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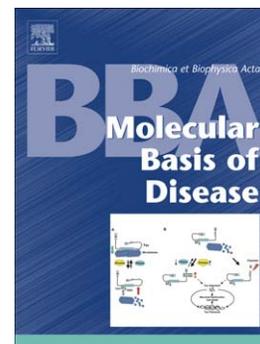
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DIABETES AND THE PERIPHERAL NERVE

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ABSTRACT

Diabetes-induced damage to peripheral nerve culminates in development of peripheral diabetic neuropathy (PDN), one of the most devastating complications of diabetes mellitus and a leading cause of foot amputation. The pathogenesis of PDN occurs as a consequence of complex interactions among multiple hyperglycemia-initiated mechanisms, impaired insulin signaling, inflammation, hypertension, and disturbances of fatty acid and lipid metabolism. This review describes experimental new findings in animal and cell culture models as well as clinical data suggesting the importance of 1) previously established hyperglycemia-initiated mechanisms such as increased aldose reductase activity, non-enzymatic glycation/glycooxidation, activation of protein kinase C, 2) oxidative-nitrosative stress and poly(ADP-ribose) polymerase activation; 3) mitogen-activated protein kinase and cyclooxygenase-2 activation, impaired Ca^{++} homeostasis and signaling, and several other mechanisms, in PDN.

Key words: diabetic neuropathy, aldose reductase, non-enzymatic glycation, oxidative-nitrosative stress, protein kinase C, poly(ADP-ribose) polymerase, mitogen-activated protein kinases, nuclear factor- κB , cyclooxygenase-2, 12/15-lipoxygenase, calcium signaling

1. INTRODUCTION.

Diabetic distal symmetric sensorimotor polyneuropathy affects at least 50% of diabetic patients, and is the leading cause of foot amputation [1]. Two largest clinical trials in subjects with Type 1 and Type 2 diabetes i.e., Diabetes Control and Complication Trial (DCCT) and UK Prospective Diabetes Study (UKPDS), indicate that intensive therapy and improved blood glucose control reduce incidence and slow progression of peripheral diabetic neuropathy (PDN) thus implicating hyperglycemia as a leading causative factor [1-3]. A number of mechanisms have been proposed to link chronic hyperglycemia to diabetes-induced deficits in motor and sensory nerve

conduction velocities (MNCV and SNCV) and small fiber sensory neuropathy. The vascular concept of PDN implies that diabetes-induced endothelial dysfunction with resulting decrease in nerve blood flow (NBF), vascular reactivity, and endoneurial hypoxia has a key role in functional and morphological changes in the diabetic nerve [4]. Endothelial changes in *vasa nervorum* have been attributed to multiple mechanisms including increased aldose reductase (AR) activity, non-enzymatic glycation and glycoxidation, activation of protein kinase C, oxidative-nitrosative stress, changes in arachidonic acid and prostaglandin metabolism [4], and, recently, decreased expression of the vanilloid receptor 1 in *vasa nervorum* [5], increased production of angiotensin (AT) II and activation of the AT1-receptor [6], activation of poly(ADP-ribose) polymerase-1 (PARP) [7], nuclear factor- κ B (NF- κ B, [8]), cyclooxygenase-2 (COX-2) [9], and others. The neurochemical concept of PDN suggests the importance of similar mechanisms in the *neural* elements of PNS i.e., Schwann cells, spinal cord oligodendrocytes, and dorsal root ganglion neurons. Other pathobiochemical mechanisms such as 1) metabolic abnormalities i.e., downregulation of Na^+/K^+ ATP-ase activity [10], "pseudohypoxia" - an increase in free cytosolic NADH/NAD⁺ ratio putatively linked to increased conversion of sorbitol to fructose by sorbitol dehydrogenase [11], changes in fatty acid and phospholipid metabolism [12], 2) impaired neurotrophic support [13,14], 3) changes in signal transduction [15], and 4) dorsal root ganglion (DRG) and Schwann cell mitochondrial dysfunction and premature apoptosis [16,17], have also been invoked. The present review of the recent findings has two major objectives i.e. 1) to evaluate new experimental evidence that supports or disproves previously formulated concepts of the pathogenesis of PDN, and 2) to characterize newly discovered mechanisms.

2. ROLE FOR ALDOSE REDUCTASE.

The sorbitol pathway of glucose metabolism consists of two reactions. First, glucose is reduced to its sugar alcohol sorbitol by NADPH-dependent AR. Then, sorbitol is oxidized to fructose by NAD-dependent sorbitol dehydrogenase (SDH). Sorbitol

accumulation in the peripheral nerve is well documented in STZ-diabetic rodents [18,19] and has recently been described in leptin-deficient (ob/ob) mice [20]. Interestingly and excitingly, increased sorbitol pathway activity manifest by sciatic nerve sorbitol and fructose accumulation is also clearly manifest in the high fat diet (HFD) model of prediabetic neuropathy characterized by impaired glucose tolerance, increased serum nonesterified fatty acid concentrations and insulin resistance, in the absence of overt hyperglycemia [21]. These findings indicating that factors, other than increased intracellular glucose concentrations, may predispose to increased sorbitol pathway activity, support and complement previous observations in models of non-diabetic conditions i.e., myocardial ischemia [22,23] and aging [24,25]. Studies of the effects of HFD on sorbitol pathway enzyme gene and protein expression may help to identify an important mechanism contributing to neuropathy and cardiovascular disease in overweight and obese individuals.

Negative consequences of the sorbitol pathway hyperactivity under diabetic conditions include intracellular sorbitol accumulation and resulting osmotic stress, and generation of fructose, a 10-times more potent glycation agent than glucose as well as fructose 1-phosphate [26]. One group reported that increased flux through SDH leads to so called “pseudohypoxia” i.e. an increased free cytosolic NADH/NAD⁺ ratio [11] whereas others [27] did not find a relation between cytosolic or mitochondrial NAD⁺/NADH redox state and SDH activity in the peripheral nerve. Two groups obtained the results indicating that increased AR, but not SDH, activity contributes to PDN [28, 29].

