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GsMTx4-D is a cardioprotectant against myocardial infarction during ischemia and reperfusion

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Abstract

GsMTx4 is a selective inhibitor of cationic mechanosensitive ion channels (MSCs) and has helped establish the role of MSCs in cardiac physiology. Inhomogeneous local mechanical stresses due to hypercontracture and swelling during ischemic reperfusion injury (IRI) likely induce elevated MSC activity that can contribute to cation imbalance. The aim of this study was to determine if the D enantiomer of GsMTx4 can act as a cardioprotectant in a mouse IRI model. Ischemia and reperfusion involved ligating a coronary artery followed by release of the ligature. GsMTx4-D was tested by either acute intravenous injection during the ischemic event or by two day pretreatment by intraperitoneal injection, both methods achieving similar results. Based on pharmacokinetic studies, GsMTx4-D dosage was set to achieve expected plasma concentrations between 50 and 5000 nM and heart tissue concentrations between 1 and 200 nM by intravenous injection. Relative to vehicle injected animals, GsMTx4-D reduced infarct area by ~40% for acute and pretreated animals for both 20 and 45 min ischemic challenges. Many indicators of cardiac output were indistinguishable from sham-treated control hearts after GsMTx4-D treatment showing improvement at both 4 and 48 h post ischemia, and premature ventricular beats immediately following reperfusion were also significantly reduced. To determine if GsMTx4-D cardioprotection could act directly at the level of cardiomyocytes, we tested its effects in vitro on indicators of IRI damage like cation influx and activation of inflammatory kinases in isolated myocytes cultured under hypoxic conditions. Hypoxia challenged cardiomyocytes treated with 10 μ M GsMTx4-D showed improved contractility and near normal contraction-related Ca^{2+} influx. GsMTx4-D inhibited indicators of ischemic damage such as the apoptotic signaling system JNK/c-Jun, but also inhibited the energy response signaling system Akt kinase. We conclude that GsMTx4-D is a potent cardioprotectant in vivo that may act directly on cardiomyocytes and potentially be useful in multidrug strategies to treat IRI.

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Disclosures

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Keywords

GsMTx4; Reperfusion injury; Mechanosensitive ion channels; Myocardial infarction; Cardiac output; Arrhythmia; Pharmacokinetics

1. Introduction

Reperfusion of ischemic cardiac tissue by percutaneous coronary intervention is a routine treatment for acute ST-elevation myocardial infarction. However, restoring blood flow to ischemic cardiac tissue can injure myocytes that were viable prior to reperfusion [1,2]. This ischemia reperfusion injury (IRI) can cause tissue death accounting for up to 50% of the final myocardial infarct (MI) area [3,4]. Despite substantial progress in understanding the mechanisms of IRI, new chemical interventions meant to reduce MI size need to be identified [1,2].

The putative causes of IRI are varied, though most models suggest that a combination of increased oxidative stress, cation imbalance [5–7] and energy depletion [8] are the primary triggers that activate multiple downstream phosphorylation cascades involved in the stress response. Pathological sarcolemmal stress associated with reperfusion of ischemic heart tissue caused by swelling and hypercontracture [9,10], has been shown to directly activate mechanosensitive signaling systems such as cation influx through mechanosensitive ion channels (MSCs) [11,12] and production of reactive oxygen species (ROS) by Nox2 [6,13].

Grammastola spatulata mechanotoxin #4 (GsMTx4) is a 35 amino acid peptide that inhibits the gating of cation selective MSCs with similar affinity in either the L or D enantiomeric forms, emphasizing its lack of stereospecificity when interacting with MSCs [14,15]. Cationic MSCs may belong to the Transient Receptor Potential (TRP) [16,17] and/or Piezo [18] families known to be expressed in cardiomyocytes and vascular tissue. In vitro measures of MSC activity in cardiac tissue, single cells and patch assays, show GsMTx4 activity in the range of 0.1–5 μ M [15,19,20]. GsMTx4 is a gating modifier that inhibits MSCs by clamping the membrane tension of the outer monolayer where its penetration depth, and thus its surface area contribution, is tension dependent [21,22].

The effects of GsMTx4-L upon normal heart function and pathology have been studied in multiple in vitro cardiac model systems (reviewed in [11]). At the concentrations mentioned above, GsMTx4-L showed no significant effects on normal heart contractility, Ca^{2+} homeostasis or automaticity [19,20,23–26], though weak effects on the length dependent slow force increase were noted in rat trabeculae [27] and human atrial myocardial strips [26]. However, it proved a significant antagonist of elevated Ca^{2+} influx occurring in many forms of cardiac pathology.

Recently, in vivo testing of GsMTx4-L in an ischemia model using pigs showed no significant improvement in infarct size, hemodynamics or arrhythmia production [28]. The synthesized peptide was tested for activity by saline perfusion of isolated rat hearts showing the ability to inhibit induced arrhythmias similar to those performed on rabbit heart [19].

However, no pharmacokinetic analysis of peptide distribution in pigs was performed prior to testing to determine the bioavailability in the heart.

