

*Review***Experimental diabetic neuropathy: an update****A.A.F. Sima, K. Sugimoto**

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Abstract

Diabetic neuropathy consists of several clinical syndromes affecting motor, sensory and autonomic nerves. Of these the most common is distal symmetric sensory polyneuropathy usually referred to as diabetic neuropathy. Animal studies, mainly in diabetic rodents, have contributed tremendously to our understanding of this disease. From these it is clear that the pathogenesis of diabetic neuropathy is multifactorial involving sequentially occurring and often closely interrelated metabolic aberrations. Major pathogenetic mechanisms include increased activity of the polyol pathway, abnormalities in vasoactive substances, non-enzymatic glycation, increased presence of free radicals, and perturbed neurotrophism. Traditionally the neuropathies accompanying Type I

(insulin-dependent) and Type II (non-insulin-dependent) diabetes mellitus have been regarded as identical. Recent investigations have, however, clearly delineated distinct differences in the functional and structural expressions of the neuropathies in the two types of diabetes. Major future challenges are the identification of the differences in underlying pathogenetic mechanisms in the two types of neuropathy and in gaining a better understanding of the hierarchy of the multifactorial mechanisms underlying the disease. This will be important for designing meaningful therapies which to date have failed miserably in diabetic neuropathy. [Diabetologia (1999) 42: 773-788]

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The peripheral nerve complications encountered in diabetes mellitus encompass a variety of abnormalities affecting both somatic and autonomic peripheral nerve function [1] and they are associated with the in-

creased morbidity and mortality seen in diabetic patients [1, 2]. The prevalence of clinically overt peripheral neuropathy is 7% within a year of diagnosis of diabetes and increases to 50% in patients with diabetes for more than 25 years [3]. If subclinical non-symptomatic neuropathy is included the prevalence approaches 100%. Diabetic neuropathies include several distinct syndromes of which symmetric sensory polyneuropathy, often associated with autonomic polyneuropathy, is the most common [1] and is usually referred to as diabetic neuropathy. The different syndromes can be separated into rapidly reversible manifestations and chronic syndromes. The latter can be further divided into symmetric polyneuropathies and focal and multifocal neuropathies [4].

The pathogenesis of diabetic neuropathy has been the subject of extensive research and some controversy over the last decade [5, 6] and has still not been ful-

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Abbreviations: ACE, Angiotensin converting enzyme; AG, aminoguanidine; AGE, advanced glycation end product; ARI, aldose reductase inhibitor; BDND, brain derived neurotrophic factor; CML, *N*^ε-[carboxymethyl]-lysine; 3DG, 3-deoxyglucosone; GLUT, glucose transporter; GSH, glutathione; IGF-1R, insulin-like growth factor-1 receptor; IR, insulin receptor; MGO, methylglyoxal; NCV, nerve conduction velocity; NF- κ B, nuclear factor κ B; NGF, nerve growth factor; NO, nitric oxide; NT-3, neurotrophin 3; NT 4/5, neurotrophin 4/5; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin.

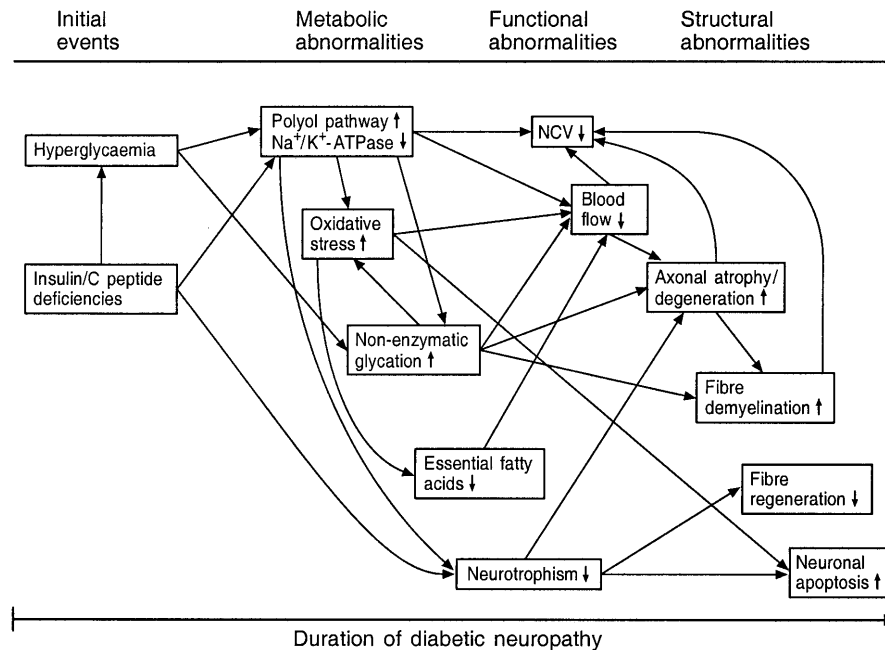


Fig. 1. The metabolic abnormalities induced by hyperglycaemia and insulin/C peptide deficiencies are multifold and appear to occur in a temporal sequence. The major metabolic abnormalities are commonly interactive and mutually perpetuating (see text). They lead to impaired nerve function and decreased blood flow, which initially are readily reversible. As structural changes emerge and progress, the functional abnormalities become increasingly less responsive to metabolic interventions

ly explored. There appears, however, to be agreement that the underlying causes of diabetic neuropathy are multifactorial, where distinct aberrations of specific metabolic pathways appear to be interrelated and mutually perpetuating [5–7]. Further, certain metabolic pathways of pathogenetic importance appear to be more relevant during particular phases of the clinical course of the disease (Fig. 1). Accumulation of sorbitol from activation of the polyol pathway seems to be an early event in both experimental [8] and human diabetic polyneuropathy [1] since increased sorbitol content is usually not found in chronically diabetic human nerve [9]. On the other hand, abnormalities in the expression of certain neurotrophic factors [10] and perturbed calcium metabolism [11] implicated in the pathogenesis of diabetic neuropathy seem to evolve more slowly and only become evident after several weeks or months of diabetes in the rat. This suggests a sequential and multi-step series of pathogenetic events in diabetic neuropathy.

Hyperglycaemia seems to be the common and an important culprit in the initiation of the various metabolic aberrations underlying diabetic neuropathy. This notion was confirmed by the results of the Dia-

betes Control and Complications Trial showing a 60% reduction in clinical neuropathy in Type I diabetic patients subjected to intensive insulin treatment for 5 years [12]. As intensive glycaemic control does not completely prevent neuropathy hyperglycaemia is probably not the only initiating metabolic abnormality responsible for diabetic neuropathy. Recent studies of the neuropathies occurring in Type I and Type II diabetic patients and animal models [5, 13] suggest that insulin or C peptide deficiencies or both as such contribute to the generally more severe diabetic neuropathy in Type I compared with that of Type II diabetes [14–16] (Fig. 1). Furthermore, only 50% of diabetic subjects develop clinically overt diabetic neuropathy [3] which strongly suggests susceptibility genes exist that predispose to the development of clinically overt diabetic neuropathy. The exploration of this possibility is, however, in its infancy for chronic complications of diabetes [17,18] and is yet to start for diabetic neuropathy.