The role for AR in PDN has been reviewed in detail [30]. New evidence for the key role of AR in functional, metabolic, and morphological manifestations of PDN has been generated in both experimental studies in animal and cell culture models of diabetes and clinical trials of AR inhibitors (ARIs). Our group demonstrated that metabolic abnormalities of early PDN, such as mitochondrial and cytosolic NAD⁺/NADH redox

imbalances and energy deficiency, can be reversed with an adequate dose of ARI i.e., the dose that completely suppressed diabetes-associated sorbitol pathway hyperactivity [31]. Of particular interest are the results implicating increased AR activity in high glucose- and diabetes-induced oxidative-nitrosative stress [31-35] and downstream events such as activations of mitogen-activated protein kinase (MAPK) [36,37], PARP [35], COX-2 [38], and NF- κ B [39]. AR inhibitors have been reported to counteract diabetes-induced loss of two major non-enzymatic antioxidants, GSH and ascorbate, lipid peroxidation, as well as nitrotyrosine formation in peripheral nerve [20,21,35], spinal cord [20], DRG [20] and epineurial arterioles [35] and superoxide production in *vasa nervorum* [35]. Our group has also demonstrated the key role for AR in diabetes-associated PARP activation in peripheral nerve, spinal cord, DRG, and high glucose-exposed human Schwann cells (HSC) [20,21,35]. AR plays a key role in diabetes-related MAPK activation in DRG neurons [13,37]. Both PARP activation and MAPK activation are involved in transcriptional regulation of gene expression, via the transcription factors NF- κ B, activator protein-1, p53, and others [40,41]. Activation of these transcription factors leads to upregulation of inducible nitric oxide synthase, cyclooxygenase-2, endothelin-1, cell adhesion molecules and inflammatory genes [40,42]. Thus, the demonstration of a major contribution of AR to oxidative-nitrosative stress and PARP and MAPK activation in tissue-sites for diabetic complications allows to predict that in the near future the link between increased AR activity and altered transcriptional regulation and gene expression will be established. Any product of genes controlled via PARP- and MAPK-dependent transcription factors, regardless of how unrelated to the sorbitol pathway this product looks from a biochemical point of view, will be affected by a diabetes-associated increase in AR activity, and amenable to control by AR inhibition. In accordance with this premise, the most recent findings have shown that increased AR activity leads to activation of NF- κ B and activator protein-1 [39], and is responsible for diabetes-induced

COX-2 upregulation in the spinal cord oligodendrocytes [38] and 12/15-lipoxygenase overexpression in sciatic nerve [21].

The role for AR in the pathogenesis of PDN is supported by findings obtained in AR-overexpressing and AR-knockout mice. Induction of STZ-diabetes in the mice transgenic for human AR resulted in a more severe peripheral nerve sorbitol and fructose accumulation, MNCV deficit, and nerve fiber atrophy than in their non-transgenic littermates [19]. Treatment of diabetic transgenic mice with the AR inhibitor (ARI) WAY121-509 significantly prevented sorbitol accumulation, MNCV slowing, and the increased myelinated fiber atrophy in diabetic AR-overexpressing mice [19]. Similar findings have been obtained in another transgenic mouse model that overexpressed AR specifically in the Schwann cells of peripheral nerve under the control of the rat myelin protein zero (Po) promoter [43]. The transgenic mice exhibited a significantly greater reduction in MNCV under both diabetic and galactosemic conditions than the non-transgenic mice with normal AR content. In contrast, AR-deficient mice appeared protected from motor nerve conduction slowing, axonal atrophy, and several metabolic manifestations of PDN [44]. These data lend further support to the important role of AR in functional, metabolic and morphological abnormalities characteristic for PDN.

The findings in transgenic and knockout mouse models are in line with new studies with structurally diverse ARIs. Coppey et al [45] implicated AR in diabetes-induced impairment of vascular reactivity of epineurial vessels, an early manifestation of PDN, which precedes motor nerve conduction slowing. An ARI treatment prevented the development of thermal hyperalgesia, an event associated with early PDN, in STZ-diabetic rats [46]. Furthermore, structurally different ARIs, at least, partially prevented thermal hypoalgesia in rats with more prolonged STZ-diabetes [46], as well HFD-fed [21] and *ob/ob*[20] mice. Tactile allodynia was not prevented by an ARI treatment in either STZ-diabetic rats [46] or *ob/ob* mice [20], although tactile withdrawal thresholds in response to light touch with flexible von Frey filaments tended to be higher in *ob/ob* mice

treated with the ARI fidarestat compared with the corresponding untreated group. New evidence supports the role of AR in the pathogenesis of advanced PDN. A 15-month AR inhibition with fidarestat dose-dependently corrected slowed F-wave, MNCV, and SNCV in STZ-diabetic rats [47]. In the same study, diabetes-induced paranodal demyelination and axonal degeneration were reduced to the normal with such low dose of fidarestat as 2 mg/kg. Other manifestations of advanced PDN such as axonal atrophy, distorted axon circularity, and reduction of myelin sheath thickness were also inhibited. In our study in *ob/ob* mice that display clearly manifest intraepidermal nerve fiber loss, a sign of small sensory nerve fiber degeneration, fidarestat treatment partially prevented diabetes-associated decrease in intraepidermal nerve fiber density [20]. The results of several recent clinical trials of ARIs are also encouraging and support applicability of the AR concept to the pathogenesis of human PDN. In particular, a clinical trial with the ARI zenarestat indicates that robust inhibition of AR in diabetic human nerve improves nerve physiology and fiber density [48]. Two double-blind placebo-controlled clinical trials of fidarestat in patients with Type 1 and Type 2 diabetes also provided a proof of efficacy of ARIs ([49], and Arezzo et al, unpublished). In these trials, fidarestat improved electrophysiological measures of median and tibial MNCV, F-wave minimum latency, F-wave conduction velocity and median SNCV (forearm and distal), as well as subjective symptoms of PDN such as numbness, spontaneous pain, sensation of rigidity, paresthesia in the sole upon walking, heaviness in the foot and hypesthesia. These findings are consistent with the results of the most recent open-label, prospective study conducted at 12 hospitals in the central area of Honshu, Japan [50]. Treatment of 22 patients with fidarestat significantly increased vibration perception threshold in the upper and lower limbs. The symptoms such as severity of numbness in the lower limbs, heaviness in the foot, coldness and hot flushes in the lower limbs, smarting pain causing difficulty in walking, sensation as if walking on sand, sensation as if walking on an uneven road, spontaneous pain in the lower limbs, and dizziness, were also improved. Recently,

