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Review

New insights into the metabolic and molecular basis for diabetic neuropathy

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Abstract. Diabetic polyneuropathy is the most common complication of diabetes mellitus. Several interactive pathogenetic mechanisms have been identified mainly in streptozotocin-induced diabetes in rats and have been ascribed to hyperglycemia. Over the last number of years it is becoming increasingly clear that diabetic neuropathy differs in type 1 and type 2 diabetes in humans and in murine models that more accurately mimic the human disorders. Beside hyperglycemia, attention is increasingly being paid to the pathogenetic roles of insulin and C-peptide deficiencies, particularly in type 1 diabetic neuropathy. There is now evidence to suggest that insulin and C-peptide deficiencies are mainly responsible for perturbations of neurotrophic factors and contribute to oxidative stress in diabetic nerve. This may also be true for apoptotic phenomena afflicting both the peripheral and central nervous systems in diabetes. The new data have lead to re-evaluations of pathogenetic components in this complex disorder, and their further exploration is likely to form a more refined basis for future therapeutic and preventive measures.

Key words. Diabetic neuropathy; hyperglycemia; insulin deficiency; C-peptide deficiency; neurotrophism; nerve regeneration; apoptosis.

Introduction

Diabetes mellitus entails several chronic disorders, which are characterized by hyperglycemia and associated with absolute or relative insulin deficiency. Diabetes affects carbohydrate, protein and lipid metabolism and is associated with progressive chronic complications. These affect the nervous systems, the retina and kidneys and are collectively referred to as microvascular complications.

Diabetic neuropathies as a group are the most common chronic complications of diabetes mellitus [1] and occur in both type 1 and type 2 diabetes. On the other hand, diabetic neuropathy remains the least understood complication. The prevalence of diabetic neuropathy varies from 10% within 1 year of diagnosis to 50% in patients with diabetes for more than 25 years [2–4], with an average prevalence of approximately 30% [5]. Diabetic neuropathy accompanying type 1 diabetes tends to occur more predictably and to progress more rapidly, resulting in a more severe neuropathy [6–8]. Diabetic neuropathies include several distinct syndromes, of which symmetric sensory polyneuropathy commonly associated with diabetic autonomic polyneuropathy are referred to as diabetic polyneuropathy (DPN). The various syndromes affecting the peripheral nervous system are separated into acute rapidly reversible syndromes and chronic progressive manifestations [6, 9]. The mechanisms underlying DPN are multiple and appear to involve genetic predispositions and several interrelated metabolic and molecular abnormalities consequent to hyperglycemia and insulin and C-peptide deficiencies [1, 10–14]. In recent

decades, several experimental drugs targeting specific mechanisms have undergone clinical testing. However, the results from these clinical trials have in general been disappointing, which in part may be due to the fact that therapeutic interventions have occurred too late in the natural history of DPN [15].

In this review I will focus on several key pathogenetic factors including the polyol pathway, oxidative stress, the role of neurotrophic factors and insulin and C-peptide deficiencies. Finally, the differences in underlying mechanisms between type 1 and type 2 DPN will be discussed which may account for the clinical differences in DPN in the two types of diabetes.

The natural history of DPN

Most data concerning the early development of DPN have been obtained from animal models. Hyperglycemic streptozotorin (STZ)-treated rats or spontaneously type 1 diabetic BB/Wor rats show, within weeks of onset, acute significant decreases in motor and sensory nerve conduction velocities (NCVs), which are associated with increased activity of the polyol pathway, decreased endoneurial blood flow and impairments of neural Na^+/K^+-ATPase and nitric oxide (NO) activities [14]. At this acute ('metabolic') stage of DPN, these early functional deficits are readily reversible and are associated with reversible structural changes consisting of nodal swellings. They are secondary to increased intra-axonal Na^+ accumulation at the node due to the impaired Na^+/K^+-ATPase activity [16, 17]. To these early metabolic changes, other additional pathogenetic components are progressively added such as the consequences of oxidative stress and the progressive decline in expression of neuroprotective trophic factors, like nerve growth factor (NGF) and the insulin-like growth factor (IGF) system [14, 18, 19]. Simultaneously, structural changes start to emerge consisting of axonal atrophy in a length-dependent manner and eventually axonal dying-back degeneration ('the structural phase'). These changes are likely to contribute to the progressively less reversible NCV defect [20]. However, type 1 DPN in humans and type 1 experimental animal models show additional structural abnormalities involving the nodal and paranodal apparatus [21, 22]. These changes consist of a progressive disruption of the paranodal ion channel barriers, 'axoglial dysjunction,' affecting especially large myelinated fibers. Experimentally, these changes are associated with lateralization of the nodal Na^+ channel and result in conduction block of affected fibers, hence contributing to the progressively less reversible nerve conduction velocity [23]. These changes do not occur in human [22] or animal models of [24, 25] type 2 diabetes, even after prolonged hyperglycemic exposure. Recent evidence suggests that these nodal and paranodal changes are associated with impaired insulin action rather than hyperglycemia. Progressive axonal degeneration, coupled with impaired regenerative capacity, in the type 1 diabetic BB/Wor rat result in a progressive nerve fiber loss, which is significantly milder in its type 2 counterpart, the BB/Z rat [24].

In human DPN, the spectrum of somatic DPN can be divided into reversible and persistent syndromes [6, 9]. The latter are classified into sensory and motor syndromes of increasing severity, which reflect the natural history of DPN. Part of the problem in staging and classifying DPN is that the progression rates of objective functional measurements are not linear and differ between nerves [26, 27] reflecting the length-dependent axonal dying-back phenomenon. The progression rate in 182 patients with established clinically overt DPN monitored over an 18-month period showed significant deterioration in vibratory perception threshold, peroneal and median F-wave latencies and in sensory NCVs in the median and ulnar nerves, whereas sural and peroneal NCVs showed no significant changes [26]. On the other hand, if the rates of decline in NCV in the sensory nerves were projected backward, they reached normal values 5–7 years prior to onset of diabetes, suggesting a steeper decline in NCVs during the early metabolic phase of the neuropathy. When correlating electrophysiological changes with quantitative structural pathology, a similar pattern arises with respect to progression rates and distribution of changes [27, 28]. Interestingly, in human DPN, only with the emergence of structural changes does DPN apparently become symptomatic. Awareness of this may in part account for the poor outcomes of several clinical trials, into which only symptomatic patients were recruited, at a time when the neuropathy is less responsive to therapeutic interventions. This pattern of non-linear and varied progression rates of DPN in various nerves is therefore important when monitoring patients longitudinally and when designing clinical neuropathy trials [27].

Differences exist in the neuropathology of DPN in the two types of diabetes. The sequence of nodal and paranodal changes, consisting of axoglial dysjunction, paranodal demyelination and intercalated internodes, is characteristic of type 1 human DPN but does not occur in type 2 DPN [22]. On the other hand, primary segmental demyelination tends to be more characteristic of type 2 DPN. These differences are likely to affect nerve function differently and are likely to account for the clinically more severe DPN in type 1 diabetic patients [7].

