Heat Shock Protein 70 Is Necessary to Improve Mitochondrial Bioenergetics and Reverse Diabetic Sensory Neuropathy following KU-32 Therapy

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ABSTRACT

Impaired neuronal mitochondrial bioenergetics contributes to the pathophysiological progression of diabetic peripheral neuropathy (DPN) and may be a focal point for disease management. We have demonstrated that modulating heat shock protein (Hsp) 90 and Hsp70 with the small-molecule drug KU-32 ameliorates psychosensory, electrophysiologic, morphologic, and bioenergetic deficits of DPN in animal models of type 1 diabetes. The current study used mouse models of type 1 and type 2 diabetes to determine the relationship of changes in sensory neuron mitochondrial bioenergetics to the onset of and recovery from DPN. The onset of DPN showed a tight temporal correlation with a decrease in mitochondrial bioenergetics in a genetic model of type 2 diabetes. In contrast, sensory hypoalgesia developed 10 weeks before the occurrence of significant declines in sensory neuron mitochondrial bioenergetics in the type 1 model. KU-32 therapy improved mitochondrial bioenergetics in both the type 1 and type 2 models, and this tightly correlated with a decrease in DPN. Mechanistically, improved mitochondrial function following KU-32 therapy required Hsp70, since the drug was ineffective in diabetic Hsp70 knockout mice. Our data indicate that changes in mitochondrial bioenergetics may rapidly contribute to nerve dysfunction in type 2 diabetes, but not type 1 diabetes, and that modulating Hsp70 offers an effective approach toward correcting sensory neuron bioenergetic deficits and DPN in both type 1 and type 2 diabetes.

Introduction

Diabetic peripheral neuropathy (DPN) is experienced by a majority of patients with type 1 or type 2 diabetes (Callaghan et al., 2012). The development of DPN is associated with a small-fiber neuropathy resulting from dysfunction of unmyelinated or thinly myelinated sensory fibers and the gradual degeneration of larger myelinated fibers. Although numerous pathologic mechanisms contribute to DPN (Farmer et al., 2012), altered mitochondrial bioenergetics (mtBE) may be a central facilitator in its development (Fernyhough et al., 2010). For example, mitochondrial function is impaired in adult sensory neurons or dorsal root ganglia isolated from diabetic rats (Huang et al., 2003, 2005; Chowdhury et al., 2010; Akude et al., 2011). Additionally, in-depth bioenergetic analysis of adult sensory neurons isolated from models of type 1 diabetic mice (Urban et al., 2012) or rats (Chowdhury et al., 2012) found substantive decreases in mtBE. However, since the etiologic development of DPN in type 1 and type 2 diabetes does not necessarily share identical mechanisms, it is unclear whether deficits in mtBE may have a kinetically similar relationship to the onset of sensory hypoalgesia that is symptomatic of DPN. Moreover, the efficacy of interventional therapies is also not identical in patients with type 1 versus type 2 diabetes. For example, tight control of blood glucose more effectively ameliorates DPN in patients with type 1 diabetes (Callaghan et al., 2012). Similarly, enalapril improved nerve blood flow and electrophysiology in a type 1 diabetic rat model (Coppey et al., 2006), but had modest effects in diabetic Zucker fatty rats, a type 2 model (Oltman et al., 2008). These results underscore that an effective therapy for DPN should be equally efficacious regardless of the underlying diabetic phenotype. To this end, we have been exploring the potential of pharmacologically manipulating molecular chaperones for treating DPN.

Heat shock protein (Hsp) 90 and Hsp70 are molecular chaperones that are critical for folding nascent proteins into their biologically active conformations (Evans et al., 2010). Heat shock protein (Hsp) 90 and Hsp70 are molecular chaperones that are critical for folding nascent proteins into their biologically active conformations (Evans et al., 2010).
has intrinsic ATPase activity. Because pharmacologic agents that inhibit the N- or C-terminal ATPase activity can prevent protein folding, inhibiting Hsp90 is an attractive target for treating malignancies, as many oncoproteins require Hsp90 for proper folding (Peterson and Blagg, 2009). However, Hsp90 also binds the transcription factor, heat shock factor 1. Upon exposure to Hsp90 inhibitors, heat shock factor 1 dissociates from Hsp90, translocates to the nucleus, and upregulates a heat shock response that promotes synthesis of cytoprotective antioxidant genes and chaperones, such as Hsp70. This response can antagonize the desired goal of cytotoxicity in treating cancers. On the other hand, inducing the heat shock response can increase molecular chaperones and decrease misfolded protein aggregates. Thus, stimulating this aspect of Hsp90 biology may have utility for treating neurodegenerative diseases (Morimoto, 2011; Zhao et al., 2012). However, developing an effective Hsp90 inhibitor for treating neurodegeneration requires establishing a therapeutic window that leads to upregulation of cytoprotective chaperones, such as Hsp70, in the absence of client protein degradation that can antagonize the protective heat shock response.

