

# Vascular Impairment of Epineurial Arterioles of the Sciatic Nerve: Implications for Diabetic Peripheral Neuropathy

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## ■ Abstract

This article reviews the impact of diabetes and its treatment on vascular function with a focus on the reactivity of epineurial arterioles, blood vessels that provide circulation to the sciatic nerve. Another focus is the relationship between the dysregulation of neurovascular function and diabetic peripheral neuropathy. Diabetic peripheral neuropathy is a debilitating disorder that occurs in more than 50 percent of patients with diabetes. The etiology involves metabolic, vascular, and immunologic pathways besides neurohormonal growth factor deficiency and extracellular matrix remodeling. In the light of this complex etiology, an effective treatment for diabetic peripheral neuropathy has not yet been identified. Current opinion postulates that any effective

treatment for diabetic peripheral neuropathy will require a combination of life style and therapeutic interventions. However, a more comprehensive understanding of the factors contributing to neurovascular and neural dysfunction in diabetes is needed before such a treatment strategy can be developed. After reading this review, the reader should have gained insight into the complex regulation of vascular function and blood flow to the sciatic nerve, and the impact of diabetes on numerous elements of vascular reactivity of epineurial arterioles of the sciatic nerve.

**Keywords:** diabetic peripheral neuropathy · epineurial arteriole · oxidative stress · inflammatory stress · vascular reactivity · calcitonin gene-related peptide · C-type natriuretic peptide · ACE · neutral endopeptidase

## 1. Introduction

Peripheral neuropathy is a multi-faceted complication of diabetes that frequently leads to foot ulcers and may progress to ulceration and necrosis necessitating limb amputations [1]. Even though it is the most common complication of diabetes, the only recommended clinical treatment is good glycemic control, which only delays the onset and slows progression [2]. Diabetic peripheral neuropathy has been described by some investigators to be a disease of the vasculature leading to nerve ischemia and altered nerve function [3-6]. Other investigators have proposed that diabetic peripheral neuropathy is caused by a combination of metabolic defects associated with an increased flux of glucose through the aldose reductase pathway, leading to a defect in Na<sup>+</sup>/K<sup>+</sup>-ATPase activity

and an alteration of signal transduction pathways in the nerve [7, 8]. Additional pathological contributors to diabetic peripheral neuropathy have been reported to include increased formation of advanced glycation endproducts, reduced neurotrophic support, and increased inflammatory and oxidative stress [9, 10]. Overall, these mechanisms and likely others cause damage to neurons, Schwann cells, and the vasculature. Ultimately, relentless damage to the nerve complex and surrounding vasculature leads to diabetic peripheral neuropathy. Given the complex etiology of diabetic peripheral neuropathy, a successful treatment will likely require a combination of early detection, lifestyle changes, and pharmaceutical interventions targeting the mechanisms deemed most responsible for the pathogenesis. Before this can occur, additional studies are needed to determine the most

relevant and targetable pathways of diabetic peripheral neuropathy.

My laboratory has focused on the role of microvascular dysfunction in the development and progression of diabetic peripheral neuropathy. Our studies in both type 1 and type 2 diabetic rats have demonstrated that impaired vascular reactivity precedes the development of nerve dysfunction, as identified by reduced nerve conduction velocity [11, 12]. Moreover, our studies have indicated that increased oxidative stress is one factor leading to impaired neurovascular function in diabetes [13, 14]. Other studies have implicated the formation of excess advanced glycation endproducts (AGEs) in the onset of microvascular complications of diabetes [15-17]. Agents that block the formation of AGEs or its receptor (RAGE) are considered to be therapeutic targets for diabetic vascular complications [18-21]. Inhibition of AGE formation has also been shown to improve peripheral neuropathy in diabetic rodents [22-24]. Additional studies have shown that inhibition of aldose reductase can improve vascular dysfunction and peripheral neuropathy in diabetic rodents [11, 25-27]. Based on this work and others, it is clear that diabetic neurovascular dysfunction is a contributing factor to diabetic peripheral neuropathy [28]. Therefore, successful treatment of diabetic peripheral neuropathy needs to include interventions that improve vascular dysfunction. This article reviews the regulation of vascular reactivity of epineurial arterioles and blood vessels that provide circulation to the sciatic nerve, and discusses the current knowledge about diabetes-induced vascular impairment in these blood vessels.

## 2. Vascular reactivity of epineurial arterioles of the sciatic nerve

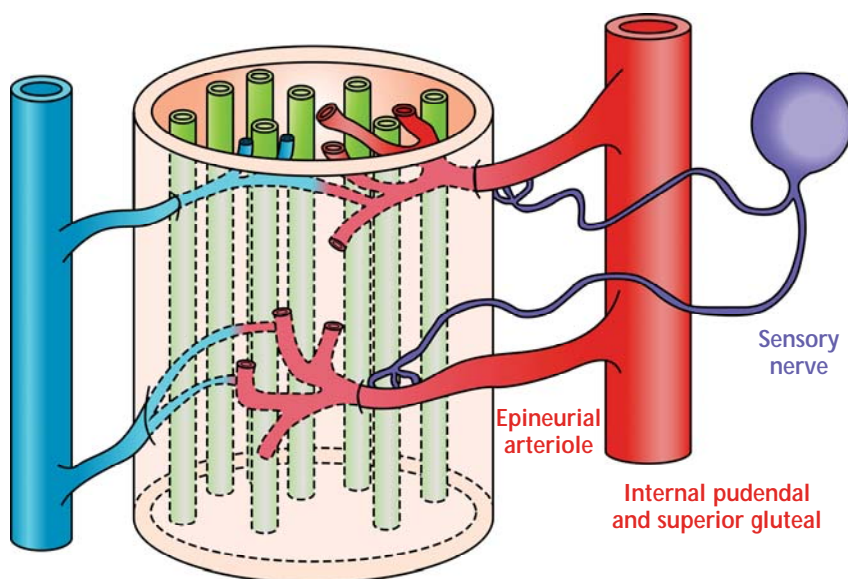
Determination of endoneurial nutritive blood flow has been a common endpoint to investigate the effect of diabetes on vascular function and diabetic peripheral neuropathy in rats, with many investigators finding an early reduction of endoneurial blood flow in the sciatic nerve of rodents with diabetes [29-35]. However, the method employed, hydrogen clearance, and the conditions maintained during the experimental procedure have contributed to controversy regarding the validity of this measurement and the contribution of changes in endoneurial blood flow to diabetic peripheral neuropathy [36-39]. Furthermore, measurement of endoneurial blood flow does not provide a direct examination of the impact of diabetes on vascular reactivity. This led us to pursue the use of video