improvement of motor and sensory nerve conduction velocity in patients with diabetic sensorimotor polyneuropathy was also found with the new ARI AS-3201 (ranirestat, [51]). The role for AR in human diabetic neuropathy is also supported by the genetic polymorphism data [30,52].

3. ROLE FOR NON-ENZYMATIC GLYCATION/GLYCOXIDATION.

Glycation is the nonenzymatic reaction of glucose, α -oxoaldehydes, and other saccharide derivatives with proteins, nucleotides, and lipids, with formation of early glycation adducts (fructosamines) and advanced glycation end-products (AGE). Formation of some AGE i.e., pentosidine and N^{ϵ} -[carboxymethyl]-lysine, combines both glycation and oxidative steps in a process termed "glycoxidation". In the last several years, the role for glycation/glycoxidation in diabetic complications including diabetic neuropathy has extensively been reviewed [53-55]. A number of new studies in animal models of diabetes and human subjects supports the role of this mechanism.

Using the state-of-the art technique i.e., liquid chromatography with tandem MS detection, Karachalias et al [56] produced evidence of accumulation of fructosyl-lysine and AGE in peripheral nerve of STZ-diabetic rats. In particular, sciatic nerve concentrations of N^{ϵ} -[carboxymethyl]-lysine and N^{ϵ} -[carboxyethyl]-lysine were markedly increased in diabetic rats compared with controls. Hydroimidazolone AGEs derived from glyoxal, methylglyoxal and deoxyglucosone were major AGEs quantitatively. The receptor for AGE (RAGE) was localized both in endothelial and Schwann cells of the peripheral nerve [57]. Recently generated RAGE^{-/-} mice appeared partially protected from diabetes-associated pain perception loss, an indicator of longstanding diabetic neuropathy [58]. Furthermore, in the same study, loss of pain perception was reversed in the diabetic wild-type mice treated with soluble RAGE. Further studies [59] revealed a dramatic and cumulative rise in RAGE mRNA and protein expression in peripheral epidermal axons, sural axons, Schwann cells, and sensory neurons within dorsal root

ganglia, correlating with progressive electrophysiological and structural abnormalities in STZ-diabetic mice. In the same study, functional and structural abnormalities of PDN, and activation of NF- κ B and PKC beta II signaling pathways were attenuated in diabetic RAGE^{-/-} mice. The findings in RAGE^{-/-} mice are in line with new “anti-glycation” agent studies that also support a pivotal role of the AGE/RAGE axis in the pathogenesis of PDN. The new inhibitor of AGE and advanced lipoxidation end-product (ALE) formation, pyridoxamine, previously reported to be effective against diabetic nephropathy and retinopathy, was found to reverse established sciatic endoneurial NBF, MNCV and SNCV deficits in STZ-diabetic rats with 8-week duration of diabetes [60]. Of interest, this correction was achieved in the absence of any significant effect of the agent on the levels of AGE/ALE, N-(carboxymethyl)lysine and n-(carboxyethyl)lysine in total sciatic nerve protein which suggests that short-term diabetes and pyridoxamine treatment target AGE/ALE in *vasa nervorum* rather than neural components of the peripheral nerve. Another new anti-glycation agent OPB-9195 reduced sciatic nerve immunoreactive AGE expression, and prevented tibial motor nerve conduction slowing, downregulation of Na⁺,K⁺-ATPase activity, and accumulation of 8-hydroxy-2'-deoxyguanosine (a marker of DNA oxidative damage) in STZ-diabetic rats with 24-week duration of diabetes [61]. High-dose therapy of thiamine and benfotiamine suppressed AGE accumulation in the peripheral nerve [62] and reversed diabetic neuropathy [63], potentially by reducing the levels of triose phosphates via activation of transketolase. Several *in vitro* studies describe adverse effects of AGE precursors and AGE *per se* in Schwann cells and DRG neurons [64-66]. In particular, methylglyoxal was found to induce rat Schwann cell apoptosis via oxidative stress-mediated activation of p38 MAPK [64]. AGE derived from glyceraldehydes and glycolaldehyde, but not from glucose, induced rat Schwann cell apoptosis, decreased cell viability and replication, decreased mitochondrial membrane potential, activated NF- κ B, and enhanced production of inflammatory cytokines i.e. TNF- α and IL- β [65]. The DRG neuron exposure to RAGE

ligand S100 was associated with increased phosphatidylinositol-3 kinase activity and enhancement of oxidative stress, and RAGE ligand-induced changes were prevented by the potent antioxidant alpha-lipoic acid [66].