Novobiocin is the prototypic ligand that binds to the C-terminal site of Hsp90, and systematic modification of novobiocin identified KU-32 (Fig. 1A) as a neuroprotective lead compound that exhibits a 500-fold divergence of Hsp70 induction from client protein degradation (Urban et al., 2010). This divergence provides an excellent therapeutic window to promote neuroprotection in the absence of toxicity; weekly administration of KU-32 reversed psychosensory, electrophysiologic, bioenergetic, and morphologic indices of DPN in diabetic mice (Urban et al., 2010, 2012). Mechanistically, KU-32 binds Hsp90 directly (Matts et al., 2011), but the drug’s neuroprotective efficacy

Fig. 1. Dose response of KU-32 in improving mechanical hypoalgesia and NCV deficits in diabetic Swiss Webster mice. (A) Structure of KU-32. (B) Swiss Webster mice were rendered diabetic with STZ and mechanical sensitivity was assessed at the indicated weeks. After 8 weeks of diabetes, mice were treated once per week with 2, 10, or 20 mg/kg KU-32 for 6 weeks and mechanical sensitivity measured weekly. One group of nondiabetic mice was treated with only 20 mg/kg KU-32 as a control. *P < 0.05 versus time-matched vehicle (Veh) + Veh; ^P < 0.05 versus time-matched STZ + Veh; #P < 0.05 versus time-matched STZ + 2 mg/kg KU-32. (C) Effect of 6 weeks of KU-32 therapy on MNCV and SNCV. *P < 0.05 versus Veh + Veh; ^P < 0.05 versus STZ + Veh; #P < 0.05 versus STZ + 2 mg/kg KU-32.
depends upon the downstream action of Hsp70 (Urban et al., 2010; Li et al., 2012).

The current study sought to determine whether deficits in mtBE in models of type 1 and type 2 diabetes show a similar relationship to the temporal onset of sensory hypoalgesia and ascertain if KU-32 improves mtBE and reverses DPN in an Hsp70-dependent manner. Our results indicate that the development of a sensory hypoalgesia preceded substantial changes in mtBE in type 1 diabetic mice. In contrast, the onset of mitochondrial dysfunction and sensory hypoalgesia were tightly correlated in type 2 diabetic mice. KU-32 improved the bioenergetic profile of sensory neurons in an Hsp70-dependent manner and effectively reversed insensate DPN in both diabetic models. These data provide novel insight into the role of mitochondrial dysfunction in the onset of insensate DPN and indicate that Hsp70 can have beneficial effects on mtBE.

Materials and Methods

Streptozotocin (STZ), carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), oligomycin, rotenone, antimycin A, Percoll, and poly(DL)ornithine were obtained from Sigma-Aldrich (St. Louis, MO). KU-32, N-(7-((2R,3R,4S,5R)-3,4-dihydroxy-5-methoxy-6,6-dimethyl-tetrahydro-2H-pyran-2-yloxy)-8-methyl-2-oxo-2H-chromen-3-yl)acetamide and trideutero KU-32 were synthesized and structural purity (>95%) verified as described previously (Huang and Blagg, 2007). Collagenase and laminin were purchased from Gibco/Invitrogen (Carlsbad, CA).

Animals. Male and female wild-type (WT) C57Bl/6 and Hsp70.1/70.3 double knockout (KO) mice on a C57Bl/6 background (Hsp70 KO) were used in the study and obtained from in-house breeding colonies (Urban et al., 2010). Male Swiss Webster mice were purchased from Harlan Laboratories (Indianapolis, IN). As a model of type 1 diabetes, 8-week-old male and female mice were fasted for 6 hours and rendered diabetic with an intraperitoneal injection of STZ (100 mg/kg) given on two consecutive days. One week after the second injection, mice were fasted as above, blood was obtained from the tail vein, and animals with fasting blood glucose (FBG) ≤ 290 mg/dl (16 mM) were deemed diabetic.

To model type 2 diabetes, heterozygous BKS.Cg-Dock7m1+/1 Leprdb/+ J mice were acquired from The Jackson Laboratory (Bar Harbor, ME) to generate animals homozygous (Leprdb/db) for the lepr mutation. Heterozygous (Leprdb/+ ) mice served as controls. At ~4–6 weeks of age, the Leprdb/mice became identifiably obese and exhibited elevated FBG compared with their heterozygous littermates. Similar numbers of male and female animals between the genotypes were enrolled in the study when they were 8 weeks old.

All animals were maintained on a 12-hour light/dark cycle with ad libitum access to water and Purina 5001 rodent chow. Preliminary dose-response studies indicated that after 8 weeks of diabetes, once a week dosing of 20 mg/kg KU-32 administered intraperitoneally in 0.2 ml of 0.1 M Captisol (b-cyclodextrin sulfobutylethers; CyDex Pharmaceuticals, Lenexa, KS) gave maximal recovery of mechanical hypoalgesia (Fig. 1B) and nerve conduction velocity deficits (Fig. 1C). All subsequent studies were performed using a once per week 20 mg/kg dosing of the drug. At the termination of each study and prior to sacrifice of the animals, FBG and hemoglobin A1c levels (A1c Now ) were determined. 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.

Fig. 2. KU-32 improves mechanical hypoalgesia and NCV deficits in Leprdb/db mice. (A) Scheme of the study design using the Leprdb/db and Leprdb/+ mice. (B) Mechanical hypoalgesia was assessed in the Leprdb/db and Leprdb/+ mice at the indicated age and weekly KU-32 therapy initiated at 10 weeks of age. KU-32 was then withdrawn in a subgroup of these mice after 4 weeks of treatment. *P < 0.05 versus time-matched Leprdb/+ vehicle (Veh); ^P < 0.05 versus Leprdb/db + Veh; # = P < 0.05 versus Leprdb/db + KU-32 withdrawal. (C) The effect of genotype and treatments on MNCV (solid bars) and SNCV (striped bars) at 18 weeks. Group colors are the same as indicated in (B). *P < 0.05 versus time-matched Leprdb/+; ^P < 0.05 versus Leprdb/db + Veh. (D) Pharmacokinetic analysis of KU-32 uptake in DRG, sciatic nerve, and foot pad.
Psychosensory and Electrophysiologic Analyses. Mechanical sensitivity was assessed in Lepr<sup>db/db</sup> mice using a nylon Semmes-Weinstein von Frey monofilament (Stoelting, Wood Dale, IL). The filament that possesses a buckling force of 1.4 g was applied to the plantar surface of the right and left hind paw. A positive response was recorded after lifting or flinching of the animal’s paw. This procedure consisted of six trials, which alternated between right and left hind paws. The percent response was obtained by determining the number of withdrawals in response to 12 separate monofilament applications (Jack et al., 2011). Alternatively, mechanical sensitivity was assessed in the type 1 animal models using a Dynamic Plantar Aesthesiometer (Stoelting Inc.) fitted with a stiff monofilament that was applied to the plantar surface at an upward force of 10 g in the Swiss Webster mice or 8 g for the C57Bl/6 and Hsp70 KO mice. Thermal sensitivity was assessed by paw withdrawal latency to a ramping, focal heat using a Hargreaves Analgesiometer (Stoelting Inc.) (Urban et al., 2010). The percent response was obtained by determining the number of withdrawals in response to 12 separate monofilament applications of withdrawals in response to 12 separate monofilament applications. The percent response was obtained by determining the number of withdrawals in response to 12 separate monofilament applications respectively. We assessed following dissipation of the proton gradient across the inner mitochondrial membrane with the protonophore FCCP (1 μM). Nonmitochondrial respiration was then assessed by conjection of 1 μM rotenone or rotenone + 1 μM antimycin A.

