Methylglyoxal mediates streptozotocin-induced diabetic neuropathic pain via activation of the peripheral TRPA1 and Nav1.8 channels

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Abstract

Objective. Methylglyoxal is known to be associated with the development of nephropathy, retinopathy, and other complications in diabetes. The present study tested the hypothesis that endogenously increased levels of methylglyoxal in diabetes are causally associated with the induction of neuropathic pain.

Materials and Methods. Streptozotocin- and methylglyoxal-induced pain models were established in rats, and the anti-nociceptive effects of the methylglyoxal scavenging agents, selective transient receptor potential channel ankyrin 1 (TRPA1) antagonist, and Nav1.8 antagonist were tested.

Results. Systemic injection of streptozotocin in rats induced a prolonged increase in plasma methylglyoxal by approximately 60%, which was correlated with the progressive development of mechanical allodynia and thermal hyperalgesia. Local subcutaneous injection of methylglyoxal into the hindpaw produced dose-dependent and biphasic flinching nociceptive responses, which resembled formaldehyde (formalin)-induced nociception. The local methylglyoxal nociception was significantly blocked by co-injection into the hindpaw of the selective transient receptor potential channel ankyrin 1 (TRPA1) antagonist, A967079, and the Nav1.8 antagonist, A803467. Co-incubation with the methylglyoxal scavengers, aminoguanidine, D-arginine, and metformin, reduced the level of free methylglyoxal by more than 90%, and injection of their incubation solutions into the hindpaw produced negligible (3–17%) nociception. Like the clinically effective anti-diabetic neuropathic pain drug gabapentin, systemic injection of aminoguanidine, D-arginine, and metformin at doses that effectively inhibit paw-injected methylglyoxal-induced nociception significantly blocked streptozotocin-induced mechanical allodynia.

Conclusion. Endogenously increased methylglyoxal may mediate diabetic neuropathic pain via activation of both TRPA1 and Nav1.8 expressed on primary afferent sensory neurons, and injection of methylglyoxal into the hindpaw may serve as a simple and robust model for testing the anti-diabetic pain drugs.

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Keywords: Methylglyoxal, Diabetic neuropathic pain, TRPA1, Nav1.8, Methylglyoxal scavengers

Abbreviations: AGE, advanced glycation end-product; TRPA1, transient receptor potential channel ankyrin 1; AMPK, AMP-activated protein kinase; DMSO, dimethyl sulfoxide; PEG400, polyethylene glycol 400; AUC, area under the curve; Emax, maximum effect; ED50, half-effective dose; HPLC, high-performance liquid chromatography; SEM, standard error of the mean.

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1. Introduction

Methylglyoxal is a physiological dicarbonyl metabolite mainly produced from the glycolysis of triose phosphates and is detoxified to the end-product D-lactate by two enzymes, glyoxalase 1 and glyoxalase 2 [1,2]. As a highly reactive α-oxoaldehyde due to the electrophilic nature of its methyl group and O-atoms [3], methylglyoxal reacts non-enzymatically with the arginine, lysine, and cysteine residues of certain intracellular proteins to yield irreversible or reversible advanced glycation end-products (AGEs) [4–7]. Hyperglycemia in diabetes leads to an increase in the formation of methylglyoxal and AGEs. Under normal conditions, plasma methylglyoxal levels are between 0.5 and 1.5 μmol/L, while its levels are increased by as much as two- to four-fold under diabetic conditions, and are significantly higher than those of other metabolites, such as glyoxal [8,9].

The endogenously increased methylglyoxal and AGEs are known to be associated with the development of nephropathy, retinopathy, and other complications in diabetes [2,10,11]. The correlation may also be particularly linked to diabetic neuropathic pain, because peripheral neurons are probably vulnerable to the accumulation of methylglyoxal due to a decrease in the expression of glyoxalase 1 [12], and the levels of plasma methylglyoxal in patients who experience diabetic pain have been found to be significantly higher in diabetic patients who do not have pain [13]. In addition, methylglyoxal modifies and activates the pain transmission target molecules expressed on peripheral sensory neurons. Methylglyoxal was reported to excite nociceptors and release neuropeptides via the activation of transient receptor potential channel ankyrin 1 (TRPA1) through the modification of its intracellular N-terminal cysteine and lysine residues [14]. Methylglyoxal was also reported to induce post-translational modification of the arginine residue of Nav1.8 channels [13]. Methylglyoxal-induced modifications were associated with increased electrical excitability and facilitated the firing of nociceptive neurons [13].