Here we investigate whether the D enantiomer of GsMTx4 (GsMTx4-D) can act as a cardioprotectant in a mouse model of IRI by monitoring infarct size, hemodynamics and production of arrhythmias. Prior to testing we performed PK analyses of plasma and cardiac tissue concentrations following different methods of peptide administration. We showed that GsMTx4-D has a long half-life in situ possibly due to its resistance to proteolytic digestion. We also showed that GsMTx4-D had no effect on normal cardiac function in telemetered free roaming ferrets whose cardiac properties are closer to humans than mice. To determine if cardioprotection was acting directly on the cardiomyocytes, we isolated cardiomyocytes and exposed them to hypoxia and showed that normal contractility and contraction related Ca^{2+} release are restored in the presence of GsMTx4-D. Further, we observed that GsMTx4-D suppressed downstream signaling systems commonly associated with IRI such as those involved in inflammation, apoptosis and energy metabolism.

2. Materials and methods

2.1. I/R model in vivo

All animal protocols in this study were approved by the Institutional Animal Care and Use Committee of the University at Buffalo-State University of New York and conform to the NIH Guide for the care and use of laboratory animals. C57Bl/6J mice (4–6 months of age) were obtained from Jackson Laboratories (Bar Harbor, MN). For the ischemia experiments, mice were anesthetized with 4% isoflurane and 100% O_2 , quickly intubated and ventilated with a respirator (Harvard apparatus, Holliston, MA, USA) [29]. The amount of isoflurane was slowly reduced to 1–3%. Buprenorphine 0.1 mg/kg was injected for analgesia. Upon absence of a pedal reflex, surgery was performed under a stereomicroscope. Core body temperature was maintained at 37 °C with a heating pad. After a left lateral thoracotomy, the left anterior descending (LAD) coronary artery was occluded just underneath the left atrium for 45 min with an 8–0 nylon suture. A small piece of gauze was used to prevent arterial injury. At the conclusion of the induced ischemia, the ligature was removed and the tissue was reperfused for 48 h before euthanizing. Following reperfusion, the chest was closed in layers. A drop of lidocaine was put on the suture site and the mouse was slowly weaned off the ventilator as it resumed spontaneous breathing. The mouse was returned to its cage and kept warm and medicated for pain (buprenorphine). Test compounds were administered via tail vein injection (saline 50 μL) 20 min before reperfusion. ECGs confirmed the hallmark ischemic ST-segment elevation during coronary occlusion (AD Instruments, Colorado Springs, CO, USA). At the conclusion of the study, mice were euthanized by IP injection of 120 mg/kg pentobarbital.

2.2. Heart rhythm analysis

We continuously recorded ECGs during reperfusion [29], providing mean heart rate, PR, QRS and QT intervals for each animal. We evaluated the incidence of arrhythmias according to the criteria of Huggins et al. [30] and Lujan and DiCarlo [31]. We counted the incidence of premature ventricular beats (PVBs), supraventricular premature beats, and arrhythmias

such as ventricular tachycardia by summing the number of events and episodes from limb lead recordings for 30 min following the initiation of reperfusion. PVBs are significant because they can lead to more serious arrhythmias such as tachycardia and fibrillation, and can also decrease cardiac output. PVBs were characterized by an early, abnormal QRS complex, often followed by a compensatory pause. Supraventricular premature beats (originating in the atria or junctional conduction pathways) were characterized by an early abnormal P wave or without a preceding P wave, followed by a normal QRS wave. Ventricular tachycardia was defined as four or more consecutive PVBs. We did not observe ventricular tachycardia or fibrillation in any of the treatment groups.

2.3. In vivo pressure-volume relationships

We measured the pressure-volume (P-V) relationships in real time in three groups of mice, including a sham group that was exposed to the open heart operation but not I/R, and two I/R (45 min ischemia/48 h reperfusion) challenged groups that were IV injected during ischemia with either vehicle or GsMTx4-D. To measure the P-V relationships, the mice were anesthetized with 4% isoflurane and 100% O₂, quickly intubated and ventilated with a respirator. The amount of isoflurane was slowly reduced to 1–1.5% until the pedal reflex was no longer observed. A horizontal incision was made inferior to the sternum and xiphoid process. The rib cage was slightly retracted and the diaphragm was gently cut away to attain an apical view of the myocardium. A 27-gauge needle was inserted into the apex of the LV free wall to create a guide-hole. A 1.2-French microtransducer pressure-volume impedance catheter (Scisense, London, ON, CA) was then inserted into the lumen of LV to assess real-time LV function. Hemodynamic parameters such as heart rate, cardiac output, and ejection fraction were measured and calculated using iWorx software (Dover, NH).

2.4. Infarct size measurement

To measure infarct size, hearts were harvested and excised for dual staining at the conclusion of reperfusion. The non-necrotic tissue in the ischemic region was stained red with 2, 3, 5-triphenyltetrazolium (TTC) and non-ischemic regions were stained blue with Evans blue. The hearts were fixed and sectioned into 1 mm slices, photographed with a Lexica MZ95 microscope and analyzed with NIH ImageJ software (<http://imagej.nih.gov/ij/>) [32]. The infarct size was calculated as the ratio of the percentage of myocardial necrosis to the ischemic Area At Risk (AAR).

