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Participation of the L-arginine–nitric oxide–cyclic GMP–ATP-sensitive K^+ channel cascade in the antinociceptive effect of rofecoxib

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Abstract

The antinociceptive effect of rofecoxib, a preferential inhibitor of cyclooxygenase-2, was assessed in the pain-induced functional impairment model in the rat. Systemic administration of rofecoxib generated a dose-dependent antinociceptive effect in rats injected with uric acid into the knee joint of the right hindlimb in order to produce nociception. Ipsilateral intra-articular pretreatment with N^G-L-nitro-arginine methyl ester (L-NAME, an inhibitor of nitric oxide (NO) synthesis), 1*H*-(1,2,4)-oxadiazolo (4,2-*a*)quinoxalin-1-one (ODQ, an inhibitor soluble guanylyl cyclase), and the ATP-sensitive potassium channel blocker glibenclamide reversed the antinociceptive effect of rofecoxib p.o. However, ipsilateral intra-articular pretreatment with L-arginine (a NO substrate), or 3-morpholino-sydnonimine-HCl (SIN-1, a non-enzymatic donor of NO), potentiated the antinociceptive effect induced by rofecoxib. The present results suggest that, in addition to cyclooxygenase-2 inhibition, the antinociceptive effect of rofecoxib could also involve activation of the L-arginine–NO–cyclic GMP (cGMP) pathway, followed by opening of ATP-sensitive K^+ channels at the peripheral level.

Keywords: Rofecoxib; Nitric oxide (NO); Cyclooxygenase

1. Introduction

Inflammatory pain involves sensitisation of nociceptors, and there is evidence that cyclic adenosine monophosphate (cAMP) may play an important role in this process (Ferreira and Nakamura, 1979). It is known that when a tissue is injured, the threshold for firing of the Aδ and C nociceptive afferents is lowered to the non-noxious range. Mechanisms involved in this lowering of the threshold include the synthesis of prostaglandins from arachidonic acid, which is released from membrane lipids via the steroid-sensitive enzyme phospholipase A_2. Then, prostaglandins act directly on specific membrane receptors located on peripheral terminals of Aδ and C fibs to promote cAMP accumulation (Smith et al., 1998). The final biochemical events responsible for the sensitisation process following the increase in the intracellular levels of cyclic AMP have not been elucidated. However, there is evidence that activation of protein kinase A and C, with subsequent phosphorylation of ion channels and modulation of cytosolic structures that control intracellular Ca^{2+} levels, may be involved (Sluka et al., 1997; Gold et al., 1998; Aley and Levine, 1999). Actually, aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) are effective because they inhibit cyclooxygenase, thus preventing sensitisation of the pain receptors.

cyclic GMP (cGMP) has been proposed to have a role in antinociception. The local injection of dibutyryl-cyclic GMP results in antinociception (Ferreira and Nakamura, 1979). Other results have shown that local administration of L-arginine produces antinociception in rats with carrageenin-induced hyperalgesia, and that this effect is blocked by nitric oxide (NO) synthase and soluble guanylyl cyclase inhibitors (Duarte et al., 1990). As a gaseous molecule, NO diffuses out from neurones and, by an action on guanylyl cyclase, stimulates cGMP formation in neighbouring cells. Depending on the expression of cGMP-controlled ion channels in target neurones, NO may act as an excitatory or inhibitory agent. NO has been impli-

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cated in the development of hyperexcitability, resulting in hyperalgesia or allodynia, by increasing nociceptive transmitters at their central terminals. The antinociceptive effects of certain NSAIDs, such as metamizole, diclofenac or ketorolac, have been suggested to involve activation of the L-arginine–NO–cGMP pathway in addition to inhibition of prostaglandin synthesis (Tonussi and Ferreira, 1994; Granados-Soto et al., 1995). The precise mechanisms by which cGMP promotes antinociception have not been defined, but a link with ATP-sensitive K^+ channels has been established, based on the finding that tolbutamide and glibenclamide reverse the peripheral antinociceptive effect of K^+ channel blockers (Soares et al., 2000). Thus, the effect of ketorolac was shown to involve activation of the NO–cyclic GMP pathway, followed by opening of ATP-sensitive K^+ channels at the peripheral level (Lázaro-Ibáñez et al., 2001).