Methylglyoxal has a similar chemical structure to formaldehyde, the simplest aldehyde compound. Local application of a diluted formaldehyde solution (formalin), especially to the hindpaw, was developed about 40 years ago, and has been extensively used to assess pain-related responses in a variety of laboratory animals, including rats, mice, cats, rabbits, guinea pigs, Octodon degus, domestic fowl, crocodiles, tortoises, toads, and primates [15]. Local subcutaneous injection of formaldehyde into the hindpaw induces biphasic pain responses. The first phase (acute phase) is thought to be a result of the direct activation of primary afferent sensory neurons, whereas the second phase (tonic phase) is proposed to reflect the combined effects of afferent input and central sensitization in the spinal dorsal horn [16]. Formaldehyde was discovered to directly activate TRPA1 channels and excite primary sensory neurons [16], and this biological property was shared by methylglyoxal [14]. Due to their similar chemical structure and biology, we hypothesized that local subcutaneous injection of methylglyoxal would also produce formaldehyde-like nociceptive responses.

In this study, we systematically tested the hypothesis that the endogenously increased levels of methylglyoxal in diabetes are causally associated with the development of neuropathic pain. First, we examined the association of plasma methylglyoxal levels and mechanical allodynia/thermal hyperalgesia in the widely used rat model of diabetes induced by systemic administration of streptozotocin [17]. We further investigated whether a local subcutaneous injection of methylglyoxal into the hindpaw would produce nociception similarly to formaldehyde, and explored the possible mechanisms associated with the activation of peripheral Nav1.8 and TRPA1 channels. Furthermore, we examined whether a series of methylglyoxal scavengers, aminoguanidine [18,19], d-arginine [20], and metformin [21], effectively blocked exogenous methylglyoxal-induced nociception and streptozotocin-induced mechanical allodynia. Our results suggest that methylglyoxal is a mediator of diabetic neuropathic pain and its local injection into the hindpaw may serve as a simple and reliable animal model for the study of anti-diabetic pain drugs.

2. Materials and Methods

2.1. Drugs

The formaldehyde (37.0–40.0% by weight in water) solution and methylglyoxal (40.0% by weight in water) solution were purchased from Sinopharm Chemical Reagent (Shanghai, China) and Aladdin (Shanghai, China), respectively. The selective TRPA1 receptor antagonist, A967079, and the Nav1.8 sodium channel blocker, A803467, were obtained from Tocris (Ellisville, MO) and Abcam (Cambridge, MA), respectively. Aminoguanidine hydrochloride, d-arginine hydrochloride, metformin hydrochloride, and streptozotocin were purchased from Sigma-Aldrich (St. Louis, MO). Streptozotocin was dissolved in the citrate buffer (pH 4.3). A967079 and A803467 were freshly dissolved in 30% dimethyl sulfoxide (DMSO) and 30% polyethylene glycol 400 (PEG400) in saline. Other reagents and drugs were freshly dissolved and diluted in normal saline solution.

2.2. Experimental Animals

Male Wistar rats (180–250 g) were obtained from the Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China). The animals were housed in a temperature- and humidity-controlled environment on a 12-h light/dark cycle (lights on 7:00 AM) and provided with food and water ad libitum. They were acclimatized to the laboratory environment for 3–5 days before entering the study. The experimental study groups were randomly assigned, and the researchers were blinded to the behavior testing. The research protocols were approved by the Animal Care and Welfare Committee of the Shanghai Jiao Tong University and followed the animal care guidelines of the National Institutes of Health. Efforts were made to reduce the number of animals used and to minimize their suffering.

2.3. Flinching Behavioral Testing in Rats

Rats were acclimatized to the observation chambers individually for about 30 min prior to testing to reduce explorative
behaviors. Following subcutaneous injection of 50 μL of formaldehyde or methylglyoxal solution into the dorsal side of the right hindpaw, the rats were immediately placed into the observation chambers. The pain-related flinching behavior was quantified by counting the incidence of flinching during a 1-min period every 5 min over 60 min for methylglyoxal and every 10 min over 90 min for formaldehyde [22].

2.4. Mechanical Allodynia and Thermal Hyperalgesia in Diabetic Rats

The rats were starved for 16 h before receiving a single intravenous injection of streptozotocin (40 mg/kg) [23,24]. Behavioral assessment of mechanical allodynia and thermal hyperalgesia was conducted as described previously [25]. The hindpaw withdrawal threshold evoked by stimulation of the hindpaw with a 2290 CE electrical von Frey hair (IITC Life Science, Woodland Hills, CA) was determined while the rats stood on a metal grid. The monofilament, which produced forces ranging from 0.1 to 90 g, was applied to the footpad with increasing force until the rats suddenly withdrew their hindlimbs. The lowest force producing a withdrawal response was considered to be the threshold, which was the mean of three repeated measurements.

