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Morphine hyposensitivity in streptozotocin-diabetic rats: Reversal by dietary L-arginine treatment

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Summary

Painful diabetic neuropathy (PDN) is a long-term complication of diabetes. Defining symptoms include mechanical allodynia (pain due to light pressure or touch) and morphine hyposensitivity. In our previous work using the streptozotocin (STZ)-diabetic rat model of PDN, morphine hyposensitivity developed in a temporal manner with efficacy abolished at 3 months post-STZ and maintained for 6 months post-STZ. As this time course mimicked that for the temporal development of hyposensitivity to the pain-relieving effects of the furoxan nitric oxide (NO) donor, PRG150 (3-methylfurox an-4-carbaldehyde) in STZ-diabetic rats, we hypothesized that progressive depletion of endogenous NO bioactivity may underpin the temporal loss of morphine sensitivity in STZ-diabetic rats. Furthermore, we hypothesized that replenishment of NO bioactivity may restore morphine sensitivity in these animals. Diabetes was induced in male Dark Agouti rats by intravenous injection of STZ (85 mg/kg). Diabetes was confirmed on day 7 if blood glucose concentrations were ≥15 mmol/L. Mechanical allodynia was fully developed in the bilateral hindpaws by 3 weeks of STZ-diabetes in rats and this was maintained for the study duration. Morphine hyposensitivity developed in a temporal manner with efficacy abolished by 3 months post-STZ. Administration of dietary L-arginine (NO precursor) at 1 g/d to STZ-diabetic rats according to a 15-week prevention protocol initiated at 9 weeks post-STZ prevented abolition of morphine efficacy. When given as an 8-week intervention protocol in rats where morphine efficacy was abolished, dietary L-arginine at 1 g/d progressively rescued morphine efficacy and potency. Our findings implicate NO depletion in the development of morphine hyposensitivity in STZ-diabetic rats.

KEYWORDS
intervention protocol, L-arginine, mechanical allodynia morphine, morphine hyposensitivity, nitric oxide, painful diabetic neuropathy, prevention protocol, STZ-diabetic rats

1 | INTRODUCTION

Painful diabetic neuropathy (PDN) is a type of peripheral neuropathic pain that develops in the long nerves in a "stocking and glove" distribution, and that affects up to 20% of patients with diabetes.[1,2] PDN symptoms include lancinating, burning and shooting pains as well as mechanical allodynia, a type of pain evoked by non-noxious mechanical stimuli such as light pressure or touch.[1,2] In our previous work, we showed that single bolus doses of the furoxan nitric oxide (NO) donor, PRG150 (3-methylfuroxan-4-carbaldehyde), evoked dose-dependent pain relief in STZ-diabetic rats. However, the efficacious bolus doses of PRG150 at 14 and 24 weeks of STZ-diabetes were 3 to 4 orders of magnitude higher than that required at 10 weeks post-STZ or in non-diabetic control rats.[3]

In other work by our group in diabetic rats assessed in longitudinal studies over prolonged periods, morphine hyposensitivity

developed in a temporal manner and was fully developed by approximately 3 months of diabetes with it being maintained for at least 6 months.[4-6] Interestingly, this time course mimicked that for progressive development of hyposensitivity to the pain-relieving effects of the furoxan NO donor, PRG150, in STZ-diabetic rats[3] as well as clinical reports of the ineffectiveness of morphine for the symptomatic relief of PDN.[1,2] Together, our previous findings suggest that temporal depletion of NO bioactivity in advanced diabetes may have a key role in the pathobiology of the associated morphine hyposensitivity.

Endogenous NO is synthesized from the precursor, L-arginine, by 3 NO synthase (NOS) enzymes, viz endothelial NOS, neuronal NOS and inducible NOS.[7] Under normal conditions in vivo, NO is a highly diffusible gas with a half-life of only a few seconds that regulates many physiological processes.[7] However, after peripheral nerve injury, ongoing ectopic firing of injured primary afferents induces central sensitization in the dorsal horn of the spinal cord that is underpinned by persistent activation of the *N*-methyl-D-aspartate (NMDA)/NOS/NO signaling cascade and glial cell activation, to markedly increase NO formation at multiple levels of the somatosensory nervous system.[8-10] In diabetes, there is increased generation of superoxide that reacts with elevated levels of NO to increase formation of the neurotoxin, peroxynitrite, resulting in nitrooxidative stress and the development of PDN.[11,12]

From the foregoing, we developed the hypothesis that progressive depletion of endogenous NO bioactivity may have a key role in the temporal development of morphine hyposensitivity in STZ-diabetic rats and that replenishment of endogenous NO bioactivity may restore morphine sensitivity in these animals. To test our hypothesis, STZ-diabetic rats were administered dietary L-arginine (NO precursor) at 1 g/d (2.5% in ground rodent chow) according to either a 15-week prevention protocol (Group 1) commencing at 3 weeks prior to the abolition morphine efficacy (9-weeks post-STZ) or an 8-week intervention protocol commencing at 14 or 30 weeks post-STZ administration (Groups 2 and 3, respectively).

