Reviews in Endocrine & Metabolic Disorders 2004;5:227–236

New Insights into the Mechanisms of Diabetic Neuropathy

Andrea M. Vincent and Eva L. Feldman
Department of Neurology at University of Michigan, Ann Arbor, MI, 48109

Key Words. **antioxidant, clinical trial, diabetes, hyperglycemia, neuropathy, oxidative stress, therapy**

Introduction

The incidence of diabetes is increasing at epidemic rates. There currently are an estimated 17 million individuals with diabetes in the United States and this number is rapidly increasing [1]. The likelihood of developing diabetes is approximately 40% in persons born after the year 2000 [2] and is more prevalent among African Americans, Hispanics and Native Americans [2]. The personal and national loss of productivity is immense [3]. The American Diabetes Association estimates that diabetes cost the United States an estimated $132 billion in 2002, $92 billion in direct medical expenditures and $40 billion due to lost work time, disability, and premature mortality [4].

The morbidity of diabetes is due to both macrovascular and microvascular complications. A diabetic individual is 2 to 4 times more likely to have accelerated atherogenesis of large vessels leading to myocardial infarction, stroke and peripheral vascular occlusive disease [3]. These macrovascular complications can occur in individuals without diabetes, although it is clear that diabetes exacerbates and accelerates atherogenesis. In contrast, microvascular complications are more unique to the diabetic state and eventually occur in nearly all patients with diabetes. Fifteen years after diagnosis, 90% of patients have evidence of diabetic retinopathy, the cause of approximately 25,000 new cases of blindness per year in the United States [5]. Diabetes is the primary cause of renal failure and accounts for 40% of new cases requiring dialysis in the United States each year [4]. More than half of all individuals with diabetes develop neuropathy [4,6] with a lifetime risk of one or more lower extremity amputations estimated at 15% [7]. The current article will focus on this last complication, diabetic neuropathy (DN), with the goal of introducing new ideas on the pathogenesis and potential treatment of this disabling complication of diabetes.

Diabetic Neuropathy (DN)

In 1983, results from the Diabetes Control and Complications Trial (DCCT) established that hyperglycemia is pivotal in the development and progression of DN [8]. The DCCT enrolled patients with type 1 diabetes. Because the primary outcome of the DCCT was retinopathy, subjects were recruited, assigned and randomized within 2 cohorts depending on baseline retinopathy status. Patients in the primary prevention cohort had no retinopathy, diabetes duration of less than 5 years and a urinary albumin excretion rate less than 40 mg/24 h. Patients in the secondary prevention cohort had mild to moderate nonproliferative retinopathy, diabetes duration of less than 15 years and a urinary albumin excretion rate less than 200 mg/24 h. Subjects in the primary and secondary prevention cohorts were randomized to intensive versus conventional therapy. During the 6.5 year mean follow-up, this translated into a 33% reduction in mean blood glucose from approximately 230 mg/dL in the conventional treatment groups to approximately 155 mg/dL in the intensive treatment groups, with respective HbA1c values of 9% versus 7.2%. DN was diagnosed by history, focused neurological examination and nerve conduction studies. The risk of developing DN was reduced in the intensive treatment group by 69% in the primary prevention cohort and 57% in the secondary prevention cohort, with an overall risk reduction of 60% [9].

After completion of the DCCT, long-term observational follow-up of 1,375 of the 1,425 DCCT patients occurred in the EDIC study [10]. DN was followed in EDIC using the Michigan Neuropathy Screening Instrument, a validated tool for the diagnosis of DN [11]. DN was assessed annually during EDIC using this instrument. While glycemic control merged in EDIC with an average HbA1c of 8% for both the intensive and conventional treatment arms, the beneficial effects of prior intensive treatment has persisted for 8 years. Patients in the initial DCCT-intensive cohort have approximately a 30% risk reduction

Corresponding author: Eva L. Feldman.
E-mail: efeldman@umich.edu

for developing DN when compared to patients in the initial DCCT-conventional cohort [12]. This phenomenon is termed metabolic memory, and implies that the salutary effects of early glycemic control persist over many years.

How Does Hyperglycemia Cause Diabetic Neuropathy?

The scientific breadth of diabetes research has expanded with the recent advances in molecular biology. These investigations have extended diabetes-related research in neuropathy to a new fundamental scientific level where formerly distinct hypotheses are now linked by common molecular mechanisms and investigative techniques. It is now generally held that all mechanisms that are contributory to the onset and progression of diabetic neuropathy are related in some way to oxidative stress [13]. This article will focus on the loss of function and survival of neurons as a cause of diabetic neuropathy, however, it is essential to consider other potential mechanisms. During diabetes there is also impaired neuronal regeneration, particularly of thinly myelinated fibers. In patients with diabetic neuropathy there is evidence of simultaneous neuronal degeneration and regeneration suggesting that there is an ongoing dynamic balance between glucose mediated injury and attempts of the neuron for self repair [14]. Studies have shown that over time the balance shifts toward degeneration and nerve fibers lose their ability to regenerate. The mechanisms leading to regenerative impairment are likely similar to those leading to neuronal dysfunction and studies linking oxidative stress to impaired regeneration would prove useful to the study of diabetic neuropathy.

The Chemisty of Oxidative Stress

The production of reactive oxygen species (ROS) is under tight control in normal neurons. There are several free radical species that are produced normally in neurons to perform specific functions, including superoxide and hydrogen peroxide [15]. While these free radicals are essential for normal cell function, they can also accelerate the neuronal dysfunction.

Superoxide

Superoxide is generated by the mitochondrial electron transfer chain when NADH is oxidized to NAD^+. It is estimated that 4% of electrons that enter the respiratory chain lead to the formation of superoxide [16]. While superoxide is essential for cell survival, excess superoxide is injurious [17]. This can occur when cells are exposed to surplus glucose that in turn impairs mitochondrial electron transfer chain by inhibiting ATP synthase. This leads to slowing of electron transfer. Slowed electron transfer leads to the increased release of electrons that combine with molecular oxygen to produce superoxide [18]. A second consequence of slowed mitochondrial electron transfer is the activation of NADH oxidase, a consequence of the inability of electron transfer to generate NAD^+. Activated NADH oxidase generates superoxide as a byproduct [19,20]. Activation of this enzyme contributes to neurovascular deficits in diabetic rats through increased ROS [21].

Hydrogen peroxide

Superoxide is converted to hydrogen peroxide and water by a family of enzymes known as superoxide dismutase (SOD). Excess superoxide leads to excess hydrogen peroxide. Hydrogen peroxide is highly diffusable across membranes [22]. Because hydrogen peroxide has the potential to easily oxidize multiple cellular components, neurons express abundant anti-oxidants that convert hydrogen peroxide to water. The anti-oxidants include catalase, glutathione and thioredoxin.