### Abbreviations:

ACE – angiotensin-converting enzyme  
ADP – adenosine diphosphate  
AGE – advanced glycation endproducts  
ASIC-2 – acid-sensing ion channel 2  
ATP – adenosine triphosphate  
NAD(P)H – nicotinamide adenine dinucleotide phosphate  
Nox – NAD(P)H oxidase  
RAGE – receptor of advanced glycation endproducts  
ROS – reactive oxygen species  
SHR – spontaneously hypertensive rat  
SOD – superoxide dismutase

microscopy to investigate the *in vitro* vasodilatory responsiveness of arterioles vascularizing the region of the sciatic nerve [40]. **Figure 1** provides an illustration of the epineurial arterioles we studied. These small vessels are generally oriented longitudinally in relation to the sciatic nerve and extend branches that penetrate the sciatic nerve. They are described as being epineurial rather than endoneurial to the sciatic nerve. The blood vessels used in our studies were second or third order branches of the internal pudendal and superior gluteal arteries [40]. The methodology used to isolate these blood vessels and to examine their vascular responsiveness *in vitro* using an organ chamber and video imaging has been previously described (**Figure 2**) [40, 41].

Relaxation of epineurial arterioles of the sciatic nerve is mediated through a variety of mechanisms, suggesting that they have an important role in regulating blood flow to the sciatic nerve. Initial studies have demonstrated that acetylcholine-mediated vascular relaxation of pre-constricted epineurial arterioles is induced by two distinct endothelium-dependent mechanisms: 1) production of nitric oxide and 2) endothelium-derived hyperpolarizing factor [40]. Both diabetes and diet-induced obesity cause an impairment of acetylcholine-mediated vascular relaxation [42-44]. With diabetes, impairment of acetylcholine-mediated vascular relaxation occurred after 1-2 weeks of onset of hyperglycemia [11, 45]. Our studies have also identified C-type natriuretic peptide and calcitonin gene-related peptide as active vasodilators of epineurial arterioles [41, 46]. C-type natriuretic peptide is localized in endothelial cells of the vessel wall, and has properties consistent with endothelium-derived hyperpolarizing factor-like activity [46]. The exogenous vasodilator activity of C-type natriuretic peptide is decreased by diabetes. Epineurial arterioles of the sciatic nerve are innervated by nonadrenergic, noncholinergic nerves that immunostain for calcitonin gene-



**Figure 1. Epineurial arterioles innervating the sciatic nerve.** Epineurial arterioles are resistance size blood vessels branching from internal pudendal and superior gluteal arteries. These blood vessels regulate blood flow to the sciatic nerve, and have unique properties including innervation by sensory nerves that contain vasoactive peptides such as calcitonin gene-related peptide. The study of these blood vessels has yielded an array of interesting findings including early impairment of vascular relaxation by diabetes. Illustration provided by Soroku Yagihashi.

related peptide; exogenous calcitonin gene-related peptide is a very potent vasodilator. In type 1 diabetic rats, the calcitonin gene-related peptide content of sensory nerves innervating the epineurial arterioles and the vasodilation response to exogenous calcitonin gene-related peptide is decreased [41]. Insulin was also found to have a weak vasodilatory effect on epineurial arterioles [44].

Vasoconstriction also contributes to the regulation of the vascular tone. Some compounds induce vasoconstriction in epineurial arterioles of the sciatic nerve, including vasopressin, neuropeptide Y, serotonin, and endothelin [47]. Epineurial arterioles are innervated by sensory nerves that contain neuropeptide Y [47]. When isolated epineurial arterioles were treated with capsaicin to induce vasodilation and the release of calcitonin gene-related peptide, it was surprising to find that capsaicin caused a concentration-dependent vasoconstriction [47]. The effect of capsaicin was transient, refractory, blocked by capsazepine, and duplicated by resiniferatoxin [47]. In long-term diabetic rats, capsaicin-induced vasoconstriction was attenuated. However, long-term diabetes did not impair vasoconstriction of epineurial arterioles in re-

sponse to vasopressin, neuropeptide Y, serotonin, or endothelin [47]. Epineurial arterioles were also found to express receptors to vanilloid-1. In long-term diabetic rats, immunoreactivity for vanilloid receptor-1 was decreased [47]. Other interesting observations were that epineurial arterioles of the sciatic nerve expressed acid-sensing ion channel 2 (ASIC-2), and that decreasing pH caused vasoconstriction, which was prevented by pre-incubating the vessels with amiloride (**Figure 3**). Based on these observations, it was concluded that vascular reactivity of epineurial arterioles that provide circulation to the sciatic nerve is regulated by several mechanisms, suggesting that control of blood flow to the sciatic nerve involves a complex interaction of vaso-regulatory compounds.