Table 1

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<th>Week</th>
<th>Group</th>
<th>Weight</th>
<th>FBG (mg/dl)</th>
<th>HbA1c (%)</th>
<th>Latency (sec)</th>
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<td>5144 ± 14</td>
<td>5</td>
<td>4.9 ± 1.4</td>
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<td>46.5 ± 2.7</td>
<td>5317 ± 108&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>4.3 ± 0.3</td>
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<td>10357 ± 111&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>20124 ± 17</td>
<td>5</td>
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<td>49.1 ± 5.0</td>
<td>10533 ± 61&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>12506 ± 103&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13</td>
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<td>50.6 ± 7.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11501 ± 73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16</td>
<td>5.5 ± 1.0</td>
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<td>18</td>
<td>db&lt;sup&gt;+&lt;/sup&gt; + Veh</td>
<td>28.0 ± 5.0</td>
<td>10122 ± 17</td>
<td>5</td>
<td>4.3 ± 0.1 (23 ± 1.1)</td>
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<tr>
<td></td>
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<td>26.9 ± 3.8</td>
<td>8137 ± 33</td>
<td>5</td>
<td>4.4 ± 0.1 (24 ± 1.1)</td>
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<td></td>
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<td>48.8 ± 5.8</td>
<td>10499 ± 78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10</td>
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<td>12504 ± 90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12</td>
<td>10.3 ± 0.4&lt;sup&gt;b&lt;/sup&gt; (89 ± 4.6)</td>
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<td>51.4 ± 8.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11495 ± 54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11</td>
<td>10.5 ± 0.4&lt;sup&gt;b&lt;/sup&gt; (91 ± 4.2)</td>
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Veh, vehicle.

<sup>a</sup>mmol of HbA1c/mol of hemoglobin.

<sup>b</sup>Paw withdrawal latency to thermal stimuli.

*P < 0.05 versus db<sup>+</sup> + Veh.
lumbar dorsal root ganglia, sciatic nerve, and plantar foot pads harvested and rapidly frozen on dry ice. Tissues were minced, homogenized in water by sonication (25 mg/g water), and spiked with 50 ng/ml tritiated KU-32 as the internal standard. The homogenate was extracted with 1 ml of i-butyl methyl ether and centrifuged. An aliquot (0.95 ml) of the supernatant was recovered and evaporated to dryness, and the resulting residue was resuspended in 0.05 ml of 20% CH₃CN. After a second centrifugation, 0.045 ml was transferred to autosampler vials. Chromatographic separation was performed using a 5-micron Agilent Zorbax SB C18 column (2.1 x 50 mm; Santa Clara, CA) and a linear gradient of CH₃CN:H₂O:formic acid (5:95:0.1) to CH₃CN:H₂O:formic acid (95:5.0:0.1) at a flow rate of 0.30 ml/min. The effluent was introduced to a Sciex API3200 Linear Ion Trap detector (Framingham, MA) using turbo ion spray in the positive ion mode. Linear calibration curves were constructed in each tissue matrix over a range of 0–1000 ng/ml, and analyze recoveries ranged from 65% to 75%.

Mitochondrial Copy Number. Real-time polymerase chain reaction (PCR) was used to measure mitochondrial copy number using primers for murine cytochrome b, cytochrome c oxidase subunit II (COII, mitochondrial genes), and β-actin (nuclear gene) (Santos et al., 2011). Forward and reverse primer sequences for mouse cytochrome b were 5'-GCAACCTTGACCCGATTCTTCGC-3' and 5'-TGAACGATTGGTGCCC-3'. Forward and reverse primer sequences for mouse COII were 5'-ATTGCCCTCCCCT-CTCTACGCA-3' and 5'-CCTAGCCTCATATCGGC-3'. PCR was performed using 25–50 ng of genomic DNA, 200 nM concentration of each primer, and the SYBR Green PCR master mix from Applied Biosystems (Grand Island, NY). Products were amplified following DNA denaturation at 95°C for 5 minutes followed by 35 cycles at 95°C for 15s, 62°C for 30s, 72°C for 30s, and a final extension at 72°C for 5 minutes. Melting curve analysis and gel electrophoresis verified the specific amplification of the expected product. Differences in gene expression were determined after normalization to β-actin using the ΔΔC(T) method.

Statistical Analysis. Student’s t tests, one-way analysis of variance (ANOVA), and repeated measures one-way ANOVA were applied for between-group comparisons. Post hoc analyses were conducted using Tukey’s test, and nonparametric data were analyzed with a Kruskal-Wallis and Dunn’s test. All data are presented as mean ± S.E.M.

