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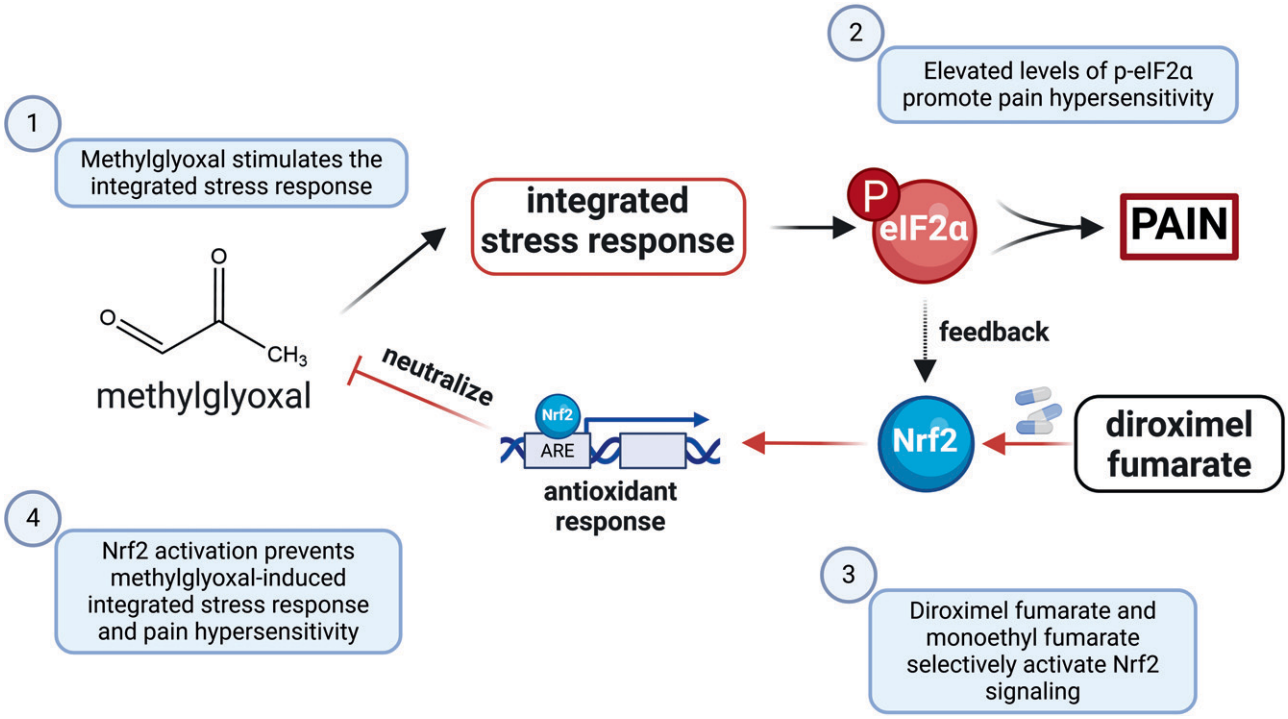

Diroximel Fumarate Acts Through Nrf2 to Attenuate Methylglyoxal-Induced Nociception in Mice and Decrease ISR Activation in DRG Neurons

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Diabetic neuropathic pain is associated with elevated plasma levels of methylglyoxal (MGO). MGO is a metabolite of glycolysis that causes pain hypersensitivity in mice by stimulating the phosphorylation of eukaryotic initiation factor 2α (p-eIF2α) and subsequently activating the integrated stress response (ISR). We first established that Zucker diabetic fatty rats have enhanced MGO signaling, engage ISR, and develop pain hypersensitivity. Since nuclear factor erythroid 2–related factor 2 (Nrf2) regulates the expression of antioxidant proteins that neutralize MGO, we hypothesized that fumarates, like diroximel fumarate (DRF), will stimulate Nrf2 signaling, and prevent MGO-induced ISR and pain hypersensitivity. DRF (100 mg/kg) treated animals were protected from developing MGO (20 ng) induced mechanical and cold hypersensitivity. Mechanistically, DRF treatment protected against MGO-induced increase in p-eIF2α levels in the sciatic nerve and reduced loss of intraepidermal nerve fiber density. Using Nrf2 knockout mice, we demonstrate that Nrf2 is necessary for the antinociceptive effects of DRF. Cotreatment of MGO (1 μmol/L) with monomethyl fumarate (10, 20, and 50 μmol/L), the active metabolite of DRF, prevented ISR in both mouse and human dorsal root ganglia neurons. Our data show that targeting Nrf2 with DRF is a strategy to potentially alleviate pain associated with elevated MGO levels.

ARTICLE HIGHLIGHTS

- Methylglyoxal (MGO) induces mechanical and cold hypersensitivity in mice that is prevented with daily treatment with diroximel fumarate (DRF).
- DRF protects against MGO-induced integrated stress response activation in the sciatic nerve and loss of intraepidermal nerve fibers.
- DRF treatment does not protect Nrf2 knockout mice from developing pain hypersensitivity, suggesting that Nrf2 is necessary for DRF's antinociceptive effects.
- Monomethyl fumarate, the active metabolite of DRF, prevents MGO-induced increase in p-eIF2α levels in mouse and human dorsal root ganglia neurons in vitro.
- Nrf2 activators, like the Food and Drug Administration–approved DRF, could be evaluated in clinical trials for diabetic neuropathic pain.

Diabetes is the most common metabolic disorder in the world, affecting around 10% of the population (1). Recent estimates have found that approximately half of all diabetic individuals eventually develop neuropathy, making diabetic neuropathic pain the most common form of neuropathic pain (2). Diabetic neuropathic pain is associated with worsening quality of life and increased burden on health care (2). Currently, diabetic neuropathic pain is treated using anticonvulsants like gabapentin and pregabalin, antidepressants like duloxetine, and opioids like tapentadol and tramadol (2,3). However, these therapies have modest efficacy and serious side effects (3,4).

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Prior studies have shown that oxidative stress and antioxidant pathways play a crucial role in the development and maintenance of diabetic neuropathy (5,6). Methylglyoxal (MGO) is a reactive dicarbonyl metabolite of glycolysis associated with elevated blood glucose levels, inflammation, and aging (7). Individuals suffering from type I and type II diabetic neuropathic pain have elevated plasma levels of MGO (~1 μmol/L), which is approximately three to six times higher than levels in patients with diabetes without neuropathic pain (8–11). MGO interacts with amino acid residues like cysteine, arginine, and lysine to produce advanced glycation end products (AGEs) and promote the synthesis of reactive oxygen species (ROS) (12–14). We have recently demonstrated that levels of MGO (1 μmol/L) associated with diabetic neuropathic pain stimulates the integrated stress response (ISR) and that pharmacologically inhibiting ISR alleviates MGO-induced mechanical hypersensitivity (15,16). ISR is an adaptive response to stressors, such as accumulated misfolded proteins and elevated ROS, that is initiated by the phosphorylation of eukaryotic initiation factor 2α (eIF2α) (17). ISR suppresses global protein synthesis and facilitates the translation of specific mRNAs, like the mRNA encoding activating transcription factor 4 (ATF4), which influence neuronal activity and viability (17). ISR also activates nuclear factor erythroid 2–related factor 2 (Nrf2), a potent transcription factor, to protect cells from and reverse oxidative stress (18,19). However, it is clear that ISR-mediated Nrf2 antioxidative response is not sufficient to prevent pain hypersensitivity, since ISR-inducing compounds that engage Nrf2 signaling, like MGO and tunicamycin, still cause pain hypersensitivity (15,18,20).

