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Research paper

KU-596 decreases mitochondrial superoxide and improves bioenergetics following downregulation of manganese superoxide dismutase in diabetic sensory neurons

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ABSTRACT

Neuronal mitochondrial dysfunction and oxidative stress are key pathophysiologic mechanisms of diabetic peripheral neuropathy (DPN). KU-596 is a small molecule modulator of heat shock protein 90 (Hsp90) that can reverse clinically relevant measures of DPN in diabetic animal models. Mechanistically, drug efficacy requires Hsp70 and correlates with improving mitochondrial maximal respiratory capacity (MRC) and decreasing oxidative stress in diabetic sensory neurons. The goal of this study was to determine if ex vivo treatment of diabetic neurons with KU-596 improves MRC by decreasing glucose-induced oxidative stress in an Hsp70-dependent manner. Sensory neurons were isolated from non-diabetic or diabetic mice wild type (WT) or Hsp70 knockout (Hsp70 KO) mice and treated with KU-596 in the presence of low or high glucose concentrations. In diabetic WT and Hsp70 KO neurons, hyperglycemia significantly increased superoxide levels, but KU-596 only decreased superoxide in WT neurons. Similarly, KU-596 significantly improved MRC in diabetic WT neurons maintained in high glucose but did not improve MRC in diabetic Hsp70 KO neurons under the same conditions. Since manganese superoxide dismutase (MnSOD) is the main mechanism to detoxify mitochondrial superoxide radicals, the cause and effect relationship between improved respiration and decreased oxidative stress was examined after knocking down MnSOD. Downregulating MnSOD in diabetic WT neurons increased hyperglycemia-induced superoxide levels, which was still significantly decreased by KU-596. However, KU-596 did not improve MRC following MnSOD knockdown. These data suggest that the ability of KU-596 to improve MRC is not necessarily dependent on decreasing mitochondrial superoxide in a MnSOD-dependent manner.

1. Introduction

Diabetes affected about 415 million adults worldwide in 2015 and about 50–60% of these patients are likely to develop diabetic peripheral neuropathy (DPN) (Ogurtsova et al., 2017). DPN is one of the most prevalent complications of diabetes and manifests as a distal, symmetric and sensorimotor neuropathy that causes positive (sharp pain) and negative (numbness, injury insensitivity) symptoms. Insensate DPN is a main cause of foot ulceration, infection and non-traumatic amputation as the loss of feeling often leads to unnoticed tissue injury and ulceration. Unfortunately, other than glycemic control (largely limited to patients with Type 1 diabetes), there are no approved drugs to help improve nerve function and resolve insensate DPN (Pop-Busui et al., 2016).

The mechanistic pathogenesis of DPN is complex but a developing consensus is that sustained hyperglycemia drives numerous secondary biochemical changes that contribute to a progressive decline in sensory neuron function and the loss of peripheral innervation that characterizes moderate to severe cases of DPN (Feldman et al., 2017). Though accumulation of advanced glycation end products, abnormal protein kinase C activity, increased oxidative stress, mitochondrial dysfunction and increased flux of glucose through polyol and hexosamine pathways are known to contribute to the development of DPN, compounds targeting these pathways have shown little efficacy in

Abbreviations: BG, basal glucose (6.1 mM); DPN, diabetic peripheral neuropathy; DRG, dorsal root ganglia; EPR, electron paramagnetic resonance; GFP, green fluorescent protein; HG, hyperglycemia (26.1 mM); Hsp70, Heat shock protein 70; Hsp70, KO, Hsp70 knockout; Hsp90, heat shock protein 90; OCR, oxygen consumption rate; MnSOD, manganese superoxide dismutase; MRC, maximum respiratory capacity; mtBE, mitochondrial bioenergetics; WT, wild-type

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clinical trials. However, targeting a single pathway or protein considered to contribute to disease progression may not be necessary to manage complex, chronic neurodegenerative diseases. Alternatively, it may prove sufficiently beneficial to pharmacologically enhance the activity of endogenous neuroprotective pathways to help neurons more effectively tolerate recurring hyperglycemic stress, which may ameliorate pre-existing insensate symptoms and impede further development of DPN (Calcutt, 2010). In this regard, we have provided extensive proof-of-concept that pharmacologic modulation of neuroprotective heat shock proteins may provide a novel, safe and effective approach toward the therapeutic management of DPN (Dobrowsky, 2016; Ma et al., 2014; Ma et al., 2015) and possibly other neuropathies (Zhang et al., 2018a).

Heat shock proteins (Hsps), such as Hsp70 and Hsp90 function as molecular chaperones that are important for folding newly synthesized proteins and for refolding or clearing damaged/denatured proteins (Pratt et al., 2015). Hsp90 contains a C-terminal domain that is essential for forming a functional Hsp90 homodimer and an intrinsic N-terminal ATPase activity which, in conjunction with a cohort of co-chaperones, directs the folding of "client" proteins into their biologically active conformations. However, Hsp90 also serves another important biologic role since it is a direct regulator of the cellular heat shock response. Hsp90 binds to heat shock factor 1 and suppresses its transactivating capacity (Vihervaara and Sistonen, 2014). Upon exposure to proteotoxic stress or binding of small molecules, conformational changes in Hsp90 disrupt its interaction with HSF1 and lead to the transcriptional induction of cytoprotective proteins, such as Hsp70 (Vihervaara and Sistonen, 2014).

Though numerous small molecules that function as N-terminal Hsp90 inhibitors have been developed to treat cancer (Woodford et al., 2016), small molecules that interact with the C-terminal of Hsp90 show strong promise as therapeutic candidates to treat neurodegeneration (Dobrowsky, 2016). Novobiocin was identified as the first ligand to interact with the C-terminal of Hsp90 (Marcu et al., 2000) and the development of diverse libraries of novobiocin derivatives identified KU-596 (ChemSpider ID 28665905) as a noviosylated fluoro-biphenyl ethylacetamide "novologue" (Kusuma et al., 2012) that demonstrates considerable efficacy in improving insensate DPN (Ma et al., 2015). Treating diabetic mice with KU-596 improved sensory hypoalgesia and this required Hsp70 since the drug could not improve sensory nerve function in diabetic Hsp70 knockout (KO) mice.

Mitochondrial dysfunction is a major pathogenic contributor to DPN and reduced respiratory chain activity is observed in diabetic sensory neurons (Chowdhury et al., 2013). An earlier generation of novobiocin derivatives increased mitochondrial bioenergetics (mtBE), decreased superoxide levels and increased the expression of Mn superoxide dismutase (MnSOD) in hyperglycemically stressed embryonic sensory neurons (Zhang et al., 2012). These data raised the possibility that novologues may require MnSOD to improve mtBE. Treating diabetic mice with KU-596 improved neuronal function, which correlated with enhanced mtBE and decreased markers of oxidative stress in diabetic dorsal root ganglia (DRG) (Ma et al., 2015). However, it was unclear if this may be a direct or indirect effect of the drug on neuronal Hsp70. Therefore, sensory neurons were isolated from diabetic WT and Hsp70 KO mice who had developed insensate DPN and treated ex vivo with KU-596 to determine if the drug could directly improve neuronal mtBE and oxidative stress. By manipulating glucose conditions and downregulating the expression of MnSOD, the current study provides evidence that KU-596 can directly decrease neuronal superoxide levels even after downregulating MnSOD in an Hsp70-dependent manner. However, downregulation of MnSOD increased mtBE and this was not enhanced by KU-596. These data support that Hsp70 is necessary for KU-596 to decrease superoxide levels and improve mtBE but that the improvement in mtBE is not necessarily dependent on MnSOD to decrease superoxide levels.