Rofecoxib (4-(4-methanesulfonylphenyl)-3-phenyl-5*H*-furan-2-one) is a preferential cyclooxygenase-2 inhibitor, as observed in numerous in vitro and in vivo assays (Chan et al., 1999). The inhibition of cyclooxygenase-2 by rofecoxib occurs via a two-step, time-dependent mechanism leading to the formation of a tightly bound inhibited complex. In contrast, rofecoxib exerts a weak inhibition of cyclooxygenase-1, which is competitive and time-independent (Chan et al., 1999). However, the compound may partly generate antinociceptive effects through the central serotoninergic system (Sandrini et al., 2002). The purpose of the present study was to determine whether the local antinociceptive activity of rofecoxib is mediated, at least in part, through the L-arginine–NO–cGMP–ATP-sensitive K^+ channel pathway.

2. Materials and methods

2.1. Animals

Female Wistar rats [Crl (WI) BR], weighing 180–200 g, were used in this study. Twelve hours before experiments, food was withheld but there was free access to water. All experimental procedures followed the recommendations of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Covino et al., 1980) and the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals (Zimmermann, 1983), and were carried out according to a protocol approved by the local Animal Ethics Committee. The number of experimental animals was kept to a minimum, and animals were housed in a climate- and light-controlled room with a 12-h light/dark cycle.

2.2. Drugs

Rofecoxib, Vioxx or 4-[4-(methylsulfonyl)phenyl]-3-phenyl-2-(5*H*)-furanone) was obtained from Merck Sharp & Dohme (D.F. Mexico). Uric acid, N^G-L-nitro-arginine methyl ester (L-NAME), N^G-D-nitro-arginine methyl ester (D-NAME), L-arginine, D-arginine, 1*H*-(1,2,4)-oxadiazolo(4,2-*a*)quinoxalin-1-one (ODQ), 3-morpholino-sydnonimine-HCl (SIN-1) and glibenclamide were purchased from Sigma (St. Louis, MO, USA).

2.3. Measurement of antinociceptive activity

Antinociceptive activity was assayed using the pain-induced functional impairment model in the rat, which has been previously described in detail (López-Muñoz et al., 1993). The animals were anaesthetised with ether before pain was induced through the injection of 0.05 ml of 30% uric acid into the knee joint of the right hind limb (intra-articular, i.a.). Immediately afterwards, an electrode was attached to the plantar surface of each hind paw between the plantar pads. Rats were allowed to recover from anaesthesia and were then placed on a cylinder, which rotated at 4 rpm for 2 min every 30 min for 4 h. The time of contact between each electrode on the limbs of the rat and the cylinder was recorded with a computer. The time of contact of the injured hind limb reached a zero value 2.5 h after the uric acid injection. At this time, rofecoxib or saline was administered. Antinociception was estimated as the recovery of the contact time.

2.4. Study design

Once the functionality index was zero, rats received saline or increasing doses of rofecoxib (10, 17 and 32 mg/kg) orally. In order to know whether rofecoxib-induced antinociception is mediated by the L-arginine–NO–cGMP–ATP-sensitive K^+ channel pathway, L-NAME (NO synthesis inhibitor), D-NAME (inactive isomer of L-NAME), L-arginine (enzymatic NO donor), D-arginine (inactive isomer of L-arginine), ODQ (NO-sensitive guanylyl cyclase inhibitor), SIN-1 (non-enzymatic NO donor) and glibenclamide (blocker of ATP-sensitive K^+ channels) were injected (0.05 ml) into the uric acid-injured knee when the functionality index was zero. Each compound was given to six animals. One hour later, animals received rofecoxib (10 or 17 mg/kg) orally. At the end of the experiment, animals were euthanised. No side effects were observed in any of the studied groups of animals.