Pathogenetic considerations

The bulk of information regarding the pathogenetic mechanisms of DPN has been obtained from experimental rodent models like the STZ-induced diabetic rat and the spontaneously type 1 diabetic BB/Wor rat [14]. These data have demonstrated that DPN is caused by a multitude of sequential and partially interacting and perpetuating mechanisms. These factors may vary during the natural history of the disease, and they appear to be different in type 1 versus type 2 DPN. Some of the key pathogenetic elements are associated with hyperglycemia (common to both types of diabetes), like activation of the polyol pathway and non-enzymatic glycation (fig. 1). Others are closely related to impaired insulin and/or C-peptide deficiency, which is commonly overlooked. For example, regulation of the expression of various neurotrophic factors and their receptors as well as that of cell-adhesive molecules appears to be less affected by hyperglycemia per se (fig. 1). The complexity of the pathogenetic components and their varying impacts on type 1 versus type 2 DPN may explain some of the problems involved in designing effective therapies, and the failures of earlier interventional clinical trials.

Polyol pathway and related metabolic derangements

Activation of the polyol pathway is an early and important mechanism in DPN. The polyol pathway is comprised of two steps: (i) the conversion of glucose to sorbitol by aldose reductase and (ii) the conversion of sorbitol to fructose by sorbitol dehydrogenase. Aldose reductase is localized to paranodal Schwann cells and endoneurial microvessels [29]. Shunting of excessive glucose through this pathway leads to intracellular accumulation of sorbitol and fructose, with compensatory depletion of other organic osmolytes like myo-inositol and taurine [30]. Depletion of the myo-inositol pool in peripheral nerves interferes with phosphoinositide turnover resulting in insufficient diacylglycerol to maintain protein kinase C (PKC) content necessary for activation of Na^+/K^+-ATPase [31–33]. Perturbations of PKC also interfere with phosphorylation of PO, a major myelin protein of peripheral nerve [34], and may play a pathogenetic role in primary segmental demyelination. Taurine acts not only as an osmolyte but also as an endogenous antioxidant and as a neurotrophic factor [35, 36]. Taurine depletion may therefore compromise antioxidative defense mechanisms and promote nerve degeneration. Polyol pathway activation promotes oxidative stress via deple-

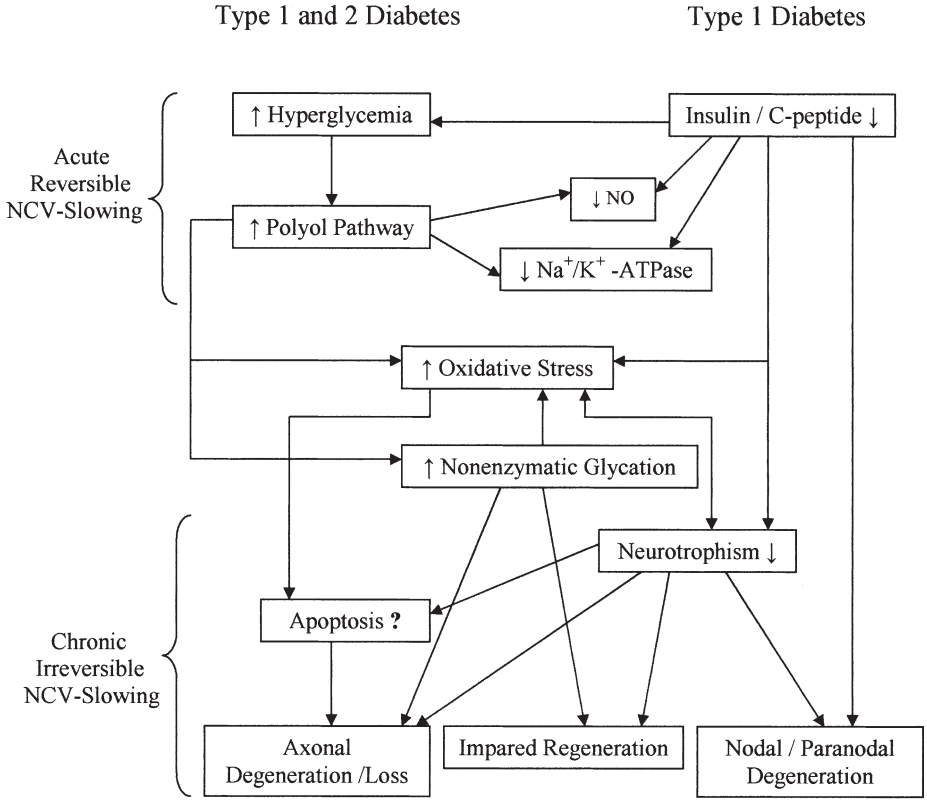

Figure 1. Simplified scheme of pathogenetic pathways involved in type 1 insulin and C-peptide deficient-diabetes versus those induced by insulin-resistant and hyperinsulinemic type 2 diabetes. Note that some of the key metabolic abnormalities of the early metabolic phase of DPN are influenced by both hyperglycemia and insulin/C-peptide deficiencies. This is also true for mechanisms resulting in structural changes ('the structural phase'), except for nodal and paranodal degeneration which appear to be a direct consequence of impaired insulin action.

tion of NADPH, a cofactor of both aldose reductase and glutathione reductase, which results in a decrease in reduced glutathione and an increase in oxidized glutathione [37]. Increased accumulation of fructose as a result of polyol pathway activity enhances oxidative stress via non-enzymatic glycation (fig. 1). On the other hand, myo-inositol depletion perturbs phosphoinositide turnover with decreased diacylglycerol synthesis and impaired activation of total PKC. This reduction is mainly due to reduced diacylglycerol-sensitive neuronal isoforms such as PKC-α and probably PKC-δ [10, 38]. On the other hand, increased activity of vascular PKC-β is believed to play a significant role in microvascular complications. The PKC-β inhibitor LY333531 dose-dependently corrects endoneurial blood flow and nerve conduction velocity in acutely diabetic STZ rats [39]. Substantially lower doses of the inhibitor in combination with vitamin E, or α-lipoic acid or γ-linolenic acid achieved the same result, suggesting a synergism between PKC-β, oxidative stress and essential fatty acid mechanisms [39]. Impaired PKC activity has been reported to play a role in reduced Na^+/K^+-ATPase activity and a blunted regenerative response in experimental diabetic nerve [40, 41]. This was recently confirmed by reports showing that treatment with both non-specific [42] and β-specific PKC inhibitors [43] prevents or normalizes NCV, decreases heart rate variability and reduces sciatic nerve blood flow in the STZ rat. Sciatic nerve Na^+/K^+-ATPase activity and *myo*-inositol depletion are also ameliorated by non-specific and β-specific PKC inhibitors, respectively, and both reduce hyperalgesia and C-fiber hyperexcitability in the STZ rat [44]. Interestingly, vasodilators and antioxidants, such as niceritrol and α-lipoic acid, ameliorate *myo*-inositol depletion in the STZ rat, possibly by improving Na^+/K^+-ATPase activity and/or energy production secondary to normalization of nutritive nerve blood flow [45, 46], suggesting that several mechanisms converge to produce these abnormalities. Hence inhibition of PKC, particularly the β isoform, may normalize the increased PKC activities in the vasculature of nerve tissue, analogous to what is seen in the retina and the kidney [47]. On the other hand, C-peptide replacement in the BB/Wo rat does not influence either the polyol pathway or oxidative stress, but it dose-dependently ameliorates the Na^+/K^+-ATPase abnormality and normalizes NO and endoneurial blood flow [48–50]. Hence, several pathways in addition to an activated polyol pathway apparently influence both Na^+/K^+-ATPase activity and endoneurial NO, two key abnormalities of the metabolic phase of DPN.

Other concomitant metabolic derangements such as carnitine deficiency [51–54] and impaired prostanoid metabolism [55] contribute to impaired Na^+/K^+-ATPase activity in experimental, and possibly human type 1 diabetic neuropathy. Decreased Na^+/K^+-ATPase activity impacts on nodal sodium channel inactivation, with decreased nodal Na^+ membrane potentials, increased intra-axonal Na^+, nodal and paranodal swelling and reduced NCV [16, 17, 56–58].