The glutathione system acts as a critical node in the antioxidant response because dicarbonyls like MGO are first neutralized by a nonenzymatic reaction with glutathione to generate hemithioacetal, which is further metabolized by glyoxalase 1 (Glo1) and glyoxalase 2 (Glo2) to generate inert D-lactate (21). Nrf2 is a transcription factor that regulates the expression of many antioxidant proteins by binding to their antioxidant response elements (ARE) (22). The gene for glutamate cysteine ligase subunit (Gclm), the rate-limiting protein in the synthesis of glutathione, and Glo1 contain an ARE in their 5′ untranslated regions, and their transcription is promoted by Nrf2 (22,23). Fumaric acid esters like diroximel fumarate (DRF), dimethyl fumarate (DMF), and their active metabolite, monomethyl fumarate (MMF), are known activators of the Nrf2 antioxidant pathway (24–26). All are Food and Drug Administration (FDA) approved for the treatment of relapsing remitting multiple sclerosis (MS), a neuroimmune and neurodegenerative disorder (26,27). Omaveloxolone, a structurally different Nrf2 activator, was recently approved for the treatment of Friedreich ataxia, a genetic neurodegenerative disorder that causes poor coordination and speech. The clinical development of these molecules suggests that activating the Nrf2 pathway is a safe strategy for neuroprotection. Building upon this, we hypothesized that DRF and pharmacological activation of the Nrf2 antioxidant pathway can be leveraged to reduce MGO-induced nociceptive hypersensitivity and signaling associated with pain in both mouse and human DRG neurons.

In addition to the production of AGEs, elevated levels of MGO can increase the production of ROS (13,28). Oxidative stress is a causative factor in the progression of diabetic neuropathy (5,6). Treating diabetic neuropathy by targeting the ROS with exogenously administered antioxidants has only been modestly effective (5,29,30), potentially because these compounds fail to reach sufficient intracellular concentrations to impede the actions of locally produced reactive oxygen. A safe and effective therapeutic targeting of this mechanism still remains elusive, but increasing the cellular capacity to scavenge reactive oxygen through Nrf2 is an attractive mechanism. Our results provide support for targeting the Nrf2 signaling pathway as a strategy for alleviating pain associated with MGO-associated neuropathy.

RESEARCH DESIGN AND METHODS

Animals

The Zucker diabetic fatty (ZDF) (Charles River Laboratories, Wilmington, MA) rat and its lean littermates were used in this study. Nrf2 knockout (Nrf2KO) mice (B6.129X1-Nfe2l2tm1Ywk/J) were obtained from Jackson Laboratories (strain 017009) and cross-bred with wild-type C57BL/6 mice over multiple generations. All experiments were performed according to guidelines established by the National Institutes of Health and the Animal Care and Use Committee at the University of Texas at Dallas (protocol 14-04). Detailed information on animals used in this study is presented in the Supplementary Materials.

Mouse and Human DRG Cultures

The protocols for culturing mouse and human DRG neurons are presented in the Supplementary Material.

Drugs and Chemicals

Methylglyoxal (~40% in H_2O) was obtained from Sigma-Aldrich (M0252). It was further dissolved in 0.9% normal saline or culturing media for in vivo and in vitro experiments, respectively. DRF (Biogen) was dissolved in 10 mmol/L citric acid/0.5% carboxymethylcellulose/0.02% Tween80, adjusted to pH 5.0; 10 mmol/L citric acid/0.5% carboxymethylcellulose/0.02% Tween80 (pH 5.0) was used as vehicle. Animals were given DRF at 60 mg/kg or 100 mg/kg as oral gavage daily throughout the experiment. For in vitro studies, MMF (651419; Sigma-Aldrich) was initially dissolved to 500 mmol/L in 100% DMSO. Subsequent dilutions of MMF were performed in culturing media. For in vitro experiments, vehicle was equivalent to DMSO concentration in the highest MMF treatment group (i.e., 0.01% DMSO).

Mouse and Rat Behavior Testing

Details of mouse and rat behavior testing are outlined in the Supplementary Materials.

Western Blotting and Carboxyethyl Lysine ELISA

Western blotting was performed as previously described (16,31) and is detailed in the Supplementary Materials. Details for *N*-ε-carboxyethyl lysine (CEL) ELISA are mentioned in the Supplementary Materials.

Immunocytochemistry

The immunocytochemistry methodology is outlined in the Supplementary Materials.

Paw Biopsy and Intraepidermal Nerve Fibers

Details of hind paw skin biopsy, intraepidermal nerve fiber (IENF) staining, and quantification are outlined in the Supplementary Materials.

Data Analysis

All results are presented as mean ± SEM. Statistical analyses are mentioned in figure legends. In general, comparisons between two groups were performed using Student *t* test with or without Welch correction, depending on the homoscedasticity of the data set. When comparing more than two conditions, one-way or two-way ANOVAs were used with or without correction for homoscedasticity of the data set. BioRender was used to build schematics. Data were graphed and analyzed on GraphPad Prism 10.

Data and Resource Availability

The data sets generated during and analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

ISR Is Activated in ZDF Rats

ZDF rats carry a mutation in the fa-type leptin receptor, leading them to spontaneously develop obesity and type II diabetes when fed a high-fat diet, ad libitum (Fig. 1*A*). ZDF rats serve as a valuable model to study diabetes, as they resemble many metabolic and physiological characteristics of human diabetes, like hyperglycemia, hyperinsulinemia, obesity, nephropathy, and neuropathy (32–34). Lean control rats only carry one mutated version of the fa receptor and are protected from obesity and hyperglycemia. Throughout our study, ZDF rats had elevated blood glucose levels and increased body weights compared with lean control rats (Fig. 1*B* and *C*). Since pain is a clinical feature of diabetes and diabetic neuropathy, we sought to assess heat, cold, and mechanical sensitivity in the ZDF rats. We found that ZDF rats progressively became hypersensitive to heat, cold, and mechanical stimuli as assessed by Hargreaves, acetone, and Randall-Selitto tests, respectively (Fig. 1*D*–*F*).

MGO has been linked to diabetic neuropathic pain in humans (8,35). To address whether MGO is increased in the DRG of ZDF rats, we quantified levels of CEL using a competitive ELISA. CEL is an MGO-specific AGE that accumulates in modified proteins of diabetes-affected tissues (36). We found that CEL levels were almost doubled in the lumbar DRGs of 16-week-old ZDF rats compared with lean controls, suggesting that MGO levels are increased in the ZDF diabetic model (Fig. 1*G*). At the same time point, we also observed an increase in phosphorylation of eIF2α (as a ratio of phospho to total-eIF2α, p/t-eIF2α) in the DRGs of 16-week-old ZDF rats, when compared with DRGs of lean control rats (Fig. 1*H*–*K*). These observations and the fact that MGO levels are elevated in human diabetes (8) formed the basis of our hypothesis that diabetes-induced MGO promotes ISR and leads to the development of pain hypersensitivity and that interfering with this pathway could pave the way for novel analgesics.

DRF Prevents MGO-Induced Mechanical and Cold Hypersensitivity

In this study, we hypothesized that DRF would prevent MGO-induced mechanical and cold pain hypersensitivity. DRF activates Nrf2 and the glyoxalase and glutathione antioxidant pathways, which are key to neutralizing MGO (Fig. 2*A*). We treated male and female mice daily with vehicle (DRF vehicle) or DRF at either 60 mg/kg (DRF-60) or 100 mg/kg (DRF-100) given orally for 5 days. On day 3, an intraplantar injection of MGO (20 ng) was also administered 1 h after oral DRF for that day. von Frey assay and acetone tests were performed to assess mechanical and cold sensitivity (Fig. 2*B*). After the first 2 days of DRF treatment, we found no change in mechanical thresholds in male and female mice in response to only DRF administration at either 60 mg/kg and 100 mg/kg, suggesting that DRF has no intrinsic effects on mechanical thresholds. Male and female mice that were administered MGO and treated with DRF vehicle developed hypersensitivity to tactile stimulation when compared with vehicle-only animals (Fig. 2*C* and *D*). DRF treatment, particularly at 100 mg/kg (DRF-100), prevented MGO-induced mechanical pain hypersensitivity. Areas under the curve of von Frey thresholds were used to directly compare each treatment group (Fig. 2*E* and *F*). Female animals were protected at both the 60 mg/kg and 100 mg/kg doses, while the male animals only benefited from a higher dose of 100 mg/kg of the drug. DRF treatment at both 60 mg/kg and 100 mg/kg completely prevented cold hypersensitivity on day 3 in both male and female mice (Fig. 2*G* and *H*). By day 5, cold sensitivity returned to baseline levels in all conditions. These results show that treatment with DRF prevents mechanical and cold hypersensitivity in response to MGO.