2.5. Data presentation and statistical evaluation

Data are expressed as the area under the curve for the time course of changes (AUC) (see López-Muñoz et al., 1993, for details). The cumulative antinociceptive effect during the whole observation period (4 h) was determined by the trapezoidal rule. All the values for each treatment are presented as the mean ± S.E.M. for six animals. Analysis of variance (ANOVA) followed by Dunnett's *t*-test was used to compare the differences between treatments. Differences

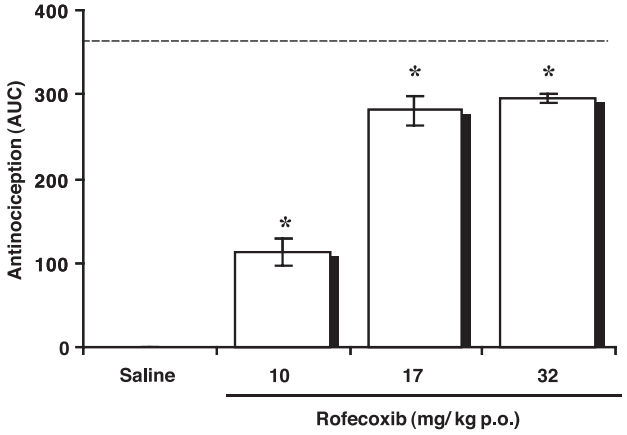

Fig. 1. Antinociceptive effect of orally administered rofecoxib in the PIFIR model, observed 4 h after treatment. Each bar represents the total antinociceptive effect expressed as the AUC (mean ± S.E.M.) for six observations. Dashed line near the top represents the maximum AUC value (375 area units) attained under these experimental conditions. *Significantly different from the corresponding saline-pretreated group ($P < 0.05$), as determined by Student's *t*-test.

were considered to achieve statistical significance when $P < 0.05$.

3. Results

3.1. Antinociceptive effect of rofecoxib

At the time the functionality index had reached zero, oral administration of rofecoxib—but not of saline—produced a dose-dependent antinociceptive effect (Fig. 1). The maximum antinociceptive effect (AUC) in this model was 375 area units (au) (López-Muñoz et al., 1993). In the present experiments, 10 mg/kg p.o. of rofecoxib generated 112.2 ± 16.2 au (i.e. 29% recovery of functionality), whereas 17 and 32 mg/kg p.o. yielded 281.2 ± 18.1 and 295.3 ± 5.2 au (75% and 78% recovery of functionality, respectively).

In order to investigate if the L-arginine–NO–cGMP–ATP-sensitive K^+ channel pathway is involved in the antinociceptive effect of rofecoxib, doses of 10 and 17 mg/kg p.o. of rofecoxib were selected, doses which would allow us to observe an increase or decrease, respectively, of the antinociceptive effect after treatment with drugs that affect the pathway.

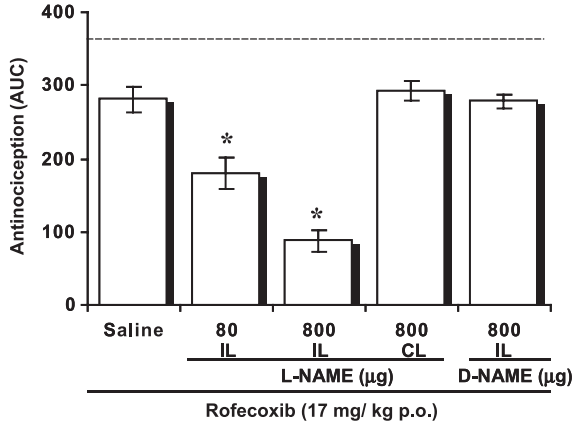

Fig. 2. Effect of the nitric oxide synthesis inhibitor, L-NAME, and its inactive isomer, D-NAME, on the antinociceptive effect to rofecoxib (17 mg/kg p.o.). Rats were pretreated with 50 µl of saline, L-NAME either in the uric acid-injured knee (ipsilateral, IL) or in the left hind limb (contralateral, CL), and with D-NAME 1 h before administration of rofecoxib. Each bar represents the total antinociceptive effect expressed as the AUC (mean ± S.E.M.) for six observations. Dashed line near the top represents the maximum AUC value (375 area units) attained under these experimental conditions. *Significantly different from the saline group ($P < 0.05$), as determined by one-way ANOVA followed by Dunnett's test.

