Glycemia-dependent Nuclear Factor κ B Activation Contributes to Mechanical Allodynia in Rats with Chronic Postischemia Pain

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Abstract

Background—Ischemia-reperfusion injury causes chronic postischemia pain (CPIP), and rats with higher glycemia during ischemia-reperfusion injury exhibit increased allodynia. Glycemia-induced elevation of nuclear factor kappa-B (NF κ B) may contribute to increased allodynia.

Methods—Glycemia during a 3 h ischemia-reperfusion injury was manipulated by: normal feeding; or normal feeding with administration of insulin; dextrose; or insulin/dextrose. In these groups, NF κ B was measured in ipsilateral hind paw muscle and spinal dorsal horn by ELISA, and SN50, an NF κ B inhibitor, was administered to determine its differential anti-allodynic effects depending on glycemia.

Results—CPIP fed/insulin rats (12.03 ± 4.9 , $N = 6$) had less allodynia than fed, fed/insulin/dextrose and fed/dextrose rats (6.29 ± 3.37 , $N = 7$, 4.57 ± 3.03 g, $N = 6$, 2.95 ± 1.10 , $N = 9$), respectively. Compared to fed rats (0.209 ± 0.022 , $N = 7$), NF κ B in ipsilateral plantar muscles was significantly lower for fed/insulin rats and significantly higher for fed/dextrose rats (0.152 ± 0.053 , $N = 6$; 0.240 ± 0.057 , $N = 7$, respectively). Furthermore, NF κ B in the dorsal horn of fed, fed/insulin/dextrose and fed/dextrose rats (0.293 ± 0.049 , $N = 6$) was significantly higher than in fed/insulin animals (0.267 ± 0.037 , $N = 6$). The anti-allodynic SN50 dose-response curves of CPIP rats in the fed/insulin/dextrose, fed/dextrose and fed conditions exhibited a rightward shift compared to the fed/insulin group. The threshold SN50 dose of CPIP fed/dextrose, fed/insulin/dextrose and fed rats (328.94 ± 92.4 , 77.80 ± 44.50 and 24.89 ± 17.20 , respectively) was higher than that for fed/insulin rats (4.06 ± 7.04).

Conclusions—NF κ B was activated in a glycemia-dependent manner in CPIP rats. Hypoglycemic rats were more sensitive to SN50 than rats with higher glycemia. The finding that

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SN50 reduces mechanical allodynia suggests that NF κ B inhibitors might be useful for treating postischemia pain.

Introduction

Ischemia-reperfusion (I/R) injury involves a prolonged period of ischemia followed by reperfusion, leading to both deleterious intracellular injuries and inflammatory reactions¹. Postischemia pain is a well-recognized consequence of I/R injury², and chronic postischemia pain (CPIP) is an animal model of long-term post-ischemia pain. Under general anesthesia, CPIP rats undergo placement of a tight-fitting tourniquet around their hind paw for a 3-h period; chronic ipsilateral and more sporadic contralateral mechanical and cold allodynia were demonstrated to follow this I/R injury for at least 4 weeks³. We have previously demonstrated that glycemic modulation during I/R injury can significantly impact allodynia in CPIP rats. Indeed, hyperglycemia (dextrose administration) during I/R injury led to significantly increased mechanical and cold allodynia for at least 3 weeks following the injury, whereas relative hypoglycemia (fasting and insulin administration) resulted in significantly decreased mechanical and cold allodynia⁴.

Nuclear factor κ B (NF κ B) is an inducible redox-sensitive transcription factor present in a latent form in the cytoplasm of almost all mammalian cells⁵⁻⁷. It regulates many aspects of cellular stress and initiates the transcription of several pro-inflammatory mediators, such as cytokines, adhesion molecules and inflammatory enzymes⁸. NF κ B activation can be modulated by different glycemic conditions. Indeed, NF κ B is activated in tissues exposed to chronic high glucose⁹. Chronic high glucose leads to increased reactive oxygen species and tumor necrosis factor- α , which mediate the degradation of inhibitor κ B- α , a regulator of NF κ B, allowing NF κ B nuclear translocation. Elevations in activated NF κ B and tumor necrosis factor- α levels were also demonstrated in healthy subjects following acute ingestion of glucose or a mixed meal^{10,11}.

In contrast, insulin has inhibitory effects on NF κ B activation. Insulin was shown to inhibit NF κ B in human aortic endothelial cells *in vitro*^{12,13} and in monocytes *in vivo*¹⁴. Similarly, infusing insulin to obese subjects increases inhibitor κ B- α levels, and decreases NF κ B¹⁴. Similar results were obtained in a rat model of type 2 diabetes mellitus, where insulin increases inhibitor κ B- α and decreases NF κ B p65 in the liver and skeletal muscle of the animals¹⁵.

NF κ B exacerbates cellular injury and inflammation in animal models of cerebral¹⁶, spinal cord¹⁷ and skeletal muscle¹⁸ I/R injury. NF κ B may enhance postischemia pain after I/R injury, and therefore might be an interesting target for intervention. In the present study, we hypothesize that the increased mechanical allodynia observed in CPIP rats with higher glycemic levels is associated with increased NF κ B activation, and that conversely, the reduced mechanical allodynia observed in relatively hypoglycemic CPIP rats is associated with reduced NF κ B activation. We also hypothesize that NF κ B inhibition will significantly reduce postischemia pain, and that groups of CPIP rats with higher glycemic levels will exhibit a rightward shift of the anti-allodynic dose-response curve after NF κ B inhibition

compared to the relatively hypoglycemic group of CPIP rats, reflecting different intrinsic levels of activated NF κ B.