2. Materials and methods

2.1. Animals

C57Bl/6NHsd wild type (WT) mice were obtained from Harlan Laboratories and the Hsp70.1/70.3 double knockout mice on a C57Bl/6N background (Hsp70 KO) mice were obtained from the Mutant Mouse Resource and Research Center. Experimental animals were generated by in-house breeding and were maintained on a 12-hour light/dark cycle with ad libitum access to water and 7022 NIH-07 rodent chow (5.2% fat). All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee and in compliance with standards and regulations for the care and use of laboratory rodents set by the National Institutes of Health.

2.2. Induction of diabetes

Male and female WT and Hsp70 KO mice were randomly assigned to either the non-diabetic or diabetic group. Mice were rendered diabetic with two intraperitoneal injections of 100 mg/kg streptozotocin (STZ) (Sigma-Aldrich, St louis, MO, #572201) freshly prepared in 50 mM sodium citrate, pH 4.5 given over two consecutive days. Control mice were treated similarly but received only the vehicle. One week after the second injection, mice were fasted for 6 h and those with a fasting blood glucose ≥290 mg/dl (16 mM) were deemed diabetic (Ma et al., 2014).

2.3. Mechanical and thermal sensitivity assessments

To monitor the development of diabetic neuropathy, the onset of a mechanical and thermal hypoalgesia was determined after the induction of diabetes. Mechanical sensitivity was assessed using a Dynamic Plantar Aesthesiometer (Stoelting Inc.) fitted with a stiff monofilament that was applied to the plantar surface at an upward force of 8 g. Thermal sensitivity was assessed by paw withdrawal latency to a ramping, focal heat using a Hargreaves Analgesiometer (Stoelting Inc.). Four responses from each animal were measured two times on each foot with 5 min between the measures. The mean of all responses from both feet was taken as the weekly measure as we have previously reported (Ma et al., 2014, 2015).

2.4. Synthesis of KU-596

KU-596, N-(2-(5-(((3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-3′-fluoro-[1,1′-biphenyl]-2-yl) ethyl)-acetamide, was synthesized and structural purity (> 95%) verified as described previously (Kusuma et al., 2012).

2.5. Adult DRG sensory neuron isolation

After 12–14 weeks of diabetes, adult sensory neurons were isolated from the non-diabetic and diabetic WT or Hsp70 KO mice as previously described (Ma et al., 2014). Mice were euthanized via gradual CO_2 asphyxiation, the spinal column dissected and the L4–L6 DRG collected from 6 to 8 mice in each group. Connective tissue was removed, and the ganglia were placed in 1 ml serum-free Ham's F10 medium (Corning, #10-070-CV). The trimmed ganglia were dissociated by incubation in 1 ml of 1.25% collagenase for 45 min at 37 °C with subsequent addition of 1 ml of 2% trypsin for 30 min at 37 °C. Digested cells were centrifuged at 1000 ×*g* for 5 min at 4 °C and the pellet was further dissociated by trituration with a fire polished glass pipette in F10 medium. The cell suspension was overlain onto a 10 ml gradient of sterile isoosmotic Percoll (0.9 ml of 10× PBS, 6.5 ml H_2O, 2.6 ml Percoll) and centrifuged at 800 ×*g* for 20 min at 4 °C to remove cell debris and myelin fragments. The cell pellet was re-suspended in fresh F10 medium with N2 supplement (Invitrogen, Carlsbad CA). Isolated

neurons were plated at a density of 5×10^3 cells/well in F10 medium with N2 supplement on 96 well plates that had been coated overnight with 0.1 mg/ml poly-DL-ornithine (Sigma, #P8638) and 2 μg/ml laminin (3 h) (Corning, #354232) (Ma et al. 2014). After plating, the adult sensory neurons were treated with 1 μM KU-596 or 0.01% DMSO overnight (~16 h) and subsequently incubated in F10 medium containing basal glucose (6.1 mM) or 26.1 mM glucose for an additional 24 h. The cells were then used to assess mtBE or superoxide production.

2.6. Mitochondrial bioenergetics assessment and MitoSOX staining

Sensory neuron mtBE were analyzed using a Seahorse XF96 Analyzer (Seahorse Biosciences, Billerica, MA). After the last incubation in basal or hyperglycemic medium, the cells were placed in bicarbonate free DMEM containing 5.5 mM glucose and 1 mM pyruvate and incubated for 1 h at 37 °C. The plate was then introduced into the Seahorse Analyzer using a 3 min mix cycle to oxygenate the medium followed by a 4 min measurement of the oxygen consumption rate (OCR). The initial four OCRs provide a measure of basal respiration prior to assessing mitochondrial dysfunction using various respiratory chain inhibitors. The portion of basal OCR that is coupled to ATP synthesis was estimated by the decrease in OCR followed by the addition of 1 μg/ml oligomycin, an ATP synthase inhibitor (Sigma #75351). Residual OCR remaining after oligomycin treatment is from uncoupled respiration (proton leak). Next, maximal respiratory capacity (MRC) and spare respiratory capacity (SRC) were assessed following dissipation of the proton gradient across the inner mitochondrial membrane with 1 μM of the protonophore FCCP (Sigma #C2920). Non-mitochondrial respiration was then assessed by co-injection of 1 μM rotenone + 1 μM antimycin A (Sigma #R8875, #A8674) (Ma et al., 2014, 2015). Oxygen consumption rates were visualized using Wave 2.6.0 and the measures were exported into the Seahorse XF Cell Mito Stress Test Report Generator software to calculate bioenergetic parameters after subtracting background and non-mitochondrial respiratory rates and normalizing to total protein per well (Brand and Nicholls, 2011).

Superoxide levels were measured in black 96-well plates using MitoSOX Red. After the last incubation in basal or hyperglycemic medium, MitoTracker Deep Red (300 nM) and MitoSOX Red (400 nM) were added to each well and the cells incubated for 15 min. After washing, only the cell bodies were imaged using an Olympus 3I Spinning Disk confocal microscope at excitation/emission wavelengths of 488/573 nm (MitoSox Red) and 644/665 nm (MitoTracker Deep Red). Fluorescence density of the red and deep red signals of 35–50 cell bodies per treatment was quantified using Image J analysis software (Zhang et al., 2012).

2.7. Knockdown of MnSOD expression by shRNA and superoxide assessment by electron paramagnetic resonance (EPR) spectroscopy animals

Recombinant adenoviruses expressing shRNA targeting mouse MnSOD (Ad-GFP-U6-mSOD2-shRNA, #shADV-272,843) or scrambled shRNA with GFP (Ad-GFP-U6-scrambled-shRNA, #1122) were purchased from Vector BioLabs (Malvern, PA). To evaluate the silencing efficiency, sensory neurons were infected immediately after plating with either the scrambled shRNA or the mouse MnSOD shRNA for 48 h. Cell lysates were prepared and protein expression was evaluated by immunoblotting with a MnSOD polyclonal antibody (Millipore-Sigma, #06-984).

Immediately after plating, the isolated sensory neurons were treated with vehicle or 1 μM KU-596 and infected with the scrambled shRNA or MnSOD shRNA adenoviruses (1:50) for 36 h. The cells were then incubated in basal glucose or 26.1 mM glucose for an additional 24 h. During the final 4 h, the cells were incubated with 100 μM mitoTEMPO-H (1-hydroxy-4-[2-triphenylphosphonio-acetamido]-2,2,6,6-tetramethylpiperidine, Sigma #SML0737). MitoTEMPO-H is a mitochondria-targeted antioxidant and is a specific spin trap for detecting mitochondrial superoxide. The cells from 8 wells were pooled together, pelleted by brief centrifugation, re-suspended in 0.2 ml of fresh F-10 medium and frozen at −80 °C until use. When performing the EPR measurements, cells were pipetted into a quartz EPR tube which was inserted into a Bruker EMX plus EPR spectrometer (Billerica, MA, USA). Settings for the spectrometer using Bruker Xenon software were: center field: 3480 G; Sweep width: 100 G; modulation amplitude: 1 G; and time constant, 0.01 s (Bakalova et al., 2015). The cells were recovered from the cuvette and total protein determined using the microBCA assay with bovine serum albumin as the standard. Relative changes in the superoxide signal were quantified by integration of the first EPR peak and normalized to total protein content.