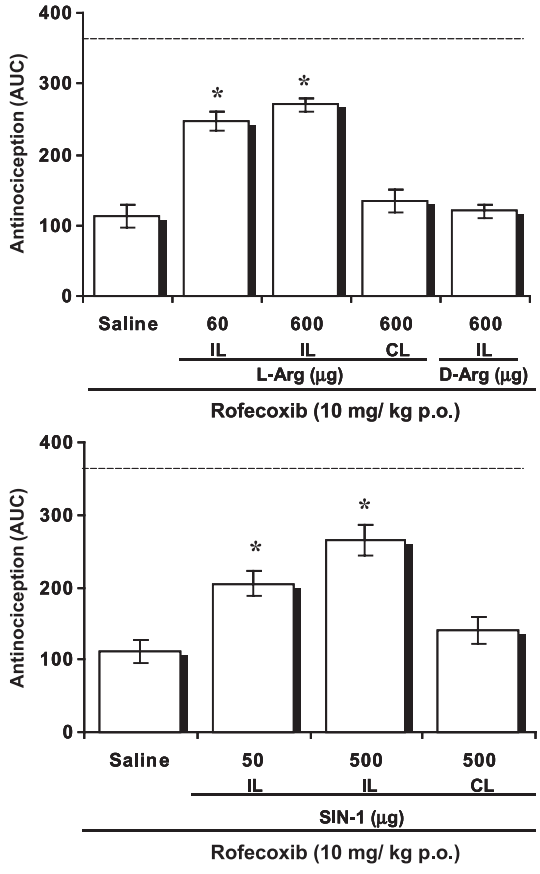

Fig. 3. Effect of the nitric oxide substrate, L-arginine (L-Arg), its inactive isomer D-arginine (D-Arg) and non-enzymatic NO donor, SIN-1, on the antinociceptive effect produced by rofecoxib (10 mg/kg p.o.). Upper panel: Rats were pretreated with 50 µl of saline, L-arginine either in the uric acid-injured knee (ipsilateral, IL) or in the left hind limb (contralateral, CL), and with D-arginine 1 h before administration of rofecoxib. Lower panel: Rats were pretreated with 50 µl of saline and SIN-1 either in the uric acid-injured knee (ipsilateral, IL) or in the left hind limb (contralateral, CL) 1 h before administration of rofecoxib (10 mg/kg p.o.). Each bar represents the total antinociceptive effect expressed as the AUC (mean ± S.E.M.) for six observations. Dashed line near the top represents the maximum AUC value (375 area units) attained under these experimental conditions. *Significantly different from the saline group ($P < 0.05$), as determined by one-way ANOVA followed by Dunnett's test.

3.2. Role of the NO–cyclic GMP pathway in rofecoxib-induced antinociception

Intra-joint articular injection of the right hind limb (ipsilateral) with L-NAME (80 and 800 μg/joint), D-NAME (800 μg/joint), L-arginine (60 and 600 μg/joint), D-arginine (600 μg/joint), SIN-1 (50 and 500 μg/joint) glibenclamide (50 and 100 μg/joint) and ODQ (10 and 100 μg/joint), and of the left hind limb (contralateral) with L-NAME (800 μg/joint), L-arginine (600 μg/joint), SIN-1 (500 μg/joint), ODQ (100 μg/joint) and glibenclamide (100 μg/joint) after acid uric injection did not produce any antinociceptive effects, i.e. the dysfunction persisted throughout the entire observation period (data not shown). Interestingly, pretreatment with L-NAME (80 and 800 μg/joint, ipsilateral) 1 h before rofecoxib administration dose dependently decreased the antinociceptive effect of rofecoxib. Furthermore, injection of 800 μg/joint of L-NAME blocked the antinociceptive effect of rofecoxib, whereas D-NAME (800 μg/joint, ipsilateral) and L-NAME (800 μg/joint, contralateral) failed to modify the antinociceptive effect of rofecoxib (Fig. 2).

