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Mechanisms of disease: The oxidative stress theory of diabetic neuropathy

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Abstract Diabetic neuropathy is the most common complication of diabetes, affecting 50% of diabetic patients. Currently, the only treatment for diabetic neuropathy is glucose control and careful foot care. In this review, we discuss the idea that excess glucose overloads the electron transport chain, leading to the production of superoxides and subsequent mitochondrial and cytosolic oxidative stress. Defects in metabolic and vascular pathways intersect with oxidative stress to produce the onset and progression of nerve injury present in diabetic neuropathy. These pathways include the production of advanced glycation end products, alterations in the sorbitol, hexosamine and protein kinase C pathways and activation of poly-ADP ribose polymerase. New bioinformatics approaches can augment current research and lead to new discoveries to understand the pathogenesis of diabetic neuropathy and to identify more effective molecular therapeutic targets.

Keywords Diabetes · Neuropathy · Oxidative stress · Bioinformatics

1 Introduction

Diabetic neuropathy is the most common complication of diabetes. While estimates vary, depending on the methods used to diagnose diabetic neuropathy, it is generally held that at least 50% of all diabetic patients will develop neuropathy in his or her lifetime [1–7]. This high prevalence of neuropathy is likely an underestimate as several recent studies report that patients with impaired fasting glucose and/or impaired glucose tolerance also exhibit neuropathy at the time of diagnosis. Diabetic neuropathy is the most common cause of foot ulcers and non-traumatic amputations in the Western world. Patients with diabetic neuropathy report a poor quality of life secondary to pain, disability and recurrent hospitalizations. It is estimated that in the United States the annual cost of diabetic neuropathy is nearly $11 billion dollars and increasing annually in parallel with the alarming increase in the incidence and prevalence of diabetes (www.diabetes.org).

There are no treatments for diabetic neuropathy other than glycemic control and diligent foot care [1, 3, 7–9]. This is in spite of ongoing research addressing the pathogenesis of the disorder, with the goal to identify mechanism based treatments. In recent years, the idea has emerged that multiple distinct metabolic pathways are impaired leading to a singular end result: enhanced cellular oxidative stress. This review will focus on the relationship between mitochondrial and cytosolic oxidative stress and the biochemical pathways that converge to enhance cellular oxidative stress and the onset and progression of diabetic neuropathy. The reader is referred to the following recent review articles for reviews of the symptoms, staging and treatment of diabetic neuropathy [1–7, 10].

1.1 Oxidative stress: reactive oxygen/nitrogen species (ROS/RNS) formation in the mitochondria

The "free radical theory" of aging was proposed by Harman in 1956. Based on his theory of aging, "the reaction of active free radicals, normally produced in organisms, with cellular constituents initiate the changes associated with

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aging." Excess generation of free radicals results in upregulation of stress signaling, negatively affecting both life quality and life span. Reactive oxygen (ROS) and reactive nitrogen (RNS) species are linked to multiple disease states [11–15], including the microvascular complications of diabetes [16–20]. Mitochondrial metabolism and the cascade of oxidative phosphorylation are highlighted as key contributors of ROS generation in many diseases.

Mitochondrial oxidative phosphorylation is the major ATP synthetic pathway in eukaryotes. In this process, electrons from reducing substrates are transferred to molecular oxygen (O_2) via respiratory chain complexes I–IV. These complexes establish a hydrogen gradient across the inner mitochondrial membrane, and the electrochemical energy of this gradient is then used to drive ATP synthesis by ATP synthase (complex V).

There are four protein complexes associated with the respiratory chain. NADH–ubiquinone oxidoreductase, or complex I, accepts electrons from NADH; these electrons are carried to succinate dehydrogenase, complex II, and used to oxidize succinate to fumarate. Electrons continue to travel down an electrochemical gradient to ubiquinol–cytochrome *c* oxidoreductase (complex III), and subsequently to cytochrome *c* oxidase (complex IV), which are finally used to reduce molecular oxygen to water. Even though the majority of molecular oxygen is reduced at complex IV to water via the respiratory chain, 1–4% of the oxygen is incompletely reduced to superoxide ($O_2 \cdot^-$) [21]. $O_2 \cdot^-$ is the most common ROS and creates other ROS/RNS via various enzymatic or nonenzymatic reactions discussed later in this review.

$O_2 \cdot^-$ generation by mitochondrial electron transport chain is mainly at complexes I and III. It is suggested that $O_2 \cdot^-$ production in complex I is via reverse electron transfer, and is predominately released into the matrix [22]. Autoxidation of the ubisemiquinone radical intermediate ($QH^{\cdot}$) at complex III is the other source of $O_2 \cdot^-$ generation. Complex III has the capacity to release $O_2 \cdot^-$ to both sides of the mitochondrial inner membrane, however, the Q site closer to the intermembrane space (Qo), is known to be the major site of $O_2 \cdot^-$ production, and the matrix side (Qi) is less likely to form $O_2{\cdot}^-$ [23, 24].

As stated above, $O_2{\cdot}^-$ is the major ROS produced in the intermembrane space or matrix of mitochondria. As a charged reactive species, $O_2{\cdot}^-$ does not readily diffuse across mitochondrial membranes. However, the mitochondrial permeability transition pore might serve as a channel for intermembranous mitochondrial $O_2{\cdot}^-$ to pass through the outer mitochondrial membrane and into the cytosol [25]. The conversion of $O_2{\cdot}^-$ to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) facilitates permeation through the generation of uncharged ROS, which easily diffuse across the membrane.

There are three isoforms of SOD, SOD 1 or copper zinc SOD (CuZn–SOD), SOD 2 or manganese SOD (Mn–SOD), and SOD 3 or extracellular CuZn–SOD (EC–SOD) [26]. CuZn–SOD (SOD 1) is found in the cytosol, nucleus, and intermembrane space of mitochondria [27], Mn–SOD (SOD 2) is expressed only in the mitochondrial matrix, and SOD 3 is located in the extracellular space. Among all SOD isoforms, SOD 2 is physiologically more important and its genetic elimination, in contrast to other isoforms, is embryonically lethal [28, 29].