2.8. Statistical analysis

One-way ANOVA, and multivariate-ANOVA were conducted for between-group comparison using Minitab Software or SAS. Post-hoc analysis was applied to determine differences between treatments using Tukey's test and Wilks' lambda test. All data are presented as mean ± SEM.

3. Results

3.1. KU-596 improves mtBE and oxidative stress in WT sensory neurons under normal and hyperglycemic conditions

WT and Hsp70 KO mice were rendered diabetic and monitored for the development of DPN by assessing the onset of a mechanical and thermal hypoalgesia. WT diabetic mice developed a significant

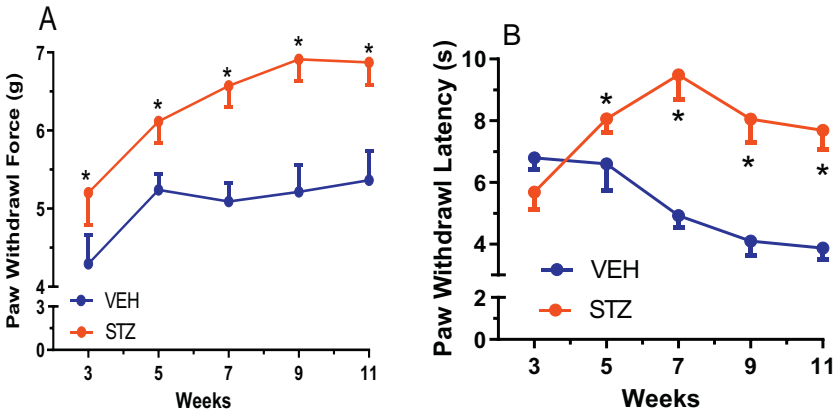

Fig. 1. Development of Mechanical (A) and Thermal (B) Hypoalgesia in Diabetic WT Mice. Mice were rendered diabetic with two injections of STZ or vehicle (Veh) and mechanical and thermal sensitivity was assessed as described in Materials and Methods. Results are mean ± SEM from 20 vehicle treated and 28 STZ treated mice. *, $p < .05$ versus time-matched non-diabetic mice.

mechanical and thermal hypoalgesia demonstrating the onset of an insensate DPN (Fig. 1A and B). Similar results were observed for the diabetic Hsp70 KO mice as previously reported (Ma et al., 2015). Although the loss of mechanical sensitivity is consistent with our prior assessments of murine DPN, some studies report the development of a mechanical allodynia in diabetic mice and this may derive from possible differences in measuring mechanical sensitivity (Biessels et al., 2014).

After 12–14 weeks, adult sensory neurons were isolated from L4 to L6 DRG of non-diabetic and diabetic WT or Hsp70 KO mice since these ganglia provide the sensory axons of the peripheral nerves that are affected in DPN (Ma et al., 2014). This time frame was chosen as our previous work found that this duration of diabetes was sufficient to promote mitochondrial dysfunction in sensory neurons (Ma et al., 2014, 2015). The isolated neurons were maintained in F-10 medium which has a basal glucose (BG) concentration of 6.1 mM. After isolation, the neurons were treated with vehicle (0.01% DMSO final concentration) or 1 μM KU-596 overnight (~16 h). The cells were then maintained for an additional 24 h in medium with BG or subjected to hyperglycemia (HG) by incubation in F-10 medium containing 26.1 mM glucose. The cells were then prepared for analysis of mtBE or superoxide production as described in Methods.

One advantage of this ex vivo cell culture model is that neurons from diabetic animals have developed metabolic changes due to chronic in vivo diabetes that cannot be adequately recapitulated using adult neurons that are only exposed to hyperglycemia and KU-596 in vitro (Akude et al., 2011). Indeed, transient hyperglycemic stress of neurons isolated from non-diabetic mice did not decrease maximum respiratory capacity (MRC) (Figs. 2A; red versus black circle and 2C; red versus black bar). MRC reflects the rate of maximal electron transport and substrate oxidation achievable in the absence of limits imposed by the inner mitochondrial membrane proton gradient. As shown previously (Zherebitskaya et al., 2009), this ex vivo culture model may more accurately reflect the in vivo response of the neurons to further episodes of glucotoxic stress.

Neurons isolated from the diabetic mice and maintained in BG had a significantly impaired MRC compared to neurons obtained from the non-diabetic mice (Figs. 2B; black circle versus black square and 2C; black stripe versus black bar). Diabetic neurons maintained in high glucose also showed a significantly impaired MRC compared to neurons obtained from the non-diabetic mice (Fig. 2C; red stripe versus black bar) but not to diabetic neurons cultured in basal glucose (Fig. 2C; red stripe versus black stripe bar). The addition of 1 μM KU-596 significantly improved OCR and the MRC of non-diabetic and diabetic neurons regardless of the glucose concentration. Similar results were seen when measuring spare respiratory capacity (SRC) and ATP production (Supplementary Figs. 1A and 1B). These data indicate that KU-596 can directly improve mtBE in the cultured diabetic neurons.

To determine the effect of diabetes and ex vivo treatment of KU-596 on mitochondrial superoxide levels, the sensory neurons were stained with MitoSOX Red and mitoTracker Deep Red (blue signal) to localize the signal to mitochondria. The intensity of the superoxide signal was normalized with the signal from mitoTracker Deep Red to rule out absorption differences between different cells.

As anticipated, superoxide staining was low in non-diabetic neurons that were maintained in BG (Fig. 3A and C). The addition of HG significantly increased superoxide levels in non-diabetic neurons (Fig. 3A and C; red versus black bar) but this did not correlate with any changes in mtBE (Fig. 2C). These data suggest that this transient mitochondrial oxidative stress was not sufficient to impair MRC.

Superoxide staining was significantly higher in diabetic neurons maintained in BG (Fig. 3B and C; black stripe versus black bar) and this correlated with the decline in MRC (Fig. 2C). Relative to non-diabetic neurons in BG, superoxide staining also remained significantly increased in diabetic neurons maintained in HG (Fig. 3C; red stripe versus black bar), but this was not associated with any further impairment in MRC (Fig. 2C). Thus, pre-existing damage induced by 12–14 weeks of diabetes may contribute to the lower MRC seen in diabetic neurons. Interestingly, treating the non-diabetic (Fig. 3A and C; purple versus red bar) or diabetic neurons (Fig. 3B and C; purple stripe versus red stripe) with KU-596 blocked the increase in superoxide levels and this correlated with an increase in MRC (Fig. 2C). Together, these data support that KU-596 can directly improve neuronal mtBE and decrease glucose-induced oxidative stress.

3.2. Hsp70 is necessary for KU-596 to improve mtBE and oxidative stress in hyperglycemically stressed sensory neurons

Mechanistically, previous data supported that Hsp70 was necessary for KU-596 to improve DPN when administered in vivo (Ma et al., 2015). However, whether Hsp70 may directly or indirectly contribute to improved neuronal function was unclear. To determine if neuronal Hsp70 was needed to improve mtBE and decrease oxidative stress, the above studies were repeated using sensory neurons isolated from non-diabetic or diabetic Hsp70 KO mice.

