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Pyritinol reduces nociception and oxidative stress in diabetic rats

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ABSTRACT

The purpose of this study was to assess the antinociceptive and antiallodynic effect of pyritinol as well as its possible mechanism of action in diabetic rats. Streptozotocin (50 mg/kg) injection caused hyperglycemia within 1 week. Formalin-evoked flinching was increased in diabetic rats as compared to non-diabetic rats. Oral acute administration of pyritinol (50–200 mg/kg) dose-dependently reduced flinching behavior in diabetic rats. Moreover, prolonged administration of pyritinol (12.5–50 mg/kg, every 2 days for 2 weeks) reduced formalin-induced nociception. 1H-[1,2,4]-oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, a guanylyl cyclase inhibitor, 2 mg/kg, i.p.), but not naltrexone (a non-selective opioid receptor antagonist, 1 mg/kg, s.c.) or indomethacin (a non-selective cycloxygenase inhibitor, 5 mg/kg, i.p.), blocked the pyritinol-induced antinociception in diabetic rats. Given alone ODQ, naltrexone or indomethacin did not modify formalin-induced nociception in diabetic rats. Oral acute (200 mg/kg) or prolonged (25 mg/kg, every 2 days for 2 weeks) administration of pyritinol significantly reduced streptozotocin-induced changes in free carbonyls, dityrosine, malondialdehyde and advanced oxidative protein products. Four to 8 weeks after diabetes induction, tactile allodynia was observed in the streptozotocin-injected rats. On this condition, oral administration of pyritinol (50–200 mg/kg) reduced tactile allodynia in diabetic rats. Results indicate that pyritinol is able to reduce formalin-induced nociception and tactile allodynia in streptozotocin-injected rats. In addition, data suggest that activation of guanylyl cyclase and the scavenger properties of pyritinol, but not improvement in glucose levels, play an important role in these effects.

1. Introduction

Diabetes is a global health problem and its prevalence is set to increase to more than 360 million worldwide by the year 2025 (Wild et al., 2004). Diabetes leads to several complications including retinopathy, nephropathy and neuropathy. Diabetic neuropathy is the most common complication affecting more than 50% of the diabetic patients. Etiology of diabetic neuropathy is complex and multifactorial. Several pathways contribute to the development of diabetic neuropathy which include: increased activation of polyol pathway, oxidative stress, advanced glycation end product formation, nerve hypoxia/ischemia, protein kinase C and reduction of nerve growth factor support (Van Dam, 2002; Obrosova, 2003; Vincent et al., 2004).

The treatment of pain in diabetic patients is frequently unsatisfactory. Anticonvulsants, tricyclic antidepressants and opioids have become the mainstay in the treatment of chronic neuropathic pain (Sindrup and Jensen, 1999). However, these drugs often have a limited effect or may cause intolerable side effects. Therefore, other options of treatment are needed.

Pyritinol is a nootropic drug (cognition-enhancing agent) used in cognitive disturbances to improve cerebral functions. This drug has been used in patients with Alzheimer's disease (Heiss et al., 1994), senile dementia (Fischhof et al., 1992), cerebral functional disorders (Herrmann et al., 1986), aging (Hartmann et al., 1993), and rheumatoid arthritis (Lemmel, 1993). Moreover, in animals, pyritinol has shown to improve sleep (Wetzel, 1990), energy metabolism (Bielenberg et al., 1986), hypoxic damage (Lun et al., 1989), learning and memory (Jaiswal et al., 1990), cholinergic deficits (Toledano and Bentura, 1994), and epilepsy (Schmidt, 1990). The fact that pyritinol and other nootropic drugs (nefiracetam, levetiracetam) have anticonvulsant effects in rats (Schmidt, 1990, Kitano et al., 2005a,b) lead us to hypothesize that pyritinol could have an effect in streptozotocin-induced pain. Moreover, pyritinol has shown to reduce oxidative stress *in vitro* (Pavlík and Pilar, 1989). It is believed that diabetes-induced hyperglycemia causes neural degeneration *via* the increased oxidative stress, among others (see above). Thus, we have hypothesized that pyritinol could reduce formalin-induced nociception and tactile allodynia in diabetic animals. Therefore, the purpose of this

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work was study the possible antinociceptive and antiallodynic effect of pyritinol as well as its possible mechanism of action in diabetic rats.

2. Materials and methods

2.1. Animals

Experiments were performed on 204 adult female Wistar rats (body weight range, 220–240 g) of 10–12 weeks of age. The animals were obtained from our own breeding facilities and had free access to drinking water, but food was withdrawn 12 h before experiments. Under this condition, we observed that streptozotocin produced a greater % of diabetic rats (80–90%). Experiments were done in normal light/dark cycle and they were started at the same time (11:00 AM). Efforts were made to minimize animal suffering and to reduce the number of animals used. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983) and were approved by our local Ethics Committee.

