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Riboflavin reduces hyperalgesia and inflammation but not tactile allodynia in the rat

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Abstract

Vitamin B_2 (riboflavin) has been proposed as a prophylactic therapy of migraine. However, so far there are no preclinical studies about the analgesic properties of this vitamin. The current study was designed to investigate the possible antinociceptive, antihyperalgesic and antiallodynic effect of riboflavin in formalin, carrageenan-induced thermal hyperalgesia, and spinal nerve ligation models, respectively. Oral riboflavin produced a dose-related antinociceptive (6.25–50 mg/kg), antihyperalgesic (25–150 mg/kg) and anti-inflammatory (50–150 mg/kg) effect. Gabapentin (100 mg/kg, positive control), but not riboflavin (150–600 mg/kg), reduced tactile allodynia in neuropathic rats. Riboflavin-induced antinociception in the formalin test was reversed by pretreatment with N^G-L-nitro-arginine methyl ester and glibenclamide, but not by N^G-D-nitro-arginine methyl ester or naloxone. Our results indicate that riboflavin is able to produce antinociception and anti-inflammatory, but not antiallodynic, effect in the rat. The effect of riboflavin could be due to the activation of K^+ channels or nitric oxide release, but not activation of opioid mechanisms.

Keywords: Riboflavin; Allodynia; Hyperalgesia; B vitamin

1. Introduction

B vitamins have been used as analgesic drugs to treat pain disorders associated with their deficiency (Marcus and Coulston, 1996). In addition, more recently it has been claimed that B vitamins are useful to relieve different pain states as carpal tunnel, migraine and premenstrual tension (Wyatt et al., 1999). Experiments in animals have shown that vitamins B_1 (thiamine), B_6 (pyridoxine) and B_{12} (cyanocobalamin) and their combination have antinociceptive activity against chemical- and heat-induced pain (Bartoszyk and Wild, 1989; Leuschner, 1992; França et al., 2001). An anti-inflammatory effect has also been reported using the carrageenan-induced edema test (Hanck and Weiser, 1985; Bartoszyk and Wild, 1989). Moreover, the individual administration of thiamine and pyridoxine produce antinociception in acetic acid-induced pain or pain induced by supramaximal electrical stimulation of afferent C fibers (Jurna et al., 1990; França et al., 2001). In contrast, negative results have also been observed. Cyanocobalamin was not able to produce antinociception in this model (França et al., 2001) and other models (Eschalier et al., 1983). A similar result has been published for thiamin and pyridoxine (Reyes-García et al., 1999).

Clinical studies have shown that vitamin B_2 (riboflavin) could have a role in migraine prophylaxis (Schoenen et al., 1994, 1998). In addition, a preclinical study has reported that riboflavin is able to reduce pain in the formalin test in mice (França et al., 2001). However, there is no information

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about the antihyperalgesic, anti-inflammatory and antiallodynic effect of this vitamin. Therefore, in this study the possible antinociceptive, antihyperalgesic, anti-inflammatory and antiallodynic effect of riboflavin in inflammatory (formalin test and carrageenan-induced thermal hyperalgesia) and neuropathic (Chung model) pain models was assessed. In addition, the effect of the opioid receptor antagonist naloxone, the nitric oxide synthesis inhibitor N^G-L-nitro-arginine methyl ester (L-NAME, Ialenti et al., 1992) and the ATP-sensitive K^+ channel blocker glibenclamide (Edwards and Weston, 1993) on riboflavin-induced antinociception was evaluated in order to gain some insight on the mechanism of action of this vitamin.

2. Material and methods

2.1. Animals

All experiments were conducted in accordance with the guidelines on ethical standards for investigation of experimental pain in animals (Zimmermann, 1983). In addition, the study was approved by the Institutional Animal Care and Use Committee (Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico City, Mexico). Female Wistar rats (weight range, 120–140 g for neuropathy studies and 180–220 g for other studies) from our own breeding facilities were used. Animals had free access to drinking water before the experiments, but food was restricted 12 h before the experiments.