### 3. Pathogenesis of diabetes and neurovascular tissue

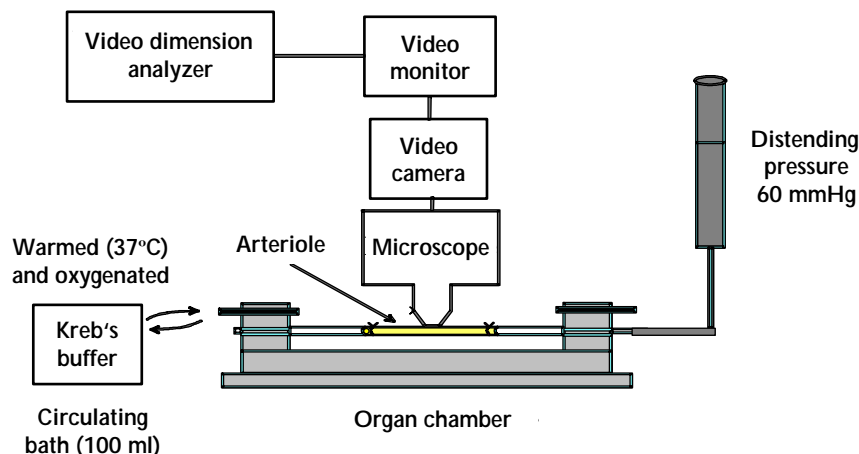
#### 3.1 Role of reactive oxygen species in diabetes-induced vascular dysfunction

Overabundance of reactive oxygen species (ROS) has long been considered to contribute to vascular dysfunction and diabetes complications [48-50]. Free radicals such as  $O_2^-$  and  $OH^\cdot$  cause vascular endothelial damage and reduced nitric oxide-mediated vasodilation. Angiotensin II formation or activity, AGE formation, and autooxidation are major sources of free radicals. Inhibition of these radicals by angiotensin-converting enzyme (ACE) inhibitors/angiotensin receptor blockers, aminoguanidine, transition metal chelators, antioxidants, or free radical scavengers have been shown to improve the diabetes-induced decrease in vascular function of epineurial arterioles, endoneurial blood flow, and neural dysfunction [13, 14, 51-58].

Impaired endothelium-dependent vasodilation has been demonstrated in various vascular beds of

animal models of diabetes and humans with type 1 and type 2 diabetes [59, 60]. Some of the mechanisms attributed to diabetes-induced endothelium dysfunction include impaired signal transduction pathways or substrate availability, impaired release or increased metabolism of vasodilatory mediators, increased release of vascular constricting factors, and decreased reactivity of the smooth muscle to vasodilatory mediators [59, 60]. The mechanisms induced by hyperglycemia/diabetes considered to contribute to vascular dysfunction are the activation of protein kinase C, increased activity of the polyol pathway, increased formation of AGEs, increased levels of angiotensin II, and abnormal mitochondria activity. Interestingly, studies by Brownlee and colleagues have suggested hyperglycemia-induced production of  $O_2^-$  by mitochondria of endothelial cells as the common link for mechanisms of diabetes-induced vascular dysfunction [61, 62]. Our studies conducted with intact vascular tissue consisting of epineurial arterioles of the sciatic nerve support the studies by Brownlee and colleagues, which were conducted with cultured endothelial cells [63]. Studies by my laboratory have provided evidence that the generation of oxidative stress, through the production of  $O_2^-$  and peroxynitrite, impairs vascular function and endothelium-dependent vascular relaxation of epineurial arterioles of the sciatic nerve from diabetic rats, which precedes the slowing of motor nerve conduction velocity [11, 13, 14, 51, 52].

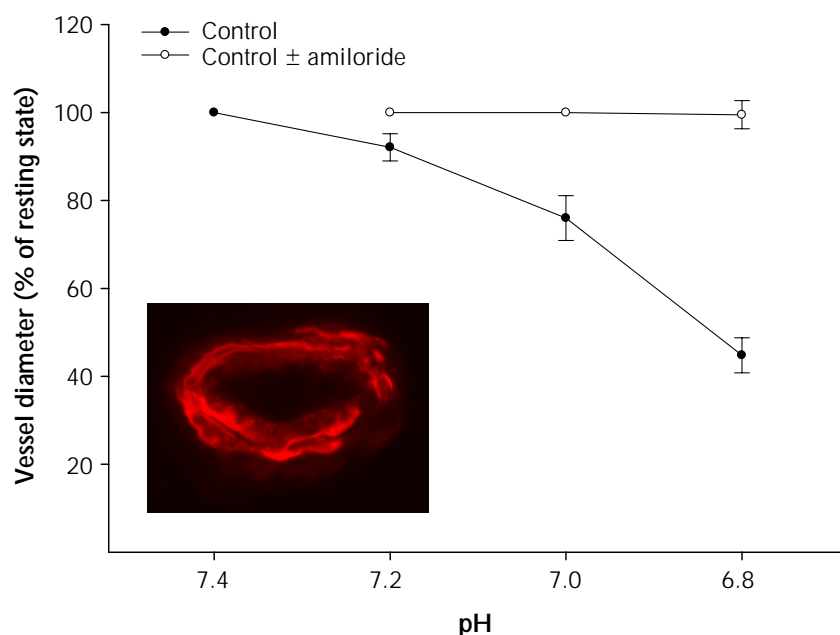
Studies designed to investigate the source of superoxide formation provided results suggesting that complex I of the mitochondrial electron transport chain and possibly NAD(P)H oxidase are responsible for the increase in  $O_2^-$  formation observed in epineurial arterioles derived from diabetic rats