Hence, as more data accumulate about the mechanisms of the evolution of this common complication of diabetes, we are facing an increasingly complex, multifaceted disease process.

In this review we will first describe the structural changes in different models of experimental diabetes and point out their differences particularly between Type I and Type II diabetes and how they relate to human diabetic neuropathy. We will also emphasise how various structural changes relate to underlying metabolic and molecular abnormalities and the extent to which structural changes possibly account for functional abnormalities.

In the second part various pathogenetic mechanisms will be dealt with in the sequence in which they seem to evolve during the clinical course of dis-

Table 1. Comparison of functional and structural abnormalities in Type I and Type II diabetic animal models

	Type I diabetes		Type II diabetes	
	STZ rat	BB/W rat	<i>db/db</i> mouse	BB/ <i>Z^{DR}</i> rat
Nerve conduction velocity deficit	+++	+++	+	+
Axonal atrophy	++	+++	+	+
Axoglial disjunction	++	+++	-	-
Paranodal demyel.	++	+++	+	+
Segmental demyel.	+	+	++	++
Wallerian degeneration	+	+	++	++
Fibre loss	-	++	-	-

+ = mild; ++ = moderate; +++ = severe; - = absent; demyel. = demyelination

ease. Again we will attempt to emphasise how these affect nerve function and structure (Fig. 1).

These data have almost exclusively been obtained from experimental studies but will as far as possible be compared with human diabetic neuropathy.

Pathology of experimental diabetic neuropathy

Various experimental diabetic models have been used in the study of the structural changes in peripheral nerve [5, 19, 20]. They have shown varied results possibly because Type I and Type II animal models tend to exhibit different structural changes [5, 21]. Furthermore, the age at which diabetes is induced in inducible rat models appears to affect the structural development of the neuropathy [20]. The most commonly used experimental model, the streptozotocin (STZ)-induced diabetic rat, is regarded as being a Type I model [19, 20] despite not requiring insulin supplementation for its survival and being confounded by severe emaciation. The STZ rat model has early functional and biochemical abnormalities similar to those in human diabetic neuropathy [22]. These will be described later in this review. In contrast to the profound biochemical and functional abnormalities, morphological changes are mild in the peripheral nerve in the STZ rat which even after extended periods fails to show apparent fibre loss or pronounced nerve fibre degeneration [22] (Table 1). Detailed morphometric examinations of peripheral nerve in the STZ rat have, however, shown reproducible changes [21, 23] such as reduction in fibre size with a proximo-distal gradient [24, 25]. Teased fibre examinations have shown nodal swelling, paranodal and segmental demyelination in a small percentage of fibres in rats diabetic for 8–12 months [24, 26, 27]. Ultrastructural examinations have also shown paranodal axo-glial disruption preceding paranodal demyelination [26] (Table 1). It has been suggested that axonal atrophy in the STZ rat is due to osmotic shrinkage [28] or retardation of normal axonal maturation [29]. These explanations are unlikely because there is no condensation of axoplasmic organelles which would be expected with osmotic shrinkage of the axon [5].

A maturational defect in axonal growth is also unlikely since treatment with aldose reductase inhibitors (ARIs) or vasoactive agents which shows no effects on body growth restores axonal size [27, 30]. The characteristic axonal atrophy is already present in distal nerves after 8 weeks of STZ diabetes whereas proximal nerve fibres show expanded axons [25], reflecting a progressive defect in axonal transport of neurofilaments. As the neuropathy progresses proximal axons also become atrophic [24], suggesting a later occurring superimposed defect in the synthesis of neuroskeletal proteins. These findings coincide with the emergence of the defects in the availability of nerve growth factor (NGF) and IGF-1 both of which are necessary for the synthetic integrity of structural proteins in the cell somata [31, 32]. In this context note that neural hypoxia, which has been invoked as an important pathogenetic factor [30], does not induce pronounced slowing of the slow component of axonal transport [33] and is therefore not likely to be responsible for the early distal axonal atrophy characteristic of the neuropathy in the STZ rat.

Distal axonal atrophy and cytoskeletal abnormalities are also seen in autonomic nerves, in which preganglionic and post-ganglionic fibres undergo degeneration and dystrophic changes [34]. These abnormalities possibly reflect a defect in the turnaround mechanisms of axonal transport. Structural examinations of the endoneurial vasculature has not been undertaken in a systematic fashion although it has been reported that microvascular densities increase after long-term treatment with evening primrose oil (containing γ -linoleic acid (GLA), dihomo GLA and dilinolein-mono GLA) [35]. It has been claimed that there is a reduction in the number of anterior horn motor neurons in STZ diabetes [36] and that dorsal root ganglion cells undergo an 18% loss in perikaryal volume [37]. Increased vacuolation of dorsal root ganglion cells has also been noted in rats with long-standing STZ diabetes [38]. These changes may reflect impaired protection against apoptosis in diabetes, mediated by decreased expressions of neurotrophic factors, particularly IGF-1 [39].

In contrast to the STZ rat, the spontaneously diabetic BB/W rat [40] develops severe structural chan-

ges in both somatic sensory and motor nerves and autonomic peripheral nerves [41–42] (Table 1) as well as in sensory central nerve tracts [43]. The diabetes in this model occurs spontaneously and rapidly around the age of 75 days due to an immune-mediated total destruction of pancreatic beta cells [40] with complete lack of insulin and C peptide. Hence, these animals require daily supplementation of insulin to survive. We have used this model extensively in our laboratory because it probably represents the best rodent model of human Type I diabetes.

The acute onset of diabetes is accompanied by an abrupt 20% decrease in nerve conduction velocity and evoked potential amplitudes [44], which subsequently level off until approximately 4 months duration of diabetes when there is a further progressive decline in nerve conduction velocity (NCV) [41, 44]. This late progressive decline in NCV coincides with progressive disruption of the paranodal Schwann cell-axon contacts (axo-glial disjunction), which constitute the paranodal ion-channel barrier [44] (Table 1). The early functional changes are reversible after insulin, ARI, or *myo*-inositol treatments [45–47]. They correlate with morphometrically detectable nodal swellings [45, 47] which are associated with intra-axonal $[Na^+]$ accumulation secondary to increased inactivation of nodal sodium channels and the early Na^+/K^+ -ATPase defect [47, 48]. These structural and metabolic abnormalities are also rapidly reversible after correction of hyperglycaemia or the polyol pathway or neural *myo*-inositol depletion [45–47].

The progressive disruption of the paranodal apparatus is associated with decreased nodal sodium permeability, probably related to redistribution of nodal sodium channels to the internodal axolemma [49]. Interestingly this paranodal structural defect is also associated with a similar redistribution of Schwann cell glucose transporter (GLUT)-1 and axonal GLUT-3 [50]. The functional consequence of these abnormalities is a slowed increase in the initial Na^+ current of the evoked potential, which eventually leads to a conduction block resulting in decreased NCV. The specific molecular events underlying these paranodal changes, characteristic of both Type I human and rodent neuropathy are not understood. Preliminary studies have shown imbalances in the expression of various cell-adhesive molecules as well as polysialic acid and E-cadherin [51]. It is not known whether the activity and signal transduction of these molecules are regulated by the co-localised, high affinity insulin receptor.