Results

KU-32 Reverses DPN in Leprdb/db Mice

Beginning at 8 weeks of age, FBG, body weight, and psychosensory measures were assessed weekly in the Leprdb/db and Leprdb/db mice. Each genotype was subdivided into two groups at 10 weeks of age and given a weekly injection of KU-32 or Captisol for up to 8 weeks (Fig. 2A). After 4 weeks of drug therapy, a subgroup of the KU-32-treated Leprdb/db mice was administered Captisol for the final 4 weeks to investigate the effects of drug withdrawal. To examine the effect of diabetes and drug treatment on mitochondrial bioenergetic (mtBE), sensory neurons were isolated from five mice prior to drug treatment and at 4 and 8 weeks after administering KU-32.

Consistent with the onset of type 2 diabetes, the Leprdb/db mice showed significant increases in total body weight and FBG and at 18 weeks of age had elevated glycated hemoglobin levels compared with age-matched Leprdb/db mice (Table 1). However, treatment with KU-32 did not affect any of these parameters in either genotype, nor did it improve glucose disposal following a 3-hour glucose tolerance test (6-hour fast and 0.5-g glucose given intraperitoneally; data not shown).

By 8 weeks of age, the Leprdb/db mice showed a significantly lower percent withdrawal response to a von Frey hair indicative of a mechanical hypoalgesia (Fig. 2B). In agreement with a prior study (Wright et al., 2007), the Leprdb/db mice did not develop a thermal hypoalgesia (Table 1) and did not show a significant decrease in intra-epidermal nerve fiber density in the foot pad at 18 weeks of age (data not shown). Weekly administration of KU-32 significantly improved mechanical sensitivity in the Leprdb/db mice, but had no effect on the Leprdb/db mice. MNCV and SNCV decreased significantly in the untreated 18-week-old Leprdb/db mice, and KU-32 therapy improved these electrophysiologic deficits (Fig. 2C).

After 4 weeks of KU-32 therapy, drug withdrawal led to a gradual redevelopment of the mechanical hypoalgesia. In contrast, MNCV and SNCV rates remained similar to those observed in the Leprdb/db mice that received the drug continuously. Although the reason for this distinction is unclear, it is not related to differences in the rate of drug clearance since pharmacokinetic analysis showed that KU-32 is rapidly distributed to and quickly cleared from dorsal root.
ganglia, sciatic nerve, and plantar foot pads, with a half-life of 1.5–2 hours (Fig. 2D).

Diabetes-Induced Hypoalgesia Coincides with Decreased Mitochondrial Function in Lepr\textsuperscript{db/db} Mice

The L4–L6 lumbar ganglia provide the cell bodies for the motor and sensory fibers that are affected in DPN and whose physiology was improved by KU-32. To determine whether the improvement in psychosensory function and NCV was associated with a change in mtBE, sensory neurons were isolated from these ganglia at 6, 8, 10, 14, and 18 weeks of age. The neurons were cultured in vitro for 2 days and the OCR was determined for 2 hours in the intact cells (Zhang et al., 2012).

At 6 weeks of age, Lepr\textsuperscript{db/db} mice showed a modest decline in maximal respiratory capacity and spare respiratory capacity (SRC), but these parameters were significantly decreased by 8 weeks (Fig. 3A) and 10 weeks (Fig. 3B) of age. To gain broader insight into the effect of diabetes on cellular respiration, the ATP-linked OCR, proton leak, SRC, and nonmitochondrial OCR were expressed as a percent of the MRC (Fig. 3B). At 10 weeks of age, Lepr\textsuperscript{db/db} showed a significant decrease in SRC and an increase in proton leak, ATP-linked OCR, and nonmitochondrial respiration, consistent with an overall alteration in mtBE.

KU-32 Therapy Improves mtBE and Sensory Hypoalgesia in Lepr\textsuperscript{db/db} Mice. Deficits in mtBE worsened by 14 weeks of age (Fig. 4, A and B) and while the extent of ATP-linked and nonmitochondrial respiration increased, SRC declined between 10 and 14 weeks of age (compare Figs. 3B

Fig. 4. KU-32 improves mitochondrial bioenergetics in Lepr\textsuperscript{db/db} mice. Sensory neurons were obtained from 14- (A and B) or 18-week- (C and D) old Lepr\textsuperscript{db/db} and Lepr\textsuperscript{+/+} mice treated with KU-32 or Captisol and prepared for bioenergetic analysis. (A) MRC was significantly impaired in untreated Lepr\textsuperscript{db/db} compared with age-matched Lepr\textsuperscript{+/+} mice, and this deficit was significantly improved by KU-32 treatment. (B) Fourteen-week-old Lepr\textsuperscript{db/db} mice have a significant decrease in SRC and an increase in ATP-linked and nonmitochondrial respiration compared with age-matched Lepr\textsuperscript{+/+} mice, and these parameters were significantly improved by KU-32 therapy. (C) At 18 weeks of age MRC remained significantly impaired in the Lepr\textsuperscript{db/db} compared with age-matched Lepr\textsuperscript{+/+} mice. However, MRC was essentially unchanged from 14-week-old untreated Lepr\textsuperscript{db/db}. (D) MRC and SRC remained significantly improved in KU-32 treated Lepr\textsuperscript{db/db} mice regardless of whether the drug was give continuously or withdrawn for the final 4 weeks of the study. In (A–D), *P < 0.05 versus Lepr\textsuperscript{+/+} + vehicle (Veh); ^P < 0.05 versus Lepr\textsuperscript{db/db} + Veh.
and 4B). The respiratory control ratio (RCR) is an indication that the mitochondria have a high capacity for substrate oxidation and ATP synthesis and a low proton leak (Brand and Nicholls, 2011). At 14 weeks of age, diabetes decreased the RCR from 14.8 ± 1.6 in the Lepr<sup>db/</sup>db mice to 6.8 ± 0.03 in the untreated Lepr<sup>db/</sup>db animals. Lepr<sup>db/</sup>db animals treated for 4 weeks with KU-32 showed a significant improvement in MRC and SRC, and the RCR increased to 12.5 ± 0.5 versus untreated Lepr<sup>db/</sup>db animals, suggesting that modulating molecular chaperones increased the capacity for mitochondrial substrate oxidation.