H_2O_2 is reduced enzymatically by catalase and glutathione peroxidase. In the mitochondrial matrix, glutathione peroxidase uses glutathione to convert H_2O_2 to water. Catalase has higher K_m for H_2O_2 compared to glutathione peroxidase, and can protect against a higher concentration of H_2O_2 [30]. Other antioxidant enzymes such as glutathione *S*-transferase and thioredoxin [31] also help in removal and inactivation of ROS formed in the mitochondria. In the presence of transition metals such as copper and iron, H_2O_2 generates hydroxyl radical (OH) via the Fenton reaction or the Haber–Weiss reaction. Hydroxyl radicals are very highly reactive and contribute significantly to local organelle damage through DNA and protein modification.

2 Regulation of mitochondrial ROS production

In the process of oxidative phosphorylation, energy carried by electrons is used by complexes I, III, and IV to pump protons out of the matrix. The resulting electrochemical gradient across the mitochondrial inner membrane is used by ATP synthase to drive the synthesis of ATP from ADP. In mitochondria, increased ATP synthesis is regulated by uncoupling proteins. Upon activation of uncoupling proteins (UCP), protons leak across the inner membrane and "uncouple" oxidative metabolism from ATP synthase, resulting in loss of ATP production. Basal and hyperglycemia-induced ROS formation are decreased in dorsal root ganglia sensory neurons that over express UCP [32]. Mitochondrial membrane permeability is increased via activation of UCP by $O_2{\cdot}^-$, resulting in decreased electrochemical potential and further reduction of $O_2{\cdot}^-$ generation. Mild mitochondrial depolarization that limits Ca^{2+} accumulation and reduces reactive species generation (e.g. by limiting nitric oxide synthase, NOS, activity) may explain the protective effect of UPC [33].

Mitochondrial ROS are also regulated by nitric oxide (NO), a diffusible gas produced by NOS. The presence of mitochondrial NOS (Mt NOS) and its activity were reported by Ghafourifar and Richter in 1997 [34]. Mt NOS is associated with the matrix face of the mitochondrial inner membrane. The activity of Mt NOS is regulated by intramitochondrial Ca^{2+} concentration, $[Ca^{2+}]m$ [34].

Elevation of $[Ca^{2+}]$m increases NO production and leads to reduction in $\Delta\psi$, while a decrease in $\Delta\psi$ releases Ca^{2+} from the mitochondria and results in Mt NOS inactivation.

Other ROS/RNS are generated in the mitochondria from interaction of $O_2^{\cdot -}$ and other reactive species. Generation of $O_2^{\cdot -}$ in the presence of NO results in peroxynitrite (ONOO−) formation. The rate of $ONOO^-$ formation is 9.5×10^{-8} M s^{-1}, which exceeds the interaction of NO with cytochrome *c* oxidase (0.8×10^{-8} M s^{-1}) [35, 36]. Ghafourifar et al. reported that peroxynitrite-induced stress promotes cytochrome *c* release from the mitochondria and results in apoptosis [37]. Mt NOS is also involved in mitochondrial dysfunction. Nitration of the tyrosine residues [38–40] of proteins and *S*-nitrosation of protein thiols are very important reactions in the mitochondria [41].

3 ROS/RNS and diabetic neuropathy

As discussed above, under normal conditions, neurons have the capacity to neutralize both ROS and RNS [42–44]. Because $O_2^{\cdot -}$ and H_2O_2 are normal products of the mitochondrial electron transport chain, SOD, catalase, and glutathione are normally sufficient to remove these metabolic byproducts (Fig. 1) [45]. However, hyperglycemia increases mitochondrial activity and subsequent $O_2^{\cdot -}$ production. A surplus production of this primary mitochondrial ROS leads to formation of RNS as outlined in the previous section. Thus, excess mitochondrial activity leads to an overwhelming production of ROS and RNS in a neuron that is already depleted of reducing equivalents and struggling with oxidative stress brought on by other metabolic and inflammatory insults (reviewed below). The buildup of ROS/RNS in the neuron coupled with the inability of the neuron to detoxify the excess ROS and RNS leads to progressive organelle, membrane and nuclear dysfunction.

Of note, mitochondria are both the source of ROS/RNS generation and also the first structures to be damaged, putting the neuron at even greater risk. Given the typical distal–proximal length dependent progression of diabetic neuropathy, axons are particularly susceptible to the