Recently, mice transgenic for human aldose reductase were shown to develop a neuropathy with structural and functional changes resembling DPN, when challenged with galactose [59]. In human type 1 patients, polymorphic microsatellites have been identified upstream of the initiation site of the aldose reductase (ALR2) gene. Several dinucleitide repeat polymorphic markers have been identified. The most prominent alleles are Z-6, Z-4, Z-2, Z, Z+2, Z+4 and Z+6 [60]. Type 1 patients with DPN show significant decreases in frequencies of the Z+2 allele. Decreased frequencies of the Z/Z+2 genotype showed a higher frequency of DPN within 5 years of diagnosis [60]. Similar dinucleotide repeat polymorphic markers upstream of the ALR2 gene have been associated with diabetic nephropathy in type 1 patients and with retinopathy in Chinese type 2 patients [61–63]. These data demonstrate that the activity of the polyol pathway is intimately involved in the pathogenesis of microvascular complications of diabetes and that genetic predispositions exist.

Non-enzymatic glycation and oxidative stress

In 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 early reversible Schiff bases and Amadori products. These then undergo chemical rearrangements to form advanced glycation end products (AGEs). In vivo, AGEs accumulate during normal aging and at an accelerated rate in diabetes in various tissues such as the lens [64], retina [65], kidney [66] and peripheral nerve [67]. The receptor for AGE (RAGE) has been cloned and identified as a member of the immunoglobulin superfamily of cell surface molecules [68]. It shows close homology with the neural cell adhesion molecule NCAM [69] and is present in the central and peripheral nervous systems [70, 71]. Its interaction with amyloid β-peptide, a possible candidate for AGE modification [72], contributes to cell death by inducing oxidative stress and activating the transcription factor NF-κB in Alzheimer's disease [73]. Therefore, accumulation of AGEs may play a crucial role in the pathogenesis of DPN through generation of oxidative stress and altered endoneurial hemodynamics.

In peripheral nerve, the glycation process is enhanced in diabetes both in humans and animal models [67, 74]. Glycation of major axonal cytoskeletal proteins such as tubulin, neurofilament and actin is likely to contribute to axonal atrophy and degeneration, and to slowing of axonal transport (fig. 1) [75–77]. Glycation of laminin, a major constituent of Schwann cell basal lamina, which is important in nerve sprouting, may contribute to impaired

nerve fiber regeneration in diabetes [78]. Myelin components such as P0, myelin basic protein and proteolipid protein are subjected to non-enzymatic glycation [79, 80] which may then be recognized and scavenged by macrophages via RAGE [81, 82], thereby probably contributing to segmental demyelination.

Increased production of fructose via activation of the polyol pathway enhances the generation of glycated proteins (fig. 1). 3-Deoxyglucosone (3-DG), a major carbonyl adduct and a potent AGE precursor, is generated directly from fructose [83]. Cross-linking of proteins by fructose occurs ten times more readily than that by glucose [84]. Furthermore, fructose is metabolized to fructose-3-phosphate and triose phosphate, which are sources for 3-DG [85] and methylglyoxal (MGO), respectively, other potent cross-linkers [86]. In this process, MGO is formed under oxidative conditions, while the formation of 3-DG does not require oxygen [87]. Interestingly, the activity of sorbitol dehydrogenase, the second enzyme of the polyol pathway, is markedly decreased by glycation [88]. Excessive production of fructose therefore likely accelerates glycation and deactivates sorbitol dehydrogenase, thereby hampering further production of fructose. The role of fructose as a glycator is further demonstrated by the ability of aldose reductase inhibitors to significantly reduce the levels of glycated proteins in aorta, lens and red blood cells [89, 90].

Oxidative stress facilitates the formation of glycoxidation products such as carboxymethyl-lysine and pentosidine [91]. There are several potential sources of oxidative stress in diabetes including altered redox status [92], dysregulation of glutathione synthesis [93] and hypoxia and ischemic reperfusion injury [94]. Glucose autoxidation and glycoxidation, which are catalyzed by trace amounts of transition metal ions, generate reactive oxygen species (ROS) [95–96]. Low-dose transition metal chelators such as deferoxamine and trientine improve nerve blood flow and nerve conduction velocity in the STZ rat [97]. Furthermore, superoxide generated not only via non-enzymatic glycation [98] but also via inactivation of Cu-Zn-superoxide dismutase [99] attenuates NO activity, leading to reduced blood flow [100, 101]. Interaction of AGEs with RAGE depletes intracellular reduced glutathione and vitamin C, thereby further enhancing oxidative stress [102, 103]. This process gives rise to breakdown of endothelial barrier functions and NF-κB-mediated gene inductions of tissue factor and endothelin-1, both of which contribute to reduced vascular blood flow [102, 104]. Tissue levels of glycoxidation products correlate with the severity of nephropathy, retinopathy and vasculopathy in diabetic patients [105–107].

Aminoguanidine inhibits the formation of AGEs [108] and has beneficial effects on the development of retinopathy, nephropathy and neuropathy. NCV slowing and myelinated fiber pathology in the STZ rat are improved after long-term treatment with aminoguanidine [109]. Impaired nerve blood flow is normalized after 8 weeks treatment with aminoguanidine, whereas it has no effect on oxygen free radical activity [110], suggesting that the hemodynamic changes can be modulated without invoking oxidative stress (fig. 1). Short-term treatment with aminoguanidine ameliorates the NCV and the Na^+/K^+-ATPase defects, but not endothelial damage as reflected by systemic thrombomodulin concentrations [111]. On the other hand, long-term aminoguanidine treatment has beneficial effects on the structural alterations of endoneurial microvessels in the STZ rat [112].

α-Lipoic acid is one of the most powerful antioxidants and acts as a coenzyme of hydrogen transfer. The suggestion has been made that α-lipoid acid may not be sufficient to eliminate the severe oxidative stress occurring in DPN [113]. A review of several clinical trials employing α-lipoic acid showed reduction in neuropathic symptoms and small improvements of autonomic function [114]. Successful antioxidant therapy will probably require multiple antioxidant compounds targeting the different mechanisms and will include α-lipoic acid, vitamin E and C, and probably agents targeting the perturbed lipid metabolism such as γ-linoleic acid,, evening primrose oil/fish liver oil and acetyl-L-carnitine [14, 97]. There is experimental evidence demonstrating a synergistic effect in this rationale of multitherapy [115, 116]. Since oxidative stress may in part be a consequence of endoneurial hypoxia, additional therapies that have proven beneficial under experimental conditions include angiotensin-converting enzyme inhibition, endothelin-1 ET_A antagonists and PKC inhibitors [116]. As pointed out earlier, correction of regional blood flow is no guarantee for correction of oxidative stress. For example, acetylcholine-induced vasodilation and improvement of endoneurial blood flow and NCV with myo-inositol, aminoguanidine or C-peptide have no effect on markers of oxidative stress [50, 117, 118]. Therefore, contrary to earlier suggestions [116, 119], the link between endoneurial hypoxia/ischemia and oxidative stress may not be all that clear.

