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Comparison of the antinociceptive activity of two new NO-releasing derivatives of the NSAID *S*-ketoprofen in rats

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1 Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase (COX) enzymes inducing analgesic, anti-inflammatory and antipyretic actions. They are not devoid of severe side effects and so, the search for new compounds with similar or higher effectiveness and a lower incidence of undesired actions is important. Nitric oxide (NO)-releasing NSAIDs resulted from this search.

2 We have compared the antinociceptive effectiveness of cumulative doses of two new NO-releasing derivatives of *S*-ketoprofen, HCT-2037 and HCT-2040, using the recording of spinal cord nociceptive reflexes in anesthetized and awake rats and after intravenous and oral administration.

3 *S*-ketoprofen and HCT-2040 were equieffective in reducing responses to noxious mechanical stimulation after i.v. administration in anesthetized animals (ID50s: 1.3 ± 0.1 and $1.6 \pm 0.2\,\mu mol\,kg^{-1}$ respectively), but did not modify wind-up. HCT-2037 was two-fold more potent (ID50 of $0.75 \pm 0.1\,\mu mol\,kg^{-1}$) in responses to mechanical stimuli and very effective in reducing wind-up ($63 \pm 17\%$ of control; $P < 0.01$; MED: $0.4\,\mu mol\,kg^{-1}$), indicating a greater activity than the parent compound.

4 In awake animals with inflammation, HCT-2037 p.o. fully inhibited mechanical allodynia, $91 \pm 12\%$ reduction, and hyperalgesia, $94 \pm 8\%$ reduction. Equivalent doses of *S*-ketoprofen only partially reduced either allodynia ($50 \pm 11\%$) or hyperalgesia ($40 \pm 4\%$). The effect on responses to noxious thermal stimulation was similar for the two compounds.

5 We conclude that the molecular changes made in the structure of *S*-ketoprofen including an NO moiety in its structure, improve the antinociceptive profile of the compound opening new perspectives in a safer use of NSAIDs as analgesic drugs.
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Keywords: Inflammation; cyclooxygenase; spinal cord; wind-up; withdrawal reflex; single motor unit

Abbreviations: COX, cyclooxygenase; DMSO, dimethyl sulfoxide; ID50, inhibitory dose 50; MAP, mean arterial pressure; MED, minimum effective dose; NO, nitric oxide; NSAIDs, nonsteroidal anti-inflammatory drugs; SMU, single motor unit

Introduction

The inhibition of the action of the cyclooxygenase (COX) enzymes, and therefore the inhibition of the synthesis of prostaglandins, is the mechanism of action of nonsteroidal anti-inflammatory drugs (NSAIDs), as described by Vane, 1971. Although this family of drugs have in common a similar mechanism of action, it is a highly heterogeneous group of compounds, with different molecular structures and therefore different chemical properties. The structure of a NSAID is critical for its rate of absorption and penetration into the central nervous system and this, in turn, determines the site of the predominant antinociceptive and anti-inflammatory effects as well as the intensity of unwanted side effects.

One of the most currently prescribed NSAIDs is ketoprofen, a compound among the group of 2-arylpropionic acids (Brune *et al*., 1992), that crosses the blood–brain barrier rapidly in humans (Netter *et al*., 1985) and that has antinociceptive actions in several models of pain (McCormack, 1994; Hummel *et al*., 1995). We have previously reported that ketoprofen is effective in reducing nociceptive spinal cord reflexes activated by mechanical and electrical stimulation in animals with inflammation (Herrero *et al*., 1997). Also, the tromethamine salt of *S*-ketoprofen, the active isomer of the NSAID, is more potent than its parent compound (Mazario *et al*., 1999) and more effective than other COX-1 or COX-2 selective inhibitors (Mazario *et al*., 2001). The inhibition of the COX enzymes by NSAIDs, including ketoprofen, is not only efficacious against pain, fever and inflammation, but also induces unwanted side effects as a consequence of the inhibition of physiological functions in which the enzymes are involved (Herrero *et al*., 2003).

