The cyclophilin inhibitor NIM-811 increases muscle cell survival with hypoxia in vitro and improves gait performance following ischemia–reperfusion in vivo

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Acute ischemia–reperfusion injury in skeletal muscle is a significant clinical concern in the trauma setting. The mitochondrial permeability transition inhibitor NIM-811 has previously been shown to reduce ischemic injury in the liver and kidney. The effects of this treatment on skeletal muscle are, however, not well understood. We first used an in vitro model of muscle cell ischemia in which primary human skeletal myoblasts were exposed to hypoxic conditions (1% O2 and 5% CO2) for 6 h. Cells were treated with NIM-811 (0–20 µM). MTS assay was used to quantify cell survival and LDH assay to quantify cytotoxicity 2 h after treatment. Results indicate that NIM-811 treatment of ischemic myotubes significantly increased cell survival and decreased LDH in a dose-dependent manner. We then examined NIM-811 effects in vivo using orthodontic rubber bands (ORBs) for 90 min of single hindlimb ischemia. Mice received vehicle or NIM-811 (10 mg/kg BW) 10 min before reperfusion and 3 h later. Ischemia and reperfusion were monitored using laser speckle imaging. In vivo data demonstrate that mice treated with NIM-811 showed increased gait speed and improved Tarlov scores compared to vehicle-treated mice. The ischemic limbs of female mice treated with NIM-811 showed significantly lower levels of MCP-1, IL-23, IL-6, and IL-1α compared to limbs of vehicle-treated mice. Similarly, male mice treated with NIM-811 showed significantly lower levels of MCP-1 and IL-1a. These findings are clinically relevant as MCP-1, IL-23, IL-6, and IL-1α are all pro-inflammatory factors that are thought to contribute directly to tissue damage after ischemic injury. Results from the in vitro and in vivo experiments suggest that NIM-811 and possibly other mitochondrial permeability transition inhibitors may be effective for improving skeletal muscle salvage and survival after ischemia–reperfusion injury.

Acute limb ischemia–reperfusion (I/R) injury is a significant clinical concern that can lead not only to local cell death and inflammation in skeletal muscle1–3 but also to systemic changes referred to as the systemic inflammatory response4,5. The systemic inflammatory response may, in turn, cause multiple organ dysfunction6,7. The pathophysiology of I/R injury emanates from two primary processes. First, oxygen deprivation leads to cell death in a subset of the skeletal muscle cell population and second, reperfusion stimulates an inflammatory response that involves the local and systemic release of inflammatory cytokines8. Recovery from I/R injury is therefore dependent at least in part on minimizing initial cell death with ischemia and then enhancing survival and recovery of cells that are “potentially salvageable” with reperfusion9. Cell survival with ischemia requires modulating levels of oxidative stress, which increases markedly as mitochondria generate abundant H2O2. Ischemia induces

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an increase in intracellular Ca++, which in turn stimulates opening of the mitochondrial permeability transition pore (mPTP) in the mitochondrial membrane. The prolonged opening of the mPTP interrupts the mitochondrial electron transport chain (membrane depolarization), disturbs mitochondrial energy production, and induces production of reactive oxygen species (ROS). These actions release different molecules from the dysfunctional mitochondria that can drive cell death. Importantly, increased ROS production is a direct inducer of inflammation by activating NF-κB pathway, pro-inflammatory cytokines, and inflammasomes.

Cyclophilin-D is a protein that can modulate structure of the mPTP. Cyclosporine-A (CsA) binds to both the cyclophilin-D component of mPTPs and the cytosolic cyclophilin-A molecules, suppressing the immune response and inhibiting the opening of mPTP. N-methyl-4-isoleucine cyclosporine (NIM-811) a non-immunosuppressive cyclophilin inhibitor, is a derivative of Cyclosporine-A (CsA), which binds to Cyclophilin-D thus preventing the development of MPT. NIM-811 has a therapeutic advantage over CsA in that it has no known systemic side effects. This difference between NIM-811 and CsA is due to the fact that NIM-811 cannot bind calcineurin. Calcineurin is a potent regulator of muscle remodeling, skeletal muscle differentiation, regeneration and fiber type specification—all functions that are crucial to muscle development, metabolism and functional adaptation. Previous work has indicated that mPTP inhibition by NIM-811 can enhance cell and tissue recovery following spinal cord injury and traumatic brain injury. It has also been found that NIM-811 can preserve renal function and lower circulating inflammatory cytokines following hindlimb I/R injury. Here we tested the hypothesis that NIM-811 can promote cell survival with ischemia directly using primary human cells. We also tested the prediction that the positive effects of NIM-811 and mPTP inhibition on cell survival would translate to muscle-specific changes in inflammation as well as improvements in gait function.