Several new studies support the presence of AGE accumulation in patients with diabetes mellitus. The AGE pyrraline immunoreactivity was more intense in the optic nerve head of diabetic subjects compared with non-diabetic controls [67]. Pronounced AGE immunoreactivity was detected in axons and myelin sheaths in 90% of patients with Type 2 diabetes but not in control subjects, and the intensity of axonal AGE positivity significantly correlated with the severity of morphological alterations characteristic for PDN [68]. In the same study, AGE positivity was clearly present in endoneurium, perineurium and microvessels of diabetic patients. Bierhaus et al [58] have demonstrated that ligands of RAGE, the receptor itself, activated NF- κ Bp65, and IL-6 colocalized in the microvasculature of sural nerve biopsies obtained from human subjects with diabetic neuropathy. Furthermore, *N*^ε-[carboxymethyl]-lysine, RAGE and NF- κ B were found in the sural nerve perineurium, epineurial vessels and endoneurial vessels of subjects with impaired glucose tolerance-related polyneuropathy [69]. Several clinical studies support the role of glycation in the pathogenesis of PDN and other diabetes complications [70-72]. In particular, it has been reported that increased accumulation of skin AGE precedes and correlates with clinical manifestations of diabetic neuropathy [70]. In another study, AGE accumulation in skin, serum, and saliva increased with progression of neuropathy, nephropathy and retinopathy [71]. Furthermore, serum *N*^ε-[carboxymethyl]-lysine concentrations were found significantly higher in children and adolescents with Type 1 diabetes and diabetes complications (background retinopathy, microalbuminuria and neuropathy) compared with the uncomplicated group [72].

4. ROLE FOR PROTEIN KINASE C ACTIVATION

PKC comprises a superfamily of isoenzymes, many of which are activated by 1,2-diacylglycerol (DAG) in the presence of phosphatidylserine. PKC isoforms

phosphorylate a wide variety of intracellular target proteins and have multiple functions in signal transduction-mediated cellular regulation. The role for PKC in the pathogenesis of PDN has been reviewed in detail [73]. PKC is activated in *vasa nervorum* of diabetic rats [74], and vessel-rich epineurial vessels of diabetic mice [75]. PKC has been reported decreased [76], unchanged [74] or increased [77] in the diabetic rat nerve, and decreased in endoneurial tissue of diabetic mouse nerve [75]. PKC activity was markedly reduced in DRG neurons of the wild-type STZ-diabetic mice and further reduced in the diabetic mice overexpressing human AR [78]. These changes were associated with reduced expression and activity of the membrane PKC- α isoform that translocated to cytosol. The membrane PKC- II β isoform expression was increased in AR-overexpressing diabetic transgenic mice, but not in the wild-type mice [78].

The experimental evidence obtained with various PKC inhibitors as well as the DAG complexing agent cremophor by several groups [74,79-81] suggests the detrimental role of PKC activation in *vasa nervorum*. The PKC inhibitors, WAY151003, chelerythrine and LY333531, as well as cremophor prevented or reversed NBF and conduction deficits (74,79,80), and the PKC inhibitor bisindolylmaleimide-1HCl corrected acetylcholine-mediated vascular relaxation in epineurial arterioles in the STZ-diabetic rat model (81). The role for neural PKC in the pathogenesis of PDN remains unclear. However, recent findings suggest that neuronal PKC may be related to diabetes-associated changes in expression, phosphorylation, and function of the vanilloid receptor 1, known to play an important role in diabetic neuropathic pain [82]. The novel PKC- β isoform selective inhibitor JTT-010 was found to ameliorate nerve conduction deficits, hyperalgesia (formalin test in its first phase) and hypoalgesia (formalin test in its second phase, tail flick test) in STZ-diabetic rats [83].

Recently, the effects of the isoform-selective PKC-beta inhibitor ruboxistaurin mesylate on endothelium-dependent and C fiber-mediated skin microvascular blood flow, sensory symptoms, neurological deficits, nerve fiber morphometry, measures of

quantitative sensory and autonomic function, nerve conduction, and quality of life were evaluated in a randomized, double-masked, single-site, 6-month study in human subjects with Type 1 and Type 2 diabetes [84]. In this cohort of patients with PDN, ruboxistaurin enhanced skin microvascular blood flow at the distal calf, reduced sensory symptoms of PDN, improved quality of life, and was well tolerated.

5. ROLE FOR OXIDATIVE-NITROSATIVE STRESS.

Enhanced oxidative stress resulting from imbalance between production and neutralization of reactive oxygen species (ROS) is a well recognized mechanism in the pathogenesis of PDN and other diabetic complications i.e., endothelial dysfunction, cataract, retinopathy, and nephropathy. Hyperglycemia and associated increase in aldose reductase activity, activations of non-enzymatic glycation/glycooxidation and protein kinase C (the latter, in *vasa nervorum* only) as well as other mechanisms lead to oxidative stress in diabetic peripheral nerve (Fig.1). Note, however, that antioxidants, including the most potent α -lipoic acid and peroxynitrite decomposition catalysts, do not reduce peripheral nerve glucose as well as sorbitol pathway intermediate concentrations ([85], reviewed in [26]) in the peripheral nerve. Recently, a significant progress has been made in the detection of diabetes-associated oxidative injury in tissue-sites for PDN. New studies [85-88] have confirmed previously established free lipid peroxidation product accumulation, GSH depletion and increase in GSSG/GSH ratio, and downregulation of superoxide dismutase (SOD) activity in the diabetic peripheral nerve. In addition, new markers of ROS-induced injury have been identified in peripheral nerve, spinal cord, *vasa nervorum* and dorsal root ganglia (DRG) in experimental PDN. Those include 4-hydroxynonenal protein adduct accumulation [81], decreased catalase and total quinone reductase activities, depletion of ascorbate and taurine and increase in dehydroascorbate/ascorbate ratio in peripheral nerve [85,86], increased production of superoxide in *vasa nervorum* [87], and accumulation of 8-hydroxy-2'-deoxyguanosine in DRG [17] of diabetic rats and mice. Accumulation of nitrotyrosine (a footprint of