The search for new compounds with a high therapeutical potential and with fewer side effects has led to the development of nitric oxide (NO) releasing derivatives of classical molecules. In fact, NO shows cytoprotective properties in the digestive mucosa, having actions similar to those produced by prostaglandins (Brown *et al*., 1992; Whittle, 1993) that may compensate the gastric damage caused by NSAIDs (Del Soldato *et al*., 1999). NO-releasing derivatives of NSAIDs have been also shown to be more potent and effective as

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antinociceptive drugs than their parent compounds, examples of this are nitroparacetamol (Al-Swayeh *et al*., 2000b; Romero-Sandoval *et al*., 2002) and nitroaspirin (Al-Swayeh *et al*., 2000a).

We have now compared the antinociceptive activity of *S*-ketoprofen with two of its NO-releasing derivatives, in order to assess if the introduction of two different NO-releasing moieties to the backbone of *S*-ketoprofen might improve its antinociceptive effectiveness and ultimately the therapeutic profile of the parent compound. The two new derivatives studied (molecular structures are illustrated in Figure 1) are: HCT-2040 ((*S*)-3-benzoyl-α-methylbenzene acetic acid 4-(nitrooxymethyl)phenylmethyl ester) and HCT-2037 ((*S*)-3-benzoyl-α-methylbenzene acetic acid 3-(nitrooxy)propyl ester). They have been evaluated in electrophysiological recordings of single motor units after intravenous administration and in anesthesia-free animals, using behavioral tests, after oral administration. We have also studied the effect of these drugs on the generation of soft-tissue inflammation and their possible effect on blood pressure.

Methods

Electrophysiological experiments

Animals and preparation Electrophysiological experiments were made on 22 adult male Wistar rats weighing 270–318 g divided into four groups: animals treated with *S*-ketoprofen ($n=6$); animals treated with HCT-2040 ($n=6$); animals treated with HCT-2037 ($n=7$) and animals treated with vehicle ($n=8$). Preparatory surgery was performed under halothane anesthesia (5% in oxygen for induction and 2% for maintenance) and consisted of the cannulation of the trachea, two superficial branches of the jugular veins (for the administration of anesthesia and drugs) and one carotid artery. After the surgery, the animal was transferred to an appropriate frame, halothane was discontinued and anesthesia was maintained with α-chloralose (Sigma) (50 mg kg^{-1} for induction and 30 mg kg h^{-1} by a perfusion pump for maintenance). The right hind limb was fixed into a Perspex block in inframaximal extension with plaster. The core temperature was maintained at 37±0.5°C by means of a homeothermic blanket, and blood pressure was monitored constantly. The preparation was left to rest for at least 1 h before the experiment started. Blood pressure was continuously monitored by means of a pressure transducer connected to the carotid artery. Animals with a systolic blood pressure under 100 mmHg before the administration of the drugs were considered unhealthy and were excluded from the experiment.

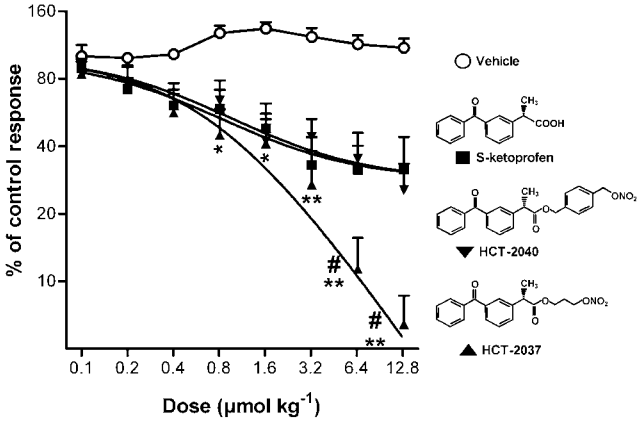

Figure 1 Effects of vehicle, *S*-ketoprofen, HCT-2040 and HCT-2037 on responses to noxious mechanical stimulation in normal rats in the SMU preparation in anesthetized animals. The administration of vehicle did not reduce the SMU responses. *S*-ketoprofen and HCT-2040 were equipotent and equieffective. HCT-2037 was two-fold more potent and induced a complete inhibition of responses. Statistical significance was calculated by one-way ANOVA with Dunnett's *post hoc* test (*$P<0.05$; **$P<0.01$, with respect to vehicle data and to control; control being the mean of the three responses previous to the first dose of each drug; $^{\#}P<0.05$ compared to *S*-ketoprofen and HCT-2040). Data are shown as mean±s.e.m.