To assess thermal hyperalgesia, the rats were placed in a plexiglass box on an elevated glass surface. Following an adaption period of not less than 30 min, radiant heat (at a low intensity) was applied to the plantar medial surface of each hindpaw until the animals suddenly withdrew or licked their paws. The hindpaw withdrawal latency was measured using a 390G Plantar Test Analgesia Meter (IITC Life Science). To avoid tissue damage, the latency cut-off time was set at 30 s. Hindpaws were tested independently three times with a 5-min interval between the trials.

2.5. Blood Sugar and Plasma Methylglyoxal Analysis of Rats

The blood sugar levels of the rats were measured on a weekly basis for a total of 4 weeks after the streptozotocin injection using a OneTouch® blood glucose meter (LifeScan, Wayne, PA). Blood was also collected from the orbit flexus under mild anesthesia at the same time points and the plasma was kept frozen until methylglyoxal measurements were made by using a simple derivatization procedure [26], in which methylglyoxal was exposed to 450 mmol/L of perchloric acid (Sinopharm Chemical Reagent) and 10 mmol/L of o-phenylenediamine (Aladdin) in the dark at room temperature for 24 h to form 2-methylquinoline. The methylglyoxal samples and external standard 2-methylquinoline (J&K Scientific, Beijing, China) were quantified in a Shimadzu LC-2010CHT high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) via a reverse C18 column (4.61D x 150 mm and a 4-μm particle diameter) (Global Chromatography, Suzhou, China).

2.6. Statistical Analysis

For the dose–response curve analysis, the parameters, i.e., the minimum effect, maximum effect (E_{max}), half-effective dose (ED_{50}), and Hill coefficient (n), were calculated by fitting nonlinear least-squares curves to the relation Y = a + bx, where x = [D]^{n}/(ED_{50}^{n} + [D]^{n}). The values of ED_{50} and b (E_{max}) were projected by yielding a minimum residual sum of squares of deviations from the theoretical curve [27]. Results were expressed as means ± standard error of the mean (SEM) and the statistical significance was evaluated by the two-tailed Student t-test and one-way or two-way repeated-measures analysis of variance (ANOVA) followed by the post-hoc Student–Newman–Keuls tests. The statistical significance criterion P value was 0.05. All data calculations and statistics analyses were carried out using the Version 5.0 GraphPad Prism Program (GraphPad Software, San Diego, CA).

3. Results

3.1. Association of Plasma Methylglyoxal Levels with Mechanical Allodynia and Thermal Hyperalgesia in Streptozotocin-Induced Diabetic Rats

Two groups of rats (six in each group) received an intravenous injection of the vehicle (the citrate buffer, pH 4.3, 1 mL/kg) or streptozotocin (40 mg/kg) by the tail vein. The blood sugar, plasma methylglyoxal level, and paw withdrawal responses to mechanical or thermal stimulus were measured before and 1, 2, 3, and 4 weeks after the injection of streptozotocin. Systemic streptozotocin caused an immediate increase in hyperglycemia of approximately 4.1-fold, which was maintained over the observation period of 4 weeks (P < 0.05 by two-way ANOVA followed by the post-hoc Student–Newman–Keuls test) (Fig. 1A). In addition, plasma methylglyoxal levels were measured in two more groups of rats (six in each group) treated with the vehicle or streptozotocin. Streptozotocin significantly increased plasma methylglyoxal levels by approximately 60%, which was maintained over the same observation period of 4 weeks (P < 0.05 by two-way ANOVA followed by the post-hoc Student–Newman–Keuls test) (Fig. 1B). Streptozotocin also caused progressive bilateral mechanical allodynia (Fig. 1C) and thermal hyperalgesia (Fig. 1D), beginning 1–2 weeks after injection and reaching plateaus at 3 weeks after injection (P < 0.05 by two-way ANOVA followed by the post-hoc Student–Newman–Keuls test).

3.2. Comparison of Methylglyoxal- and Formaldehyde-Induced Nociception

To compare formaldehyde- and methylglyoxal-induced nociceptive responses, 12 groups of rats (six in each group) received a subcutaneous injection of 50 μL of saline, formaldehyde (2.0, 6.7, 13.3, 20, and 33.3 μmol, i.e., 50 μL of 0.2%, 1%, 2%, 3%, and 5% by volume) or methylglyoxal (2.0, 6.7, 13.3, 20, and 33.3 μmol, i.e., 50 μL of 0.7%, 2.4%, 4.8%, 7.2%, and 12% by volume) into the right hindpaw, and the nociceptive behaviors were measured for 90 min. As shown in Fig. 2A, local injection of formaldehyde produced a characteristic biphasic flinching response consisting of an initial, rapidly decaying acute phase (within 5 min after injection) followed by a slowly rising and long-lived (10–90 min after injection) tonic phase. The quiescent period was within
5–15 min between the acute and tonic phases. Similarly, local injection of methylglyoxal into the paw also produced biphasic flinching responses with the same acute response but a shorter quiescent period (within 5–10 min after injection) and a shorter tonic period (within 60 min after injection) (Fig. 2B).