2 | RESULTS

2.1 | Mechanical hypersensitivity in the hindpaws of STZ-diabetic rats

The mean (± SEM) paw withdrawal thresholds (PWTs) in the bilateral hindpaws of drug-naïve STZ-diabetic Dark Agouti (DA) rats were significantly lower ($P \leq .05$) than those for control non-diabetic rats (11.9 ± 0.2 g). Specifically, in Group 1 STZ-diabetic DA rats (Figure 1), the mean (±SEM) PWTs decreased significantly ($F_{(3,71)} = 265.4$, $P \leq .05$; one-way ANOVA with Tukey's multiple comparisons test) from 11.9 (± 0.2) g in non-diabetic rats to 6.8 (± 0.3) g by 9 weeks post-STZ administration. In Group 2 STZ-diabetic rats (Figure 1), the mean (± SEM) hindpaw PWTs at 14 and 24 weeks post-STZ administration were significantly reduced ($F_{(2,11)} = 14.6$, ($P \leq .05$) one-way ANOVA with Tukey's multiple comparisons test) to 3.8 (± 0.2) g and 3.1 (± 0.3) g respectively. These data show that mechanical allodynia was fully developed (PWTs ≤ 6 g) for at least 24 weeks post-STZ administration. In Group 3 rats (Figure 1), the mean (± SEM) hindpaw PWT at 24 weeks post-STZ administration was 3.1 (± 0.1) g which differed significantly ($P > .05$; unpaired *t* test) from that in non-diabetic rats at 11.9 (± 0.2) g. Overall, dietary L-arginine treatment of STZ-diabetic rats for up to 15 weeks did not reverse mechanical allodynia in the bilateral hindpaws (see details in Supplementary Results).

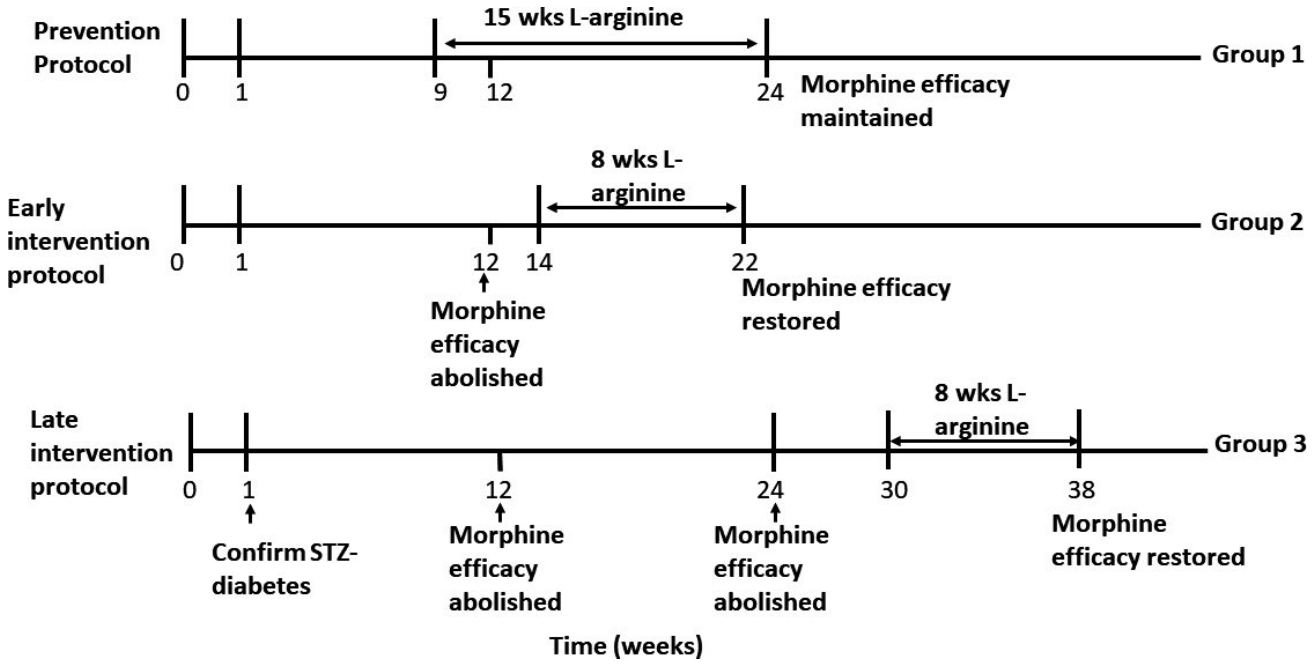

FIGURE 1 Schematic diagram of the 3 L-arginine dosing protocols used in the present work

