Short-term hyperglycemia produces oxidative damage and apoptosis in neurons

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SPECIFIC AIMS

This study aims to confirm the hypothesis that oxidative stress is a primary mediator of dorsal root ganglia (DRG) neuron injury in an in vitro model of diabetic neuropathy by exploring *1)* the mechanisms of glucose-induced oxidative stress leading to programmed cell death, *2)* the antioxidant capacity of the neurons, and *3)* the protective ability of exogenous antioxidants.

PRINCIPAL FINDINGS

1. A 2 h exposure to hyperglycemia is sufficient to induce DRG neuron cell death

We previously demonstrated that increasing the concentration of glucose in the culture media by 20 mM caused DRG neuron mitochondrial dysfunction and programmed cell death. In these earlier studies, the glucose concentration remained elevated for 6–24 h. In diabetes in animals, however, the period of hyperglycemia is likely to be short. To mimic the in vivo diabetic state, glucose (20 mM) was added for 2 h only, returned to baseline levels, then programmed cell death was assessed after 6–24 h. The peak of caspase activation at 6 h and TUNEL at 24 h was the same whether DRG neurons were exposed to 20 mM added glucose for 2 h, 6 h, or 24 h. These data demonstrate that a short period of hyperglycemia, often experienced in the diabetic state, is sufficient to produce DRG neuron injury.

2. Significant oxidative stress occurs in DRG neurons within a 2 h period

If oxidative stress were a primary mediator of glucose-induced DRG neuron injury, then oxidative stress should be present within the first 2 h of hyperglycemia. Oxidative stress was assessed in DRG neurons over 0–6 h in response to exposure to 20 mM added glucose. Aconitase is an enzyme that is sensitive to inhibition by superoxide through oxidative alterations of the iron-sulfur center. Aconitase activity significantly decreased in DRG neurons within 1 h of exposure to 20 mM glucose, but not to 20 mM mannitol, used as an osmotic control. The assay for thiobarbituric acid reactive substances (TBARS) in lysed DRG neurons demonstrated a statistically significant increase in lipid peroxidation within 1 h of adding 20 mM glucose compared with untreated control cultures (**Fig. 1*A***). In contrast, 20 mM mannitol did not alter TBARS in the DRG neurons. A secondary measure of lipid peroxidation was performed by Western blot for 8-isoprostane F2α (Fig. 1*B*). Again, lipid peroxidation was found to increase within 1 h exposure to 20 mM added glucose, and remained elevated at 6 h. These assays have not been previously reported in DRG neurons. We demonstrate that the TBARS assay is reliable in tissue despite reports that measurements are subject to error in plasma.

3. NADH oxidase is activated after hyperglycemia in DRG neurons

Activation of NADH oxidase indicates there is excess NADH in the cell that must be converted to NAD+ to allow glycolysis to continue. This enzyme produces superoxide as a by-product. Two peaks of NADH oxidase activity were observed 1 and 5 h after application of 20 mM glucose. Using the NADH oxidase inhibitor diphenylene iodonium, we demonstrate that the early peak of reactive oxygen species in hyperglycemia is partially produced by NADH oxidase but that the later peak results almost entirely from NADH oxidase activity.

4. DRG neurons activate an antioxidant response to hyperglycemia-induced oxidative stress

Cells exposed to oxidative stress may respond by increasing the expression of antioxidant enzymes. Although the antioxidant response is reported to be limited in neurons, we predicted that hyperglycemia-induced oxidative stress would increase the activities of superoxide dismutase (SOD), catalase, and glutathione (GSH) recycling enzymes in DRG neurons. SOD and catalase activities increased 3 h after application of 20

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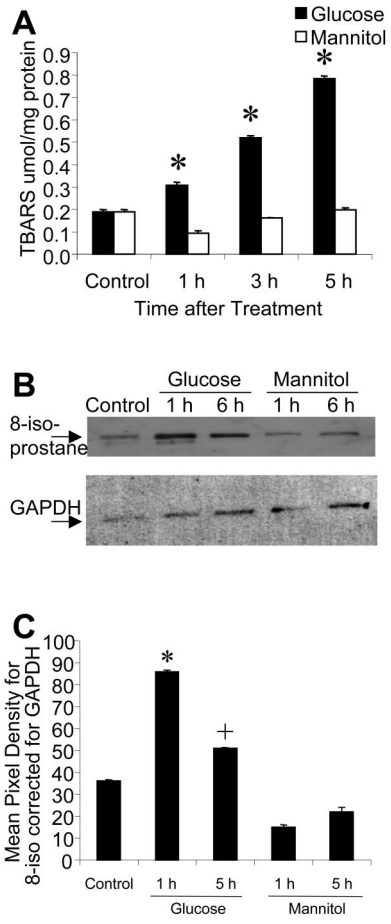