In contrast to the response in WT mice, diabetes suppressed but did not significantly decrease MRC versus the non-diabetic Hsp70 KO mice (Fig. 4A and C, black stripe versus black bar). As expected, KU-596 treatment could not improve MRC in either non-diabetic (Fig. 4A and C; purple versus red bar) or diabetic neurons (Fig. 4B and C; purple stripe versus red stripe) subjected to HG treatment. Rather unexpectedly, KU-596 treatment improved MRC in non-diabetic and diabetic Hsp70 KO neurons that were incubated only in BG; similar results were seen when measuring SRC and ATP production (Supplementary Figs. 1C and 1D). These data suggest that KU-596 may have some Hsp70-independent effects to improve mtBE under certain cellular conditions, but this response is lost in the diabetic neurons incubated in HG. Thus, hyperglycemia is shifting efficacy to improve mtBE to become more Hsp70-dependent, possibly due to the role of Hsp70 in regulating mitophagy (Drew et al., 2014).

When examining superoxide, it was notable that the MitoSox Red signal was slightly higher in the non-diabetic Hsp70 KO neurons relative to WT cells (Fig. 5A versus Fig. 3A). The activities of SOD1 and SOD2 have been reported to be decreased in Hsp70 KO mice and this might account for the higher basal superoxide levels in the non-diabetic Hsp70 KO sensory neurons (Afolayan et al., 2014; Choi et al., 2005). This is also consistent with findings in muscle of Hsp70 KO mice (Drew et al., 2014). However, neither incubation in HG nor KU-596 caused any significant change in superoxide levels in neurons from non-diabetic Hsp70 KO mice (Fig. 5A and C). Neurons from diabetic Hsp70 KO mice maintained in BG showed a small increase in superoxide compared to their non-diabetic counterparts (Fig. 5B and C, black stripe versus black bar), but it did not recapitulate the magnitude observed in WT mice (Fig. 3C). Exposing the diabetic Hsp70 KO neurons to HG significantly increased superoxide levels but KU-596 was unable to decrease this response, which correlated with the lack of improvement in MRC. The lack of efficacy in decreasing superoxide and improving MRC in the diabetic Hsp70 KO neurons supports that neuronal Hsp70 is a key component in the mechanism of action of KU-596.

3.3. KU-596 decreases mitochondrial oxidative stress and improves mtBE following MnSOD knockdown

To determine if KU-596 improved mtBE by reducing superoxide production, WT neurons were treated with recombinant adenoviruses expressing shRNA against murine MnSOD or a scrambled shRNA. Infection of adult neurons with the MnSOD shRNA adenovirus decreased the expression of MnSOD by about 60% (Fig. 6A). To determine the effect of decreasing MnSOD expression on the production of superoxide radicals, we employed electron paramagnetic resonance (EPR) spectroscopy using mito-TEMPO-H, a mitochondria selective spin trap. This approach was necessary since the MnSOD shRNA virus co-

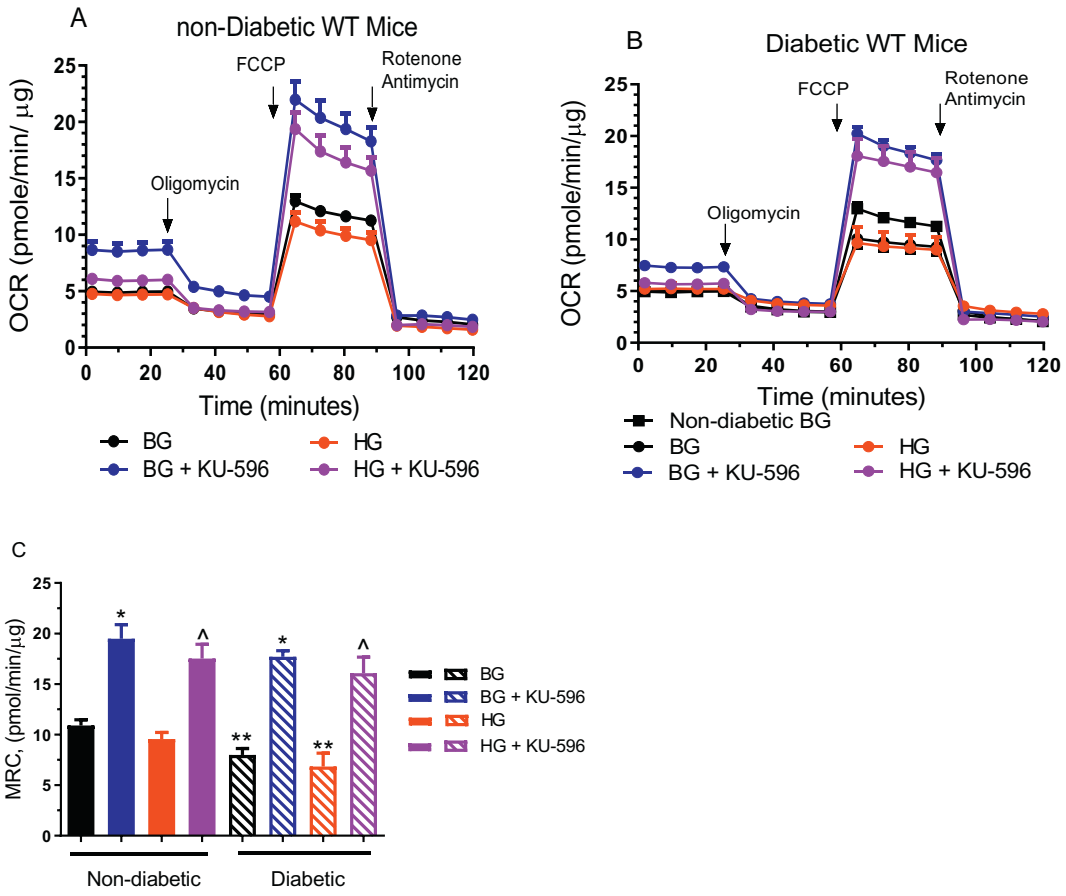

Fig. 2. KU-596 improves mtBE in hyperglycemically stressed WT diabetic neurons. Adult sensory neurons were isolated from non-diabetic or diabetic WT mice and treated +/− 1 µM KU-596 overnight in medium containing 6.1 mM basal glucose (BG). Hyperglycemia (HG) was then induced in some cultures for 24 h by the addition of 20 mM excess glucose. **A)** KU-596 improves OCR in WT non-diabetic sensory neurons under BG and HG conditions. **B)** Diabetes decreased sensory neuron OCR compared to non-diabetic mice and this deficit was improved by KU-596. Oligomycin, FCCP and rotenone/antimycin A were added immediately after completion of the 4th, 8th and 12th OCR measures (arrows), respectively. **C)** Quantification of MRC. *, $p < .05$ vs BG; ^, $p < .05$ vs HG; ** $p < .05$ vs non-diabetic BG. Results are from 4 to 5 wells of neurons per group that were obtained from 8 non-diabetic or diabetic mice.

expressed EGFP for visualizing the efficiency of viral infection. When using MitoSox Red to detect superoxide, this resulted in an overlap between the green and red fluorescence signals.

Neurons from non-diabetic and diabetic WT mice were prepared as described above and maintained in BG medium for the knockdown experiments. In non-diabetic sensory neurons, knockdown of MnSOD increased the mitochondrial superoxide signal about two-fold (Figs. 6B, red versus black line and 6D; red versus black bar) and KU-596 was unable to significantly lower this signal. Consistent with the MitoSox data, diabetic neurons treated with the scrambled shRNA showed an increased superoxide signal compared to that present in similarly treated non-diabetic neurons (Figs. 6C pink versus black line and 6D, black stripe versus black bar). Downregulating MnSOD expression in the diabetic neurons further increased the superoxide signal about 1.5-fold (Fig. 6C, red versus black line; 6D, red versus black stripe bar). Surprisingly, KU-596 significantly decreased the magnitude of the superoxide signal in diabetic cells infected with the scrambled (Fig. 6D, blue stripe versus black stripe bar) as well as the MnSOD shRNA (Fig. 6D, purple stripe versus red stripe bar). These data suggest that MnSOD may not be necessary for the drug to decrease mitochondrial superoxide in diabetic WT neurons, though incomplete knockdown may leave a sufficient reservoir of enzyme to account for this effect. As anticipated, KU-596 had no effect on the superoxide signal in Hsp70 KO neurons treated with scrambled or MnSOD shRNA (data not shown).