2.2 | Dietary L-arginine prevents loss of morphine efficacy in STZ-diabetic rats

2.2.1 | Group 1: L-arginine prevention protocol

The dose-response curve for single subcutaneous (s.c.) bolus doses of morphine in STZ-diabetic DA rats at 9 weeks post-STZ administration is shown in Figure 2. The corresponding mean (± SEM) dose that produced a 50% response (ED_{50}) was 6.1 (± 0.3) mg/kg in agreement with that for STZ-diabetic DA rats at 3 weeks post-STZ reported previously by our laboratory.[4] Additionally, the mean (± SEM) ED_{50} for s.c. morphine (7.0 ± 0.5 mg/kg) after 3 weeks of dietary L-arginine treatment (12 weeks post-STZ) did not differ significantly ($F_{(2,54)} = 2.9$, $P > .05$; one-way ANOVA with Tukey's multiple comparison test) from that determined in Group 1 STZ-diabetic DA rats just prior to initiation of the dietary L-arginine intervention at 9 weeks post-STZ (Figure 2). Thus, administration of dietary L-arginine for as little as 3 weeks prevented the abolition of morphine efficacy (Figure 2) that otherwise occurs at 12 weeks post-STZ as previously reported by our laboratory.[4,5] After 15 weeks of dietary L-arginine treatment in the same animals (24 weeks post-STZ), acute s.c. morphine efficacy was maintained (Figure 2) in contrast to its abolition in STZ-diabetic DA rats at 24 weeks post-STZ that were fed a standard rodent chow diet.[4] Specifically, after 15 weeks of dietary L-arginine, the s.c. morphine dose-response curve was shifted to the left (Figure 2) such that the mean (± SEM) ED_{50} was 5.0 (± 0.9) mg/kg (Figure 2) *c.f.* 7.0 (± 0.5) mg/kg in the same animals at 12 weeks post-STZ administration ie, after 3 weeks treatment with dietary L-arginine.

2.3 | Dietary L-arginine restores morphine efficacy and potency in STZ-diabetic rats

2.3.1 | Group 2: L-arginine early intervention protocol

Administration of the s.c. morphine ED_{50} (6.1 mg/kg) determined at 9 weeks post-STZ in Group 1 rats (Figure 2), to Group 2 diabetic rats at 14 weeks post-STZ, showed that the anti-allodynic efficacy of morphine was abolished in these animals (Figure 3). Specifically, the mean (± SEM) extent and duration of anti-allodynia (percent maximum possible effect area under the curve; %Maximum Possible Effect [MPE] AUC) was only 5.2 (± 2.5) %MPE.h (Figure 3). However, after 4 weeks of the dietary L-arginine intervention in these animals (18 weeks post-STZ), morphine efficacy was restored (Figure 3) such that the mean (± SEM) %MPE AUC for a single s.c. dose of morphine at 6.1 mg/kg was 109.8 (± 28.6) %MPE.h (Figure 3). Four weeks later after 8 weeks of the dietary L-arginine intervention (22 weeks post-STZ), morphine efficacy was not only retained in these animals (Figure 3) but there was a significant increase in the potency of single s.c. bolus doses of morphine at 6.1 mg/kg ($F_{(2,11)} = 14.5$; $P \leq .05$; one-way ANOVA with Tukey's multiple comparison test) (Figure 3). Additionally, the mean (± SEM) %MPE AUC was 149.5 (± 9.5) %MPE.h which did not differ ($F_{(3,16)} = 11.5$, $P > .05$; one-way ANOVA with Tukey's multiple

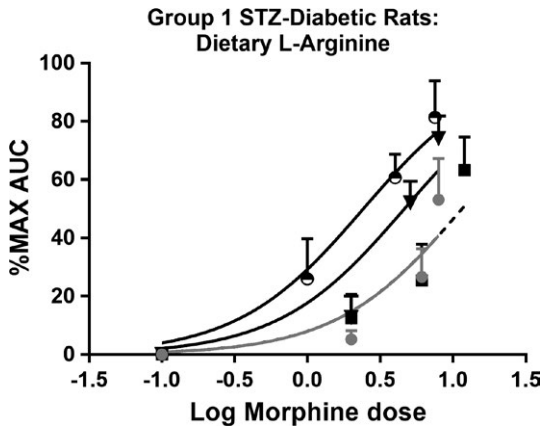

FIGURE 2 Dietary L-arginine (1 g/d mixed in ground rodent chow) administered as a prevention protocol prevented temporal development of morphine hyposensitivity in adult male STZ-diabetic Dark Agouti (DA) rats. Specifically, there was a 2-fold rightward shift in the dose-response curve for single s.c. bolus doses of morphine at (—●—) 9 weeks post-STZ administration relative to that for non-diabetic male DA rats. Administration of dietary L-arginine for 3 weeks (12 weeks post-STZ) prevented the abolition of morphine efficacy that otherwise occurred in similar animals.[4,5] Administration of the dietary L-arginine intervention for 15 weeks in the same STZ-diabetic animals not only prevented the development of morphine hyposensitivity, but it increased morphine potency by ~50%. (—●—) Non-diabetic; (—●—) 9 weeks post-STZ; pre L-Arg; (-■-) 12 weeks post-STZ; 3 weeks L-Arg; (—▼—) 24 weeks post-STZ; 15 weeks L-Arg