DRF Protects Against MGO-Induced ISR and Intraepidermal Denervation

We followed up our behavioral observations with a separate set of experiments to uncover how DRF treatment can be protective against MGO-induced pathology. Since diabetes predominantly affects the nerves, we dissected the sciatic nerve

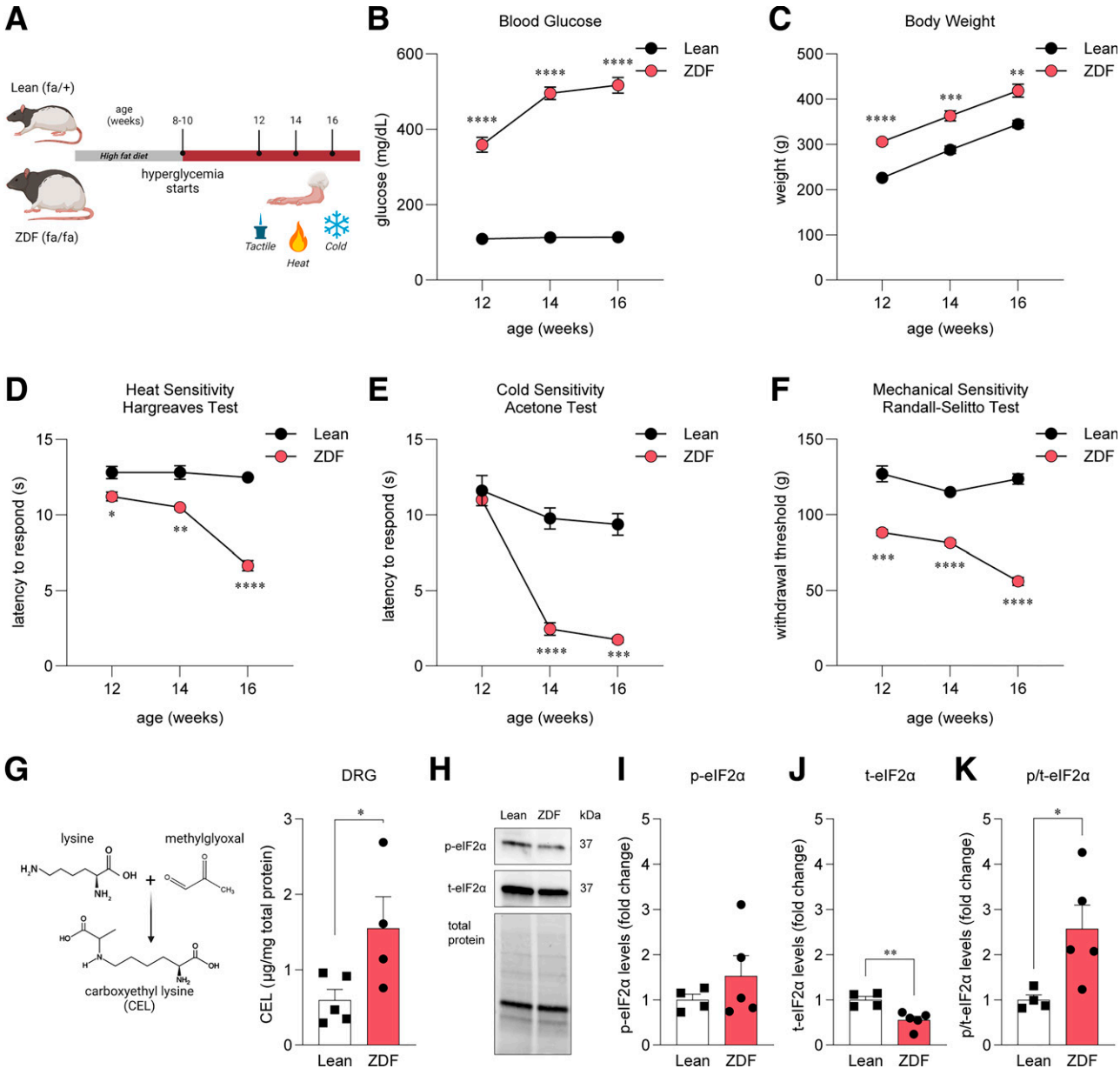

Figure 1—ZDF rats develop hyperglycemia and pain hypersensitivity that correlate with increased CEL and enhanced ISR in DRG. *A*: Male ZDF rats carry a mutation in the leptin receptor (fa/fa) and develop hyperglycemia, obesity, and neuropathy when fed a high-fat diet. We tested ZDF rats for mechanical, thermal, and cold hypersensitivity at 12, 14, and 16 weeks of age. *B* and *C*: ZDF rats have elevated blood glucose levels and increased weight when compared with lean (fa/+) controls. *D–F*: Similarly, ZDF rats develop thermal, cold, and mechanical hypersensitivity by 16 weeks of age. *G*: Since MGO has been linked to neuropathic pain in diabetes, we quantified the levels of CEL, an MGO-specific AGE, in DRG of ZDF and lean rats. We found that CEL levels were significantly increased in DRG of 16-week-old ZDF rats. *H–K*: At the same time point, phosphorylation of eIF2α was also increased in ZDF rat DRG. These experiments link hyperglycemia, MGO signaling, and ISR in an animal model. For *B–F*, significance was calculated using repeated measures two-way ANOVA followed by Sidak multiple comparison test. For *I–K*, statistical significance was determined using unpaired *t* test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

from vehicle-only, MGO-treated, and MGO plus DRF (100 mg/kg) treated mice 24 h after the MGO injection (Fig. 3*A*). Based on our previous experiment, this time point represented the greatest difference between the control and treatment groups. Using Western blotting, we found that phosphorylation of eIF2α (p/t-eIF2α) was increased in the sciatic nerves of MGO-treated mice, and this effect was abrogated with DRF treatment (Fig. 3*B* and *C*). Subsequent investigation revealed that Gclm, a target of Nrf2 and the rate-limiting step in glutathione synthesis (22), was significantly elevated in the sciatic nerve of DRF-treated mice (Fig. 3*D* and *E*). This suggests that DRF treatment stimulates an antioxidant response and prevents the engagement of MGO-induced ISR, a diabetes and pain-associated mechanism, in vivo.