The recording of withdrawal reflexes as single motor units (SMU) has been described in detail several times and it has been shown to be useful in the study of the antinociceptive activity of different compounds (Herrero & Headley, 1991; Herrero *et al*., 1997; De Felipe *et al*., 1998; Mazario & Herrero, 1999). Briefly, SMUs activated by noxious mechanical and electrical stimulation (wind-up) were recorded by means of a bipolar tungsten electrode inserted percutaneously into muscles of the right hindlimb. Isolation of motor units was performed by moving the electrode with a micromanipulator, while a mild pressure was applied to the paw. Motor units with a steady firing rate were selected for experiments. The activity was evoked in 3 min cycles consisting of 10 s of mechanical stimulation (0.2 N above the threshold over an area of 14 mm^2) and 16 electrical stimuli (2 ms width, 1 Hz, two times the threshold intensity for the recruitment of long latency responses; Herrero & Cervero, 1996) applied to the most sensitive area of the cutaneous receptive field of the unit. Electrical stimulation was used to study the phenomenon of wind-up (see Herrero *et al*., 2000 for review). Mechanical stimulation was performed by a computer-controlled pincher device, and threshold force was considered as the minimum force needed to obtain sustained firing over the period of 10 s of stimulation. The mean force used for mechanical stimulation was 844±0.1 mN, and the mean intensity for electrical stimulation was 4.7±0.8 mA. At the end of the experiment, the animals were killed with an overdose of sodium pentobarbital (Euta-Lender, Normon).

Drugs and analysis of data *S*-ketoprofen (MW: 254.3), HCT-2040 (MW: 419.4) and HCT-2037 (MW: 357.4) were dissolved in dimethyl sulfoxide (DMSO, Sigma) and polyethylene glycol (1 : 1) (Panreac) in a concentration of 50 mM, and diluted in saline for intravenous administration. Initial dose was 0.1 μmol kg^{-1} (25 μg kg^{-1} for *S*-ketoprofen, 42 μg kg^{-1} for HCT-2040 and 35 μg kg^{-1} for HCT-2037) and was chosen according to the effect observed with other ketoprofen derivatives in similar experiments (Mazario *et al*., 2001; Gaitan & Herrero, 2002). In all the cases, drugs were injected i.v. in log 2 cumulative doses every seven cycles of stimulation (21 min) until an inhibition to or below 25% of control response was observed with the most effective drug. All drugs were kindly supplied by NicOx S.A. (Sophia-Antipolis, France).

The data from mechanical stimulation were pooled and expressed as percentage of control; control being the mean of the three cycles previous to the administration of the first dose.

Data from electrical stimulation were analyzed by counting the responses evoked between 150 and 650 ms after each pulse. These responses were previously considered as C-fiber-mediated inputs in the same preparation (Herrero & Cervero, 1996). Only data from the last two cycles of stimulation (18–21 min) after each dose were used for analysis. The collection of data and the stimulation protocol were performed using commercial software (CED, U.K.; Spike 2 for Windows™). Different tests were performed for statistical analysis using commercial software (GraphPad Prism and GraphPad Instat for Windows™). One-way ANOVA with Dunnett's *post hoc* test was used for comparing between mechanical stimulation and control and for the analysis of wind-up curves. Comparison of responses to noxious mechanical stimulation between groups was made using the nonparametric Mann–Whitney *U*-test. The comparison of inhibitory dose 50 (ID50s) and the analysis of the changes observed in blood pressure were made by the Student's *t*-test. Data are expressed as mean ± s.e.m.

