with primaquine in nondiabetic rats on (A) sciatic nutritive endoneurial blood flow; (B) sciatic mean endoneurial oxygen tension; (C) sciatic motor conduction velocity and (D) plasma angiotensin converting enzyme concentration. N, nondiabetic control; NP nondiabetic group treated with primaquine (0.5 mg kg^{-1} day^{-1}); D, diabetic control group, DA, diabetic group treated with probucol (1 g kg^{-1} day^{-1}); from [34].

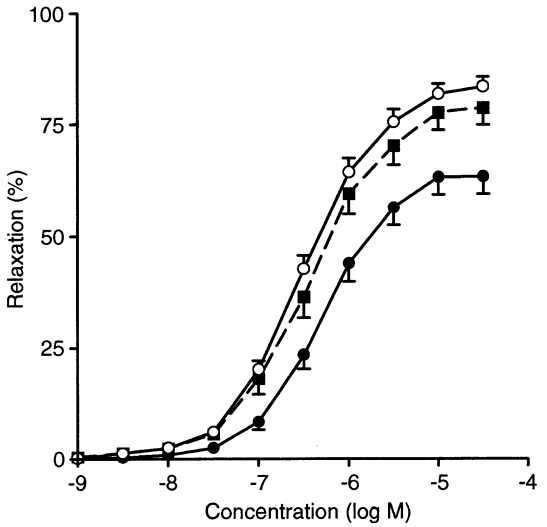

Fig. 3. Effects of diabetes and antioxidant treatment with butylated hydroxytoluene on endothelium-dependent relaxation of phenyephrine precontracted aortas to acetylcholine in vitro. Open circles, nondiabetic control group; filled circles, 8 week streptozotocin-diabetic group, filled squares, diabetic group treated with butylated hydroxytoluene (1 g kg^{-1} day^{-1}) from diabetes induction; from [22].

prooxidant due to autoxidation [32,52]. Under physiological conditions, it has been suggested that vitamin C helps to recycle vitamin E from its radical form [53], however, with the pharmacological doses used in the treatment of diabetic rats there was no evidence of a synergistic effect of vitamin C and E cotreatment. Instead, their effects were additive as if they were acting independently in lipid and aqueous phases [32].

Another natural antioxidant, α-lipoic acid, was at least ten times more effective than vitamin E in preventing or correcting blood flow and NCV defects in diabetic rats [14,40]. Both *R* and *S* enantiomers had similar efficacy on impaired nerve function and perfusion [40], as well as for the inhibition of lipid peroxidation of neural tissues in vitro [39]. α-Lipoic acid also prevented a reduction in sciatic nerve GSH, thereby preserving endogenous antioxidant protection [14]. Effects were also noted for small fiber function. In the isolated corpus cavernosum preparation from diabetic rats, chronic α-lipoic acid treatment prevented the development of a diabetic deficit in vasorelaxation to nitrergic nerve fiber stimulation [54]. Short-term multicenter studies in patients have demonstrated that α-lipoic acid has some efficacy against symptomatic and cardiac autonomic neuropathy [55,56].

The relatively high potency of α-lipoic acid compared to other scavengers could be due to the additional property of transition metal chelation. Low doses of deferoxamine (relatively specific for iron) and trientine (relatively specific for copper) corrected sciatic nerve blood flow and NCV deficits in diabetic rats [41]. Furthermore, trientine treatment prevented the impaired maturation of NCV in young diabetic and galactosemic rats and improved regenerative responses to nerve damage [50]. In vessels such as aorta, chronic trientine and deferoxamine treatment prevented the development of defective NO-mediated endothelium-dependent relaxation [42,57]. Thus, transition metal catalyzed ROS production makes an important contribution to nerve and vascular dysfunction in experimental models related to diabetes.