Apart from axo-glial disjunction and paranodal demyelination, the neuropathy in the BB/W rat is characterised by progressive distal axonal atrophy of both myelinated and unmyelinated fibres similar to, but more severe than, that in the STZ rat [41, 52]. By a year of diabetes there is approximately a 50% loss of myelinated fibres in the sural nerve [41]. Hence,

these changes are similar to those in Type I human diabetic neuropathy [13]. In addition to the peripheral nerve changes, the BB/W rat shows progressive distal axonal atrophy and fibre loss in the dorsal columns of the spinal cord [53], underpinning a true central-peripheral dying back phenomenon characterising diabetic neuropathy.

Axonal atrophy and degeneration occur in both myelinated and unmyelinated fibres of the vagus nerve accompanied by a progressive decline in RR interval variability [54]. Similar changes occur in the penile and distal myenteric parasympathetic nerves [42] and in the nerves innervating the urinary bladder [55]. In these autonomic circuits notably the sensory afferent limb is consistently affected first and progressively more severely involved compared with the efferent parasympathetic limb [55].

Examination of the optic nerve in the BB/W rat has shown axonal atrophy and paranodal axo-glial disjunction similar to that seen in somatic peripheral nerve [56], although they occur at a slower pace than in peripheral nerves. These changes which are responsive to ARI treatment correlate with prolonged latencies of visual evoked potentials [56]. Mononeuropathic changes resulting from endoneurial vascular occlusions by platelets, probably secondary to abnormal prostacyclin metabolism, have been reported in the BB/W rat [57].

As in the STZ rat, nerve regeneration is impaired in the BB/W rat [58], which is associated with perturbations of neurotrophic factors such as NGF and IGF-1 and their receptors [59] (Fig. 1). Note that although regeneration is highly promoted by ARI treatment in previously axotomised BB/W rats [58], the increased number of regenerated fibres do not attain functional and structural maturity even 220 days after axotomy [59]. As in non-treated diabetic BB/W rats, those treated with ARI fail to show maturation of the paranodal apparatus of regenerated fibres, which most likely accounts for the failure to attain normal NCVs and amplitudes in regenerated fibres [58].

From the above it is apparent that the spontaneously diabetic BB/W rat develops a spectrum of neuropathic syndromes which generally resemble those that develop in Type I human diabetic neuropathy [13]. Although, underlying metabolic abnormalities are similar to those of the STZ rat, its advantage lies in the development of structural changes similar to those in human disease, which hence can be correlated with functional and metabolic aberrations as outlined above. The STZ rat by comparison can be regarded as in-between a Type I and Type II diabetic model since it is not insulin dependent but capable of producing sufficient insulin (and C peptide) for its survival.

Several Type II diabetic models have been investigated. The *db/db* mouse shows full penetrance of a

Type II hyperinsulinaemic diabetic syndrome associated with obesity. Nerve conduction velocity decreases with onset of diabetes and is reversed by sufficient insulin treatment [60]. In contrast to other diabetic rodent models, in the *db/db* mouse motor NCV appears to be more consistently affected than sensory NCV [61].

Structurally this model shows a mild but progressive axonal atrophy but no pronounced fibre loss or paranodal changes [60, 62] (Table 1). The fibre atrophy seen in this model was suggested [62] to be due to a maturational defect as reflected by decreased long bone growth. These data could not, however, be replicated by others [60]. Endoneurial capillaries in the *db/db* mouse are normal in appearance, although thickening and duplication of endothelial basal laminae have been noted. The fatty Wistar diabetic rat (WKY/Ncp) has proved a useful model for Type II diabetes [63, 64]. It shows decreased motor NCV and structural changes occurring at 10–11 months of diabetes consisting of axonal degeneration, paranodal and segmental demyelination [63]. The WBN/Kob rat is a selectively inbred spontaneously diabetic model that develops chronic pancreatitis and non-insulin requiring diabetes. Structural studies of this model have shown mild axonal atrophy, segmental demyelination and pronounced endoneurial vascular changes [65].

A relatively new model with spontaneous onset of Type II diabetes, the obese BB/Z^{DR} rat [66], shows mild deficits in NCV and progressive mild axonal atrophy but no nerve fibre loss even after 14 months of diabetes. Instead, it shows relatively strong segmental demyelination and Wallerian degeneration compared with the Type I diabetic BB/W rat (Table 1). This, like all of the Type II rodent animal models, fails to show paranodal changes or axo-glial disjunction which is in keeping with the lack of this structural abnormality in Type II human diabetic neuropathy [13]. It therefore seems to set Type I and Type II diabetic neuropathies apart not only neuropathologically but also translating into differences in NCV.

Thus, several animal models representing either Type I or Type II diabetes develop neuropathologic changes. Despite similar hyperglycaemic levels, differences exist in their expression of morphologic changes. Type I BB/W rats show severe axonal and paranodal changes, less expressed in Type II models which instead tend to show more pronounced demyelinating and vascular changes. The commonly used STZ rat shows structural changes between those of the Type I and Type II models.

Pathogenetic mechanisms

The polyol pathway and Na⁺/K⁺-ATPase. An early and extensively investigated metabolic pathway in diabetic neuropathy is the increased activation of the

polyol pathway in which excessive glucose is shunted by the high K_m aldose reductase to form sorbitol and fructose [6]. Accumulation of the organic osmolyte sorbitol results in compensatory depletions of other osmolytes such as *myo*-inositol and taurine [67]. *Myo*-inositol depletion is by perturbation of phosphoinositide metabolism [6], in part responsible for the concomitant reduction in Na^+/K^+ -ATPase activity, which appears to be important in explaining the early reversible nerve conduction defect in experimental diabetes [68, 69]. It has been suggested that impaired activation of neural protein kinase C by diminished phosphoinositide derived diacylglycerols mediates the effect of *myo*-inositol depletion on Na^+/K^+ -ATPase activity [70,71]. It is notable that in galactosaemic rats or mice who develop a diabetes-like neuropathy, the NCV defect is associated with an increase in Na^+/K^+ -ATPase [72, 73], suggesting that in these models factors other than impaired Na^+/K^+ -ATPase activity underlie the functional defect. Alternatively, increased Na^+/K^+ -ATPase in galactosaemic neuropathy may represent endothelial Na^+/K^+ -ATPase activity rather than neuronal.

Furthermore protein kinase C inhibitors have an unexpected effect on the hyperexcitability of nociceptive C-fibres in STZ diabetic rats [74], suggesting that protein kinase C isoforms are affected differently in diabetic peripheral nerve. Na^+/K^+ -ATPase activity in diabetic rats can be affected through several perturbed metabolic pathways some of which are associated with activation of the polyol pathway such as nitric oxide synthesis [6], pseudohypoxia [75] or prostacyclin deficiency [76, 77]. This explains not only the effects of aldose reductase inhibition and *myo*-inositol supplementation on Na^+/K^+ -ATPase and NCV in experimental diabetic neuropathy but also those of prostacyclin analogues or their precursors [77], acetyl-L-carnitine [8, 78] or C peptide [15].