peroxynitrite-induced protein nitration) has been documented in peripheral nerve, spinal cord, *vasa nervorum*, and DRG in both Type 1 and Type 2 diabetic rodent models [20,21,88-93] indicating that diabetes creates not just oxidative, but oxidative-nitrosative stress in PNS. Using specific markers for certain cell types of PNS, we localized immunoreactive nitrotyrosine in endothelial and Schwann cells of peripheral nerve, as well as neuronal and glial cells of dorsal root ganglia, spinal cord neurons, oligodendrocytes and astrocytes (V.R. Drel and I.G. Obrosova, unpublished) Thus, nitrosative stress affects all major cell targets for PDN. We have also found that 1) nitrosative stress is more severe in STZ-diabetic mice than in rats with similar duration of diabetes (compare [91] and [92]), 2) inducible nitric oxide synthase (iNOS) is the most important source of nitric oxide for peroxynitrite formation in the peripheral nerve, but not DRG neurons [94], and 3) STZ-diabetic iNOS^{-/-} mice are, at least, partially protected from nerve conduction deficits and small sensory nerve fiber neuropathy that are clearly manifest in wild-type mice with diabetes of similar severity and duration [94]. Enhanced nitrosative stress has been documented in plasma of human subjects with PDN [95]. Furthermore, we found highly variable accumulation of nitrotyrosine in peripheral blood monocytes, which correlated with fasting blood glucose, glycosylated hemoglobin, and plasma C-reactive protein levels, in diabetic patients [96].

Numerous new studies reveal the important role of oxidative stress in nerve functional, metabolic, neurotrophic and morphological abnormalities characteristic for PDN. The role for ROS in diabetes-associated nerve conduction and blood flow deficits has been demonstrated in studies with the “universal” antioxidant DL- α -lipoic acid [4,85,97,98] which is known to combine free radical and metal chelating properties with an ability (after conversion to dehydrolipoic acid) to regenerate levels of other antioxidants i.e., GSH, ascorbate, α -tocopherol, catalase, glutathione peroxidase. It has also been confirmed with other antioxidants including the potent hydroxyl radical scavenger dimethylthiourea, HESD [99], the SOD mimetic M40403 [87], the NAD(P)H

oxidase inhibitor apocynin [100], and the xanthine oxidase inhibitor allopurinol [101]. Furthermore, diabetes-induced MNCV, SNCV, and nerve blood flow deficits, reduced reactivity of epineurial arterioles, and small sensory nerve fiber degeneration were alleviated by a peroxynitrite decomposition catalyst treatment [89-92,102].

Several reports suggest involvement of oxidative-nitrosative stress in the mechanisms underlying diabetic neuropathic pain and abnormal sensory responses. Studies in the “mature” short-term rat model of STZ-diabetes revealed thermal and mechanical hyperalgesia (exaggerated pain state) which was corrected by lipoic acid [103], and alleviated by the hydroxyl radical scavenger dimethylthiourea [99]. In our studies [89-92,103], a peroxynitrite decomposition catalyst treatment counteracted thermal and mechanical hypoalgesia in STZ-diabetic, NOD, and *ob/ob* mice, as well as mechanical hyperalgesia in rats short-term STZ-diabetes. The mechanisms underlying diabetes-associated tactile allodynia have not been studied in detail; however, a beneficial effect of niterapone, an inhibitor of catechol-O-methyltransferase and antioxidant, suggests the involvement of oxidative-nitrosative stress [104].

Two groups produced experimental evidence of an important role for oxidative stress in diabetes-associated impairment of neurotrophic support to the peripheral nerve by demonstrating that 1) diabetes and prooxidant treatment caused NGF and NGF-regulated neuropeptide i.e., substance P and neuropeptide Y, deficits in the sciatic nerve that were, at least partially, counteracted by α -lipoic acid [105]; 2) taurine alleviated oxidative stress and prevented diabetes-induced NGF deficit in the sciatic nerve of STZ-diabetic rats [86].

Evidence for the important role of oxidative-nitrosative stress in PDN in human subjects with diabetes mellitus is emerging. Oral treatment with alpha-lipoic acid improved symptomatic diabetic polyneuropathy in SYDNEY and SUDNEY 2 clinical trials [106,107]. Increased plasma nitrotyrosine content has been reported to directly correlate with redistribution of sudomotor responses, an early sign of sympathetic nerve

dysfunction, in patients with Type 1 diabetes [95]. In a recent neuropathy study [108], plasma antioxidant capacity for peroxynitrite (assessed by the pholasin test) correlated with the neurological impairment score of the lower limbs (NIS-LL) in type 1 diabetic patients. Note, however, that another study from the same group [109] did not reveal any correlations between plasma antioxidant capacity for peroxynitrite and a number of individual measures of nerve function in patients with both types of diabetes, and the authors had to perform the multiple linear regression analysis to identify the lag time for peroxynitrite disappearance from circulation as an independent factor associated with NIS-LL. New studies are needed to reveal if monocyte nitrosylated protein expression (an index of intracellular nitrosative stress) can be used as a reliable marker of the presence, severity and progression of PDN, and if peroxynitrite decomposition catalysts display better efficacy against diabetic neuropathy than conventional antioxidants.