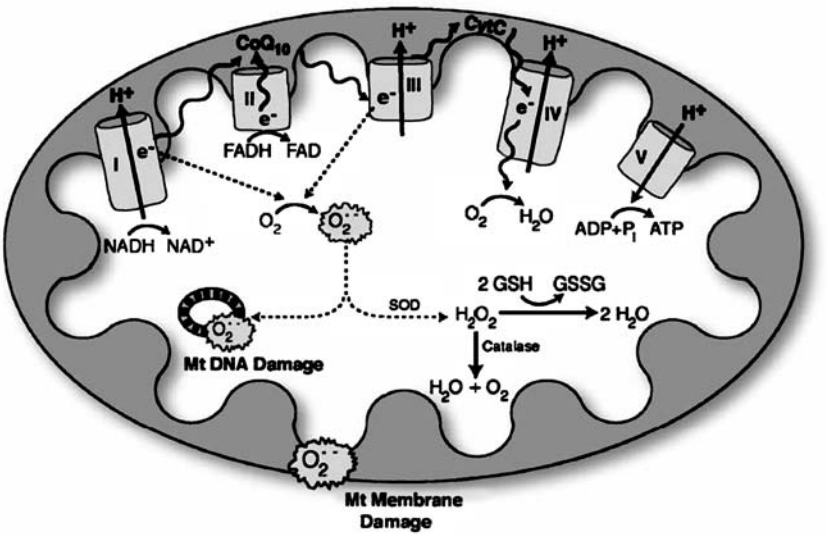

Fig. 1 Oxidative stress and mitochondrial dysfunction [45]. Hyperglycemia increases production of reactive oxygen species (ROS) in mitochondria. NADH and $FADH_2$ produced from the tricarboxylic acid cycle transfer to the mitochondria, where they serve as electron donors to the mitochondrial membrane-associated redox enzyme complexes. The electrons (e^-) are shuttled through oxidoreductase complexes I, II, III and IV (cytochrome *c*), until they are donated to molecular oxygen, forming water. The electron transfer into complexes I, III and IV by NADH (and $FADH_2$ via complex II to complex III) produces a proton gradient at the outer mitochondrial membrane, generating a potential between the inner mitochondrial membrane and outer mitochondrial membrane. This potential drives ATP synthesis, and is crucial for mitochondrial viability, function, and normal metabolism. As electrons are passed from complex II to complex III, however, ROS are produced as by-products. The levels of ROS produced during normal oxidative phosphorylation are minimal, and they are detoxified by cellular antioxidants such as glutathione, catalase and superoxide dismutase. The hyperglycemic cell, on the other hand, shuttles more glucose through the glycolytic and tricarboxylic acid cycles, providing the cell with an over-abundance of NADH and $FADH_2$ electron donors. This produces a high proton gradient across the inner mitochondrial membrane, which increases the turnover of the initial complexes, and thereby produces increased levels of radicals. Accumulation of these radicals, or ROS, is severely detrimental to mitochondrial DNA, mitochondrial membranes and the whole cell. Abbreviations: *Cyto-c* cytochrome *c*, *CoQ10* coenzyme Q10, e^- electrons, *GSH* glutathione, *GSSG* oxidized glutathione, H_2O_2 hydrogen peroxide, $O_2^{\cdot -}$ superoxide, P_i phosphate, *SOD* superoxide dismutase

metabolic and vascular imbalances that lead to diabetic neuropathy [45]. Axons are susceptible to hyperglycemia not only because of their direct access to nerve blood supply, but also because of their large population of mitochondria. Mounting evidence suggest that axons are as, if not more, susceptible to ROS and RNS mediated damage, in part because of their dependence on local mitochondria for energy. As these mitochondria become progressively dysfunctional, axons undergo energy failure which in turn precipitates axonal degeneration [45–47].

Mitochondria are also critical regulators of cell survival signaling pathways, and not surprisingly, oxidative damage to mitochondrial DNA, proteins, and membranes initiates signaling pathways that subsequently lead to apoptosis. Prior to the onset of frank apoptosis, mitochondria damaged by oxidative stress are destroyed via a localized process called mitoptosis. Mitoptosis is regulated, in part, by shifting the balance in the normal mitochondrial fission/fusion equilibrium. The fission of mitochondria is initiated by dynamin related protein 1 (Drp1), which translocates from the cytosol to the mitochondria during times of stress [48]. Excess mitochondrial fission leads to mitoptosis, which then may progress to apoptosis. Drp1 is elevated in *in vitro* and *in vivo* models of diabetic neuropathy [45], further promoting mitochondrial dysfunction, energy failure and axonal degeneration.

While numerous studies document the presence of axonal dystrophy and apoptosis in diabetic sensory neurons [49], some studies have failed to detect apoptosis in high glucose treated sensory neurons in culture [50, 51]. A hypothesis that accounts for this is that neurons, with support from trophic factors and antioxidants provided by surrounding glia, are initially able to undergo successful repair. Eventually, though, the cycle of glucose mediated ROS/RNS accumulation results in mitochondrial damage and the injury cascade outlined above: energy failure and axonal degeneration [52, 53].

4 The intersection of ROS/RNS and other metabolic pathways

Excess production of mitochondrial ROS/RNS is a pivotal step in hyperglycemia-induced nerve damage in diabetic neuropathy. Several other metabolic pathways become perturbed in the nervous system due to continued hyperglycemia. Each of these pathways contributes to the neuronal and axonal injury present in diabetic neuropathy, and also to enhanced levels of oxidative stress present in the diabetic nervous system. In the following sections, each of these pathways will be discussed, and the reader is provided with one or more references for thorough reviews on the individual pathways. Figure 2 is a schematic of the interaction of the pathways in the generation of diabetic neuropathy; the pathways are discussed in the order they are presented in Fig. 2, from left to right.

As the reader reviews the other metabolic pathways believed to produce diabetic neuropathy, it is critical to remember that diabetic neuropathy results from both hyperglycemia-induced damage to nerve cells and axons *per se* and from neuronal ischemia caused by hyperglycemia-induced decreases in neurovascular flow. We have summarized recent animal models of experimental diabetes and neuropathy with evidence of peripheral nervous system oxidative stress in Table 1.

4.1 Advanced glycation endproducts (AGE) pathway

Advanced glycation endproducts (AGEs) are non-enzymatically created adducts between reducing sugars or oxaldehydes and proteins, DNA, or lipids [72, 73]. AGEs are thus heterogenous, and are found both inside and outside the cell, where their formation interferes with multiple aspects of cell function. Reactive dicarbonyls are the precursor molecules to AGEs that spontaneously form covalent bonds with proteins or lipids, and are synthesized through three pathways: glucose oxidation, which forms glyoxal; degradation of fructose-lysine adducts (Amadori products); and formation of methylglyoxal through the abnormal metabolism of glycolytic intermediates. Methylglyoxal is highly reactive and causes vascular endothelial cells to become more sensitive to damage [74]. Extracellular formation of protein AGEs not only disrupt cellular adhesion (through interference with cell surface protein/extracellular matrix interactions), but also activate a specific cell-surface receptor for the AGEs, known as RAGE [75].