The polyol pathway is also linked to oxidative stress, since this is considerably diminished in sciatic nerve and lens by ARI treatment of diabetic or rats fed with galactose [79, 80] (Fig. 1). Aldose reductase of the polyol pathway requires NADPH as a cofactor as does the glutathion redox cycle [30] and nitric oxide synthase in the arginine → citrulline + nitric oxide pathway [6]. Thus, when the polyol pathway is activated, a competitive NADPH deficit will impact on the recycling of glutathione (GSH) from oxidised glutathion (GSSG) (Fig. 2) and the synthesis of nitric oxide (Fig. 3). It has been shown that oxidation of sorbitol to fructose by sorbitol dehydrogenase increases the ratio of NADH:NAD⁺, resulting in an increased lactate:pyruvate ratio and “pseudohypoxia” [75] and that inhibition of sorbitol dehydrogenase improves vascular and neural dysfunction [81]. These results have not, however, been reproduced by others [82, 83]. Treatment with acetyl-L-carnitine, which has no effect on the polyol pathway, has been shown to cor-

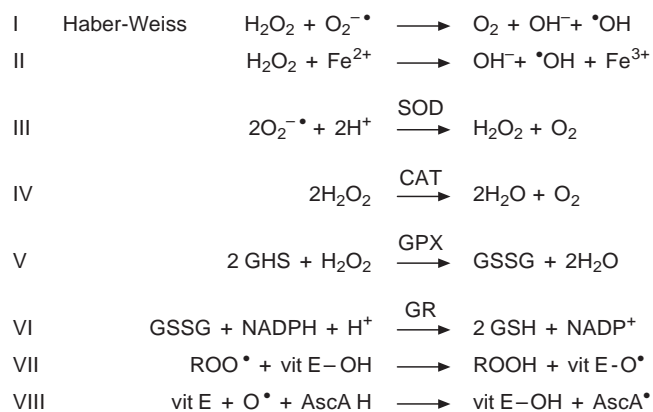


Fig. 2. Sequence of the formation of hydroxyl radicals ($\bullet OH$) and the ROS scavenging system. In the Haber-Weiss reaction (I) superoxide $O_2^{\bullet -}$ is reduced by hydrogen peroxide, H_2O_2 , to form hydroxyl radicals ($\bullet OH$). This reaction is catalysed by transition metal ions, mainly iron or copper, the Fenton reaction (II). Superoxide $O_2^{\bullet -}$ is reduced to H_2O_2 in the presence of superoxide dismutase (SOD) (III), which is further reduced to water and molecular oxygen in the presence of catalase (CAT)(IV). Hydrogen peroxide, H_2O_2 is reduced by reduced glutathione (GSH) in the presence of glutathione peroxidase (GPX)(V). The oxidised glutathione (GSSG) so formed is reduced back to reduced glutathione in the presence of glutathione reductase (GR) and NADPH (VI). Peroxyl radicals (ROO^\bullet) are reduced by vitamin E (vit E-OH) (VII), and oxidised vitamin (Vit E- O^\bullet) is reduced, probably by ascorbic acid (AscAH)

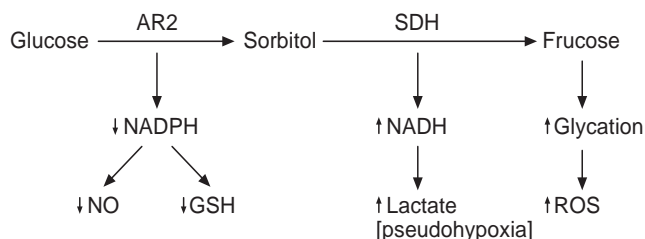


Fig. 3. Relations between the activated polyol pathway, NO production, reduced glutathione (GSH) availability, pseudohypoxia, non-enzymatic glycation and ROS production

rect neural Na^+/K^+ -ATPase [8, 84], blood flow [85, 86], NCV as well as vasoactive prostaglandins [8]. An additional mechanism by which Na^+/K^+ -ATPase activity is impaired in diabetic nerve is by C peptide or insulin deficiency or both because C-peptide treatment, which does not influence hyperglycaemia, returns Na^+/K^+ -ATPase and NCV to normal in diabetic nerve [14, 15] (Fig. 1). Whether this effect by C peptide is due to activation of the insulin receptor signalling pathway intermediaries or alternate pathways is not yet known [87].

These findings suggest that multiple, not necessarily related to the polyol pathway, metabolic aberrations are responsible for decreased neural Na^+/K^+ -ATPase. The absence of detectable demyelination or nerve fibre degeneration in association with the rapid NCV

slowing in acutely diabetic rats [41, 45], suggest a biochemical or biophysical mechanism or both. Direct measurement of reduced nerve Na^+/K^+ -ATPase activity associated with decreased NCV as well as their corrections by dietary *myo*-inositol [45, 69], ARI treatment [69] acetyl-L-carnitine [8] or C-peptide supplementation [14, 15] suggests a close link. This is further supported by voltage clamp studies in acute diabetic neuropathy in the diabetic BB/W rat documenting a corresponding reduction in the resting axolemmal membrane Na^+ potential, increased inactivation of nodal Na^+ -channels and a multifold increase in intra-axonal $[Na^+]$ [47–48]. These bioelectrical abnormalities are associated with morphometrically detectable nodal axonal swellings, which are reversible after *myo*-inositol supplementation or ARI treatment [45–47]. It therefore appears that the first step of the polyol-pathway activation is important for the acute effects of hyperglycaemia in diabetic nerve.

In the galactosaemic rat model, galactose is converted to galactitol by aldose reductase, which is, however, a poor substrate for sorbitol dehydrogenase and is therefore not metabolised further by the second step of the pathway. Importantly, galactosaemia gives rise to ARI-preventable abnormalities similar to those of diabetes such as NCV slowing, vascular dysfunction, impaired regenerative capacity, nodal axonal swelling and paranodal axo-glial disjunction [88, 89]. Similar results were obtained from feeding galactose to mice transgenic for the human aldose reductase gene, who develop slowed NCV and severe nerve fibre atrophy [90]. Composite Na^+/K^+ -ATPase activity in peripheral nerve is, however, increased in galactosaemic animals but returns to normal after treatment with ARI [73].

From experimental diabetic neuropathy it is therefore apparent that the activation of the polyol pathway by glucose plays an important part in the early pathophysiology of this disorder not only by exerting effects on sorbitol, *myo*-inositol and Na^+/K^+ -ATPase metabolism but also through secondary mechanisms affecting generation of reactive oxygen species, perturbing nitric oxide synthesis and promoting non-enzymatic glycation (Figs. 1, 3) [6, 30].

It should, however, be emphasised that increased polyol-pathway activity does not appear to be an essential pathogenetic mechanism in diabetic neuropathy, since diabetic mice accumulate only low polyol concentrations in sciatic nerve and still develop neuropathy [91]. Recent studies on the BB/W rat suggest that the accumulation of sorbitol and associated *myo*-inositol depletion diminish as the neuropathy progresses and that secondary or other metabolic mechanisms or both become more prominent [8]. Whether this is also true for human diabetic neuropathy is not known but, if so, it would in part explain the generally disappointing results from clinical neuropathy trials using aldose reductase inhibitors [92].