The nociceptive responses to formaldehyde and methylglyoxal were dose-dependent. A dose–response analysis in molar base showed nearly the same acute nociceptive response for formaldehyde and methylglyoxal, with potencies of 5.5 μmol (50 μL of 0.9% by volume) and 6.2 μmol (50 μL of 2.2% by volume), and efficacies of 21 and 20 flinches/min, respectively (Fig. 2C). In contrast, methylglyoxal-induced tonic phase nociception (summarized as the area under the curve (AUC)\textsubscript{5–60 min}) was significantly less than that of formaldehyde (summarized as the AUC\textsubscript{10–90 min}) (\(P < 0.05\) by the two-tailed Student t-test), mainly due to its shorter duration (Fig. 1D). The ED\textsubscript{50} values for formaldehyde and methylglyoxal were 9.2 μmol (50 μL of 1.5% by volume) and 13.5 μmol (50 μL of 4.8% by volume), and their E\textsubscript{max} values were 1351 and 644 flinches∗min, respectively. Methylglyoxal at 20 μmol (50 μL of 7.2% by volume) displayed a maximal nociceptive effect and was selected for later experiments.

3.3. Inhibition of Methylglyoxal-Induced Nociception by Local Injection of TRPA1 and Nav1.8 Blockers

To explore whether methylglyoxal-induced nociception acted through peripheral TRPA1, the selective TRPA1 blocker, A967079\[28,29\], was injected locally into four groups of rats (six in each group). Three groups of rats received a local subcutaneous injection of methylglyoxal (20 μmol) dissolved in 50 μL of the vehicle (30% DMSO and 30% PEG400 in saline) with or without A967079 (30 and 100 μg), and then the nociceptive responses were immediately measured. As shown in Fig. 3A, local injection of methylglyoxal produced acute and tonic biphasic flinching responses. Local co-injection with A967079 blocked both methylglyoxal-induced acute and tonic flinching responses in a dose-dependent manner. A967079 at 100 μg blocked the acute and tonic responses by 55% (Fig. 3B) and 52% (Fig. 3C), respectively (\(P < 0.05\) by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test). To exclude the possibility that A967079 anti-nociception was due to its subcutaneous absorption and a systemic effect, an additional group of rats received a local injection of methylglyoxal (20 μmol) into the ipsilateral paw and 100 μg of A967079 into the contralateral
Subcutaneous injection of A967079 into the contralateral paw did not significantly affect either acute or tonic flinching responses induced by methylglyoxal (Fig. 3).

To explore whether methylglyoxal-induced nociception was also associated with peripheral Nav1.8 activity, the selective Nav1.8 sodium channel blocker, A803467, was injected locally into four groups of rats (six in each group). Three groups of rats received a subcutaneous injection of methylglyoxal (20 μmol) dissolved in the vehicle (30% DMSO and 30% PEG400 in saline) with or without A803467 (3 and 10 μg) into the paw and flinching responses were measured immediately after. As shown in Fig. 4A, local injection of methylglyoxal produced acute and tonic biphasic flinching responses. Local co-injection with A803467 blocked both acute and tonic methylglyoxal-induced flinching responses in a dose-dependent manner. A803467 at 10 μg blocked the acute and tonic responses by 36% (Fig. 4B) and 90% (Fig. 4C), respectively (P < 0.05 by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test).

To confirm their scavenging activities, aminoguanidine, D-arginine, and metformin at 1200 mmol/L were incubated with methylglyoxal (400 mmol/L) at 37 °C for 3 h [20]. HPLC analysis showed that the free methylglyoxal level in the control sample was 385 ± 0.8 mmol/L (n = 3). Incubation with aminoguanidine, D-arginine, and metformin reduced the free methylglyoxal level by 99.4% (0.7 ± 0.05 mmol/L), 97.3% (3.0 ± 0.04 mmol/L), and 92.1% (8.4 ± 0.2 mmol/L), respectively (P < 0.05 by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test).

To test the residual nociceptive responses to the methylglyoxal solution after its incubation with aminoguanidine, two groups of rats (six in each group) received a subcutaneous injection of methylglyoxal solution alone or methylglyoxal/aminoguanidine incubation solution, and the tonic nociceptive flinching response was measured immediately after. To assess the possible anti-nociceptive effect by the same amount of aminoguanidine due to its systemic absorption, an additional group of 6 rats received a subcutaneous injection of methylglyoxal alone into the ipsilateral paw and aminoguanidine into the contralateral paw. As shown in Figs. 5A and B, incubation with aminoguanidine (33 mg/kg, equal to the amount dissolved in the methylglyoxal incubation solution) significantly reduced methylglyoxal (20 μmol)-induced tonic nociception.