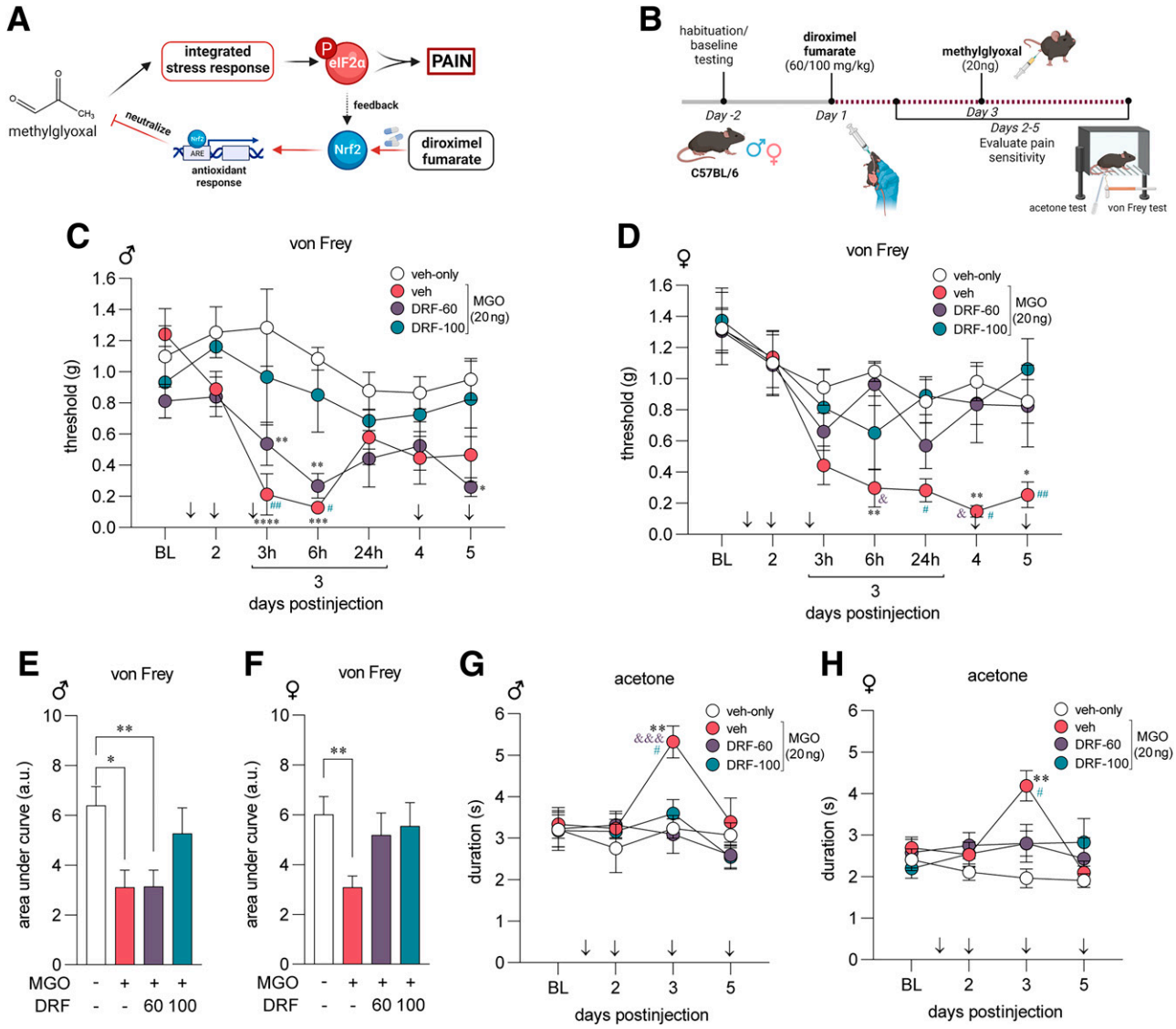

Figure 2—DRF pretreatment protects against MGO-induced pain hypersensitivity. *A*: MGO stimulates ISR and promotes the phosphorylation of eIF2α. DRF is a prodrug and is metabolized to MMF, which activates the Nrf2 antioxidant response neutralizing MGO. *B*: Male (n = 8 per condition) and female (n = 8 per condition) mice were treated with DRF (60 or 100 mg/kg) for 5 days. MGO (20 ng) was administered in an intraplantar injection on the third day, 1 h after oral DRF administration for the day. Mechanical and cold sensitivity was assessed using von Frey and acetone tests, respectively. Acetone test was performed an hour after von Frey testing was complete. We observed that male (*C*) and female (*D*) mice had reduced von Frey thresholds in response to MGO injection on day 3. Daily treatment with DRF prevented MGO-induced mechanical hypersensitivity following MGO injection on day 3. *E* and *F*: Using area under the curve of von Frey thresholds, we determined that DRF at a dose of 100 mg/kg was effective at preventing MGO-induced tactile hypersensitivity in males, while both 60 mg/kg and 100 mg/kg doses were effective in females. MGO produced cold hypersensitivity in male (*G*) and female (*H*) mice, which was abrogated in DRF-treated mice at both doses. For *C*, *D*, *G*, and *H*, significance was calculated with repeated measures two-way ANOVA with Tukey multiple comparison test. *, &, and #: $P < 0.05$; ** and ##: $P < 0.01$; *** and &&&: $P < 0.001$; ****$P < 0.0001$. *indicates comparison with veh-only, & indicates comparison with MGO + DRF-60, and # indicates comparison with MGO + DRF-100. For *E* and *F*, significance was calculated with a one-way ANOVA with Brown-Forsythe correction. *$P < 0.05$, **$P < 0.01$.

Loss of intraepidermal innervation, as quantified by the number of free nerve endings crossing the dermal-epidermal junction, is a well-established diagnostic marker of diabetic neuropathy (37,38). We next sought to assess whether MGO reduced IENF density, mirroring diabetic neuropathy, and whether DRF treatment was protective. To this end, we immunostained skin biopsies from the hind paw for protein gene product 9.5 (PGP9.5) as a marker for peripheral nerve fibers. We found that MGO injection in the paw caused a significant reduction in the number of IENF crossings from dermis to epidermis, and treatment with DRF prevented the loss of IENFs following MGO (Fig. 3*F–I*). These data imply that an intraplantar MGO injection recapitulates neuropathic features of diabetic neuropathy and that pretreatment with DRF protects against neuropathy.

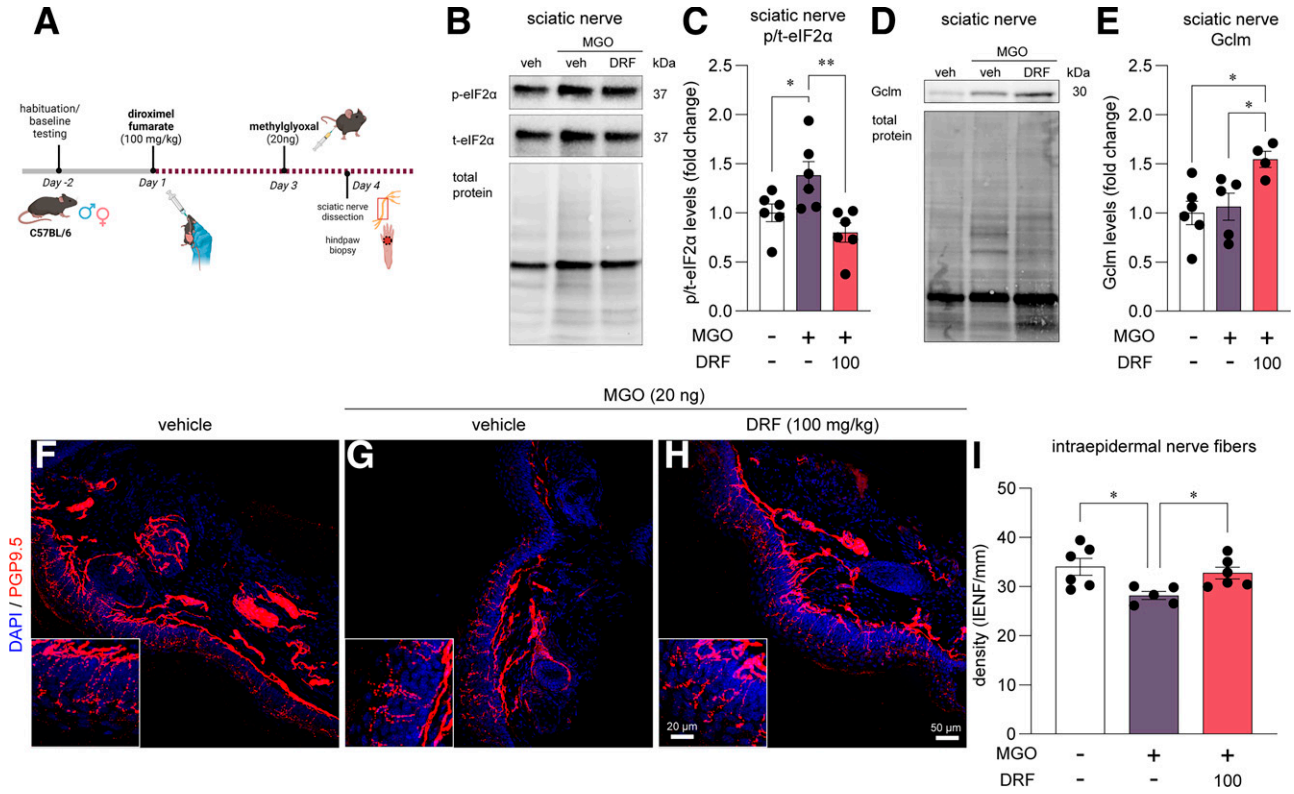

Figure 3—DRF prevents MGO-induced ISR in the sciatic nerve and the loss of IENF. *A*: Male (n = 3) and female (n = 3) mice were treated with DRF for 2 days prior to a single MGO (20 ng) injection. The following day, 24 h later, sciatic nerves and hind paw biopsies were obtained. *B* and *C*: MGO injection in the hind paw increased phosphorylation of eIF2α in the sciatic nerve, which was prevented in animals treated with DRF (100 mg/kg). *D* and *E*: Moreover, DRF treatment increased protein levels of Gclm, a direct transcriptional target of Nrf2 and a critical node in glutathione synthesis. *F–I*: Hind paw skin biopsies were stained for PGP9.5, and IENF density was quantified as number of dermal-epidermal crossings per millimeter of tissue. We found that DRF treatment protected against MGO-induced reduction in IENF density. Statistical significance was determined using one-way ANOVA. $^*P < 0.05$, $^{**}P < 0.01$.