Cellular Injury Due to ROS

Both excess superoxide and hydrogen peroxide are injurious to neurons. When superoxide combines with nitric oxide (NO) highly reactive peroxynitrite forms that attacks and disrupts proteins and lipids [23]. Superoxide can also attack the iron sulfur center of particular enzymes leading to enzyme inhibition. Several key enzymes are sensitive to this type of attack including complexes 1–3 of the electron transfer chain and aconitase of the TCA cycle [23].

Unchecked, hydrogen peroxide can react with free iron and produce hydroxyl radicals. Lipids are major targets of hydroxyl radical attack [24]. Lipids undergo peroxidation and form what are known as lipid peroxides. Lipid peroxides are directly toxic to cells and mediate cell death. When proteins and nucleic acids undergo peroxidation and nitrosylation, they can accumulate and overload the ability of the cell to recycle them [25]. In addition, damage to nucleic acids can activate the mechanisms of apoptosis [25]. Collectively, accumulation of damaged proteins, lipids and nucleic acids lead to loss of neuronal function.

There is also now well established oxidative modification by superoxide and hydrogen peroxide of transcription factors that lead to decreased expression of many key proteins needed for cell survival, including complex 1 and bcl-2 [24]. In turn, oxidative stress may increase the gene expression of several pro-apoptotic proteins including JNK kinase, poly ADP polyribose polymerase and cyclooxygenase-2 [26]. Mitochondrial in neurons may be particularly sensitive to oxidative damage [27,28]. This in turn could impair the normal energy regulatory function in neurons which is highly critical to their survival. Thus,

in neurons, oxidative stress leads to damage of proteins, lipids, transcription factors and mitochondrial DNA as well as increased expression of pro-apoptotic proteins. These interlinked processes are injurious to the nervous system, slowing axonal transport, altering neuronal signaling and promoting neuronal dysfunction.

Cellular Anti-Oxidant Defense

An anti-oxidant is defined as a compound that can terminate radical chain reactions by donating at least one hydrogen atom to a free radical [29]. A second type of anti-oxidant prevents the initiation of a free radical chain reaction. Cells maintain needed levels of anti-oxidants, frequently referred to as the cellular anti-oxidant potential, through both de novo synthesis as well as dietary intake. Excess ROS can deplete the anti-oxidant potential of a cell resulting in oxidative stress and the sequelae of unchecked ROS [30]. Vitamin C and vitamin E are the common dietary anti-oxidants for mammalian cells [31]. Vitamin C (ascorbic acid) scavenges an array of ROS including, hydroxyl, alkoxyl, peroxyl, superoxide anion, hydroperoxyl radicals and reactive nitrogen radicals such as nitrogen dioxide, nitroxide, and peroxynitrite at very low concentrations. In addition ascorbic acid regenerates other antioxidants such as α-tocopheroxyl, urate and β-carotene radical cation from their radical species [29]. The principal role of vitamin E (the generic name for the family of tocopherol related compounds) as an antioxidant is in scavenging lipid peroxyl radical intermediates and thereby blocking lipid peroxidation [31]. Tocopherols also react with other reactive oxidants, including singlet oxygen, alkoxyl radicals, peroxynitrite, nitrogen dioxide, ozone, and superoxide.

Glutathione (GSH) is the most important cellular antioxidant for neurons [32]. As discussed above, SOD is a key enzyme in cellular detoxification. SOD detoxifies superoxide into hydrogen peroxide, which is reduced in the mitochondria by GSH. Reduction (detoxification) of hydrogen peroxide generates an oxidized glutathione disulfide. To regenerate glutathione, glutathione disulfide is reduced by NADPH [33]. In diabetes, conversion of glucose to sorbitol is linked to the oxidation of NADPH to $NADP^+$. This leads to depletion of the NADPH needed for regenerating GSH [13,34]. GSH is also necessary to keep protein thiol groups in their reduced form and to combine with lipid peroxides to form an inert lipid hydroxyl group in water [32].

Anti-oxidant potential of the cells is augmented by the presence of enzymes that synthesize and maintain antioxidant molecules [35]. These include enzymes that synthesize and regenerate GSH and the closely related thioredoxin. In addition, there are enzymes that detoxify specific ROS. SOD and catalase are the key enzymes present, each with specific ROS targets. SOD converts superoxide to hydrogen peroxide and water. Catalase converts hydrogen peroxide to water and therefore its activity is required when SOD is active.

Production of ROS in Diabetes

The pathogenesis of diabetic neuropathy has been a source of controversy and intense investigation. While there are several theories on how excess glucose injures the nervous system, 3 areas have been the focus of the most intense investigation. These are (1) accumulation of advanced glycation end products (AGEs), (2) unchecked activity of the polyol pathway, and (3) activation of protein kinase (PK) C. Oxidative stress provides a unifying mechanism for these divergent theories, and is now well documented to occur as a result of AGE formation, polyol pathway activity and PKC activity in experimental and clinical diabetes [18,36]. The resultant changes in the redox status of the cell are augmented by glucose-mediated injury to mitochondria through excessive activation of glycolysis, further promoting a cycle of oxidative stress and cellular injury [37]. These pathways are summarized in Figure 1.

Advanced Glycation Endproducts (AGEs) and ROS Formation

Glycation is the result of non-enzymatic addition of glucose or other saccharides to proteins, lipids and nucleotides [36]. Glucose can bind to protein amino groups which first form a shift base that then progress to an Amadori product and result in the formation of AGEs and subsequent cross linking of proteins [38]. While formation of a shift base and an Amadori product are reversible, formation of AGEs is irreversible. In diabetes, excess glucose results in accelerated AGE production [36]. AGE formation in cells leads to intra and extracellular cross linking of proteins and protein aggregation [39]. These reactions require transition metal and ions as catalysts and depletion of the transition metal capacity in diabetes may lead to further AGE formation [36]. AGEs bind to a number of receptor proteins, including the receptor for advance glycation end products (RAGE) [40]. RAGE has multiple definite downstream signaling targets and is the main receptor through which AGE signaling is mediated. Downstream signaling of AGE RAGE interaction include activation of mitogen activated protein kinases (MAP kinases), p21 ras, NFκB and multiple other intermediates [40]. When AGEs interact with RAGE there is a production of reactive oxygen species in part by activation of NADPH oxidase and relocalization of pro-oxidant molecules at the cell surface