Activation of RAGE by extracellular AGEs leads to activation of the transcription factor nuclear factor kappa B (NF-κB), which regulates gene expression, apoptosis and inflammation (Fig. 2). RAGE activation in diabetic animal models contributes to the onset and progression of diabetic neuropathy. When RAGE knockout mice are made diabetic with streptozotocin (STZ), there is a significant improvement in both electrophysiological and anatomical markers of diabetic neuropathy, compared to the STZ control animals. The diabetic RAGE knockout mice also have decreased expression of NF-κB and protein kinase C in peripheral nerves, and particularly in Schwann cells [73]. RAGE activation in neurons also induces NADPH oxidase activity, which further promotes mitochondrial oxidative stress and dysfunction [76]. The confluence of data strongly suggest that RAGE is a therapeutic target for the treatment of diabetic neuropathy [73, 76–81]. For a detailed dis-

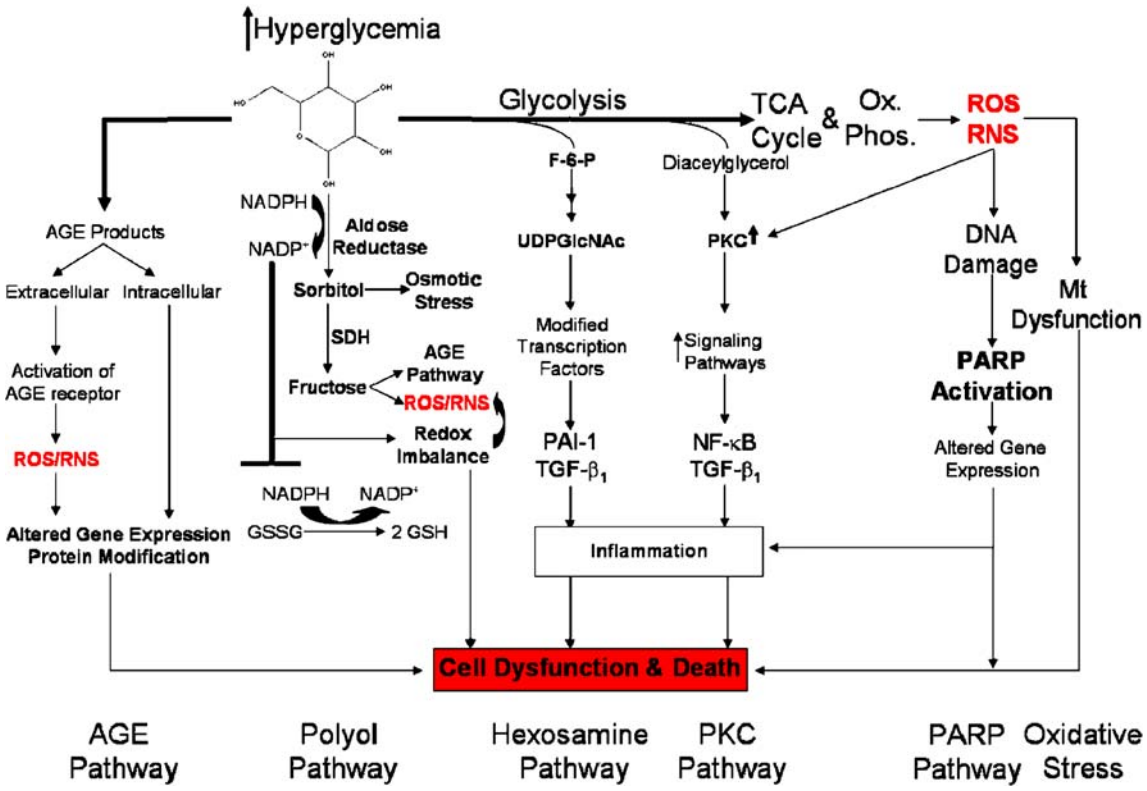

Fig. 2 Schematic of hyperglycemic effects on biochemical pathways in diabetic neuropathy [46]. Excessive glucose metabolism generates excess NADH and leads to overload of the electron transport chain causing oxidative stress, damage to Mt, activation of PARP. PARP activation by ROS acts in conjunction with the hexosamine and PKC pathway to induce inflammation and neuronal dysfunction. A combination of oxidative stress and hyperglycemia activate the detrimental pathways of AGE, polyol, hexosamine and PKC pathways which lead to redox imbalance, gene expression disturbances, and further oxidative stress. These pathways also induce inflammation and neuronal dysfunction. Abbreviations: *NF-κB* nuclear factor kappa B, *PARP* poly(ADP-ribose) polymerase, *PKC* protein kinase C, *AGE* advanced glycation endproducts, *RNS* reactive nitrogen species, *ROS* reactive oxygen species, *GSH* glutathione, *GSSG* oxidized glutathione, *UDPGlcNAc* UDP-*N*-acetylglucosamine, *VEGF* vascular endothelial growth factor. (Reprinted from [46] copy right 2008 with permission from Elsevier)

cussion on the role of AGE and RAGE in the pathogenesis of diabetic neuropathy and the potential therapeutic efficacy of blocking RAGE in the treatment of diabetic neuropathy, the reader is referred to an excellent 2008 review by Sugimoto and colleagues [82].