Material and methods

Animals

The present study was conducted in male Long Evans rats (300–500 g, Charles River, Senneville, Quebec, Canada). Following their arrival, rats were allowed a minimum of 3 days to acclimatize before the start of the experiment. Rats were housed in groups of two to four, had unlimited access to food and water, and were exposed to a 12-h light:dark cycle (lights on 7:00 AM). Experiments were approved by the McGill Animal Care and Ethics committees, and were performed in accordance with the ethical guidelines of the Canadian Council on Animal Care and the International Association for the Study of Pain¹⁹.

Chronic post-ischemia pain induction

I/R injury of the left hind paw was induced as described³. Rats were deeply anesthetized with an intraperitoneal bolus (55 mg/kg) of sodium pentobarbital, followed by a 2-h continuous infusion (27.5 mg/kg/h, intraperitoneal). After anesthesia induction and verification of its depth by confirming the absence of pedal reflexes, a Nitride 70 Durometer O-ring (O-rings, Seattle, WA) with a 5.5 mm internal diameter was placed around the rat's left hind paw, proximal to the ankle and left in place for 3h, after which it was cut to allow reperfusion. The termination of the pentobarbital infusion was timed so that rats would recover within 60 min after reperfusion. Sham surgery consisted of pentobarbital anesthesia alone.

Glycemia-modulating treatment conditions

Rats undergoing I/R or sham injury were divided into four treatment groups, in order to evaluate different glycemic conditions. The first group of rats had normal access to food and water (fed; seven CPIP/six sham-treated). The second group of rats had normal access to food and water, but also received subcutaneous injections of insulin diluted in normal saline (fed/insulin; six CPIP/seve Sham-treated), according to a sliding scale designed for this experiment (baseline glycemia: 6: 0.5 international units (IU)/kg; > 6 7: 1 IU/kg; > 7 8: 1.5 IU/kg; > 8 9: 2 IU/kg; > 9 10: 2.5 IU/kg; because none of the rats had a baseline glycemia > 10, it was not necessary to further extend the insulin sliding scale). The total dose of insulin was administered in two half-doses, the first one immediately prior to ischemia, and the second one 60 min after ischemia induction. The maximal volume of insulin solution administered was 50 μ l. A third group of rats had normal access to food and water, but also received insulin according to the above-described sliding scale, as well as dextrose (fed/insulin/dextrose; six CPIP/eight Sham-treated). Dextrose was dissolved in deionised water (final concentration: 40%) and administered as 1 ml intraperitoneal injections at baseline, 60, and 120 min following the induction of ischemia. A fourth group of rats (fed/dextrose; nine CPIP/eight sham-treated) consisted of normally fed animals given dextrose according to the above schedule in order to produce a relative hyperglycemia.

Glucose measurements

Glycemic levels were measured with a glucometer (*FreeStyle mini*, Abbott Diabetes Care, Mississauga, Ontario, Canada). Blood was withdrawn from the extremity of the rat tail, from a submillimetric incision, by an observer blinded to treatment. We did not measure local glucose levels, since we determined in preliminary trials that sampling blood from the hind limb produced local injury that produced significant allodynia on its own. Volume of blood withdrawn varied between 10.0 and 40.0 μ l. Blood samples were withdrawn at baseline, and then 30, 90, and 150 min after the placement of the tourniquet (ischemia phase), as well as 20, 40, and 60 min following the removal of the tourniquet (reperfusion phase).

Drugs

Insulin R (Eli Lilly Canada Inc., Toronto, Ontario, Canada) was diluted in sterile normal saline to obtain a final concentration of 10 IU/ml. SN50 (Enzo Life Sciences International Inc., Plymouth Meeting, PA) was diluted in normal saline to a concentration of 50 ng/ μ l. Solutions were made freshly prior to the experiment. The inactive control peptide of SN50 (Enzo Life Sciences International Inc.) stored at -20°C was similarly diluted in normal saline to a concentration of 50 ng/ μ l, and freshly diluted to a final concentration of 30 ng/ μ l prior to the experiment.

Tissue sampling and preparation for ELISA

Four CPIP and four sham groups of rats fed (seven CPIP and six sham), fed/insulin (six CPIP and seven sham), fed/insulin/dextrose (six CPIP and eight sham) and fed/dextrose (nine CPIP and eight sham), were tested for mechanical allodynia on day 2 post-I/R procedure by an observer blinded to treatment. Six animals randomly selected from each group (seven from CPIP fed rats) were killed by decapitation following a lethal dose of sodium pentobarbital. Muscle samples (12–30 mg wet weight each) of the superficial plantar layer (*flexor hallucis brevis*, *flexor digiti minimi* and *flexor digitorum brevis*) and spinal cord samples (10–20 mg wet weight each) at the lumbar (L3–L6) segments, ipsilateral to the tourniquet placement, were immediately extracted. Spinal cord samples were sectioned in half horizontally to isolate the dorsal part containing the dorsal horns, and then sectioned in half vertically at the midline to separate the ipsilateral from the contralateral side. Samples were flash frozen in isopentane (-45°C), put on dry ice, and kept at -80°C until processing.