Results

Effectiveness of NIM-811 in treating ischemic myoblasts. We evaluated the effects of NIM-811 on the viability of ischemic human myoblasts by treating cells with 0–20 μM of NIM-811 for 2 h following the exposure to hypoxia for 6 h. MTS assays showed there was a statistically significant, dose-dependent, difference in cell number between the control group and treated groups (Fig. 1A). Furthermore, the LDH data indicate that 5 μM NIM-811 was significantly more effective in ameliorating ischemic injury compared to higher doses (10–20 μM) of NIM-811 (Fig. 1B).

Identification of elastic lasso tourniquet-induced ischemia–reperfusion model. Inducing limb ischemia in a rodent model using a tourniquet is a well-established technique. A laser doppler imager was used to measure blood flow in the ischemic left limb to verify the muscle ischemia. Once we applied the tourniquet, the blood flow remained steady during the 90 min of ischemia to about 1% of the baseline. At the beginning of the reperfusion phase, once the tourniquet is released, an increase in the blood flow to approximately 10% of the baseline was observed in the control group. However, in the NIM-811 treated group, the tourniquet release led to an increase in blood flow to approximately 50% of the baseline in both female (Fig. 2A–D) and male (Fig. 3A–D) mice. Pretreatment with NIM-811 (10 mg/kg) before ischemia significantly improved blood flow during reperfusion.
Mice treated with NIM-811 had enhanced limb function and performance. There were no statistically significant differences between male and female animals for distance, speed, or gait scores (Table 1). However, both the speed (P = 0.0051) and the limb function (gait score; P = 0.0096) were significantly enhanced with NIM-811 treatment (Table 2).

Histological changes in skeletal muscle. TA muscle cross-sectional area of the intact fibers did not show significant mean differences between ischemic and non-ischemic limbs within control and treated group of both sexes (Fig. 4A–D). Histological analysis of muscle cell morphology in both male and female animals does, however, show that muscles from the ischemic limbs of control (vehicle) treated mice display a disordered, irregular muscle fiber morphology (Fig. 4E,F). In contrast, muscle fibers from the ischemic limbs of NIM-811 treated mice are more regular, and degeneration is not so obvious (Fig. 4G,H).

4-HNE staining for lipid peroxidation as a marker of oxidative stress. NIM-811 significantly reduced (P < 0.05) 4-HNE staining in muscles from ischemic limbs of female mice treated with NIM-811 (Fig. 5A); however, in male mice there, was no significant difference between the male ischemic control muscle fibers compared to the ischemic NIM-811 treated muscle fibers (Fig. 5B). Immunohistochemistry analysis of muscle cells in both male and female animals does show that muscles from the ischemic limbs of control (vehicle) treated mice display a higher immunoreactivity to 4-HNE compare to ischemic limb from the NIM-811 treated group in female mice (Fig. 5C,E). However, that was not the case in muscle fibers from the ischemic limbs of control (vehicle) treated mice compared to the ischemic limb from the NIM-811 treated group in male mice (Fig. 5D,F).
Figure 3. Laser Doppler imaging of male mice models of hindlimb ischemia. (A) Blood flow measurements of ischemic left hindlimbs in male vehicle (control)-treated mice subjected to 90 min ischemia the hindlimb baseline (pre-ischemia) flux, as well as the reperfusion flux, were significantly ****P = 0.0001 higher than ischemic flux. (B) Representative Laser Doppler images in control mice during baseline, ischemia, and reperfusion. (C) Blood flow measurements of ischemic left hindlimbs in male mice treated with NIM-811 subjected to 90 min ischemia the hindlimb baseline (pre-ischemia) flux, as well as the reperfusion flux, was significantly ****P = 0.0001 higher than ischemic flux. Moreover, the reperfusion flux was significant **P = 0.0006 vs. baseline (pre-ischemia). (D) Representative Laser Doppler images in male mice treated with NIM-811 during baseline, ischemia, and reperfusion.