The role of oxidative stress and decreased blood flow.

Diabetes causes increased oxidative stress which can be defined as a higher production of reactive oxygen species (ROS) as well as an impaired endogenous capacity to scavenge free radicals [7, 93, 94]. Increased production of ROS leads to increased peroxidation of lipid membranes, proteins and DNA with important consequences for cell function and structure. A notable target for increased pro-oxidant activity in diabetes is the vascular system in part explaining the increased propensity for atherogenesis and cardiovascular disease [30]. In the microvascular complications of diabetes, oxidative stress affects endothelial cell function and vascular reactivity, contributing to impaired blood flow and oxygenation in peripheral nerve (Fig.1). The mechanisms underlying the increased presence of ROS and impaired activity of antioxidant factors in diabetes are multifold and not completely understood [93, 94].

Molecular oxygen becomes toxic when reduced products like superoxide, $O_2^{\cdot-}$, hydrogen peroxide and the hydroxyl radical $\cdot OH$ are formed through the Harber-Weiss reaction (Fig.2). Further contributions are provided by autoxidation of molecules like glucose, ascorbic acid, thiols and catecholamines enhanced by transition metal ions, particularly iron and copper [95].

Increased production of ROS is associated with ischaemia-reperfusion effects induced by the conversion of xanthine to uric acid by xanthine oxidase [96]. Reactive oxygen species are generated from the electron transport chains in mitochondria and by activated phagocytes.

Polyunsaturated fatty acids are particularly vulnerable to free radical damage because the fatty acid radical adds oxygen to form a peroxy radical which by oxidising further fatty acids perpetuates the process [30]. Another particular target for ROS is the glycolytic and mitochondrial phosphorylation of ADP with profound effects on cell energy metabolism [97].

Among the free radical defence mechanisms, superoxide dismutase (SOD) scavenges superoxide and is located in cytosol and mitochondria. The hydrogen peroxide that is formed is converted to water and molecular oxygen (Fig.2). Glutathione peroxidase is an enzyme that eliminates both lipid hydroperoxides and hydrogen peroxide. Most free radical scavengers, however, are compounds which react with free radicals. An important compound is α -tocopherol (vitamin E) that reacts with lipid peroxy and hydroxyl radicals (Fig.2). It interrupts the self-perpetuating chain reaction of lipid peroxidation. Other lipid soluble antioxidants are ubiquinone and carotenoids. Water soluble antioxidants include ascorbic acid, cystine and uric acid.

Oxidative stress causes a series of changes in endothelial cell function and gene expression in diabetes.

The vascular renin-angiotensin system is up regulated in several vascular systems including vasa nervorum [98, 99]. Circulating plasma angiotensin converting enzyme (ACE) activity is increased in diabetic rats and patients [100]. This increase is prevented in STZ rats by the antioxidant probucol [101]. Another vaso-genic factor increased by oxidative stress is endothelin-1, probably by nuclear factor- κB (NF- κB) [102].

Both endothelin-1 and angiotensin II are potent vasoconstrictors and therefore probably contributors to reduced peripheral nerve blood flow in diabetes.

Endothelin-1 antagonists, such as the ET_A receptor antagonists BQ123 and BM5 182874, restore the decreased blood flow and NCV in streptozotocin STZ rats, whereas the ET_A/ET_B antagonist basentan has a lesser effect [103, 104]. This is probably because stimulation of the ET_B receptor releases nitric oxide (NO) and prostacyclin, causing vasodilation which offsets the ET_A -mediated vasoconstriction [103]. Activation of NF κB also increases adhesion of leucocytes to endothelial cells and therefore contribute to increased incidence of thrombosis in diabetes [105]. α -Tocopherol and probucol have inhibitory effects on leucocyte adhesion [106].

Reduced nutritive blood flow resulting in endoneurial hypoxia has been implicated as an important and early pathogenetic factor in diabetic neuropathy in both patients and animal models [7, 107]. This notion is strongly supported by experimental data, since blockade of the renin-angiotensin system with either ACE-inhibitors or angiotensin II AT_1 receptor antagonists reduces impaired blood-flow, endoneurial oxygenation and NCV in STZ diabetic rats [98, 99, 108]. The same regimen also prevents the increased resistance to ischaemic conduction failure and restores the blunted regenerative capacity of nerve fibres to normal [99]. In addition ACE and AT_1 inhibition partially protects against deficits in NO-mediated vessel relaxation and vessel hypertrophy [109].

Oxidative stress affects endothelium-dependent vessel relaxation which is evident even in acute hyperglycaemia. Peroxynitrite formed from the neutralising effect of superoxide on NO has a toxic effect on endothelial cells [110]. The reduction in acetylcholine mediated endothelium-dependent relaxation of aortic rings incubated in high glucose can be partially prevented by the antioxidant probucol or by SOD [111, 112].

Likewise impaired endothelium-dependent relaxation of corpus cavernosum, coronary vessels and aorta can be prevented by a variety of radical scavengers, such as α -tocopherol, dimethylthiourea and acetyl cysteine, which also correct impaired peripheral nerve blood flow and NCV [113]. Acetyl cysteine and α -tocopherol have, in addition, a protective effect on axonal atrophy and blunted nerve fibre regeneration characteristic of diabetic neuropathy [114, 115].