2.5. Isolation of mouse cardiomyocytes

Cardiomyocytes were enzymatically isolated as previously described [33,34]. Briefly, mice were given 100 units of heparin (Sagent Pharmaceuticals, Schaumburg, IL) for anticoagulation, followed by 100 mg/kg sodium pentobarbital IP for anesthesia. The hearts were excised and fastened onto cardiomyocyte perfusion apparatus (Radnoti, Monrovia, CA). Hearts were perfused at 37 °C with a Ca²⁺-free Krebs-Henseleit based (KHB) buffer (pH 7.4) containing: 0.6 mM KH₂PO₄, 0.6 mM Na₂HPO₄, 10 mM HEPES, 14.7 mM KCl, 1.7 mM MgSO₄, 120.3 mM NaCl, 4.6 mM NaHCO₃, 30 mM taurine, 10 mM glucose, and 10 mM 2,3-butanedione monoxime. The buffer was oxygenated with 95% O₂/5% CO₂. After several minutes of stabilization, the hearts were digested with fresh buffer containing 0.067 mg/mL Liberase Blendzyme 4 (Roche, Indianapolis, IN) for 15 min without 2,3-

butanedione monoxime. After digestion, hearts were removed and minced to disperse the cardiomyocytes in fresh Ca^{2+} -free-KHB buffer. Extracellular Ca^{2+} was added incrementally to 1 mM. Only rod-shaped myocytes with clear edges were selected for pharmacological testing and cell contractility studies.

2.6. Hypoxia treatment

Isolated mouse cardiomyocytes were separated into two treatment groups (normal and hypoxic). Hypoxic cells were kept at 37 °C in a sealed chamber under a humidified atmosphere of 5% CO_2 and 95% N_2 for 15 min, with or without GsMtx4-D. Normal cells were placed into a water jacketed incubator at 37 °C under normal atmospheric oxygen levels during the same period.

2.7. Cardiomyocyte shortening/relaxation measurement

The mechanical properties of ventricular myocytes were assessed using the SoftEdge MyoCam system (IonOptix Corporation, Milton, MA, USA) [35]. In brief, myocytes were placed in a chamber mounted on the stage of an inverted microscope (Olympus, IX-70, Center Valley, PA, USA) and incubated at 25 °C in a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 Glucose, and 10 HEPES, at pH 7.4. The cells were stimulated with suprathreshold voltage at a frequency of 0.5 Hz, using a pair of platinum wires placed on opposite sides of the chamber and connected to a stimulator (FHC Inc., Brunswick, NE, USA). Images of the myocytes being studied were displayed on a computer monitor using an IonOptix MyoCam camera. IonOptix SoftEdge software was used to capture changes in sarcomere length during contraction and relaxation. Sarcomere shortening and lengthening were assessed using the following indices: peak shortening amplitude (PS) during contraction indicative of peak ventricular contractility; and maximal velocities of shortening/relengthening, maximal slope (derivative) of shortening and relengthening phases, indicative of the maximal velocities of ventricular pressure increase/decrease.

2.8. Intracellular Ca^{2+} transient measurements

Isolated cardiomyocytes were loaded with Fura-2/AM (2 μM) for 20 min, and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). Myocytes were placed on an Olympus (St. Louis, MO, USA) IX-70 inverted microscope and imaged through a 40 $\times$ Fluor oil objective. Cells were illuminated by either 340 or 380 nm excitation, while being stimulated to contract at 0.5 Hz. Fluorescence emission was detected at 480 and 520 nm by a photomultiplier tube [33] and high speed sampling using the interpolated numerator method.

2.9. Immunoblotting analysis and antibodies

Immunoblotting was performed as previously described [29,36]. Isolated cardiomyocyte proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Rabbit antibodies against phospho-Akt (Ser⁴⁷³) (p-Akt), phospho-c-Jun (Ser [63]) (p-c-Jun), c-Jun, phospho-JNK (Thr¹⁸³/Tyr¹⁸⁵) (p-JNK), JNK, and GAPDH were purchased from Cell Signaling (Danvers, MA, USA).

Akt1/2/3 antibody was purchased from Santa Cruz (Santa Cruz, CA, USA). The densities of immunoblot bands were analyzed using ImageJ software.

2.10. Statistical analysis

Data are expressed as means $\pm$ SE. Statistical significance was analyzed with either a Student's *t*-test or one-way ANOVA where appropriate. For multi-pairwise comparisons the Tukey post hoc test was performed to measure individual group differences of interest. The *F* and α significance levels of 0.1, 0.05 and 0.01 are indicated.

3. Results

3.1. GsMTx4-D reduces myocardial infarction size during I/R

We first tested three different doses of the peptide by acute administration during ischemia challenge. We performed preliminary pharmacokinetic (PK) testing to determine the distribution of the peptide in the heart and plasma over 24 h by both IV (fast absorption) and subcutaneous (slow absorption) administration using LC/MS/MS (see Supporting Fig. S1). Dosages were chosen to achieve target concentrations between 0.1 and 5 μ M based on in vitro tests of MSC activity in cardiac tissue [15,19,20]. Based on the PK analysis, IV administration of GsMTx4-D at 0.1, 1 and 10 mg/kg was expected to produce concentrations of 0.05–5 μ M and 2–200 nM in the plasma and heart tissue respectively. While these analyses cannot substitute for PK analysis in larger animals with different peptides, they do suggest that previous IR testing of GsMTx4-L in pigs may have only been ~1 nM in the heart tissue, well below the expected concentration needed to inhibit MSCs.