Not surprisingly, MRC and SRC remained significantly impaired in the 18-week-old Lepr<sup>db/</sup>db animals compared with the Lepr<sup>db/</sup>db mice, and continued therapy with KU-32 improved MRC and SRC, but not really beyond levels observed after 4 weeks of drug administration (Fig. 4C). This result was similar to the continued increase in nerve electrophysiology that was observed 4 weeks after drug administration was terminated (Fig. 4D). This result was similar to the continued increase in nerve electrophysiology that was observed 4 weeks after drug withdrawal.

Lastly, to determine whether the improved bioenergetics might be due to an increase in mitochondrial biogenesis, the levels of cytochrome b and cytochrome c oxidase subunit II (mitochondrial genes) in the DRG from the 18-week-old mice were assessed by quantitative PCR and normalized to the nuclear gene, β-actin (Santos et al., 2011). The expression of both genes was expressed as the fold of the level present in vehicle-treated Lepr<sup>db/</sup>db mice. However, no differences were observed in the untreated (1.01 ± 0.08, n = 3) or KU-32 treated (0.98 ± 0.06, n = 4) Lepr<sup>db/</sup>db mice.

**Hsp70 Is Necessary to Improve Mitochondrial Bioenergetics following KU-32 Therapy**

We have shown previously that Hsp70 was necessary for the beneficial effect of KU-32 on improving insensitive DPN (Urban et al., 2010). To examine whether the improvement in mtBE also required Hsp70, we compared the effect of KU-32 therapy on WT and Hsp70 KO mice rendered diabetic with STZ (Table 3). Diabetic WT and Hsp70 KO mice developed a mechanical hypoaesthesia (Fig. 6, A and B) and MNCV deficits (Fig. 6, C and D). Thermal sensitivity and SNCV responded similarly (Supplemental Fig. 2, A–D). After 12 weeks of diabetes, the sensory deficits were well established, and although initiating weekly KU-32 therapy improved all the sensory endpoints in the WT mice, this response was totally absent in the drug-treated, diabetic, Hsp70 KO mice.

**Diabetes-Induced Hypoaesthesia Precedes Decreased Mitochondrial Function in a Model of Type 1 Diabetes**

Because it is unclear whether changes in mtBE and sensory function follow similar patterns in type 1 and type 2 diabetes, this was examined using Swiss Webster mice rendered diabetic with STZ (Table 2). By 12 weeks of diabetes, the mice developed a significant mechanical (Fig. 5A) and thermal (Supplemental Fig. 1A) hypoaesthesia. However, these early sensory deficits were not associated with a significant impairment in mitochondrial respiration since SRC was significantly impaired only after 16 weeks of diabetes (Fig. 5B).

To determine whether improved sensory function following treatment with KU-32 was linked to an improvement in mtBE, drug therapy was initiated at week 17, and sensory neurons were harvested 1, 3, and 5 weeks after drug administration. Prior to drug treatment, the mice showed significant decreases in nerve conduction velocities (Supplemental Fig. 1B) along with the thermal and mechanical hypoaesthesia. Although 1 week of KU-32 administration did not increase the diabetes-induced decline in mitochondrial respiration, continued treatment revealed a tight temporal correlation between an improved SRC (Fig. 5B) and recovery from the mechanical hypoaesthesia (Fig. 5C), MNCV (Fig. 5D), and SNCV deficits (Supplemental Fig. 1C).

