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Modulating molecular chaperones improves sensory fiber recovery and mitochondrial function in diabetic peripheral neuropathy

Michael J. Urban [a,b], Pan Pan [a], Kevin L. Farmer [a], Huiping Zhao [c], Brian S.J. Blagg [c], Rick T. Dobrowsky [a,b,*]

[a] Department of Pharmacology and Toxicology, The University of Kansas, Lawrence, KS, USA
[b] Program in Neuroscience, The University of Kansas, Lawrence, KS, USA
[c] Department of Medicinal Chemistry, The University of Kansas, Lawrence, KS, USA

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ABSTRACT

Quantification of intra-epidermal nerve fibers (iENFs) is an important approach to stage diabetic peripheral neuropathy (DPN) and is a promising clinical endpoint for identifying beneficial therapeutics. Mechanistically, diabetes decreases neuronal mitochondrial function and enhancing mitochondrial respiratory capacity may aid neuronal recovery from glucotoxic insults. We have proposed that modulating the activity and expression of heat shock proteins (Hsp) may be of benefit in treating DPN. KU-32 is a C-terminal Hsp90 inhibitor that improved thermal hypoalgesia in diabetic C57Bl/6 mice but it was not determined if this was associated with an increase in iENF density and mitochondrial function. After 16 weeks of diabetes, Swiss Webster mice showed decreased electrophysiological and psychosensory responses and a >30% loss of iENFs. Treatment of the mice with ten weekly doses of 20 mg/kg KU-32 significantly reversed pre-existing deficits in nerve conduction velocity and responses to mechanical and thermal stimuli. KU-32 therapy significantly reversed the pre-existing loss of iENFs despite the identification of a sub-group of drug-treated diabetic mice that showed improved thermal sensitivity but no increase in iENF density. To determine if the improved clinical indices correlated with enhanced mitochondrial activity, sensory neurons were isolated and mitochondrial bioenergetics assessed *ex vivo* using extracellular flux technology. Diabetes decreased maximal respiratory capacity in sensory neurons and this deficit was improved following KU-32 treatment. In conclusion, KU-32 improved physiological and morphologic markers of degenerative neuropathy and drug efficacy may be related to enhanced mitochondrial bioenergetics in sensory neurons.

Diabetic peripheral neuropathy (DPN) is a common neurodegenerative complication of diabetes that has proven difficult to manage pharmacologically due to its multifactorial etiology (Yorek, 2011). Despite the efficacy of a plethora of small molecule inhibitors that target a single etiologic contributor to DPN in rodent models, the biochemical and temporal complexity underlying the progression of DPN in humans has proved challenging for translating the success in animal models to its clinical management (Calcutt et al., 2009). Thus, considerable need exists to identify novel pharmacologic targets or therapeutic paradigms that, in combination with good glycemic control, will help patients overcome the difficulties of slowing or reversing the progression of DPN. Examples of such approaches lie in improving dyslipidemia (Vincent et al., 2009), using a dual specificity vasopeptidase inhibitor to block angiotensin converting enzyme and neutral endopeptidase to improve neural and vascular deficits in DPN (Yorek, 2008) and the recent demonstration that hydroxyflavones may target multiple mechanisms that contribute to DPN (Stavniichuk et al., 2011). An additional and relatively unexplored paradigm is that pharmacologically modulating the activity of molecular chaperones will promote a broadly cytoprotective response that improves psychosensory, electrophysiological, biochemical and morphological indices of DPN.

Heat shock proteins 90 and 70 (Hsp90 and Hsp70) are two molecular chaperones that are critical for the proper folding of nascent proteins. Many neurodegenerative diseases (for example, Alzheimer's and Parkinson's disease) can be considered as protein-conformational disorders since the accumulation of specific mis-folded or aggregated proteins is a primary contributor to their etiology (Muchowski and Wacker, 2005). Although the accumulation of any one specific misfolded or aggregated protein is not associated with the development of DPN, hyperglycemia can increase the oxidative modification of amino acids (Akude et al., 2009; Obrosova, 2009) leading to impairments in protein folding (Muchowski and Wacker, 2005), increased

Abbreviations: DPN, diabetic peripheral neuropathy; FBG, fasting blood glucose; FCCP, carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone; Hsp, heat shock protein; iENF, intraepidermal nerve fiber; MNCV, motor nerve conduction velocity; MRC, maximal respiratory capacity; SNCV, sensory nerve conduction velocity; SRC, spare respiratory capacity; STZ, streptozotocin.