Table 1. Descriptive statistics of gait measures by sex and treatment group and the two-factor ANOVA F-test for the sex by treatment interaction.
Cytokine changes in serum and skeletal muscle. There was a significant elevation in serum levels of IL-10, IL-27, and GM-CSF, and a significant decrease in IL-1α, in the female mice pre-treated with NIM-811 compared to the control group at 24 h of reperfusion (Fig. 6A–D). There was a significant elevation in serum levels of IL-27 and a considerable reduction in IL-1α in the male mice pre-treated with NIM-811 compared to their control group at the same time point (Fig. 6E,F).

Cytokine data from muscle lysates of the ischemic limbs of female mice treated with NIM-811 showed significantly lower levels of pro-inflammatory factors IL-1α, IL-1β, IL-17α, MCP-1, INF-β, and IL-6 (Fig. 7A–F) compared to muscle lysates from the ischemic limbs of vehicle-treated mice. Additionally, muscle lysates from

| Gait measure | Variable | Level | Mean  | SD    | F    | p-value |
|--------------|----------|-------|-------|-------|------|---------|
| Distance     | Sex      | Female| 13.90 | 4.78  | 0.69 | 0.4178  |
|              |          | Male  | 12.23 | 4.73  |      |         |
|              | Treatment| Control| 11.27 | 3.83  | 3.22 | 0.0905  |
|              |          | NIM-811| 14.87 | 4.99  |      |         |
| Speed        | Sex      | Female| 0.19  | 0.11  | 0.38 | 0.5484  |
|              |          | Male  | 0.16  | 0.18  |      |         |
|              | Treatment| Control| 0.09  | 0.07  | 10.31| 0.0051  |
|              |          | NIM-811| 0.26  | 0.15  |      |         |
| Gait score   | Sex      | Female| 2.30  | 0.82  | 0.34 | 0.5675  |
|              |          | Male  | 2.10  | 0.99  |      |         |
|              | Treatment| Control| 1.70  | 0.67  | 8.50 | 0.0096  |
|              |          | NIM-811| 2.70  | 0.82  |      |         |

Table 2. Two-factor main effects ANOVA model results on gait measures.

Figure 4. Histology of muscle tissue. (A–D) Morphometric analysis of muscle fiber cross-sectional area of the TA muscles intact fibers in female and male mice does not differ significantly between the ischemic limbs of vehicle (control) and NIM-811 treated mice. (E–H) Hematoxylin and eosin (H&E)/trichome sections of TA muscle fibers from the ischemic limbs of vehicle-treated female and male mice show some shrinkage, necrosis, and irregular morphology compared to non-ischemic limb from the same group. The muscle fiber morphology was more regular, and staining was more uniform from the ischemic limbs of NIM-811 treated mice, most of the muscle cell was still located in the muscle membrane, and degeneration less frequent compared to the non-ischemic limb from the same group. Scale bar = 100 μm.
the ischemic limbs of female mice treated with NIM-811 showed significantly lower levels of IL-23 compared to the non-ischemic limb of the same group (Fig. 7G). Similarly, male mice treated with NIM8-11 showed lower levels of MCP-1 and IL-1α in muscle lysates from the ischemic limb compared to lysates from ischemic limbs of vehicle-treated mice (Fig. 8A,B).

**LDH levels in muscle tissue.** The tissue LDH concentration was lower at the ischemic gastrocnemius muscle compared with non-ischemic muscle in the female control group. However, the pretreatment with NIM-811 leads to a significant decrease in the LDH concentration in ischemic muscle compared to the non-ischemic muscle from the same group (Fig. 8C).

**TGF-β1 protein expression in gastrocnemius muscle.** ELISA analysis of ischemic muscle lysates showed a significant increase in the TGF-β1 level compared to the non-ischemic muscle in the female control group. This increase between the ischemic and non-ischemic muscle was not significant in the female mice treated with NIM-811 (Fig. 8D).