**Figure 2. Vascular reactivity by videomicroscopy.** This device is used to examine vascular reactivity of epineurial arterioles of the sciatic nerve. Isolation of these blood vessel branches of the superior gluteal and internal pudendal arteries (50 to 150  $\mu$ m internal diameter and 1-2 mm in length) is carried out by carefully dissecting and trimming fat and connective tissue. Both ends of the isolated vessel segment (in yellow) are then cannulated with glass micropipettes filled with physiological saline solution (PSS), and secured with nylon monofilament sutures. The pipettes are then attached to a single pressure reservoir (initially set at 0 mmHg) under conditions of "no flow". The organ chamber containing the cannulated vessels is fixed to the stage of an inverted microscope. Attached to the microscope are a closed-circuit television camera, a video monitor, and a video caliper. The organ chamber is connected to a rotary pump, which continuously circulates 37°C oxygenated PSS at 30 ml/min. The pressure within the vessel is increased to 60 mmHg over a period of 30 min. Internal vessel diameter (resolution of 2  $\mu$ m) can be measured by manually adjusting the video micrometer. Vasodilation studies are performed with vessels preconstricted with U46619 to 30-50% of resting diameter.

[63]. Furthermore, pretreatment of epineurial arterioles from diabetic rats with the protein kinase C inhibitor bisindolylmaleimide (GF 109203X) improved acetylcholine-mediated vascular relaxation, but did not prevent the increase in  $O_2^-$  formation, suggesting that activation of protein kinase C by oxidative stress is downstream of  $O_2^-$  formation [63]. We have also demonstrated that treatment of diabetic rats with three different types of antioxidants or chelators ( $\alpha$ -lipoic acid, hydroxyethyl de-feroxamine, or M40403) prevents the diabetes-induced increase in  $O_2^-$  and peroxynitrite formation in aorta and epineurial arterioles of the sciatic nerve and diabetes-induced vascular and neural dysfunction, thereby providing additional evidence that increased oxidative stress contributes to diabetes-induced vascular and neural disease [13, 14].

Studies from other laboratories have provided further evidence that antioxidants may prevent



**Figure 3.** Effect of pH on vasoconstriction of epineurial arterioles of the sciatic nerve. Vascular relaxation in response to pH is examined using the videomicroscopy system, as described in **Figure 2**. Epineurial arterioles are incubated with or without amiloride for 30 min before examining the effect of pH on vascular reactivity. As shown, a decrease in pH causes vasodilation of epineurial arterioles that is totally prevented by amiloride. The image is a representation of immunostaining for ASIC 2 in a cross section of an epineurial arteriole of the sciatic nerve. These data demonstrate the expression and functionality of ASIC in epineurial arterioles of the sciatic nerve.

vascular complications in diabetes. Treating diabetic rats with tempol, a stable superoxide dismutase mimic compound, abolished the diabetes-induced increase in vascular  $O_2^-$ , malondialdehyde, and 8-epi-prostaglandin  $F(2\alpha)$  production, and also the impairment in relaxation of aortic rings to acetylcholine [64]. Cameron and colleagues have demonstrated that treatment of diabetic rats with  $\alpha$ -lipoic acid, or the metal chelators hydroxyethyl starch deferoxamine, or trientine prevent the diabetes-induced impairment in vascular relaxation associated with hyperalgesia and neurovascular deficits [54, 55, 65-67]. In addition, Keegan *et al.* found that treating diabetic rats with  $\alpha$ -lipoic acid improves endothelium-dependent vascular relaxation of corpus cavernosum smooth muscle [57]. These studies imply that increased  $O_2^-$  formation via the mitochondrial electron transport chain and perhaps NAD(P)H oxidase are partially responsible for reduced vascular reactivity observed in epineurial arterioles of the sciatic nerve and other vascular tissues in diabetic rats [63]. Because

metal chelators and OH scavengers have been demonstrated to effectively prevent diabetes-induced vascular and neural dysfunction, it is likely that the formation of OH may also contribute to impairment of vascular reactivity and nerve function in diabetes [51, 54, 55, 58, 65-68].

Besides antioxidants, therapies designed to improve nitric oxide formation have been shown to be beneficial in improving diabetes-induced vascular dysfunction. Treatment of diabetic rats with arginine or a tetrahydrobiopterin derivative improved vascular function in diabetic rats [69-75]. This implies that reduced availability of nitric oxide due to increased degradation, such as scavenging of nitric oxide by  $O_2^-$  to form peroxynitrite or reduced production of nitric oxide in the diabetic state, may lead to vascular impairment. Studies performed in my laboratory

support the role of peroxynitrite in the pathogenesis of diabetic vascular and neural complications. Our studies demonstrated that treating diabetic rodents with a protein nitration inhibitor or peroxynitrite decomposition catalyst prevents vascular dysfunction and improves peripheral neuropathy in diabetic rodents [76, 77].