### TABLE 2

<table>
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<tr>
<th>Week</th>
<th>Treatment</th>
<th>Weight</th>
<th>FBG</th>
<th>HbA&lt;sub&gt;1c&lt;/sub&gt;</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Veh + Veh</td>
<td>34.1 ± 2.5</td>
<td>127 ± 12</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>STZ + Veh</td>
<td>27.3 ± 1.1</td>
<td>540 ± 96*</td>
<td>—</td>
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<tr>
<td>12</td>
<td>Veh + Veh</td>
<td>34.0 ± 1.7</td>
<td>122 ± 10</td>
<td>5.0 ± 0.4 (31 ± 4.4)</td>
<td>6</td>
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<tr>
<td></td>
<td>STZ + Veh</td>
<td>30.3 ± 3.9</td>
<td>600*</td>
<td>12.9 ± 0.2* (117 ± 2.2)</td>
<td>7</td>
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<tr>
<td>16</td>
<td>Veh + Veh</td>
<td>43.0 ± 3.1</td>
<td>117 ± 6</td>
<td>5.4 ± 0.7 (36 ± 7.7)</td>
<td>4</td>
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<tr>
<td></td>
<td>STZ + Veh</td>
<td>28.6 ± 4.1</td>
<td>573 ± 54*</td>
<td>11.7 ± 0.9* (104 ± 9.8)</td>
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</tr>
<tr>
<td>18</td>
<td>Veh + Veh</td>
<td>43.8 ± 3.1</td>
<td>114 ± 15</td>
<td>4.7 ± 0.2 (28 ± 2.2)</td>
<td>4</td>
</tr>
<tr>
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<td>29.3 ± 4.2</td>
<td>564 ± 35*</td>
<td>9.9 ± 1.8* (85 ± 19.7)</td>
<td>4</td>
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<tr>
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<td>Veh + KU-32</td>
<td>39.2 ± 2.2</td>
<td>138 ± 18</td>
<td>4.6 ± 0.3 (27 ± 3.3)</td>
<td>4</td>
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<tr>
<td></td>
<td>STZ + KU-32</td>
<td>30.8 ± 1.7</td>
<td>589 ± 21*</td>
<td>10.7 ± 0.8* (93 ± 8.7)</td>
<td>4</td>
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<tr>
<td>20</td>
<td>Veh + Veh</td>
<td>40.2 ± 3.9</td>
<td>125 ± 9</td>
<td>4.9 ± 0.2 (30 ± 2.2)</td>
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<td>STZ + Veh</td>
<td>32.7 ± 2.8</td>
<td>600*</td>
<td>12.1 ± 0.6* (109 ± 6.6)</td>
<td>4</td>
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<tr>
<td></td>
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<td>41.4 ± 2.6</td>
<td>120 ± 1</td>
<td>5.2 ± 0.6 (33 ± 6.6)</td>
<td>4</td>
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<td>STZ + KU-32</td>
<td>33.0 ± 6.0</td>
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<td>12.1 ± 0.9* (109 ± 9.8)</td>
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<td>22</td>
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<td>44.0 ± 0.6</td>
<td>123 ± 10</td>
<td>5.1 ± 0.1 (32 ± 1.1)</td>
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<td>STZ + Veh</td>
<td>30.3 ± 2.6</td>
<td>600*</td>
<td>10.2 ± 1.7* (88 ± 8.6)</td>
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<td>120 ± 1</td>
<td>4.8 ± 0.3 (29 ± 3.3)</td>
<td>4</td>
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<td>35.9 ± 0.9</td>
<td>600*</td>
<td>10.7 ± 1.6* (93 ± 17.5)</td>
<td>3</td>
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</tbody>
</table>

Veh, vehicle.

*μmol of HbA<sub>1c</sub>/μmol of hemoglobin.

*P < 0.05 versus Veh + Veh.
diabetes-induced decline in mtBE. On the other hand, 3–6 weeks of KU-32 therapy improved MRC and SRC in diabetic WT mice but neither respiratory parameter was altered by the drug in diabetic Hsp70 KO mice. Importantly, the lack of efficacy of KU-32 in the Hsp70 KO mice is unlikely to result from differences in drug uptake or metabolism, since similar levels of KU-32 were present in DRG, sciatic nerve, and foot pads of WT and Hsp70 KO mice (data not shown). These data

**Fig. 5.** Sensory hypoalgesia precedes the onset of mitochondrial bioenergetic deficits in a type 1 model of diabetes. Swiss Webster mice were rendered diabetic with STZ, mechanical sensitivity measured at the indicated weeks, and sensory neurons isolated after 10, 12, and 16 weeks of diabetes to assess mitochondrial function. (A) Diabetes induced a mechanical hypoalgesia that was maximal after 13 weeks and was reversed by KU-32 treatment. *P < 0.05 versus time-matched vehicle (Veh) + Veh; ^P < 0.05; ^^P < 0.01; ^^^P < 0.001, versus time-matched STZ + Veh for all P values. (B) SRC was significantly decreased after 16 weeks of diabetes, and initiation of KU-32 therapy led to a time-dependent recovery of SRC that correlated with improvements in mechanical hypoalgesia (C) and MNCV (D). *P < 0.05 versus Veh + Veh; ^P < 0.05 versus STZ + Veh; ^#P < 0.05 versus STZ + 1 week KU-32.

**TABLE 3**

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Treatment</th>
<th>C57Bl/6 Weight</th>
<th>C57Bl/6 FBG</th>
<th>C57Bl/6 HbA1c</th>
<th>Hsp70 KO Weight</th>
<th>Hsp70 KO FBG</th>
<th>Hsp70 KO HbA1c</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>g</td>
<td>mg/dl</td>
<td>% (mmol/mol)</td>
<td>g</td>
<td>mg/dl</td>
<td>% (mmol/mol)</td>
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<tr>
<td>15</td>
<td>Veh + Veh</td>
<td>28.6 ± 3.4</td>
<td>149 ± 38</td>
<td>4.7 ± 0.2 (28 ± 2.2)</td>
<td>12</td>
<td>25.9 ± 3.1</td>
<td>137 ± 38</td>
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<tr>
<td></td>
<td>STZ + Veh</td>
<td>20.7 ± 3.8</td>
<td>546 ± 75*</td>
<td>9.9 ± 1.58 (85 ± 17)</td>
<td>9</td>
<td>22.9 ± 3.4</td>
<td>431 ± 114*</td>
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<tr>
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<td>Veh + KU-32</td>
<td>24.4 ± 2.8</td>
<td>163 ± 47</td>
<td>4.5 ± 0.2 (26 ± 2.2)</td>
<td>12</td>
<td>24.2 ± 2.9</td>
<td>133 ± 18</td>
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<td></td>
<td>STZ + KU-32</td>
<td>21.6 ± 2.9</td>
<td>506 ± 98*</td>
<td>8.0 ± 1.5* (64 ± 17)</td>
<td>8</td>
<td>23.6 ± 3.9</td>
<td>478 ± 68*</td>
</tr>
<tr>
<td>18</td>
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<td>25.5 ± 1.5</td>
<td>163 ± 45</td>
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<td>23.7 ± 3.1</td>
<td>127 ± 15</td>
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<td>STZ + Veh</td>
<td>25.0 ± 2.7</td>
<td>419 ± 104*</td>
<td>8.2 ± 0.7* (66 ± 7.7)</td>
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<td>25.4 ± 2.7</td>
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<td>Veh + KU-32</td>
<td>27.0 ± 0.6</td>
<td>151 ± 18</td>
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<td>25.3 ± 2.3</td>
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<td>STZ + KU-32</td>
<td>24.0 ± 2.2</td>
<td>415 ± 161*</td>
<td>8.5 ± 1.9* (69 ± 21)</td>
<td>3</td>
<td>22.9 ± 2.9</td>
<td>413 ± 57*</td>
</tr>
</tbody>
</table>