Next, the effect of MnSOD downregulation and KU-596 treatment on mtBE in neurons from non-diabetic and diabetic mice was assessed. In non-diabetic neurons treated with scrambled shRNA, KU-596 did not improve mitochondrial respiration and downregulating MnSOD in the absence or presence of KU-596 had little effect on mtBE (data not shown). As expected, diabetic sensory neurons treated with scrambled shRNA showed a markedly diminished MRC compared to similarly treated non-diabetic neurons (Fig. 7A, black stripe versus black bar), and this deficit was significantly improved by KU-596 (Fig. 7A, blue versus black stripe bar). Curiously, MRC was significantly increased following MnSOD downregulation in diabetic neurons and treating these neurons with KU-596 did not affect the MRC (Fig. 7A, red and purple stripe bars). Since KU-596 significantly decreased the superoxide signal in diabetic neurons treated with MnSOD shRNA (Fig. 6D), these data suggest that the ability of KU-596 to improve MRC (Fig. 2C) is not necessarily dependent on decreasing mitochondrial superoxide in a MnSOD-dependent manner.

To determine if the increase in MRC following downregulation of MnSOD in diabetic neurons was mainly driven by the increase in superoxide, the cells were treated with mito-TEMPO-H to scavenge superoxide. Knockdown of MnSOD in diabetic neurons significantly increased MRC once again and this was associated with a small increase in proton leak (Supplementary Fig. 2A). Treatment with mito-TEMPO-H significantly decreased MRC (Fig. 7B, red cross stripe versus red angle stripe bar) while increasing the magnitude of proton leak and decreasing coupling efficiency (Supplementary Figs. 2A and 2B). This data suggests that the increase in superoxide levels following knockdown of MnSOD is associated with an elevation in MRC. However, KU-596 was

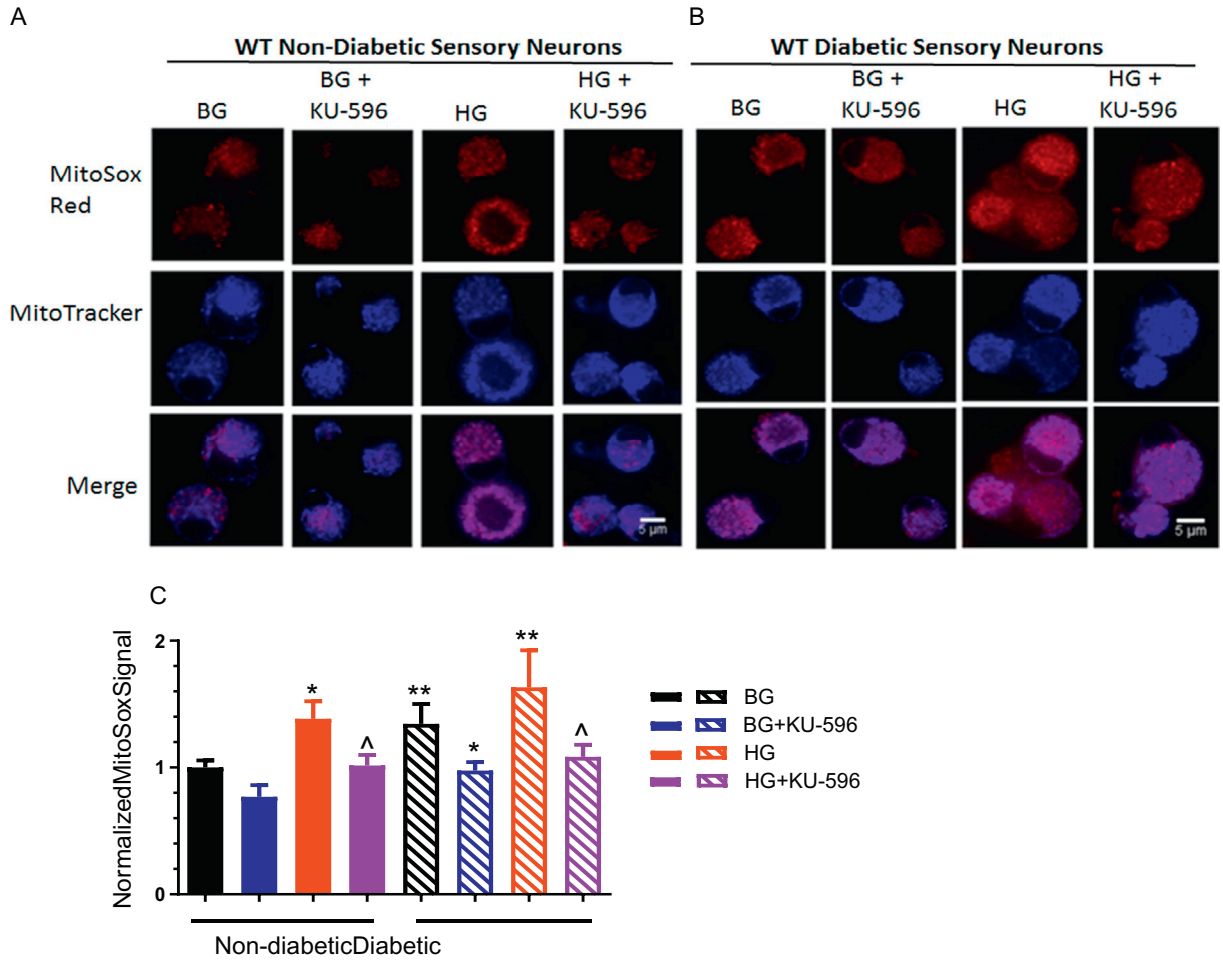

Fig. 3. KU-596 decreases mitochondrial superoxide levels in hyperglycemically stressed WT neurons. Cells were treated as described in Fig. 2 and mitochondrial superoxide assessed by confocal microscopy after staining the cells with MitoSox Red and MitoTracker Deep Red. **A)** Oxidative stress was increased in non-diabetic sensory neurons subjected to HG and this was decreased by KU-596 treatment. **B)** Diabetes increased the level of superoxide production regardless of glucose concentration and this was significantly decreased by KU-596. **C)** Quantification of MitoSOX staining intensity normalized to MitoTracker Deep Red staining intensity. *, $p < .05$ vs BG; ^, $p < .05$ vs HG; **, $p < .05$ vs non-diabetic BG. Results are an average of at least 36 imaged neurons per treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unable to further improve MRC or affect proton leak following MnSOD knockdown in either the presence or absence of mito-TEMPO-H.

4. Discussion

A critical need exists for developing novel therapies to treat insensate DPN by interfering with disease relevant mechanisms of action. Our prior work has provided proof-of-principle for the in vivo efficacy of modulating Hsp90 activity with KU-596 as a potential translational approach toward treating insensate DPN. Although in vivo treatment with KU-596 improved sensory neuron mtBE and decreased transcriptomic markers of oxidative stress in an Hsp70-dependent manner, the drug also decreased the activation of inflammatory pathways in diabetic mice (Ma et al., 2014). Thus, it was unclear if KU-596 is capable of directly improving mtBE and oxidative stress in diabetic sensory neurons. The ex vivo assessment of drug efficacy in neurons isolated from diabetic animals allowed us to address this issue. Although we cannot rule out potential contributions from contaminating cells in the neuronal preparations, our data support that KU-596 can directly improve mtBE and decrease superoxide levels in diabetic sensory neurons in an Hsp70-dependent manner.