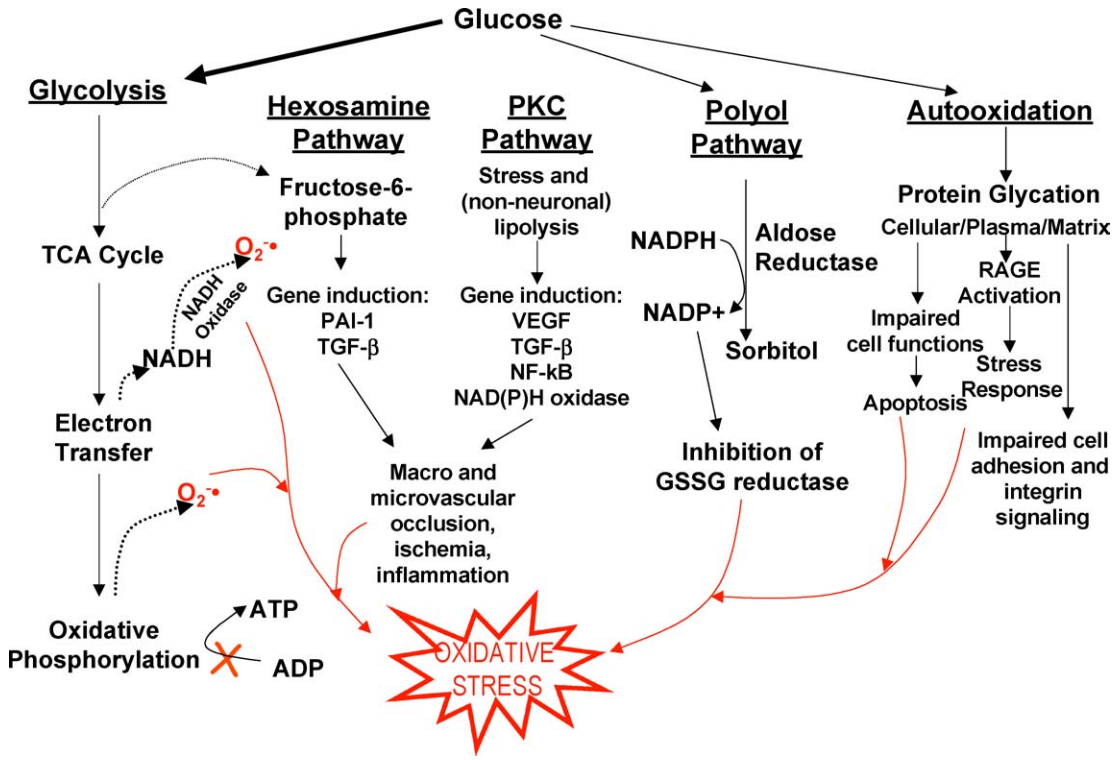

Fig. 1. *Mechanisms leading to oxidative stress in hyperglycemia. The normal end-point of glucose metabolism is the generation of ATP from ADP. In the presence of excess glucose ADP becomes rate-limiting, and the pathway becomes clogged. ROS then result at several points, including escape of electrons in the mitochondrial transfer chain to generate superoxide, and NADH oxidase is activated also producing superoxide. When glycolysis slows, fructose 1-6-bisphosphate is shunted into the hexosamine pathway that produces oxidative stress. Excess glucose is diverted to the polyol pathway as well as activating intracellular and extracellular glycation reactions.*

[41]. AGEs also cause a depletion of glutathione. If AGE accumulation is blocked and/or AGE RAGE interaction is prevented, there is a decrease in cellular oxidative stress and a restoration of nerve conduction deficits in the streptozotocin rat [36].

The Polyol Pathway

Excess glucose can be converted to sorbitol by aldose reductase [13]. This first step in the polyol pathway is linked to the oxidation of NADPH to $NADP^+$. This leads to depletion of NADPH needed for regenerating glutathione [42]. Thus, early after the induction of diabetes, metabolic defects lead to loss of NADPH, which limits the nerve's ability to scavenge reactive oxygen species, promoting a vicious cycle of oxidative stress. Sorbitol accumulation can also result in cellular osmotic stress that may alter the anti-oxidant potential of the cell and increase ROS [34]. The aldose reductase pathway has been the target of multiple experimental and clinical studies. Aldose reductase inhibitors have been successful in experimental diabetes, however, they have not proven effective in clinical diabetes [7,34]. This could be due in part to the ability of the drug to enter the nerve. Those aldose reductase inhibitors that do penetrate the nerve do decrease nerve sorbitol levels and in one clinical trial there is evidence of enhanced axonal regeneration [43]. In parallel, recent genetic studies link polymorphisms of the aldose reductase gene to an increased risk of diabetic complications [44].

PKC Activation

The role of PKC activity in the peripheral nervous system is less clear than its well-established role in the retina, kidney and the microvasculature [45]. It is generally held that the potential effects of the PKC pathway on the pathogenesis of diabetic neuropathy is more likely due to its contribution to vascular blood flow [46]. PKC is exquisitely sensitive to a cell's redox status. Anti-oxidants can bind the catalytic domain of PKC and inhibit the enzyme's activity. In contrast, pro-oxidants interact with the regulatory domain of PKC to activate the enzyme [47]. PKC activation results in MAP kinase activation and the phosphorylation of transcription factors that can increase the gene expression of multiple cellular stress related genes such as c-Jun kinases and the heat shock proteins [48]. These in turn

can damage the cell. In streptozotocin diabetic rats, inhibition of PKC can normalize blood flow, restore nerve conduction velocities and reduce nerve oxidative stress [49]. There is currently an ongoing trial of a PKC blocker in the treatment of a separate microvascular complication of diabetes, retinopathy [45].

Mitochondria, Oxidative Stress, and ROS

In the diabetic state, AGE accumulation, polyol pathway activity and PKC activation all lead to increased systemic and nervous system oxidative stress. Augmenting all of these processes is the generation of ROS under acute hyperglycemic episodes that over-activate the normal pathway of glucose metabolism. Once a cell has achieved a maximal level of ATP, oxidative phosphorylation shuts down, and intermediates in the pathway accumulate [50]. Electrons escape from the electron transfer pathway to produce superoxide. Excessive mitochondrial NADH is converted back to NAD+ with the generation of superoxide as a by-product, and fructose-6-phosphate is diverted from glycolysis to the hexosamine pathway that leads to inflammation and vascular disease. The net result of all these consequences of hyperglycemia is the accumulation of ROS that leads to cellular damage and eventually to cellular apoptosis [27,51]. This later apoptotic process appears to occur in neurons via ROS mediated injury to mitochondria [27,28]. In cultured DRG neurons, we have demonstrated how hyperglycemia produces apoptosis through mitochondrial oxidative stress through a novel hypothesis involving uncoupling proteins. Uncoupling proteins relieve hyperglycemia-induced electron transfer stress that produces mitochondrial hyperpolarization. DRG neurons express uncoupling protein 3 (UCP3), but this protein is downregulated in diabetes. Increasing UCP3 expression decreases hyperglycemia-induced superoxide formation and subsequent cell death (Fig. 2).

There are two key events in apoptosis that occur at the mitochondrial level: (1) mitochondrial permeability transition with change in mitochondrial membrane potential ($\Delta\Psi_M$) leading to mitochondrial membrane depolarization (MMD), and (2) release of proapoptotic factors like cytochrome c and Ca^{2+} [27,52]. Mitochondrial permeability transition occurs when large pores open in the inner mitochondrial membrane; these pore openings cause osmotic swelling, that in turn disrupt the integrity of the outer mitochondrial membrane and lead to changes in mitochondrial membrane potential ($\Delta\Psi_M$) [53]. These events culminate in mitochondrial membrane depolarization (MMD) and release of proapoptotic factors into the cytoplasm that activate the caspase cascade [27,37]. Superoxide anion is the major ROS generated in mitochondria. Superoxide formation results in mitochondrial lipid, DNA and protein peroxidation and nitrosylation that in turn leads to $\Delta\Psi_M$ and MMD [18]. In the diabetic state, high glucose leads to ROS formation in neurons followed by mitochondrial permeability transition, mitochondrial swelling, $\Delta\Psi_M$ and MMD [27].