Veh, vehicle.  
*mmol of HbA1c/mol of hemoglobin.  
*P < 0.05 versus Veh + Veh.
support the conclusion that Hsp70 is required to improve mtBE and sensory function following KU-32 therapy.

**Discussion**

**Mitochondrial Dysfunction and the Onset of DPN in Type 2 Diabetes.** Similar to previous results in models of type 1 diabetes (Chowdhury et al., 2012; Urban et al., 2012), we observed deficits in multiple bioenergetic parameters in sensory neurons obtained from Leprdb/db animals, a genetic model of type 2 diabetes. Although prior results have clearly implicated mitochondrial dysfunction in the development of insensate DPN in type 1 diabetes (Fernyhough et al., 2010; Chowdhury et al., 2013), our results are the first to temporally link the kinetics of onset of the bioenergetic deficits with the developing hypoalgesia in a model of type 2 diabetes.

The Lepr<sup>db/db</sup> mice showed significant deficits in MRC and SRC 4–6 weeks after the onset of hyperglycemia, which correlated with the rapidly developing sensory hypoalgesia. MRC measures the rate of maximal electron transport activity and substrate oxidation that the sensory neurons can achieve in the absence of limitations imposed by the proton gradient across the inner mitochondrial membrane. The decrease in MRC suggests that electron transport in the diabetic mitochondria was impaired or that availability of substrates such as glucose and pyruvate was limiting. Although substrate availability might be affected by decreased activity of glycolysis and the tricarboxylic acid cycle as observed in 24-week-old Lepr<sup>db/db</sup> mice (Hinder et al., 2013b), the rate of extracellular acidification, a measure of glycolytic activity, was relatively similar between the Lepr<sup>db/db</sup> and Lepr<sup>d/+</sup> mice (data not shown). The similar levels of extracellular acidification suggest that the deficit in ATP production is either not sufficient to stimulate glycolysis or the diabetic neurons do not effectively revert to glycolysis to help produce ATP. Indeed, as observed by others (Chowdhury et al., 2012), the percent of the basal OCR that was attributable to ATP-linked respiration increased in the 10–18-week-old Lepr<sup>db/db</sup> compared with the Lepr<sup>d/+</sup> mice. Presumably, since the maximal rate of electron transport and substrate oxidation is compromised in the diabetic neurons, a greater portion of total respiration is commitment toward ATP-production to meet cellular demands.

SRC provides an indication of how close a cell is functioning to its bioenergetic limit (Sansbury et al., 2011). The progressive decline in SRC suggests that the diabetic neurons have a diminished energetic reserve to respond to the continued metabolic challenges associated with the chronic hyperglycemia and dyslipidemia in the Lepr<sup>db/db</sup> mice. Because KU-32 significantly improved SRC and NCV even after 4 weeks of drug withdrawal, moderate improvements in respiratory function via modulating chaperones may have long-term benefits for nerve electrophysiology. For example, decreased Na<sup>+</sup>/K<sup>+</sup> ATPase activity is associated with slowing of MNCV in DPN (Coppey et al., 2001), and improved mitochondrial function may contribute to ameliorating this deficit.

Lastly, an unexpected outcome of our study was the significant improvement in SRC in the nondiabetic Lepr<sup>d/+</sup> mice treated with KU-32 for 8 weeks. The underlying reason for this improvement is unclear but may be related to the decreased mitochondrial respiratory capacity that was observed in the vehicle-treated 18-week-old Lepr<sup>d/+</sup> mice.
(compare black squares in Fig. 4, A and C). The decreased respiratory capacity between untreated 14- and 18-week-old Leprdb/db mice was observed in the testing of two separate groups of animals from different litters and suggests that the Lepr mutation may have an age-related effect on mitochondrial function.

**Mitochondrial Dysfunction and the Onset of DPN in Type 1 Diabetes.** In the type 1 model, diabetic mice developed a mechanical and thermal hypoalgesia between 6–12 weeks in the absence of significant decreases in SRC. However, mitochondrial respiration was significantly reduced after 16 weeks of diabetes. These data suggest that a fundamental difference may exist in the temporal role of altered mtBE in contributing to the early onset of hypoaalgiesia in type 1 versus type 2 diabetes. Because both models showed relatively similar levels of hyperglycemia, differences in the kinetics of onset of the bioenergetic decline may be influenced by insulin resistance, which is coincident with mitochondrial dysfunction in tissues such as oxidative skeletal muscle and liver of the Leprdb/db mice (Holmstrom et al., 2012). Alternatively, dyslipidemia is often associated with type 2 diabetes and has been suggested to contribute to DPN (Vincent et al., 2009). However, genetic deletion of apolipoprotein E to increase dyslipidemia in Leprdb/db mice did not exacerbate DPN assessed at 6 months of age (Hinder et al., 2013a), suggesting that dyslipidemia may not significantly worsen sensory neuron mitochondrial dysfunction in type 2 diabetes.