Nuclear extracts were prepared according to a protocol from a commercially supplied kit (Active Motif, Carlsbad, CA). Briefly, tissue samples from the ipsilateral paw muscles (left paw in sham-treated animals) and the ipsilateral dorsal horn (left dorsal horn in sham-treated animals) were diced into very small pieces using a clean razor blade. Pieces were collected in a prechilled container. On ice, tissues were disrupted and mechanically homogenized in a lysis buffer. Samples were then centrifuged at 850 g in a microcentrifuge pre-cooled to 4°C for 10 min. The pellets were then resuspended in a hypotonic buffer, after which they were incubated for 15 min on ice. Detergent was added and the suspension was centrifuged for 30 s at 14,000 g in a microcentrifuge precooled to 4°C . The supernatant was stored at -80°C . Nuclear-enriched pellets were resuspended in a lysis buffer and were vortexed for 10 s at maximal intensity. They were then incubated for 60 min on ice, followed by 30 s vortexing at maximal intensity, and then concentrated by centrifugal filtration at 14,000 g for 20 min.

using cellulose filters with a 30 kDa cut-off (Microcon YM-30, Millipore Corp., Billerica, MA). The resulting nuclear fractions were aliquoted and stored at -80°C until further analysis. Total protein content of nuclear extracts was determined according to the Bradford method²⁰.

Measurement of NF κ B by ELISA

In order to measure NF κ B levels, we used a commercially supplied p65 NF κ B transcription factor binding assay (Active Motif, Carlsbad, CA), according to the protocol described by the manufacturer. Duplicates of nuclear samples containing 25 μg total protein (volume 20–30 μL) were deposited in buffer in wells (final volume 150 μL) containing the NF κ B consensus site oligonucleotide sequence (5'-GGGACTTTC-3') and incubated overnight at 4°C . The N-terminus of NF κ B is significantly conserved; consequently, the NF κ B consensus motif of the assay binds both human and rat p65. After three washes with 200 μL of wash buffer, 100 μL of diluted rabbit NF κ B antibody (1:1,000) was added to each well and incubated for 60 min at room temperature. After three washes with 200 μL of wash buffer, 100 μL of horse radish peroxidase-conjugated antibody (1:1,000) was added to the wells, after which the samples were incubated for 60 min at room temperature. Colorimetric detection was then used to semi quantitatively assess nuclear (translocated) NF κ B p65 levels, measured as net absorbance (total absorbance minus background and negative control levels) on a spectrophotometer at 450 nm (Molecular Devices, Sunnyvale, CA).

NF κ B inhibition with SN50

The NF κ B inhibitor SN50 was used to investigate the involvement of NF κ B in mechanical allodynia in relatively hyperglycemic CPIP rats *versus* normo- and relatively hypoglycemic CPIP rats. SN50 is a cell-permeable synthetic peptide which inhibits the translocation of the active NF κ B complex into the nucleus, in cell cultures²¹ and *in vivo*²².

The effects of SN50 on NF κ B inhibition were studied in CPIP rats ($N = 7$ per group) in each of the four glycemic conditions described above (*i.e.*, fed, fed/insulin, fed/insulin/dextrose and fed/dextrose). Five different drug treatments were tested for each group. Each group of rats received four doses of SN50 intrathecally, every other day (four different SN50 doses between 6.66 ng and 1,000 ng) and 600 ng of inactive control peptide (on the last day of treatment). The first SN50 treatment was administered on day 2 postprocedure, following testing for mechanical allodynia (baseline score), and the last treatment was given on day 10. Intrathecal injections (all 20 μL) were performed by lumbar puncture at L5 – L6 level. SN50 injections were performed under isoflurane anesthesia (2.5 – 3.5 % in O_2). Rats were then returned in their cages for a 20-min recovery period, after which they were placed in clear acrylic cages (25 cm X 12.5 cm with a height of 16 cm), fitted with a stainless steel wire mesh floor for a 10–20 min acclimatisation period before allodynia testing. Rats were tested for mechanical allodynia immediately before, and then 30, 75, 120, 165, and 210 min after SN50 administration by an observer blinded to treatment.

Mechanical allodynia

Ipsilateral mechanical allodynia was assessed by measuring paw-withdrawal thresholds (PWTs) (50% withdrawal threshold) to von Frey filaments, according to a modification of

the method described by Chaplan *et al.*²³. Briefly Semmes-Weinstein monofilaments (Stoelting Co., Wood Dale, IL) were applied to the plantar surface of the hind paw for 10 s or until the rat exhibited a flexion reflex. Filaments were applied in ascending (following no response) or descending (following a response) order of strength to determine the filament closest to the threshold of response. Filaments were applied thus until a pattern of responses showing three changes (rats responding or not) was obtained²⁴. The minimal stimulus intensity was 0.25 g, and the maximum was 15 g.

Rotarod performance

In order to rule out any sedative effects or motor disturbances elicited by SN50, rats were tested by being placed on a rotating drum (7 cm diameter), 23 cm above the floor of a metal enclosure (11 cm wide, 42 cm long and 40 cm high) (Rotarod, IITC, Woodland Hills, CA). Normal, untreated rats were trained for 5 consecutive days, in which they underwent six trials, each separated by a 20-min recovery period. Each trial began by placing the rat on the stationary cylinder and then linearly increasing cylinder rotation from 0 to 30 rpm over the trial duration of 2 min. Time was measured until the rat fell from the rotating rod or to the end of the trial (latency to fall). On day 6, performance was recorded on the rotarod starting 30 min after injection of 600 ng of intrathecal SN50, using the experimental paradigm described above. Reported rotarod latency scores were based on the average of the six trials performed on each test day.