DRF Requires Nrf2 to Counteract the Pain-Promoting Effects of MGO

Endoplasmic reticulum (ER) pathology is a canonical feature of elevated MGO (15,39). MGO stimulates PERK to phosphorylate eIF2α and Nrf2 to instigate ISR and antioxidant mechanisms, respectively. We have previously shown that p-eIF2α signaling is critical for developing mechanical hypersensitivity in multiple models of neuropathic pain, including MGO-induced hypersensitivity (15). To delineate whether the protective effects of Nrf2 arm affects the duration and intensity of nociceptive hypersensitivity following MGO administration, we treated wild-type and global Nrf2KO animals with a single intraplantar injection of MGO (20 ng) or vehicle (saline) and followed these animals for 20 days. Since we found no evidence of sex-specific effects of DRF (Fig. 2), we performed these experiments with an equal number of male (n = 4) and female (n = 4) mice in each group. We found that MGO induced robust tactile hypersensitivity in wild-type (n = 8) and Nrf2KO (n = 8) animals compared with the vehicle-treated group and that effect was comparable between genetic strains (Fig. 4*A* and *B*). We directly compared the response to MGO using areas under the curve of von Frey thresholds of each genotype and found no significant difference in the response of Nrf2KO and wild-type mice to MGO administration (Fig. 4*C*).

We next sought to answer whether DRF's antinociceptive effects are mediated specifically by Nrf2. We treated male and female Nrf2KO mice (n = 8) and their wild-type littermates (n = 8) with 100 mg/kg of DRF (DRF-100) and MGO following the same treatment paradigm as outlined in Fig. 2. We discovered that Nrf2KO animals developed MGO-induced mechanical hypersensitivity that was not prevented with DRF treatment (Fig. 5). On the contrary, wild-type littermates were protected from MGO-induced tactile hypersensitivity (Fig. 5). Our data taken from Figs. 4 and 5 suggest that Nrf2 is not necessary for the development, maintenance, or resolution of MGO-induced mechanical hypersensitivity while pharmacologically promoting Nrf2 signaling plays an important role in preventing it.

MMF Suppresses MGO-Induced ISR and Reduces p-eIF2α in Mouse and Human Sensory Neurons

DRF is a prodrug that is metabolized in the small intestines to the active compound, MMF, and an inactive compound, 2-hydroxyethyl succinimide (26,40). We have previously shown that MGO promotes ISR by phosphorylating eIF2α

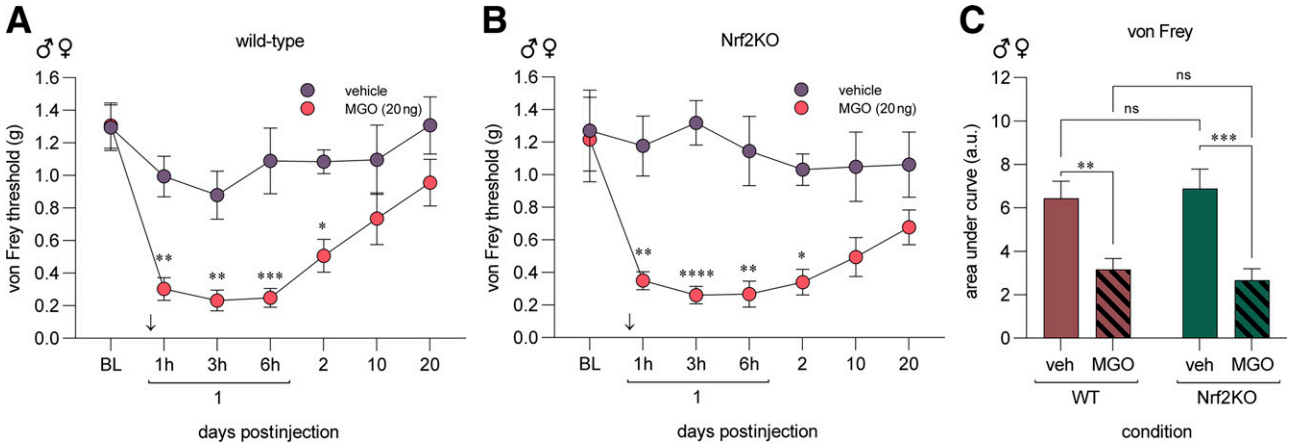

Figure 4—MGO induces mechanical pain hypersensitivity in Nrf2KO mice. Wild-type (*A*) and Nrf2KO (*B*) animals were injected with MGO (20 ng) or vehicle (saline) in the left paw. Mechanical sensitivity was assessed using the von Frey test for up to 20 days after injection. MGO (20 ng) injection produced mechanical hypersensitivity in both wild-type and Nrf2KO mice. *C*: Area under the curve of von Frey thresholds was used to directly compare the response of wild-type and Nrf2KO animals to MGO injection. Each condition included four male and four female mice for a total of eight animals. For *A* and *B*, statistical analysis was performed with repeated measures two-way ANOVA followed by Sidak multiple comparison test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. In *C*, two-way ANOVA with Tukey test was used. $**P < 0.01$, $***P < 0.001$.

and that reversing or preventing ISR alleviates pain associated with MGO (15,16). To investigate whether MMF inhibits ISR, we treated sensory neuron cultures from independent male (n = 2) and female (n = 2) mouse dorsal root ganglia (DRG) with MGO (1 μmol/L) and MMF (10, 20, and 50 μmol/L) or vehicle for 24 h. Afterward, cells were fixed with formalin and processed for immunohistochemistry. We observed an increase in p-eIF2α immunoreactivity in cells treated with MGO compared with vehicle-treated cells (Fig. 6*A* and *B*). This increase in p-eIF2α levels was prevented when MGO-treated cultures were cotreated with MMF in a concentration-dependent manner (Fig. 6*C* and *D*).

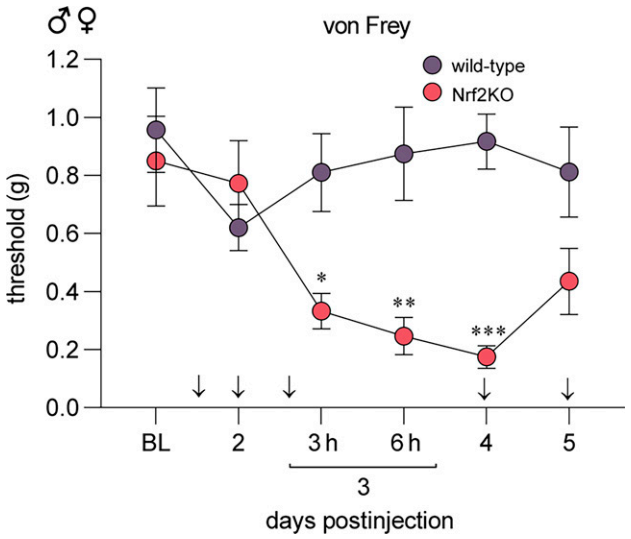

Figure 5—DRF treatment fails to prevent mechanical hypersensitivity in Nrf2KO mice. Wild-type and Nrf2KO animals were treated with DRF at 100 mg/kg based on the paradigm outlined in Fig. 2*A*. MGO (20 ng) was injected into the paw on day 3 since the start of oral DRF treatment in wild-type (n = 8, four males and four females) and Nrf2KO (n = 8, four males and four females) littermates. Nrf2KO animals developed mechanical hypersensitivity compared with the wild-type animals, suggesting that DRF treatment was unable to prevent MGO-induced hypersensitivity in Nrf2KO animals. A repeated measures two-way ANOVA was used followed by Sidak post hoc test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

To expand the clinical relevance of our findings, we sought to replicate our murine findings in human sensory neurons from cultured DRGs recovered from organ donors (Fig. 7*A*–*C*). MGO (1 μmol/L) treatment elevated p-eIF2α immunoreactivity in human sensory neurons, and cotreating them with MGO (1 μmol/L) and MMF (10, 20, and 50 μmol/L) prevented the increase in p-eIF2α levels (Fig. 7*D*). Similar to mouse DRG neurons, we observed a significant reduction in p-eIF2α immunoreactivity at 20 and 50 μmol/L MMF concentrations (Fig. 7*D*). Hence, mouse and human DRG neurons in vitro are protected from MGO-induced ISR by MMF.