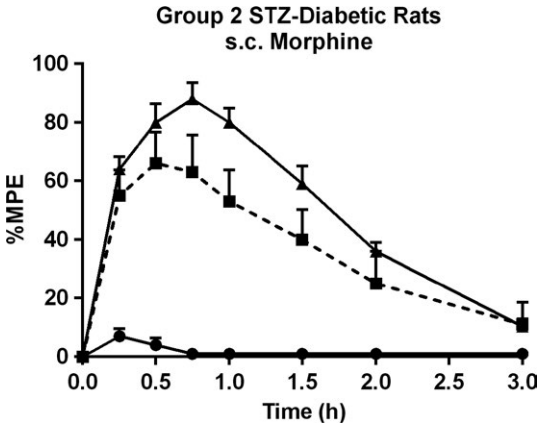

FIGURE 3 Administration of dietary L-arginine according to an intervention protocol rescued morphine efficacy and potency in adult male STZ-diabetic DA rats exhibiting marked morphine hyposensitivity at 14 weeks post-STZ. Specifically, mean (± SEM) response (% maximum possible effect, %MPE) vs time curves for single s.c. bolus doses of morphine at 6.1 mg/kg administered to STZ-diabetic rats at 14 weeks post-STZ and then at 4 and 8 weeks after initiation of a dietary L-arginine intervention (18 and 22 weeks post-STZ respectively) in the same animals showed restoration of morphine efficacy and a progressive increase in potency over the 8-week L-arginine treatment period. (—●—) 14 weeks post-STZ; (-■-) 18 weeks post-STZ with 4 weeks L-Arg; (—▲—) 22 weeks post-STZ with 8 weeks L-Arg

comparison test) from that for s.c. morphine at 6.1 mg/kg in opioid-naïve non-diabetic control rats (136.9 ± 16.1%MPE.h).

2.3.2 | Group 3: L-arginine late intervention protocol

In STZ-diabetic DA rats where morphine efficacy had been abolished since 12 weeks post-STZ administration,[4] an 8-week dietary L-arginine intervention at 1 g/d commencing at 30 weeks post-STZ (Figure 1), resulted in restoration of morphine efficacy (Figure 4). Specifically, after 4 weeks of dietary L-arginine, the mean (± SEM) %MPE AUC value evoked by an s.c. bolus dose of morphine at 6.1 mg/kg was 62.2 (± 15.8) %MPE.h (Figure 4) which did not differ significantly ($F_{(4,23)}$ = 31.1, P > .05; one-way ANOVA with Tukey's multiple comparison test) from that (63.6 ± 7.4%MPE.h) evoked in Group 1 diabetic rats at 9 weeks post-STZ and fed a standard rodent chow diet (Figure 2). Continuation of the late dietary L-arginine intervention for a further 4 weeks to 8 weeks in the same animals (30-38 weeks post-STZ) (Figure 4) resulted in a significant increase ($F_{(4,23)}$ = 31.1, P ≤ .05; one-way ANOVA with Tukey's multiple comparison test) in morphine's anti-allodynic potency (Figure 4). Specifically, the mean (± SEM) %MPE AUC evoked by a single s.c. bolus dose of morphine at 6.1 mg/kg was 117.1 (± 15.4) %MPE-h which was approximately 2-fold larger than the respective mean (± SEM) %MPE AUC value after 4 weeks of the late dietary L-arginine intervention in the same animals (Figure 4).

2.3.3 | Dietary L-arginine: No effect on morphine efficacy or potency in control non-diabetic rats

Mean (± SEM) food intake for L-arginine treated STZ-diabetic rats was 43.2 (± 0.2) g/d whereas control non-diabetic rats treated with dietary L-arginine consumed 20.5 (± 0.7) g of food per day. Thus, in order to give comparable doses of L-arginine to both STZ-diabetic and non-diabetic weight-matched control rats, the concentration of L-arginine in the ground rodent chow was 2-fold higher for non-diabetic rats at 5% *c.f.* 2.5% for STZ-diabetic animals. For non-diabetic male DA rats, the mean (± SEM) ED_{50} at 3.0 (± 0.2 mg/kg) for the antinociception (%MPE) dose-response curve (Figure 5) evoked by single s.c. bolus doses of morphine did not differ significantly (P > .05; unpaired t test) from that (2.5 ± 0.2 mg/kg) for non-diabetic male DA rats administered L-arginine (1 g/d) for 1 week prior to antinociceptive testing (Figure 5). Thus, chronic administration of dietary L-arginine did not alter morphine antinociception in opioid-naïve non-diabetic male DA rats (Figure 5).