Statistics

Glycemic values (mmol/L of glucose), PWT (g) and ELISA results are expressed as mean $\pm$ 0.95 confidence intervals. The choice of the appropriate analysis was based on sample size, variance homogeneity (Levene test) and on data distribution (Kolmogorov-Smirnov test of normality). Statistical differences amongst the CPIP groups were assessed using two-way ANOVA for 1 repeated measurement (groups by time during I/R). Analysis of PWT measurements from rats used for NF κ B measurements was similarly performed by two-way repeated-measures ANOVA. Analysis of PWTs obtained after drug treatment was performed by three-way ANOVA with 2 repeated measures (treatment group by drug dose by time after drug administration). If significant heterogeneity of variance was detected (Levene test $P < 0.05$), the ANOVA was performed using square root-transformed observations (raw, untransformed data are graphically presented). When appropriate, Tukey's tests were used for *post hoc* pair-wise comparisons between groups. Within group comparisons of test values to the corresponding baseline scores were also done using Dunnett's test. An analysis of covariance (ANCOVA) was conducted on the linear regression models (area under the curve *vs.* log dose of SN50), and a student's *t*-test (two-tailed) was conducted on the linear regression intercepts to assess shifts in the dose-response curve. Daily rotarod performance was analysed using one-way repeated measures ANOVA followed by Tukey's post-hoc comparisons.

The test used and the degrees of freedom are indicated with each test statistic in the Results section. Results were considered significant if $P < 0.05$. Statistics were performed using SPSS (Statistical Package for Social Sciences, IBM, 2004, Chicago, IL) or Statistica (Version 6, StatSoft, Tulsa, OK) software.

Results

NF κ B levels in muscle and spinal cord for different glycemic conditions

Glycemia during I/R injury—As shown in figure 1A, the four different groups of CPIP rats which underwent tissue extraction on day 2 postprocedure displayed statistically significant differences in glycemia during the I/R injury ($F(18, 120) = 13.909$, $P < 0.0001$, ANOVA Group X Time interaction). While the mean glycemia of the four groups did not differ at baseline ($P > 0.05$, Tukey's), tests insulin administration significantly lowered the blood glucose of the fed/insulin group below baseline values throughout the ischemic period at 30 min ($P < 0.001$), 90 min ($P < 0.001$), 150 min ($P < 0.001$), and throughout the period from 20, 40 and 60 min after reperfusion ($P < 0.01$ for each, Tukey's tests). The glycemic level of the fed/insulin/dextrose group was less than baseline values only at 30 min postischemia ($P < 0.05$, Dunnett's test). In contrast, dextrose administration to fed animals increased glucose levels compared to baseline at 90 min postischemia ($P < 0.05$). Rats in the fed group did not exhibit significant variations in mean glycemia at any time point. Compared to the fed group, rats of the fed/insulin group had lower glucose at 30 during ischemia ($P = 0.0343$, Tukey's tests), but not thereafter. Rats in the fed/dextrose group had significantly higher glucose levels compared to the fed group at 90 min of ischemia ($P = 0.0318$). Rats in the fed/insulin/dextrose group had higher glycemic values at 90 and 150 min of ischemia when compared to rats in the fed/insulin group ($P = 0.0037$ and $P = 0.0017$, respectively). Rats in the fed/insulin/dextrose group did not differ from rats in the fed group at any time point (Tukey's tests).

Effects of glycemic treatments on PWTs—The results of the PWT testing 48 h after the ischemic challenge are displayed in figure 1B. The PWTs varied significantly depending on glycemic treatment and injury (sham vs. CPIP) (Group by treatment interaction: $F(3,46) = 4.0943$, $P = 0.0117$, ANOVA). In the fed, fed/insulin/dextrose and fed/dextrose groups, CPIP animals displayed lower PWT compared to sham-treated rats (Tukey's test $P = 0.0263$, $P < 0.0001$ and $P < 0.001$, respectively). There was no difference in PWT between CPIP and sham-treated animals in the fed/ins group. The CPIP animals in the fed/insulin group exhibited significantly higher PWTs compared to those in the fed group ($P = 0.0049$). The fed/insulin CPIP rats also exhibited significantly higher PWTs in comparison to CPIP rats in the fed/dextrose group ($P = 0.003$ and $P < 0.0001$, respectively). There was no significant difference in the PWTs between the fed and the fed/insulin/dextrose groups, but PWTs in the fed/dextrose group were significantly lower than CPIP rats in the fed group ($P = 0.0009$). PWTs did not differ between groups for Sham-treated rats. The relationship between mean glucose level measured during the ischemia period and each animal's PWT is displayed in figure 1C. As shown, PWTs were negatively related to glucose levels, with a highly significant R^2 value.

NF κ B levels in the ipsilateral plantar muscles and dorsal horn—As shown in figure 2A, NF κ B levels in the ipsilateral hind paw muscles varied significantly depending on glycemic treatment ($F(3,42) = 7.633$, $P = 0.0004$) and injury condition ($F(1,42) = 95.332$, $P < 0.0001$). NF κ B P65 levels were significantly higher in CPIP animals compared to sham-treated rats in the fed, fed/insulin/dextrose and fed/dextrose groups (Tukey's test $P = 0.0004$,

$P = 0.0003$ and $P = 0.0002$, respectively). CPIP rats of the fed/insulin rats did not differ from the sham-treated control animals. The CPIP animals of the fed, fed/insulin/dextrose and fed/dextrose groups also exhibited significantly higher NF κ B levels in the plantar muscles compared to those of the fed/insulin group ($P = 0.038$; $P = 0.024$; $P = 0.001$), but did not differ from the fed/insulin/dextrose group ($P > 0.05$).