2.2. Induction of diabetes

Rats were injected with streptozotocin (50 mg/kg, i.p.) (Research Biochemical International, Natick, MA, USA) to produce experimental diabetes (Courteix et al., 1993). Control animals (age-matched) received distilled water. Diabetes was confirmed 1 week after injection by measurement of tail vein blood glucose levels with the glucose meter Ascensia ELITE (Bayer, Mexico City). Two weeks after streptozotocin injection, glycemia was again determined and only animals with a final blood glucose level ≥300 mg/dl were included in the study. Experiments were started with numbers greater than six considering that only 80–90% of the streptozotocin-treated rats became hyperglycemic or survived at 2 weeks. However, the survival percentage after 4–8 weeks decreases to about 70%. Thus, groups had to be started considering this fact.

2.3. Assessment of nociception

Nociception in non-diabetic and diabetic (2 weeks) rats was assessed using the 0.5% formalin test (Juárez-Rojop et al., 2006). The rats were placed in open plexiglas observation chambers for 30 min to allow them to acclimate to their surroundings; then they were removed for formalin administration. Fifty μl of diluted formalin (0.5%) were injected subcutaneously into the dorsal surface of the right hind paw with a 30-gauge needle. The animals were returned to the chambers and nociceptive behavior was observed immediately after formalin injection. Mirrors were placed in each chamber to enable unhindered observation. Nociceptive behavior was quantified as the number of flinches of the injected paw during 1-min periods every 5 min, up to 60 min after injection (Wheeler-Aceto and Cowan, 1991). Flinching was readily discriminated and was characterized as a rapid and brief withdrawal, or as a flexing of the injected paw. Formalin-induced flinching behavior was biphasic (Wheeler-Aceto and Cowan, 1991). The initial acute phase (0–10 min) was followed by a relatively short quiescent period, which was then followed by a prolonged tonic response (15–60 min). Animals were used only once and at the end of the experiment they were sacrificed in a CO_2 chamber.

2.4. Assessment of allodynia

Tactile allodynia was tested in diabetic rats 4 to 8 weeks after streptozotocin injection as previously described (Chaplan et al., 1994). Rats were transferred to a clear plastic, wire mesh-bottomed cage and allowed to acclimatize for 30 min. von Frey filaments (Stoelting, Wood Dale, IL) were used to determine the 50% paw withdrawal threshold using the up-down method of Dixon (1980). A series of filaments, starting with one that had a buckling weight of 2 g, was applied in consecutive sequence to the plantar surface of the right hind paw with a pressure causing the filament to buckle. Lifting of the paw indicated a positive response and prompted the use of the next weaker filament whereas that absence of a paw withdrawal after 5 s indicated a negative response and prompted the use of the next filament of increasing weight. This paradigm continued until four more measurements had been made after the initial change of the behavioral response or until 5 consecutive negative (assigned a score of 15 g) or four consecutive positive (assigned a score of 0.25 g) responses had occurred. The resulting scores were used to calculate the 50% response threshold by using the formula: 50% g threshold = $10^{(Xf+\kappa\partial)}/10000$, where Xf = the value (in log units) of the final von Frey filament used, κ = the value (from Table in Chaplan et al., 1994) for the pattern of positive and/or negative responses, and ∂ = the mean difference (in log units) between stimulus strengths. Behavioral tests (threshold assessment) were performed immediately before and every 30 min until 5 h after drug administration. Allodynia was considered to be present when paw withdrawal thresholds were <4 g. Diabetic rats not demonstrating allodynia were not further studied.

2.5. Determination of free carbonyls

Quantification of free carbonyls was done using a method proposed elsewhere (Dalle-Done et al., 2003). One-hundred μl of plasma were mixed with 1 ml of 2,4-dinitrofenilhidrazine 10 mM in HCl 2.5 M. Samples were incubated at room temperature, in darkness, and stirred up every 15 min during 1 h. Then, they were precipitated with a 20% solution of trichloacetic acid, centrifuged for 10 min at 3500 rpm, and then washed again with 10% solution of trichloacetic acid for the collection of precipitated protein. The samples were centrifuged for 10 min at 3500 rpm, and washed again with a 10% solution of trichloacetic acid, for the collection of precipitated protein. Finally, the precipitated was washed with a 3 ml solvent mixture (1:1) of ethanol plus ethyl acetate in order to eliminate the excedent 2,4-dinitrofenilhidrazine. The product was centrifuged again, and the new precipitate was dissolved in 1 ml of guanidine 6 M in a potassium phosphate solution 20 nM, and incubated for 10 min at 37 °C. Samples were analyzed spectrophotometrically, in a wavelength of 370 nm. The coefficient of molar extinction of 2,4-dinitrofenilhidrazine is $\varepsilon = 22{,}000/M^{-1}\,cm^{-1} = 22{,}000/10^6$ nmol/ml, and it was used to calculate the concentration of free carbonyls, expressed in osazone/ml plasma, corrected for mg of protein, quantified according with Lowry's method (Lowry et al., 1951).