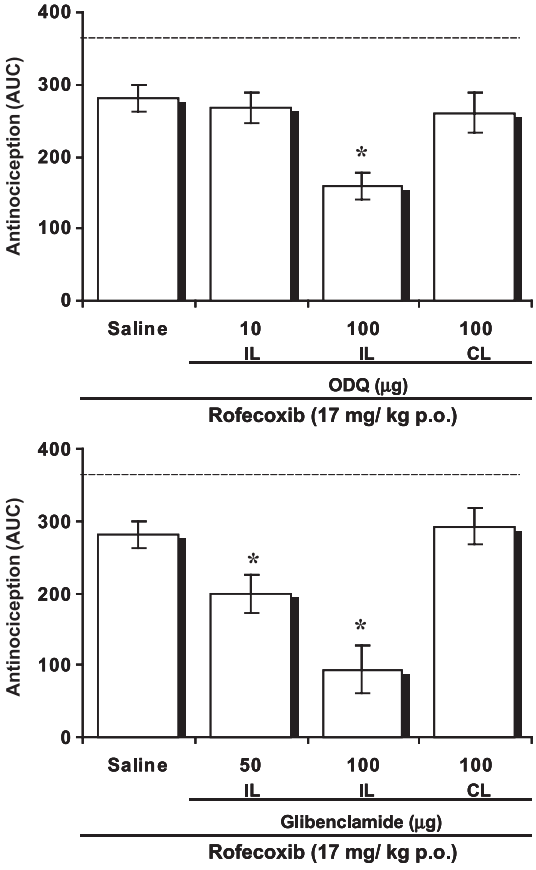

Fig. 4. Effect of the guanylyl cyclase inhibitor, ODQ, and a selective blocker of ATP-sensitive K^+ channel, glibenclamide, on the antinociceptive effect produced by rofecoxib (17 mg/kg p.o.). Upper panel: Rats were pretreated with 50 μl of saline and ODQ either in the uric acid-injured knee (ipsilateral, IL) or in the left hind limb (contralateral, CL) 1 h before administration of rofecoxib. Lower panel: Rats were pretreated with 50 μl of saline and glibenclamide in the uric acid-injured knee (ipsilateral, IL) 1 h before administration of rofecoxib. Each bar represents the total antinociceptive effect expressed as the AUC (mean ± S.E.M) for six observations. Dashed line near the top represents the maximum AUC value (375 area units) attained under these experimental conditions. *Significantly different from the corresponding saline-pretreated group ($P < 0.05$), as determined by Student's *t*-test.

Ipsilateral injection of the NO substrate, L-arginine, 1 h before rofecoxib administration increased the antinociceptive effect of rofecoxib in a dose-dependent manner. Pretreatment with 60 or 600 μg/joint of L-arginine increased the antinociceptive effect of rofecoxib, i.e. the functionality index, by 66% and 72%, respectively. In contrast, contralateral administration of L-arginine (600 μg/joint) did not modify the antinociceptive effect of rofecoxib, whereas pretreatment with D-arginine (600 μg/joint) ipsilaterally had the effect as rofecoxib alone (Fig. 3, upper panel). Pretreatment with the NO donor, SIN-1 (50 and 500 μg/joint, ipsilateral), increased the antinociceptive effect of rofecoxib. As noticed with L-arginine and L-NAME, contralateral administration of SIN-1 had no effect on the antinociceptive effect of rofecoxib (Fig. 3, lower panel).

The effect of the NO-sensitive guanylyl cyclase inhibitor, ODQ, is shown in Fig. 4 (upper panel).The dose 100 μg/joint (ipsilateral) of ODQ decreased by 43% the antinociceptive effect of rofecoxib; contralateral administration of ODQ was without effect on rofecoxib-induced antinociception. Finally, ipsilateral but not contralateral pretreatment with glibenclamide (100 μg/joint) decreased the antinociceptive effect of rofecoxib by 66% (Fig. 4, lower panel).

4. Discussion

Rofecoxib is a selective COX-2 inhibitor which has little or no effect on the COX-1 isoenzyme: it has approximately 1000-fold greater selectivity for COX-2 than for COX-1 (Chan et al., 1999). In the present study, rofecoxib generated a dose-dependent antinociceptive effect in the pain-induced functional impairment model in the rat; there is some evidence suggesting that COX-1 and COX-2 participate in this nociceptive model (Ventura-Martínez et al., 2000). Prostaglandin synthesis inhibition is involved in the antinociceptive effects of rofecoxib, but an additional mechanism of action cannot be excluded. The purpose of this work was to analyse the role of L-arginine–NO–cGMP–ATP-sensitive K^+ channels in the peripheral antinociceptive effect induced by rofecoxib in the pain-induced functional impairment model in the rat.