Behavioral experiments

Animals and experimental protocol Behavioral experiments were made in 20 adult male Wistar rats weighing 250–320 g. The animals were divided into three groups according to the treatment performed: (1) *S*-ketoprofen 1–8 μmol kg^{-1} (0.25–2 mg kg^{-1}, $n=6$); (2) HCT-2037 1–8 μmol kg^{-1} (0.35–2.8 mg kg^{-1}, $n=6$); (3) equivalent amounts of the vehicle used for 1–8 μmol kg^{-1} ($n=8$). The animals were placed in individual boxes on a 12 h light–dark cycle and were provided with food and water *ad libitum*. In all cases, the animals were allowed to habituate for 4 days to the testing environment and for the necessary time to be relaxed when handled by the experimenter.

Inflammation was induced 16 h before the experiment under brief halothane anesthesia (5% in oxygen for induction, 2% for maintenance) by the intraplantar administration of 100 μl of carrageenan λ (10 mg ml^{-1} in distilled water, Sigma) in the right hind paw. Another 100 μl of saline were injected in the left hind paw as a control for inflammation. Tests were made immediately before the administration of carrageenan, 16 h after the induction of inflammation, to assess the level of sensitization due to inflammation, and 30 min after the administration of each dose, to assess the effect of the drugs in nociceptive responses. Each test consisted in a mechanically evoked intensity–response curve, noxious thermal stimulation and measurement of paw volume. In most animals, mechanical stimulation was first made and was followed by thermal stimulation. The order of stimuli was changed in at least two animals per group observing no differences between the two experimental protocols. Intensity–response curves for mechanical stimulation were established by placing the animals on a grid and applying 10 stimuli on the plantar surface of each paw with each of the following Von Frey filaments: 60, 100, 200, 300 and 500 mN. The methods have been described in detail elsewhere (Mazario *et al*., 2001) and were adapted from those described by Gilchrist *et al*. (1996). A response was considered as positive when a withdrawal of the paw, due to the application of the filament, was observed. The development of thermal hyperalgesia was assessed by measuring paw withdrawal latencies to 55°C radiant heat generated by an infra-red source (Hargreaves *et al*., 1988). Animals were placed in a clear plastic chamber and were allowed to acclimatize for 5 min before testing (Ugo Basile plantar test). The heat source was positioned under the floor directly underneath the tested paw and the maximum cutoff time was set to 15 s to avoid tissue damage. Both paws were tested two times for each rat with an interval of 2–3 min between tests. The effectiveness of carrageenan in the induction of inflammation and the possible anti-inflammatory effect of the drugs were assessed by measuring the volume of the paw by plethysmometry (Letica plethysmometer) before the administration of carrageenan or saline, and after each of the tests performed. The behavior of the animals was observed continuously throughout the experiment and no abnormal postures, face washing, wall climbing, forepaw treading, wet-dog shakes, jumping or mastication were observed in any case.

Drugs and analysis of data *S*-ketoprofen and HCT-2037 were dissolved in a small amount of DMSO (Sigma, 1 mg 10 μl^{-1}) and administered p.o. in suspension in α-methylcellulose 0.5% in a constant volume of 0.5 ml. The initial dose was administered 16 h after the induction of inflammation and was chosen according to the results observed in SMU experiments. Cumulative log 2 doses were administered every 30 min until a complete reversal of hyperalgesia to precarrageenan level was observed with one of the compounds. Preliminary experiments showed that maximum effect was always observed between 15 and 20 min after the administration of the drugs and the effect remained stable for at least 45 min.