Altered neurotropism

There is now overwhelming evidence indicating that impaired neurotrophic support is involved in diabetes-related neuronal dysfunctions (fig. 1) [18, 19]. NGF is selectively trophic to small-fiber sensory and sympathetic ganglion neurons [120]. In the STZ rat, reduced expression of NGF mRNA in muscle and skin [18] and impairment of its retrograde axonal transport [121, 122] lead to impaired neurotrophic support of NGF-dependent neurons. The cause of NGF reduction in diabetic tissues is not known. Several mechanisms have been proposed. Oxidative stress has regulatory effects on NGF gene expression and sensitivity, and the vitamin D metabolic, 1,25-

dihydroxyvitamin D3, which is reduced in diabetic rats, induces NGF mRNA in vitro [19, 123, 124]. Supplementation with antioxidant taurine corrects oxidative stress and NGF deficits [125]. Conversely, NGF can block the induction of ROS and stabilize the mitochondrial membrane potential [126]. Besides abnormalities in the synthesis and transport of NGF, alterations in NGF receptors are likely to mediate reduced responses. The low-affinity NGF receptor p75 apparently undergoes an increased turnover in diabetic nerve [127]. Expression of the high-affinity Trk A receptor is markedly reduced both at the mRNA and protein levels in diabetic dorsal root ganglia (DRG) [128]. NGF administration prevents the reduction of neuropeptides like substance P and calcitonin gene-related peptide in DRG neurons and sciatic nerve of the diabetic rat [129, 130]. These neuropeptides are confined to small-fiber sensory neurons, mediating nociceptive, or thermoreceptive sensation [131].

The expression of neurotrophin-3 (NT-3), which is trophic for sympathetic neurons and sensory neurons of large-diameter fibers [132, 133], is also reduced in diabetic muscle. Administration of NT-3 ameliorates only sensory NCV deficits, but not those of motor nerves [134]. Reduced expressions of the high-affinity receptors in the respective neurons [134, 135] and decreased synthesis of these neurotrophins contribute to nerve dysfunction in DPN. Recent clinical trials have, however, been inconclusive with respect to the effect of recombinant human NGF [136].

IGFs have neurotrophic actions on sensory, sympathetic and motor neurons [137]. Reduction in systemic IGF-I levels and increased IGF-I-binding protein levels contribute to impaired IGF-I activity in type 1 diabetic patients [138]. In the type 1 STZ rat and BB/Wor rat, IGF-I mRNA expression is dramatically reduced in peripheral nerve, DRG and spinal cord [139–142]. Interestingly, in the BB/Wor rat, the IGF-I receptor is upregulated in peripheral nerve but downregulated in DRG and in the brain [128, 142–144]. These abnormalities are significantly milder in the spontaneously type 2 diabetic BB/Z rat and unaltered in the fa/fa rat [128, 143, 145]. However, in the latter model, IGF-II mRNA expression is reduced in sciatic nerve, spinal cord and brain [145]. In the STZ rat, subcutaneous infusion of IGF-I or IGF-II prevents the progression of hyperalgesia [146] and local administration of IGFs protects against impairments in sensory nerve regeneration [147]. The abnormalities in IGF-I and IGF-I receptor expression in peripheral nerve, DRG and brain in the type 1 BB/Wor rat are normalized by C-peptide replacement [148, 149], and this is believed to be related to a PI-3 kinase-mediated effect on NF-κB [150].

High glucose induces apoptotic changes in cultured DRG neurons and Schwann cells, which are prevented by IGF-I [151]. This finding has been confirmed by in vivo experiments both in 1-month STZ diabetes and in acute hyperglycemia induced by continuous glucose infusion (6–10 h) in rats [152]. Rats with more longstanding (12–15 months) STZ diabetes show similar apoptotic structural changes in DRG neurons [153]. Beside IGF deficiencies, oxidative stress in this animal model may contribute to mitochondrial changes and the initiation of programmed cell death. However, these in vivo findings have become controversial because of the high incidence of claimed apoptotic activity in DRG cells, coupled with an unaltered number of sensory fibers in corresponding peripheral nerve.

Insulin and C-peptide actions

Hyperglycemia, although an important etiological factor, is not the sole culprit in the development of diabetic complications. Increasingly attention is being paid to insulin and/or C-peptide deficiencies. Both insulin and C-peptide exert neuroprotective and antiapoptotic effects [48, 149].

Insulin is synthesized by pancreatic β cells as a single-chain precursor, proinsulin. The A and B chains of proinsulin are joined by a connecting peptide, C-peptide. Proinsulin is cleaved by membrane-bound proteases into equimolar amounts of insulin and C-peptide which are released into the circulation via secretory granules [154]. C-peptide was initially believed to have no biological activity. Although it has no glucose-lowering effects, a number of physiological effects have been reported [48, 148, 155].

The physiological role of the proinsulin C-peptide has received increasing attention, and the potential therapeutic value of C-peptide replacement in preventing type 1 diabetic complications is seriously being considered. In patients with type 1 diabetes, autonomic nerve function as measured by heart rate variability improves after administration of C-peptide [156]. A subgroup of the latter patients with signs of sensory neuropathy exhibited improved temperature threshold discrimination [157]. None of these effects were seen in patients who received insulin therapy alone. C-peptide stimulates nerve Na^+/K^+-ATPase activity, resulting in improved electrolyte balance and enzyme state. Additionally, the effects may be the consequence of improved endoneural blood flow. Several studies demonstrate an effect of C-peptide on NO release. C-peptide stimulates endothial NO synthase (eNOS) with release of NO from bovine aortic endothelial cells in a concentration-dependent manner, an effect that is abolished by NOS inhibitors [158]. This is in keeping with the finding that C-peptide induces increased forearm blood flow in type 1 diabetic patients, which is blocked by a NO synthase blocker [159] and the demonstration of a C-peptide concentration-dependent dilatation of rat skeletal muscle arterioles in the presence of insulin [160]. In the BB/Wor rat, C-peptide prevents the decrease in en-

doneurial blood flow but interestingly without affecting oxidative stress [50]. This effect was eliminated by a NO inhibitor. C-peptide has no effect on blood glucose levels nor does it affect the polyol pathway [48, 161].

C-peptide elicits concentration-dependent stimulation of Na^+/K^+-ATPase activity in a variety of tissues including renal tubular cells, rat sciatic nerve, pancreatic islets, granulation tissue and red blood cells (fig. 1) [155, 161]. Further support for C-peptide effects on Na^+/K^+-ATPase is provided by its effect on rat sciatic nerve Na^+/K^+-ATPase in type 1 diabetic BB/Wor rats treated with C-peptide for 8 months, an effect that is dose dependent [48, 161]. This was associated with partial correction of NCV and paranodal swelling, secondary to axonal Na^+ accumulation. In the diabetic BB/Wor rat, the expression of both insulin receptor and IGF-I receptor mRNA and protein in peripheral nerve and brain tissue was normalized by C-peptide replacement and the diabetes-induced hippocampal apoptosis was prevented by C-peptide replacement [149]. In the same model, C-peptide prevents in a dose-dependent fashion nodal changes and axonal degeneration and dose-dependently promotes nerve regeneration [161].

In peripheral nerve, insulin has failed to promote metabolic effects such as glucose and amino acid uptake [162, 163]. Physiological concentrations of insulin, however, stimulate neurite outgrowth and [^{3}H]-thymidine incorporation, and are required for survival of sensory and sympathetic neurons [164, 165]. Insulin upregulates and stabilizes neurofilament and tubulin mRNAs during neurite outgrowth in neuroblastoma cells in a dose-dependent manner [166, 167]. In the STZ rat, local unilateral administration of insulin to sciatic nerve results in an increased number of small myelinated fibers and prevention of NCV slowing in the treated nerve, suggesting that insulin is involved in peripheral nerve fiber regeneration and repair in this model [168]. Insulin is required for NGF to exert its protective effects on human neuroblastoma cells [169].

