Animal Models of Diabetic Complications Consortium
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Mitochondrial SOD as a Target for Diabetic Neuropathy

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Table of Contents

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

Rodent models of diabetes fail to develop changes that closely resemble human diabetic nephropathy or neuropathy. While the reasons for the resistance of rodents to full-blown complications are likely multiple, they may include an increased resistance to oxidative stress or the absence of important genetic susceptibility genes. Our general strategic approach to this dilemma is to accelerate the injury of diabetes by predisposing critical cells within the peripheral nervous system to glucose-mediated oxidative injury.

Recent Progress and Major Accomplishments

We generated *in vitro* evidence in support of the role of antioxidant enzymes in resistance to diabetic neuropathy and made progress in the development of tissue-specific antioxidant gene knockout to develop new models of diabetic neuropathy. We have systematically explored DRG neuron degeneration under conditions of decreased or increased SOD2. We showed that increased expression of SOD2 in cultured DRG neurons decreases susceptibility to hyperglycemia-induced oxidative stress and cell injury (Fig. 1).

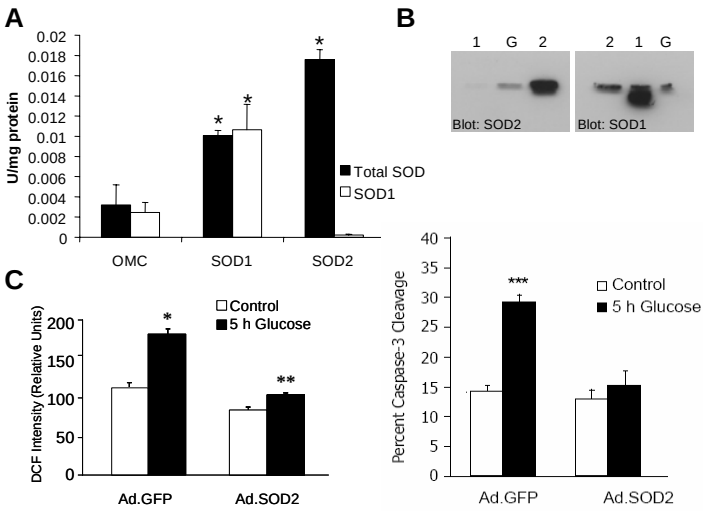

Fig. 1. SOD2 Protects Sensory Neurons from $O_2^{\cdot-}$ Mediated Damage. A) Adenoviral infection specifically increases SOD1 or SOD2 in DRG neurons. The graph shows SOD activity in lysates from Ad.OMC (a comparison mitochondrial protein) Ad.SOD1 or Ad.SOD2-infected DRG neurons. Ad.SOD2 specifically upregulated the activity of SOD2 but not SOD1. *p<0.01 compared to Ad.OMC-infected DRG neurons. B) Western blots of lysates of (1) Ad.SOD1-infected, (2) Ad.SOD2-infected, or (G) Ad.GFP-infected DRG neurons that are probed for SOD1 or SOD2. C) Oxidation of non-fluorescent CM-H_2DCFDA to green fluorescent DCF indicates an increase in oxidative stress, particularly H_2O_2 generation following treatment with 20 mM added glucose that is blocked in SOD2 transfected cells. *p<0.01 compared with untreated control; **p<0.01 compared with Ad.GFP/glucose. D) SOD2, but not GFP overexpression, prevents 20 mM glucose-induced caspase-3 activation. ***p < 0.001 compared to untreated control.

As a corollary, we cultured DRG neurons from adult mice with decreased expression of SOD2, mice with heterozygous knockout of the SOD2 gene. In culture, these DRG neurons showed evidence of increased oxidative stress under basal glucose conditions and greater hyperglycemia-induced oxidative stress and cell injury (Fig. 2).