3.4. Inhibition of Methylglyoxal-Induced Nociception by Methylglyoxal Scavengers

To confirm their scavenging activities, aminoguanidine, D-arginine, and metformin at 1200 mmol/L were incubated with methylglyoxal (400 mmol/L) at 37 °C for 3 h [20]. HPLC analysis showed that the free methylglyoxal level in the control sample was 385 ± 0.8 mmol/L (n = 3). Incubation with aminoguanidine, D-arginine, and metformin reduced the free methylglyoxal level by 99.4% (0.7 ± 0.05 mmol/L), 97.3% (3.0 ± 0.04 mmol/L), and 92.1% (8.4 ± 0.2 mmol/L), respectively (P < 0.05 by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test).

To test the residual nociceptive responses to the methylglyoxal solution after its incubation with aminoguanidine, two groups of rats (six in each group) received a subcutaneous injection of methylglyoxal solution alone or methylglyoxal/aminoguanidine incubation solution, and the tonic nociceptive flinching response was measured immediately after. To assess the possible anti-nociceptive effect by the same amount of aminoguanidine due to its systemic absorption, an additional group of 6 rats received a subcutaneous injection of methylglyoxal alone into the ipsilateral paw and aminoguanidine into the contralateral paw. As shown in Figs. 5A and B, incubation with aminoguanidine (33 mg/kg, equal to the amount dissolved in the methylglyoxal incubation solution) significantly reduced methylglyoxal (20 μmol)-induced tonic nociception.

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To test the residual nociceptive responses to the methylglyoxal solution after its incubation with aminoguanidine, two groups of rats (six in each group) received a subcutaneous injection of methylglyoxal solution alone or methylglyoxal/aminoguanidine incubation solution, and the tonic nociceptive flinching response was measured immediately after. To assess the possible anti-nociceptive effect by the same amount of aminoguanidine due to its systemic absorption, an additional group of 6 rats received a subcutaneous injection of methylglyoxal alone into the ipsilateral paw and aminoguanidine into the contralateral paw. As shown in Figs. 5A and B, incubation with aminoguanidine (33 mg/kg, equal to the amount dissolved in the methylglyoxal incubation solution) significantly reduced methylglyoxal (20 μmol)-induced tonic nociception.
In contrast, the contralateral injection of aminoguanidine did not significantly alter methylglyoxal-induced flinching response. In addition, the same treatment regimens showed that incubation with D-arginine (63 mg/kg) and metformin (50 mg/kg), which were equal to the amount dissolved in the methylglyoxal incubation solution, reduced methylglyoxal-induced tonic flinching responses by 97% and 83%, respectively (P < 0.05 by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test), whereas the contralateral injection of the same doses of D-arginine or metformin slightly (30%) inhibited or did not significantly inhibit methylglyoxal nociception (Figs. 5C–F).

To validate whether systemic methylglyoxal scavengers blocked methylglyoxal-induced nociception further, four groups of rats (six in each group) received a subcutaneous injection of saline (1 mL/kg), aminoguanidine (50 mg/kg), D-arginine (300 mg/kg), or metformin (250 mg/kg) followed by a challenge with methylglyoxal 30 min later. Local injection of methylglyoxal (20 μmol) produced profound acute and tonic nociceptive flinching responses (Fig. 6A) but, in contrast, markedly reduced methylglyoxal-induced tonic flinching responses by 50%, 53%, and 67%, respectively (P < 0.05 by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test) (Fig. 6C).

3.5. Inhibition of Streptozotocin-Induced Painful Diabetic Neuropathy by Systemic Injection of Methylglyoxal Scavengers

To explore whether the methylglyoxal scavengers blocked painful diabetic neuropathy, four groups of rats (six in each group), approximately 4 weeks after injection of streptozotocin to induce diabetes, received a subcutaneous injection of saline (1 mL/kg), aminoguanidine (50 mg/kg), D-arginine (300 mg/kg), and metformin (250 mg/kg), respectively. Paw withdrawal thresholds were recorded before and after drug administration for 4 h. Subcutaneous injection of aminoguanidine, D-arginine, and metformin exhibited similar significant blockage effects on methylglyoxal-induced mechanical allodynia in a time-dependent manner; all three methylglyoxal scavengers reached a peak of anti-nociception 1 h after injection, with inhibitory rates of approximately 44%, 55% and 56%, respectively (P < 0.05 by two-way ANOVA followed by the post-hoc Student–Newman–Keuls test) (Fig. 7A).
For a comparison, the clinically effective anti-diabetic neuropathic pain drug, gabapentin [31–33], was also tested in two groups of diabetic rats (six in each group), approximately 4 weeks after streptozotocin injection. A subcutaneous injection of gabapentin (50 mg/kg) reversibly blocked streptozotocin-induced mechanical allodynia by a similar degree (by approximately 67% inhibition) to the methylglyoxal scavengers (P < 0.05 by two-way ANOVA followed by the post-hoc Student–Newman–Keuls test) (Fig. 7B).