DISCUSSION

In this study, we show that ZDF diabetic rats develop pain hypersensitivity, have increased CEL, an MGO-specific AGE, and elevated phosphorylation of eIF2α in DRG. We also demonstrate that DRF prevents MGO-induced mechanical and cold hypersensitivity in mice and protects against the engagement of ISR and loss of IENF. Using Nrf2KO mice, we demonstrate that endogenous Nrf2 signaling in response to MGO (1 μmol/L) does not affect the initiation, maintenance, or resolution of MGO-induced mechanical pain hypersensitivity. However, the antinociceptive effect of DRF is lost in Nrf2KO mice, suggesting that DRF's effects on Nrf2 are essential for reducing mechanical hypersensitivity in mice. At a concentration of MGO observed in the plasma of

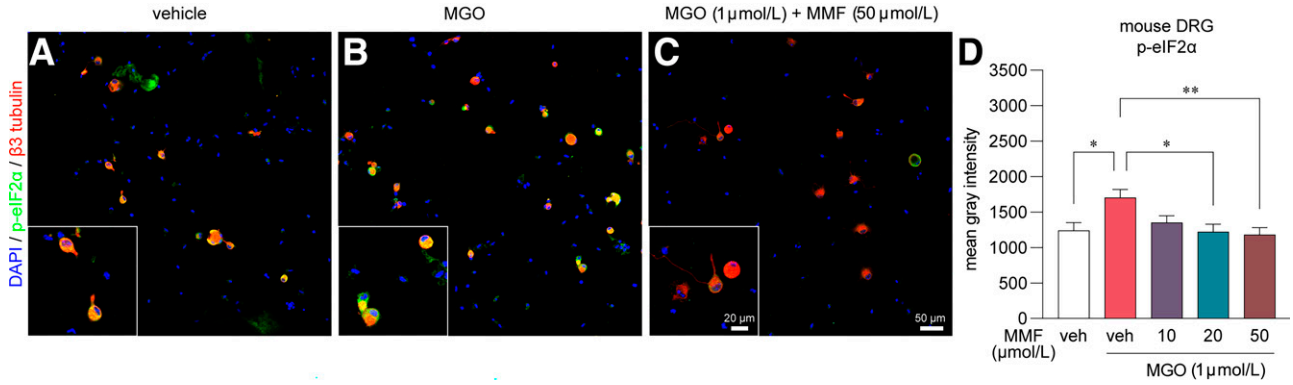

Figure 6—MMF prevents MGO-evoked increases in p-eIF2α levels in mouse DRG neurons. *A–C*: Representative images of immunohistochemistry of mouse DRG neurons cultured for 24 h and subsequently treated with vehicle only, MGO (1 μmol/L), or MGO (1 μmol/L) plus MMF (10, 20, or 50 μmol/L). *D*: We observed a significant increase in the levels of p-eIF2α when cells were treated with MGO as compared with vehicle-treated cells. MMF treatment with MGO prevented elevation of p-eIF2a, particularly at 20 μmol/L and 50 μmol/L concentrations. Neurons were identified by their expression of β3 tubulin. Vehicle-only (n = 18), MGO (n = 37), MGO + 10 μmol/L MMF (n = 20), MGO + 20 μmol/L MMF (n = 16), and MGO + 50 μmol/L MMF (n = 32). One-way ANOVA was used to calculate significance. *$P < 0.05$, **$P < 0.01$.

people with diabetic neuropathic pain (1 μmol/L), MGO activates ISR and elevates levels of p-eIF2α (15,16). This effect is significantly blunted in cultured mouse and human DRG neurons when MGO is coadministered with the active metabolite of DRF, MMF. Our data collectively provide evidence for targeting the Nrf2 antioxidant system for treating MGO-induced nociception and suggest that drugs targeting this pathway, like DRF, could be amenable for pain treatment in people with diabetic neuropathy and elevated MGO levels.

Here, we demonstrate that the Nrf2 signaling pathway can be leveraged to fortify and protect against ongoing oxidative and dicarbonyl stress responsible for MGO-induced pain hypersensitivity. Nrf2 is a master transcription factor that regulates the transcription of other antioxidant genes, particularly those with an ARE element in their promoter region (22). Nrf2 also regulates bioenergetics and mitochondrial function to fine-tune mitochondrial integrity and oxidative stress (41). Nrf2 activators, DMF and monoethylfumarate, in cultured human astrocytes engage the glutathione and glyoxalase pathways that peak 12 to 24 h after administration (42). We thus hypothesized that pretreatment with DRF will prime the antioxidant system such that exogenous MGO is promptly neutralized, protecting the cells against the detrimental effects of MGO. Although not directly addressed in our work, this hypothesis is testable in future experiments.

The interaction between ISR and the Nrf2 pathway is complex, with feedforward and feedback mechanisms. Conditions of ER stress prime PERK to phosphorylate both eIF2α and Nrf2, dissociating Nrf2 from its inhibitor Keap1, and facilitating its nuclear translocation (19). A recent report also found that ER stress-induced ATF4 increases *Nrf2* transcription, increasing the global pool of Nrf2 to counteract ROS (18). In addition, MGO at high concentrations (1 mmol/L) can covalently modify Keap1

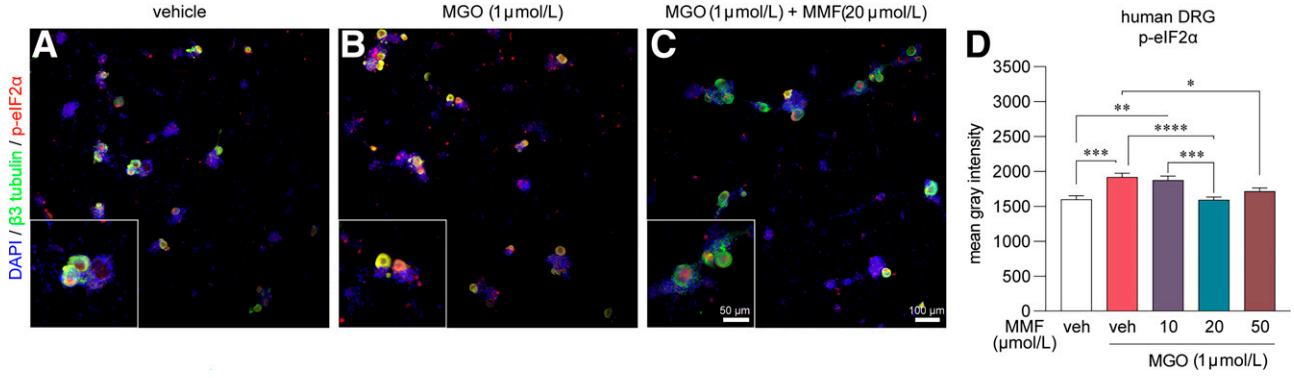

Figure 7—MMF prevents MGO-evoked increases in p-eIF2α levels in human DRG neurons. *A–C*: Representative images of human DRG neurons obtained from organ donors treated with vehicle, MGO (1 μmol/L), and MGO + MMF. *D*: MGO (1 μmol/L) increases p-eIF2α in human DRG neurons, which is prevented with 20 μmol/L and 50 μmol/L MMF cotreatment. Vehicle only (n = 170), MGO (n = 152), MGO + 10 μmol/L MMF (n = 125), MGO + 20 μmol/L MMF (n = 176), and MGO + 50 μmol/L MMF (n = 111). One-way ANOVA was used to calculate statistical significance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

to form dimers and promote Nrf2 activity (43). Based on these prior observations, we expected that MGO-induced mechanical hypersensitivity would be more pronounced or take longer to resolve in Nrf2KO mice than in wild-type animals. To determine how endogenous Nrf2 signaling affects the characteristics of MGO-induced mechanical hypersensitivity, we treated Nrf2KO mice with MGO and assessed their mechanical hypersensitivity for up to 20 days. Surprisingly, we found no difference in tactile hypersensitivity between wild-type animals and Nrf2KO animals. This suggests that the endogenous activation of Nrf2 antioxidant systems is not sufficient to prevent or alleviate pain hypersensitivity and that an exogenous application of Nrf2 activators is required to galvanize the antioxidant pathway. One possible reason for this is that the kinetics of the ISR and Nrf2 antioxidant responses are different. ER stress and ROS potently activate ISR in the first few hours, while Nrf2-mediated antioxidant response requires roughly 24 h (18). Coupled with the observation that exogenous MGO induces pain hypersensitivity in the first few hours, it is likely that ISR-dependent pain-causing mechanisms have already been set in motion before the endogenous antioxidant Nrf2 response takes effect.