### 3.2 Prevention of oxidative stress as treatment for diabetic vascular and neural dysfunction

Many therapeutic approaches for the prevention of diabetic peripheral neuropathy have been applied successfully in animal models of diabetes, indicating that the etiology of the disease is multifaceted and complex. This has led investigators to propose a wide range of mechanisms for the cause of diabetic peripheral neuropathy. Some of the different treatments that have been demonstrated to improve or prevent peripheral neuropathy in diabetic animal models include:

- Aldose reductase inhibitors
- Aminoguanidine
- Antioxidants
- Inhibitors of protein kinase C
- Vasodilators
- Nerve growth factor
- $\gamma$ -linolenic acid
- Omega-3 polyunsaturated fatty acids derived from fish oils
- Acetyl L-carnitine
- Myo-inositol
- Gangliosides
- Inhibitors of poly(ADP-ribose) polymerase and others [78-92].

At this time, not all of these treatment protocols have been applied to human diabetes, although the results from those that have been used have been disappointing. This raises the question of why these treatment protocols generally failed in human trials after being successful in diabetic animal models. There are many reasons that could explain this unfavorable outcome, including the following:

1. Due to side effects or other concerns the dosage of the drugs used in human studies may have been inadequate and the efficacy too low to be effective against the targeted mechanism compared to animal studies. This is a valid concern and outcomes from studies using aldose reductase inhibitors, nerve growth factor, and antioxidants may have been influenced by this problem.
2. The targeted mechanisms for treating diabetic peripheral neuropathy in animal models may be incorrect, and have no direct contribution to diabetic peripheral neuropathy in humans. This appears unlikely as each of the compounds mentioned above, and their targeted mechanisms, are based on valid scientific data from both animal and human studies. However, whether each of the targeted mechanisms plays a significant role in the development of diabetic peripheral neuropathy in humans is a point of conjecture. Also, it is unlikely that one single mechanism is solely responsible for the development of diabetic peripheral neuropathy, and that combination therapy targeting two or more mechanisms will probably be necessary to treat diabetic peripheral neuropathy.
3. The patients participating in the human studies were inappropriate for the targeted mechanism because their complications have

progressed to the point that the symptoms or endpoints being examined are not readily reversible. This is certainly a valid concern in studies of human diabetic peripheral neuropathy. Measurement of nerve conduction and symptom scores are the standard endpoints for many clinical trials. It takes many years of diabetes to develop deficits in nerve conduction velocity. It is possible that drug intervention in human diabetic peripheral neuropathy will likely have minimal benefit when patients have clinical symptoms that took many years to develop. The reason may be that most of these patients will probably have nerve damage that is either irreversible or only slowly reversed. Positive clinical outcomes would likely take longer to achieve than the period applied in the clinical trial. For instance, many of the clinical trials conducted with aldose reductase inhibitors were designed with a year of treatment or less. Considering the severity of the progression of diabetic peripheral neuropathy in patients who participated in these studies this treatment period is likely too short.

4. Outcomes were based on invalid endpoints. For instance, measurement of sorbitol for interventions using aldose reductase inhibitors may not be a good outcome predictor for the patient. Measurement of sorbitol may provide good evidence of the current metabolism, but may not reflect the severity or progression of the disease. In the case of human diabetic peripheral neuropathy, treatment with aldose reductase inhibitors may provide control of glucose flux through the aldose reductase pathway, and measurement of the level of sorbitol in tissue samples or cells may provide information on the efficacy of aldose reductase inhibitor treatment, but likely will not provide any indication of the present extent of nerve damage or progression of the disease. In order to better analyze the progression of diabetic peripheral neuropathy several relevant markers of nerve function and biological status including oxidative stress will probably be required.

These issues and failures of clinical trials have led to attempts to identify earlier markers for diabetic peripheral neuropathy. Promising research in animal models and humans have focused on examining density of the sensory nerves in the skin or cornea as an early and sensitive biomarker for diabetic peripheral neuropathy [93, 94]. Our work

has indicated that loss of corneal nerves in the region of the whorl of the sub-basal plexus and penetration of the cornea epithelium are early markers for diabetic peripheral neuropathy [95, 96].

One of the most promising approaches for intervention and treatment of diabetic peripheral neuropathy and other diabetic complications is still the prevention of oxidative stress [97]. A variety of antioxidants including vitamin E have beneficial effects in treating diabetic peripheral neuropathy in diabetes patients and diabetic animal models [98-100]. More recently,  $\alpha$ -lipoic acid has shown promise as a potential antioxidant treatment for diabetic peripheral neuropathy [101-103]. Our studies have demonstrated that  $\alpha$ -lipoic acid provides a good protection against oxidative stress in diabetic rats of 4-6 week duration [13]. The treatment of diabetic rats with  $\alpha$ -lipoic acid significantly improved the diabetes-induced decrease in endoneurial blood flow, endothelium-dependent vascular relaxation in arterioles that provide circulation to the region of the sciatic nerve, and motor nerve conduction velocity.  $\alpha$ -lipoic acid treatment also reduced the production of superoxide by the aorta and superoxide and peroxynitrite by arterioles that provide circulation to the region of the sciatic nerve. Treatment of diabetic rats with  $\alpha$ -lipoic acid prevented the diabetes-induced increase in thiobarbituric acid-reactive substances in serum, and significantly improved lens glutathione levels.