We tested the effects of GsMTx4-D on infarct size in three separate experiments with different modes of peptide administration, ischemic challenge and reperfusion time, all showing similar results. In one experiment, C57Bl/6 mice were subjected to 45 min occlusion of the LAD followed by 48 h of reperfusion (Fig. 1). GsMTx4-D and vehicle (saline) were administered through tail vein injection 20 min before reperfusion. There was no death or morbidity observed in any group. At the conclusion of reperfusion, the animals were euthanized and the hearts were stained with TTC and Evans Blue (Fig. 1A). The ratios of the AAR to total myocardial area were similar among the four cohorts (Fig. 1B). All GsMTx4-D dosages produced a significant decrease in myocardial infarction size compared to control (Fig. 1C). The 1 mg/kg dosage produced the greatest decrease, up to 35%. In a similar experiment using a 20 min ischemia challenge and analyzing infarct size after 4 h of reperfusion, again 1 mg/kg of GsMTx4-D produced the greatest (40%) decrease in infarct size compared to vehicle control (Supporting Fig. S2). This cardioprotective effect of GsMTx4-D was not limited to acute administration as it also decreased infarct size by 38% after 24 h of reperfusion when animals were pretreated for two days prior to ischemic challenge with a 50 mg/kg dose/day by IP injection (Supporting Fig. S3). The PK analysis showed that GsMTx4-D persisted in the heart tissue for up to 8 days after a single injection (Supporting Fig. S1C) which is likely due to the resistance of D amino acid peptides to proteolytic digestion.

3.2. GsMTx4-D improves cardiac function after I/R

Real time P-V relationships were monitored by left ventricular catheterization in sham-operated controls and acute IV injected animals with either vehicle or 1.0 mg/kg GsMTx4-D (Fig. 2A–C).

Animals were injected with vehicle or GsMTx4-D 20 min prior to reperfusion during the 45 min ischemia challenge. Catheterization was performed 48 h after reperfusion. Ventricular dilation, the rate of ventricular pressure change and the ejection fraction are considered particularly important indicators of IRI damage and long term prognosis for heart failure [37,38]. Comparing the vehicle injected mice to the sham treated animals, the ischemic challenge significantly reduced the ejection fraction (Fig. 2D) and the maximal dP/dt (Fig. 2G), and produced increased end systolic and diastolic volumes (Fig. 2E and F). Mice injected with GsMTx4-D showed improvement in all these indicators compared to vehicle. Other indicators (cardiac output, heart rate, and stroke volume, Fig. 2H, J and K respectively) were not statistically different from sham for either the vehicle or GsMTx4-D injected groups. End systolic pressure was not significantly different from either the sham or the vehicle injected animals but trended toward higher pressures (Fig. 2I). In other experiments, where mice were pretreated with GsMTx4-D two days prior to a 20 min ischemic challenge, we observed similar positive indicators on the hemodynamics (Supporting Fig. S4). Ejection fraction, end systolic volume, stroke volume and cardiac output all showed improvement with GsMTx4 pretreatment.

To assess potential adverse effects of GsMTx4-D on normal cardiac hemodynamics, we injected the peptide the tail vein of free roaming telemetered ferrets at three different concentrations (the highest concentration was 4.3 mg/kg, see Supporting data Fig. F1A–D in “Ferret Study”). We chose Ferrets because their cardiac function is more closely associated with that of humans than are mouse cardiac properties [39]. GsMTx4-D showed no significant changes in any hemodynamic parameters at any concentration compared to vehicle injected animals.

3.3. GsMTx4-D reduce I/R induced arrhythmia

Heart rate and rhythm were monitored by ECG to confirm the hallmark ischemic ST-segment elevation during coronary occlusion and the gradually increasing inversion of the T-wave during reperfusion (Fig. 3A). The probability of mechanically induced arrhythmias increases during ischemia and reperfusion [40,41], and increased incidence of premature beats (PB) (Fig. 3B) and arrhythmias are indicators of the severity of I/R damage [42].

No ventricular tachycardia was observed during 45 min of ischemia or in the subsequent 30 min of reperfusion in controls or GsMTx4-D treated animals. However, we did observe PVB and supraventricular PBs occurring within 30 min of reperfusion following the ischemic challenge. While 0.1 and 1 mg/kg GsMTx4-D reduced the incidence of premature events, only IV injection of 10 mg/kg GsMTx4-D caused a significant reduction ($\alpha = 0.1$) compared to vehicle (Fig. 3C). Considering that PVBs are indicative of more severe damage, these events were analyzed separately (Fig. 3D). We observed results similar to those for the total incidence of premature beats. Similar results for premature events were obtained in

experiments using 20 min ischemic challenge (Supporting Fig. S5A and B). Here both 1 and 10 mg/kg doses produced significant reductions in premature beats. In the experiments using 45 min ischemia, isoflurane was used to anesthetize the mice, and no change in heart rate was observed either during the ischemic challenge or during the subsequent 30 min of reperfusion (Fig. 3E and F respectively). However, in the 20 min ischemia experiments we used pentobarbital to anesthetize which caused a significant suppression of the heart rate (Supporting Fig. 5C, normal mouse resting heart rate is 500–600 bpm). In GsMTx4 treated mice the pentobarbital suppression was reduced causing a mild increase in heart rate (Supporting Fig. 5D) suggesting some interaction between these two compounds. In free roaming ferrets GsMTx4-D at 4.3 mg/kg had no effect on cardiac electrical activity over 24 h (Supporting data Fig. F2A–D, “Ferret Study”).