4.2 Polyol pathway

The polyol pathway converts glucose to fructose through a two-step reduction/oxidation: First, aldose reductase reduces glucose to sorbitol, and then sorbitol dehydrogenase oxidizes sorbitol to fructose (Fig. 2). Both aldose reductase and sorbitol dehydrogenase are prevalent in tissues prone to diabetic complications. The aldose reductase pathway is susceptible to over activation by a mass-action effect of hyperglycemia, which results in imbalances of two of the pathways metabolites, NADPH and sorbitol. Excess glucose flow through the pathway causes consumption of NADPH, which is required for regeneration of reduced glutathione [83, 84]. The depletion of glutathione secondary to excess aldose reductase activity thus renders the cell susceptible to oxidative stress, as discussed above. Increased production of sorbitol causes the intracellular environment to become hypertonic, and leads to compensatory efflux of other osmolytes such as myo-inositol (MI, important in signal transduction) and taurine (an antioxidant) [19, 85]. Intracellular reducing potential is further diminished by the second step in the polyol pathway, the production of fructose [86]. Hyperglycemia-driven production of excess fructose promotes glycation and further depletion of NADPH. Finally, activation of aldose reductase may also increase formation of diacylglycerol, which activates the deleterious protein kinase C pathway (discussed below) [87, 88]. Several studies of the human aldose reductase gene revealed polymorphisms associated with susceptibility to diabetic complications. Patients with a

Table 1 Rodent models of diabetic neuropathy and oxidative stress

Rodent model of diabetes	Duration of diabetes	Neuropathy assessment	OX stress measurements	Reference
STZ-induced				
DBA/2J mice	24 weeks	NCV behavior	NT HODEs	Wiggin et al. [54]
Wistar rats	6 and 12 weeks	MNCV	Hexokinase	Gardiner et al. [55]
Sprague–Dawley rats	8 weeks, 2 or 8 weeks endaravone	MNC, NBF behavior	LPO, SOD catalase	Saini et al. [56]
Sprague–Dawley rats	6 weeks untreated, 2 weeks reseratrol	MNCV, NBF behavior	MDA, peroxynitrite catalase	Kumar et al. [57]
Sprague–Dawley rats	4–12 weeks untreated, 12 weeks enalapril/L-158809	MNCV, NBF	$O_2^{\cdot-}$	Coopey et al. [58]
Male Wistar rats	2 weeks untreated, 2 weeks ISO	Behavior	NT, PAR O^{2-}	Ilnytska et al. [59]
Sprague–Dawley rats	2 weeks untreated, 10 weeks fidarestat	MNCV, NBF	GSH, 8-OHdG sorbitol	Kuzumoto et al. [60]
Sprague–Dawley rats	6 weeks untreated, 2 weeks U83836E	MNCV, NBF behavior	SOD, catalase MDA	Sayyed et al. [61]
Sprague–Dawley rats	6 weeks untreated, 2 weeks troxol	MNCV, NBF behavior	SOD catalase LPO, NOS	Sharma and Sayyed [62]
Sprague–Dawley rats	4, 12, 52 weeks	MNCV, SNCV	8-OHdG	Schmeichel et al. [63]
C57BL6 mice	8 weeks untreated, 1 weeks FP15	MNCV, SNCV	NT, PCr/Cr, sorbitol, glucose, fructose	Obrosova et al. [64]
COX-2 KO mice	24 weeks	MNCV, SNCV, INFD	MDA, $O_2^{\cdot-}$, GSH, PG	Kellogg et al. [65]
AR KO mice	4, 8, 12 weeks	MNCV, SNCV	JNK activation, GSH, $O_2^{\cdot-}$, DNA damage, sorbitol, 8-OHdG	Ho et al. [66]
Galactose-induced Wistar rats, PARP KO mice	4 weeks untreated, 2 weeks 3-aminobenzamide, 13 weeks	MNCV, SNCV, NBF	PAR, PCr/Cr, nerve energy failure	Obrosova et al. [67]
Spontaneous				
ZDF rats	8–40 weeks old	MNCV, NBF	NT, $O_2^{\cdot-}$	Oltman et al. [68]
BKS.Cg-m+/+*Lepr*^{db}/J (BKS-db/db), C57BL/6J STZ-induced	24 weeks old	MNCV, SNCV, behavior, INFD	NT	Sullivan et al. [52]
B6.V-Lep^{ob}*/J*	8 weeks old untreated, 3 weeks FP15/FeTMPS	MNCV, SNCV, behavior, INFD	NT, PAR	Vareniuk et al. [69]
B6.V-Lep^{ob}*/J*	5 weeks old untreated, 6 weeks fidarestat	MNCV, SNCV, behavior, INFD	NT, PAR	Drel et al. [70]
C57BL6/J high-fat diet	16 weeks old high-fat diet	MNCV, SNCV, behavior, INFD	NT, 4-HNE, PAR, 12/15-lipoxygenase, Sorbitol	Obrosova et al. [71]

4-HNE Aminoacid-(4)-hydrosynonenal adducts, *8-OHdG* 8-hydroxy-2′-deoxyguanosine, *AR* aldose reductase, *FeTMPS* Fe(III) tetra-mesitylpophyrin octasulfonate, *FP15* Fe(III) tetrakis-2-(*N*-trethylene glycol monomethyl ether)-pyridyl porphyrin, *ISO* 1,5-isoquinolinediol, *LPO* lipid peroxidation, *MDA* malondialdehyde, *MNCN* motor nerve conduction velocities, *NBF* nerve blood flow, *NT* nitrotyrosine, $O_2^{\cdot-}$ superoxide, *PAR* poly(ADP)-ribose, *PCr/Cr* phosphocreatinine/creatinine ratio, *PG* prostaglandin content, *SNCV* sensory nerve conduction velocities, *SOD* superoxide dismutase, *STZ* streptozotocin, *ZDF* Zucker diabetic fatty

"high aldose reductase expression" genotype are commonly found to have early diabetic neuropathy while patients with a "low aldose reductase expression" genotype are less susceptible to neuropathy [89–91].

The polyol pathway has and continues to be a target of drug intervention in the treatment of diabetic neuropathy. Aldose reductase inhibitors block the formation of sorbitol preventing NADPH depletion. This leaves sufficient NADPH for glutathione production allowing neurons to mount a cellular defense against ROS/RNS mediated damage. It is now generally believed that it is this mechanism of action that underlies the salutary effects of

aldose reductase inhibitors. Peter Oates recently published a thorough review of the polyol pathway and the past and current aldose reductase inhibitor trials in man [92]. As of yet, none of these compounds have shown efficacy in a Phase 3 trial of diabetic neuropathy.