2.6. Determination of malondialdehyde

Malondialdehide was measured according to the method of Yagi (1998) based in the quantification of reactive compounds to thiobarbituric acid, which are markers of lipid peroxidation. The procedure was done mixing 400 μl of buffer Tris-preset 7.2 mM at a pH of 8.0 with 100 μl of plasma and 1 ml of acid thiobarbituric 0.375% in HCl 0.2 N. The mixture was warmed at 90 °C during 15 min. Later on 0.5 ml de HCl 0.2 N were added and the solution was analyzed spectrophotometrically at 532 nm wavelength in a Perkin Elmer UV/VIS spectrophotometer model B050-9914 at 25 °C, using 1,1,3,3-tetramethoxipropane as standard.

2.7. Determination of dityrosines

Quantification of dityrosines was done according with the method proposed by Lehrer and Pasman (1967) using a plasma sample (10 μl) re-suspended in a 6 M urea solution in $NaHCO_3$ 0.1 M, pH 9.8, incubated at 23 °C during 30 min. The excitation spectrum for fluorescence of dityrosines was 280 to 370 nm wavelength. A spectrofluorometer PTI (Photon Technology International), registering the emission at 405 nm was used.

Table 1
Effects of the intraperitoneal administration of streptozotocin on blood glucose levels and body weight in rats 15 days after treatment

Treatment	Glucose (mg/dl)		Body weight (g)	
	Initial value	Final value	Initial value	Final value
Saline	70.3±4.2	90.6±1.3[a]	233.9±1.7	286.6±11.2[a]
Streptozotocin	68.6±1.4	433.0±37.3[a]	221.7±4.0	214.9±7.7

Data are the mean±S.E.M. of 6 animals.
[a] Significantly different from the initial value group ($P<0.05$), as determined by the Student's *t*-test.

2.8. Determination of advanced oxidative protein products

Advanced oxidative protein products were determined using the technique of Capeillére-Blandin (Capeillére-Blandin et al 2004). 200 µl of plasma (1:5 dilution in polybenzamine solution, PBS) was mixed with 20 µl of glacial acetic acid. The mixture was stirred for 2 min and an optical density was read at 340 nm.

2.9. Study design

Independent groups of animals (204 total, $n=6$) were used for each experimental condition. In order to define the best time to administer pyritinol, diabetic rats received an oral administration of vehicle (carboxymethyl cellulose 0.5%) or pyritinol (200 mg/kg, p.o.) at 30 and 60 min before formalin injection (50 µl). These times were selected from pilot experiments in our laboratory. Since the best antinociceptive effect was observed at the 30 min pretreatment (data not shown), dose–response curve for acute administration of pyritinol was carried out giving vehicle or increasing doses of pyritinol (50–200 mg/kg, p.o.) 30 min before formalin injection into the right paw. Moreover, other groups of rats received prolonged administration of pyritinol (12.5–50 mg/kg, p.o. every 2 days for 2 weeks). These animals were then submitted to the 0.5% formalin test 24 h after the last pyritinol or vehicle administration.

In an attempt to determine the possible mechanism of antinociceptive action of acute pyritinol in diabetic rats, indomethacin (a non-selective cyclooxygenase inhibitor 5 mg/kg, i.p.), naltrexone (a non-selective opioid receptor antagonist, 1 mg/kg, s.c.) or 1H-[1,2,4]-oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, a guanylyl cyclase inhibitor, 2 mg/kg, i.p.) was administered 20 min before pyritinol (200 mg/kg, p.o.) administration and the formalin-induced nociceptive behavior was assessed. Doses of these drugs were selected from previous studies (Whiteside et al., 2004; Arreola-Espino et al., 2007) and from pilot experiments in our laboratory.

To establish whether pyritinol-induced antinociception was mediated by its scavenging properties, non-diabetic and diabetic rats received either acute (200 mg/kg, p.o.) or prolonged (25 mg/kg, p.o. every 2 days for 2 weeks) administration of pyritinol. At the end of the study, rats were sacrificed and blood was obtained directly from the heart. Blood samples were used to determine free carbonyls, dityrosine, malondialdehyde and advanced oxidative protein products.

For the study of allodynia, rats received the oral administration of vehicle (carboxymethyl cellulose 1%) or increasing doses of pyritinol (50–200 mg/kg, p.o.) and withdrawal threshold in diabetic (4–8 weeks) and non-diabetic rats was measured for the next 5 h. Observer was unaware of the treatment in each animal.