The insulin signaling comprises two major pathways: the phosphatidylinositol 3-kinase (PI 3-kinase) pathway and the mitogen-activated protein (MAP) kinase pathway (fig. 2). The PI 3-kinase pathway is linked to metabolic effects such as glucose transport, glycolysis, glycogen synthesis and protein synthesis. The MAP kinase pathway is associated with cell proliferation and differentiation [for a review see ref. 170].

Recently, we demonstrated that peripheral nerve and brain mainly express the high-affinity isoform of the insulin receptor, which in peripheral nerve is localized to the nodal and paranodal apparatus [171, 172]. These specialized plasma membranes of myelinated fibers possess a highly organized molecular structure with a non-uniform clustering of several molecules including ion channels [173], Na^+/K^+-ATPase [174], glucose transporter

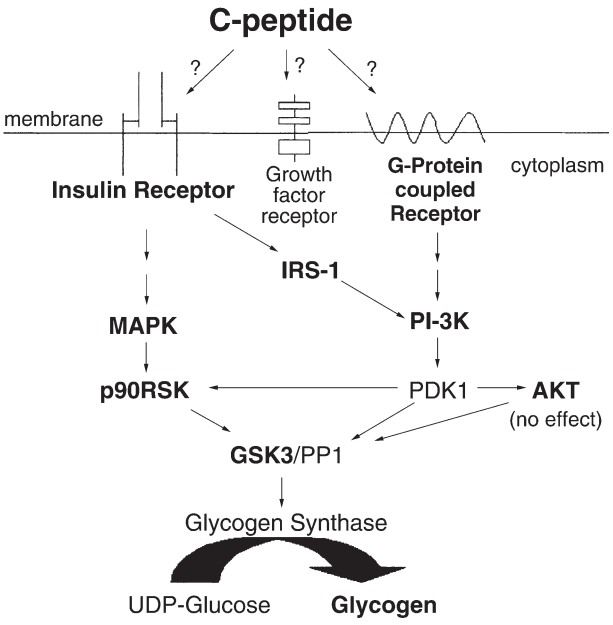

Figure 2. Flow chart of C-peptide signaling. Although the receptor binding of C-peptide remains elusive, C-peptide shows a signaling pattern similar to that of insulin. This is further supported by the ability of C-peptide to phosphorylate the insulin receptor and to synergize signaling effects of submaximal insulin doses. C-peptide alone increases glycogen synthesis and amino acid uptake (not shown).

[175], aldose reductase [29] and the cell adhesion molecules, caspr and ankyrin$_G$ [176–178]. These molecules are involved in the integrity of normal nodal function and structure and their colocalization with the insulin receptor suggests that insulin influences their biological activities.

Proinsulin C-peptide is known to enhance the effects of insulin [160, 179–181] and phosphorylates the insulin receptor [180, 181]. C-peptide signals through the insulin- signaling pathway and mediates glucogen synthesis and amino acid uptake on its own and enhances the same effects by insulin within a narrow concentration range [180]. We have therefore suggested that C-peptide interacts with the insulin receptor, although others have suggested that C-peptide binds to a specific membrane receptor [182]. C-peptide substitution in the type 1 diabetic BB/Wor rat prevents and ameliorates paranodal molecular abnormalities, and related nerve conduction deficits [48, 161, 183]. These findings indicate that impaired insulin and/or C-peptide availability play important pathogenic roles in the abnormalities of the nodal and paranodal apparatus characteristic of type 1 human and murine DPN [183].

The molecular components of the node of Ranvier and the paranodal apparatus and their interactive regulation are complex and not fully understood. Voltage-gated Na channels are located in the nodal apparatus and are responsible for action potential initiation and conduction.

They consist of a pore-forming α subunit and two auxiliary subunits: β_1 and β_2. Interactions between spectrin, actin and contactin [184, 185] with ankyrin$_G$ and the Na channel β subunits appear to be critical for Na channel and Na^+/K^+-ATPase enrichment at the node [186–188]. The β_1 subunit interacts with receptor tyrosine phosphatase β(RTPT$_\beta$), which in turn interacts with contactin [189, 190]. RTPT$_\beta$ belongs to a class of protein tyrosine phosphatases which exhibit features common to cell surface receptors. The extracellular domain has hallmarks characteristic of adhesion molecules, whereas the cytoplasmic portion contains two tyrosine phosphatase domains [191]. RTPT$_\beta$ is dependent on insulin and NGF signaling.

In the paranode, caspr is a major molecular component of the tight junctions. These are also associated with spectrin, actin and contactin, which via the β_1 Na channel subunit and RTPT$_\beta$ interact with caspr [191-193]. The type 1 diabetic BB/Wor rat shows a downregulation of several key molecules, such as contactin, the β_1 Na channel subunit and caspr, which is not seen in the type 2 BB/Z rat and is prevented by replenishing insulinomimetic C-peptide in type 1 diabetic rats [183]. Hence these data suggest that insulin and C-peptide deficiencies perturb the expression of crucial nodal and paranodal molecules and that impaired insulin action is likely to interfere with their assembly, thereby leading to the progressive disruption of the paranodal apparatus, which characterizes type 1 DPN in humans and rodents alike. This is further supported by the intimate colocalization of the insulinreceptor with paranodal tight junctions and the nodal membrane [171].

Cognitive function may be regulated by insulin signaling [194], since water maze training upregulates insulin receptor mRNA and the synaptic membrane insulin receptor protein in the rat hippocampus [195]. Chemically induced disruption of the brain insulin receptor causes persistent cognitive dysfunction in the rat [196]. In Alzheimer's disease, impaired insulin actions have been invoked in the genesis of this type of dementia [197–199]. Recent clinical data have demonstrated a duration-dependent cognitive decline in insulin-deficient type 1 diabetic subjects [200, 201]. Data from our laboratory have shown a downregulation of both insulin and IGF-I receptor expression in hippocampus of the BB/Wor rat. These changes were associated with increased neuronal apoptosis and loss of pyramidal cell neurons, and impaired water maze performances [144], and were significantly but not fully prevented by long-term C-peptide replacement [149].

Nerve regeneration

The progressive nerve fiber loss characterizing DPN [1, 202] is in part due to impaired regenerative responses to the degenerative process [203]. Nerve regeneration is a complex spatio-temporal sequence of events involving immediate early gene responses such as IGF-I $\rightarrow$ c-fos $\rightarrow$ NGF [141, 204], elaboration of interleukins and cytokines, macrophage recruitment, Wallerian degeneration, induction of cytoskeletal protein synthesis and, finally, axonal sprouting, elongation and maturation [205]. Several of these components of nerve fiber regeneration are perturbed in type 1 diabetes, such as altered immediate early gene responses [141], delayed Wallerian degeneration, delayed onset and rate of regeneration and impaired maturation of regenerated fibers [206]. Cytoskeletal proteins synthesized in DRG and transported into the axon are affected by DPN. Both perturbed synthesis and slowed transport of neurofilaments have been described. Neurofilaments are the most abundant structural components in axons and determine their size and NCVs [207–210]. They are intermediate filaments and comprise three subunit proteins: low-, medium- and high-molecular-weight neurofilaments [211, 212]. The importance of neurofilaments in determining axonal caliber and thereby conduction velocity has been demonstrated by analysis of a recessive mutation resulting from a premature translation terminator of the low-molecular neurofilaments gene [213]. Suppressed expression of neurofilaments leads to inhibition of axonal radial growth with consequent reduction in NCVs. Post-translational changes of neurofilaments such as increased JNK-mediated phosphorylation [214, 215] are likely to have consequences for the normal assembly of the axonal cytoskeleton and contribute to axonal degeneration. Tubulins are major components of microtubules. They display extensive heterogeneity, especially in the nervous system, where several neurospecific isotubulins have been demonstrated [216, 217]. Together with actin filaments, microtubules play important roles in directional outgrowth of neurites, such as growth cone advance and polarity [218]. Alterations in microtubule proteins affecting the assembly and stability of microtubules are therefore likely to modify axonal function and to influence neuronal remodeling and regeneration.