* Corresponding author at: Department of Pharmacology and Toxicology, The University of Kansas, 5064 Malott Hall, 1251 Wescoe Hall Dr., Lawrence, KS 66045, USA. Fax: +1 785 864 5219.
E-mail address: dobrowsky@ku.edu (R.T. Dobrowsky).

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interaction with molecular chaperones (Pratt et al., 2010) and decreased mitochondrial function (Fernyhough et al., 2010). Moreover, Hsp70 and Hsp90 are also components of the cellular heat shock response (HSR). Numerous conditions that promote cell stress lead to the Hsp90-dependent induction of the HSR which, in part, promotes the transient up-regulation of Hsp70 to aid the refolding or clearance of aggregated and damaged proteins (Pratt et al., 2010). Hsp70 upregulation can also prevent neuronal apoptosis (Bienemann et al., 2008) and decrease oxidative stress in neurodegenerative disorders (Chaudhury et al., 2006). Importantly, pharmacologic inhibitors of Hsp90 can induce Hsp70 and have shown potential in treating neurodegenerative diseases (Luo et al., 2007) and DPN (Urban et al., 2010).

Hsp90 contains a C-terminus ATP binding domain that weakly binds the antibiotic novobiocin (Marcu et al., 2000). Through systematic modification of the coumarin ring pharmacophore of novobiocin, KU-32 (Fig. 1) was identified as a C-terminal Hsp90 inhibitor whose ability to promote a heat shock response requires an acetamide substitution on the coumarin ring (Matts et al., 2011). Consistent with this effect, KU-32 protected against glucose-induced death of unmyelinated embryonic sensory neurons and ameliorated neuregulin-induced demyelination of myelinated Schwann cell and sensory neuron co-cultures in an Hsp70 dependent manner (Urban et al., 2010). Importantly, KU-32 is readily bioavailable and weekly treatment of diabetic mice with 20 mg/kg KU-32 reversed multiple clinical indices of DPN, including thermal hypoalgesia. Loss of thermal sensation in the feet involves dysfunction or loss of small intra-epidermal nerve fibers (iENF) that respond to thermal stimuli (Beiswenger et al., 2008a, 2008b). Loss of iENFs has been reported in both Type 1 and Type 2 diabetics (Boucek et al., 2005; Pittenger et al., 2005) as well as individuals with impaired glucose tolerance (Smith et al., 2001). Thus, interventions that can improve fiber recovery or preserve the function of remaining fibers may be particularly beneficial to help manage human DPN. However, diabetic rodents can show pharmacologic recovery of thermal sensation in the absence of appreciable increases in iENF density (Obrosova et al., 2010; Stavniichuk et al., 2011). In our previous study, it was unclear if the recovery of thermal sensitivity after KU-32 treatment was associated with an increase in iENF density since untreated diabetic mice did not exhibit a significant loss of iENFs (Urban et al., 2010). Additionally, though the efficacy of KU-32 required expression of Hsp70, little insight was gained into how KU-32 may affect neuronal physiology. It is well appreciated that increased oxidative stress and mitochondrial dysfunction contribute to the pathogenesis of DPN (Chowdhury et al., 2011; Obrosova, 2009). We have shown recently that prolonged diabetes downregulated numerous mitochondrial proteins in dorsal root ganglia and that decreases in the mitochondrial proteome correlated with a decrease in mitochondrial respiratory capacity (Akude et al., 2011; Chowdhury et al., 2011). Therefore, the current study addressed whether the improvement in DPN by KU-32 is associated with a recovery of iENF density and an enhanced bioenergetic profile of adult sensory neurons using Swiss Webster mice as a genetically outbred strain that develops severe DPN and shows rapid changes in iENF density in response to STZ-induced diabetes (Beiswenger et al., 2008a, 2008b; Kennedy and Zochodne, 2005).