Fumaric acid esters obtained from fungi have been used to treat inflammatory conditions since medieval times (26). Next-generation fumaric acid esters were developed in the late 1980s (26). Most recently, DRF received FDA approval for relapsing remitting MS (27). In clinical trials, DRF was found to be superior to its predecessor, DMF, because of its improved side effect profile, particularly a reduction in adverse gastrointestinal effects and abdominal pain because of a lack of methanol byproduction created by esterase activity (40). We have previously shown that DMF's antinociceptive effects are dependent on Nrf2 activity in a rodent model of traumatic nerve injury (25). Since DMF and DRF share the same active metabolite, MMF, we speculated that DRF's antinociceptive effects are specifically due to its activity on Nrf2. A recently published study found that DRF changed the transcriptome of cultured human astrocytes that was consistent with Nrf2 activation; however, no causal link was presented (24). Our data provide evidence that DRF's antinociceptive effects in the MGO-induced pain model are specifically mediated by Nrf2, as DRF was unable to prevent MGO-induced mechanical hypersensitivity in global Nrf2KO mice. Contrary to our findings, DMF reduced pain hypersensitivity in a rodent model of complex regional pain syndrome through an Nrf2-independent mechanism (44). This suggests that the specificity of DMF/DRF-Nrf2 signaling may depend on the pain model being studied.

Skin biopsies are routinely used, in combination with clinical assessments of neuropathy, to quantify the loss of IENF density as a diagnostic criterion for diabetic small fiber neuropathy (37,38,45). Here, we show that MGO injection in the hind paw reduces IENF density, and this is prevented in animals treated with DRF. These observations suggest that *1*) MGO (20 ng) causes neuropathy similar to that observed in diabetic conditions, and *2*) engaging the Nrf2 pathway by DRF treatment protects against MGO-induced neuropathy. We have previously shown that rodent $IB4^+$ nonpeptidergic nociceptors are more susceptible to engaging ISR following MGO treatment than other neuronal subtypes (15). Using our current approach, we cannot discern whether MGO specifically causes the loss of $IB4^+$ nonpeptidergic innervation of the epidermis and whether peptidergic and large-diameter fibers are also affected. Moreover, the current approach of quantifying the crossing of $PGP9.5^+$ fibers into the epidermis does not consider more nuanced morphology metrics like branching, swellings, beadings, and varicosities, which have been previously described in patients with neuropathy (45–49). Morphology metrics are more sensitive to diabetic pathology and can provide early detection of nerve fiber degeneration, as may be the case with nerve fiber swellings (46,47) and increased branching complexity (48). While not addressed in this report, these metrics can be tested in future experiments.

While our data provide promising results for an Nrf2 activator in reducing the pain-promoting effects of MGO, it comes with some limitations. First, we acknowledge that we have yet to demonstrate that DRF treatment reduces pain hypersensitivity, ISR, and p-eIF2α levels in the ZDF diabetic model; however, our experiments with MGO in mice and on human neurons provide clear support for this hypothesis. Second, because of a strong body of literature (21,23) that suggests that Nrf2 transcriptionally controls Gclm and Glo1 expression, we reasoned that a possible mechanism of action of DRF is that Nrf2 activation neutralizes MGO before it can be pathogenic. However, we have not quantified levels of MGO or AGEs following DRF treatment to specifically test this hypothesis. Our data here present a strong rationale for future experiments addressing whether pretreatment with Nrf2 activators neutralizes MGO. Third, our experiments with DRF were prevention experiments where we pretreated animals with DRF prior to the MGO injection. In a clinical setting, preventative pain medicines are rarely prescribed. Whether DRF can reverse already established MGO-induced pain hypersensitivity remains to be tested. Because DRF is clinically available, we contend that the in vivo and in vitro data we provide are strong support for planning of a future clinical trial (50). Further development of Nrf2 activators that locally deliver the drug at sites of oxidative stress while limiting off-target side effects provides a promising avenue for treating neuropathic pain (50).

Since MGO and its byproduct, CEL, can be measured in patient populations, our work provides a strong rationale for testing Nrf2 activators in people with diabetic neuropathic pain specifically associated with elevated MGO concentrations. This approach is feasible because levels of MGO and MGO-specific AGEs like CEL can easily be assessed from routine blood chemistry analysis of diabetic patients, stratifying a patient population that would ideally respond to DRF treatment. Having been FDA-approved,

DRF has already demonstrated itself to be safe and well-tolerated, so such clinical trials could be conducted.