**Discussion**

Acute limb ischemia can cause severe pain, poor limb function, and even compromise patient survival. Skeletal muscle is highly susceptible to ischemic insult, and ischemia may cause irreversible muscle damage depending on the duration of impaired perfusion. Limb reperfusion may, in turn, cause further muscle damage with the release of myoglobin, LDH, and other intracellular muscle contents. Systemic immune response and mitochondrial dysfunction are the major contributors to loss of myocytes during myocardial ischemia and subsequent reperfusion. A variety of pharmacological therapies have been proposed for the treatment of limb IR injury. CsA was widely investigated in previous studies during ischemia-reperfusion. The main limitation of this drug is that it is non-specific. Besides binding to cyclophilin D, a key component of the mitochondrial transition pore, it also binds to cytosolic cyclophilin A. Cyclophilin A has several molecular targets within the cellular survival/death pathways that lead to a wide range of adverse effects such as immunosuppression, nephrotoxicity, and hepatotoxicity. For these reasons, finding a safe and effective small molecule that can prevent ischemia-reperfusion injury is essential. In the present study, we used a more specific and potent inhibitor of the MPT pore named N-methyl-4-isoleucine-cyclosporin (NIM-811). NIM-811 is non-immunosuppressive and does not
interact with the cytosolic cyclophylin A. We report here that inhibition of the MPT pore can protect ischemic human myoblasts in an in vitro model of ischemia–reperfusion. MTS assay showed that there was a statistically significant difference in cell numbers between all treated groups and the control group. Furthermore, the LDH assay results suggest that NIM-811 was highly effective in ameliorating the ischemic injury compared to those treated with vehicle.

Muscle fiber necrosis, disintegration and edema were detectable in the muscles from ischemic limbs of vehicle (control) treated mice whereas muscles from the ischemic limbs of NIM-811 treated mice showed less obvious pathological changes. This may explain the greater speed and the limb function (gait score) with NIM-811 treatment compared to the vehicle-treated group. Staining for lipid peroxidation demonstrated that NIM-811 treatment significantly reduced levels of oxidative stress in skeletal muscle from ischemic hindlimbs. Since NIM-811 and CsA cannot act as direct antioxidants because they lack the required chemical structure for that action, they may indirectly, via the reduction in oxidative damage, maintain mitochondrial homeostasis. Fewer free radicals and less oxidative damage were observed as a result. Moreover, we observed greater reperfusion in vivo with NIM-811 treatment, suggesting that this drug may preserve the microvasculature response, which might positively impact long-term graft function after limb reperfusion. Importantly we also observed significant sex differences in the response of muscle tissue to NIM-811 following ischemia–reperfusion. Specifically, NIM-811 treatment significantly reduced 4-HNE staining in muscles from females but not in males, which was also reflected in sex differences in cytokine levels (see below). There are now documented examples of sex differences in the response of muscle tissue to NIM-811 following ischemia–reperfusion.

Figure 6. Serum cytokine levels in female and male mice after hindlimb ischemia–reperfusion and treatment with NIM-811. (A–C) Serum IL-10, IL-27 and GM-CSF levels increased significantly in the serum of female mice treated with NIM-811 compared to control group. (D) Serum IL-α level decreased significantly in the serum of female mice treated with NIM-811 compared to control group. (E) Serum IL-27 level increased significantly in the serum of male mice treated with NIM-811 compared to control group. (F) Serum IL-α level reduced in the serum of male mice treated with NIM-811 compared to control group. *P < 0.05, **P < 0.01 and ***P < 0.001.
differences in MPT with ischemia–reperfusion. For example, cardiac mitochondria of females are thought to be less susceptible to MPT than male mitochondria, whereas female brain mitochondria may be more susceptible to MPT. Our data suggest that small molecules such as NIM-811 that target MPT may be more effective in females in the setting of ischemia–reperfusion injury.