Fig. 4 – Inhibitory effects of a local injection of the selective Nav1.8 sodium channel blocker, A803467, on methylglyoxal-induced pain in rats [A]. Three groups of rats received a local injection of methylglyoxal (20 μmol) dissolved in 50 μL of the vehicle (30% DMSO and 30% PEG400 in saline) with or without A803467, and an additional group of rats received a subcutaneous injection of methylglyoxal (20 μmol) in the ipsilateral paw and of 10 μg of A803467 in the contralateral paw. Nociceptive behavior, quantified by counting the number of flinches in 1-min periods, was considered as acute (B) and tonic (C) phases as measured by AUC5–60 min. Data are presented as means ± SEM (n = 6 in each group). * Denotes a statistically significant difference compared with the vehicle control group (P < 0.05 by one-way ANOVA followed by the post-hoc Student–Newman–Keuls test).

4. Discussion

A number of studies have revealed that plasma methylglyoxal levels are increased in diabetic patients in the presence or absence of pain [8,9,13]. It is known that an increase in methylglyoxal induces the overproduction of reactive oxidative stress [34,35] and the formation of AGEs by reacting with the arginine, lysine, and cysteine residues of selective intracellular proteins [10,11], both of which have been reported to be closely related to the induction of diabetic neuropathy. Streptozotocin-induced diabetes is commonly used as an experimental metabolic model of neuropathic pain which is sensitive to the clinically effective anti-diabetic pain drug, gabapentin [31,33]. In this study, we showed that streptozotocin significantly increased the formation of methylglyoxal by approximately 60% within 7 days or less after injection, and the increase was maintained over an observation period of 4 weeks. The increase in plasma methylglyoxal level was associated with (although faster) the progressive development of painful neuropathy accompanied by hypersensitivity and allodynia to mechanical and thermal stimulation. Mechanical allodynia and thermal hyperalgesia began approximately 1–2 weeks after streptozotocin injection and lasted for at least 1 month. The role of methylglyoxal in the mediation of diabetic pain was further supported by the fact that exogenous methylglyoxal induced robust flinching nociception. In naïve rats, local subcutaneous injection of methylglyoxal (2–20 μmol/paw) into the hindpaw produced dose-dependent, biphasic nociception characterized by paw flinching. Our results were consistent with a recent finding that intraplantar administration of methylglyoxal (250 nmol/paw) induced nociceptive behaviors in mice [14]. It is possible that the local concentration of methylglyoxal after hindpaw injection is higher than that in streptozotocin-induced diabetic rats. It was reported that systemic administration of methylglyoxal (5 μg) produced thermal (cold and heat) and mechanical hyperalgesia in mice [36], in which the increased plasma
concentration (estimated to be 1.7 μmol/L) was approximately 3-fold higher than that in our streptozotocin-induced diabetic rats (+0.6 μmol/L). The relatively lower concentration required to induce pain in diabetic rats may be attributed to its longer exposure of methylglyoxal, by which the development of diabetic pain is slower than that of methylglyoxal production induced by streptozotocin.

The causal correlation between increased levels of methylglyoxal and the induction of diabetic neuropathic pain was further confirmed by the intervention study with a series of methylglyoxal scavengers, aminoguanidine, D-arginine, and metformin. Aminoguanidine reacted with methylglyoxal to form triazines under physiological conditions [18,19], prevented the irreversible modification of plasma and intracellular protein and thus attenuated the development of hypertension [18,37]. Both D-arginine and L-arginine showed a high affinity for methylglyoxal and prevented methylglyoxal-induced oxidative stress, endothelial dysfunction, and the formation of AGEs [20].