Finally, to establish whether pyritinol-induced antinociception was consequence of an improvement in either weight or blood glucose levels, the weight and glucose levels of diabetic animals treated with either acute or chronic pyritinol and vehicle were compared at the end of the study (2 weeks).

2.10. Drugs

Streptozotocin, 1H-[1,2,4]-oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) and naltrexone were obtained from Sigma (St. Louis, MO, USA). Streptozotocin was freshly dissolved in distilled water (obtained from a Milli-Q water system), protected from light and immediately administered. Pyritinol was dissolved in carboxymethyl cellulose 0.5% and given orally by a gastric tube at a volume ratio of 2 ml/kg. ODQ and indomethacin were dissolved in 25% dimethyl sulfoxide, while naltrexone was dissolved in 0.9% isotonic saline.

2.11. Data analysis and statistics

All results are presented as the mean±S.E.M. for 6 animals per group. For the formalin test, curves were made for the mean number of flinches against time. The area under the number of flinches against time curves (AUC) for both phases was calculated according to trapezoidal rule. For allodynia, curves were constructed plotting the 50% threshold for paw withdrawal as a function of time. From these plots, area under the 50% threshold withdrawal against time curve (AUC) was computed.

Student's *t*-test was used to compare 2 treatments, while one-way analysis of variance (ANOVA) followed by Tukey's test was used to compare differences between more that 2 treatments. Differences were considered to reach statistical significance when $P<0.05$.

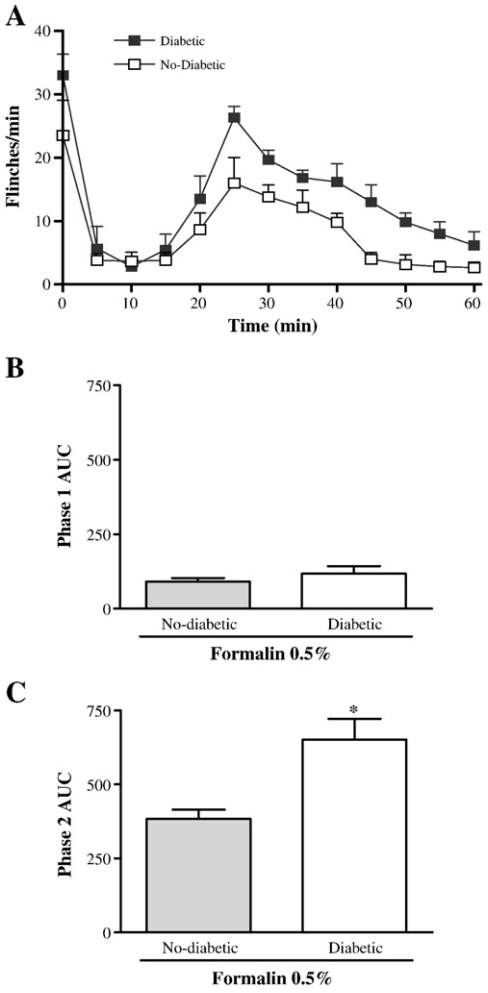

Fig. 1. A) Time course of the nociceptive behavior induced by subcutaneous injection of 0.5% formalin to non-diabetic and diabetic rats. Panels B and C show formalin-induced nociceptive behavior, in non-diabetic and diabetic rats during phases 1 and 2, respectively. The later data are expressed as the area under the number of flinches against time curve (AUC). Data are the mean±S.E.M. of 6 animals. *Significantly different from the non-diabetic group ($P<0.05$), as determined by Student's *t*-test.

3. Results

3.1. Streptozotocin injection and formalin-evoked flinching behavior in non-diabetic and diabetic rats

Streptozotocin, but not saline, injection caused hyperglycemia (Table 1). Streptozotocin-treated rats also showed a significant increase in body weight after 15 days of treatment (Table 1). Moreover, animals increased ingest of food and water intake and displayed polyuria (data not shown).

Both the streptozotocin-induced diabetic (2 weeks) and non-diabetic groups exposed to 0.5% formalin exhibited the biphasic pattern of this test, characterized by periods of flinching of the injected hind paw separated by a period of decreased activity. Formalin-evoked flinching was increased in diabetic rats as compared to non-diabetic rats (Fig. 1A). The overall analysis of formalin-evoked flinching as AUC showed significant difference ($P<0.05$) between the diabetic and non-diabetic groups only during the second phase (Fig. 1B–C). Since streptozotocin mainly increased nociception during phase 2 of the formalin test, in the following studies only phase 2 was further analyzed.