Interestingly, KU-596 treatment improved mtBE in non-diabetic and diabetic Hsp70 KO sensory neurons, but only when the cells were incubated in medium containing a basal glucose concentration. When subjected to hyperglycemia, KU-596 could not improve MRC in either non-diabetic or diabetic Hsp70 KO neurons. This suggests KU-596 may have some Hsp70-independent effects but that hyperglycemia is shifting efficacy to improve mtBE to become more Hsp70-dependent. The reason for this distinction is unclear but Chiosis and colleagues have provided an elegant demonstration that stress-induced changes in Hsp90 chaperone complexes can affect the sensitivity of various tumors to N-terminal Hsp90 inhibitors (Rodina et al., 2016). Since the interaction of small molecules with Hsp90 can be altered by its association with various client proteins and co-chaperones (Joshi et al., 2018), diabetic stress may promote the formation of Hsp90 protein complexes whose binding by KU-596 leads to an Hsp70-dependent mode of action. For example, expression of the Myc oncoprotein can promote the formation of an Hsp90 protein network that imparts sensitivity to select N-terminal inhibitors (Rodina et al., 2016). Moreover, using saturation transfer distance NMR spectroscopy to analyze the interaction of KU-596 with Hsp90 has identified that it functions as an allosteric modulator that can elicit global conformational changes, which can extend to the N-terminal region in the Hsp90 dimer and potentially affect co-chaperone interactions (Kumar Mv et al., 2018). However, additional work is needed to determine if diabetes can similarly induce changes in protein partners that interact with Hsp90 and affect the pharmacology of C-terminal Hsp90 inhibitors such as KU-596.

MitoSox red staining and EPR spectroscopy showed that diabetes

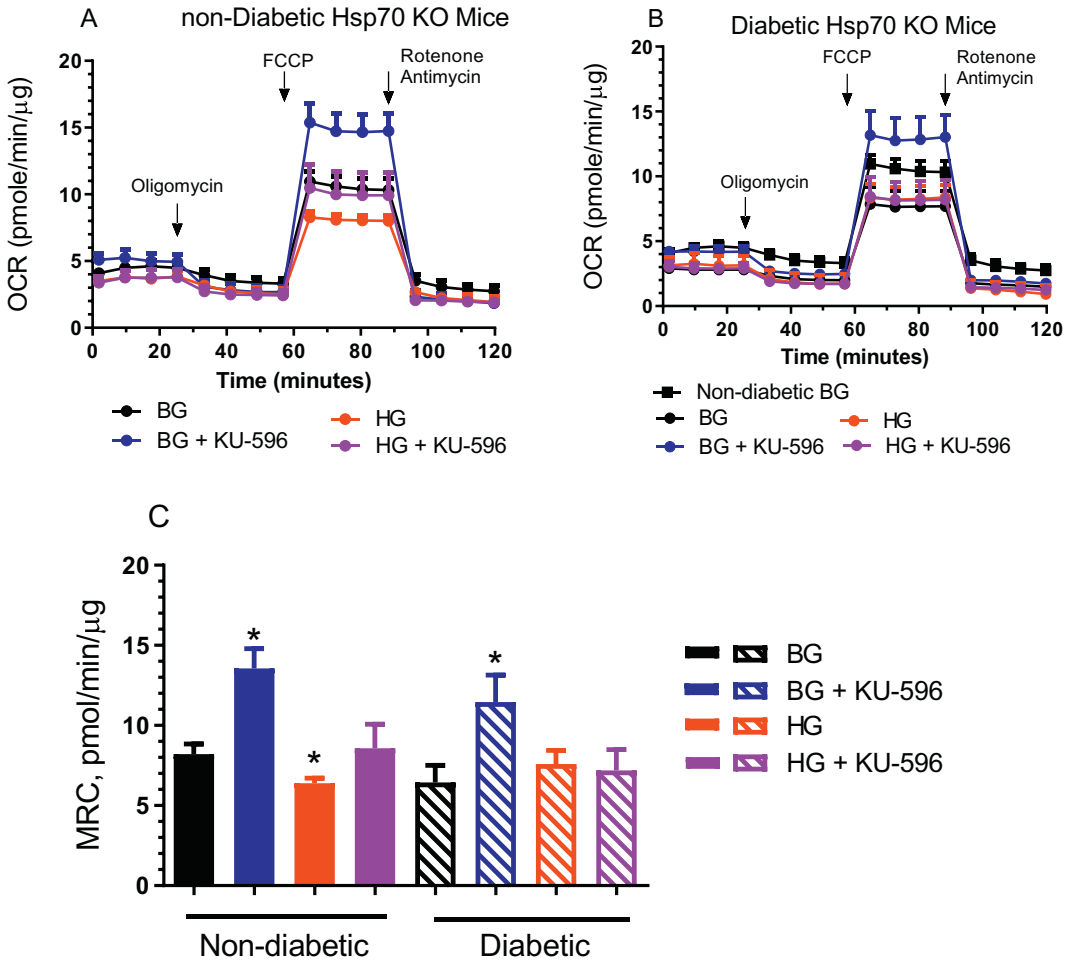

Fig. 4. KU-596 does not improve mBE in hyperglycemically stressed Hsp70 KO neurons. Adult sensory neurons were isolated from non-diabetic or diabetic Hsp70 KO mice and treated ± 1 μM KU-596 overnight in medium containing 6.1 mM basal glucose (BG). Hyperglycemia (HG) was then induced in some cultures for 24 by the addition of 20 mM excess glucose. KU-596 improved OCR in non-diabetic **(A)** and diabetic **(B)** sensory neurons under BG condition but not after the cells were subjected to HG stress. Oligomycin, FCCP and rotenone/antimycin A were added immediately after completion of the 4th, 8th and 12th rate measures (arrows), respectively. **C)** Quantification of MRC. *, $p < .05$ vs BG. Results are from 4 to 5 wells of neurons per group that were obtained from 6 non-diabetic or diabetic mice.

increased superoxide levels in WT diabetic neurons incubated in either basal or high glucose medium and this increase was significantly attenuated by KU-596 treatment. Although the source of the increased superoxide in diabetic neurons is unclear, it may arise from aberrant activity of the respiratory complexes and/or via the interaction of NAPH oxidase 4 with mitochondria, which can increase mitochondrial oxidative stress in some cell types (Mori et al., 2018). Although NADPH oxidase 4 has been implicated in contributing to oxidative stress in DPN, this has been limited to Schwann cells (Zhang et al., 2018b) and its contribution to mitochondrial oxidative stress in diabetic sensory neurons needs further investigation.

MnSOD is the primary mechanism for removing mitochondrial superoxide radicals and knockdown of MnSOD by about 60% increased mitochondrial superoxide levels in both non-diabetic and diabetic neurons. However, downregulation of MnSOD did not prevent KU-596 from significantly attenuating superoxide levels in diabetic neurons. Although we cannot rule out that the downregulation of MnSOD was insufficient to prevent this effect, the magnitude of the decrease approximated that observed in WT neurons (Fig. 3C). Thus, these data suggest that the drug-induced decrease in mitochondrial superoxide levels is not necessarily dependent on MnSOD. Curiously, downregulation of MnSOD was associated with an increase in mitochondrial respiration in diabetic sensory neurons. The increase in superoxide levels following MnSOD downregulation seemed responsible for the enhanced respiration in the diabetic sensory neurons since scavenging mitochondrial superoxide with mitoTEMPO-H attenuated the rate of respiration. Downregulation of MnSOD has been reported to increase mtBE in normal kidney cells and this was attributed to an increase in mitochondrial biogenesis (Marine et al., 2014). However, we did not determine if mitochondrial biogenesis was increased in the diabetic sensory neurons. Since KU-596 decreased superoxide levels after MnSOD knockdown, these data suggest that the ability of KU-596 to improve MRC is not necessarily dependent on decreasing mitochondrial superoxide.