NO is a controversial neuromediator in nociception. It is able to produce pronociceptive or nociceptive effects in central or peripheral pain mechanisms. Intraplantar administration of L-arginine (NO precursor) can reduce (Duarte et al., 1990; Nakamura et al., 1996; Duarte and Ferreira, 2000) or induce (Aley et al., 1998) the hyperalgesia in the rat paw pressure test. However, low doses of intraplantar L-arginine increase the first phase, while higher doses reduce the second phase of the mouse response to intraplantar formalin,

thereby suggesting that the role of NO in peripheral nociception depends on the tissue level (Kawabata et al., 1994). Intraplantar SIN-1 reduced prostaglandin E_2-induced hyperalgesia (Cunha et al., 1999). Explanations for the controversial results on the involvement of NO in nociceptive processing include drug specificity, dose, route of administration, distribution and pharmacokinetics, as well as local conditions associated with the primary disorders that follow tissue injure (Luo and Cizkova, 2000).

Pretreatment with L-NAME and ODQ blocked in a dose-dependent manner the antinociceptive effect of meloxicam in the formalin test whereas glibenclamide decreased the antinociceptive effect of diclofenac and dipyrone in prostaglandin E_2-induced hyperalgesia (Alves and Duarte, 2002). It has been proposed that this dual effect depends on the dose and pain model under study (Sousa and Prado, 2001). Thus, a systemic dose of 17.8 mg/kg (p.o.) of rofecoxib was selected in order to establish a condition as that would allow us to observe potential decreases in the antinociceptive effect with our pain-induced functional impairment model in the rat. In the same way, the systemic dose of 10 mg/kg (p.o.) rofecoxib was chosen in order to detect potential increases in the antinociceptive response.

In this way, we found that when L-arginine, SIN-1, L-NAME, ODQ and glibenclamide were individually injected into the dysfunctional hind limb, they did not generate hyperalgesic effects and neither increased or decreased the antinociceptive effect by themselves (data not shown). These results allowed us to exclude the possibility that the prevention or potentiation of rofecoxib-induced antinociception was due to a hyperalgesic or nociceptive effect exerted by the drug pretreatments. These results are in line with the observation that intraplantar NOS inhibitors are ineffective in the rat paw pressure test (Aley et al., 1998), the formalin test (Granados-Soto et al., 1997) or in the pain-induced functional impairment model in the rat (Granados-Soto et al., 1995). In our experimental model, pretreatment with L-NAME, L-arginine, and methylene blue before the uric acid injection into the knee was ineffective in altering the progressive dysfunction (data not shown). Thus, NO does not induce hyperalgesia in the pain-induced functional impairment model in the rat. An explanation for this includes the concentration of NO donors or inhibitors used. Local application of drugs producing low NO or high NO concentrations can reduce or increase pain, respectively, through activation of guanylate cyclase pain (Prado et al., 2002). Different pain models may generate different pain states and, therefore, may reveal differential effects of drugs that interfere with NO production.

The effect of rofecoxib was blocked in a dose-dependent manner by local administration of the NO synthesis inhibitor, L-NAME, but not by its inactive isomer, D-NAME, or by saline. These results suggest that local NO release may have a role in the peripheral antinociception induced by rofecoxib. In addition, the nitric oxide donor SIN-1, and L-arginine, which did not produce antinociception by themselves, were able to induce significant antinociceptive effect in combination with rofecoxib. These results agree with those previously reported for L-arginine and cGMP, which failed to produce any effect when given alone but significantly potentiated the effects produced by several antinociceptive drugs (Nozaki-Taguchi and Yamamoto, 1998). In contrast, there are reports showing that, after local administration, NO donors or cGMP analogues are able to produce antinociception by themselves without requiring co-administration of an analgesic agent. The effect of these drugs was blocked by L-NAME or methylene blue administered locally (Duarte et al., 1990). These discrepancies in the effectiveness of NO donors could be explained by the nature of the inflammatory stimuli and the different models of pain used.