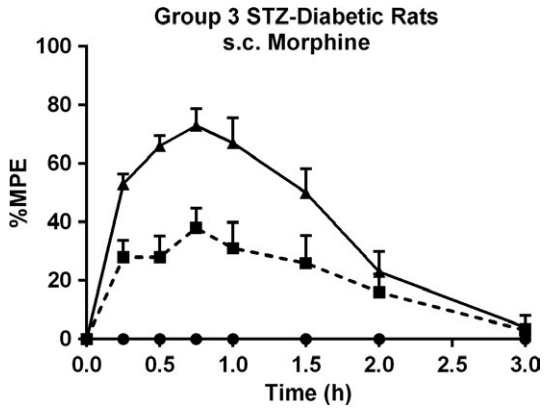

FIGURE 4 Dietary L-arginine administered as an intervention protocol rescued morphine efficacy and potency in adult male STZ-diabetic DA rats exhibiting abolition of morphine efficacy at 24 weeks post-STZ. Specifically, mean (± SEM) response (% maximum possible effect, %MPE) vs time curves for single s.c. bolus doses of morphine at 6.1 mg/kg administered to STZ-diabetic rats at 24 weeks post-STZ and then again at 4 and 8 weeks after L-arginine treatment initiation (34 and 38 weeks post-STZ respectively) showed restoration of morphine efficacy and a progressive increase in potency over the 8-week L-arginine treatment period. (—●—) 24 weeks post-STZ; (-■-) 34 weeks post-STZ with 4 weeks L-Arg; (—▲—) 38 weeks post-STZ with 8 weeks L-Arg

2.4 | General health

Following induction of diabetes with STZ, there was a 12%-13% decrease in mean (± SEM) body weight (see details in Supplementary Results online) and there was a marked increase in blood glucose concentrations (from 6 to >20 mmol/L) (Supplementary Results). Dietary administration of L-arginine at 1 g/d mixed in rodent chow to STZ-diabetic rats for up to 15 weeks did not adversely alter body weight (P > .05) and it had no significant effect on elevated blood glucose concentrations (P > .05) (see details in Supplementary Results).

3 | DISCUSSION

Our findings show for the first time that dietary administration of the endogenous NO substrate, L-arginine at 1 g/d according to either a 15-week prevention protocol (Figure 1) or an 8-week intervention protocol (Figure 1) in STZ-diabetic rats modulated the extent and

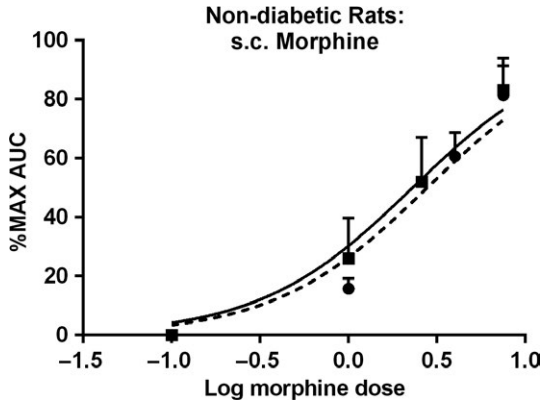

FIGURE 5 Administration of dietary L-arginine to non-diabetic adult male DA rats for 1 week did not alter morphine sensitivity relative to that for similar animals fed standard rodent chow. Specifically, the dose-response curve for single s.c. bolus doses of morphine in groups of non-diabetic male Dark Agouti rats administered either (i) rodent chow supplemented with 5% L-arginine for 1 week or (ii) standard rodent chow for 1 week, did not differ significantly (P > .05; unpaired t test). (—●—) No L-Arg; (-■-) 1 week L-Arg

duration of pain relief evoked by single s.c. bolus doses of morphine in these animals (Figs 2-4). Specifically, 15 weeks of dietary L-arginine given according to a prevention protocol initiated at 9 weeks post-STZ administration in rats, prevented the abolition of morphine efficacy that otherwise occurred at 12 weeks post-STZ (Figure 2) in comparable rats fed a standard rodent chow diet.[4,5] Not only was morphine efficacy maintained in these animals, but the potency of morphine did not differ significantly ($P > .05$) from that in the same animals prior to commencement of the dietary L-arginine treatment (Figure 2).

Intriguingly, initiation of a dietary L-arginine (1 g/d) intervention after morphine efficacy had been abolished in STZ-diabetic rats (ie, at 14 weeks post-STZ for Group 2; Figure 1), progressively rescued morphine efficacy and potency over an 8-week treatment period (Figure 3). Administration of the same dietary L-arginine regimen (1 g/d for 8 weeks) to STZ-diabetic rats commencing at 30 weeks post-STZ, also progressively restored morphine efficacy and potency in the bilateral hindpaws of these animals (Figure 3).

In weight-matched control, non-diabetic rats, dietary L-arginine at 1 g/d had no significant ($P > .05$) effect on morphine potency relative to that for control non-diabetic rats fed a standard rat chow diet (Figure 5). Our findings are aligned with previous work by others[13] whereby a single intraperitoneal (ip) bolus dose of L-arginine at 200 mg/kg given 10 minutes prior to morphine had no significant effect on levels of antinociception in mice.