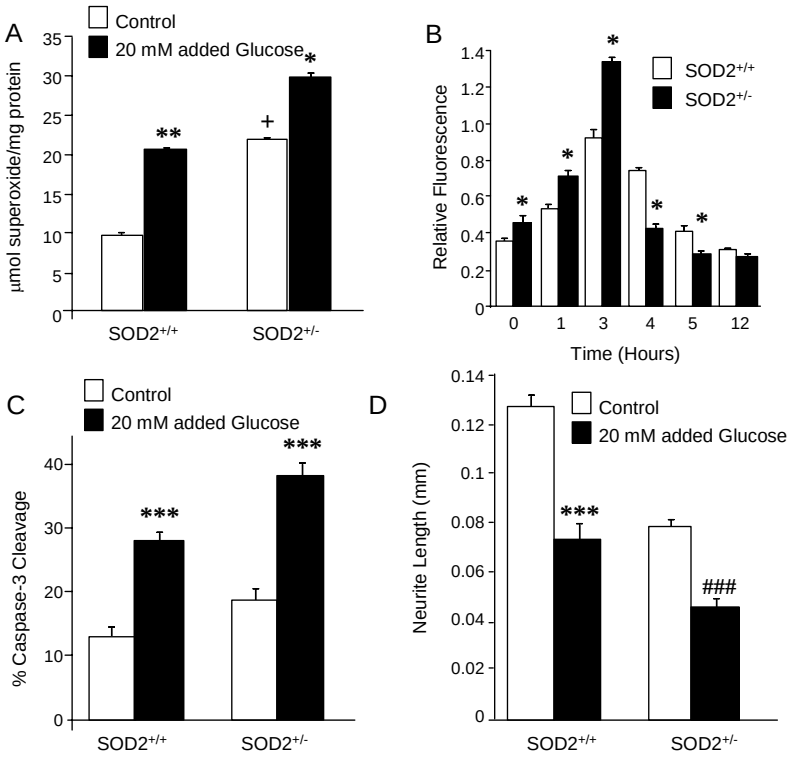

Fig. 2. Decreased Expression of SOD2 Increases Glucose-Mediated $O_2^{\cdot-}$ and Apoptosis. DRG neurons from adult SOD2$^{+/+}$ and SOD2$^{+/-}$ mice were exposed to 20 mM added glucose. A) $O_2^{\cdot-}$ generation was measured using an *in vitro* Amplex Red oxidation reaction at 5 h. **p < 0.001 compared to SOD2$^{+/+}$ in basal glucose; +p < 0.01 compared to SOD2$^{+/+}$ in basal glucose; *p < 0.05 compared to SOD$^{+/-}$ in basal glucose. B) DRG neurons were loaded with TMRM (50 nM), then mitochondrial membrane potential assessed through the increase in red fluorescence. In the presence of 20 mM glucose, hyperpolarization was greater and depolarization occurred earlier in SOD2$^{+/-}$ compared to SOD2$^{+/+}$ neurons. C) Caspase-3 activation was determined by counting the percent of DRG neurons labeled with a fluorescent caspase-3 substrate (CaspaTag). Glucose-induced caspase-3 activation was increased in both the SOD2$^{+/-}$ and SOD$^{+/+}$ cultures, compared to basal glucose, ***p < 0.001. D) Neurites were measured in DRG neurons 12 h after plating in control or hyperglycemia (20 mM added glucose) media. Mean neurite length was shorter in 20 mM added glucose than basal glucose, ***p < 0.001 for SOD2$^{+/+}$ and ###p < 0.05 for SOD2$^{+/-}$.

These profound changes in sensory neurons *in vitro* led us to explore the effects of decreased SOD2 on the development of diabetic neuropathy. Diabetic neuropathy was assessed in SOD2$^{+/-}$ C57BL/6J mice and their SOD2$^{+/+}$ litter mates following streptozotocin (STZ) treatment. These animals, while hyperglycemic, did not display any signs of diabetic neuropathy (Fig. 3). Neither did they display evidence of oxidative stress as examined by measuring total radical antioxidant potential (TRAP). Thus, the loss of one copy of SOD2 was not sufficient to increase oxidative stress *in vivo*, despite our observations *in vitro*. It is possible that the mice adapted to the loss of one copy of the SOD2 gene by upregulating other antioxidant defense systems.