Our laboratory was the first group to propose glucose-mediated apoptosis as a fundamental mechanism in diabetic complications, including neuropathy. We have reported that apoptosis occurs in dorsal root ganglion neurons (DRG) and Schwann cells (SC) from diabetic rats [27]. Approximately 30% of DRG from diabetic rats express cleaved capsase-3 and are TUNEL positive. These markers of apoptosis are confirmed by ultrastructural analysis with electron microscopy (EM). EM also reveals that DRG from diabetic animals contain swollen mitochondria with disrupted inner cristae structure [51]. Our observations in DRG from diabetic animals are corroborated by other laboratories [37,54] and the concept of glucose induced cell death as a mediator of diabetic complications is now expanded to include other complication prone tissues, including the retina, kidney, vascular endothelium, and fibroblasts [25,55,56].

Oxidative Stress as the Final Common Pathway in Neuropathy

In summary, unchecked, ROS produce (1) lipid, DNA and protein peroxidation [57,58], (2) ischemia and reduced nerve blood flow [46,59], and (3) cellular apoptosis [27,28,37,51]. These alterations in cellular metabolism result in peripheral nervous system damage and the signs and symptoms of neuropathy [60].

In the diabetic rat, measures of oxidative stress and reduced levels of circulating antioxidants parallel neuropathy and blocking oxidative stress in the diabetic animal prevents the development of neuropathy [58]. Antioxidants restore normal blood flow and sciatic and saphenous nerve conduction velocities in STZ diabetic rats [61,62]. Treatment with insulin decreases ROS activity in diabetes and prevents experimental diabetic neuropathy [63,64]. Growth factors may also serve as anti-oxidants and this function may contribute to their role as possible therapeutic entities in diabetic neuropathy [65,66]. *In vitro*, nerve growth factor (NGF) protects neuronal cells from ROS-triggered apoptosis [67,68]. In parallel, IGF-I also protects primary and transformed neurons from oxidative stress and cell death [69,70]. The mechanism(s) of action underlying the neuroprotective effects of growth factors are unknown. These data in experimental diabetes are paralleled by recent progress in clinical diabetes.

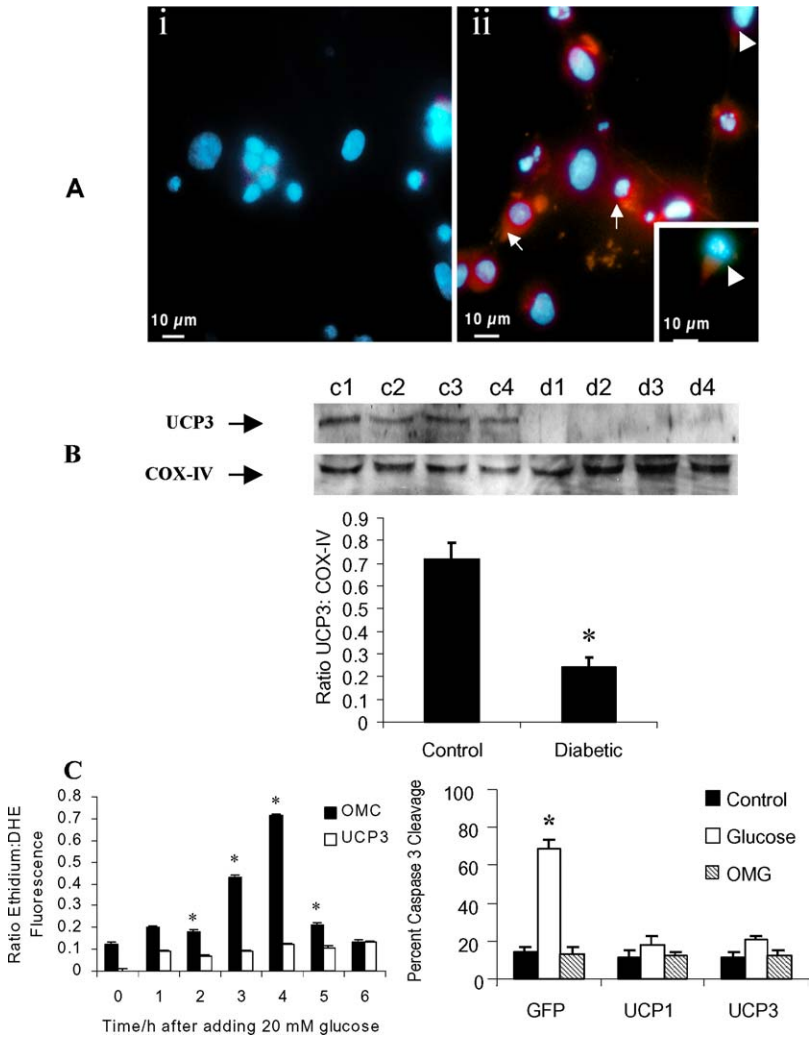

Fig. 2. *A Increasing concentrations of glucose induce cleavage of caspase-3, oxidative stress and apoptosis that are blocked by uncoupling proteins. (A) Increased glucose induces cleavage of caspase-3 in cultured DRG neurons. DRG neurons were cultured in control media (i) or media containing 20 mM added glucose (ii). After 6 h, the cultures were fixed and stained with 5 μg/ml CM1 to indicate cleaved (active) caspase-3 (orange-red), and 1 μg/ml bisbenzamide to indicate nuclear chromatin (blue). i. Immunofluorescent image showing normal diffuse nuclear staining in the DRG neuron (blue). ii. Immunofluorescent image showing cleaved caspase-3 in the cytoplasm (white arrows) with some of the nuclei showing aggregation of chromatin consistent with apoptosis (white arrowheads). ii shows high magnification of a single DRG neuron with punctate diffuse CM1 staining (cleaved caspase-3) in the cytoplasm, with chromatin clumping consistent with dissolution of the nucleus (white arrow head). (B) A. DRG from 4 week STZ diabetic rats were probed by Western blotting for UCP3 protein with probing for COX-IV as a loading control. DRG from 8 diabetic and 8 weight-matched control animals were used and protein concentration was equalized before loading. The blot illustrates 4 control and 4 diabetic rats. The bar graph shows the mean UCP densitometry for 8 control and 8 diabetic (*diabetic<control animals, $p < 0.01$). (C) DRG neurons were infected either with Ad.OMC (a mitochondrial protein; oxomaleate carrier) or Ad.UCP3 and exposed to high-glucose. Superoxide was assessed through oxidation of DHE to ethidium. The ethidium:DHE ratio was significantly decreased in UCP compared with OMC overexpressing controls at the time points shown (*$p < 0.01$). (D) Cultured DRG neurons were infected with adenovirus containing UCP1, UCP3, or GFP for 24 h prior to the application of 20 mM extra glucose or 20 mM osmotic control o-methyl glucopyranose (OMG). Neurons were then fixed at 6 h and stained for caspase-3 cleavage. Caspase-3 staining in control GFP-expressing neurons increases in 20 mM added glucose, but not in the presence of 20 mM OMG. In contrast, overexpression of UCP1 or UCP3 completely prevents caspase 3 cleavage caused by 20 mM added glucose. (Taken from Russell et al. [27]; Vincent et al. [28])*