6. ROLE FOR IMPAIRED NEUROTROPHIC SUPPORT.

Impaired neurotrophic support plays an important role in PDN, and a number of neurotrophic factors including insulin, IGF-1, nerve growth factor (NGF), ciliary neurotrophic factor, neurotrophin-3 (NT-3), sonic hedgehog protein, and prosaposin-derived peptide have been reported to have a beneficial effect on multiple manifestations of diabetes-induced peripheral nerve damage (recently reviewed in [110]). Recently, insulin and IGF-1 have been shown to play an important role in providing a remote neurotrophic support to unmyelinated intraepidermal nerve fibers [111]. Central intrathecal delivery of IGF-1 and insulin for one month after two months of untreated diabetes promoted intraepidermal nerve fiber regeneration and increased fiber density. In a similar fashion, intrathecal administration of NGF and NT-3 increased myelinated innervation of the dermal footpad of STZ-diabetic mice, which suggests that deficits in both neurotrophic factors play an important role in diabetes-associated impairment of myelinated cutaneous innervation [112]. Neuregulins (NRGs), the growth factors that bind to Erb receptor tyrosine kinases in Schwann cells, are known to promote cell

survival, mitogenesis, and myelination in undifferentiated SCs, but also to induce demyelination of myelinated SC. A recent elegant study in co-cultures of SC and DRG neurons revealed the role of hyperglycemia-induced increase in Erb B2 activity and concomitant downregulation of the protein caveolin-1 in NGG-induced SC demyelination [113]. The study provides rationale for development of principally novel therapeutic approaches, to counteract demyelination, a major structural disorder associated with human PDN.

7. ROLE FOR POLY(ADP-RIBOSE)POLYMERASE ACTIVATION.

The enzyme poly(ADP-ribose) polymerase (PARP) is localized in the nucleus and mitochondria, and cleaves nicotinamide adenine dinucleotide (NAD⁺) with formation of nicotinamide and ADP-ribose residues which are attached to nuclear proteins and to PARP itself, with formation of poly(ADP-ribosyl)ated protein polymers [114]. Until recently, a conservative view on PARP activation as a phenomenon arising from free radical- and peroxynitrite-induced DNA single-strand breakage hampered studies of the role for other mechanisms in excessive poly(ADP-ribosylation) in pathological conditions associated with oxidative stress including diabetic complications. However, several new studies [115-117] including those from the our laboratory [117] revealed that 1) in some tissues, e.g., peripheral nerve, PARP activation may precede rather than result from oxidative-nitrosative stress, and 2) PARP activation does not necessarily require DNA single-strand breakage, and may occur due to phosphorylation by phosphorylated ERK. PARP activation leads to 1) NAD⁺ depletion and energy failure [7,114,118]; 2) changes of transcriptional regulation and gene expression [40,42,114]; 3) poly(ADP-ribosylation) and inhibition of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) resulting in diversion of the glycolytic flux towards several pathways implicated in diabetes complications [119]. Recent studies of our group [7,118,120,121] revealed that PARP activation is an early and fundamental mechanism of PDN. It is clearly manifest in peripheral nerve, *vasa nervorum*, and DRG neurons of

STZ-diabetic rats [7,92,118,120,121] as well as peripheral nerves, spinal cord, and DRG of STZ-diabetic [91,121], *ob/ob* [20,90], and HFD-fed [21] mice. Using endothelial and Schwann cell markers and double immunostaining [7], we localized PARP activation in endothelial and Schwann cells of diabetic rat nerve. Our group was first to develop the Western blot analysis of poly(ADP-ribosyl)ated proteins in rat sciatic nerve [122]; using this approach, we found poly(ADP-ribosyl)ated protein abundance increased by 74% in rats with 4-wk duration of STZ-diabetes compared with non-diabetic controls. Furthermore, we found accumulation of poly(ADP-ribosyl)ated proteins to develop very early, i.e. within ~12-24 h of exposure of cultured human endothelial and Schwann cells to high glucose [117]. PARP-1 protein abundance was not affected by high glucose or PARP inhibitor treatment in either cell type consistent with the current knowledge on PARP-1 as abundantly expressed enzyme with very minor, if any, transcriptional regulation [114].

Using several structurally unrelated PARP inhibitors and PARP-deficient (PARP^{-/-}) mice, we revealed the important role for PARP activation in MNCV and SNCV deficits, neurovascular dysfunction, sensory neuropathy, peripheral nerve energy failure, intraepidermal nerve fiber loss, and axonal atrophy associated with peripheral diabetic neuropathy [7,118,120,121]. PARP^{-/-} mice were protected from both diabetic and galactose-induced MNCV and SNCV deficits and nerve energy failure that were clearly manifest in the wild-type (PARP^{+/+}) diabetic or galactose-fed mice [7]. Furthermore, whereas diabetic wild-type mice developed clearly manifest thermal and mechanical hypoalgesia, tactile allodynia, and intraepidermal nerve fiber loss, diabetic PARP^{-/-} mice did not display any manifestations of small sensory nerve fiber neuropathy, and maintained normal intraepidermal nerve fiber density [121]. The data in PARP^{-/-} mice are consistent with the findings obtained in pharmacological studies. Two structurally unrelated PARP inhibitors, 3-aminobenzamide and 1,5-isoquinolinediol, reversed established nerve blood flow and conduction deficits and energy deficiency in STZ-

diabetic rats [7]. Alleviation of diabetes-associated neurovascular dysfunction and nerve conduction deficits was also achieved with a relatively weak PARP inhibitor, nicotinamide [123]. Another PARP inhibitor, PJ34, essentially corrected nerve conduction deficits and energy deficiency despite relatively modest (17%) reversal of nerve blood flow deficit [118]. The latter is consistent with normalization of free NAD^+/NADH ratio, an index of tricarboxylic cycle activity and glucose utilization, in the peripheral nerve mitochondrial matrix of PJ34-treated diabetic rats [118]. In the same study, PJ34 treatment counteracted decrease in free cytosolic NAD^+/NADH ratio, accumulation of lactate and glutamate, and depletion of α -glutarate in diabetic peripheral nerve. Nerve glucose and sorbitol pathway intermediate concentrations were similarly elevated in PJ34-treated and untreated rats which is consistent with the downstream localization of PARP activation, consequent to increased AR activity, in the pathogenesis of diabetic complications [26]. In our most recent study [121], the orally active PARP inhibitor 10-(4-methylpiperazin-1-ylmethyl)-2H-7-oxa-1,2-diaza-benzo[de]anthracen-3-one (GPI-15427; formulated as a mesilate salt, 30 mg kg^{-1} day $^{-1}$) in the drinking water for 10 weeks after the first 2 weeks without treatment) at least partially prevented PARP activation in peripheral nerve and DRG neurons, as well as thermal hypoalgesia, mechanical hyperalgesia, tactile allodynia, exaggerated response to formalin, and, most importantly, intraepidermal nerve fiber degeneration in streptozotocin-diabetic rats. We have also reported neuroprotective effects of PARP inhibitor-containing combination therapies [122]. In particular, combined treatments with low-dose PARP inhibitor and one of two vasodilators i.e., the ACE-inhibitor lisinopril and β -adrenoceptor agonist salbutamol, resulted in reversal of neurovascular dysfunction, SNCV deficit (MNCV deficit was reversed by salbutamol-, but not lisinopril-containing drug combination) as well as thermal and mechanical hyperalgesia in rats with short-term STZ-diabetes [122]. Theoretically, PARP activation can contribute to diabetic neuropathic pain and abnormal sensory responses via several mechanisms including but not limited to 1) upregulation of