Figure 1. Lipid peroxidation increases within 1 h after hyperglycemia in DRG neurons. *A)* The TBARS assay demonstrates that MDA increases significantly within 1 h of adding 20 mM glucose compared with untreated control cultures, $P < 0.05$. TBARS continues to increase significantly between 1–3 h and 3–5 h, $P < 0.05$. In contrast, 20 mM mannitol does not alter TBARS in DRG neurons. *B)* Comparison of 8-isoprostane F2α in untreated control cultures with 1 or 6 h exposure to 20 mM glucose demonstrates a significant increase within 1 h, which remains elevated at 6 h. This effect is not present with mannitol treatment. Membranes were reprobed with GAPDH as a loading control. *C)* Blot illustrated in panel *B* analyzed by densitometry. The corrected mean pixel density for 1 h exposure to 20 mM glucose is 3-fold higher than in the control ($P<0.001$). 5 h after application of 20 mM glucose, 8-isoprostane F2α remains higher than the control ($P<0.05$).

mM glucose. The increase in catalase activity was 5-fold over control levels whereas the increase in SOD was more modest. The concentration of GSH decreased from 18.1 ± 3 μmol/mg protein to 10.8 ± 2 μmol/mg protein by 3 h after application of 20 mM glucose. This suggests that while GSH is being oxidized through significant oxidative stress, DRG neurons retained the ability to regenerate GSH. Taken together, these data suggest that DRG neurons are capable of an antioxidant response that could constitute a therapeutic target against diabetic neuropathy.

To further assess the intrinsic antioxidant capacity of DRG neurons, we modified the assay of total radical antioxidant potential (TRAP) for application to lysed DRG neurons. Because DRG neurons mount an antioxidant response, we predicted that overall TRAP would not decrease significantly in glucose-treated DRG neurons. The dependability of the assay was confirmed by pretreating DRG neurons with the antioxidant α-lipoic acid. After antioxidant treatment, basal TRAP increased by 3-fold. Yet application of 20 mM

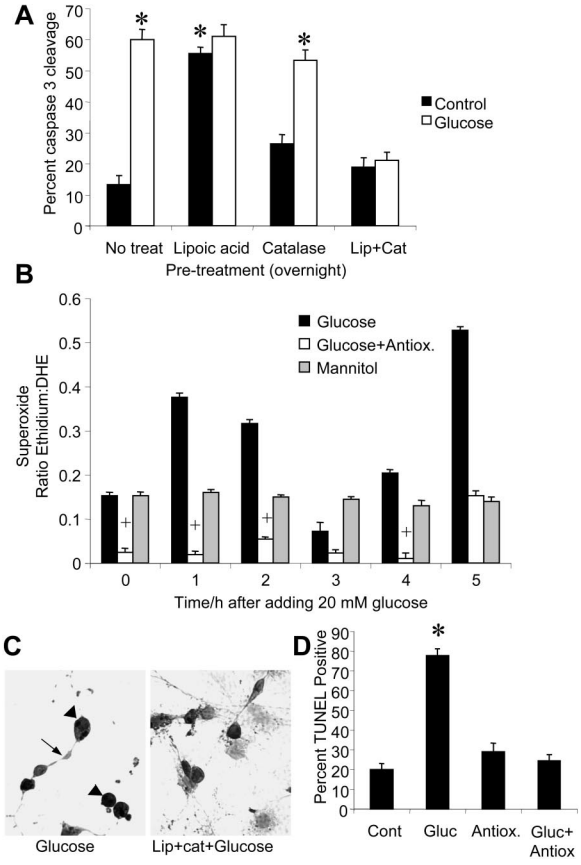

Figure 2. Exogenous antioxidants prevent glucose-induced oxidative stress and programmed cell death in cultured DRG neurons. *A)* Glucose-induced activation of caspase-3 was assessed by immunohistochemistry in the presence of α-lipoic acid (100 μM), catalase (100 U/mL), or both. *Glucose or α-lipoic acid not treated with catalase significantly increased caspase-3 activation, $P < 0.001$. *B)* Superoxide formation in intact DRG neurons was assessed by loading with DHE (3 μM). The effects of glucose (20 mM) alone, glucose plus α-lipoic acid (100 μM) and catalase (100 U/mL) (glucose+Antiox), and mannitol (20 mM) on DHE oxidation were determined for up to 5 h. $^{+}$Antioxidant treatment prevents the glucose-induced increases as well as basal levels of superoxide generation at 0, 1, 2, and 4 h. Mannitol does not alter superoxide generation in the DRG neurons. *C)* Programmed cell death evidenced by DNA fragmentation (TUNEL assay) was determined in DRG neurons 24 h after glucose treatment ± antioxidant treatment. The increase in TUNEL positivity (brown nuclei) by glucose was prevented in the presence of catalase-treated α-lipoic acid. Evidence of condensed chromatin (arrowhead) and beading of neurites (arrow) also suggest that glucose-induced cell death involves programmed cell death. *D)* Quantitation of the TUNEL staining in panel *C* demonstrates that glucose significantly increases DNA fragmentation in DRG neurons ($P<0.001$), but application of catalase-treated α-lipoic acid (Antiox) completely prevents glucose-induced programmed cell death.