3.4. Contractility and Ca^{2+} response in isolated cardiomyocytes following hypoxia

To determine if the in vivo cardioprotective effect could be a direct effect on cardiomyocytes, we isolated cardiomyocytes and exposed them to hypoxic conditions for 15 min in the presence of GsMTx4-D and measured contractility and Ca^{2+} signaling. Hypoxia induces cell swelling and other physiological changes that may induce MSC activity [9]. Cardiomyocyte contractility was examined with an extracellular $[\text{Ca}^{2+}]$ of 1.0 mM and a stimulus frequency of 0.5 Hz. Hypoxia did not change the resting sarcomere length, nor did 10 μM GsMTx4-D (Fig. 4A and B). Hypoxia also did not significantly reduce peak shortening length (Fig. 4C), though significant decreases in the velocity of contraction and relaxation were observed (Fig. 4D and E). GsMTx4-D had no effect on the contractile function in normoxic cells. However, it provided protection against the decline in function observed in hypoxia treated cells by inhibiting the reduction of contraction and relaxation rates.

As a known cationic MSC inhibitor, GsMTx4 has been shown to protect against stress dependent loss of contraction strength and the contractile Ca^{2+} release in dystrophic skeletal muscle [43,44], and reduces the elevated resting Ca^{2+} levels in dystrophic cardiomyocytes [27]. We measured the Ca^{2+} influx in hypoxic cardiomyocytes during contraction by preloading the cells with Fura-2AM. Cardiomyocytes in normoxic conditions showed a rapid increase in intracellular Ca^{2+} levels that closely paralleled the contraction rate and strength (Fig. 5A). Resting Ca^{2+} levels were unchanged after hypoxia (Fig. 5B). However, the peak Ca^{2+} influx was reduced (Fig. 5C) and the influx activation and deactivation rates were slower (Fig. 5D and E respectively). As with the contractile function, GsMTx4-D had no effect on the Ca^{2+} response in normoxic cells. However, with the hypoxic cells it produced nearly complete protection from the reduction in peak Ca^{2+} response and slowing of the Ca^{2+} kinetics. This suggests that, while Ca^{2+} influx through MSCs is undetectable during normal contractile activity, there is increased MSC permeability in hypoxic cells that plays an important role in regulating the Ca^{2+} release mechanism.

3.5. Downstream signaling systems – indicators of IR damage in isolated cardiomyocytes

Clearly GsMTx4 inhibition of cation selective MSCs can affect Ca^{2+} influx which can trigger activation of multiple downstream kinase cascades like the inflammatory and apoptotic signaling systems, key indicators of IRI. To determine if GsMTx4-D affects these

downstream systems we measured the phosphorylation changes on both isoforms of JNK and one of its targets, transcription factor c-Jun (Fig. 6A). Activation of JNK/c-Jun system by phosphorylation triggers apoptosis in hypoxic cells [45,46]. In normoxic cells, GsMTx4-D had no effect on phosphorylation of either the 54 or 46 kDa forms of JNK (Fig. 6C and D, respectively) or c-Jun (Fig. 6E). Hypoxia caused a significant increase in JNK phosphorylation that was inhibited by the inclusion of 10 μ M GsMTx4-D during the hypoxic challenge. c-Jun phosphorylation increased during hypoxia and also appears to be reduced by GsMTx4-D treatment, though the difference did *not* reach statistical significance ($\alpha = 0.1$).

Activation of the cardioprotective kinase Akt has been linked to cell growth and increased metabolism, and to improved cell survival during ischemia [46,47]. The active form of Akt kinase is phosphorylated, and that is triggered by hypoxia (Fig. 6B and F). GsMTx4-D had no effect on Akt phosphorylation in normoxic cells, but significantly decreased phosphorylation under hypoxic conditions (Fig. 6F). Thus, GsMTx4-D cardioprotective effects appear not to be generated by increased metabolic function signaled by activating Akt kinase.

4. Discussion

4.1. GsMTx4-D mechanism of cardioprotection and its therapeutic potential

GsMTx4 is the most potent specific inhibitor of cation selective MSCs and has become an important tool for the study of cardiac pathologies [11] which often involve mechanically induced cation imbalance. In vitro studies of isolated human trabeculae show that cationic MSCs have a minimal role in normal cardiac function [26]. This was evident in the free roaming Ferret study in the supporting data section. But under high cardiac demand, or after pathologically induced mechanical changes, the MSC contribution can be much more significant. For example, in normal mouse cardiomyocytes, GsMTx4 only partially inhibited the Ca^{2+} dependent slow component of stretch-induced increase in cardiac force, and had no effect on the Frank-Starling mechanism [27]. However, under pathological conditions like ischemia, many changes occur in the mechanical properties of the cardiomyocytes including disruption of cytoskeletal components critical to regulating cortical stress [9,10,48,49] that can lead to aberrant MSC activity. Loss or compromise of cortical stress regulation and subsequent increased MSC activity has also been observed in skeletal muscle pathology [50,51].