tumor necrosis factor-alpha (TNF- α) and other inflammatory genes; 2) activation of p38 MAP kinase in the spinal cord and Schwann cells; and 3) Ca⁺⁺-regulated excitotoxic insults, all of which have been implicated in the pathogenesis of painful neuropathy (124-126).

8. ROLE FOR MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) ACTIVATION.

Numerous findings indicate that ROS and reactive nitrogen species cause MAPK activation [13,15,127], and increasing evidence supports the importance of MAPKs in the pathogenesis of PDN. ERK, p38 MAPKs and JNK are activated in DRG neurons of STZ-diabetic rats [13,15]. Sural nerve JNK activation and increases in total levels of p38 and JNK have been observed in patients with both Type 1 and Type 2 diabetes [13,15]. MAPKs are implicated in aberrant neurofilament phosphorylation, a phenomenon involved in the etiology of the diabetic sensory polyneuropathy [128]. Fernyhough et al [128] have reported a 2-3-fold elevation of neurofilament phosphorylation in lumbar DRG of STZ-diabetic and spontaneously diabetic BB rats, as well as 2.5-fold elevation in neurofilament M phosphorylation in sural nerve of BB rats. Diabetes-induced 3-4-fold increase in phosphorylation of a 54-kDa isoform of JNK in DRG and sural nerve correlated with elevated c-Jun and neurofilament phosphorylation. p38 activation in DRG neurons of STZ-diabetic rats is prevented by the ARIs sorbinil and fidarestat which suggests the important role of AR in diabetes-related alterations in MAPK signaling [13, 129]. The p38 MAPK inhibitor SB239063 corrected MNCV and SNCV deficit in STZ-diabetic rats thus implicating p38 MAPK in motor and sensory nerve dysfunction [129]. In the same animal model, the p38- α MAPK inhibitor SD-282 counteracted mechanical allodynia, C-, but not Adelta-fiber-mediated thermal hyperalgesia, and attenuated flinching behavior during the quiescent period and the second phase of the formalin response [130]. Spinal p38 MAPK has also been implicated in the neuropathic pain induced by inflammation [131].

9. ROLE FOR NF- κ B ACTIVATION.

Both PARP-1 and MAPKs are involved in transcriptional regulation of gene expression, via the transcription factors NF- κ B, activator protein-1, p53, and others [40,41,114]. Activated NF- κ B has been identified in perineurium, epineurial vessels and endoneurium in sural nerve biopsies of subjects with impaired glucose tolerance [69] and overt diabetes [58]. NF- κ B activation was also found in isolated Schwann cells cultured in high glucose medium compared with those in low glucose [132], and such activation was prevented by the ARI fidarestat. Activation of NF- κ B and other transcription factors by high glucose and oxidative stress [133] leads to upregulation of inducible nitric oxide synthase, cyclooxygenase-2, endothelin-1, cell adhesion molecules and inflammatory genes [40,42]. Growing evidence indicates that the afore-mentioned transcription factors and their target genes are involved in the pathogenesis of diabetic complications, and, in particular, PDN [8]. Thus, it is not surprising that inhibition of NF- κ B by pyrrolidine dithiocarbamate and the serine protease inhibitor N-alpha-tosyl-L-lysine chloromethylketone (TLCK) i.e. I κ B protease activity-blocking agent, corrected nerve conduction and blood flow deficits in STZ-diabetic rats [8]. In the same animal model, TLCK also partially reversed gastric autonomic neuropathy [8].

10. ROLES FOR CYCLOOXYGENASE-2 (COX-2) and 12/15-LIPOXYGENASE (12/15-LO) ACTIVATION.

Evidence for the important role of arachidonic acid metabolic pathways i.e. COX-1 and COX-2, cytochrome p450 epoxygenase and lipoxygenases in diabetes complications is emerging. The lipid products of these pathways include thromboxane, prostaglandins, leukotrienes, lipoxins, epoxyeicosatrienoic (EET) acid, 12-hydroperoxy-eicosatetraenoic acid (12-HPETE), 12-HETE, 15-HETE and a whole variety of their derivatives. COX-1 protein expression was reported unchanged in the diabetic peripheral nerve [9] and reduced in the spinal cord [134] whereas COX-2 protein expression was