$\alpha$ -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 [104-106]. However, in its reduced form, as dihydrolipoic acid, it is a good scavenger of superoxide and prevents initiation of lipid peroxidation [104-106]. *In vivo*,  $\alpha$ -lipoic acid can be converted into dihydrolipoic acid [102, 104]. This reaction requires NAD(P)H, which is reduced in diabetes due to the increased flux of glucose through the aldose reductase pathway [107, 108]. Therefore, one potential form of combination therapy for the treatment of diabetic peripheral neuropathy may be combining an aldose reductase inhibitor with  $\alpha$ -lipoic acid. This combination should promote the formation of dihydrolipoic acid, thereby enhancing the antioxidant potential of  $\alpha$ -lipoic acid, and possibly providing a synergistic effect [25]. In a study of diabetic peripheral neuropathy in streptozotocin-induced diabetic rats conducted by Nakamura *et al.*, the authors found that treating diabetic rats with the aldose reductase inhibitor NZ-314 improves nerve function and reduces oxidative stress [109]. They concluded that

the efficacious effect of aldose reductase inhibition on diabetic peripheral neuropathy may be mediated by decreasing oxygen free radicals [109]. This would agree with our studies, demonstrating that treating diabetic rats with sorbinil improves glutathione levels presumably by correcting the redox imbalance [52]. We postulate that a similar mechanism may apply for the conversion of  $\alpha$ -lipoic acid to dihydrolipoic acid. Correcting the redox imbalance in diabetic rats with an aldose reductase inhibitor may promote the production of dihydrolipoic acid *in vivo* [25].

The development of superoxide dismutase mimetics are another class of drugs with potential for treatment of diabetic complications, including diabetic peripheral neuropathy [14, 110, 111]. Because of limitations associated with enzyme therapies these non-peptidyl compounds may offer advantages resulting in better clinical therapies and outcomes for diseases mediated by  $O_2^-$  radicals such as diabetes [112]. In our studies, we demonstrated that treating diabetic rats with M40403 inhibits the generation of superoxide by aorta and epineurial arterioles of the sciatic nerve, the formation of peroxynitrite by epineurial arterioles of the sciatic nerve, the reduction in endoneurial blood flow, the slowing of motor nerve conduction velocity, and the impairment of endothelium-dependent vasodilation of epineurial arterioles. The treatment also improved the diabetes-induced increase in serum thio-barbituric-reactive substances and sciatic nerve-conjugated diene level, two additional markers of oxidative stress [14]. M40403 is a prototypic example of a stable, low molecular weight, manganese-containing, non-peptidic molecule possessing the function and catalytic rate of native superoxide dismutase (SOD) enzymes, but with the advantage of being a much smaller molecule (molecular weight 483 vs. 30,000 for M40403 and the native enzyme, respectively) [14, 112, 113].

Another form of treatment for diabetic peripheral neuropathy, yet to be thoroughly examined, is the intervention with ACE inhibitors and/or angiotensin receptor antagonists. It has been demonstrated that these drugs have antioxidant properties, neuroprotective potential, and may reduce the accumulation of AGEs [114-119]. Angiotensin II causes endothelium dysfunction by increasing NAD(P)H oxidase-mediated vascular  $O_2^-$  production [120, 121]. It has been shown that two isoforms of the NAD(P)H oxidase family (Nox), Nox1 and Nox4, are involved in the vascular oxidative stress pathways in response to angiotensin II [121]. The renin-angiotensin system is highly acti-

vated in patients with type 2 diabetes. Evidence suggests that intrarenal renin-angiotensin system activation is responsible for non-ACE-dependent angiotensin II production within the kidney and for the resulting low-renin state in patients with diabetes [122]. Hyperglycemia (both acute and sustained) and obesity activate the renin-angiotensin system [122]. A wide range of evidence now exists demonstrating that blocking of the renin-angiotensin system in diabetic patients reduces the progression of diabetic nephropathy [122]. However, little information is available whether treatment of diabetic patients with ACE inhibitors or angiotensin receptor antagonists improves or prevents the progression of diabetic neurovascular and neural disease. To provide an answer as to whether blocking the renin-angiotensin system can prevent diabetic peripheral neuropathy two issues must be addressed:

1. Does vascular dysfunction cause diabetic peripheral neuropathy?
2. Can ACE inhibitors and/or angiotensin receptor antagonists ameliorate diabetic vascular dysfunction and hence neuropathy [123]?

Existing data suggests that vascular dysfunction contributes significantly to the development and progression of diabetic peripheral neuropathy [13, 14, 52, 53]. It has also been demonstrated that treatment with ACE inhibitors improves endothelial dysfunction and reduces oxidative stress in diabetes [114, 118, 124]. Therefore, it seems likely that ACE inhibitors and/or angiotensin receptor antagonists may improve diabetic peripheral neuropathy. In studies by Cameron and colleagues with diabetic animal models, treatment of diabetic rats with an ACE inhibitor or with an angiotensin receptor antagonist improved motor and sensory nerve conduction velocities, increases endoneurial blood flow, and stimulated endoneurial angiogenesis [125, 126]. Likewise, Aggarwal *et al.* have demonstrated that treating streptozotocin-induced diabetic rats with lisinopril, an ACE inhibitor, improves diabetic peripheral neuropathy [127]. We have demonstrated that treating diet-induced obese and diabetic rats with enalapril improves glucose utilization and endpoints associated with diabetic peripheral neuropathy, including impaired vascular relaxation to acetylcholine and calcitonin gene-related peptide [128-133]. In studies using streptozotocin-induced diabetic rats, we demonstrated that treating diabetic rats with

enalapril or the angiotensin receptor blocker L158809 improves diabetes-induced vascular and neural dysfunction [133]. Significant improvement was observed even when treatment was initiated 12 weeks after the onset of hyperglycemia.