Intracellular cation overload is a significant contributor to myocyte oncosis during the ischemic event and during the return to ionic homeostasis upon reperfusion [1]. During reperfusion, rapid osmotic, pH and energy changes lead to cell swelling and hypercontracture that place high stress on a structurally impaired sarcolemma. A likely scenario is that GsMTx4 slows cation influx through MSCs into cardiomyocytes during both ischemia and reperfusion. This inhibition would decrease cation overload, speed ionic equilibration and reduce hypercontracture. GsMTx4 may also affect vascular tone by inhibiting contraction of VSMCs in the microvasculature [52–54] within the AAR where swelling can lead to decreased blood flow [1].

Interestingly, while GsMTx4 is a known inhibitor of cation influx, it protects from the loss in controlled Ca^{2+} release associated with excitation-contraction coupling when the hypoxic cardiac tissue is under high pathological stress (c.f. Fig. 5). This result is similar to those in studies on dystrophic skeletal muscle where GsMTx4-L protected against loss of Ca^{2+} release after eccentric-contraction induced injury [43,44]. In dystrophic cardiomyocytes, GsMTx4-L reduces the resting intracellular Ca^{2+} levels associated with elevated cation leak in older animals that leads to loss of contraction strength and cardiomyopathy [27]. It is likely that protection from ischemia induced sarcolemma cation leak through MSCs extends to components of the sarcoplasmic reticulum. However, we cannot rule out GsMTx4-D effects on other sarcolemma components involved in Ca^{2+} release such as the Na/Ca exchanger and Ca^{2+} ATPase. In our hypoxic treatment experiments, we did not observe a change in baseline Ca^{2+} levels. However, there was a strong reduction in the rates of Ca^{2+} release and removal.

While these studies demonstrate the acute (1–2 days) cardioprotective effects of GsMTx4-D, its inhibition of JNK/cJun activation in isolated hypoxic cardiomyocytes suggests a potential long term benefit as well. This effect was directly on myocytes separated from the influence of endothelial cells and leukocytes known to play a role in activation of the inflammatory response in situ. Blocking cation flow through MSCs during ischemia may inhibit activation of downstream inflammatory and apoptotic signaling systems.

Surprisingly, GsMTx4-D *inhibited* phosphorylation of the pro-growth Akt kinase whose activation is also correlated with cardioprotection. This may not be detrimental for clinical use of GsMTx4 to treat IRI since there are a number of other therapeutic agents already under study that directly activate the Akt kinase and the complimentary AMPK system that also increases cellular metabolism [46]. For example, volatile anesthetics like isoflurane are cardioprotective and may act through improved mitochondrial function by activation of Akt and other kinases [55,56]. *Most importantly, GsMTx4-D provided significant cardioprotection even in the presence isoflurane and may act synergistically in combination with these other agents.*

While the precise mechanisms need further investigation, these studies clearly show that GsMTx4-D substantially reduced I/R induced infarct size whether by pretreatment (IP injection) or acute (IV) administration during the ischemic challenge. These cardioprotective properties lasted for at least 48 h in the pretreated animals, and that include stages when “late phase” apoptotic cell death begins to occur [10]. These results are in stark contrast to those observed in Barrabes et.al. [28] where the L form of GsMTx4 was used to treat an IRI model in pigs. As stated in the PK results above, it is highly likely that these tests using 58 $\mu\text{g/kg}$ (2 fold less than the lowest concentration used here) were too low to produce a significant effect. This is supported by the observation that the effective refractory period in pigs treated with GsMTx4 was unchanged from control saline injected animals. Effective refractory period was the same indicator used by the authors in isolated rat hearts to show peptide function.

We observed no overt signs of GsMTx4-D toxicity in the heart during these studies, consistent with the lack of toxicity reported in earlier in vitro studies using the L form of

GsMTx4 on cardiac tissue [19,26]. GsMTx4-D reduced the incidence of I/R induced PVBs, reduced left ventricular dilation and increased ejection fraction. We observed a transient (first 15 min after reperfusion) concentration-dependent increase in HR in the ischemia challenged mice anesthetized with pentobarbital, but the HR was the same as controls after 24 and 48 h of reperfusion. No effect on HR was observed in isoflurane anesthetized mice, nor in normal free roaming ferrets which have cardiac properties more closely associated with humans [39].

4.2. GsMTx4 targets may include multiple mechanosensitive signaling systems

The specific target(s) of GsMTx4 in cardiac tissue need to be rigorously determined. However, both the L and D enantiomers have been shown to inhibit mechanosensitive Piezo channels expressed in heterologous systems [57], and tests with the L form suggest it may also inhibit TRP channels in the heart [27,58]. GsMTx4 inhibits endogenous cationic MSCs and exogenously expressed Piezo 1 channels in outside-out patches with a K_D of $\sim 0.5 \mu\text{M}$. The D enantiomer shows strong inhibition of endogenous MSCs in muscle cell types such as chick heart cells [14] and mouse skeletal muscle myotubes [43,51].