Clinical Parameters of Oxidative Stress in Diabetes

In type 1 patients, there is evidence of systemic oxidative stress prior to the onset of micro or macrovascular complications [35]. Thus, systemic and tissue parameters of oxidative stress are assessed to determine the risk of complications as well as the efficacy of therapeutic interventions. Antioxidant potential and biomarkers of peroxidation correlate with the level of glycemic control [71]. As

the duration of both type 1 and type 2 diabetes progresses, patients show decreased antioxidant potential, although consistent changes in individual antioxidant molecules are not characterized [72]. Serum vitamins E and C tend to decrease in diabetic patients, and the decrease is greater in diabetic patients with angiopathy [73]. Diabetic patients generally display increased SOD activity, plasma lipid peroxides, and glutathione peroxidase and reductase and a decrease in GSH and α-tocopherol [74]. In contrast, after complications such as kidney disease develop, platelet SOD activity decreases [75]. Another strong indicator of diabetic kidney disease is the presence of the peroxidated DNA adduct 8-OH-2-deoxyguanosine [76]. Urinary 8-isoprostane F2α and plasma malondialdehyde generally increase in diabetes [72]. Lipid peroxidation correlates with AGE formation in type 2 diabetes and to a greater extent in type 1 diabetes [71]. Plasma protein carbonyls lipid peroxidation, and non-protein thiol levels are all increased to a greater extent in diabetes patients with complications than diabetes patients with no complications [77].

Mechanistic Treatment of Diabetic Neuropathy

Treatment strategies that halt oxidative stress decrease cell injury and, in many cases, restore function in cell culture and animal models of diabetic complications. Oxidative stress therefore is a therapeutic target in the treatment of diabetic complications. Therapies may be designed to block; (1) the initial phase of glucose-induced oxidative stress, (2) the free radicals that are generated by oxidative stress, or (3) the cellular damage produced by the accumulation of free radicals. A second approach can involve enhancing the natural antioxidant response of the cell, making it less susceptible to oxidative damage. Interestingly, diabetic patients with marked kidney disease tend to have an impaired renal anti-oxidant response [75], suggesting that innate ability to respond to oxidative stress may underlie differential susceptibility to complications.

Clinical trials in diabetic patients reveal complex interactions of drugs in the pathways of glycemic control and oxidative stress mechanisms. The widely-used glycemia lowering drugs gliclazide and metformin possess additional *in vivo* anti-oxidant properties [78]. Attributing the prevention of diabetic complications to anti-oxidant effects rather than reduced hyperglycemia is difficult to prove. One study compared the effects of gliclazide that promotes anti-oxidant status with glibenclamide that is not an anti-oxidant [59]. Gliclazide but not glibenclamide improves both plasma anti-oxidant potential and nitric oxide-mediated vasodilation in type 2 diabetic patients. Despite difficulty of interpretation in many cases, the abundance of antioxidant therapy trials makes a strong case for the importance of oxidative stress in the development of diabetes complications.

Very few studies have examined neuropathy as an endpoint for therapeutic benefit in antioxidant clinical trials. The most widely studied antioxidant in this area is α-lipoic acid. This compound is approved for the prevention of diabetic neuropathy in Europe [79]. Ongoing examinations of cohorts of patients demonstrate decreased neuropathic symptoms including pain, paresthesia, and numbness with 600 mg/d doses [80]. Patients receiving 1200 mg/d have greater improvement in these symptoms, but increased adverse effects. Beneficial effects are noted even in patients with poor glycemic control [81]. α-Lipoic acid is both fat and water-soluble, and so decreases oxidation in multiple tissues and plasma through recycling other antioxidants and chelating transition metals [82].

Vitamin E is a lipid phase antioxidant that also can improve insulin action and decrease glycated hemoglobin [83]. α-Tocopherol (the major dietary vitamin E supplement) was applied in a small double blind placebo-controlled trial in diabetic patients with moderately severe neuropathic symptoms [84]. The trial demonstrated improvements in nerve conduction velocity. Electrophysiological testing was performed at the start and after 6 mo treatment with 900 mg/d vitamin E or placebo.

Vitamin C is the most prominent antioxidant in the plasma. As such, it is not expected to provide significant benefit directly against neuropathy, although chronic administration, greater than 4 mo, of 1g/day vitamin C not only decreases plasma free radicals but also leads to increases in cellular GSH [85]. Generally, vitamin C is applied in clinical trials in combination with other antioxidants.

As already stated, many antioxidants have been clinically tested in diabetes, but neuropathy itself was not examined. Since the same mechanisms that produce cellular injury through oxidative stress are likely to mediate neuropathy, we include a list of some of the more common antioxidants. Allopurinol [86] and flavonoids [87] are both biologically active antioxidants. In clinical trials these correct plasma oxidative deficits such as plasma lipid peroxidation, and decreased plasma HbA1c without altering plasma glucose. L-Arginine decreases evidence of urinary lipid peroxidation [88], and zinc that promotes the levels of glutathione peroxidase also promotes plasma antioxidant potential [89]. Many further clinical studies of α-lipoic acid and vitamins E and C have demonstrated lower plasma lipid peroxidation and urinary 8-isoprostanes [90], with α-lipoic acid being the most potent. These compounds also reduce oxidative stress in the eye [91] and improve vascular function [92].

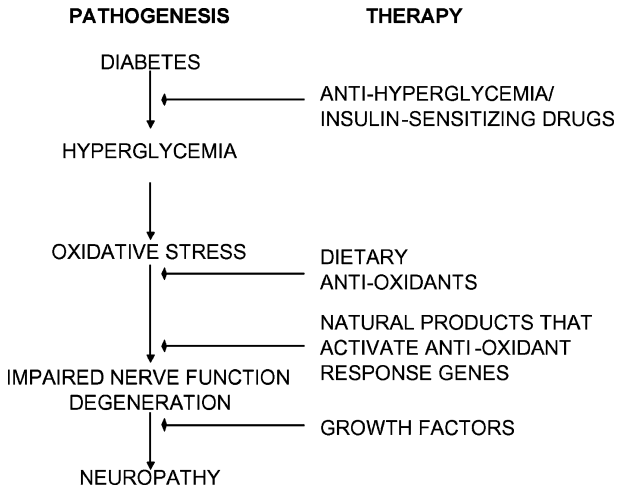

Fig. 3. *The pathway leading to diabetic neuropathy presents several therapeutic targets. Overwhelming evidence that hyperglycemia-induced oxidative stress is key to the development of diabetic neuropathy suggests multiple therapeutic targets. Combination therapies aimed at multiple targets, therefore, should provide potent protection against this devastating disease.*

To date, a thorough examination of long-term anti-oxidant therapy with correlations to glycemia control, oxidative stress, and neuropathy has not been completed. Despite this lack we have demonstrated an abundance of evidence supporting the need to promote anti-oxidant status in diabetic patients. An increasing literature describes the potential to regulate the innate anti-oxidant response, although no conclusive evidence for a therapeutic benefit against diabetic neuropathy has been presented [93]. Because anti-oxidant systems are intricately balanced, and since novel strategies to promote anti-oxidant status are under development, we anticipate that combination therapies targeting multiple anti-oxidant systems will produce a breakthrough in the treatment and prevention of diabetic neuropathy (Fig. 3).