The restoration of morphine efficacy in STZ-diabetic rats administered the L-arginine dietary intervention for 8 weeks occurred despite the ongoing neuropathic pain state that remained unaltered in these animals during the 8 weeks of L-arginine treatment. The mechanism by which L-arginine progressively rescued morphine efficacy and potency over the 8-week treatment period in STZ-diabetic rats without altering the underlying peripheral neuropathic pain state, is unclear. However, NO formed from the dietary L-arginine, may have attenuated perineurial hypoxia and the associated oxidative stress, as well as reducing otherwise elevated levels of diacylglycerol and activated protein kinase C (PKC), that are produced as a consequence of persistent hyperglycaemia in diabetes.[14,15] Of particular importance to the present work, PKC mechanisms are implicated in desensitization of the opioid receptor[16-22] as well as the development of diabetic complications.[16] A role for PKC in the development of opioid hyposensitivity in diabetes is supported by observation that supraspinal pretreatment of STZ-diabetic rats with the PKC inhibitor, calphostin C, prevented the development of opioid hyposensitivity.[23,24]

In recent work by others in STZ-diabetic rats exhibiting opioid hyposensitivity, MOP receptor immunoreactive primary sensory neurons were co-localized with activated PKC isoforms as well as with the receptor for advanced glycation end products (RAGE).[16] Using a range of measures including administration of a selective PKC inhibitor, intrathecal RAGE siRNA, or inhibition of advanced glycation end product (AGE) formation to prevent both RAGE-dependent PKC activation and desensitization of the MOP receptor, opioid analgesic efficacy was restored.[16] In other work in rats with advanced diabetes at 12 weeks post-STZ, the inhibitory effects of intraplantar bolus doses of morphine on capsaicin-induced nocifensive behaviour were impaired.[25] Also, there was a markedly reduced inhibitory effect of morphine on capsaicin-induced TRPV1 current in DRG neurons from these animals.[25] These changes were associated with a loss of functional MOP but not TRPV1 receptors in primary sensory neurons with all changes reversed by intrathecal infusion of nerve growth factor (NGF).[25] NO plays a role in NGF-mediated neurotrophic responses. Hence, it is plausible that chronic dietary administration of L-arginine in STZ-diabetic rats to replenish in vivo NO bioactivity and modulate NGF-TrkA signaling pathways,[26] underpins the rescue of morphine analgesic efficacy in advanced diabetes herein.

In the Zucker Diabetic Fatty rat model of Type 2 diabetes, morphine hyposensitivity in animals with advanced diabetes at 29 weeks of age, was underpinned by reduced MOP receptor function in the spinal cord that appeared to be due to reduced basal G-protein activity.[6] In other work in rats with advanced diabetes (12 weeks post-STZ administration), hyposensitivity to the pain-relieving effects of single intrathecal (i.t.) bolus doses of the MOP receptor agonist, fentanyl, was accompanied by a reduction in MOP immunoreactivity in small diameter sensory neurons, a reduction in membrane-bound MOP binding sites and impaired MOP receptor G-protein coupling in the dorsal horn of the spinal cord.[27] Pretreatment with intrathecal bolus doses of NGF reversed these changes and rescued opioid responsiveness.[25]

Although dietary supplementation with L-arginine restored morphine efficacy and potency in STZ-diabetic rats herein, this treatment had only a minor effect on baseline PWTs. These findings are in contrast to work by others where administration of L-arginine as a prevention protocol in the drinking water (2.6 g/L for 3 weeks; water consumption 276 mL/d) prevented the development of both mechanical and thermal hypersensitivities in the hindpaws of STZ-diabetic rats.[28] This difference may be because L-arginine treatment commenced at the time of diabetes onset rather than at 9, 14 or 30 weeks post-STZ administration as was done in the present work.

As L-arginine has the potential to stimulate hormonal secretion including that of growth hormone, glucagon, prolactin and insulin,[29] we assessed the effect of chronic dietary L-arginine on blood glucose levels in rats with advanced STZ-induced diabetes. Importantly, we show that irrespective of when the dietary L-arginine intervention was initiated in the interval 9-30 weeks post-STZ administration, it did not alter the markedly elevated blood glucose levels (>20 mmol/L) in these animals (Supplementary Results). Our findings are aligned with other work whereby intravenous injection of a bolus dose of L-arginine (150 mg/kg) followed by chronic infusion (10 mg/kg per minute for 60 minutes), did not significantly alter ($P > .05$) blood glucose levels in either non-diabetic or STZ-diabetic rats.[30] Furthermore, chronic L-arginine administration in the drinking water at 1.25 mg/mL for 4 weeks commencing at 12 weeks post-STZ administration or at 1.0 mg/mL for 12 weeks after diabetes induction[31,32] did not significantly alter ($P > .05$) hyperglycaemia in these animals, mirroring the profoundly elevated blood glucose levels measured in STZ-diabetic rats herein.

Irrespective of the cellular mechanisms involved in the restoration of morphine efficacy in rats with advanced STZ-diabetes and given dietary L-arginine treatment at 1 g/d for either 15 weeks according to a prevention protocol or 8 weeks according to an intervention protocol,

our findings suggest that NO donors may restore opioid analgesic efficacy in patients with advanced diabetes. If so, it may be possible to provide improved pain relief in diabetic patients whose PDN is poorly relieved by clinically available drug treatments recommended by the Neuropathic Pain Special Interest Group of the International Association for the Study of Pain, such as the gabapentinoids and the tricyclic antidepressants.[33]

In summary, our findings are the first to provide in vivo evidence linking depletion of NO bioactivity with the development of morphine hyposensitivity in the STZ-diabetic rat model of PDN. Specifically, treatment of STZ-diabetic rats with dietary L-arginine at 1 g/d for 15 weeks according to a prevention protocol prevented the abolition of morphine efficacy that otherwise occurred in STZ-diabetic rats at 12 weeks post-STZ administration. Additionally, treatment of STZ-diabetic rats that were insensitive to the pain-relieving effects of single s.c. bolus doses of morphine, with dietary L-arginine at 1 g/d for 8 weeks according to an intervention protocol progressively rescued morphine efficacy and potency in these animals without significantly altering the underlying peripheral neuropathic pain state.