4.3 Hexosamine pathway

As with the polyol pathway, excess available glucose causes a mass action increase in flux through the hexosamine pathway. Under normal circumstances, a small amount of the glycolytic intermediate fructose-6 phosphate is shunted from glycolysis to the hexosamine pathway. The hexosamine pathway converts fructose-6 phosphate to glucosamine-6 phosphate by glutamine fructose-6 phosphate amidotransferase [93]. Glucosamine-6 phosphate is then converted to uridine diphosphate-*N*-acetyl glucosamine (UDP-GlcNAc), which is the obligatory substrate for *O*-GlcNAc transferase, attaching *O*-GlcNAC to the serine and threonine residues of transcription factors and altering gene expression [16]. Thus, a hyperglycemia-driven increase in flux through this pathway results in abnormalities in gene expression [16, 94, 95]. An increased understanding of *O*-GlcNAC biology also suggests that *O*-GlcNACcylation regulates the nutrient sensing role of the hexosamine pathway and has a role in insulin resistance and macrovascular complications [96, 97].

Sp1 is one transcription factor implicated in diabetic complications that is subject to modification by UDP-GlcNAc. Sp1 regulates the expression of many glucose-induced "housekeeping" genes, including tissue type plasminogen activator inhibitor-1 (PAI-1) and transforming growth factor-β1 (TGF-β1) [16, 98]. Interest in plasminogen activator and PAI-1 is based on the premise that impaired fibrinolysis in small neural blood vessels promotes nerve ischemia, leading to oxidative stress and the signs and symptoms of diabetic neuropathy. Data to support this idea come primarily from studies in man. Plasminogen activator expression is lower by four to six fold in the epineurial and endoneurial microvessels in sural nerves from patients with diabetic neuropathy compared to control nerve biopsies. This lower expression would promote thrombosis and nerve ischemia [99]. This idea is further supported by data from men with type 1 diabetes in the Epidemiology of Diabetes Interventions and Complications Study (EDIC); patients with diabetic neuropathy had higher serum levels of plasminogen activator/PAI-I complexes than those men without neuropathy [100]. Type 2 patients who are obese have higher PAI-I levels which may contribute to the high incidence of diabetic neuropathy in this population [101, 102]. In experimental animals, PAI-I blocks nerve regeneration [103]. More work is needed on experimental models of diabetic neuropathy to fully understand the role of plasminogen activator/PAI-I complexes.

Over expression of TGF-β1 is associated with diabetic nephropathy and contributes to microvascular damage by stimulating collagen matrix production and suppressing mitogenesis of mesangial cells [104, 105]. A recent study by the Russell laboratory identifies a role for TGF-β and other TGF isoforms in experimental diabetic neuropathy. After 12 weeks of STZ diabetes, TGF-β isoforms are increased in the dorsal root ganglia and sciatic nerves of rodents with neuropathy. In parallel, TGF-β isoforms applied directly to dorsal root ganglia cultures *in vitro* block neurite outgrowth [106]. Collectively, these new findings suggest TGF-β may be a potential new target for diabetic neuropathy, similar to its role in diabetic nephropathy.

4.4 Protein kinase C (PKC) pathway

Hyperglycemia stimulates over-activation of the protein kinase C (PKC) pathway by increasing synthesis of diacylglycerol (DAG), which activates PKC. The PKC β-isoform in particular has been linked to the development of retinopathy, nephropathy, and cardiovascular disease [107–109]. Hyperstimulation of PKC causes the overexpression of the angiogenic protein vascular endothelial growth factor (VEGF), PAI-1, NF-κB, and TGF-β, supporting a role for PKC activation in the pathogenesis of diabetic neuropathy (Fig. 2). Studies in STZ diabetic rats report that inhibitors of PKC-β improve measures of diabetic neuropathy, including sciatic blood flow and nerve conduction velocity, [85, 110]. While the exact mechanisms by which PKC-β contributes to diabetic neuropathy require further study, PKC-induced vasoconstriction, altered capillary permeability, hypoxia, and nerve basement membrane thickening are all thought to be involved [107, 108]. Overexpression of PKC isoforms also directly induces insulin resistance which can further contribute to the onset of diabetic neuropathy [111, 112]. Treatment of patients with symptomatic diabetic neuropathy with a PKC inhibitor, ruboxistaurin, did not result in clinical improvement [113, 114], which could be due to the fact the drug can not penetrate the blood nerve barrier [108]. A recent review completely discusses the role of PKC and diabetic micro- and macrovascular complications and the therapeutic efficacy of PKC inhibitors [108].

4.5 Poly-ADP ribose polymerase (PARP) pathway

PARP, a nuclear enzyme closely associated with oxidative-nitrosative stress, is expressed in sensory neurons, Schwann cells, and endothelial cells. While hyperglycemia, free

radicals, and oxidants stimulate PARP activation, PARP also causes oxidative stress (Fig. 2) [115]. PARP cleaves nicotinamide adenine dinucleotide (NAD^+) to nicotinamide, and also removes ADP-ribose residues attached to nuclear proteins [116]. PARP's catalytic activity causes a number of deleterious effects, including changes in gene expression, increases in free radical and oxidant concentration, NAD^+ depletion, and shunting of glycolytic intermediates to other pathogenic pathways that can lead to PKC activation and AGE formation [64, 117–119]. In experimental diabetes, these varied effects result in neurovascular abnormalities, neuropathy, decreased nerve conduction velocity, thermal and mechanical hyperalgesia, and tactile allodynia [44, 59, 67, 120–122]. Several recent reviews outline the role of PARP activation in diabetic neuropathy and discuss emerging new PARP targeted therapies [123, 124].