One of the systemic complications following limb IR is the local inflammatory response that can contribute to tissue damage. Different inflammatory mediators and proinflammatory cytokines are thought to be released during this process. In our experiments, IL-1α showed a considerably lower value in the serum of the NIM-811 treated-group in male mice, and IL-1α and IL-1β were both reduced in the female mice with NIM-811 treatment. Previous work has shown that the pro-inflammatory cytokine IL-1 is involved in the pathogenesis of a wide range of inflammatory diseases. IL-1 blockade is the standard of care for the treatment of “autoinflammatory diseases”, a family of conditions characterized by dysfunction of monocytes/macrophages and recurrent bouts of debilitating inflammation. Serum levels of several anti-inflammatory cytokines such as IL-10 and GM-CSF were significantly elevated in NIM-811 treated female mice, and IL-27 was significantly elevated with NIM-811 treatment in both sexes. These findings indicate that enhanced muscle structure and function in the ischemic limb with NIM-811 treatment had not only local but systemic effects, suggesting that NIM-811 may promote cellular function in multiple organ systems following ischemic injury.

We also observed marked changes in the levels of inflammatory cytokines in skeletal muscle tissue lysates with NIM-811 treatment. Pro-inflammatory cytokines IL-17α, IL-23, IL-6 as well as INF-β were significantly lower in gastrocnemius muscles from ischemic limbs of NIM-811 treated female mice. Moreover, the lower IL-1-α serum levels of NIM-811 treated male mice, and lower serum IL-1-α and IL-1-β in female NIM-811 treated mice, corresponded to similar decreases in skeletal muscle IL-1-α and IL-1-β levels with NIM-811 treatment. MCP-1 level was also significantly lower in ischemic muscles from NIM-811 treated mice compared to ischemic muscles from vehicle-treated mice. In skeletal muscle, MCP-1 is known to promote macrophage infiltration after (severe) tissue damage and represents a molecular link in the crosstalk between adipose tissue and skeletal muscle. Previous findings have documented elevated MCP-1 in response to different stressors, and our data showing lower MCP-1 in muscle with NIM-811 is consistent with an overall decrease in the inflammatory response. TGF-β1 is a major contributor to fibrosis after injury in various tissues and is increased after injury in regenerating muscle. We found no significant effect on muscle TGF-β1 with NIM-811 treatment. Differentiating between total and active forms of TGF-β1 present in the muscles of each experimental group may be needed to provide additional, more relevant information for comparison.

Our study demonstrated that NIM-811 was capable of enhancing the levels of anti-inflammatory cytokines in muscle tissue, decreasing levels of pro-inflammatory cytokines, and improving limb function after ischemia–reperfusion. These findings are consistent with previous work demonstrating positive effects of NIM-811 on cell survival and tissue function following spinal cord injury and traumatic brain injury. Together these results...
indicate that NIM-811 or similar molecules that target the mPTP could be useful in the trauma setting, and perhaps improve patient outcomes following the injury to multiple organs and tissues.

Material and methods

Hypoxic cell culture and treatment of primary human myoblasts. Primary human skeletal myoblasts purchased from ThermoFisher Scientific (Catalog # A11440) were cultured as monolayers in Dulbecco's Modified Eagle Medium (DMEM/High Glucose), supplemented with 5% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (Pen-Strep) as we described previously41. The medium was changed after 24 h to discard non-adherent cells. Media was changed every 2 days, and the cells were expanded until passage 4 to 6. The expanded myoblast cells were then seeded in 48-well plates at a density of 7000 cells/well and allowed to attach and grow for 24 h. The culture medium was changed the following day to DMEM containing 4.5 g/L glucose without l-glutamine and phenol red. Myoblasts were then exposed to hypoxia condition (1% O2 and 5% CO2) for 6 h.

A stock solution of N-methyl-4-isoleucine cyclosporine NIM-811 (NIM-811; MedChemExpress, NJ, USA) was prepared by dissolving in DMSO at a concentration of 1 mM. As we described previously41, different volumes of the stock solution were added to myoblast cultures during the last 20 min of the hypoxia-period to obtain (0–20 µM) final concentrations of NIM-811 in the culture medium (phenol red-free DMEM supplemented with 1% FBS). At the end of the hypoxia period the myoblast cultures were transferred to a normoxic incubator for an additional 2 h.