2.2. Measurement of antinociceptive activity

2.2.1. Formalin test

Antinociception was assessed using the formalin test (Wheeler-Aceto and Cowan, 1991). Rats were placed in open Plexiglas observation chambers for 30 min to allow them to accommodate to their surroundings; then they were removed for formalin administration. Fifty μl of diluted formalin (1%) were injected s.c. into the dorsal surface of the right hind paw with a 30-gauge needle. Animals were then returned to the chambers and nociceptive behavior was observed immediately after formalin injection. Mirrors were placed 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; Malmberg and Yaksh, 1992; Aguirre-Bañuelos and Granados-Soto, 2000). Flinching was readily discriminated and was characterized as rapid and brief withdrawal or flexing of the injected paw. Formalin-induced flinching behavior is biphasic. The initial acute phase (0–10 min) is followed by a relatively short quiescent period, which is then followed by a prolonged tonic response (15–60 min). At the end of the experiment the rats were sacrificed in a CO_2 chamber.

2.2.2. Thermal hyperalgesia

To assess thermal hyperalgesia, a paw thermal stimulator previously described (Dirig et al., 1997) was used. The device consisted of a glass surface upon which the rats were placed individually in Plexiglas cubicles. The glass surface temperature was maintained at 30 ± 0.1 °C. The thermal nociceptive stimulus originated from a focused projection bulb was manually manipulated to permit the stimulus to be delivered separately to both hind paws of each test subject. This stimulus was positioned under each footpad before and after carrageenan injection (50 μl, 10 mg/ml). A timer was automatically actuated with the light source, and the response latency was defined as the time required for the paw to show an abrupt withdrawal. In all cases, a cut-off of 20 s was employed to avoid tissue injury. Rats were acclimated to the test chamber for 20–30 min prior to testing. Testing was carried out immediately before and every 30 min up to 4 h after drugs administration. At the end of experiments the rats were killed in a CO_2 chamber.

2.2.3. Carrageenan-induced paw edema

Paw edema was induced as previously described (Winder et al., 1957). Briefly, 50 μl of carrageenan (10 mg/ml, Type IV, Lambda) in saline was injected subcutaneously into the left hind paw. The volume of the edema (ml) was measured with the aid of a plethysmometer (Plethysmometer 7150, Ugo Basile, Italy). Measurements were made immediately before and 1, 2, 3, 4, 5 and 6 h after carrageenan injection to determine the differences in paw volume, up to the tibiotarsal joint.

2.2.4. Neuropathic pain model

For the neuropathy studies, rats were prepared according to the method of Kim and Chung (1992). Animals were anesthetized with a mixture of ketamine/xylazine (45/12 mg/kg, i.p.). After surgical preparation and exposure of the dorsal vertebral column, the left L5 and L6 spinal nerves were exposed and tightly ligated with 6-0 silk suture distal to the dorsal root ganglion. For sham operated rats, the nerves were exposed but not ligated. The incisions were closed, and the animals were allowed to recover for 12 days. Rats exhibiting motor deficiency (such as paw-dragging) were discarded from testing. Tactile allodynia was determined by measuring paw withdrawal in response to probing with a series of calibrated fine filaments (von Frey filaments). The strength of the von Frey stimuli ranged from 0.4 to 15 g. Withdrawal threshold was determined by increasing and decreasing stimulus strength eliciting paw withdrawal (Chaplan et al., 1994). The stimulus intensity required to produce a response in 50% of the applications for each animal was defined as "50% withdrawal threshold". All nerve-ligated rats were verified to be allodynic (responding to a stimulus of less than 4 g). Rats not demonstrating allodynia were not further studied (less than 5%).

2.2.5. Motor co-ordination test

Two independent groups of rats ($n = 6$, each) were examined for motor co-ordination in a treadmill apparatus (rotarod) after receiving saline or the highest effective dose used of riboflavin (150 mg/kg, p.o.). Animals were placed upon a cylinder (7 cm diameter) rotating at a speed of 10 rpm. Rats were trained to walk on the cylinder for three consecutive sessions and on the fourth, they received the drug treatment (time 0) and the number of falls during a 5-min period was counted after 1 and 4 h.

2.3. Drugs

Riboflavin and gabapentin were a gift of Merck SA de CV (Mexico City). Naloxone, carrageenan (Type IV, Lambda), glibenclamide, N^G-L-nitro-arginine methyl ester (L-NAME) and N^G-D-nitro-arginine methyl ester (D-NAME) were purchased from Research Biochemical International (Natick, MA, USA). Gabapentin, naloxone, N^G-L-nitro-arginine methyl ester and N^G-D-nitro-arginine methyl ester were dissolved in saline, whereas that glibenclamide was dissolved in dimethylsulfoxide (DMSO) 50%. Riboflavin was dissolved in saline.