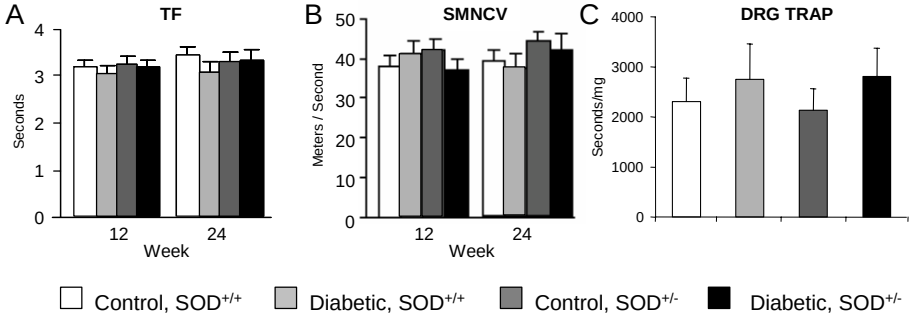

Fig. 3. Measures of Nerve Function and Antioxidant Potential in SOD2$^{+/+}$ and SOD2$^{+/-}$ Mice on a C57BL/6 Background. A) Tail flick (TF) latencies are measured at 12 and 24 weeks post induction of diabetes. B) Sciatic Motor Nerve Conduction Velocity (SMNCV) was assessed at 12 and 24 weeks. C) TRAP was measured in DRG after 24 weeks. No differences were found between the four experimental groups.

While these results were at first unexpected, these same animals had no evidence of diabetic nephropathy or retinopathy. These data were submitted to the AMDCC website on

www.amdcc.org. Our *in vivo* data parallel one of the main findings of the AMDCC i.e. that there is significant variability among the susceptibility of different mouse strains to develop diabetic complications at different levels of glycemia. We concluded that the lack of diabetic complications in the C57BL/6J SOD2$^{+/+}$ and SOD2$^{+/-}$mice is potentially due to the method of diabetes induction. The STZ protocol developed by the AMDCC is 55 mg/kg over 5 days for a total STZ dose of 250 mg/kg; this method results in hyperglycemia in the range of human diabetes, around 200 to 250 mg/dl serum glucose. This is very different from other studies of diabetic complications on rodent models that routinely use one dose of 200 mg/kg of STZ producing more extreme hyperglycemia in the range of 500 mg/dl that do develop slowed motor nerve conduction velocities (Breyer et al, 2005. J Am.Soc.Nephrol. 16, 27-45). These data suggest that the C57BL/6J mice require extremely high nonphysiologic glucose serum levels to develop diabetic neuropathy.

We next pursued our studies in a genetic model of type 2 diabetes, the db/db mouse. In contrast to the STZ C57BL/6J animals, in the first 12 weeks of diabetes, SOD2$^{+/+}$ db/db and SOD2$^{+/-}$ db/db mice displayed behavioral evidence of diabetic neuropathy, with prolonged tail flick times of greater than 10 sec, highly statistically significant from nondiabetic SOD2$^{+/+}$ db^{+} and SOD2$^{+/-}$ db^{+}mice (Fig. 4). In addition, sciatic motor nerve conduction velocities declined in the SOD2$^{+/+}$ db/db compared with non-diabetic litter mates and there was a robust further decline in the SOD2$^{+/-}$ db/db animals (Fig. 4). The SOD2$^{+/+}$ mice displayed 10-fold higher TRAP than the SOD2$^{+/-}$ mice regardless of the diabetic state. This was a surprising result suggesting that these mice may be highly dependent upon the regulation of SOD2 activity to resist obesity-induced oxidative stress. In SOD2$^{+/-}$ mice, db/db animals displayed a significant decrease in TRAP compared with db^{+} mice, suggesting that the antioxidant capacity is overwhelmed in diabetes in these mice and may contribute to the development of diabetic neuropathy.