4 | MATERIALS AND METHODS

4.1 | In vivo experimentation

4.1.1 | Animals

Ethics approval was from the Animal Ethics Committee of The University of Queensland (Brisbane, Australia). Male DA rats were purchased from the Central Animal Breeding House, The University of Queensland. Rats were housed in pairs in a temperature controlled room (21 ± 2°C; mean ± SD) with a 12 h/12 h light/dark cycle. Rats had free access to rodent chow and water, and were acclimatized prior to initiation of experimentation.

4.2 | Reagents and materials

L-arginine hydrochloride, streptozotocin, citric acid and trisodium citrate were from Sigma Aldrich (Sydney, Australia). Morphine hydrochloride was from the Pharmacy Department, Royal Brisbane and Women's Hospital (Brisbane, Australia). Sodium benzylpenicillin (Benpen) vials containing 600 mg of powder, ketamine hydrochloride (Ketamav-100) vials (100 mg/mL) and xylazine hydrochloride (Ilium Xylazil-20) vials (20 mg/mL) were from Abbott Australasia Pty Ltd (Sydney, Australia). Sodium chloride ampoules were from Delta West Pty. Ltd. (Perth, Australia). Single lumen polyethylene tubing (0.5 mm internal diameter) was from Critchley Electrical Products Pty. Ltd. (Auburn, Australia). Blood glucose testing strips (Glucostix) were from The University of Queensland Campus Pharmacy (Brisbane, Australia) and a glucometer Precision Q.I.D was from Medisense Australia Pty Ltd (Melbourne, Australia). Medical grade CO_2 and O_2 were purchased from BOC Gases Australia Ltd. (Brisbane, Australia).

4.3 | Induction of diabetes with Streptozotocin (STZ)

Anaesthesia was induced with a mixture of ketamine (100 mg/kg, ip) and xylazine (16 mg/kg, ip) to facilitate insertion of a polyethylene cannula (pre-filled with 0.1 mL of sterile saline) into the right common jugular vein. Cannulae were tested for correct placement by withdrawing a small amount of blood. Diabetes was induced in rats by injection of STZ (85 mg/kg in 0.1 mol/L citrate buffer at pH 4.5) via the jugular vein cannula.[3-5] Benzylpenicillin (60 mg s.c.) was administered and rats were monitored during surgical recovery. Diabetes was confirmed on day 7 post-STZ in individual rats if daily water intake was ≥100 mL and blood glucose levels (BGLs) were ≥15 mmol/L. Non-diabetic rats were used in the control experiments.

4.4 | L-arginine dietary administration

A group of control non-diabetic rats received dietary L-arginine at 1 g/d (5% mixed with ground rodent chow) for 7 days. This L-arginine dose was used previously by others in hypertensive non-diabetic rats.[34] A 2-fold lower concentration of L-arginine (2.5% mixed with ground rodent chow) was used in STZ-diabetic rats because their mean (± SEM) food consumption was 2-fold higher at 43.2 (± 0.2) g/d compared with 20.5 (± 0.7) g/d for the non-diabetic group.

4.5 | Dosing solutions and dose administration

A stock solution of morphine (45 mg/mL as the free base in sterile saline) was prepared for s.c. bolus dose administration and aliquots were frozen at approximately −20°C until required. On each dosing occasion, an aliquot was thawed and serially diluted with sterile saline to produce the required dosing solution. Rats received a single s.c. injection (100 μL) of morphine into the scruff of the neck, using a 250 μL Hamilton syringe.

4.6 | Treatment groups

This study comprised 3 groups of STZ-diabetic DA rats and 1 group of non-diabetic DA rats. Morphine bolus dose administration was according to a "washout" protocol with 4 days of washout between successive doses and each rat received a maximum of 3 doses.

4.6.1 | Group 1 STZ-diabetic rats: L-arginine prevention protocol

Group 1 DA rats (n = 25; 256 ± 3.6 g, mean ± SEM) received an intravenous dose of STZ (85 mg/kg) and were studied longitudinally over a 6-month period. For each testing session, rats received single s.c. doses of morphine. Individual animals received one of three bolus doses of morphine (Figure 1) to produce dose-response curves at 9, 12 and 24 weeks post-STZ. PWTs were measured in the bilateral hindpaws at regular intervals over a 3-hour post-dosing period using Von Frey filaments. At 9 weeks post-STZ in Group 1 STZ-diabetic rats, dietary L-arginine at 1 g/d mixed with ground rodent chow was

initiated and continued until study completion at 24 weeks post-STZ administration.