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Author Contributions. M.S.Y. designed and performed experiments and analyzed data. M.M.M., B.J.W., V.T., L.H., R.A., D.R., and J.Z. performed experiments. J.L. generated Nrf2KO animals and maintained the colony. M.S.Y., M.C., P.M.G., and T.J.P. supervised the study. M.S.Y. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Koye DN, Magliano DJ, Nelson RG, Pavkov ME. The global epidemiology of diabetes and kidney disease. Adv Chronic Kidney Dis 2018;25:121–132
2. Feldman EL, Callaghan BC, Pop-Busui R, et al. Diabetic neuropathy. Nat Rev Dis Primers 2019;5:41
3. Schreiber AK, Nones CF, Reis RC, Chichorro JG, Cunha JM. Diabetic neuropathic pain: Physiopathology and treatment. World J Diabetes 2015;6:432–444
4. Finnerup NB, Attal N, Haroutounian S, et al. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. Lancet Neurol 2015;14:162–173
5. Sifuentes-Franco S, Pacheco-Moisés FP, Rodríguez-Carrizalez AD, Miranda-Díaz AG. TherRole of oxidative stress, mitochondrial function, and autophagy in diabetic polyneuropathy. J Diabetes Res 2017;2017:1673081
6. Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis of diabetic neuropathy. Endocr Rev 2004;25:612–628
7. Allaman I, Bélanger M, Magistretti PJ. Methylglyoxal, the dark side of glycolysis. Front Neurosci 2015;9:23
8. Bierhaus A, Fleming T, Stoyanov S, et al. Methylglyoxal modification of Nav1.8 facilitates nociceptive neuron firing and causes hyperalgesia in diabetic neuropathy. Nat Med 2012;18:926–933
9. Andersen ST, Witte DR, Dalsgaard E-M, et al. Risk factors for incident diabetic polyneuropathy in a cohort with screen-detected type 2 diabetes followed for 13 years: ADDITION-Denmark. Diabetes Care 2018;41:1068–1075
10. Al-Saoudi E, Christensen MMB, Nawroth P, et al. Advanced glycation end-products are associated with diabetic neuropathy in young adults with type 1 diabetes. Front Endocrinol (Lausanne) 2022;13:891442
11. Shuck SC, Achenbach P, Roep BO, et al. Methylglyoxal products in pre-symptomatic type 1 diabetes. Front Endocrinol (Lausanne) 2023;14:1108910
12. Eberhardt MJ, Filipovic MR, Leffler A, et al. Methylglyoxal activates nociceptors through transient receptor potential channel A1 (TRPA1): a possible mechanism of metabolic neuropathies. J Biol Chem 2012;287:28291–28306
13. Polykretis P, Luchinat E, Boscaro F, Banci L. Methylglyoxal interaction with superoxide dismutase 1. Redox Biol 2020;30:101421
14. Beisswenger PJ. Methylglyoxal in diabetes: link to treatment, glycaemic control and biomarkers of complications. Biochem Soc Trans 2014;42:450–456
15. Barragán-Iglesias P, Kuhn J, Vidal-Cantú GC, et al. Activation of the integrated stress response in nociceptors drives methylglyoxal-induced pain. Pain 2019;160:160–171
16. Yousuf MS, Sahn JJ, Yang H, et al. Highly specific σ2R/TMEM97 ligand FEM-1689 alleviates neuropathic pain and inhibits the integrated stress response. Proc Natl Acad Sci U S A 2023;120:e2306090120
17. Yousuf MS, Shiers SI, Sahn JJ, Price TJ. Pharmacological manipulation of translation as a therapeutic target for chronic pain. Pharmacol Rev 2021;73:59–88
18. Sarcinelli C, Dragic H, Piecyk M, et al. ATF4-dependent NRF2 transcriptional regulation promotes antioxidant protection during endoplasmic reticulum stress. Cancers (Basel) 2020;12:569
19. Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol Cell Biol 2003;23:7198–7209
20. Inceoglu B, Bettaieb A, Trindade da Silva CA, Lee KSS, Haj FG, Hammock BD. Endoplasmic reticulum stress in the peripheral nervous system is a significant driver of neuropathic pain. Proc Natl Acad Sci U S A 2015;112:9082–9087
21. Kammerscheit X, Hecker A, Rouhier N, Chauvat F, Cassier-Chauvat C. Methylglyoxal detoxification revisited: role of glutathione transferase in model cyanobacterium *Synechocystis* sp. strain PCC 6803. mBio 2020;11:10.1128/mbio.00882-20
22. Tonelli C, Chio IIC, Tuveson DA. Transcriptional regulation by Nrf2. Antioxid Redox Signal 2018;29:1727–1745
23. Xue M, Rabbani N, Momiji H, et al. Transcriptional control of glyoxalase 1 by Nrf2 provides a stress-responsive defence against dicarbonyl glycation. Biochem J 2012;443:213–222
24. Swindell WR, Bojanowski K, Chaudhuri RK. Transcriptomic analysis of fumarate compounds identifies unique effects of isosorbide di-(methyl fumarate) on NRF2, NF-kappaB and IRF1 pathway genes. Pharmaceuticals 2022;15:461
25. Li J, Ma J, Lacagnina MJ, et al. Oral dimethyl fumarate reduces peripheral neuropathic pain in rodents *via* NFE2L2 antioxidant signaling. Anesthesiology 2020;132:343–356
26. Hoogendoorn A, Avery TD, Li J, Bursill C, Abell A, Grace PM. Emerging therapeutic applications for fumarates. Trends Pharmacol Sci 2021;42:239–254
27. Wang Y, Bhargava P. Diroximel fumarate to treat multiple sclerosis. Drugs Today (Barc) 2020;56:431–437
28. Chan C-M, Huang D-Y, Huang Y-P, et al. Methylglyoxal induces cell death through endoplasmic reticulum stress-associated ROS production and mitochondrial dysfunction. J Cell Mol Med 2016;20:1749–1760
29. Kharitonova T, Shvarts YG, Verbovoy AF, Orlova NS, Puzyreva VP, Strokov IA. Efficacy and safety of the combined metabolic medication, containing inosine, nicotinamide, riboflavin and succinic acid, for the treatment of diabetic neuropathy: a multicenter randomized, double-blind, placebo-controlled parallel group clinical trial (CYLINDER). BMJ Open Diab Res Care 2022;10:e002785
30. Ziegler D, Low PA, Litchy WJ, et al. Efficacy and safety of antioxidant treatment with α-lipoic acid over 4 years in diabetic polyneuropathy: the NATHAN 1 trial. Diabetes Care 2011;34:2054–2060
31. Yousuf MS, Samtleben S, Lamothe SM, et al. Endoplasmic reticulum stress in the dorsal root ganglia regulates large-conductance potassium channels and contributes to pain in a model of multiple sclerosis. Faseb J 2020;34:12577–12598
32. Garcia-Perez E, Schönberger T, Sumalla M, et al. Behavioural, morphological and electrophysiological assessment of the effects of type 2 diabetes mellitus on large and small nerve fibres in Zucker diabetic fatty, Zucker lean and Wistar rats. Eur J Pain 2018;22:1457–1472
33. Otto KJ, Wyse BD, Cabot PJ, Smith MT. Longitudinal study of painful diabetic neuropathy in the Zucker diabetic fatty rat model of type 2 diabetes:

impaired basal G-protein activity appears to underpin marked morphine hyposensitivity at 6 months. Pain Med 2011;12:437–450
34. Pugsley MK, Brooks MB, Fishman CE, et al. Use of the ZDF rat to model dietary fat induced hypercoagulability is limited by progressive and fatal nephropathy. J Pharmacol Toxicol Methods 2021;107:106933
35. Düll MM, Riegel K, Tappenbeck J, et al. Methylglyoxal causes pain and hyperalgesia in human through C-fiber activation. Pain 2019;160:2497–2507
36. Lieuw-A-Fa MLM, van Hinsbergh VWM, Teerlink T, et al. Increased levels of N(epsilon)-(carboxymethyl)lysine and N(epsilon)-(carboxyethyl)lysine in type 1 diabetic patients with impaired renal function: correlation with markers of endothelial dysfunction. Nephrol Dial Transplant 2004;19:631–636
37. Timar B, Popescu S, Timar R, et al. The usefulness of quantifying intraepidermal nerve fibers density in the diagnostic of diabetic peripheral neuropathy: a cross-sectional study. Diabetology &Amp; Metabolic Syndrome 2016;8:31
38. Divisova S, Vlckova E, Srotova I, et al. Intraepidermal nerve-fibre density as a biomarker of the course of neuropathy in patients with type 2 diabetes mellitus. Diabet Med 2016;33:650–654
39. Kırça M, Yeşilkaya A. Methylglyoxal stimulates endoplasmic reticulum stress in vascular smooth muscle cells. J Recept Signal Transduct Res 2022; 42:279–284
40. Palte MJ, Wehr A, Tawa M, et al. Improving the gastrointestinal tolerability of fumaric acid esters: early findings on gastrointestinal events with diroximel fumarate in patients with relapsing-remitting multiple sclerosis from the phase 3, open-label EVOLVE-MS-1 study. Adv Ther 2019;36: 3154–3165
41. Holmström KM, Kostov RV, Dinkova-Kostova AT. The multifaceted role of Nrf2 in mitochondrial function. Curr Opin Toxicol 2016;1:80–91
42. Brennan MS, Matos MF, Li B, et al. Dimethyl fumarate and monoethyl fumarate exhibit differential effects on KEAP1, NRF2 activation, and glutathione depletion in vitro. PLoS One 2015;10:e0120254
43. Bollong MJ, Lee G, Coukos JS, et al. A metabolite-derived protein modification integrates glycolysis with KEAP1-NRF2 signalling. Nature 2018; 562:600–604
44. Guo T-Z, Shi X, Li W, Wei T, Kingery WS, Clark JD. Dimethyl fumarate reduces oxidative stress and pronociceptive immune responses in a murine model of complex regional pain syndrome. Anesthesia Analgesia 2021;132: 1475–1485
45. Mangus LM, Rao DB, Ebenezer GJ. Intraepidermal nerve fiber analysis in human patients and animal models of peripheral neuropathy: a comparative review. Toxicol Pathol 2020;48:59–70
46. Lauria G, Morbin M, Lombardi R, et al. Axonal swellings predict the degeneration of epidermal nerve fibers in painful neuropathies. Neurology 2003;61:631–636
47. Gibbons CH, Griffin JW, Polydefkis M, et al. The utility of skin biopsy for prediction of progression in suspected small fiber neuropathy. Neurology 2006;66:256–258
48. Lauria G, Holland N, Hauer P, Cornblath DR, Griffin JW, McArthur JC. Epidermal innervation: changes with aging, topographic location, and in sensory neuropathy. J Neurol Sci 1999;164:172–178
49. Ebenezer GJ, Hauer P, Gibbons C, McArthur JC, Polydefkis M. Assessment of epidermal nerve fibers: a new diagnostic and predictive tool for peripheral neuropathies. J Neuropathol Exp Neurol 2007;66:1059–1073
50. Avery TD LJ, Turner DJL, Rasheed MSU, et al. Site-specific drug release of monomethyl fumarate to treat oxidative stress disorders. Nat Biotech 4 November 2024 [Epub ahead of print]. DOI: 10.1038/s41587-024-02460-4