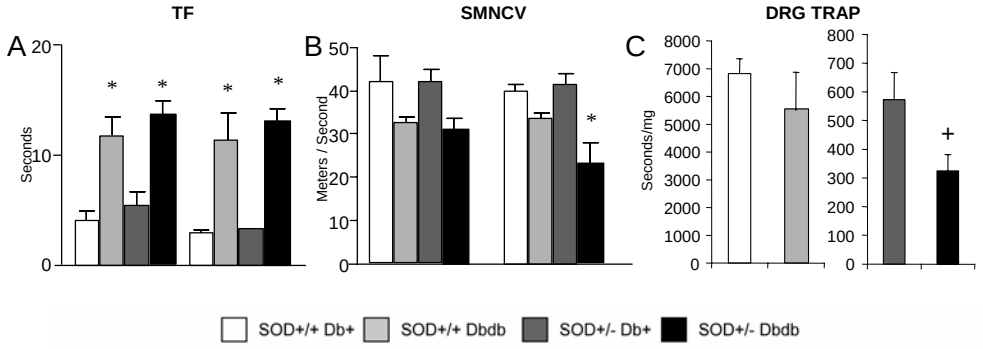

Fig. 4. Measures of Nerve Function and Antioxidant Potential in SOD2$^{+/+}$ and SOD2$^{+/-}$ Mice on a db+ or db/db Background. Nerve function and antioxidant potential was assessed using tail flick latency (TF) at 8 and 12 weeks (A), Sciatic Motor Nerve Conduction Velocity (SMNCV) at 12 and 24 weeks (B), and DRG TRAP at 24 weeks (C). In A, TF latencies are significantly longer in db/db, *p < 0.001. In B, a significant difference in SMNCV is only detected at 24 weeks between the db^{+} and db/db SOD2$^{+/-}$ mice (*p < 0.001). In C, TRAP was significantly lower in db/db mice compared with db^{+} ($^{+}$p<0.05).

The SOD2$^{+/+}$ db/db and SOD2$^{+/-}$ db/db mice also displayed loss of small intraepidermal nerve fibers in the footpads (Fig. 5). This anatomical assessment of neuropathy is part of the standard neuropathy phenotyping recommended by the AMDCC (www.amdcc.org) and we confirm that it is reliable, sensitive and is highly correlative with nerve conduction studies. In agreement with the observed changes in the sciatic motor nerve conduction velocities, the SOD2$^{+/-}$ db/db mice had even fewer PGP 9.5 positive fibers i.e. a lower IENF than the SOD2$^{+/+}$ db/db mice (p < 0.05). The anticipated differences between epidermal nerve fiber densities in diabetic and control animals (regardless of SOD2 expression) were also in accordance with published reports in human patients Yasuda et al, 1985, Acta Pathol Jpn. 35, 1-8; Levy et al

1989, Diabetologia 32, 427-433; Kennedy et al 1996, Neurology 47, 1042-1048; Arezzo, 1999, Am.J.Med. 107, 9S-16S; Christianson et al 2003, Exp Neurol. 179, 188-199.

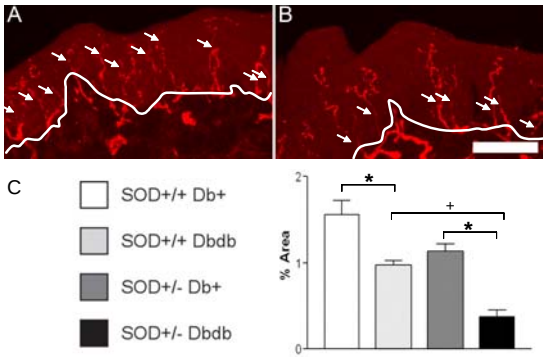

Figure 5. Measures of IENFD in SOD2$^{+/+}$ and SOD2$^{+/-}$ Mice on a C57BL/6 db+ or db/db Background. Representative SOD2$^{+/+}$ db+ (A) and SOD2$^{+/-}$ db/db (B) images are shown to illustrate the flattened confocal mouse footpad sections processed for PGP 9.5 immunofluorescence. The white line in A identifies the division between dermis and epidermis and the arrows indicate IENF that cross this division. Fewer fibers are evident in the diabetic footpad (B) compared with the non-diabetic control (A). Bar = 50 m. In C, IEFD expressed as percent of total epidermal area. *p < 0.01 between SOD2$^{+/+}$ db+ and SOD2$^{+/+}$ db/db, and between SOD2$^{+/-}$ db^{+} compared to SOD2$^{+/-}$ db/db. +p < 0.05 between SOD2$^{+/+}$ db/db compared to SOD2$^{+/-}$ db/db.