We also found that treatment with enalapril is more effective in preventing/reversing diabetes-induced vascular and neural dysfunction than treatment with L158809. In contrast to reports suggesting synergy in treatment of diabetic nephropathy or hypertension by combination of ACE inhibitors with an angiotensin II receptor blocker, we found no evidence of synergy in the preventing/reversal of vascular and neural dysfunction related to diabetic peripheral neuropathy when we combined enalapril and L158809 [134-136]. We found that treatment of diabetic rats with enalapril or L158809 prevented/reversed superoxide formation by the aorta [132]. However, enalapril treatment was only partially effective and treatment with L158809 was non-effective in preventing/reversing superoxide formation in epineurial arterioles of the sciatic nerve. This is consistent with previous studies that have demonstrated that these drugs can prevent angiotensin II stimulation of NAD(P)H oxidase activity in large vessels associated with hypertension or diabetes, but subsequently increased the formation of superoxide [119, 120]. Our previous studies have suggested that the mitochondria may be the primary contributor to superoxide formation by epineurial arterioles derived from diabetic rats [63]. Therefore, it appears that enalapril and to a greater extent L158809 may have a limited capacity for preventing superoxide formation and oxidative stress in resistance size vessels.

Vasopeptidase inhibitors are a new class of drugs that simultaneously inhibit neutral endopeptidase and ACE activity [137]. Neutral endopeptidase is found in many tissues including vascular and renal tissue, and its activity is increased by fatty acids and glucose in human microvascular cells [138-142]. Interestingly, neutral endopeptidase is activated by protein kinase C, which is increased by diabetes in vascular tissues including endothelial cells [143, 144]. Neutral endopeptidase degrades natriuretic peptides, adrenomedullin, bradykinin, endothelin, and calcitonin gene-related peptide [145]. Therefore, the use of vasopeptidase inhibitors would likely promote expression of calcitonin gene-related peptide and C-type natriuretic peptide by blocking their degradation and thus, improving vascular functions. In this regard, vascular conductance in the

femoral artery of streptozotocin-induced diabetic rats is improved by a vasopeptidase inhibitor [146]. Vasopeptidase inhibitors have also been reported to decrease matrix metalloproteinases and AGE accumulation/formation in type 2 diabetes and to improve wound healing [147-149]. Finally, it has been reported that neutral endopeptidase is responsible for 50% of the degradation of glucagon-like peptide 1 [150].

In summary, there is great potential for the treatment of diabetic complications with vasopeptidase inhibitors. However, there is no information about the potential benefits of vasopeptidase inhibitors in diabetic peripheral neuropathy. To address this issue we performed studies using diet-induced obese rats, streptozotocin-induced diabetic rats, and Zucker diabetic fatty rats treated with ilepatril, a vasopeptidase inhibitor [46, 128, 129, 151-154]. These studies demonstrated for the first time that treatment of obese or diabetic rats (modeling types 1 or 2 diabetes) with a vasopeptidase inhibitor significantly improved vascular and neural function. Correction of acetylcholine-mediated vascular relaxation in epineurial arterioles, endoneurial blood flow, and motor nerve conduction velocity was nearly 100%, while sensory nerve conduction velocity was improved, and thermal hypoalgesia was prevented by about 75%. Ilepatril also prevented loss of sensory nerves in the skin and cornea [155]. Vasopeptidase inhibitor treatment also significantly reduced oxidative stress in the aorta and epineurial arterioles. Overall, the level of efficacy with ilepatril was the greatest we have observed compared to a variety of other interventions ranging from antioxidants, ACE inhibitors, angiotensin receptor antagonists, aldose reductase inhibitors, aminoguanidine, or myo-inositol [11-14, 25].

There are likely several reasons for this level of efficacy:

1. As we have demonstrated, epineurial arterioles of the sciatic nerve express both C-type natriuretic peptide and neutral endopeptidase. Moreover, in diabetes the expression of neutral endopeptidase was increased [46]. It has been demonstrated that neutral endopeptidase decreases the bioactivity of C-type natriuretic peptide, and that local concentrations of vasoactive peptides in the vessel wall are regulated by the neutral endopeptidase cleavage pathway in the immediate vicinity of their target cells [145, 156]. Therefore, in epineurial arterioles of obese and diabetic rats increased expression of neutral endopeptidase can decrease C-type natriuretic peptide bioactivity, and treatment of diabetic rats with ilepatril protects C-type natriuretic peptide from degradation.
2. As we previously demonstrated, treatment with antioxidants protects vascular and neural function in streptozotocin-induced diabetic rats [13, 14]. Since vasopeptidase inhibitors contain ACE inhibitor activity and the ACE inhibitor enalapril improved diabetes-induced vascular and neural dysfunction and reduced oxidative stress in vascular tissue (as we have also demonstrated), it is likely that ilepatril efficacy is partially due to antioxidant properties [128, 129, 131, 133]. This was supported by studies demonstrating that increased formation of superoxide and nitrotyrosine in epineurial arterioles of diabetic rats is significantly reduced by treatment with ilepatril [46].
3. It has been demonstrated that another target for neutral endopeptidase is calcitonin gene-related peptide [157]. Neutral endopeptidase plays a major role in the inactivation of calcitonin gene-related peptide released from sensory fibers. It actively degrades calcitonin gene-related peptide in human skin, whereas inhibition of neutral endopeptidase increases circulating plasma levels of calcitonin gene-related peptide [158-160]. We have demonstrated that epineurial arterioles of the sciatic nerve are innervated by sensory nerves that contain calcitonin gene-related peptide, and that calcitonin gene-related peptide is an important regulator of vascular tone and blood flow in the sciatic nerve [41]. Furthermore, we have demonstrated that the bioactivity of exogenous calcitonin gene-related peptide and calcitonin gene-related peptide expression are decreased in type 1 and type 2 diabetic rats [41, 45]. Since neutral endopeptidase expression is increased in diabetes, the decrease in calcitonin gene-related peptide bioactivity in diabetes could partially be due to increased degradation. Overall, a decrease in C-type natriuretic peptide and calcitonin gene-related peptide bioactivity *in vivo* would likely compromise vascular tone, impair blood flow, induce ischemia, and subsequently decrease neural function. The likelihood of vasopeptidase inhibitors becoming a treatment option however is very small.