Fig. 5 – Inhibitory effects of a local injection of the incubation solution of aminoguanidine (A, B), D-arginine (C, D), and metformin (E, F), on methylglyoxal (MG)-induced pain in rats. Aminoguanidine, D-arginine, and metformin were incubated with methylglyoxal at a 3:1 ratio at 37 °C for 3 h. Two groups of rats received a local injection of 50 μL of methylglyoxal alone, the methylglyoxal/aminoguanidine, methylglyoxal/D-arginine, or methylglyoxal/metformin incubation solution. Additional groups of rats received a subcutaneous injection of methylglyoxal (20 μmol) alone in the ipsilateral paw and of aminoguanidine (33 mg/kg), D-arginine (63 mg/kg), or metformin (50 mg/kg) in the contralateral paw. Tonic nociceptive behavior was quantified by counting the number of flinches in 1-min periods and was measured by the area under the curve (AUC)5–60 min. Data are presented as means ± SEM (n = 6 in each group). * Denotes a statistically significant difference compared to the methylglyoxal alone group (P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test).
Metformin is a first-line anti-hyperglycemic drug that has been widely prescribed for diabetic patients and significantly reduced their plasma methylglyoxal levels [21] by forming a metformin-methylglyoxal adduct characterized as a triazepinone and reducing the production of AGEs [38]. Using the HPLC technology, we confirmed that the co-incubation of aminoguanidine, D-arginine, or metformin with methylglyoxal dramatically reduced the free methylglyoxal content by more than 90%. In addition, a subcutaneous injection of the methylglyoxal sample after incubation with aminoguanidine, D-arginine, or metformin into the paw induced negligible (3–17%) residual flinching nociception. The results were supported by a recent study in which thermal hyperalgesia induced by systemic methylglyoxal was reduced by the synthetic peptide GERP, which was designed with 10 arginine residues per molecule to act as an effective scavenger of methylglyoxal [13]. More importantly, systemic injection of aminoguanidine, D-arginine, or metformin, at doses that effectively inhibit methylglyoxal-induced tonic nociception in the hindpaw, markedly blocked diabetes-induced mechanical allodynia. Gabapentin exhibited similar effects in this model. The findings were supported by recent studies, in which metformin and the arginine-enriched peptide, GERP, were able to attenuate mechanical hyperalgesia, heat hyperalgesia, and cold allodynia in streptozotocin-induced painful neuropathy in rats and mice [13,39]. All of these results suggest that methylglyoxal could be a potential target molecule for the treatment of metabolic neuropathic pain, and that methylglyoxal scavengers could serve as therapeutic interventions. However, this does not seem to be supported by a recent clinical survey study [40], in which orally administered metformin was not shown to be associated with lower pain scores in diabetic patients or even in neuropathic pain patients, but the investigation was not a prospective study and the dose of metformin used (an average of 1432 mg) may not be sufficiently high. Given that metformin is relatively safe and is effective in animal pain models, a double-blinded, random-controlled clinical trial should be performed to investigate the efficacy of higher doses of metformin as a potential analgesic in metabolic neuropathic pain patients, while using plasma methylglyoxal as a biomarker.

However, although the anti-nociceptive effects of D-arginine in addition to aminoguanidine and metformin are correlated to their methylglyoxal scavenging effects, it is difficult to exclude the possibility that other mechanisms are also involved in these agents’ anti-nociception. Indeed, aminoguanidine is also an inhibitor of inducible nitric oxide synthase [41], which could induce methylglyoxal to form peroxynitrite [34,42], and has been claimed to be involved in neuropathic pain [43–45] and inflammatory pain [46,47]. In addition, metformin has multiple effects, including inhibiting

![Fig. 6 – Inhibitory effects of a subcutaneous injection of the methylglyoxal scavengers, aminoguanidine, D-arginine, and metformin, on methylglyoxal-induced pain in rats (A). Rats received a subcutaneous injection of saline (1 mL/kg), aminoguanidine (50 mg/kg), D-arginine (300 mg/kg), or metformin (250 mg/kg) followed by a challenge with methylglyoxal (20 μmol) 30 min later. Nociceptive behavior, quantified by counting the number of flinches in 1-min periods, was considered as acute (B) and tonic (C) phases and measured by the area under the curve (AUC)5–60 min. Data are presented as means ± SEM (n = 6 in each group). * Denotes a statistically significant difference compared with the saline control group (P < 0.05 by one-way ANOVA followed by post-hoc Student-Newman-Keuls test).](image-url)
channels. Intraplantar injection of methylglyoxal induced sensory neurons through a specific and direct action on TRPA1 cysteine and lysine residues [14], which consist with earlier findings that formaldehyde activated primary afferent sensory neurons through a specific and direct action on TRPA1 channels [54]. Intraplantar injection of methylglyoxal induced nociceptive behaviors in wild-type but not in TRPA1−/− mice [36]. On the other hand, methylglyoxal was also reported to enhance sensory neuronal excitability by the post-translational modification of the arginine residue of Nav1.8 channels, which are also located in the peripheral sensory neurons and to modulate nociceptive transmission and transduction under conditions of pathophysiological pain [55,56]. Systemic treatment of wild-type mice with methylglyoxal reduced nerve conduction velocity and induced thermal and mechanical hyperalgesia, which were lost in Nav1.8 knockout (Snx10−/−) mice or small interfering RNA-induced Nav1.8 knockout mice [13]. Our comparative study, entailing the local co-application of either the selective TRPA1 antagonist A967079 [28,29], or Nav1.8 antagonist A803467 [30] with methylglyoxal, confirmed that the activation of both peripheral TRPA1 and Nav1.8 contributed to methylglyoxal nociception. Local co-injection of A967079 (100 μg) into the paw blocked both acute and tonic methylglyoxal-induced nociception in the ipsilateral paws by 55% and 52%, respectively. Similarly, local co-injection of A803467 (10 μg) into the paw also suppressed both acute and tonic nociception by 36% and 90%, respectively. In contrast, local injections of the same doses of A803467 or A967079 into the contralateral paws did not inhibit methylglyoxal nociception, thus excluding the possibility of systemic or central effects. It expects that blockade of TRPA1 and Nav1.8 channels by A967079 and A803467 would produce synergistic anti-nociception. Our hypothesis of an independent bi-mechanism is reasonable, because methylglyoxal is a fast, non-specific and non-enzymatic modulator of the arginine, cysteine, and lysine residues of selective intracellular proteins [4–7], including TRPA1, Nav1.8, and possibly other peripheral receptors, channels, enzymes, and transporters, which are involved in pain transmission and transduction.