Acknowledgments

The authors would like to thank Ms. Judy Boldt for excellent secretarial support. This work was supported by the Juvenile Diabetes Research Foundation (JDRF) Center for the Study of Complications in Diabetes, NIH NS42056, NIH NS38849, NIH NS36778 and the Program for Understanding Neurological Diseases (ELF).

References

1. Polydefkis M, Griffin JW, McArthur J. New insights into diabetic polyneuropathy. *JAMA* 2003;290:1371–1376.
2. Narayan KM, Boyle JP, Thompson TJ, Sorensen SW, Williamson DF. Lifetime risk for diabetes mellitus in the United States. *JAMA* 2003;290:1884–1890.
3. Jagger C, Goyder E, Clarke M, Brouard N, Arthur A. Active life expectancy in people with and without diabetes. *J Public Health Med* 2003;25:42–46.
4. Feldman EL, Stevens MJ, Russell JW, Greene DA. Somatosensory neuropathy. In: Porte D, Jr., Sherwin RS, Baron A, eds. *Ellenberg and Rifkin's Diabetes Mellitus*. McGraw Hill, 2002.
5. Gardner TW, Antonetti DA, Barber AJ, LaNoue KF, Levison SW. Diabetic retinopathy. More than meets the eye. *Surv Ophthalmol* 2002;47(Suppl 2):S253–S262.
6. Vinik AI, Erbas T, Pfeifer MA, Feldman EL, Stevens MJ, Russell JW. Diabetic autonomic neuropathy. In: Porte D, Jr., Sherwin RS, Baron A, eds. *Ellenberg and Rifkin's Diabetes Mellitus.* McGraw Hill, 2002.
7. Feldman EL, Stevens MJ, Russell JW, Greene DA. Diabetic neuropathy. In: Becker KL, ed. *Principles and Practice of Endocrinology and Metabolism*. Philadelphia, Lippincott Williams & Wilkins, 2001.
8. Molitch ME, Steffes MW, Cleary PA, Nathan DM. Baseline analysis of renal function in the Diabetes Control and Complications Trial. The Diabetes Control and Complications Trial Research Group. *Kidney Int* 1993;43:668–674.
9. The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993;329:977–986.
10. Epidemiology of Diabetes Interventions and Complications (EDIC). Design, implementation, and preliminary results of a long-term follow-up of the Diabetes Control and Complications Trial cohort. *Diabetes Care* 1999;22:99–111.
11. Feldman EL, Stevens MJ, Thomas PK, Brown MB, Canal N, Greene DA. A practical two-step quantitative clinical and electrophysiological assessment for the diagnosis and staging of diabetic neuropathy. *Diabetes Care* 1994;17:1281–1289.
12. Greene D, Martin C, Feldman E, Stevens M, Cleary P. Neuropathy in patients with type 1 diabetes four years after a trial of intensive therapy in The Diabetes Control and Complications Trial. *Diabetes Care* 2004; submitted.
13. Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocr Rev* 2003; in press.
14. Kennedy JM, Zochodne DW. The regenerative deficit of peripheral nerves in experimental diabetes: Its extent, timing and possible mechanisms. *Brain* 2000;123(Pt 10):2118–2129.
15. Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radic Biol Med* 1995;18:775–794.
16. Fridovich I. Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 1995;64:97–112.
17. Lee MH, Hyun DH, Jenner P, Halliwell B. Effect of proteasome inhibition on cellular oxidative damage, antioxidant defences and nitric oxide production. *J Neurochem* 2001;78:32–41.
18. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001;414:813–820.
19. Wu DC, Teismann P, Tieu K, Vila M, Jackson-Lewis V, Ischiropoulos H, Przedborski S. NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. *Proc Natl Acad Sci USA* 2003;100:6145–6150.
20. Li JM, Shah AM. ROS generation by nonphagocytic NADPH oxidase: Potential relevance in diabetic nephropathy. *J Am Soc Nephrol* 2003;14:S221–S226.
21. Cotter MA, Cameron NE. Effect of the NAD(P)H oxidase inhibitor, apocynin, on peripheral nerve perfusion and function in diabetic rats. *Life Sci* 2003;73:1813–1824.
22. Antunes F, Cadenas E. Estimation of H2O2 gradients across biomembranes. *FEBS Lett* 2000;475:121–126.