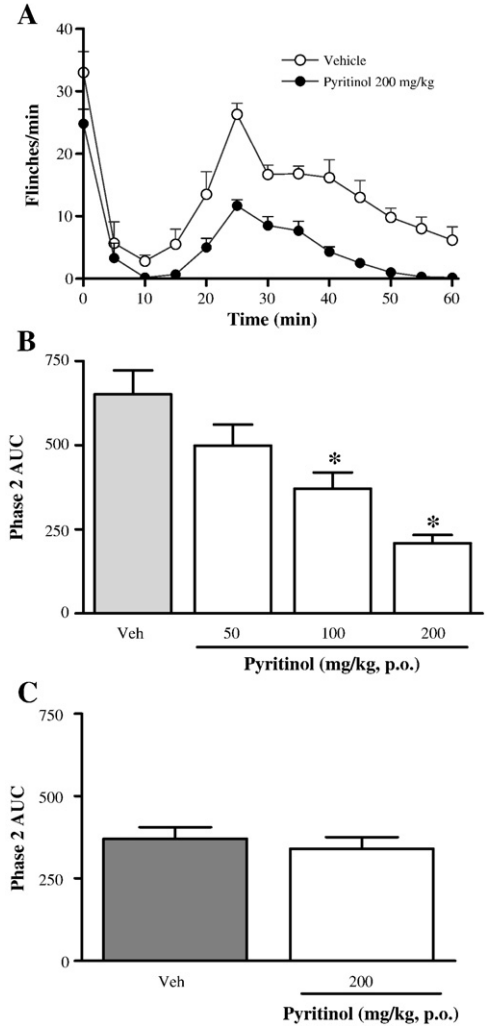

Fig. 2. A) Time course of the antinociceptive effect of pyritinol (200 mg/kg, p.o.) in diabetic rats submitted to the subcutaneous injection of 0.5% formalin. Panel B shows the dose–response curve for the anti-hyperalgesic effect of pyritinol in diabetic rats. Panel C depicts the effect of pyritinol in non-diabetic rats. In these plots, data are expressed as the area under the curve of the number of flinches against time (AUC) of phase 2. Bars are the mean ± S.E.M. for 6 animals. *Significantly different from the vehicle group ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

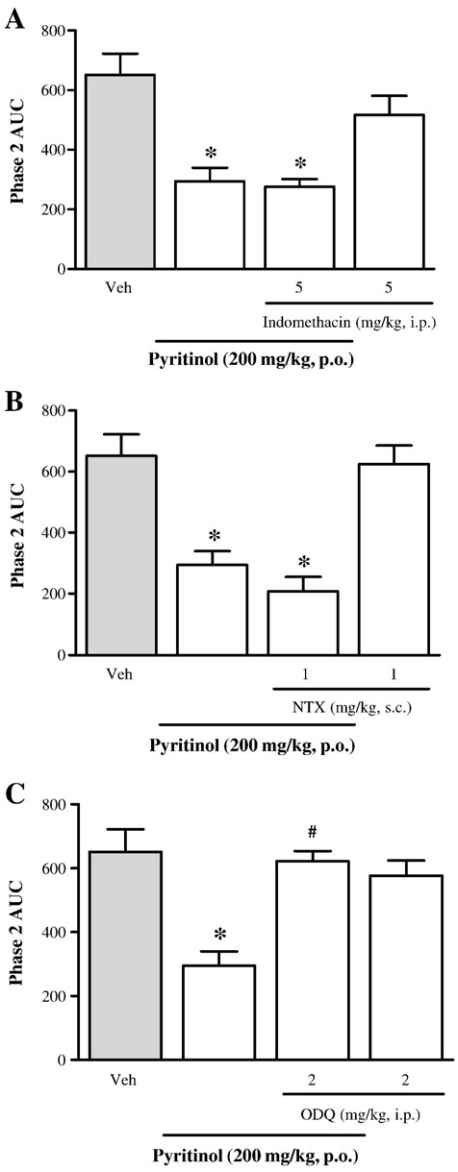

Fig. 3. Effect of indomethacin (panel A), naltrexone (NTX, panel B) and ODQ (panel C) on the antinociceptive effect of pyritinol in diabetic rats submitted to the 0.5% formalin test. Data are expressed as the area under the curve of the number of flinches against time (AUC) of phase 2. Bars are the mean ± S.E.M. for 6 animals. *Significantly different from the vehicle group ($P<0.05$) and #significantly different from pyritinol group, as determined by analysis of variance followed by Tukey's test.

3.2. Antinociceptive effect of acute administration of pyritinol in diabetic rats

Oral administration of pyritinol (200 mg/kg) significantly ($P<0.05$) reduced formalin-induced nociceptive behavior at 30 and 60 min pretreatment. However, the best antinociceptive effect was observed with the 30 min pretreatment (data not shown) and this pretreatment time was used in the following experiments. Under this condition, pyritinol significantly (Fig. 2A, $P<0.05$) and dose-dependently (Fig. 2B) reduced flinching behavior during phase 2 in diabetic rats. Contrariwise, administration of pyritinol, at the greatest dose tested (200 mg/kg, p.o.), did not modify formalin-induced flinching behavior in non-diabetic animals (Fig. 2C). Moreover, acute treatment with this dose (200 mg/kg, p.o.) did no affect glucose levels (pyritinol, 430 ± 12.3 versus vehicle, 433 ± 37.3 mg/dl) in diabetic rats. Considering this result, non-diabetic rats were no further tested with the different inhibitors used in diabetic animals.