In a series of experiments we have previously shown significantly delayed and suppressed immediate early gene responses, impaired macrophage recruitment and Wallerian degeneration in the type 1 BB/Wor rat [141–143, 206, 207, 219]. These abnormalities were associated with impaired upregulation of tubulin and a lack of normal downregulation of neurofilament expression in DRG associated with a downregulation of IGF-I, trkA and p75 in DRG cells [142]. This led us to suggest that tubulin expression exerts a negative feedback on neurofilament expression to facilitate the early transport of tubulins to initiate the growth cone [142]. The sequence of abnormalities ultimately resulted in impaired axonal extension and caliber growth. Interestingly, parallel studies in the iso-

hyperglycemic and hyperinsulinemic type 2 BB/Z rat failed to demonstrate any major abnormalities in this sequence of events and showed more robust nerve fiber regeneration [128, 143]. C-peptide-replaced type 1 rats exhibited only mild changes compared to non-replaced animals [148]. C-peptide normalized the immediate early gene response and the expression of neurotrophic factors, their receptors and tubulin and neurofilaments in DRG neurons. This has led us to suggest that impaired nerve regeneration is a more prominent phenomenon in type 1 DPN and may contribute to the more severe clinical expression of DPN in this type of diabetes. Impaired nerve regeneration appears to be mainly the result of impaired insulin action rather than hyperglycemia.

Apoptosis

In vivo studies

Apoptosis is involved in the development of type 1 diabetes [220, 221] and several apoptotic pathways have been implicated in pancreatic β cell destruction in type 1 diabetes [222]. It occurs in diabetic retinopathy [223], nephropathy [224], encephalopathy [144, 149], and endothelial cells [225]. Recently, several reports have described apoptosis of DRG in the STZ-diabetic rat model [152, 153, 226]. Russell et al. [152] reported in 1-month STZ-diabetic rats, 34% apoptotic L5-DRG neurons as assessed by TUNEL stain. This finding was associated with nuclear ultrastructural abnormalities of chromatin condensation, clumping, and fragmentation and ballooning of mitochondria. Surprisingly, neuronal densities of DRG revealed a 2.7-fold increase in diabetic animals [152]. In in vitro studies, the authors described increased caspase 3 activation that correlated with glucose concentrations and suggested that hyperglycemia-induced oxidative stress promotes mitochondrial changes, leading to apoptosis. This was further elaborated by Srinivasan et al. [226], who in 3- to 6-week diabetic STZ rats demonstrated increased positive mitochondrial membrane potentials in diabetic DRG neurons. These findings were coupled with translocation of cytochrome C from mitochondria to the cytoplasm and decreased immunohistochemical identification of Bcl-2. Two weeks of treatment with insulin to achieve euglycemic levels normalized the mitochondrial membrane potentials and the apoptotic indices [226]. In this study, the apoptotic index in DRG was 9% and only twice that of age-matched control animals. The authors concluded that oxidative stress leads to mitochondrial injury as a specific target for DRG neuronal apoptosis. In a recent study, Low and collaborators [153] investigated L5 DRG neurons from 1-, 3-, and 12-month diabetic STZ rats. Semiquantitative immunocytochemical evaluation of TUNEL-positive, caspase 3-positive and 8-hydroxy-2[1]-deoxyguanosine (a product of oxidative DNA damage) labeled neurons, revealed apoptotic indices of 8, 7 and 11% at the three time points. Based on reduced cytochrome oxidase stainability in 12-month STZ rats, the authors concluded that oxidative stress in diabetes leads to impaired mitochondrial function and apoptosis [153].

In none of the above studies were apoptotic activities correlated with reliable morphometric analyses of either DRG populations or sensory nerve fiber loss, which in previous studies by other investigators have been normal or only minimally affected in the STZ rat [227, 228]. This apparent discordance between apoptosis of neuronal somata and almost intact axonal populations has been explained by delayed apoptosis or 'apoptosis lente' [229], by which the cell enters into apoptosis (TUNEL and caspase 3 positivity), but final execution of the cell is delayed through DNA repair and other mechanisms.

DRG neuronal apoptosis has not been examined in the spontaneously diabetic BB/Wor or BB/Z rats. However, we have examined hippocampal neuronal apoptosis, which in the BB/Wor rat occurs at 8 month of diabetes as assessed by LM-PCR DNA laddering, caspase 3 activity, Bax and Bc1-xL, and is associated with a 37% neuronal loss of the hippocampal CA_1 region [144]. In the iso-hyperglycemic type 2 BB/Z rat with the same duration and magnitude of hyperglycemic exposure, we have no evidence suggesting apoptotic activity in the hippocampus. Replacement of insulinomimetic C-peptide in the type 1 BB/Wor rat significantly but not fully prevents hippocampal apoptosis, neuronal loss and cognitive dysfunction [149]. These data, as well as the data on DRG by Srinivasan open up the possibility that insulin deficiency may be more important than hyperglycemia in diabetes-related apoptosis. Correlations between nerve fiber loss in sural nerves and impaired insulin availability or hyperglycemia show a highly significant exponential relationship with impaired insulin action but not with hyperglycemia (fig. 3). However, this nerve fiber loss is unlikely to solely reflect DRG neuronal apoptotic death.

Apoptosis in in vitro models mimicking the diabetic condition

Human neuroblastoma cells (SH-SY5Y) as well as primary DRG neurons, Schwann cells and hippocampal neurons undergo apoptosis when exposed to hyperosmolar conditions [152, 230, 231]. Both high mannitol and glucose induce apoptosis, indicating a hyperosmolar effect, although high glucose within pathophysiological concentration ranges causes more severe apoptosis, suggesting an additional glucotoxic effect. We [232, 233] and others [234–236] have demonstrated that IGF-I protects SH-SY5Y cells from apoptosis via a PI-3 kinase-dependent bi-directional regulation of p38 and JNK kinase