Increased mitochondrial superoxide can promote the clearance of damaged mitochondria via a lysosome-mediated autophagy pathway termed mitophagy (Lemasters, 2005). For example, MnSOD overexpression decreased superoxide levels and autophagy while MnSOD knockdown increased mitochondrial superoxide levels and autophagy in HeLa cells (Chen et al., 2009). Though crosstalk between mitophagy, redox signaling and mitochondrial dysfunction is still not well understood, recent evidence suggests that mitochondrial superoxide production and membrane depolarization help execute mitophagy downstream of parkin recruitment (Xiao et al., 2017). Mitophagy is controlled by a variety of proteins including PTEN-induced putative kinase 1 (PINK1) and parkin. PINK1 is a mitochondria-targeted serine–threonine kinase that regulates mitochondrial homeostasis. Initially, PINK1 is stabilized by mitochondrial depolarization, and direct

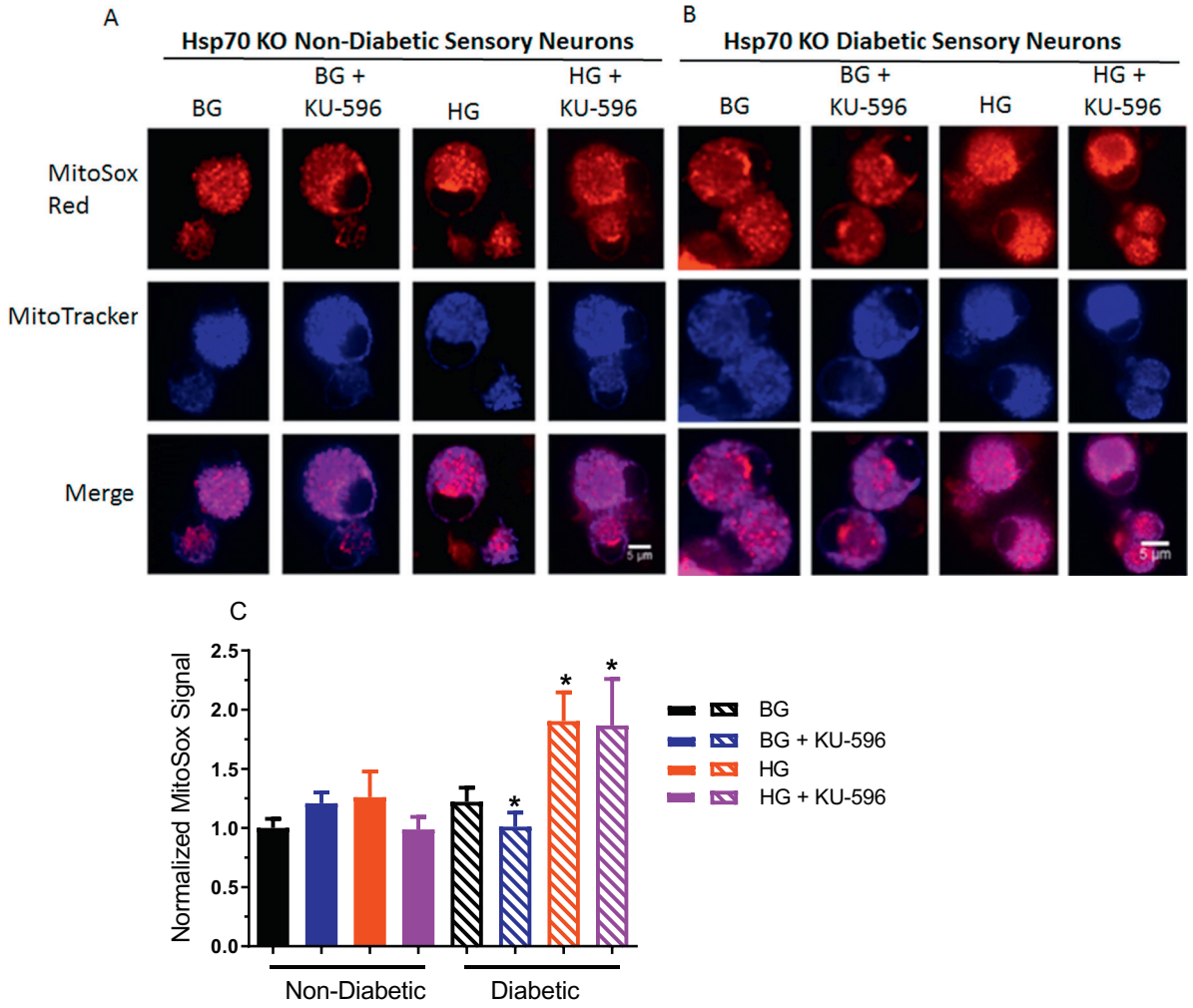

Fig. 5. KU-596 does not decreases mitochondrial superoxide levels in hyperglycemically stressed Hsp70 KO neurons. Cells were treated as described in Fig. 4 and mitochondrial superoxide assessed by confocal microscopy after staining the cells with MitoSox red and MitoTracker Deep Red. **A)** Oxidative stress was not increased in non-diabetic Hsp70 KO sensory neurons subjected to HG stress. **B)** HG stress increased superoxide production in diabetic Hsp70 KO neurons and KU-596 treatment was unable to attenuate this response. **C)** Quantification of MitoSOX staining intensity normalized to MitoTracker Deep Red staining intensity. *, $p < .05$ vs BG. Results are an average of at least 39 imaged neurons per treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

or indirect PINK1 activation leads to the phosphorylation of parkin and its recruitment into mitochondria (Kim et al., 2008; Wang et al., 2012). Interestingly, Hsp70 is necessary for the recruitment of parkin to mitochondria to facilitate mitochondrial clearance of damaged proteins (Drew et al., 2014). Prior results have shown that Hsp70 rapidly translocates to mitochondria following depolarization and is essential for the recruitment of parkin (Drew et al., 2014). It is well documented that mitochondria from diabetic sensory neurons undergo significant depolarization (Akude et al., 2011; Fernyhough, 2015; Zherebitskaya et al., 2009). We hypothesize that the Hsp70-dependence of KU-596 in diabetic neurons subjected to hyperglycemic stress may be due to an enhanced depolarization of the damaged mitochondria by oxidative damage and the recruitment of Hsp70 to the organelle to facilitate a parkin-mediated mitophagy. Enhanced clearance of the damaged mitochondria may indirectly contribute to the changes in superoxide levels and mtBE observed with KU-596 treatment.

In summary, the current experiments support that KU-596 can directly improve oxidative stress and mtBE in diabetic sensory neurons in an Hsp70-dependent manner. However, MnSOD does not seem critical toward these actions of the drug. Mitophagy is emerging as an important contributor to the development of diabetic nephropathy (Higgins and Coughlan, 2014; Huang et al., 2016), but its role in affecting sensory neuron function in DPN is not well defined. Mito QC mice express a fluorescence-based mitophagy reporter protein that enables the subcellular visualization and quantification of mitophagy in tissues using confocal microscopy (McWilliams et al., 2016). Crossing these mice onto various knockout backgrounds (McWilliams et al., 2018) provides a powerful approach to address mechanisms by which mitophagy may contribute to the development of DPN and to assess the potential of modulating mitophagy as a therapeutic target to manage DPN. Current efforts are directed at determining if recruitment of Hsp70 to depolarized mitochondria may provide a pivotal mechanism in the efficacy of KU-596 by facilitating a parkin-dependent mitophagy that contributes to the drug's ability to improve physiologic parameters of DPN.