23. Brown GC, Borutaite V. Nitric oxide, cytochrome c and mitochondria. *Biochem Soc Symp* 1999;66:17–25.
24. Rosen P, Nawroth PP, King G, Moller W, Tritschler HJ, Packer L. The role of oxidative stress in the onset and progression of diabetes and its complications: A summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes Metab Res Rev* 2001;17:189–212.
25. Yorek MA. The role of oxidative stress in diabetic vascular and neural disease. *Free Radic Res* 2003;37:471–480.
26. Du X, Matsumura T, Edelstein D, Rossetti L, Zsengeller Z, Szabo C, Brownlee M. Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J Clin Invest* 2003;112:1049–1057.
27. Russell JW, Golovoy D, Vincent AM, Mahendru P, Olzmann JA, Mentzer A, Feldman EL. High glucose induced oxidative stress and mitochondrial dysfunction in neurons. *FASEB J* 2002;16:1738–1748.
28. Vincent AM, Olzmann JA, Brownlee M, Sivitz WI, Russell JW. Uncoupling proteins prevent glucose-induced neuronal oxidative stress and programmed cell death. *Diabetes* 2004;53:726–734.
29. Mukai K, Nishimura M, Kikuchi S. Stopped-flow investigation of the reaction of vitamin C with tocopheroxyl radical in aqueous triton X-100 micellar solutions. The structure-activity relationship of the regeneration reaction of tocopherol by vitamin C. *J Biol Chem* 1991;266:274–278.
30. Ceriello A, Bortolotti N, Motz E, Pieri C, Marra M, Tonutti L, Lizzio S, Feletto F, Catone B, Taboga C. Meal-induced oxidative stress and low-density lipoprotein oxidation in diabetes: The possible role of hyperglycemia. *Metabolism* 1999;48:1503–1508.
31. Di Mambro VM, Azzolini AE, Valim YM, Fonseca MJ. Comparison of antioxidant activities of tocopherols alone and in pharmaceutical formulations. *Int J Pharm* 2003;262:93–99.
32. Ueno Y, Kizaki M, Nakagiri R, Kamiya T, Sumi H, Osawa T. Dietary glutathione protects rats from diabetic nephropathy and neuropathy. *J Nutr* 2002;132:897–900.
33. Anderson ME. Glutathione: An overview of biosynthesis and modulation. *Chem Biol Interact* 1998;111/112:1–14.
34. Obrosova IG, Minchenko AG, Vasupuram R, White L, Abatan OI, Kumagai AK, Frank RN, Stevens MJ. Aldose reductase inhibitor fidarestat prevents retinal oxidative stress and vascular endothelial growth factor overexpression in streptozotocin-diabetic rats. *Diabetes* 2003;52:864–871.
35. Marra G, Cotroneo P, Pitocco D, Manto A, Di Leo MA, Ruotolo V, Caputo S, Giardina B, Ghirlanda G, Santini SA. Early increase of oxidative stress and reduced antioxidant defenses in patients with uncomplicated type 1 diabetes: A case for gender difference. *Diabetes Care* 2002;25:370–375.
36. Thornalley PJ. Glycation in diabetic neuropathy: Characteristics, consequences, causes, and therapeutic options. *Int Rev Neurobiol* 2002;50:37–57.
37. Schmeichel AM, Schmelzer JD, Low PA. Oxidative injury and apoptosis of dorsal root ganglion neurons in chronic experimental diabetic neuropathy. *Diabetes* 2003;52:165–171.
38. Ulrich P, Cerami A. Protein glycation, diabetes, and aging. *Recent Prog Horm Res* 2001;56:1–21.
39. King RH. The role of glycation in the pathogenesis of diabetic polyneuropathy. *Mol Pathol* 2001;54:400–408.
40. Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. *J Biol Chem* 1997;272:17810–17814.
41. Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL. Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. *Am J Physiol Endocrinol Metab* 2001;280:E685–E694.
42. Feldman EL, Stevens MJ, Greene DA. Treatment of diabetic neuropathy. In: Mazzaferri EL, Bar RS, Kreisberg RA, eds. *Advances in Endocrinology and Metabolism*. Chicago: Mosby Year Book, 1994.
43. Greene DA, Arezzo JC, Brown MB. Effect of aldose reductase inhibition on nerve conduction and morphometry in diabetic neuropathy. Zenarestat Study Group. *Neurology* 1999;53:580–591.
44. Oates PJ, Mylari BL. Aldose reductase inhibitors: Therapeutic implications for diabetic complications. *Expert Opin Investig Drugs* 1999;8:2095–2119.
45. Hammes HP, Du X, Edelstein D, Taguchi T, Matsumura T, Ju Q, Lin J, Bierhaus A, Nawroth P, Hannak D, Neumaier M, Bergfeld R, Giardino I, Brownlee M. Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. *Nat Med* 2003;9:294–299.
46. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 2000;404:787–790.
47. Gopalakrishna R, Jaken S. Protein kinase C signaling and oxidative stress. *Free Radic Biol Med* 2000;28:1349–1361.
48. Srivastava AK. High glucose-induced activation of protein kinase signaling pathways in vascular smooth muscle cells: A potential role in the pathogenesis of vascular dysfunction in diabetes (review). *Int J Mol Med* 2002;9:85–89.
49. Sisson JC, Lynch JJ, Johnson J, Jaques S, Wu D, Golgos G, Lucchesi BR, Wieland DM. Scintigraphic detection of regional disruption of adrenergic neurons in the heart. *Am Heart J* 1988;116:67–76.
50. Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M. Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci USA* 2000;97:12222–12226.
51. Russell JW, Sullivan KA, Windebank AJ, Herrmann DN, Feldman EL. Neurons undergo apoptosis in animal and cell culture models of diabetes. *Neurobiol Dis* 1999;6:347–363.
52. Nangle MR, Cotter MA, Cameron NE. Protein kinase Cbeta inhibition and aorta and corpus cavernosum function in streptozotocin-diabetic mice. *Eur J Pharmacol* 2003;475:99–106.
53. Srinivasan S, Stevens MJ, Wiley JW. Diabetic peripheral neuropathy: Evidence for apoptosis and associated mitochondrial dysfunction. *Diabetes* 2000;49:1932–1938.
54. Cheng C, Zochodne DW. Sensory neurons with activated caspase-3 survive long-term experimental diabetes. *Diabetes* 2003;52:2363–2371.
55. Tomlinson DR. Mitogen-activated protein kinases as glucose transducers for diabetic complications. *Diabetologia* 1999;42:1271–1281.
56. Solini A, Chiozzi P, Falzoni S, Morelli A, Fellin R, Di Virgilio F. High glucose modulates P2X7 receptor-mediated function in human primary fibroblasts. *Diabetologia* 2000;43:1248–1256.
57. Cameron NE, Cotter MA. Effects of protein kinase Cbeta inhibition on neurovascular dysfunction in diabetic rats: Interaction with oxidative stress and essential fatty acid dysmetabolism. *Diabetes Metab Res Rev* 2002;18:315–323.
58. Maritim AC, Sanders RA, Watkins JB, III. Effects of alpha-lipoic acid on biomarkers of oxidative stress in streptozotocin-induced diabetic rats. *J Nutr Biochem* 2003;14:288–294.
59. De Mattia G, Laurenti O, Fava D. Diabetic endothelial dysfunction: Effect of free radical scavenging in Type 2 diabetic patients. *J Diabetes Complications* 2003;17:30–35.