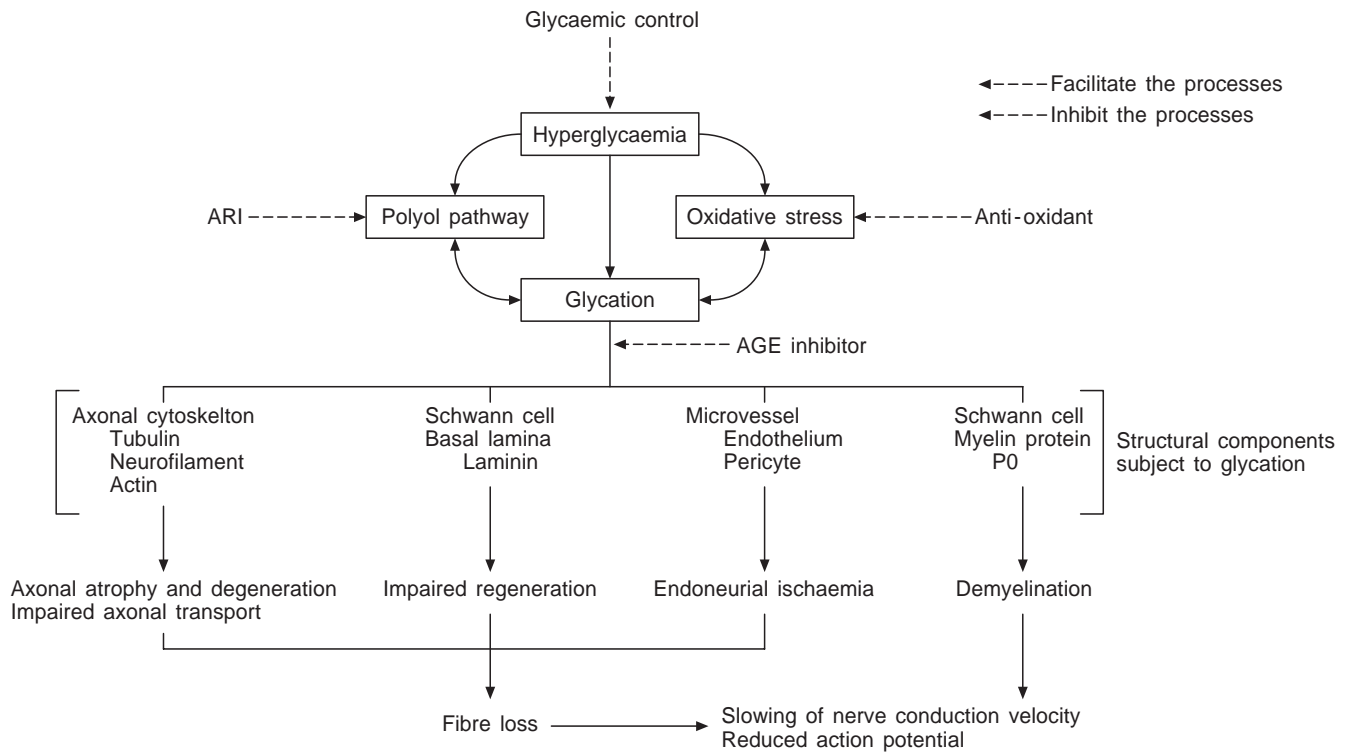


Fig. 4. Pathogenetic mechanisms possibly related to non-enzymatic glycation. Consequences of neuropathic changes developing as a result of non-enzymatic glycation of structural components are shown. Axonal cytoskeletal proteins such as tubulins, neurofilaments, and actin are subject to non-enzymatic glycation. Glycation of these structural proteins may contribute to impaired axonal transport, axonal atrophy and degeneration. Myelin proteins such as PO, myelin basic protein, and proteolipid protein are also subjected to glycation and may be scavenged by macrophages, possibly leading to segmental demyelination. Deposition of AGE's-proteins in Schwann cells may result in dysregulation of trophic support of nerve fibres and impaired synthesis of myelin proteins. Modification of laminin by glycation may impair fibre regeneration. Localisation of glycated proteins such as CML in endoneurial microvessels possibly relate to microvascular changes such as thickened vessel walls and narrowed vascular lumina through cellular responses of both endothelial cells and pericytes to increased oxidative stress

Furthermore, α -lipoic acid has a preventive effect on the impaired function of nitrergic vasodilator fibres innervating corpus cavernosum, suggesting a potential treatment for diabetes associated impotence [113].

The contribution by autoxidation of monosaccharides and advanced glycation events to ROS production, catalysed by transition metal ions, is important in peripheral nerve. Treatment with transition metal chelators rapidly corrects nerve blood flow and NCV in diabetic rats and rats fed with galactose [30].

Hence, impaired nutritive blood flow in diabetic nerve is an early event that undoubtedly contributes to the pathogenesis of diabetic neuropathy [30, 116].

Several lines of evidence implicate oxidative stress, induced by increased production of ROS as well as impaired endogenous free radical scavenging capacity, as major causative factors.

The role of non-enzymatic glycation in diabetic neuropathy. In the process of non-enzymatic glycation, reducing sugars such as glucose, fructose or galactose initially react with free amino groups of proteins, lipids or nucleic acids to form the early reversible products such as Schiff bases and ketamines or Amadori adducts. In addition autoxidative glycosylation takes place through the Wolff pathway forming glycoxidation products [117, 118]. Critical intermediaries in the formation of advanced glycation end products (AGEs) are 3-deoxyglucosone (3DG) from fructose-lysine and glyoxal and methylglyoxal (MGO) from either Amadori compounds, Schiff base intermediaries or direct oxidation of sugars [119, 120]. With time these products undergo chemical rearrangement, dehydration and fragmentation reactions and cross-linking to form irreversible AGEs. In vivo, AGEs accumulate ubiquitously in the body including peripheral and central nervous systems. A variety of neurodegenerative disorders including Alzheimer's disease, Parkinson disease, diffuse Lewy body disease and amyotrophic lateral sclerosis show increased non-enzymatic glycation of tau protein, the constituents of Lewy bodies as well as Cu^{++} , Zn^{++} -superoxide dismutase contributing to cell death by the generation of oxidative stress.

It has been shown that the glycation process is enhanced in peripheral nerve in both diabetic patients

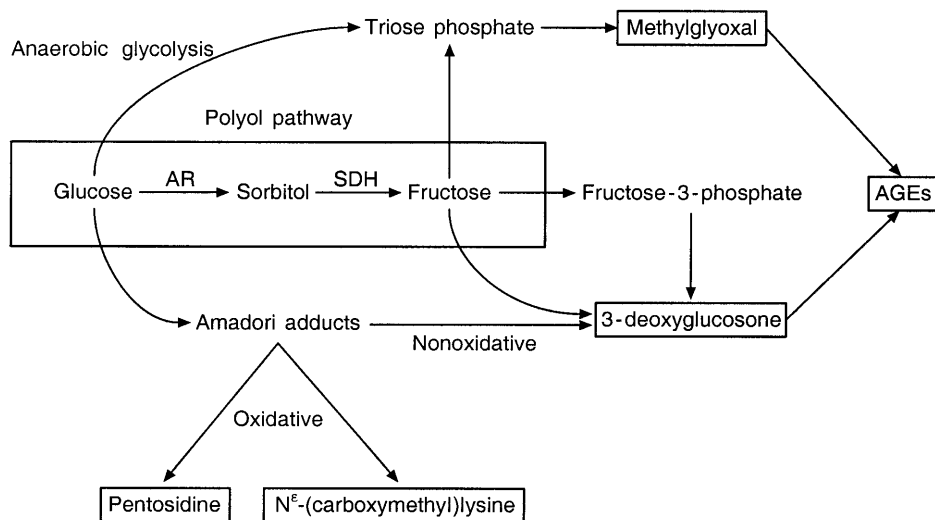


Fig. 5. Relations between polyol pathway and non-enzymatic glycation. 3-Deoxyglucosone is induced by amino groups of proteins reacting with fructose and fructose-3-phosphate. Fructose-3-phosphate is produced by phosphokinase from fructose. Methylglyoxal is formed by amino groups reacting with triose phosphate. Triose phosphate is produced during anaerobic glycolysis and by the reaction of fructokinase with fructose. Reactive oxygen species are not primarily involved in the formation of either 3DG or MGO. On the other hand, both N^{ϵ} -[carboxymethyl] lysine and pentosidine are oxidation products initiated by the reaction of free lysine with glucose. AR, aldose reductase; SDH, Sorbitol dehydrogenase

and animals [121, 122]. Studies in diabetic rats have shown that myelin components such as P0, myelin basic protein and proteolipid protein are subject to non-enzymatic glycation [122, 123] (Fig. 4). It has been suggested that peripheral nerve myelin modified by AGEs becomes recognised and scavenged by macrophages via putative AGE-specific receptors [124]. Such interactions between peripheral nerve myelin and macrophages are likely to contribute to segmental demyelination in diabetes by digestion of myelin proteins (Fig. 1). Several axonal cytoskeletal proteins such as tubulin, neurofilament and actin also undergo glycation [125–127] (Fig. 4). These axonal components have essential roles in the maintenance of axonal function and structure. Their modification by glycation causes alterations of axonal structures, slowed axonal transport, axonal atrophy and degeneration (Fig. 1). Laminin, a major constituent of Schwann cell basal laminae, functions as a promotor and guidance for regenerating fibres. Glycated laminin (Fig. 4) loses its ability to promote nerve fibre regeneration, a phenomenon that probably contributes to impaired nerve fibre regeneration in diabetes [128].