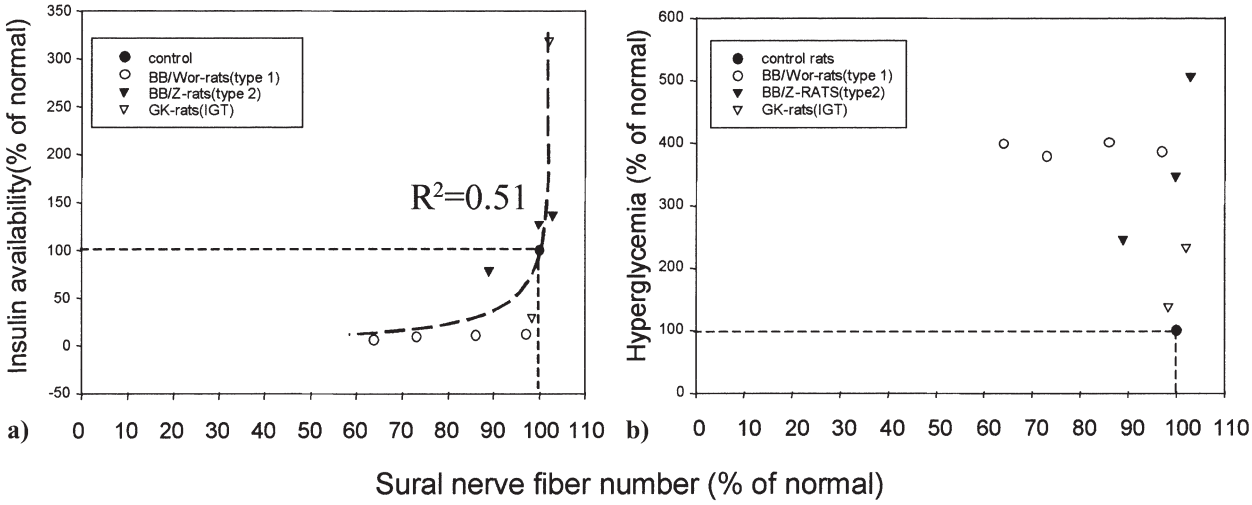

Figure 3. Relationships between insulin availability (*a*) and hyperglycemia (*b*) and sural nerve fiber loss in animal models of type 1 and type 2 diabetes and impaired glucose tolerance (IGT). A significant exponential relationship is seen between insulin deprivation and fiber loss, whereas no relationship was found between hyperglycemia and fiber loss. However, progressive fiber loss is unlikely to be due to neuronal DRG apoptosis, and is more likely to reflect progressive dying-back axonal degeneration in the insulinopenic BB/Wor rat.

[232, 233, 237]. IGF-I-mediated neuroprotection against oxidative stress in primary cultures of rat cerebellar granular cell cultures and hypothalamic GT 1-7 cells was found to be associated with activation of NF-κB in a PI-3-kinase-dependent manner [238], indicating a close relationship between PI-3 kinase and NF-κB in cell survival. Elevation of Bcl-2 family expression by NF-κB has been suggested as one mechanism underlying its antiapoptotic effect [239].

The role of insulin as an antiapoptotic hormone was recently demonstrated. Originally, this effect was believed to be mediated via the IGF-Ireceptor (IGF-IR). Several recent studies, however, have revealed that it exerts its antiapoptotic signaling through activation of its own receptor. In Chinese hamster ovary (CHO)-R cells, insulin serves an antiapoptotic function through a Raf-1-dependent pathway that leads to NF-κB activation via a PI-3-kinase-dependent pathway [240]. Recent data from our laboratory have demonstrated an antiapoptotic effect of insulin on SH-SY5Y cells grown in high glucose [150]. This was mediated via stimulation of PI-3 kinase and p38 MAP kinase activation, disinhibition of I-κB and translocation of NF-κB, promotion of $Bc1_2$ expression and inhibition of JNK phosphorylation [241]. These effects of insulin alone were significantly enhanced by the addition of insulinomimetic C-peptide [241]. Although we cannot exclude the possibility that these antiapoptotic effects by IGF-I, insulin and C-peptide may be mediated via possible effects on oxidative stress or mitochondrial dysfunction, eventhough C-peptide does not affect oxidative stress in peripheral nerve [50], they may also provide unrelated antiapoptotic functions as indicated by the effect of insulin and C-peptide on the Fas pathway [241, 242].

Apoptotic pathways

Our understanding of mechanisms involved in apoptosis of neuronal components in diabetes is still in its infancy. Several apoptotic pathways have been described (fig. 4) and there is some evidence to suggest that most of them may be involved in diabetes. Apoptosis of DRG has been described as being associated with mitochondrial dysfunction, oxidative stress and impaired IGF-I action (152, 153, 236).

Apoptosis can be initiated by activation of death receptors, mitochondrial dysfunction, endoplasmic reticulum (ER) abnormalities and alterations in calcium homeostasis (fig. 4). Apoptotic signals converge toward a common death pathway, for which the Bcl-2 family proteins act as regulators [243]. There are two classes of Bcl-2 family proteins with opposite effects on apoptosis the antiapoptotic members of the Bcl-2 family (e.g. Bcl-2, Bcl-xL) which protect cells against apoptosis, and the proapoptotic members (e.g. Bax, Bcl-xS) which promote programmed cell death [244–248]. The decision for cells to undergo apoptosis depends partly on the balance between these regulatory proteins.

Caspases are the main executioners of apoptosis. The family of caspase proteases exists as inactive proenzymes and are activated by two distinct mechanisms. The first is 'the caspase cascade': a previously activated caspase activates inactive procaspases, such as caspase 3, 6 and 7 [249]. The second is 'the induced proximity': binding of ligands to death receptors which activates an initiator caspase, such as caspase 8, 9 and 12.

Mitochondria play an important role in regulation of apoptosis (fig. 4). Mitochondrial membrane changes may lead to disruption of the inner transmembrane potential (DeltaPsi) and release of cytochrome C, apoptosis-induc-