**Modulating Hsp70 Improves mtBE and DPN.** Although the onset of a thermal and mechanical hypoalgesia does not require a significant change in mtBE in the type 1 model, the improvement in the psychosensory and electrophysiologic measures of nerve function in both diabetic animal models tightly correlated with an increase in mtBE following KU-32 therapy. The absence of this correlation in the diabetic Hsp70 KO mice provides the first evidence that modulating Hsp70 is a necessary effector for KU-32 to improve the bioenergetic profile of the sensory neurons and nerve function. This benefit was unrelated to any effect of KU-32 or Hsp70 on improving glucose disposal or any of the metabolic parameters in either model. However, a clear limitation of this work is that although a tight, Hsp70-dependent correlation exists between improved mtBE and the various measures of nerve function, a causal relationship between them is not established. Thus, we cannot rule out that Hsp70 may have independent and parallel effects on mtBE and other targets that contribute to improving psychosensory and electrophysiologic function following KU-32 therapy.

Although the etiology of DPN is not attributed to the accumulation of a specific misfolded or aggregated protein, the efficacy of Hsp70 in improving mtBE suggests that diabetic mitochondria are undergoing some level of proteotoxic stress. Because KU-32 can decrease oxidative stress and increase mitochondrial and cytosolic chaperones in
modulating Hsp70 to enhance neuronal mtBE might rely on the extent of Hsp70 induction since overexpression of Hsp70 in HeLa cells inhibited oxidative phosphorylation and augmented anaerobic glycolysis (Wang et al., 2012). As certain malignant phenotypes are characterized by Hsp70 overexpression and increased glycolytic rates, the efficacy of modulating Hsp70 to enhance neuronal mtBE might rely on pharmacologic agents that are weak or cell-selective inducers of Hsp70. Although glycolytic activity decreases in diabetic nerve (Hinder et al., 2013b), additional work will be needed to determine if long-term modulation of Hsp70 might negatively affect neuronal oxidative phosphorylation and be a liability in treating DPN.

In summary, numerous approaches toward treating insensitive DPN have centered on inhibiting specific pathogenic pathways linked to glucotoxicity, with limited translational success (Calcott et al., 2009). The pharmacologic management of insensitive DPN is difficult since no single etiologic event has been unequivocally identified to contribute to disease development in a temporally and/or biochemically uniform fashion over the long natural history of the disease. Since pharmacologic modulation of endogenous neuroprotective chaperones does not rely on targeting a specific pathogenic enzyme (i.e., aldose reductase) or pathway (i.e., oxidative stress) that contributes to DPN, it provides a venue to pharmacologically modulate endogenous mitochondrial dysfunction and abrogates the pathogenesis of diabetic neuropathy.

Acknowledgments

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Authorship Contributions

Participated in research design: Dobrowsky, Ma, Farmer.

Conducted experiments: Ma, Farmer, Pan, Urban.

Contributed new reagents or analytic tools: Zhao, Blagg.

Performed data analysis: Ma, Farmer, Pan, Urban, Dobrowsky.

Wrote or contributed to the writing of the manuscript: Dobrowsky, Ma, Farmer.

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Jack MM, Ryals JM, and Wright DE (2011) Characterisation of glyoxalase I in diabetic mice (Zhang et al., 2012). In this regard, the dependence on Hsp70 for the tight correlation between improved mtBE and the physiologic measures of DPN adds to treating DPN.


Muchowski PJ and Wacker JL (2005) Modulation of neurodegeneration by molecular chaperones does not rely on targeting a specific pathogenic enzyme (i.e., aldose reductase) or pathway (i.e., oxidative stress) that contributes to DPN, it provides a venue to pharmacologically modulate endogenous mitochondrial dysfunction and abrogates the pathogenesis of diabetic neuropathy.


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Heat Shock Protein 70 is Necessary to Improve Mitochondrial Bioenergetics and Reverse Diabetic Sensory Neuropathy Following KU-32 Therapy

Jiacheng Ma, Kevin L. Farmer, Pan Pan, Michael J. Urban, Huiping Zhao, Brian S.J. Blagg and Rick T. Dobrowsky

Journal of Pharmacology and Experimental Therapeutics

Supplemental Figure 1- Effect of Diabetes and KU-32 Therapy on Thermal Hypoalgesia, NCV and Mitochondrial Bioenergetics. Swiss Webster mice were rendered diabetic with STZ and thermal sensitivity assessed at the indicated weeks. After 17 weeks of diabetes, KU-32 was given weekly and at 1, 3 and 5 weeks after drug administration, NCV was measured and sensory neurons were isolated to assess mitochondrial bioenergetics. A) Diabetes induced a thermal hypoalgesia that was reversed by KU-32 treatment. *, p< 0.05, **, p< 0.01 vs time-matched Veh + Veh; ^, p< 0.05 vs time-matched STZ + Veh. B) Both MNCV and SNCV were decreased after 16 weeks of diabetes (0 weeks KU-32) and drug therapy improved these deficits. *, p< 0.05 vs Veh + Veh; ^, p< 0.05 vs STZ + Veh; #, p< 0.05 vs STZ + 1 week KU-32. (C) Correlation between recovery of SRC and improvement in SNCV.
Supplemental Figure 2- Hsp70 is Necessary for KU-32 to Reverse DPN. C57Bl/6 (A, C) and Hsp70 KO (B, D) mice were rendered diabetic with STZ and at 12 weeks of diabetes, KU-32 was given weekly for 6 weeks. Thermal sensitivity (A, B) was assessed at the indicated weeks and SNCV (C, D) was assessed after 15 (3 weeks KU-32) and 18 weeks (6 weeks KU-32) of diabetes. *, p< 0.05 vs Veh + Veh; ^, p< 0.05 vs STZ + Veh. Symbol and bar colors in legend are shared between (A and B) and (C and D), respectively.