2.4. Study design

One group of rats received an oral administration of vehicle (saline) or increasing doses of riboflavin (1–50 mg/kg) 30 min before formalin injection. In order to assess the antihyperalgesic effect, a second group of rats was pretreated with an oral administration of vehicle (saline) or increasing doses of riboflavin (6.25–150 mg/kg) immediately after carrageenan injection. To assess the anti-inflammatory effect of riboflavin, a third group of rats were randomly divided and treated with saline or increasing doses of the vitamin (50–150 mg/kg, p.o.) and the carrageenan-induced paw edema volumes were measured in these rats. A fourth group (neuropathic rats) received an oral administration of riboflavin (150–600 mg/kg) 30 min before withdrawal thresholds were assessed. Oral gabapentin (100 mg/kg) was used as a positive control in neuropathic rats. In order to assess the possible mechanism of action of the vitamin, the effect of pretreatment with naloxone (2 mg/kg, s.c.), N^G-L-nitro-arginine methyl ester, N^G-D-nitro-arginine methyl ester (3 mg/kg, s.c.) or glibenclamide (8 mg/kg, s.c.) on the antinociceptive activity of riboflavin was assessed in the formalin test in a fifth group of animals.

Doses were selected on the basis of previous studies (França et al., 2001) and on pilot experiments in our conditions. Observer was unaware of the treatment in each animal.

2.5. Data analysis and statistics

All results are presented as means ± S.E.M. for at least six animals per group. For the formalin test, dose–response data are presented as the percentage of antinociception of the area under the number of flinches against time curve. %Antinociception was calculated with the following equation: %Antinociception=((Saline – post-compound)/saline) × 100. For the thermal hyperalgesia test, dose–response data are presented as the percentage of antinociception in maximum possible effect (%MPE). %MPE was calculated with the following equation: %MPE=((Saline – post-compound)/cut-off (20 s) – saline) × 100. Inflammation is represented as the volume of the edema (ml) as a function of time. In the spinal nerve ligated rats, curves were constructed plotting the threshold for paw withdrawal as a function of time. One-way analysis of variance (ANOVA), followed by Tukey's test was used to compare differences between treatments. Inflammation data were compared by two-way analysis of variance for dose and time. Differences were considered to reach statistical significance when $P < 0.05$.

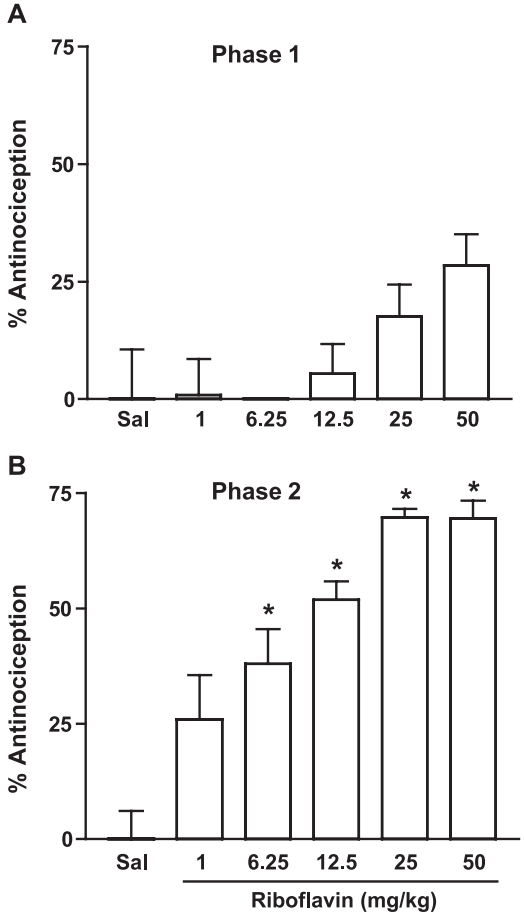

Fig. 1. Antinociceptive effect produced by oral administration of riboflavin during phase 1 (A) and 2 (B) of the formalin test. Rats were pretreated with saline (Sal) or riboflavin 30 min before formalin injection. Data are expressed as the percentage of antinociception. Bars are the means ± S.E.M. of six animals. *Significantly different from vehicle ($P < 0.05$), as determined by analysis of variance followed by Tukey's test.