Authorship contributions

Participated in research design: ZY, RTD; *Conducted experiments*: ZY; *Contributed reagents:* ZZ, BSJB; *Performed data analysis*: ZY, RTD; *Wrote or contributed to the writing and editing of the manuscript: ZY*, BSJB, RTD.

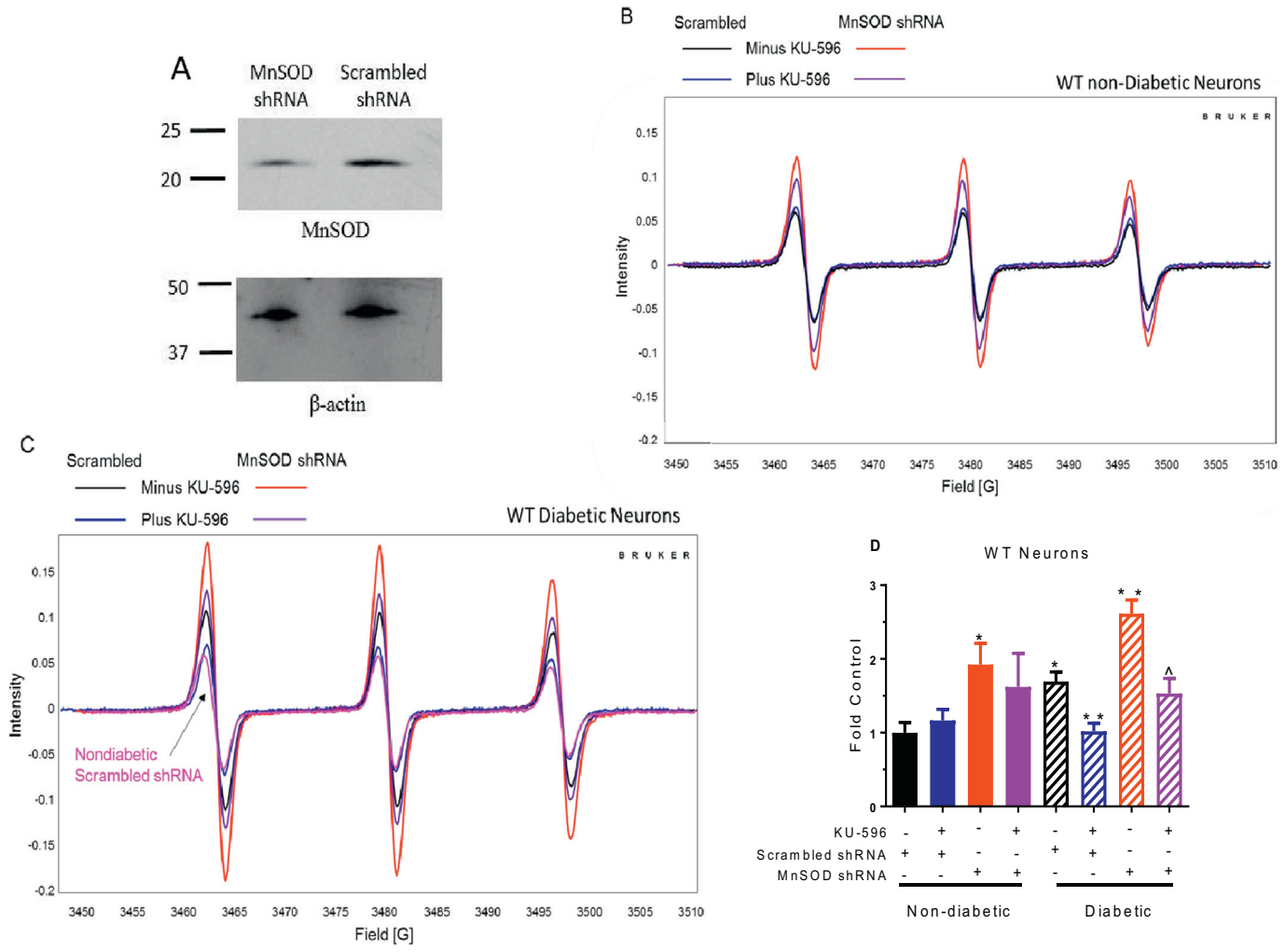

Fig. 6. KU-596 decreased superoxide levels in diabetic neurons following downregulation of MnSOD. A) MnSOD expression in sensory neurons after infection with MnSOD shRNA adenovirus. **(B & C)** EPR assessment of the effect of MnSOD downregulation and KU-596 treatment on the superoxide signal in **(B)** non-diabetic and **(C)** diabetic neurons from WT mice. **D)** Quantification of the area under the curve of the first superoxide signal peak, normalized to total protein and expressed as the fold of the non-diabetic plus scrambled shRNA control. *, $p < .05$ versus non-diabetic plus scrambled shRNA; **, $p < .05$ versus diabetic neurons plus scrambled shRNA; ^, $p < .05$, vs diabetic neurons plus MnSOD shRNA. Results are from two separate experiments in which 3–4 wells of neurons per group were obtained from 6 non-diabetic or diabetic mice.

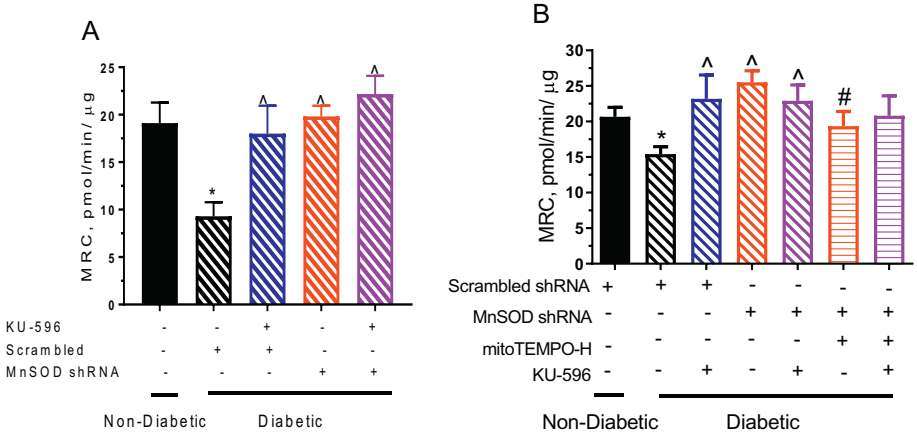

Fig. 7. KU-596 does not improve MRC following downregulation of MnSOD in diabetic neurons. A) Quantification of MRC in diabetic WT diabetic neurons in the presence and absence of KU-596 following MnSOD downregulation. *, $p < .05$ versus non-diabetic neurons treated with scrambled shRNA; ^, $p < .05$, versus diabetic neurons treated with scrambled shRNA; **B)** Quantification of MRC in diabetic neurons in the presence and absence of mito-TEMPO-H and KU-596 following MnSOD downregulation. *, $p < .05$, versus non-diabetic neurons treated with scrambled shRNA. ^, $p < .05$, versus diabetic neurons treated with scrambled shRNA; #, $p < .05$, versus diabetic neurons treated with MnSOD shRNA. For each figure, the results are from 4 to 5 wells of neurons per group obtained from 6 non-diabetic or diabetic mice.

Conflicts of interest

Drs. Blagg and Dobrowsky are named inventors on US and foreign patent applications claiming KU-596 and related compounds, which are among a series of patents and patent applications licensed to Reata Pharmaceuticals by the University of Kansas.

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