We have shown that GsMTx4 associates superficially with membranes under low tension, and inserts deeper when membrane tension increases acting as an area/tension clamp for the outer monolayer [21,22]. Recent electron micrographic studies of the Piezo1 channel structure place the likely mechanosensing domains for these channels on the outer monolayer [59]. So it is not surprising that GsMTx4 inhibits gating of these channels. We also predict that GsMTx4 buffering of tension in the outer monolayer would transfer tension to the cytoplasmic monolayer. Interestingly, GsMTx4 appears to potentiate 2P domain channels [21] which are found in cardiomyocytes [60,61] and are predicted to have gating elements sensitive to tension changes in the cytoplasmic monolayer [62,63].

Like all tarantula venom derived peptides, other channel types show sensitivity to the L form of GsMTx4 at relatively high concentrations (IC_{50} 's for various voltage gated channels were in the range of 10–50 μM [64]). Based on our PK analysis, we predict the GsMTx4-D concentration in the heart at the time of reperfusion was 1–2 μM after two IP pretreatments, and $<0.5 \mu\text{M}$ for the highest dose by IV injection during ischemia. The predicted concentration after IV injection is similar to those used to inhibit atrial fibrillation in rabbit hearts (170 nm) [19] and arrhythmias in rat cardiac tissue sheets (0.4 μM) [20]. These concentrations are likely too low to significantly affect voltage gated channel properties. Multiple studies have shown that, under normal mechanical stress conditions, the L form of GsMTx4 has no detectable effect on cardiac electrical or functional activity at concentrations $<5 \mu\text{M}$ [23] (and reviewed in Friedrich et.al. [11]).

With GsMTx4 functioning as a tension clamp of the outer monolayer, it is possible that it may also affect enzymatic mechanosensitive signaling molecules. For example, Nox2 (gp91^{phox}) and gp22^{phox} subunits are transmembrane proteins that occur as heterodimers and form part of a multi-subunit complex with other cytoplasmic components in the active form [65]. The active complex rapidly (<2 second resolution) responds to mechanical stress in both cardiac and skeletal muscles [13], and its activity is sensitive to sarcolemmal mechanical integrity and microtubule assembly [66]. GsMTx4-L inhibits the Ca^{2+} influx

triggered by ROS production [67]. However, it is unclear whether mechanically sensitive ROS production is affected by elevated cytoplasmic Ca^{2+} in a positive feedback loop, or if the sarcolemma located Nox2 is directly mechanosensitive. Hypothetically, if the stability of the Nox2 assembly is increased by higher lipid tension, then GsMTx4 might also inhibit ROS production. GsMTx4's protection against contractile strength loss and the corresponding reduction in Ca^{2+} release and removal rates are reminiscent of the protective effects observed by gp91ds inhibition of Nox2 mediated oxidative stress during pathological mechanical stress in cardiomyocytes [13]. Nox2-dependent ROS production in isolated cardiomyocytes activates the pro-growth and metabolism kinase Akt [68], and is a key signaling agent of inflammatory pathways involved in JNK/cJun activation [69]. Further studies are needed to determine the mechanosensitive properties of Nox2 and whether GsMTx4 affects ROS production.

5. Conclusions

In summary, GsMTx4-D represents the first member of a new class of drugs for treatment of ischemic reperfusion injury. GsMTx4-D improved all measures of IRI as shown by decreased infarct area, increased cardiac output and decreased incidence of arrhythmias. At the cellular level it improved contractile function and blocked the detrimental effects on SR Ca^{2+} release and uptake mechanisms. It also blocked apoptotic signaling systems which may produce improved long term cardiomyocyte survival. GsMTx4-D activity appears to be most pertinent when cells experience pathological stress since there is little effect on cardiac electrical or functional properties in the normal state. GsMTx4-D may also be useful in reperfusion events occurring with transplantation, angina, cardiac surgery [3] and traumatic injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yjmcc.2016.07.005>.

Nonstandard abbreviations and acronyms

MSC Mechanosensitive ion channel

LCMSMS	Liquid chromatography tandem mass spectrometry
IRI	Ischemia reperfusion injury
I/R	Ischemia reperfusion
P-V	Pressure volume
TTC	2, 3, 5-Triphenyltetrazolium dye
AAR	Area at risk
JNK	c-Jun N-terminal kinase
ROS	Reactive oxygen species

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Fig. 1.

Acute administration of GsMTx4-D during 45 min I/R reduces infarct size after 2 days of reperfusion (time line at top). Hearts from isoflurane anesthetized mice were subjected to 45 min ischemia followed by 48 h of reperfusion. At the conclusion of reperfusion, the tissue was dual stained with TTC and Evans Blue. The white area shows the myocardial infarction, red and white areas show area at risk (AAR), and the blue area shows the non-ischemic tissue. GsMTx4-D was administered at 0.1 (n = 3), 1 (n = 3), 10 (n = 4) mg/kg and compared with vehicle (saline 50 μ L, n = 4) injection administered through tail vein 20 min before initiating reperfusion. Panel A shows representative sections of myocardial infarction. (B) shows the ratio of area at risk (AAR) to total myocardial area, and (C) shows the ratio of infarct area to AAR (Vehicle = $45 \pm 0.7\%$, GsMTx4-D 0.1 mg/kg = $34 \pm 1.2\%$, 1 mg/kg = $29 \pm 1.2\%$, 10 mg/kg = $34 \pm 3.2\%$). Values are means $\pm$ SE. * denotes $P < 0.05$ vs. vehicle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Acute administration of GsMTx4-D during 45 min ischemia improved cardiac function after 2 days of reperfusion. The effects of GsMTx4-D on left ventricular function, was determined by comparing parameters from the P-V relationships obtained by left ventricular catheterization. GsMTx4-D (1 mg/kg) and vehicle (saline 50 μ L) were injected by tail vein into mice anesthetized with isoflurane during 45 min ischemia, 20 min before reperfusion. (A–C) show representative PV loops obtained from the 3 treatment groups – sham, vehicle and GsMTx4 1 mg/kg respectively. Cardiac functional parameters extracted from the P-V loops are shown in (D–K). Values are means $\pm$ SE from sham (n = 5), vehicle (n = 5) and GsMTx4-D (n = 4) injected animals. Statistically significant changes in vehicle injected