Ilepatril and other vasopeptidase inhibitors were found to cause angioedema in a small number of human subjects, and further development is unlikely. However, Novartis is developing LCZ696, a first-in class dual angiotensin II receptor blocker and neutral endopeptidase inhibitor [161]. This combination drug has been shown to reduce hypertension in spontaneously hypertensive rats (SHRs) [162]. In human studies, LCZ696 was found to be superior to enalapril in reducing the risks of death and hospitalizations for heart failure [163, 164]. The efficacy of LCZ696 in this study was so high that the clinical trial was stopped earlier than anticipated. It is believed that this drug may become available for testing of other purposes such as diabetes complications. My laboratory is currently negotiating with Novartis with the intent to test LCZ696 as a treatment for diabetic vascular and neural complications.

Another potential treatment for diabetic vascular and neural disease that may involve protection of neural tissue from oxidative stress is inhibition of protein kinase C and poly ADP ribose polymerase [165-172]. The protein kinase C family includes at least 12 isoforms and a number of other proteins with homology to either the phorbol ester/diacylglycerol domain or the protein kinase C-terminal region [165]. One specific isoform, protein kinase C- $\beta_2$ , has been found to be activated in retina, heart, and aorta of diabetic rats [165]. Using a specific inhibitor of the protein kinase C- $\beta_2$  isoform, researchers found that retinal and renal vascular reactivity is normalized and renal function is improved in diabetic rats [165]. Recently, studies have demonstrated that protein kinase C- $\beta_2$  inhibition also improves nerve conduction velocity and endoneurial blood flow in diabetic rats [167]. At present, the mechanism responsible for protein kinase C- $\beta_2$ -mediated vascular dysfunction in diabetes is unknown. However, it has been shown that high concentrations of vitamin E can reverse some of the changes in retinal and renal vessels caused by diabetes and inhibit the activation of protein kinase C- $\beta_2$  by diabetes or hyperglycemia [166]. Furthermore, Brownlee and colleagues have demonstrated that the activation of protein kinase C by hyperglycemia in cultured bovine aorta endothelial cells is downstream of  $O_2^-$  generation, indicating that increased oxidative stress may influence the activation of protein kinase C in vascular tissue [62].

Another possible downstream mediator of hyperglycemia-induced oxidative stress is poly ADP ribose polymerase. Poly ADP ribose polymerase is

an abundant nuclear enzyme of eukaryotic cells that participates in DNA repair in response to genotoxic stress [169, 170]. When activated by DNA single-stranded breaks, poly ADP ribose polymerase initiates an energy-dependent cycle by transferring ADP ribose units from  $NAD^+$  to nuclear proteins. This process results in rapid depletion of the intracellular  $NAD^+$  and ATP pools by slowing the rate of glycolysis and mitochondrial respiration, and eventually leading to cellular dysfunction and death [170]. Overactivation of poly ADP ribose polymerase represents an important mechanism of tissue damage in various pathological conditions associated with oxidant stress, including diabetes [169]. Recently, Pacher *et al.* have reported that the activation of poly ADP ribose polymerase contributes to the development of endothelium dysfunction in streptozotocin-induced diabetic mice [170]. In our studies, inhibition of poly ADP ribose polymerase reduced oxidative stress in epineurial arterioles of the sciatic nerve and aorta, and improved diabetic peripheral neuropathy [171, 172]. The development and use of specific inhibitors of poly ADP ribose polymerase have indicated that poly ADP ribose polymerase may be a novel target for the intervention of diabetes-induced endothelial dysfunction [169-174].

#### 4. Conclusions

In summary, neurovascular dysfunction in diabetes is closely related to the development and progression of diabetic peripheral neuropathy. We have found that impaired endothelium-dependent vascular relaxation by epineurial arterioles of the sciatic nerve precedes the development of diabetic peripheral neuropathy. The regulation of vascular reactivity of epineurial arterioles of the sciatic nerve involves the interaction of a number of vasoactive mediators; many of which are affected by diabetes. The diabetes-induced dysregulation of vascular reactivity of epineurial arterioles of the sciatic nerve is also complex, and likely involves an increase in oxidative stress and activation of ACE and neutral endopeptidase. Preventing diabetes-induced increase in oxidative stress using antioxidants or drugs having antioxidant properties and inhibiting downstream mediators of oxidative stress have been shown to improve vascular activity and neural function in diabetic animal models, indicating that an increase in oxidative stress in diabetes is associated with neural dysfunction.

Future studies should focus on determining the most effective therapeutic approach, timing and dosage of antioxidant therapy for preventing the

diabetes-induced increase in oxidative stress and thus, the development and/or progression of diabetes complications including diabetic peripheral neuropathy [97]. In patients, an effective treatment for diabetic peripheral neural will likely require early intervention, prior to the development of severe clinical symptoms, with a combination of drugs that target several mechanisms most highly associated with the development and progression

of diabetic peripheral neuropathy. The development of reliable early clinical endpoints or markers of diabetic peripheral neuropathy will also be required in order to effectively evaluate the efficacy of these drugs and outcomes during the clinical studies.

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