As illustrated in figure 2B, dorsal horn NF κ B levels were significantly different between the groups ($F(1,42) = 28.267$, $P < 0.0001$). NF κ B P65 levels were significantly higher in the ipsilateral dorsal horn of CPIP compared to sham-treated rats in the fed, fed/insulin/dextrose and fed/dextrose groups ($P = 0.0441$, $P = 0.0068$ and $P = 0.0080$, respectively). CPIP rats of the fed/insulin rats did not differ from the sham-treated control animals. CPIP rats of the fed/dextrose group had higher P65 levels than CPIP rats of the fed/insulin group ($P = 0.0246$). No significant difference was found between the fed rats and the fed/insulin rats or between the fed rats and the fed/insulin/dextrose rats.

Intrathecal SN50 treatment

Glycemia during the I/R injury—As depicted in figure 3A, the four groups of CPIP rats that underwent treatment with SN50 had varying mean glycemia during the ischemia and the subsequent reperfusion period (Group by time interaction: $F(18,150) = 8.38$, $P < 0.00001$). The mean group glucose values did not differ at baseline. Glycemia in the fed group remained constant throughout the ischemia and reperfusion periods when compared to their baseline values (Dunnett's test $P > 0.05$). Glycemia was significantly reduced compared to baseline in fed/insulin rats at every time point during ischemia injury and reperfusion ($P < 0.001$, Dunnett's test), and of the fed/insulin/dextrose rats only at 30-min postischemia ($P < 0.05$, Dunnett's test). Conversely, glycemia was increased compared to baseline in fed/dextrose rats at 90 min into the ischemic period ($P = 0.0002$). Glycemic levels of the fed/insulin/dextrose rats were significantly higher than those of the fed rats at 20 min after reperfusion ($P = 0.0242$, Tukey's test). Glycemia in the fed/insulin rats was significantly lower than that of the fed/dextrose group after 90 and 150 min of ischemia ($P = 0.0021$ and $P = 0.0251$, respectively, Tukey's tests), and significantly lower than that of the fed/insulin/dextrose group also at 90 and 150 min of ischemia ($P < 0.0003$ and $P = 0.0009$, respectively), and at 30 and 40 min of the reperfusion period ($P = 0.0001$, $P = 0.0132$, respectively, Tukey's tests).

Effects of glycemic treatment on PWTs for SN50-treated rats—As illustrated in figure 3B, significant differences were observed in PWT scores between the four different glycemic conditions at predrug baseline 2 days after I/R injury ($F(1,41) = 81.614$, $P < 0.0001$). PWTs were lower in CPIP animals than in the corresponding sham-treated rats in the fed, fed/insulin/dextrose and fed/dextrose groups ($P = 0.0179$, $P = 0.0002$ and $P = 0.0002$, respectively). There was no difference in PWTs between CPIP and sham-treated animals in the fed/insulin group). The fed/insulin group exhibited significantly higher PWTs than the fed/insulin/dextrose and the fed/dextrose groups ($P = 0.0161$ and $P = 0.0031$, respectively). However, there was no significant difference in PWTs between the fed and the fed/insulin/dextrose group ($P > 0.05$). The relationship between mean glucose level measured during the ischemia period and each animal's PWT is displayed in figure 3C. As

shown, once again PWTs were negatively related to glucose levels, with a highly significant R^2 value.

PWTs after treatment with SN50—As stated in the Methods section, the CPIP animals of each glycemic treatment group received four different doses of SN50 and a single dose of an inactive control peptide corresponding to the scrambled sequence of SN50. A common dose of 200 ng SN50 was administered to each group. The PWTs of the fed/insulin group (fig. 4A) varied significantly as a function of the time and SN50 dose in a dose-dependent manner (ANOVA Dose X Time interaction $F(20,120) = 1.66$, $P = 0.050$). PWTs in this group did not change significantly over the course of testing after the administration of control peptide or SN50 6.66 ng. After the administration of 22.22 ng SN50, the PWTs of the fed/insulin group were significantly higher than baseline values at 30 and 75 min ($P = 0.011$ and $P < 0.0001$, Dunnett's test). With 66.66 ng SN50, PWTs were significantly elevated compared to baseline at 30 and 75 min post-injection ($P = 0.012$ and $P = 0.003$, respectively, Dunnett's test). Finally, administration of 200 ng SN50 to the fed/insulin group led to significant increases in PWTs above baseline throughout the testing period at 30 ($P = 0.012$), 75 ($P = 0.010$), 120 ($P = 0.007$), 165 ($P = 0.006$) and 210 min postinjection ($P = 0.006$, Dunnett's tests).

The PWTs of the fed group (fig. 4B) also varied as a function of time and SN50 dose in a dose-dependent manner (ANOVA Dose X Time interaction; $F(20,140) = 2.760$, $P = 0.0003$). PWTs in this group did not significantly change at any point post-drug compared to the baseline levels after control peptide, 22.22 or 66.66 ng SN50. Administration of 200 ng SN50 resulted in a significant increase of the PWTs compared to baseline at 30 and 75 min postdrug administration ($P < 0.0001$ and $P < 0.0001$, respectively, Dunnett's test). Finally, at 600 ng SN50, the PWTs were significantly higher than the baseline values for the whole duration of the testing period, at 30 ($P = 0.004$), 65 ($P < 0.0001$), 120 ($P = 0.001$), 165 ($P < 0.0001$) and 210 min postinjection ($P < 0.0001$, Dunnett's test).