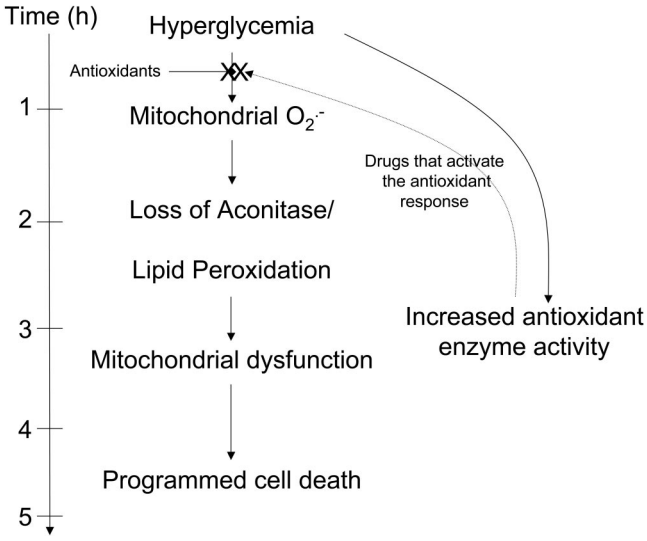

Figure 3. Model. The schematic diagram illustrates the time course of glucose-induced oxidative stress, the antioxidant response, and programmed cell death in DRG neurons. The data suggest two therapeutic targets against mitochondrial superoxide ($O_2^{\cdot-}$) and resulting oxidative stress that will protect neurons against hyperglycemic injury. Further studies are required into these therapeutic targets to develop the optimum treatment regimen for diabetic neuropathy and potentially for the other complications of diabetes.

glucose did not decrease TRAP in the whole DRG neuron cell lysate, suggesting a robust antioxidant response to hyperglycemic insult. Since our overarching hypothesis contends it is mitochondrial oxidative stress that produces DRG neuron injury, DRG neurons were then fractionated and TRAP was assessed in enriched mitochondria or the cytosol. A differential effect on TRAP was observed in the two fractions. After exposure of the intact DRG neuron cultures to 20 mM glucose, TRAP decreased in the mitochondria but increased in the cytosol after 3 h. These data strongly support our contention that rapid development of mitochondrial oxidative stress is key to glucose-induced DRG neuron injury.

5. Exogenous antioxidants prevent glucose-induced oxidative stress and programmed cell death in cultured DRG neurons

The potent antioxidant α-lipoic acid (100 μM) was used to assess the potential for antioxidants to prevent glucose-induced DRG neuron programmed cell death. A prominent finding of this work was that α-lipoic acid requires pretreatment with catalase before adding to cell cultures to remove hydrogen peroxide that is generated during solubilization (**Fig. 2*A***). Preloading the DRG neurons with catalase-treated α-lipoic acid significantly decreased basal superoxide formation and completely prevented glucose-induced superoxide formation (Fig. 2*B*). Subsequent DRG neuron programmed cell death also was prevented in the antioxidant-treated DRG neurons, assessed by TUNEL staining for DNA degradation (Fig. 2*C*).

CONCLUSIONS AND SIGNIFICANCE

These studies provide strong evidence for the role of oxidative stress, particularly in the mitochondria, in the development of diabetic neuropathy. First, only 2 h of hyperglycemia leads to subsequent neuron programmed cell death, although the peak of caspase-3 cleavage and DNA degradation occur 5–6 h subsequent to the glucose insult. In contrast, significant oxidative stress is evident within the 2 h period of hyperglycemia that leads to DRG neuron programmed cell death. This oxidative stress apparently is instrumental in producing the injury, since preloading DRG neurons with the antioxidant α-lipoic acid prevents both oxidative stress and DRG neuron death.

A second finding of this study is that the DRG neurons can mount an antioxidant response. By 3 h after a hyperglycemic insult, a transient increase in SOD and catalase activities is detected. At this time, however, DRG neurons are already committed to die. The question remains whether the response would be similarly transient if the DRG neurons were not already entering programmed cell death. Further studies will establish the nature of this antioxidant response: whether it can be activated by pharmacological agents and whether preactivation of this response will prevent DRG neuron death in the presence of a hyperglycemic insult.

The data demonstrate that it is rapid hyperglycemia-induced acute oxidative stress that leads to DRG neuron injury. We can suggest from these data why fluctuations in glucose produce greater cell injury than exposure to a consistently high level of glucose. Because the neurons possess the ability to adapt to the hyperglycemic environment, this explains why longer periods of hyperglycemia do not produce greater injury. But when glucose levels decrease, the transient response to oxidative stress is turned off. The cells revert to their original state and again are vulnerable to a rapid increase in the concentration of glucose.

In conclusion, exposure to hyperglycemia results in rapid oxidative stress in DRG neurons, which produces mitochondrial dysfunction and programmed cell death. This finding should be key to the development of treatments to prevent or reverse diabetic neuropathy as well as oral antioxidant therapy; specific targeting of the DRG neuron innate antioxidant response could provide superior protection against this disease. FJ