3. Results

3.1. Effects of riboflavin

Formalin injection produced a typical pattern of flinching behavior. The first phase started immediately after administration of formalin and then diminished gradually in about 10 min. The second phase started at 15 min and lasted until 1 h. Oral administration of riboflavin dose-dependently reduced flinching behavior during phase 2 ($P<0.05$), but not in phase 1, and this was interpreted as antinociception (Fig. 1). A 25 mg/kg dose produced the maximal (about 70%) antinociceptive effect and greater doses (75 mg/kg) were not able to further increase the antinociception in this model. Administration of carrageenan, but not saline, in the right paw produced a hyperalgesic response which lasted about 6 h (Fig. 2A). Oral administration of riboflavin, but saline, produced a significant antihyperalgesic effect ($P<0.05$, Fig. 2B). A 75 mg/kg dose of riboflavin produced the maximal (about 45%) antihyperalgesic effect and higher doses (150 mg/kg) were not able to further increase

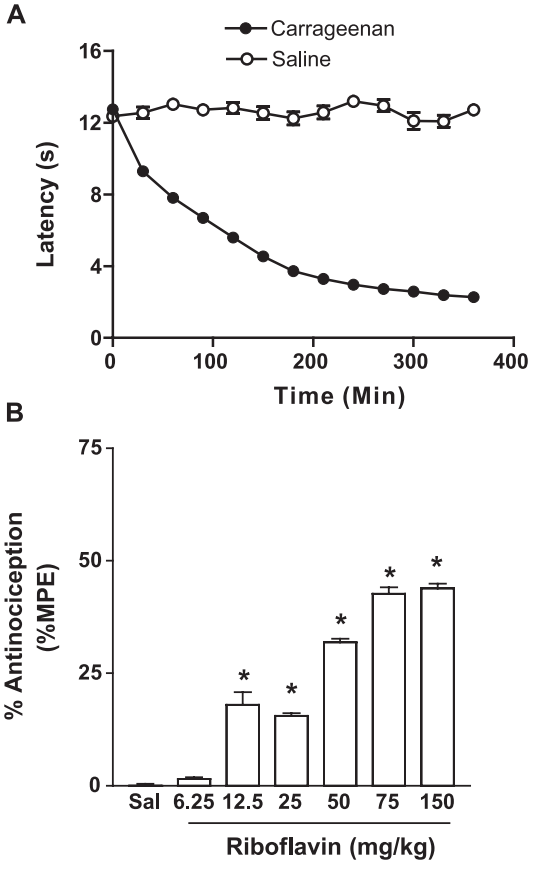

Fig. 2. (A) Hyperalgesic effect of carrageenan in rats submitted to thermal stimulus. (B) Antihyperalgesic effect of oral riboflavin in carrageenan-induced thermal hyperalgesia. Rats were pretreated with saline (Sal) or riboflavin 30 min before carrageenan injection. Data are expressed as the percentage of antinociception of the maxim possible effect (MPE). Bars are the means ± S.E.M. for six animals. *Significantly different from saline ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

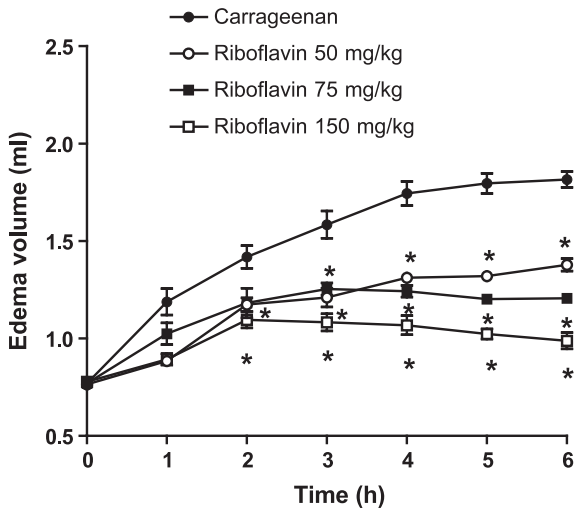

Fig. 3. Anti-inflammatory effect elicited by oral administration of riboflavin in the carrageenan-induced paw edema test. Rats were treated with vehicle or riboflavin before the carrageenan injection. Data are expressed as the edema volume (ml) as a function of time. Data are the means ± S.E.M. of six animals. *Significantly different from carrageenan ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