Rats of the fed/insulin/dextrose group (fig. 4C) also displayed dose-dependent effects of SN50 (ANOVA Dose X Time interaction; $F(20,120) = 3.614$, $P < 0.0001$). Administration of 100 or 200 ng SN50 did not significantly change the PWTs at any time point. SN50 400 ng significantly raised the PWTs of the fed/insulin/dextrose rats over the baseline values at 30 ($P < 0.0001$) and 75 min postinjection ($P < 0.0001$, Dunnett's tests), whereas 600 ng SN50 raised PWTs significantly at 30 ($P < 0.0001$), 75 ($P < 0.0001$), 120 ($P < 0.0001$) and 210 min postinjection ($P < 0.0001$, Dunnett's tests).

Rats of the fed/dextrose group (fig. 4D) displayed the lowest responsiveness to SN50 in the dose-response profile over time ($F(20,120) = 2.1743$, $P = 0.0053$). Administration of 200 ng SN50 had no significant effect on PWTs, while 400 ng SN50 only increased PWTs at 75 min post-injection ($P = 0.0086$, Dunnett's test). The PWTs were elevated above pre-drug baseline values at 75 and 120-min postinjection ($P = 0.0065$ and $P = 0.0135$, respectively) after 800 ng SN50. Following administration of 1,000 ng SN50, PWTs were significantly elevated above baseline values for every time point from 30 to 210 min postinjection ($P = 0.0190$, $P = 0.0002$, $P = 0.0004$, $P = 0.0003$ and $P = 0.0042$, respectively).

Figure 5A illustrates that the four CPIP groups reacted differently to the common 200 ng dose of SN50 ($F(10,90) = 2.380$, $P = 0.015$, ANOVA Group X Time interaction). The fed/insulin group exhibited significantly higher mechanical thresholds compared to the fed/insulin/dextrose group at 30 ($P = 0.013$), 165 ($P = 0.010$) and 210 min postinjection ($P = 0.007$, Tukey's tests), and compared to the fed group at 210 min postinjection ($P = 0.035$, Tukey's test). The fed group exhibited significantly higher mechanical thresholds compared to the fed/insulin/dextrose group only at 30 min postinjection ($P = 0.026$, Tukey's test).

Each group exhibited consistent baseline (predrug) PWTs, so there was no residual effect of SN50 before the administration of the next dose or any other interaction between day of testing and mean group PWTs (two way ANOVA, $F(12,96) = 0.6216$, $P > 0.05$, and $F(4,96) = 5.243$, $P > 0.05$ for the Days by treatment group interaction and Days effects, respectively).

As illustrated in figure 5B, administration of different SN50 doses to the four groups produced log dose-response curves with slopes that did not differ significantly ($F(1) = 5.05$, $P > 0.05$, ANCOVA, but with statistically significant shifts relative to each other ($F(2) = 7.51$, $P < 0.01$, ANCOVA). The log dose-response curve of the fed/insulin/dextrose was shifted significantly to the right relative to the fed/insulin group, with a significantly lower x-intercept for the later ($4.06 \text{ ng} \pm 7.04$ vs. $77.80 \text{ ng} \pm 44.50$, $P = 0.0262$). The x-intercept of the log dose-response curve from the fed/insulin/dextrose group did not differ from that of the fed group (77.80 ± 44.50 vs. 24.89 ± 17.20 , respectively, $P = 0.088$), but was also higher than that of the fed/insulin group ($P = 0.0345$). The x-intercept for the log dose-response curve from the rats in the fed/dextrose condition ($328.94 \text{ ng} \pm 92.94$) was also significantly higher than that of the fed/insulin groups ($P = 0.031$). The x-intercept value from the fed and fed/insulin did not differ from each other.

Effect of SN50 on rotarod performance—As illustrated in figure 5C, the rats' rotarod performance improved throughout the 5-day training period ($F(5, 210) = 2.661$, $P = 0.023$, ANOVA). Following the administration of 600 ng SN50 on day 6, rats stayed on the rotarod for an average of 65.14 ± 5.67 s, which was not significantly different from the performance achieved on day 5 (54.82 ± 5.22).

Discussion

The current study has replicated our earlier study showing that varying glycemic levels at the time of I/R injury are associated with different post-injury mechanical allodynia. The current study further demonstrates that varying glycemic levels at the time of I/R is associated with varying NF κ B levels in the ipsilateral hind paw muscles and ipsilateral dorsal horn of the spinal cord; NF κ B levels being higher in relatively hyperglycemic groups. These results suggest that NF κ B plays a role in the pathophysiology of post-I/R mechanical allodynia in CPIP rats. Accordingly, administration of SN50 resulted in a reduction of post-lesion mechanical allodynia in a manner associated with the glycemia present during the I/R injury, with the dose-response curve of the fed/insulin group being significantly shifted to the left in comparison to the fed/insulin/dextrose, fed and fed/dextrose groups.

The relationship between glycemia and NF κ B levels in the CPIP rat

The four treatment groups included in the study differed in terms of glycemic values during I/R injury. Different glycemic values during I/R injury led to significantly different NF κ B levels, in both the ipsilateral hind paw and the ipsilateral dorsal horn, suggesting that glycemic level affected not only peripherally activated NF κ B, but also that glycemia influenced the central activation of NF κ B, presumably induced by the peripheral I/R injury. Thus, tight glycemic control at the time of the injury seems to result in reduced NF κ B levels peripherally and spinally, whereas higher glycemia led to higher NF κ B levels. These changes in NF κ B levels were present 2 days post-I/R injury in both muscle and spinal cord.