Following these studies, we further examined the role of mouse strain on the development of diabetic neuropathy. We examined diabetic neuropathy in streptozotocin (STZ)-induced [B6] and spontaneous type 1 diabetes [B6Ins2^{Akita}] and spontaneous type 2 diabetes [B6-db/db, BKS-db/db]. Diabetic neuropathy was defined using the criteria of the AMDCC. Despite persistent hyperglycemia, the STZ-treated B6 and B6Ins2^{Akita} mice were resistant to neuropathy. In contrast, diabetic neuropathy developed in both type 2 diabetes models: the B6-db/db and BKS-db/db mice. This effect required an increased fat diet in B6-db/db mice but was evident on standard mouse chow in BKS-db/db mice. These data support the hypothesis that genetic background and diet influence the development of diabetic neuropathy and should be considered when developing new models of the disease. In the BKS-db/db mice we were able to demonstrate TUNEL staining of DNA fragmentation and increased nitrotyrosine as evidence of oxidative stress (Fig. 6).

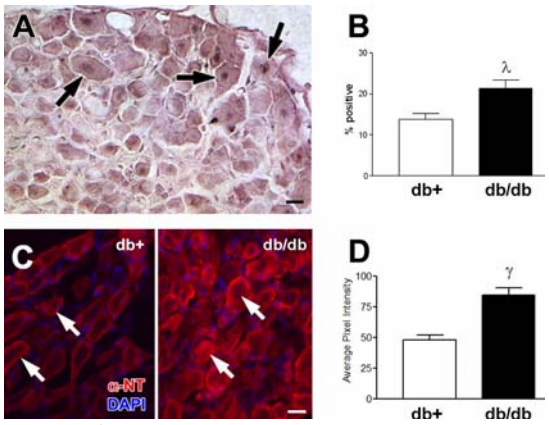

Fig. 6. Damaged DNA was measured by TUNEL staining. A) TUNEL positive sensory neurons (arrows) were detected in the lumbar DRG of BKS-db/db. B) Increased number of TUNEL labeled DRG in BKS-db/db mice at 24 weeks, λp < 0.05. Five animals/group and >150 neurons/animal were counted. Results are expressed as the percent TUNEL positive out of total neurons counted. Localization of nitrated proteins was measured by nitrotyrosine immunofluorescence (-NT). C) -NT reveals an increase in nitrated proteins within DRG neurons (arrows) from BKS-db/db compared to BKS-db^{+} mice [nuclei stained with DAPI]. D) Histograms of the fluorescence signal indicate a relative increase in the intensity of NT-immunofluorescence in DRG from BKS-db^{+} versus BKS-db/db mice (γp<0.01). Bar = 20 μm. Open bars represent the nondiabetic measurements and the black bars represent the diabetic measurements.

Plans for the Upcoming Year

We are beginning our manipulations of the expression of catalase per Aim 1 of the grant application. Initially, we will explore the effects of overexpression or underexpression of catalase alone on hyperglycemic injury in DRG neuron cultures. Currently available catalase knockout mice will be used to generate catalase deficient DRG neuron cultures. Concurrently, we will supply the full length catalase gene in a shuttle vector to the Gene Vector Core at the University of Iowa and they will construct an adenovirus that will permit rapid and efficient catalase gene transfer to our cultured DRG. We anticipate that since the total knockout of catalase produces mild to no phenotype, we will be able to obtain these mice and directly cross them with our Nes-cre-Sod2<lox>BKLSdbdb and db+ mice and backcross to the BKLSdbdb background. We anticipate sending the catalase shuttle vector to the Gene Vector Core by October 2007. We anticipate that we will obtain a breeding colony of catalase knockout mice by December 2007.

Preliminary Milestones for 2009 and Beyond

We are anticipating the identification of even more significant antioxidant genes that may afford significant resistance to oxidative injury in currently available mouse strains. We are considering the lipoic acid synthase gene that is beginning to be characterized in mice. We and others have demonstrated the powerful protective capacity of lipoic acid against diabetic neuropathy. A mitochondrial lipoic acid synthase gene was identified that is essential for normal development and provides mitochondrial protection (Yi X and Maeda N.Mol Cell Biol (2005) 25(18):8387-92). We anticipate that an inducible knockdown of this gene will make the DRG neurons highly susceptible to diabetes complications. Potentially, this inducible knockdown will also need to be tissue-specific.