60. Feldman EL. Oxidative stress and diabetic neuropathy: A new understanding of an old problem. *J Clin Invest* 2003;111:431–433.
61. Pieper GM, Siebeneich W. Oral administration of the antioxidant, N-acetylcysteine, abrogates diabetes-induced endothelial dysfunction. *J Cardiovasc Pharmacol* 1998;32:101–105.
62. Cameron NE, Cotter MA, Maxfield EK. Anti-oxidant treatment prevents the development of peripheral nerve dysfunction in streptozotocin-diabetic rats. *Diabetologia* 1993;36:299–304.
63. Simmons Z, Feldman EL. Update on diabetic neuropathy. *Curr Opin Neurol* 2002;15:595–603.
64. Greene DA, Obrosova I, Stevens MJ, Feldman EL. Pathways of glucose-mediated oxidative stress in diabetic neuropathy. In: Packer L, Rosen P, Tritschler HJ, King GL, Azzi A, eds. *Antioxidants in Diabetes Management*. New York: Marcel Dekker, 2000.
65. Park DS, Morris EJ, Stefanis L, Troy CM, Shelanski ML, Geller HM, Greene LA. Multiple pathways of neuronal death induced by DNA-damaging agents, NGF deprivation, and oxidative stress. *J Neurosci* 1998;18:830–840.
66. Leinninger GM, Vincent AM, Feldman EL. The role of growth factors in diabetic peripheral neuropathy. *J Periph Nerv Sys* 2004;9:26–53.
67. Thippeswamy T, Morris R. Nerve growth factor inhibits the expression of nitric oxide synthase in neurones in dissociated cultures of rat dorsal root ganglia. *Neurosci Lett* 1997;230:9–12.
68. Estévez AG, Radi R, Barbeito L, Shin JT, Thompson JA, Beckman JS. Peroxynitrite-induced cytotoxicity in PC12 cells: Evidence for an apoptotic mechanism differentially modulated by neurotrophic factors. *J Neurochem* 1995;65:1543–1550.
69. Russell JW, Feldman EL. Insulin-like growth factor-I prevents apoptosis in sympathetic neurons exposed to high glucose. *Horm Metab Res* 1999;31:90–96.
70. van Golen CM, Feldman EL. Insulin-like growth factor I is the key growth factor in serum that protects neuroblastoma cells from hyperosmotic-induced apoptosis. *J Cell Physiol* 2000;182:24–32.
71. Kalousova M, Skrha J, Zima T. Advanced glycation end-products and advanced oxidation protein products in patients with diabetes mellitus. *Physiol Res* 2002;51:597–604.
72. Vessby J, Basu S, Mohsen R, Berne C, Vessby B. Oxidative stress and antioxidant status in type 1 diabetes mellitus. *J Intern Med* 2002;251:69–76.
73. Skrha J, Prazny M, Hilgertova J, Weiserova H. Serum alpha-tocopherol and ascorbic acid concentrations in Type 1 and Type 2 diabetic patients with and without angiopathy. *Clin Chim Acta* 2003;329:103–108.
74. Seghrouchni I, Drai J, Bannier E, Riviere J, Calmard P, Garcia I, Orgiazzi J, Revol A. Oxidative stress parameters in type I, type II and insulin-treated type 2 diabetes mellitus; insulin treatment efficiency. *Clin Chim Acta* 2002;321:89–96.
75. Ceriello A, Morocutti A, Mercuri F, Quagliaro L, Moro M, Damante G, Viberti GC. Defective intracellular antioxidant enzyme production in type 1 diabetic patients with nephropathy. *Diabetes* 2000;49:2170–2177.
76. Tsukahara H, Sekine K, Uchiyama M, Kawakami H, Hata I, Todoroki Y, Hiraoka M, Kaji M, Yorifuji T, Momoi T, Yoshihara K, Beppu M, Mayumi M. Formation of advanced glycosylation end products and oxidative stress in young patients with type 1 diabetes. *Pediatr Res* 2003;54:419–424.
77. Cakatay U, Telci A, Salman S, Satman L, Sivas A. Oxidative protein damage in type I diabetic patients with and without complications. *Endocr Res* 2000;26:365–379.
78. Gargiulo P, Caccese D, Pignatelli P, Brufani C, De Vito F, Marino R, Lauro R, Violi F, Di Mario U, Sanguigni V. Metformin decreases platelet superoxide anion production in diabetic patients. *Diabetes Metab Res Rev* 2002;18:156–159.
79. Ametov AS, Barinov A, Dyck PJ, Hermann R, Kozlova N, Litchy WJ, Low PA, Nehrdich D, Novosadova M, O'Brien PC, Reljanovic M, Samigullin R, Schuette K, Strokov I, Tritschler HJ, Wessel K, Yakhno N, Ziegler D. The sensory symptoms of diabetic polyneuropathy are improved with alpha-lipoic acid: The SYDNEY trial. *Diabetes Care* 2003;26:770–776.
80. Ziegler D, Reljanovic M, Mehnert H, Gries FA. Alpha-lipoic acid in the treatment of diabetic polyneuropathy in Germany: Current evidence from clinical trials. *Exp Clin Endocrinol Diabetes* 1999;107:421–430.
81. Borcea V, Nourooz-Zadeh J, Wolff SP, Klevesath M, Hofmann M, Urich H, Wahl P, Ziegler R, Tritschler H, Halliwell B, Nawroth PP. alpha-Lipoic acid decreases oxidative stress even in diabetic patients with poor glycemic control and albuminuria. *Free Radic Biol Med* 1999;26:1495–1500.
82. Packer L, Tritschler HJ, Wessel K. Neuroprotection by the metabolic antioxidant alpha-lipoic acid. *Free Radic Biol Med* 1997;22:359–378.
83. Narayan KM, Gregg EW, Fagot-Campagna A, Engelgau MM, Vinicor F. Diabetes–a common, growing, serious, costly, and potentially preventable public health problem. *Diabetes Res Clin Pract* 2000;50(Suppl 2):S77–S84.
84. Tutuncu NB, Bayraktar M, Varli K. Reversal of defective nerve conduction with vitamin E supplementation in type 2 diabetes: A preliminary study. *Diabetes Care* 1998;21:1915–1918.
85. Paolisso G, Balbi V, Volpe C, Varricchio G, Gambardella A, Saccomanno F, Ammendola S, Varricchio M, D'Onofrio F. Metabolic benefits deriving from chronic vitamin C supplementation in aged non-insulin dependent diabetics. *J Am Coll Nutr* 1995;14:387–392.
86. Butler R, Morris AD, Belch JJ, Hill A, Struthers AD. Allopurinol normalizes endothelial dysfunction in type 2 diabetics with mild hypertension. *Hypertension* 2000;35:746–751.
87. Keenoy B, Vertommen J, De L, I. The effect of flavonoid treatment on the glycation and antioxidant status in Type 1 diabetic patients. *Diabetes Nutr Metab* 1999;12:256–263.
88. Lubec B, Hayn M, Kitzmuller E, Vierhapper H, Lubec G. L-Arginine reduces lipid peroxidation in patients with diabetes mellitus. *Free Radic Biol Med* 1997;22:355–357.
89. Faure P, Benhamou PY, Perard A, Halimi S, Roussel AM. Lipid peroxidation in insulin-dependent diabetic patients with early retina degenerative lesions: Effects of an oral zinc supplementation. *Eur J Clin Nutr* 1995;49:282–288.
90. Davi G, Ciabattoni G, Consoli A, Mezzetti A, Falco A, Santarone S, Pennese E, Vitacolonna E, Bucciarelli T, Costantini F, Capani F, Patrono C. *In vivo* formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: Effects of improved metabolic control and vitamin E supplementation. *Circulation* 1999;99:224–229.
91. Peponis V, Papathanasiou M, Kapranou A, Magkou C, Tyligada A, Melidonis A, Drosos T, Sitaras NM. Protective role of oral antioxidant supplementation in ocular surface of diabetic patients. *Br J Ophthalmol* 2002;86:1369–1373.
92. Beckman JA, Goldfine AB, Gordon MB, Garrett LA, Keaney JF, Creager MA. Oral antioxidant therapy improves endothelial function in type 1 but not type 2 diabetes mellitus. *Am J Physiol Heart Circ Physiol* 2003;285:H2392–H2398.
93. Halat KM, Dennehy CE. Botanicals and dietary supplements in diabetic peripheral neuropathy. *J Am Board Fam Pract* 2003;16:47–57.