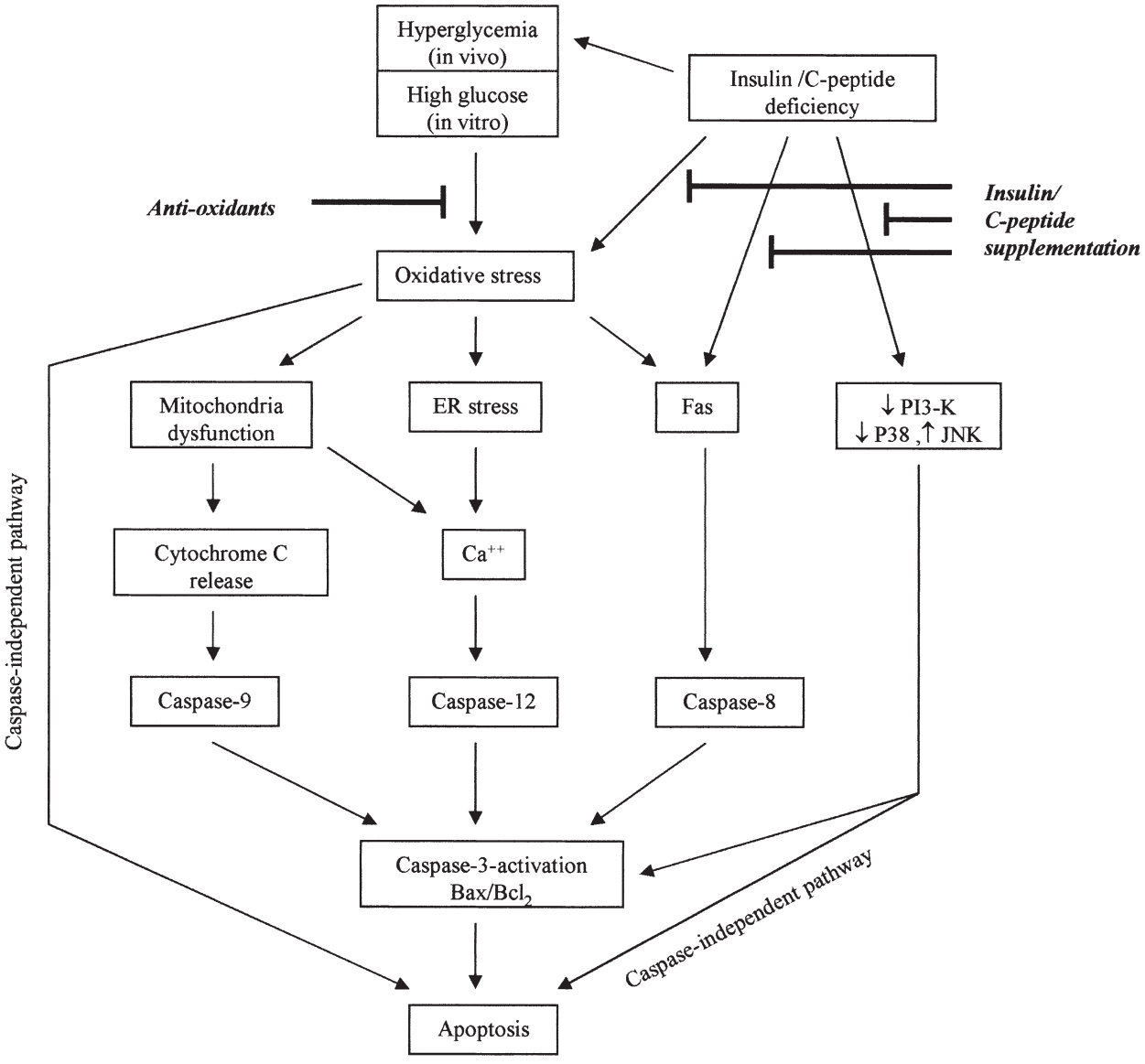

Figure 4. Potential apoptotic pathways induced by hyperglycemia in vivo or high glucose concentrations in vitro and by insulin/C-peptide deficiencies. Several possible caspase-mediated pathways have been implicated, as have caspase-independent pathways. Also indicated are potential inhibitors of pathways induced by oxidative stress and those potentiated by insulin/C-peptide deficiencies. Although apoptosis has been demonstrated in various cell systems exposed to a diabetic milieu, the role of apoptosis as a major factor in DPN remains controversial.

ing factor (AIF), DNase and caspase 2 and 9 [250–252], via the permeability transition (PT) pore. Cytochrome C activates caspase 9, together with the cytosolic factors dATP (or ATP) and Adaf-1, and subsequently caspase 3 [253], whereas AIF directly activates caspase 3 and induces apoptosis [254, 255].

A further mechanism by which reactive oxidants may cause cell death involves peroxynitrite and hydrogen peroxide, which cause single-strand DNA breakage triggering the activation of poly-ADP-ribose synthetase (PARS) (256). PARS inhibitors prevent this mechanism in cardiac myoblasts, endothelial cells and neurons (257–259).

Alterations in intracellular calcium homeostasis are commonly observed during apoptosis. Marked elevations of $[Ca^{2+}]_i$ activate hydrolytic enzymes, lead to exaggerated energy expenditure, impaired energy production, and initiate cytoskeletal degradation, ultimately resulting in cell death [260]. Over time in diabetes, there is a progressive increase in cytoplasmic Ca^{2+} in DRG neurons of the type 1 BB/Wor rat, which is not prevented by nimodipine [261–263]. An elevated cytoplasmic Ca^{2+} concentration has been described as a cause of cell injury [264–267]. However, the mechanism by which calcium might regulate cell death is still poorly understood.

Ischemia-induced hyperglutaminergic activity has been implicated as an excitotoxic apoptotic mechanism in neuronal cells [268, 269]. This is most likely mediated by Ca^{2+} influx with subsequent mitochondrial dysfunction [268], although other mechanisms such as K^+ efflux have been proposed [270]. We have previously shown that inhibition of GCP II (NAALADase), an enzyme responsible for the hydrolysis of NAAG to NAA and glutamate, partially prevents nerve conduction showing, hyperalgesia and axonal atrophy in the BB/Wor rat [271], which, however, are probably not the results of neuronal apoptosis.

ER participates in protein synthesis and trafficking, cellular responses to stress and intracellular Ca^{2+} signaling. Recent studies have shown that ER plays important roles in apoptosis [272, 273]. As the largest store of releasable calcium in the cell, alterations in ER calcium homeostasis contribute to neuronal apoptosis and excitotoxicity, and are being linked to the pathogenesis of several neurodegenerative disorders, including Alzheimer's disease and stroke [for a review see ref. 274].

Death receptors are a subgroup of the tumor necrosis factor (TNF)/NGF receptor superfamily. So far, six members of the death receptor family have been identified: TNF-R1, Fas, DR3, TRAIL-R1, TRIL-R2 and DR6. Recently, Kim et al. [275] showed that TNF induces apoptosis via increasing calcium release from the ER and by suppressing Bcl-2 expression, suggesting a caspase-independent mechanism. Recent studies from our laboratory have demonstrated increased expression of Fas coupled with increased Ac-DEVD-AMC-inhibitable activity of caspase 3 in 8-month diabetic hippocampi from the BB/Wor rat, suggesting a Fas-mediated apoptotic component [276]. Undoubtedly, apoptotic cell death or degeneration ('apoptosis lente') are involved in the microvascular and neurological complications of diabetes. Some studies indicate that these mechanisms may differ in type 1 and type 2 diabetes. However, this is a rapidly evolving area with only recently emerging concepts within the realm of diabetes.

Summary and conclusions

Diabetic syndromes are a diverse group of chronic metabolic disorders, which have in common abnormal glucose handling and, commonly, hyperglycemia. It is therefore not surprising that over the last few decades, hyperglycemia has been regarded as the major and sometimes the only culprit underlying the microvascular complications accompanying the two major types of diabetes; insulin-deficient type 1 and insulin-resistant type 2 diabetes. As repeatedly pointed out in this review, the hyperglycemic (but still insulin-sufficient) STZ-induced diabetic rat has provided the bulk of our knowledge regarding underlying metabolic and molecular aberrations producing DPN. Based on this information, we have extrapolated the findings to human diabetes regardless of type. During the last two decades, however, slowly and reluctantly, the concept is being accepted that the complications differ with respect to pathogenetic mechanisms, metabolic and molecular perturbations as well as the structural and clinical expressions of DPN in type 1 and type 2 diabetes. By employing animal models that more closely mimic either type 1 or type 2 diabetes, we and others have suggested that beside hyperglycemia, insulin and C-peptide deficiencies play important pathogenetic roles, which cannot be disregarded. These differences appear to account for the more severe functional, structural and symptomatic DPN in type 1 diabetes. Mechanistically, insulin and C-peptide deficiencies appear to have greater impacts on perturbations of neurotrophic factors like NGF and IGFs than does hyperglycemia. In addition, the inherent neuroprotective effects of insulin and C-peptide themselves must be considered. Deficits in insulin action and/or signaling are likely to have a greater impact on apoptotic phenomena, if they do indeed occur in neuronal perikarya of the peripheral nervous system. Recently, oxidative stress has received overwhelming attention as a hyperglycemia-induced pathogenetic phenomenon which is probably correct, except for the fact that insulin-deficiency-induced abnormalities of trophic factors are likely to contribute to the overall burden of oxidative stress in DPN.

In conclusion, I believe that we have in the past been totally focused on hyperglycemia as being the sole evil in DPN, and that other potential mechanisms have been largely overlooked. Thus in the years to come we must differentiate hyperglycemia-induced mechanisms from those that can be ascribed to abnormalities in insulin/C-peptide action per se. This information will enable us to tailor design appropriate therapies for disorders as diverse as the diabetic neuropathies and the microvascular complications and, hopefully, thereby improve and refine the treatment and prevention of the dreaded complications of diabetes.

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