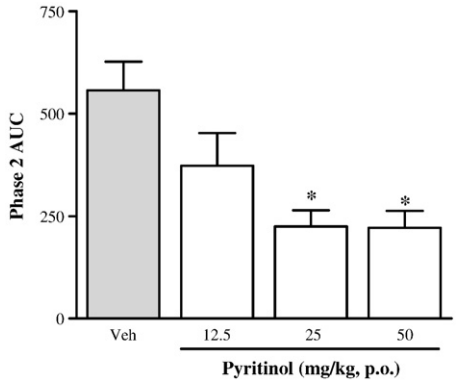

Fig. 4. Effect of prolonged (12.5–50 mg/kg, p.o. every 2 days for 2 weeks) administration of pyritinol in diabetic rats submitted to the 0.5% formalin test 24 h after the last vehicle or pyritinol treatment. Data are expressed as the area under the curve of the number of flinches against time (AUC) of phase 2. Bars are the mean±S.E.M. for 6 animals. *Significantly different from the vehicle group ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

3.3. Effect of indomethacin, naltrexone and ODQ on the antinociceptive effect of pyritinol in diabetic rats

Subcutaneous administration of indomethacin (5 mg/kg) or naltrexone (1 mg/kg) was not able to block pyritinol-induced antinociceptive activity in diabetic rats (Fig. 3A–B). In marked contrast, ODQ (2 mg/kg, s.c.) significantly reduced the antinociceptive effect of pyritinol in diabetic rats (2 weeks, Fig. 3B). At the greatest tested doses, indomethacin, naltrexone or ODQ did not modify formalin-induced nociception in diabetic rats (Fig. 3).

3.4. Antinociceptive effect of prolonged administration of pyritinol in diabetic rats

Oral administration of pyritinol, but not vehicle, every 2 days during 2 weeks significantly reduced formalin-induced flinching behavior, assessed 24 h after the last administration of pyritinol or vehicle, in diabetic rats (Fig. 4). This effect was observed with lower doses (25–50 mg/kg) than those used in the acute (100–200 mg/kg) administration protocol. Under this protocol, pyritinol 50 mg/kg (412±9.6 mg/dl) did not modify blood glucose levels compared to animals treated with vehicle (414±22.2 mg/dl). Likewise, pyritinol was not able to affect weight (217.0±8.1 g versus 214.2±8.4 g) in diabetic rats.

3.5. Antioxidative effect of either acute or prolonged administration of pyritinol in diabetic rats

Two weeks after streptozotocin treatment, a significant increase in free carbonyls, dityrosines, malondialdehyde or advanced oxidative protein products was observed. This increase was markedly reduced by acute (200 mg/kg, p.o.) or chronic (25 mg/kg every 2 days for 2 weeks, p.o.) treatment with pyritinol (Figs. 5 and 6). Pyritinol did not significantly modify free carbonyls, dityrosines, malondialdehyde or advanced oxidative protein products in non-diabetic rats.

3.6. Antiallodynic effect of pyritinol in diabetic rats

Four to 8 weeks after diabetes induction, tactile allodynia was observed in the streptozotocin-injected rats compared to the distilled water-injected rats. On this condition, oral administration of pyritinol (200 mg/kg), but not vehicle (carboxymethyl cellulose 1%), significantly