the antihyperalgesic effect. In addition, to its antinociceptive and antihyperalgesic effect, riboflavin significantly reduced in a dose ($P<0.01$)- and time ($P<0.01$)-dependent manner carrageenan-induced edema (Fig. 3). Riboflavin, at doses greater (150–600 mg/kg) than those used to reduce

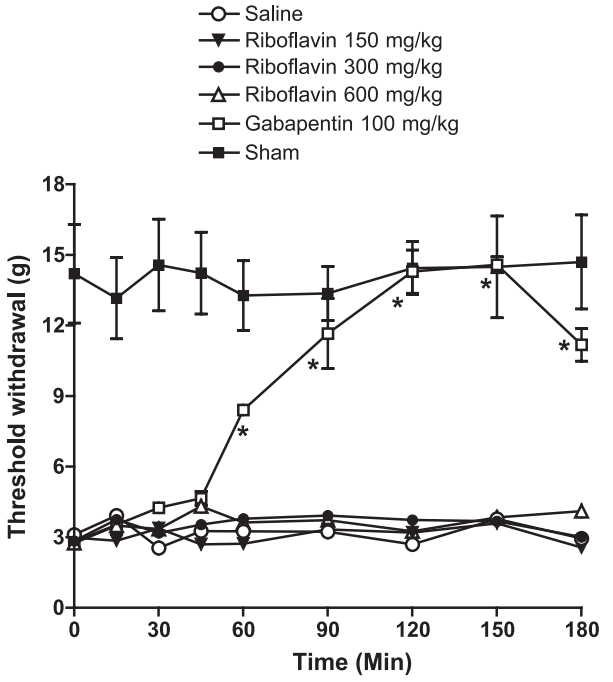

Fig. 4. Effect of oral administration of riboflavin and gabapentin on rats submitted to ligation of L5 and L6 spinal nerves. Rats were treated with saline or increasing doses of riboflavin before starting thresholds evaluations. Data are the mean ± S.E.M. for six animals. *Significantly different from saline ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

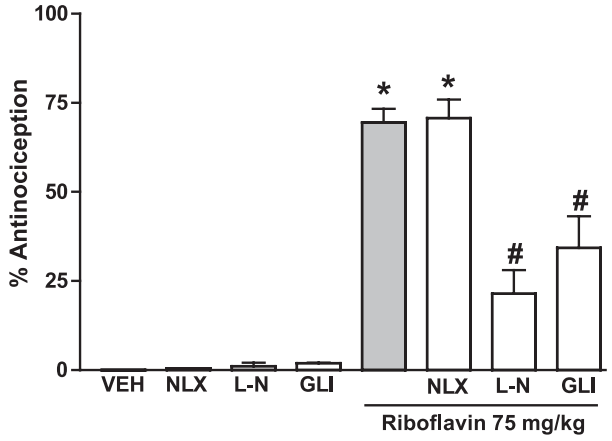

Fig. 5. Effect of naloxone (NLX), N^G-L-nitro-arginine methyl ester (L-N) and glibenclamide (GLI) on riboflavin-induced antinociception. Rats were pretreated (− 40 min) with naloxone, N^G-L-nitro-arginine methyl ester and glibenclamide, then they received riboflavin 30 min before formalin injection. Data are expressed as the percentage of antinociception. Data are the means ± S.E.M. of six animals. *Significantly different from vehicle (VEH, $P < 0.05$) and #significantly different from riboflavin, as determined by analysis of variance followed by Tukey's test.

inflammation, thermal hyperalgesia or formalin-induced flinching, was not able to reduce tactile allodynia in the spinal nerve ligation model. In contrast, oral gabapentin (100 mg/kg, used as positive control) significantly increased threshold withdrawal indicating a reduction of tactile allodynia in this model (Fig. 4).