The lower activated NF κ B levels in the fed/insulin group are likely secondary to a lower glycemic level more so than anti-inflammatory effects of insulin, as insulin administration to the fed/insulin/dextrose group did not prevent the increase in NF κ B levels. This was also observed in a previous study examining the effects of lowering or increasing glycemia during I/R injury on CPIP⁴. Consequently, a lower glycemia *per se* seems more protective than other direct insulin effects in terms of NF κ B levels. Importantly, the same relationship between these two groups was exhibited in the measures of mechanical allodynia, where insulin's antiallodynic effects were lost when dextrose was added during I/R injury. These results are consistent with our previously published results showing that allodynia in CPIP rats is lowered as much by fasting as by insulin treatment, and that giving dextrose to fasted CPIP rats eliminated the benefit produced by fasting⁴. We predict, therefore, that fasting would also reduce NF κ B levels in CPIP rats.

Glycemia, NF κ B levels and post-ischemia pain

As we saw previously, different mean glycemic values between the fed, the fed/insulin the fed/insulin/dextrose and the fed/dextrose groups during I/R injury led to significantly different post-ischemia allodynia with a protective effect of lower glycemia on mechanical allodynia. Because higher glycemic levels are correlated with higher NF κ B levels, NF κ B may be the mechanism by which high glycemia worsens postischemic pain in the CPIP rat model. Indeed, activated NF κ B has been demonstrated to play a role in several pain models with different etiologies, such as inflammatory²⁵, neuropathic^{26,27}, or postischemic²⁸ pain models. NF κ B is activated by proinflammatory cytokines in the spinal cord^{29,30}. Activated NF κ B induces the transcription of inflammatory genes³¹, and plays a key role in signalling pathways involving pain mediators, such as cyclooxygenase-2, dynorphin, c-fos and neuronal nitric oxide synthase^{32–35}. NF κ B thus perpetuates spinal cord inflammatory responses, which further facilitate pain transmission, central sensitisation and chronic pain states³⁶.

SN50 and the reduction of postischemia pain

The different response profiles of each experimental group to SN50 also points towards an important role of NF κ B in the pathophysiology of CPIP, and the influence of glycemia on CPIP. The fed/insulin group (leftward shift) achieved significantly higher von Frey thresholds at a lower SN50 dose than the fed, fed/insulin/dextrose or fed/dextrose groups. Furthermore, this effect was maintained for a longer period. This shows that rats with lower glycemia and NF κ B levels require lower doses of SN50. This suggests that less inhibition of

NF κ B translocation is needed to alleviate CPIP. The dose-response findings are in accordance with a role of NF κ B in allodynia induced by CPIP²⁸. Furthermore, the varying sensitivity, and duration of responses to SN50 in the various groups implies that glycemia-related increased mechanical allodynia depends on NF κ B activity.

In the present study, SN50's effects on mechanical allodynia were maintained up to day 8 post-I/R. This finding is important, since in a previous study showed that NF κ B levels in CPIP rats were normalized in muscle 7 days following the I/R, but were still elevated in spinal cord tissue, suggesting that the central pathology was still ongoing, and that its inhibition can still interrupt the inflammatory cascade involved in nociception²⁸. Although, we do not feel it is necessary to reconfirm the action of SN50 as a NF κ B inhibitor, it would be useful to determine the relationship between allodynia and NF κ B levels for each glycemic condition at later time points.

One may question the specificity of SN50, as at high concentrations it has been shown to block the nuclear import of not only NF κ B, but also of other transcription factors such as activator protein 1, signal transducers and activator of transcription and nuclear factor of activated T-cells³⁷. However, these observations occurred in cell lines and may not be transposable to *in vivo* experiments. Furthermore, the doses we used in our experiments were also significantly less than the 210 μ g/mL used in the *in vitro* study.

Concluding remarks

In conclusion, we demonstrated that the differences in mechanical allodynia thresholds between the three groups of CPIP rats with varying glycemic levels are correlated with different NF κ B levels present in the ipsilateral paw muscles and the ipsilateral dorsal horn of these rats, and that these different NF κ B levels are modulated by the glycemia present during the I/R injury. Furthermore, the antiallodynic dose-response effects of SN50 were shifted to the left in relatively hypoglycemic rats, and to the right in relatively hyperglycemic rats. These observations could have important applications for postischemic pain conditions: by maintaining a tight glycemic control during I/R injury, less NF κ B will be activated, and an analgesic response to an NF κ B inhibitor will be attainable with smaller doses and for longer periods of time.

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Fig 1.

Mean ($\pm$ 0.95 confidence intervals) glycemic levels at different time points during the ischemic and reperfusion (I/R) periods (**A**), and mean ($\pm$ 0.95 confidence intervals) ipsilateral paw withdrawal thresholds (PWTs, g) on day 2 post-IR injury (**B**), for chronic post-ischemia pain (CPIP) and Sham-treated rats in the fed, fed/insulin (fed/ins), fed/insulin/dextrose (fed/ins/dex) and fed/dextrose (fed/dex) groups. **A**) The fed/ins group displayed lower glycemic values compared to their baseline throughout testing (** $P < 0.01$), while the glycemic levels of the fed/ins/dex group were below baseline only at 30 min after ischemia onset (* $P < 0.05$). Rats in the fed/dex group had elevated glucose levels compared to baseline 90 min after ischemia onset (* $P < 0.05$). The glycemic levels of the fed/ins rats were significantly lower than the glycemia of the fed rats at 30 min of ischemia († $P < 0.05$); and the fed/ins/dex group at 90 and 150 min of ischemia (§§ $P < 0.01$); **B**) PWTs of CPIP fed, fed/ins/dex and fed/dex rats were significantly lower than those of sham-treated animals subjected to the same glycemic condition (* $P < 0.05$; ** $P < 0.01$). PWTs of CPIP fed/ins rats

were higher than those of fed rats and PWTs of fed/dex rats were lower than those of fed rats ($\dagger P < 0.05$). C) Overall relation between the mean blood glucose measured throughout the 180 min of ischemia and the PWT recorded 2 days post-injury for CPIP rats in the various glycemic conditions.