Assaying viability and proliferation of ischemic primary human myoblasts. After the ischemic human myoblasts were treated with NIM-811 as detailed above, the MTS and LDH assays were performed as we showed previously41, different volumes of the stock solution were added to myoblast cultures during the last 20 min of the hypoxia-period to obtain (0–20 µM) final concentrations of NIM-811 in the culture medium (phenol red-free DMEM supplemented with 1% FBS). At the end of the hypoxia period the myoblast cultures were transferred to a normoxic incubator for an additional 2 h.
lov scale) as 0 = no movement; 1 = barely perceptible movement, no weight-bearing; 2 = frequent and vigorous
once the mice were recovered from anesthesia. Limb function was assessed using the clinical use score (Tar-
sis. Semi-quantitative assessments of limb function after applying the ischemia/reperfusion were performed
ation; (2) Images digitization; (3) Export of distance traveled as well as speed data to a spreadsheet; (4) Data analy-
frame at 15 f/s using the Kinovea version 0.8.24. The procedure included four steps: (1) Kinovea frame calibra-
to recover from anesthesia. Mice were then videotaped walking on trackway and their gait score was recorded.

Limb perfusion measurement. Isoflurane was delivered by face mask, 2% for induction, and 1% for
maintenance, along with continuous oxygen at 2 l/min while the mice were kept on a heating pad to maintain the
body temperature at 37 °C. Following induction of anesthesia, the fur was removed from the left hindlimb with a
hair removal cream and the skin cleaned with 70% ethanol. The Laser Doppler imager (Moor Instruments, Wilmingtom, DE) was used to assess limb perfusion.24,45 The animal remained on the warming table under
isoflurane anesthesia during the 90 min of ischemia. The Laser Doppler source was mounted on a movable rack
10 cm above the mouse hindlimb, and blood movement was detected and processed by the laser beam (780 nm)
to provide a computerized, color-coded image. Mean flux values representing tissue perfusion were calculated
from the relative flux (in U/cm²) in the areas corresponding to the plantar aspect of the hindlimb using image
analysis software (Laser Doppler perfusion measure, V3.08, Moor Instruments). Baseline images were obtained
from each mouse immediately after the induction of anesthesia and hair removal to assess the limb ischemia.
Ischemia was induced on the proximal thigh with ORBs 4.5 oz (American Orthodontics, Sheboygan, WI). This
diameter was chosen based on measurements of the thigh of CD-1 mice (28–35 g) mice, and the previous study
uses this tourniquet to induce limb ischemia in a rodent model.23 Another Laser Doppler image was obtained
30 min into the procedure to assess limb ischemia. Data were expressed as percent basal perfusion in the limbs.

Mouse hindlimb was scanned under isoflurane anesthesia at three intervals: (1) right after the induction of
anesthesia (baseline), (2) 30 min after applying of rubber band ischemia and (3) right after the removal of
the rubber band and start of reperfusion. The NIM-811 treated group received boluses of NIM-811 at a concentra-
ton of (10 mg/kg) 10 min before reperfusion, and 3 h into the reperfusion period. Control mice received boluses
of PBS with the same concentration of DMSO, 10 min before reperfusion, and 3 h into the reperfusion period.

Video recording and gait score analysis. After the 90 min of the ischemic period, mice were allowed
to recover from anesthesia. Mice were then video-taped walking on trackway and their gait score was recorded.
Mice were filmed using Digital Video Camcorder (GoPro Hero8). The video recordings were analyzed frame-by-
frame at 15 f/s using the Kinovea version 0.8.24. The procedure included four steps: (1) Kinovea frame calibra-
tion; (2) Images digitization; (3) Export of distance traveled as well as speed data to a spreadsheet; (4) Data analy-
ysis. Semi-quantitative assessments of limb function after applying the ischemia/reperfusion were performed
once the mice were recovered from anesthesia. Limb function was assessed using the clinical use score (Tar-
lov scale) as 0 = no movement; 1 = barely perceptible movement, no weight-bearing; 2 = frequent and vigorous
movement, no weight-bearing; 3 = supports weight, may take 1 or 2 steps; 4 = walks with the only mild deficit;
5 = normal but slow walking and 6 = full and fast walking.44,45

Tissue and blood processing. After 24 h of reperfusion, the animals were euthanized and about one
mL of whole blood was collected from each mouse heart. The blood kept was on ice for 2–3 h, centrifuged for
30 min at 1000 rpm, and serum collected for cytokine array. Skeletal muscle tissue samples collected at the
time of death from both hindlimbs included tibialis anterior (TA) and gastrocnemius muscles. TA muscles
were fixed in 10% neutral buffered formalin and prepared for histology. Gastrocnemius muscles were flash-frozen
in liquid nitrogen and stored at –70 °C for lactate dehydrogenase assay (LDH), cytokine array, and enzyme-linked
immunosorbent assay (ELISA).