The antinociceptive effect is related to the elevated levels of cyclic GMP (Cunha et al., 1999), and NO activates guanylate cyclase and the subsequent production of cGMP. We confirm that cGMP is associated with antinociception because pretreatment with ODQ, a soluble guanylyl cyclase inhibitor, blocked in a dose-dependent manner the antinociceptive effect of rofecoxib. Thus these results confirm the participation of the NO–cGMP pathway in the antinociceptive effect of rofecoxib, as is the case for other NSAIDs (Duarte et al., 1990; Tonussi and Ferreira, 1994; Granados-Soto et al., 1995; Lorenzetti and Ferreira, 1996). However, activation of this pathway does not appear to be required in the antinociceptive action of all NSAIDs. In the pain-induced functional impairment model in the rat, local L-NAME administration failed to block the antinociception produced by acetaminophen (López-Muñoz et al., 1996). These results show that there are differences in the mechanisms of action of different compounds classified in the same pharmacological group.

Interesting results have shown that NO can activate different types of K^+ channels in different types of tissues by an increase in cGMP (Carrier et al., 1997). In addition, it has been shown that morphine induces antinociception by participation of ATP-sensitive channels in peripheral antinociception (Rodrigues and Duarte, 2000). It has been reported that glibenclamide, an ATP-sensitive potassium channel blocker (Amoroso et al., 1990), reduces the antinociceptive effects of the NO donor, sodium nitroprusside (Soares et al., 2000), suggesting a link between activation of the L-arginine–NO–cGMP pathway and potassium channel opening. Thus this work showed that ODQ and glibenclamide were able to decrease rofecoxib-induced antinociceptive effects. In this way, the antinociceptive effect of NO may be explained by its interaction with guanylyl cyclase and the subsequent production of cGMP; this latter in turn would activate ATP-sensitive K^+ channels. These results are similar to those reported recently showing that the opening of K^+ channels is involved in the peripheral antinociceptive effects produced by certain NSAIDs, such as ketorolac (Lázaro-Ibáñez et al., 2001). Thus, the antinociceptive effect of rofecoxib could result from the inhibition of cyclooxygenase-2 and

the activation of the NO–cGMP–ATP-sensitive K^+ channel pathway. Potassium channel opening results in an outward leakage of potassium, producing hyperpolarisation followed by nociceptor desensitisation.

Several observations indicate that activation of the L-arginine–NO–cGMP pathway may result in hyperalgesia rather than antinociception. In such cases, local or systemic administration of L-NAME produces a dose-dependent antinociceptive effect (Haley et al., 1992; Malmberg and Yaksh, 1993; Aley et al., 1998). The nociceptive or inflammatory role of the NO–cyclic GMP pathway has been described after its activation by bradykinin, substance P and carragenin (Kawabata et al., 1994). Hence, a simple explanation for these conflicting observations may be that the role of this pathway varies among the groups of primary sensory neurones activated by different types of nociceptive stimuli (Cunha et al., 1999). However, further investigation is still required to fully elucidate these issues.

The antinociceptive effect of rofecoxib could result from the inhibition of cyclooxygenase-2 and the activation of the NO–cyclic GMP–ATP-sensitive K^+ channel pathway. However, other actions of rofecoxib have been reported recently that may also contribute to its antinociceptive effect, including activation of the serotoninergic system (Sandrini et al., 2002).

In conclusion, rofecoxib produced dose-dependent antinociceptive effects in the pain-induced functional impairment model in the rat. L-NAME and ODQ prevented these effects whereas a NO donor produced potentiation. These results strongly suggest that, in addition to the inhibitory action on prostaglandin synthesis, activation of the NO–cGMP pathway plays an important role in the peripheral antinociceptive action of rofecoxib. Although it is generally accepted that "cross-talk" between products of the cyclooxygenase and NO synthase pathways occurs, reports in the literature are controversial regarding whether NO activates or inhibits prostaglandin production. It is known that NO stimulates cyclooxygenase activity in RAW 264.7 murine macrophages (Salvemini et al., 1993) and that nitric oxide inhibits prostaglandin synthesis in chondrocytes and lipopolysaccharide stimulated macrophages (Amin et al., 1997; Habib et al., 1997). In our study, we cannot exclude that the ability of NO to inhibit prostaglandin release may be associated with a decrease in cyclooxygenase-2 expression.

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