4.6 Inflammation

Elevated blood levels of inflammatory proteins, including C-reactive protein and TNF-α, are associated with neuropathy [125, 126]. Hsp 27, part of the TNF-α signaling pathway that leads to release of the inflammatory mediators cyclooxygenase-2 (Cox-2), IL-6, and IL-8, was recently found by the Eurodiab study to be elevated in the blood of diabetic patients with neuropathy [127]. As discussed in the previous sections of this review, some inflammatory mediators like TNF-α and TGF-β are regulated by hyperglycemia-driven abnormalities in metabolism and signaling [17, 20]. Excess glucose-mediated activity in the hexokinase and PKC pathways results in activation of signaling intermediates and modified transcription factors, ultimately increasing TGF-β and NF-κB [16]. Similarly, formation of the AGE methylglyoxal results in covalently modified transcription factors that can lead to aberrant expression of inflammatory proteins, particularly a repressor of angiotensin II called Sp3 [74]. The resulting increase in available angiotensin II activates vascular endothelial cells [74]. Activated endothelial cells in the endoneurium recruit inflammatory cells, leading to local cytokine production, reduced blood flow, and generation of reactive oxygen species [58].

RAGE activation by extracellular AGEs also affect inflammation by causing the upregulation of NF-κB [73], which in turn upregulates Cox-2 [128]. Cox-2 activation results in a feed forward loop: Cox-2 stimulates production of prostaglandin E2 and ROS, which go on to further activate NF-κB. NF-κ-B/Cox-2 upregulation is present in the vasculature and peripheral nerves of animal models of diabetes [129]. Either blocking Cox-2 upregulation with a drug or genetic knock-down prevents multiple aspects of diabetic neuropathy, including blood flow and nerve conduction deficits, glutathione depletion, and TNFα upregulation [65, 130].

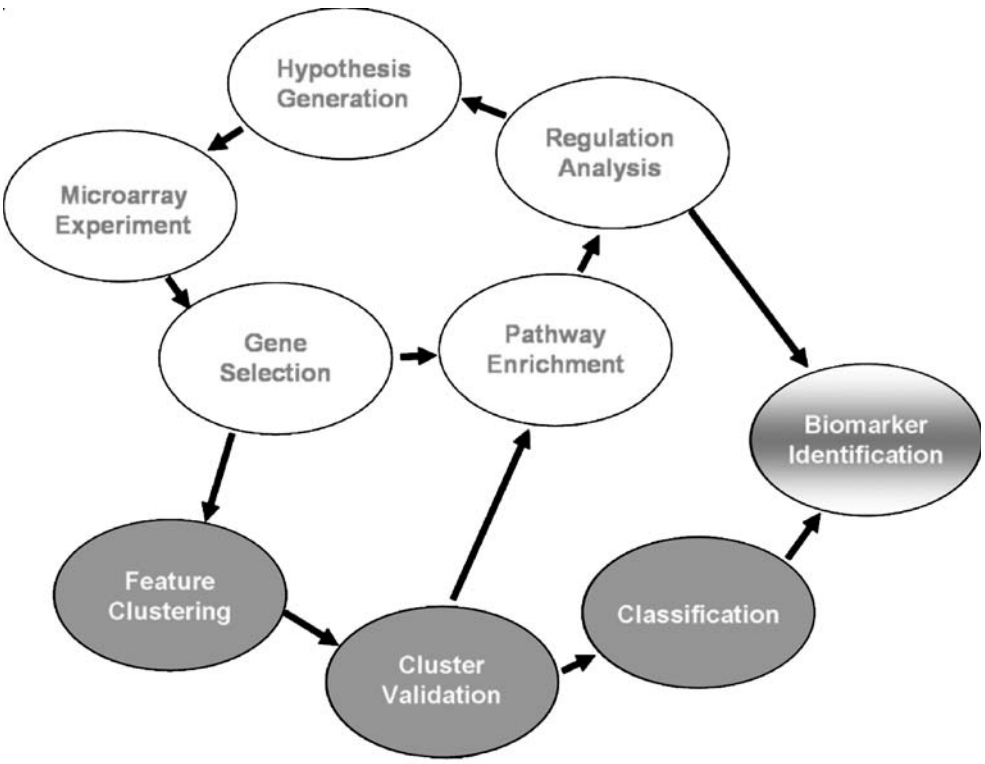

Fig. 3 Methodologies in biomarker research [46]. Activities in white are hypothesis driven and attempt to identify biomarkers based on the disequilibrium of identified targets in diabetic neuropathy, leading to an abnormal accumulation of products, such as modified proteins or small molecules. Activities in grey are discovery oriented and seek to identify features of the data set that are predictive of diabetic neuropathy without necessarily corresponding to a single target. (Reprinted from [46] copy right 2008 with permission from Elsevier)

NF-κB participates in a second vicious cycle of inflammation, in which it both induces and is induced by inducible nitric oxide synthase (iNOS) [131, 132]. NO produced by the excess of iNOS contributes to microvascular damage by diminishing the blood supply to nerves [133, 134]. Moreover, NO contributes to axon and myelin degeneration following injury, damages growth cones, and is involved in the development of neuropathic pain [133, 135].

NF-κB appears to be the keystone of the inflammatory pathways that participate in the development of diabetic neuropathy. Chronic NF-κB activation appears to render neurons and blood vessels more susceptible to ischemia–reperfusion injury [136]. The subsequent extensive infiltration of macrophages is further intensified by NF-κB-stimulated release of cytokines from endothelial cells, Schwann cells and neurons [137]. The activation of macrophages leads to further production of cytokines, as well as proteases and ROS that lead to myelin breakdown, cellular oxidative damage, and impairment of nerve regeneration [138–140]. Cameron and Cotter [141] have recently reviewed the role of NF-κB in diabetic neuropathy and the new therapies targeted at decreasing inflammation to halt progression of diabetic neuropathy.

5 The search for novel therapeutic targets

Glucose control remains the only disease-modifying therapy for diabetic neuropathy [7, 18, 47]. We propose a bioinformatics approach as the next important paradigm in examining the causes and potential treatments of diabetic neuropathy. This novel paradigm will provide insight into disease pathogenesis and identify viable targets for disease-modifying treatments. Analysis of genomic and proteomic data from patients and animal models of diabetic neuropathy will not only validate or refute current hypotheses but will also lead to new ideas to further enhance our understanding of disease onset and progression.