Increased flux through the first half of the polyol pathway, catalyzed by aldose reductase, contributes to oxidative stress. The reductions in nerve GSH content found in diabetes and galactosemia are rapidly corrected by ARI treatment [45], which also prevented an increase in nerve malondialdehyde, a marker of lipid peroxidation, in diabetic rats [58]. ARIs prevent or correct nerve blood flow and NCV defects in diabetic rats [59,60]. They improve endothelium-dependent relaxation in vessels from diabetic animals or vessels from nondiabetic animals acutely exposed to elevated glucose concentrations [3]. One polyol pathway linked mechanism that could be important for tissue antioxidant protection is the requirement of aldose reductase for NADPH as a cofactor, which is depleted by high polyol pathway flux. NADPH is also required by glutathione reductase for maintaining GSH concentrations, therefore, competition for NADPH in diabetes can diminish GSH levels. This can be restored by ARI treatment or by antioxidants such as α-lipoic acid which directly recycle GSH. Another potential effect of the polyol pathway is increased AGE formation. Thus, ARI treatment reduces tissue

AGE accumulation [61], perhaps by inhibiting the synthesis of fructose or by decreasing elevated flux through the pentose phosphate pathway; processes which produce sugars that are better glycating agents than glucose. Alternatively, ARI induced increases in tissue GSH and antioxidant capacity will reduce AGE formation by glycoxidation [62]. The AGE reactions are a source of ROS, therefore any reduction in them will decrease oxidative stress. Aminoguanidine irreversibly binds to reactive carbonyl intermediates, thus blocking AGE formation. Chronic aminoguanidine treatment has similar consequences to antioxidants and ARIs in improving NCV and blood flow in diabetic rats, effects which are blocked by NO synthase inhibitor cotreatment [3,4,63,64].

Thus, it is clear that antioxidant strategies based on the use of ROS scavengers and transition metal chelators can be very effective against experimental models of diabetic neuropathy and vasculopathy. The high levels of reducing sugars in these models means that a 'scavenger alone' approach requires massive drug doses which would be unacceptable if directly translated to patients. However, it is possible that in patients with better glycemic control, more physiological doses for example, of vitamin E, could be effective. The use of agents with additional properties targeting the mechanisms of ROS production, such as α-lipoic acid, or combined therapy with drugs that improve endogenous protection mechanisms, such as ARIs could be an appropriate future strategy.

3. Interaction between antioxidants and essential fatty acids

Polyunsaturated fatty acids are a major target for ROS damage. This includes *n*-6 and *n*-3 essential fatty acids, which are necessary for normal membrane structure and fluidity, and eicosanoid production. The common dietary sources are linoleic acid (*n*-6) and/or α-linolenic acid (*n*-3) which are further metabolized by a series of desaturation and elongation steps to produce several polyunsaturated fatty acids, including arachidonic acid (*n*-6) and eicosapentaenoic acid (*n*-3) which are major precursors of prostanoids, leukotrienes and other mediators. Diabetes reduces the rate limiting desaturation steps, particularly delta-6 desaturation which converts linoleic acid to γ-linolenic acid (GLA) and α-linolenic acid to stearidonic acid. Thus, the reduced availability of essential fatty acid intermediates in diabetes is further exacerbated by increased destruction due to elevated ROS.

For vasa nervorum diabetes reduces the synthesis of the vasodilator prostacyclin, mainly due to reduced availability of arachidonic acid [4]. Nerve blood flow and NCV deficits in diabetic rats may be corrected by treatment with *n*-6 metabolites downstream of the delta-6 desaturation step, particularly GLA and arachidonic acid [65]. Compared to *n*-6 components, *n*-3 essential fatty acids only have modest effects on nerve dysfunction in diabetic rats [3]. Thus, the vascular endothelium NO deficit in diabetes is compounded by a prostacyclin defect as well as enhanced vasoconstriction due to angiotensin II and endothelin-1 [3,4,46,47]. Normally, these systems interact to provide an integrated local system for nerve blood flow control. However, diabetes disrupts several mechanisms simultaneously, which disintegrates control and shifts the balance towards vasoconstriction. These systems are mutually interactive. Chronic treatment of nondiabetic rats with moderate doses of cyclooxygenase or NO synthase inhibitors caused modest NCV deficits. However, with joint treatment, there was a 5-fold amplification of NCV defects compared to that expected for simple summation, suggesting that compensatory synergy between prostanoid and NO systems normally makes vasa nervorum relatively resistant to a single inhibitory action [4].