Recent studies have indicated that not only glucose but also glucose-6-phosphate, products of the

pentose phosphate pathway as well as trioses and fructose produced through the polyol pathway, participate in the generation of glycated proteins [129]. Fructose for instance is a much more potent glyicator than glucose or galactose [129, 130]. One of the major carbonyl adducts and a potent AGE precursor, 3DG, is formed directly from fructose [129] (Fig. 5). Cross-linking of proteins, which is the most characteristic feature of AGEs, is promoted by fructose. Fructose can be metabolised to fructose-3-phosphate and triose phosphate by phosphokinase and fructokinase, respectively. These metabolites become potential sources of 3DG and MGO (Fig. 5) [131, 132] both of which are potent cross-links causing tissue damage through oxidative stress [133]. Hence these metabolites promote the formation of AGEs, which when involving structural neuronal proteins interfere with their polymerisation and normal function. On the other hand, it has been reported that the activity of sorbitol dehydrogenase, the second enzyme in the polyol pathway, is considerably suppressed by glycation [134]. It is therefore possible that excessive production of fructose deactivates sorbitol dehydrogenase through increased glycation, thereby preventing further production of fructose and subsequent accumulation of AGEs. In addition, intervention studies with ARIs in diabetic patients and animals have shown a pronounced reduction in the tissue concentrations of glycated proteins in aorta, lens and erythrocytes [135, 136]. Although there are convincing data supporting a linkage between the polyol pathway and glycation in a variety of tissues, there is little showing this in nervous tissues. In the process of glycation and oxidation reactions glycoxidation products are formed such as N^{ϵ} -[carboxymethyl]-lysine (CML) and pentosidine which are considered markers for both glycative as well as oxidative stress (Fig. 5) [94]. Other than the oxidative stress induced directly by non-enzymatic glycation, oxidative stress is also enhanced by glycation and inactivation of Cu-

Zn-superoxide dismutase [137]. Binding interactions between AGEs and their specific receptors such as the receptor for advanced glycation and end products has been suggested as being the source of oxidative stress, by depleting intracellular GSH and vitamin C, resulting in the induction of NF- κ B in endothelial cells [138]. Antioxidants like lipoic acid can suppress the activation of NF- κ B in this process [138]. In a recent study [139], we showed that immunoreactive CML is localised to perineurial basal laminae, axons, Schwann cells and endoneurial microvessels in human peripheral nerve. The staining intensities were significantly increased in diabetic patients ($p < 0.01$) and correlated with myelinated fibre loss ($p < 0.0005$). Note, however, that CML is also formed during lipid peroxidation reactions. It has been shown [140] that aminoguanidine (AG) inhibits the formation of AGEs in aortic collagen in diabetic rats. Numerous studies in rats have confirmed the beneficial effect of AG on the development of retinopathy, nephropathy and neuropathy. In particular, improvements in nerve conduction velocity and morphometric variables after long-term treatment with AG have been documented [141]. The beneficial effect of AG was not immediate but became appreciable only after a prolonged lag period. The mechanism by which AG exerts its effect is not fully clarified and has been a matter of some controversy [118]. It appears, however, that AG inhibits cross-linking but it is not clear whether this is due to entrapment of reactive carboxyl compounds or other glycoxidative intermediaries. It can therefore be said that AGEs and glycoxidation products participate in glycative and oxidative damage leading to cytotoxicity, cell injury and to weakened antioxidant defences. This cycle of events is not, however, brought about by AGEs alone but also by reaction products derived from lipid peroxidation.

Changed neurotrophism in diabetic neuropathy. Aberrations in the synthesis and availability of neurotrophins and neurotrophic factors or their receptors have been implicated in the pathogenesis of diabetic neuropathy [10, 142].

The neurotrophins, which regulate a variety of neuronal proteins, are synthesized by the neuronal target cells and delivered to the neuronal perikarya by retrograde axonal transport. Nerve growth factor is one of the most researched neurotrophins which also include neurotrophin 3 (NT-3), neurotrophin 4/5 (NT-4/5) and brain derived neurotrophic factor (BDNF). It is synthesized in the periphery by muscle and skin and is transported retrogradely to a subset of sensory neurons and sympathetic ganglia [143, 144]. Likewise BDNF and NT-3 are transported by retrograde transport to dorsal root ganglia and motor neurons in adult rats [144]. Retrogradely transported NGF binds to two receptors; the high affinity trkA

and the low affinity p75-receptor. In diabetic rats there is reduced availability of NGF that possibly reflects both decreased synthesis as well as decreased axonal transport [31, 59, 145]. In addition the mRNA expression of trkA in dorsal root ganglia and that of p75 in sciatic nerve are down regulated in diabetic rats [59]. These changes do not occur suddenly but gradually and are established in rats that have had diabetes for 12 weeks [142, 146] (Fig. 1).

Nerve growth factor, other neurotrophins as well as IGF-1 are involved in the regulation of several neuronal proteins. Nerve growth factor regulates the synthesis of medium and low molecular weight neurofilaments, as shown by an increase in neurofilament mRNA in axotomised dorsal root ganglia after treatment with NGF [32, 147] a function that it shares with insulin [148]. As neurofilaments are the major structural determinants for the maintenance of axonal size [24], this implies that deficient neurotrophic factors in diabetic neuropathy are in part responsible for the characteristic axonal atrophy (Fig. 1). Nerve growth factor also appears to regulate the synthesis of substance P [142]. It is believed that NGF exerts this action through the regulatory protein preprotachykinin-A which regulates transcription of substance P.

After nerve injury, the synthesis of NGF in target tissues is taken over by Schwann cells and fibroblasts at the site of injury. This response is thought to be induced by early transforming gene responses, such as *c-fos* and *c-jun* [142]. The subsequent early "emergency" response in local NGF synthesis is believed to induce interleukin 1 release from recruited macrophages which in turn sustain a more long-lasting production of NGF by Schwann cells and probably fibroblasts [149]. In diabetic nerve both the immediate gene responses as well as the sustained local mRNA expressions of NGF and trk A are considerably perturbed, which contribute to the delayed initiation and decreased sustenance of nerve fibre regeneration in diabetes [58, 142]. The perturbed regeneration is mediated by impaired induction of neurofilament and growth-associated protein 43 (GAP-43) synthesis [150]. Nerve growth factor has been used in clinical neuropathy trials with some success in improving thermal perception [151]. A drawback, however, is the mechanical and thermal hyperalgesia caused by subcutaneous injections, probably reflecting its nociceptive role through substance P.