2. Collaboration:

Collaborations With Other AMDCC Groups and JAX:

Besides our active and continued collaboration with Dr. Brosius, Dr. Dale Abel from the University of Utah will send us a newly developed mouse for neuropathy phenotyping. We will employ the methods outlined in our progress report. We are available to phenotype potentially interesting models for the presence of neuropathy for all AMDCC investigators; this can be coupled with nephropathy phenotyping and/or returning the tissues of interest to the home institution.

We also continue to interact actively with Dr. Brosius and share animal models for phenotyping of nephrology and neuropathy. Coordination of nephropathy and neuropathy phenotyping begins with the birth of the animals and continues through their assignment to an experimental group to final tissue collection. We are collaborating with two of Dr. Brosius' co-investigators to identify biomarkers for diabetic neuropathy. Dr Sub Pennathur performs state-of-the-art GC-mass spec analysis of mouse plasma and complication-prone tissue for oxidized lipid and protein products. The oxidative stress molecular signatures help determine the mechanism leading to oxidative stress and the efficacy of any interventions. Dr. Matthias Kretzler performs RNA expression studies in microarray format. Again, the gene expression profiles permit the discovery of disease mechanisms and the effect of treatment as well as the broader effect of a single gene manipulation.

In our efforts to improve our mouse models, Jax began strain development for us in May. We sent mice with flox/flox sod-2 mice on a C57Bl/6 background to be backcrossed with BLKS/db+ mice expressing Cre recombinase under the control of nestin (neuronal) promoter. Jax implanted embryos into several C57Bl/6 female mice in May 2007 and mice are breeding. In addition, they began backcrossing the nes-cre Tg mouse onto BLKS/db+. The first experiment generated limited embryos and may not produce live pups, but will be repeated. Finally, B6-Tg(Nes-Cre) mice are ordered and these will be backcrossed onto the BKS/db+ background within the next month. These experiments will yield Nes-cre-Sod2<lox>BKLSdbdb and db+ mice that have a genetic background that is susceptible to diabetic neuropathy (BKS-db/db) with the added complete knockout of SOD2 in the sensory neurons. We hypothesize that these mice will develop significantly greater diabetic neuropathy than previous models.

Listed below are the major collaborative projects related to the consortium goals but independent of AMDCC:

1. We have IRS -/- mice from Dr. Kahn at the Joslin Clinic and the SOD1-/- mice from Dr. John Faulkner at the University of Michigan. In both instances, these mice are 2 years old and are being phenotyped.
2. Dr. Feldman is the Principal Investigator for several collaborative grants investigating the etiology, pathogenesis and treatment of diabetic polyneuropathy. .
3. Dr. Feldman is an investigator in neuropathy aspects of the multi-institutional Epidemiology of Diabetes Interventions and Complications (EDIC) study.

3. Address previous EAC comments:

NOT APPLICABLE THIS YEAR

4. Publications:

Abstracts:

Vincent, Am, Russell JW, Sullivan K, Brosius FC, Feldman EL (ENDO 2007) SOD2 Protects Neurons from Injury in Cell Culture and Animal Models of Diabetic Neuropathy.

Sullivan KA, Hayes JM, Wiggin TD, Backus C, Oh SS, Lentz SI, Brosius FC, Feldman EL: Mouse Models of Diabetic Neuropathy, (PNS 2007).

Manuscripts:

Sullivan KA, Hayes JM, Wiggin TD, Backus C, Oh SS, Lentz SI, Brosius FC, Feldman EL: Mouse Models of Diabetic Neuropathy, *Neurobiology of Disease*, in press.

Vincent AM, Russell JW, Sullivan KA, Mentzer AE, Schin ML, Backus C, Hayes JM, McLean LL, Burke K, Brosius FC, Feldman EL: SOD2 Protects Neurons from Injury in Cell Culture and Animal Models of Diabetic Neuropathy, *Experimental Neurology*, in press.

Kern TS, Berkowitz BA, Feldman EL. National Institute of Diabetes and digestive and Kidney Diseases (NIDDK) Meeting Summary: Advances Toward Measuring Diabetic Retinopathy and Neuropathy: From the Bench to the Clinic and Back Again. *Journal of Diabetes and Its Complications*, submitted.