animals vs sham experiments is denoted as * ($P < 0.05$), and statistically significant improvements for GsMTx4-D compared to vehicle injected mice is denoted [†] ($P < 0.05$).

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Fig. 3.

GsMTx4-D suppressed I/R induced PVBs. (A) Representative ECG: baseline, ischemia and reperfusion. Hearts from isoflurane anesthetized mice were subjected to 45 min ischemia followed by reperfusion. The characteristic elevated ST-segments during coronary occlusion and T-wave inversion during reperfusion were observed. (B) PVB and supraventricular PB occurring within 30 min of initiating reperfusion were counted and examples of these events are indicated by arrows above the data records. (C) Summary of total PVB and supraventricular PB incidence in the presence of different concentrations of GsMTx4-D. (D)

Incidence of the more severe premature beats of ventricular origin only. There was no change in heart rate following ischemia with the different GsMTx4 treatments (E and F). Values are means $\pm$ SE from independent experiments (vehicle n = 12, GsMTx4-D 0.1 mg/kg n = 3, 1 mg/kg n = 4 and 10 mg/kg n = 7). Statistical significance is denoted as * (P < 0.1) vs. vehicle.

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Fig. 4.

Contractile properties of cardiomyocytes from vehicle and GsMTx4-D treatment after being exposed to hypoxia. (A) Representative sarcomeric length change recordings are shown during contractions stimulated at a frequency of 0.5 Hz. Recordings are shown from vehicle and 10 μM GsMTx4-D treatment for both normoxic and hypoxic treated cells. (B) Resting sarcomere length was unchanged by any of the treatments. (C) Peak sarcomeric length change during contraction. (D) Shows the maximal velocity of shortening ($+dL/dt$), and (E) shows the maximum velocity of relaxation ($-dL/dt$). Values are means $\pm$ SE, $n = 85$ –117 cells per group derived from 3 to 4 mice each, * $P < 0.05$ and ** $P < 0.01$ hypoxia vehicle versus normoxia vehicle; $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$ hypoxia GsMTx4-D versus hypoxia vehicle.

Fig. 5.

GsMTx4-D protects against hypoxia induced loss of intracellular Ca^{2+} release in contracting cardiomyocytes. (A) 340/380 ratio fluorescent imaging records of cardiomyocytes loaded with Fura2-AM Ca^{2+} indicator. Representative recordings of contraction induced intracellular Ca^{2+} changes are shown during contractions stimulated at a frequency of 0.5 Hz. Recordings are shown from vehicle and 10 μM GsMTx4-D treatment for both normoxic and hypoxic treated cells. (B) Resting intracellular Ca^{2+} was unchanged by any of the treatments. (C) Peak Ca^{2+} changes during contraction. (D) Shows the maximum rate of Ca^{2+} change during contraction ($+dF/dt$), and (E) shows the maximum rate of Ca^{2+} change during relaxation ($-dF/dt$). Values are means $\pm$ SE, $n = 60$ –100 cells per group derived from 3 to 4 mice each, * $P < 0.05$ and ** $P < 0.01$ hypoxia vehicle versus normoxic vehicle; † $P < 0.05$ and †† $P < 0.01$ hypoxia GsMTx4-D versus hypoxia vehicle.

Fig. 6.

GsMTx4-D (10 μ M) inhibits activation of JNK/cJun apoptotic signaling system and activation of Akt cell growth and metabolism signaling systems during hypoxia treatment. Representative immunoblots of homogenates from isolated cardiomyocytes are shown from cells in normoxic and hypoxic conditions, and in the absence or presence of GsMTx4-D (A and B). (A) Shows the total amounts of the 46 and 54 kDa forms of JNK and c-Jun (t-JNK and t-c-Jun – which represent loading controls) and the relative amounts of their respective phosphorylated states (p-JNK and p-c-Jun). (B) Shows the total amounts of 3 isoforms of Akt kinase (t-Akt 1/2/3) and the amount of the 60 kDa phosphorylated form (p-Akt). GAPDH shown as a loading control. Normalized averages $\pm$ SE (3 mice, separate experiments each) of phosphorylated:total protein concentration ratio are shown compared

to normoxic conditions without GsMTx4 for 54 kDa JNK (C), 46 kDa JNK (D), c-Jun (E), and Akt (F). *, $P < 0.05$ and **, $P < 0.01$ hypoxia vehicle vs normoxic vehicle, and † , $P < 0.05$ hypoxia GsMTx4_D vs hypoxia vehicle.

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