4.6.2 | Group 2 STZ-diabetic rats: L-arginine early intervention protocol

Group 2 STZ-diabetic DA rats (n = 17, 239.7 ± 4.9 g, mean ± SEM) received an intravenous injection of STZ (85 mg/kg) and were studied longitudinally over a 6-month period. Individual rats (n = 6) received the ED_{50} dose of s.c. morphine (6.1 mg/kg) determined in Group 1 rats at 9 weeks post-STZ to evaluate the acute anti-allodynic responses at 14, 18 and 22 weeks post-STZ administration (Figure 2). PWTs were measured in the bilateral hindpaws at regular intervals over a 3-hour post-dosing period as for Group 1. At 14 weeks post-STZ administration, an L-arginine dietary intervention at 1 g/d mixed with ground rodent chow was initiated and continued for 8 weeks (Figure 2).

4.6.3 | Group 3 STZ-diabetic rats: L-arginine late intervention protocol

Group 3 STZ-diabetic DA rats (n = 6, 228.8 ± 4.2 g, mean ± SEM) were a sub-group of the same rats used by Nielsen et al.[4] These had previously received single s.c. bolus doses of morphine at 3, 9, 12 and 24 weeks post-STZ administration.[4] Commencing at 30 weeks post-STZ, these animals received dietary L-arginine mixed with ground rodent chow at 1 g/d for 8 weeks (Figure 3). After 4 and 8 weeks of dietary L-arginine, rats received a single bolus dose of s.c. morphine at 6.1 mg/k (ED_{50} in STZ-diabetic rats at 9 weeks post-STZ in Group 1). PWTs were measured in the bilateral hindpaws at regular intervals over a 3-hour post-dosing period as for Groups 1 and 2.

4.6.4 | Control non-diabetic rats

A group of opioid-naïve, non-diabetic DA rats (n = 18; 215.0 ± 2.0 g, mean ± SEM) received one of three bolus doses (Figure 4) of s.c. morphine. PWTs were measured in the bilateral hindpaws at regular intervals over a 3-hour post-dosing period. Opioid-naïve control DA rats that had received dietary L-arginine at 1 g/d mixed in ground rodent chow for 1-week (n = 18; 236.8 ± 2.5 g, mean ± SEM) also received a single s.c. bolus dose of morphine and underwent antinociceptive testing (Figure 4). As STZ-diabetic rats eat twice as much as non-diabetic rats, the concentration of L-arginine mixed with ground rodent chow administered to control non-diabetic rats was doubled to 5% to ensure that L-arginine intake was similar between STZ-diabetic rats and the control non-diabetic rats.

4.7 | Assessment of paw withdrawal thresholds in the bilateral hindpaws

Mechanical allodynia in the bilateral hindpaws, a defining symptom of neuropathic pain, was assessed using calibrated Von Frey filaments (2-20 g). In brief, rats were placed individually into wire mesh cages (20 cm × 20 cm × 20 cm) and allowed to acclimatize. Von Frey filaments were used to measure the lowest mechanical threshold to evoke a brisk hindpaw withdrawal reflex starting with the 2 g filament. The absence of a response after 5 seconds prompted application of the next filament of increasing force. A score of 20 g was given to animals that did not respond to any of the filaments. Pre-drug (morphine) paw withdrawal thresholds (PWTs) were the mean of 3 readings taken ~5 minutes apart. Assessment of bilateral hindpaw PWTs was determined pre-dose and at the following times post-morphine administration: 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 hours.

4.8 | Data analysis

The Von Frey scores for individual rats were converted to the percentage of the Maximum Possible Effect (%MPE), using the following formula:

$$\%\text{MPE} = \frac{(\text{Post- dose threshold} - \text{Pre- dose threshold})}{(\text{Maximum threshold} - \text{Pre- dose threshold})} \times \frac{100}{1}$$

where maximum threshold = 20 g.

%MPE vs time curves were constructed for individual rats. The area under the %MPE vs time curve (%MPE AUC value; %MPE.h) was calculated using trapezoidal integration. The mean (± SEM) percentage maximum AUC (%Max AUC) for each morphine dose was plotted vs the log dose to produce dose-response curves. ED_{50} doses (mean ± SEM) were estimated using non-linear regression of the %Max AUC vs log dose values as implemented in the GraphPad Prism (v 6.0) statistical analysis package.

4.9 | Statistical analysis

A one-way ANOVA with Tukey's multiple comparison test as implemented in the GraphPad Prism statistical analysis package was used to assess differences in body weights, blood glucose levels and bilateral hindpaw PWTs between the treatment groups. One-way ANOVA with Tukey's multiple comparison test was also used to compare the ED_{50} values for morphine for the various treatment groups in STZ-diabetic rats. Unpaired *t* tests were used to compare various parameters in non-diabetic rats administered dietary L-arginine at 1 g/d for 1 week compared with similar animals fed standard rodent chow. The statistical significance criterion was $P < .05$. For statistical comparisons using one-way ANOVA, *F* values are reported as $F_{(df \text{ of treatment, residual})}$.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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