3.2. Effect of naloxone, N^G-L-nitro-arginine methyl ester and glibenclamide on riboflavin-induced antinociception

Subcutaneous pretreatment with glibenclamide (8 mg/kg) and N^G-L-nitro-arginine methyl ester, but not naloxone, N^G-D-nitro-arginine methyl ester or vehicle, significantly reversed ($P < 0.05$) the antinociceptive effect induced by the oral administration of riboflavin (50 mg/kg) (Fig. 5). Given alone, inhibitors were not able to modify formalin-induced nociception.

3.3. Effect of riboflavin on motor co-ordination

In the group of saline only 1 (of 6) rats had a fall from the rota-rod. Riboflavin at the highest dose used (150 mg/kg, p.o.) did not modify the number of falls as compared with the saline group, thus suggesting that this vitamin does not have unspecific motor effects in the rats either at 1 or 4 h after treatment.

4. Discussion

The present study demonstrated that oral administration of riboflavin is able to reduce inflammation, thermal hyperalgesia and formalin-induced nociception, but not tactile allodynia in rats. In general, effective doses to reduce inflammation were greater than those to reduce thermal hyperalgesia and these were higher that those to reduce flinching behavior in the formalin test. Our results agree with a previous report showing that riboflavin reduces nociceptive and inflammatory effect induced by formaldehyde in mice (França et al., 2001). Therefore, our results confirm previous observations indicating that riboflavin is an antinociceptive and anti-inflammatory drug against inflammatory painful stimulus. However, we also observed that besides the antinociceptive effect in the formalin test, riboflavin was able to produce an antihyperalgesic effect in a model of carrageenan-induced thermal hyperalgesia. In contrast, riboflavin did not modify tactile allodynia of spinal nerve ligated rats, whereas that oral administration of the antiallodynic drug gabapentin, used as positive control, produced a significant reduction of tactile allodynia in the same model. Taken together, data suggest that riboflavin is able to reduce inflammatory, but not neuropathic pain in the rat. Differences to reduce one type of pain, but not other, could be related with the different patophysiology of inflammatory and neuropathic pain. A similar profile is observed with anti-inflammatory and opioid drugs, which are also ineffective to reduce tactile allodynia in animals (Lashbrook et al., 1999) and humans (Backonja and Glanzman, 2003) with neuropathic pain.

Riboflavin is a water-soluble vitamin that functions as co-enzyme for several flavoproteins involved in the electron transport chain in mitochondria (Scholte et al., 1995). So far, the principal use of riboflavin is to prevent migraine headaches (Schoenen et al., 1994, 1998; Schoenen, 1999). It has been claimed that riboflavin exerts its beneficial effects in migraine by increasing complex I and II activity and mitochondrial energy metabolism (Scholte et al., 1995; Schoenen et al., 1998). However, there is no conclusive evidence on this point. In our study, the antinociceptive effect of riboflavin was not modified by subcutaneous pretreatment with the opioid receptor antagonist naloxone, thus suggesting that opioid mechanisms do not participate in its antinociceptive effect in this model. On the other hand, a nitric oxide synthesis inhibitor N^G-L-nitro-arginine methyl ester, but not its inactive isomer N^G-D-nitro-arginine methyl ester, and the ATP-sensitive K^+ channel blocker glibenclamide significantly reversed riboflavin-induced antinociception in the formalin test. These data support the idea that riboflavin could produce its antinociceptive effect through the release of nitric oxide and opening of ATP-sensitive K^+ channels, as it is the case for some anti-inflammatory drugs (Granados-Soto et al., 1995; Islas-Cadena et al., 1999; Aguirre-Bañuelos and Granados-Soto, 2000; Lázaro-Ibáñez et al., 2001; Ortiz et al., 2002, 2003). However, with the present data we cannot discard that other effects, such as an increase in mitochondrial energy metabolism (Scholte et al., 1995), inhibition of adenylyl cyclase (Daly et al., 1998) or inhibition of the synthesis and/or action of inflammatory

mediators (França et al., 2001) could also contribute to the antinociceptive effect of this vitamin.

In summary, we have shown in this study that oral administration of riboflavin produces antinociception in inflammatory, but not neuropathic, pain models. In addition, riboflavin-induced antinociception seems to be related with the opening of ATP-sensitive K^+ channels and nitric oxide release, but not with activation of opioid mechanisms.

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