TA muscles were paraffin-embedded and cross-sections cut at the 2–3-μm thickness and stained with Masson
tricrome. Stained slides were examined under optical microscopy at 20× magnification. Muscle fiber cross-
sectional area (CSA) was measured using Image J software.55 Fifty random fields were analyzed using a cool color
camera and RS image software program (Ocular Image Acquisition Software, QImaging, Canada). Muscle fibers
were scored as uninjured as having well-defined borders, consistency of texture, and uniformity throughout the
fiber without holes or breaks. Total areas are measured using the “analyze” and “measure” functions which will
report area, minimum, maximum, and the mean.
Immunohistochemistry study. Paraffin-embedded serial sections of TA muscle were deparaffinized, rehydrated, blocked in with 5% nonfat dried milk, and incubated with immunoglobulin G-purified polyclonal rabbit antibodies specific for 4-HNE overnight at 4°C. Immunoreactivity was detected using a secondary biotinylated goat anti-rabbit antibody (1:200). Avidin/biotinylated horseradish peroxidase kit (Pierce, Rockford, IL) was used to visualize the interactions between the primary and secondary antibodies. Immunostaining procedure in the absence of primary antibody was used to eliminate the non-specificity immunoreactivity that might be a result of nonspecific interactions of the secondary antibody.

LDH assay for assessment of the cellular injury. Degree of cellular injury was assessed based on the activity of LDH in protein extracts from gastrocnemius muscle in ischemic and non-ischemic limb in both control and NIM-811 treated groups as showed previously47. LDH level was assessed spectrophotometrically by monitoring NADH oxidation at an absorbance at 450 nm (Cytotoxicity Detection Kit MAK066, Sigma-Aldrich), according to the manufacturer’s instructions.

Cytokine antibody array. Cytokine Bead Array Mouse Inflammation 13-Plex Panel (Bio-Legend, San Diego, CA, USA) was used for estimating the differential amount of cytokines in pooled protein extracts from gastrocnemius muscle as well as serum, accordingly to the manufacturer’s instructions.

Determination of TGF-beta1 protein. Total TGF-beta1 was determined by ELISA, the kit according to manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA), on the gastrocnemius muscle from both limbs of NIM-811 treated group as well as the control group in male and female mice. Briefly, a 500 μl of a solution containing 1% Triton X-100, 20 mM Tris/Hydrochloric acid (HCl) pH 8.0, 137 mM sodium chloride (NaCl), 10% glycerol, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenyl-methyl-sulfonyl-urea, 1% aprotinin, and 15 μg/ml leupeptin was used to homogenized a 10–20 mg of gastrocnemius muscle. To activate latent TGF-beta 1 activity 1 N HCl 1:1 was added to 1 μg total protein. Optical density was determined within 30 min of adding stop solution by using a microplate reader set at 450 nm.48

Statistical analysis. Statistical analysis was performed using SAS 9.4 and statistical significance was assessed using an overall alpha level of 0.05. Means and standard deviations within sex, limb, treatment, and measurement time (where appropriate) were determined for all outcome measures. All post hoc pairwise comparisons were performed using a Bonferroni adjustment to the overall alpha level for the number of pairwise comparisons made. Repeated measures mixed models were used to examine differences between treatments (NIM-811 versus control) by sexes and limbs (ischemic versus control). For LDH blood flow, a repeated measures mixed model was used to examine differences over time (pre-ischemia, during ischemia, reperfusion) between treatments and sexes on the ischemic limb. To examine whether differences in distance, speed, and gait score between treatment groups and sex, two-factor ANOVA was used. Main effects of treatment group and sex as well as the two-factor interaction were included in each ANOVA model.

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Author contributions
K.B.E. wrote the manuscript and did the experiments, M.W.H. design the experiments and reviewed the manuscript, M.K. helped in the performance of ischemic reperfusion experiment, J.W. did all the statistical analysis of the experiments, B.M. and F.O. helped in reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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