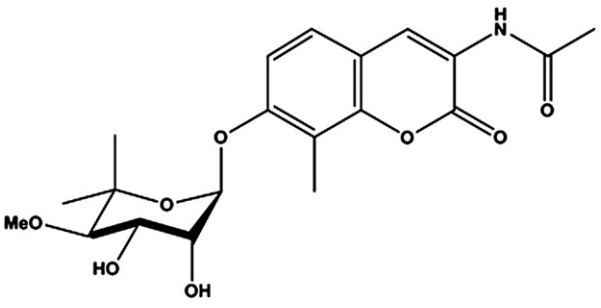

Fig. 1. Structure of KU-32.

Methods

Materials

Streptozotocin (STZ), carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), oligomycin 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], was synthesized and structural purity (>95%) verified as described (Huang and Blagg, 2007). Collagenase and laminin were obtained from Gibco/Invitrogen (Carlsbad, CA).

Induction of diabetes and drug treatments

Six-week old, outbred Swiss Webster mice were purchased from Harlan Laboratories (Indianapolis, IN) and diabetes induced at 8 weeks of age. After 6 h of food withdrawal, mice were injected with 100 mg/kg STZ dissolved in 0.2 ml of sterile 0.1 M sodium citrate in phosphate buffered saline. The STZ injection was repeated the next day and control mice received two injections of the vehicle. One week after the last injection, mice with fasting blood glucose (FBG) >290 mg/dl (One-Touch Ultra glucometer) were deemed diabetic. After 16 weeks, control and diabetic animals were given a once per week intra-peritoneal injection (0.2 ml) of 5 mM Captisol (CyDex Pharmaceuticals, Lenexa, KS) or 20 mg/kg KU-32 in 5 mM Captisol for 10 weeks. All animals were maintained on a 12 h light/dark cycle with ad libitum access to water and Purina diet 5001 rodent chow. FBG and HbA1c levels (A1C Now^{+}) were determined prior to euthanizing the animals.

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) and in compliance with standards and regulations for care and use of laboratory rodents set by the National Institutes of Health. To comply with veterinary oversight and IACUC approval, we also employed a body condition score (BCS) to assess an overall decline in body condition that was unlikely to spontaneously improve (Ullman-Cullere and Foltz, 1999). In a mouse, a BCS1 indicates advanced muscular wasting and extreme loss of subcutaneous fat deposits yielding prominent indentations between vertebrae and sharp protuberances of spinal processes, the ileum and sacrum. This is an extreme that we tried to avoid since death would be imminent. A mouse of BCS1^{+} status had less severe fat loss and with milder bony protuberances. If the mouse showed hunched posture, closed or sunken eyes and was lethargic, this was all taken into consideration in conjunction with a veterinary consult to euthanize the animal since it was unlikely to spontaneously improve.

Measures of nerve conduction velocity (NCV), mechanical and thermal sensitivity

Motor (MNCV) and sensory (SNCV) NCV measurements were performed on deeply anesthetized mice using a TECA™ Synergy N2-EMG Monitoring System as we have previously described in detail (McGuire et al., 2009). A Dynamic Plantar Aesthesiometer (Stoelting Inc., Wood Dale, IL) fitted with a stiff monofilament was used to assess mechanical sensitivity. Preliminary experiments indicated that applying the filament to the plantar surface at an upward force of 10 g was necessary to provide a sufficient dynamic range to detect mechanical hypoalgesia in the diabetic Swiss Webster mice. Thermal sensitivity was assessed by paw withdrawal latency to a ramping focal radiant heat using a Hargreaves Analgesiometer (Stoelting Inc.,