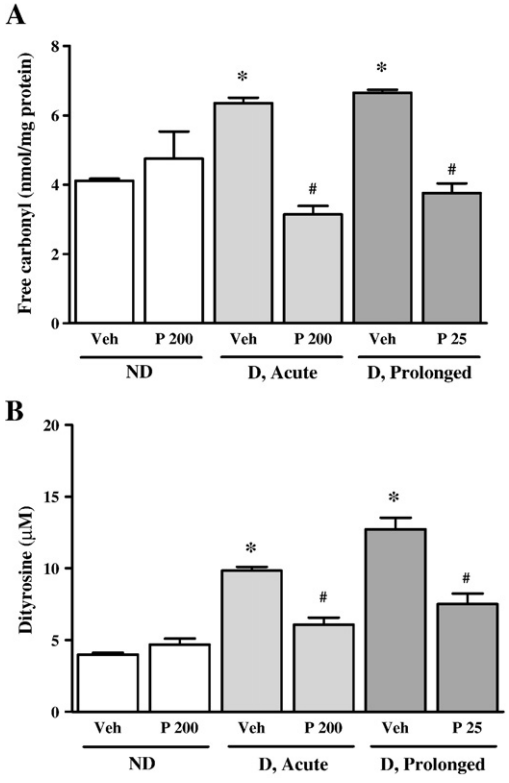

Fig. 5. Effect of either acute (200 mg/kg, P 200) or prolonged (25 mg/kg every 2 days for 2 weeks, P 25) administration of pyritinol on free carbonyls (A) and dityrosine (B) in blood of non-diabetic and diabetic rats. Bars are the mean±S.E.M. for 6 animals. *Significantly different from the vehicle of the non-diabetic group ($P<0.05$) and #significantly different from the vehicle of the diabetic group ($P<0.05$), as determined by Student's t-test.

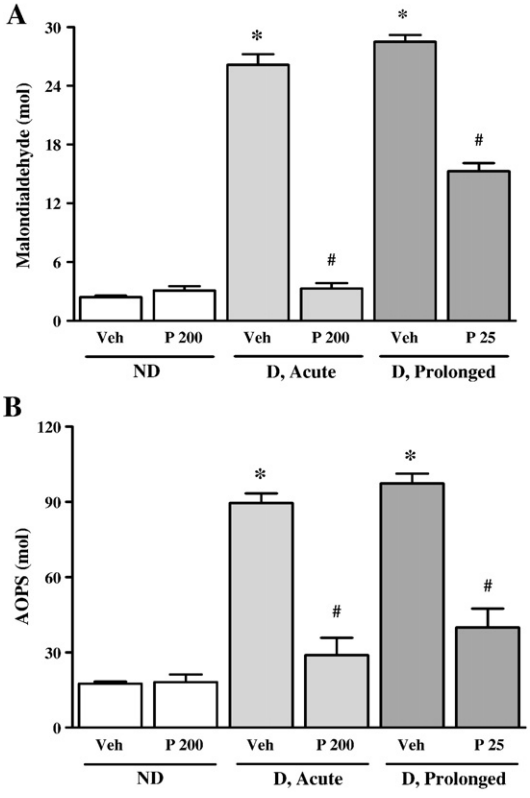

Fig. 6. Effect of either acute (200 mg/kg, P 200) or prolonged (25 mg/kg every 2 days for 2 weeks, P 25) administration of pyritinol on malondialdehyde (A) and advanced oxidative protein products (AOPS, B) in blood of non-diabetic and diabetic rats. Bars are the mean±S.E.M. for 6 animals. *Significantly different from the vehicle of the non-diabetic group ($P<0.05$) and #significantly different from the vehicle of the diabetic group ($P<0.05$), as determined by Student's t-test.

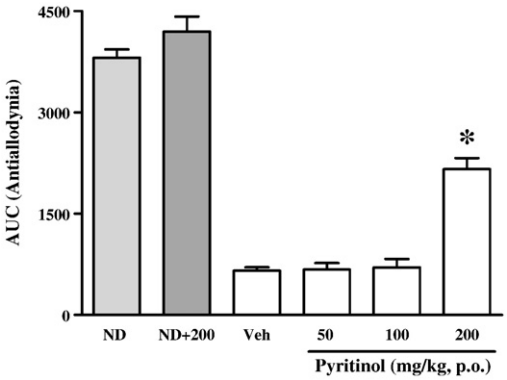

Fig. 7. Antiallodynic effect of pyritinol in diabetic rats. After diabetes induction, animals were allowed to develop tactile allodynia for 4 to 8 weeks. Animals were treated with oral pyritinol and withdrawal threshold was measured for the next 5 h. Data are expressed as the 50% threshold withdrawal against time curve (AUC). Data are the mean ± S.E.M. for 6 animals. *Significantly different from the vehicle (Veh) group ($P<0.05$), as determined by analysis of variance followed by the Tukey's test. ND: Non-diabetic rats; ND+200: Non-diabetic rats + pyritinol 200 mg/kg, p.o.

($P<0.05$) increased the withdrawal threshold in diabetic, but not in non-diabetic, rats (Fig. 7).

4. Discussion

4.1. Antinociceptive and antiallodynic activity of pyritinol in diabetic rats

In this study, we have shown that either acute or prolonged oral administration of pyritinol is able to reduce formalin-induced nociceptive behavior and tactile allodynia in diabetic rats. To our knowledge, this is the first report about the antinociceptive and antiallodynic activity of pyritinol. However, our data agree with observations showing that nefiracetam, another nootropic drug, reduces mechanical hyperalgesia in streptozotocin-treated mice (Rashid and Ueda, 2002). Thus, our results in diabetic rats indeed support this study by demonstrating that pyritinol has antinociceptive and antiallodynic activity in diabetic rats.