To date, only two animal studies (and no human studies) have addressed alterations in gene expression within the peripheral nervous system under hyperglycemic stress and/or a common treatment for diabetes [54, 142]. Price et al. [142] performed microarray analyses on Wistar rat dorsal root ganglion neurons, 1, 4, and 8 weeks post-STZ-induced diabetes. Diabetic neuropathy was confirmed by slowed nerve conduction velocities at weeks 4 and 8 [142]. The induction of diabetes, prior to the onset of neuropathy correlated with the upregulation of genes involved in glucose metabolism [142]. By week 4, glutathione transferase was upregulated secondary to conditions of oxidative stress [142]. We recently reported (2008) that genes functionally relevant to metabolism, mitochondria, metal ion binding and general cellular regulation are significantly differentially expressed in the sciatic nerves of type 1 diabetic and healthy mice [54]. The changes in mitochondrial gene expression were linked to the presence of both NF-κB and AP1 binding sites in the proximal promoter, a configuration that was ten-fold over-represented in the regulated mitochondrial genes compared to the overall distribution of transcription factor binding sites in mouse promoter regions. The diabetic mice were treated with Rosiglitazone, which reduced the development of neuropathy, and decreased oxidative stress in the nerve [54]. Genes that were significantly regulated by diabetes then returned to normal levels by Rosiglitazone treatment were analyzed for common transcription factor binding sites, and the results were cross checked in healthy mice treated with Rosiglitazone to determine the direction of causation. Two

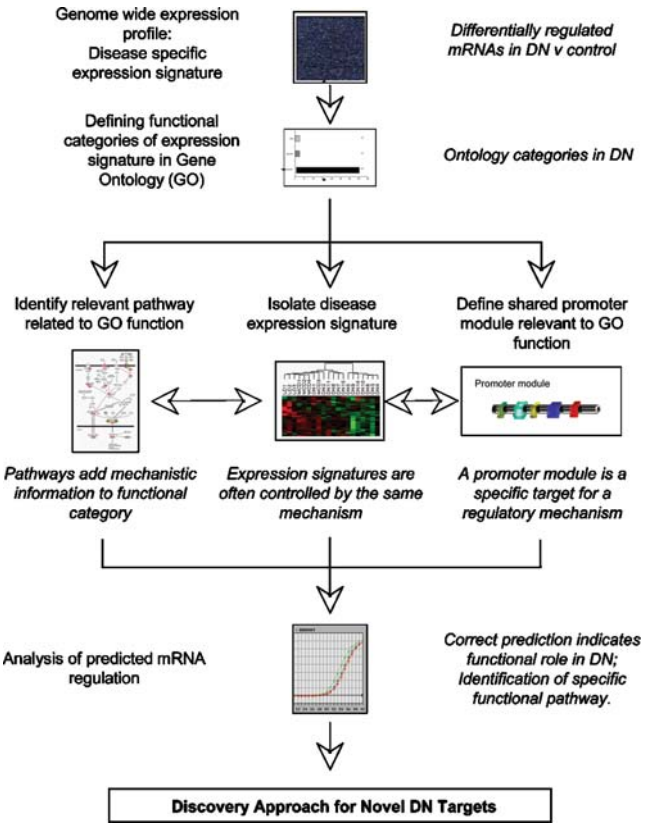

Fig. 4 Discovery approach for novel targets in diabetic neuropathy (DN) [46]. Genome wide expression profiling of neural tissues from animal models with diabetic neuropathy (DN) will yield differentially regulated transcripts. Analyses of these data using Gene Ontology (GO) will provide the data needed to define categories of genes that are functionally related providing a molecular signature for diabetic neuropathy. Further analyses of these data can define relevant pathways related to functional gene categories and shared promoter modules among members of different gene categories, providing one or more specific targets for disease regulation. These targets can be verified at the mRNA level, confirming the identification of a novel disease target. (Reprinted from [46] copy right 2008 with permission from Elsevier)

site combinations, SP1F and ZBPF, and a configuration of two EGRF sites were found to be significant by both approaches [54]. We and others are now making a comprehensive effort to establish the molecular signatures of neural tissues including peripheral nerve and sensory and sympathetic ganglia, from genome wide screening of RNA from human patients and animal models with and without diabetic neuropathy [52, 143]. Our approach for diabetic neuropathy is presented in Fig. 3.

The feasibility, power, and utility of using a discovery/bioinformatics approach to uncover disease mechanisms is described for chronic kidney disease by Kretzler and colleagues [144–147]. Human renal biopsies examined by Affymetrix™ microarray analyses and real-time RT-PCR revealed differentially expressed genes between healthy and diseased tissue. These genes were mapped onto known cellular pathways that predicted regulatory elements controlling the observed changes. The regulatory elements were then used to predict the downstream effects of gene expression, including the potential biomarkers of chronic renal disease. We propose a similar comprehensive approach be applied to diabetic neuropathy to advance our understanding of disease pathogenesis and development of disease-modifying therapies. Of special interest is how this approach, in parallel, leads to biomarker discovery. As outlined in Fig. 4, the first step is to employ microarray analyses and confirmatory Q-PCR to analyze gene expression of relevant neuronal and Schwann cell markers followed by validation techniques to detect enriched pathways. These data provide the information needed to predict proteins and macromolecules influenced by gene expression. The use of clustering and classification analysis, while maintaining high standards of mathematical validation, is a valuable tool in discovering the most useful target genes, proteins and macromolecules.

6 Summary

Ongoing research suggests that multiple metabolic and vascular pathways intersect to produce systemic and neural oxidative stress that underlies the onset and progression of diabetic neuropathy. Therapies based on the mechanisms discussed in the current review have not yet been successful in ameliorating disease progression. We suggest that a new bioinformatics approach to diabetic neuropathy provides promise for the future identification of more promising molecular targets.

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