Systemic injection of streptozotocin produced immediate and prolonged hyperglycemia and induced progressive, long-lasting thermal hyperalgesia and mechanical hyperalgesia/tactile allodynia and diabetes within 2–8 weeks [17]. The animal model mimics the symptoms of painful diabetic neuropathy and is widely used in preclinical studies of diabetes and diabetic pain, but takes about 3–5 weeks to set up. A fast and reliable animal model of diabetic pain is therefore needed, particularly for testing the anti-diabetic pain drugs. Methylglyoxal, also known as acetyl formaldehyde, shares similar chemical properties with formaldehyde. Although methylglyoxal produced a shorter duration of pain and had a higher ED₅₀ value than formaldehyde, it shared the same characteristics of providing a simple procedure and robust nociceptive biphasic responses. In addition, the formaldehyde (formalin) test is a well-studied animal model and its relatively known mechanisms and signal transduction pathways would be helpful to illustrate anti-diabetic drugs in the methylglyoxal test. For example, formaldehyde-induced tonic pain but not acute nociception is known to be derived from the combined effects of afferent input and central sensitization in the spinal dorsal horn [16], and is associated with pain hypersensitivity states, including painful diabetic neuropathy [57,58]. Our data showed that the systemic injection of all of three methylglyoxal scavengers, aminoguanidine, d-arginine, and metformin, significantly blocked methylglyoxal-induced tonic pain, but had almost no effect on methylglyoxal-induced acute nociception. The

mitochondrial respiration and gluconeogenesis, activating AMP-activated protein kinase (AMPK), increasing insulin sensitivity, antagonizing the action of glucagon, and increasing fatty acid oxidation [48]. Metformin was reported to induce AMPK activation in the spinal cord or in the sciatic nerves, and decreased the nociceptive behaviors in neuropathic pain [49,50], inflammatory nociception [51], and painful diabetic neuropathy [39].

TRPA1 is a member of the transient receptor potential family of cation channels that is highly expressed by a subset of peripheral C-fiber nociceptors and has been increasingly presented as a target molecule for the treatment of pain hypersensitivity states [14,36,52,53]. Methylglyoxal was found to excite nociceptors and release neuropeptides via the activation of TRPA1 channels by modifying their intracellular N-terminal cysteine and lysine residues [14], which consists with earlier findings that formaldehyde activated primary afferent sensory neurons through a specific and direct action on TRPA1 channels [54]. Intraplantar injection of methylglyoxal induced
feasibility of the methylglyoxal test to study anti-diabetic pain was further validated by the following evidence. (1) Endogenously increased methylglyoxal is highly correlated with diabetes and diabetic pain, and methylglyoxal-induced pain may be directly linked to human conditions; and (2) Methylglyoxal scavengers are effective in blocking exogenous methylglyoxal-induced pain (particularly tonic pain) and streptozotocin-induced metabolic neuropathic pain, which is probably mediated by endogenously released methylglyoxal.

Author Contributions

Y.X.W., H.Q. and C.Y. wrote the manuscript. H.Q., C.Y., N.G. and Y.X.W. researched and analyzed data. All authors have read and approved the final manuscript.

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Conflict of Interest

All authors declare no conflict of interest.

REFERENCES