The proposed mechanisms to explain the actions on pyritinol in cognitive function disturbances include the increase of glucose utilization and cyclic GMP content as consequence of an increase in the activity of the cholinergic system (Greiner et al., 1988). The mechanisms of antinociceptive and antiallodynic activity of pyritinol in diabetic rats are unknown. This prompted us to attempt to find the possible mechanisms of action of pyritinol in this condition.

4.2. Effect of indomethacin, naltrexone or ODQ on the antinociceptive effect of pyritinol in diabetic rats

The antinociceptive effect of pyritinol was diminished by ODQ. Since ODQ is a guanylyl cyclase inhibitor (Moro et al., 1996), our data suggest that pyritinol-induced antinociception in diabetic rats could be due to activation of guanylyl cyclase. This activation would lead to the synthesis of cyclic GMP, which would then activate protein kinase G and finally it could open potassium channels (Han et al., 2002). Our data agree with previous observations showing that pyritinol increases cyclic GMP as consequence of activating the cholinergic system in the cortex (Greiner et al., 1988). On marked contrast, subcutaneous administration of the non-selective opioid receptor antagonist naltrexone and the non-selective cyclooxygenase inhibitor indomethacin did not affect the antinociceptive effect induced by the acute administration of pyritinol. These data suggest that neither opioid receptors nor inhibition of prostanoid synthesis plays an important role in pyritinol-induced antinociception in diabetic rats. The lack of effect of naltrexone and indomethacin is not due to the doses used in this work, as these doses have been reported to be effective in several studies (Whiteside et al., 2004; Arreola-Espino et al., 2007). Our data contrast with previous studies demonstrating that acute spinal inhibition of cyclooxygenase 2 significantly alleviated formalin-induced flinching behavior in diabetic rats. However, it has been suggested that the relatively ineffective treatment of painful diabetic neuropathy by systemically delivered non-steroidal anti-inflammatory drugs, such as indomethacin, may be due to insufficient quantity of drugs crossing the blood brain barrier to gain access to spinal sites of action, and spinal targeting of cyclooxygenase 2 (Ramos et al., 2007).

4.3. Oxidative stress

Diabetes-induced hyperglycemia plays a critical role in the development and progression of diabetic neuropathy. It is believed that one of the mechanisms by which hyperglycemia produces neural degeneration is *via* the increased oxidative stress that accompanies diabetes (Callaghan et al., 2005). Oxidative stress causes vascular impairment leading to endoneurial hypoxia resulting in impaired neural function and reduced conduction velocity (Pop-Busui et al., 2006). Thus, from the above studies it is convincing to assume that the amelioration of oxidative stress using antioxidants can be beneficial in diabetic neuropathy. These studies and the antioxidant properties of pyritinol (Pavlík and Pilar, 1989) prompted us to assess whether pyritinol-induced antinociception could be due to the reduction of oxidative stress induced by streptozotocin in diabetic rats. Accordingly, streptozotocin treatment increased oxidative stress, as demonstrated by the increase of 4 damage markers, namely free carbonyls, dityrosine, malondialdehyde and advanced oxidative protein products, in 2-weeks diabetic rats. In these animals, acute or prolonged (2 weeks pretreatment) administration of pyritinol significantly reduced free radicals. To our knowledge, this is the first report about the scavenger or antioxidant effect of pyritinol in diabetic rats. However, previous reports have shown that pyritinol is able to protect cell proteins by scavenging free radicals *in vitro* (Pavlík and Pilar, 1989). Based on these results, it is tempting to suggest that the antinociceptive activity of pyritinol could be, at least in part, due to its scavenger properties. The fact that the reduction in free radicals correlates with an antinociceptive effect in diabetic animals treated with either curative or preventive administration of pyritinol supports our suggestion, although other mechanisms might also be involved. Moreover, systemic or intrathecal administration of other free radical scavengers is able to reduce tactile allodynia in other models of neuropathic pain in rats (Obrosova et al., 2001; Kim et al., 2004; Gao et al., 2007). It is likely that pyritinol could produce its effects through an improvement in blood glucose levels; however, acute or prolonged administration of pyritinol was not able to reduce glucose levels in streptozotocin-induced diabetic rats. Thus, this effect can be excluded as a part of its mechanisms of action.

In conclusion, oral administration of pyritinol to diabetic rats significantly reduces formalin-induced nociception and tactile allodynia. The antinociceptive effects are completely prevented by the guanylyl cyclase inhibitor ODQ, but not by the non-selective opioid receptor antagonist naltrexone and the non-selective cyclooxygenase inhibitor indomethacin. Thus, data suggest the possible participation of cyclic GMP in the antinociceptive activity of pyritinol in diabetic rats. This mechanism along with the ability of pyritinol to scavenger free radicals could explain the antinociceptive activity of this drug in diabetic rats.

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