increased in both tissues [9, 38]. COX-2 activity was also increased in the diabetic spinal cord, and was localized to spinal cord oligodendrocytes [38]. Selective COX-2 inhibitors were reported to prevent motor nerve conduction and endoneurial nutritive blood flow deficits, peripheral nerve oxidative stress and inflammation [9,135], as well as spinal hyperalgesia (exaggerated flinching response in the formalin pain test) in the diabetic rats [38]. Diabetic COX-2 deficient mice were protected from MNCV and SNCV slowing, intraepidermal nerve fiber loss, and oxidative damage that was clearly manifest in the diabetic wild-type mice [135]. One of the most interesting members of the lipoxygenase family is 12/15-LO, a nonheme iron-containing dioxygenase that forms 12-HPETE and 12- and 15-HETEs and oxidizes esterified arachidonic acid in lipoproteins (cholesteryl esters) and phospholipids [136,137]. 12/15-LO is abundantly expressed in endothelial cells (e.a., aortic and retinal endothelial cells), smooth muscle cells, monocyte/macrophages, as well as renal mesangial cells, tubular epithelial cells, and podocytes, and the enzyme expression is increased under diabetic and hyperglycemic conditions [136,137]. Recent *in vivo* and cell culture studies revealed that high glucose-induced 12/15-LO activation affects multiple metabolic and signal transduction pathways, transcriptional regulation and gene expression [136,137]. The major consequences include increased free radical production and lipid peroxidation, MAPK and NF- κ B activation, inflammatory response, excessive growth, as well as adhesive and chemoattractant effects. Recent findings from our group suggest an important role for 12/15-LO activation in peripheral DN [138]. In particular, we found that 1) 12/15-LO is abundantly expressed in mouse peripheral nerve and its expression increases in diabetic conditions; 2) 12/15-LO^{-/-} mice with both Type 1 (streptozotocin-induced) and Type 2 (high fat diet-induced) diabetes develop less severe peripheral DN than wild-type mice; 3) some manifestations of peripheral DN in STZ-diabetic mice are reversed by a short-term 12/15-LO inhibitor treatment; 4) 12/15-LO is abundantly expressed in human Schwann cells (HSC), one of the major cell targets in human DN, and its overexpression

is clearly manifest after a short-term (24 h) exposure to high glucose; and 5) 12/15-LO overexpression in high glucose-exposed HSC contributes to activation (phosphorylation) of all three subtypes of MAPKs including p38 MAPK, ERK 1/2 and JNK-1, recently implicated in the pathogenesis of both experimental and human DN [21,138]. These results provide the rationale for detailed studies of the role for 12/15-LO pathway in diabetic neuropathic changes.

11. ROLES FOR ALTERED Ca^{++} HOMEOSTASIS AND SIGNALING.

Growing evidence suggests that diabetic sensory neuropathy is associated with abnormal Ca^{++} homeostasis and signaling in DRG neurons [139,140]. Enhanced Ca^{++} influx via multiple high-threshold calcium currents is present in sensory neurons of several rat models of diabetes mellitus, including the spontaneously diabetic BioBred/Worcester (BB/W) rats and STZ-diabetic rats [139]. Sensory neurons of STZ-diabetic rats have increased mRNA expression of voltage-gated calcium channels [140]. Enhanced calcium entry in diabetes is also linked to the impairment of G-protein-coupled modulation of calcium channel function [139]. Increased AR activity and taurine deficiency contribute to diabetes-related enhancement of voltage-dependent calcium currents in DRG neurons [141,142]. These two mechanisms probably converge at the level of oxidative stress and resulting PARP activation known to promote $[\text{Ca}^{2+}]_i$ accumulation and impair calcium signaling.

Evidence for important role of $[\text{Ca}^{2+}]_i$ in neuropathic pain of different origin, and, in particular, diabetic neuropathic pain is emerging. Luo et al [143] found that injury type-specific calcium channel $\alpha_2\delta_1$ subunit upregulation in rat neuropathic pain models (mechanical nerve injuries, diabetic neuropathy, chemical neuropathy) correlated with antiallodynic effect of the anticonvulsant gabapentin. Furthermore, a recent 6-week, randomized, double-blind, multicenter clinical trial in 246 patients with painful diabetic neuropathy revealed that pregabalin, a new drug that interacts with the $\alpha_2\delta_1$

protein subunit of the voltage-gated calcium channel, is a efficacious and safe treatment for diabetic neuropathic pain [144]. Similar findings have been obtained in another 12-week, randomized, double-blind, multicenter, placebo-controlled trial of pregabalin that revealed a significant pain relief in patients with chronic postherpetic neuralgia or painful diabetic neuropathy [145].

12. ROLE FOR Na^+/H^+ -EXCHANGER-1 ACTIVATION.

Recent studies from our group suggest an important role for Na^+/H^+ -exchanger-1 (NHE-1) in PDN [146]. The NHE-1 specific inhibitor cariporide, at least, partially prevented MNCV and SNCV deficits, thermal hypoalgesia, mechanical hyperalgesia, and tactile allodynia in STZ-diabetic rats. STZ-diabetic NHE-1 \pm mice developed less severe early PDN than the wild-type mice. Increased NHE-1 protein expression and activity were found early (~24 h) after exposure of human endothelial and Schwann cells to high glucose thus suggesting that this mechanism can be of importance in human PDN.

13. CONCLUSION.

Multiple mechanisms are involved in the pathogenesis of PDN. New findings support the role for previously discovered mechanisms such as increased AR activity, non-enzymatic glycation/glycooxidation, PKC activation, oxidative stress, and impaired neurotrophic support in functional and morphological abnormalities in the diabetic nerve. Evidence for the important role for nitrosative stress, MAPK activation, PARP activation, COX-2 activation and Ca^{++} signaling is emerging. Several newly discovered mechanisms include activations of NF- κ B, the 12/15-lipoxygenase pathway and Na^+/H^+ exchanger-1. Most of the pathogenetic mechanisms converge at the level of oxidative-nitrosative stress which is likely to be a central mechanism in PDN (Fig.1). Studies of the roles for individual mechanisms leading to oxidative-nitrosative stress and PDN, and their interactions with each other are in progress.

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FIGURE LEGENDS.

1. Interactions among pathogenetic mechanisms of diabetic neuropathy triggered by hyperglycemia.

ACCEPTED MANUSCRIPT