Fig. 2. p65 nuclear factor kappa B (NFκB) levels (density absorbance measures, arbitrary units (AU) $\pm$ 0.95 confidence intervals) on day 2 post-ischemic/reperfusion injury in the ipsilateral plantar muscles (**A**), and in the ipsilateral dorsal horn (**B**), of chronic post-ischemia (CPIP) and sham animals in the fed/insulin (fed/ins), fed, fed/insulin/dextrose (fed/ins/dex) and fed/dextrose (fed/dex) groups. **A**) NFκB levels were significantly higher in the ipsilateral plantar muscles of the fed, fed/ins/dex and fed/dex groups compared to the sham group (* $P < 0.05$; ** $P < 0.01$) and the fed/ins group (# $P < 0.05$; ## $P < 0.01$). **B**) NFκB levels were significantly higher in the ipsilateral dorsal horn of the fed, fed/ins/dex and fed/dex groups when compared to their respective sham-treated group (* $P < 0.05$; ** $P < 0.01$). The CPIP rats in the fed/dex group also displayed NFκB P65 levels higher than those of the fed/ins group (# $P < 0.05$).

Fig. 3.

Mean (± 0.95 confidence intervals) glycemic levels at different time points during the ischemia and reperfusion periods (**A**), and mean (± 0.95 confidence intervals) pre-drug ipsilateral paw withdrawal thresholds (PWTs, g) on day 2 post-I/R injury (**B**), for chronic post-ischemia pain (CPIP) and sham-treated rats in the fed, fed/insulin (fed/ins), fed/insulin/dextrose (fed/ins/dex) and fed/dextrose (fed/dex) groups that would later receive SN50. **A**) The fed/insulin group had glycemic values lower than pre-ischemia baseline throughout the ischemia and reperfusion periods (** $P < 0.01$). The glycemic values of the fed/ins/dex group was lower than baseline only at 30 min post-ischemia (* $P < 0.05$). The fed/dex group displayed blood glucose levels higher than baseline at 90 min of ischemia (* $P < 0.05$). The

glycemic levels of the fed/ins rats were also lower than those of the fed/dextrose rats at 90 and 150 min of ischemia ($\dagger P < 0.05$; $\dagger\dagger P < 0.01$), and the fed/ins/dex rats from 90–150 min of ischemia and 20–40 min of reperfusion ($\S P < 0.05$; $\S\S P < 0.01$). Glycemic values of the fed/ins/dex group were higher than in the fed group at 20 min of the reperfusion period ($\ddagger\dagger P < 0.01$). **B)** PWTs of CPIP rats were lower than those of sham rats ($* P < 0.05$; $** P < 0.01$) in the fed, fed/ins/dex and fed/dex rats. PWTs of the fed/ins CPIP rats were higher than those of fed/ins/dex rats and fed/dex rats ($\dagger P < 0.05$). **C)** Overall relation between the mean blood glucose measured throughout the 180 min of ischemia and the PWT recorded 2 days post-injury for CPIP rats in the various glycemic conditions that would later receive SN50.

Fig. 4.

Paw withdrawal thresholds (mean $\pm$ 0.95 confidence intervals) for fed/insulin (fed/ins) (A), fed (B), fed/insulin/dextrose (fed/ins/dex) (C), and fed/dextrose (fed/dex) (D) chronic post-ischemia pain rats at different doses of SN50. Doses of SN50 required to raise paw-withdrawals (i.e., anti-allodynia) were lowest for fed/ins rats in A, higher for fed rats in B, and even higher for fed/ins/dex in C. The thresholds different from the pre drug baseline values are indicated by * $P < 0.05$ and ** $P < 0.01$.

Fig. 5.

Paw withdrawal thresholds (PWTs, mean $\pm$ 0.95 confidence intervals) at the common dose of 200 ng of SN50 in chronic post-ischemia pain (CPIP) rats (**A**) and log dose-response curves (Area under the curve (AUC) -PWT $\times$ min $\pm$ 0.95 confidence intervals) (**B**) for the groups of fed, fed/insulin (fed/ins) fed/insulin/dextrose (fed/ins/dex) and fed = dextrose (fed/dex) CPIP rats. (**C**) rotarod performance in control rats displayed for five daily training trials and for the post-SN50 administration on the sixth (filled bar). **A**) The PWTs of the fed/ins group were significantly higher than the fed/ins/dex group at 30, 165 and 210 min post-injection (* $P < 0.05$; ** $P < 0.01$) and the fed group at 210 min post-injection (†† $P < 0.01$).

The PWTs of the fed group were significantly higher than in the fed/ins/dex group at 30 min post-injection ($\ddagger P < 0.05$). The PWTs in the fed/dex group did not differ from baseline values at any time point. **B)** Log dose response curve for the area under the curve of the paw withdrawal threshold after-SN50 for the 4 groups of CPIP rats. The slopes of the linear regressions were not statistically different ($P > 0.05$). The fed, fed/ins/dex and fed/dex log dose-response curve exhibit a significant rightward shift compared to the fed/ins group. **C)** Time (sec $\pm$ 0.95 confidence intervals) spent on the rotarod before and after treatment with SN50 600 ng (the highest dose administered). The drug did not affect rotarod performance (performance on day 6 is not statistically different than the performance on day 5, $P > 0.05$).