In addition to NGF, NT-3 might be a potentially useful neurotrophin in the treatment of the mainly sensory diabetic neuropathy. Improvement of sensory NCV in diabetic rats after treatment for 4 weeks with human recombinant NT-3 has been shown [142].

Apart from the neurotrophins, there are systemically circulating peptides, such as insulin-like growth factor-1 (IGF-1), insulin and C peptide that have neuroprotective effects on peripheral nerve. Insulin-like

growth factor-1 is synthesized predominantly in the liver but is also produced in Schwann cells of peripheral nerve, where it has autocrine and paracrine functions. In diabetes IGF-1 derived from both systemic and from peripheral nerve shows a progressive decline, which are established in the spontaneously diabetic BB/W rat with a 6–8 week duration of diabetes [10, 152].

Both insulin and C peptide become rapidly deficient in Type I diabetes, in contrast to Type II diabetes which may even show increased levels. This difference between the two types of diabetes, may explain why functional and structural neuropathy in Type I models is more severe than in Type II diabetic models [21]. Insulin-like growth factor-1, insulin and C peptide all have neurotrophic actions on sensory, autonomic and motor neurons [14, 153]. It has been postulated that decreased insulin concentrations secondarily impair IGF-1 synthesis [10] (Fig. 1). The insulin and IGF-1 receptors (IR; IGF-1R) are structurally similar, both being comprised of $\alpha_2\beta_2$ heterotetramers, where the extracellular α -subunits contain the ligand binding domains. The IGF-1R is present on peripheral nerve somata, neurites and Schwann cells, whereas the IR is localised to the paranodal Schwann cell and the node of Ranvier and endoneurial vessels. Recent studies from our laboratory have shown that C peptide enhances the insulin receptor activity [87]. It has also been shown that C peptide binds to a specific membrane receptor (Wahren, personal communication). Insulin-like growth factor-1 promotes the synthesis of the low molecular weight and medium molecular weight neurofilaments [48], whereas insulin promotes phosphorylation of neurofilaments, necessary for their polymerisation and exportation from the somata to the neurites [10]. Both insulin and IGF-1 increase α - and β -tubulin mRNA in vitro by stabilising their transcripts, a property they share with NGF [148, 154]. Insulin growth factor-1 appears to have a more pronounced effect on β -tubulins. It has been shown that IGF-1 enhances growth cone motility by promoting reorganisation of actin and activation of focal adhesion proteins [155]. These data are likely to explain ARI-treated rats and humans [46, 58, 92] showing an appreciable increase in nerve fibre regeneration, which in the BB/W rat is associated with a sixfold up regulation of nerve IGF-1 mRNA [59]. Insulinlike growth factor-1 also has a protective effect on glucose induced apoptosis which appears to be mediated by activation of mitogen-activated protein kinases [156] (Fig. 1).

Both insulin and C peptide have neuroprotective effects. Supplementation of C peptide in Type I diabetic rats has no effect on hyperglycaemia, but restores Na^+/K^+ -ATPase to normal in both peripheral nerve [15] and renal tubule cells and NCV [14, 15], suggesting a mode of action different from that of insulin.

Therefore it appears that perturbations of various neurotrophic factors are of pathogenetic importance particularly in the development of structurally recognisable changes secondary to abnormalities in the synthesis of structural proteins. This is in keeping with the observation that changes in neurotrophic support do not occur suddenly but gradually as diabetic neuropathy progresses from a metabolic to a structural phase (Fig. 1).

Essential fatty acids. Production of vasoactive prostanoids is compromised in diabetes [30, 157]. This is believed to be due to impaired desaturation particularly of the Δ -6 step in their synthesis with subsequent deficits in the ω -6 essential fatty acids. The depressed desaturation, particularly in the liver, is probably due to a combination of hyperglycaemia, hypoinsulinaemia and oxidative stress [158]. The consequences of the impaired Δ -6 desaturation step are reduced tissue and plasma concentrations of γ -linoleic acid and arachidonic acid with impaired synthesis of cyclooxygenase products such as vasodilatory and antiplatelet aggregation prostaglandins [30]. These abnormalities lead to increased vascular tone and decreased endoneurial blood flow.

Synthesis of prostaglandins PGI_2 and PGE_2 is reduced in sciatic nerve secondary to diminished arachidonic acid [8, 158]. This deficit is overcome by treating diabetic rats with evening primrose oil, resulting in restoration of vasoactive prostaglandins and preventing decreased nerve blood flow, nerve hypoxia and impaired NCV [30, 159, 160] (Fig. 1). Long-term treatment with evening primrose oil also promotes endoneurial vascularisation in diabetic and galactosaemic rats [161, 162]. These effects are blocked by cyclooxygenase inhibition underlining the importance of prostanoid synthesis for these effects [35]. These data support the notion that impairment of essential fatty acid synthesis in diabetes exerts its main effect on the endoneurial vasculature. These effects are, however, potentiated by increased blood flow secondary to the stimulatory effect of prostanoid synthesis on NO release.

Another aspect of abnormal fatty acid metabolism involves reduced plasma and tissue L-carnitine content in experimental diabetes [87, 163]. Treatment with L-carnitine prevents the development of diabetic neuropathy [8, 163].

In diabetic neuropathy acetyl-L-carnitine improves nerve regeneration and prevents structural and morphometric abnormalities [8]. The mechanisms by which L-carnitine acts are not fully clear but it appears to have several effects. It improves cellular energy metabolism by promoting mitochondrial transport of long-chain fatty acids for β -oxidation, it restores neural Na^+/K^+ -ATPase, NO synthesis and prostaglandins to normal [8, 84], improves nerve blood flow [86] and may act as an antioxidant [85].

It is therefore evident that essential fatty acid metabolism perturbing vasoactive prostanoids have important pathogenetic functions in diabetic neuropathy. These mechanisms often act in concert with additional pathogenetic elements such as the polyol pathway and non-enzymatic glycation, with consequences for normal vessel function, although this is not the only target tissue.

Conclusion

Several animal models have proven to be useful for the study of diabetic neuropathy. It is apparent that diabetic neuropathy is a multifactorial disorder in which various metabolic abnormalities are interrelated but appear to occur sequentially during the clinical course of the disease. Moreover, it is also evident that the neuropathies accompanying Type I and Type II diabetes are different. The usually milder neuropathies in Type II models, despite similar degrees of hyperglycaemia, suggest that aetiological factors other than hyperglycaemia are important in the pathogenesis of this disorder.

Differences in the availability of systemic insulin or C peptide or both in the two types of diabetes are likely to be of pathogenetic importance. Lack of insulin or C peptide or both appears to contribute to the more severe axonal atrophy, loss of axons and to the development of axo-glial disjunction in Type I diabetes. This agrees with the beneficial effect of C-peptide supplementation on diabetic neuropathy in Type I diabetic patients and animal models. Therefore one of the challenges in the next years will be to understand the mechanistic differences between Type I and Type II diabetic neuropathy, since this possibly will translate into different treatment strategies in the two types of diabetic neuropathy. Another challenge will be to gain a better understanding of the sequence and precise interrelations between various metabolic and molecular events to establish a hierarchy as to their relevance.

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