Recent studies have shown that it is possible to make use of these synergistic interactions to gain a potential therapeutic advantage in diabetic rats. Thus, an experiment comparing the effects of treatment with low dose GLA or the probucol analogue, BM150639, either alone or in combination, showed that there was an approximately 7.5-fold increase in drug efficacy on NCV and blood flow for joint treatment [66]. ARIs also had synergistic interactions with GLA-containing

evening primrose oil to give a 10-fold increase in drug effects [64]. This approach has been followed up with the development of new drugs containing antioxidants and GLA. Although vitamin C was not very effective in correcting NCV and blood flow deficits in diabetic rats [32] it nevertheless enhanced GLA action. Thus the novel compound, ascorbyl-6-GLA, was 4.4-times more potent than GLA alone [67]. Recent attention has focused on α-lipoic acid and GLA combinations, an example of which is shown in Fig. 4. The effects on endoneurial blood flow of low doses of GLA and α-lipoic acid were not statistically significant, however, when combined, blood flow was in the nondiabetic range. NCV changes paralleled those for blood flow. A similar efficacy was noted for the novel drug, GLA-lipoic acid [40].

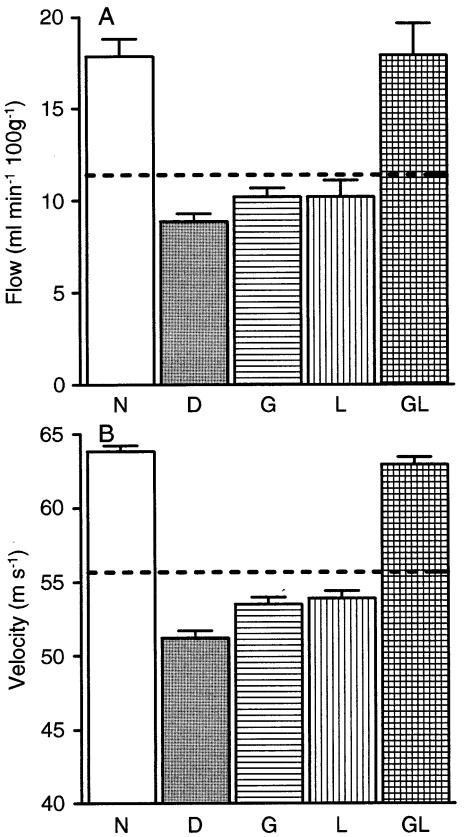

Fig. 4. Effects of low doses of γ-linolenic acid and α-lipoic acid treatment, alone and in combination, on (A) sciatic endoneurial blood flow and (B) sciatic motor conduction velocity in streptozotocin-diabetic rats. N, nondiabetic control group; D, 8 week diabetic control group; G or L, 8 week diabetic group treated for the final 2 weeks with γ-linolenic acid (20 mg kg^{-1} day^{-1}) or α-lipoic acid (20 mg kg^{-1} day^{-1}); GL, 8 week diabetic group given joint γ-linolenic acid-α-lipoic acid treatment for 2 weeks. The horizontal dashed lines indicate the expected levels of blood flow or conduction velocity for simple addition of drug effects. The GL group markedly exceeded this level for both measures, indicating a synergistic drug interaction; from [40].

4. Conclusions

Antioxidant studies have shown that oxidative stress makes a marked contribution to the etiology of nerve dysfunction in experimental diabetes. Neurovascular effects predominate in the short term; ROS cause vascular endothelium dysfunction which reduces NO mediated vasodilation and increases local vasoconstrictor production and reactivity. This reduces nerve perfusion, causing endoneurial hypoxia which results in conduction deficits. Autoxidation of glucose and its metabolites, and other transition metal catalyzed reactions such as advanced glycation are important sources of ROS. Polyol pathway activity contributes to oxidative stress by compromising the glutathione redox cycle. There are powerful synergistic interactions between ROS/NO and *n*-6 essential fatty acid/prostanoid mechanisms in the control of nerve blood flow. One promising direction for future research and therapeutic intervention is to take advantage of this effect, using combined antioxidant-GLA treatment.

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