The experimenter was blind to the treatment of animals. Data obtained with 60 and 80 mN force hairs were analyzed separately, since the responses obtained with them were below the threshold intensity in tests performed before the administration of carrageenan. An increase in the responses obtained with these stimuli due to the carrageenan-induced inflammation was considered as allodynia, and, therefore, a reduction of these responses after the administration of a drug was considered as a reduction or attenuation of allodynia. Data obtained with 100–300 mN force hairs were also analyzed separately, since the responses obtained with them were situated between stimuli near threshold intensity and stimuli that produce, or are close to, saturation of responses in tests performed before the administration of carrageenan. The increase in responses observed with these filaments, due to the administration of carrageenan, was considered as hyperalgesia, and the reduction of responses in this range was considered as inhibition or attenuation of hyperalgesia. Data were pooled and expressed as mean ± s.e.m. The intensity–response curves were compared using the one-way ANOVA for repeated measures with the Dunnett's post-test (GraphPad Prism and GraphPad Instat for Windows). Comparison of hyperalgesia, allodynia, latency and paw volume was made with of the nonparametric Mann–Whitney *U*-test.

Either in electrophysiological or in behavioral experiments, the animals were used for one procedure only and were humanely killed on completion of testing by an overdose of pentobarbitone. European Union legislation regulating animal experiments was followed and all efforts were made to minimize suffering and to reduce the number of animals used.

Results

Electrophysiological experiments

Mechanical stimulation Mean number of spikes evoked by 10 s of noxious mechanical stimulation was very similar in the three experimental groups before the administration of any drug: 301 ± 35 in animals treated with *S*-ketoprofen; 308 ± 36 in animals treated with HCT-2040 and 345 ± 42 in animals treated with HCT-2037. Pooled data of results observed with the three drugs tested against noxious mechanical stimulation in normal animals are shown in Figure 1. The administration of equivalent doses of the vehicle ($n=8$) caused a nondose-dependent and nonsignificant increase of SMU responses to noxious mechanical stimulation (Figure 1) with a maximum of $134 \pm 9\%$ of control response with the equivalent amount used to dissolve 1.6 μmol kg^{-1} of the drugs. Equivalent doses of 3.2, 6.4 and 12.8 μmol kg^{-1} gave values of 123 ± 12, 114 ± 11 and $110 \pm 10\%$, respectively. The administration of *S*-ketoprofen ($n=6$) induced a dose-dependent reduction of the nociceptive responses evoked by mechanical stimulation. This reduction was significant for doses of 1.6 μmol kg^{-1} (0.41 mg kg^{-1}) and higher. The highest dose used of 12.8 μmol kg^{-1} (3.28 mg kg^{-1}) reduced the responses down to $31 \pm 12\%$ of control response ($P<0.01$ vs control and vs vehicle). The ID50 was 1.3 ± 0.1 μmol kg^{-1} (0.33 mg kg^{-1}). The administration of HCT-2040 ($n=6$) also induced a significant and dose-dependent reduction of the responses of SMUs to mechanical stimulation, this was very similar to that observed with the parent compound (Figure 1). The reduction was significant from doses of 1.6 μmol kg^{-1} (0.67 mg kg^{-1}), with a maximum effect of $25 \pm 7\%$ of control ($P<0.01$ vs control and vs vehicle), observed with the highest dose used of 12.8 μmol kg^{-1} (5.4 mg kg^{-1}). The ID50 was 1.6 ± 0.2 μmol kg^{-1} (0.67 mg kg^{-1}).

The reduction of responses to noxious mechanical stimulation observed after the administration of HCT-2037 ($n=7$) was more potent and effective than that observed with *S*-ketoprofen and HCT-2040. In this case, the reduction was significant from doses of 0.8 μmol kg^{-1} (0.28 mg kg^{-1}) and the ID50 was 0.75 ± 0.1 μmol kg^{-1} (0.26 mg kg^{-1}), about two-fold lower than that observed with the other two compounds. The maximum effect observed was $6 \pm 2\%$ of control response ($P<0.01$ vs control and $P<0.001$ when compared to vehicle) and in all cases no recovery was observed in 45 min of recording after the administration of the highest dose. The reduction of responses observed with 6.4 and 12.8 μmol kg^{-1} resulted significantly higher than that observed with *S*-ketoprofen or HCT-2040 (Figure 1).

Electrical stimulation: wind-up Figure 2 shows pooled data of the responses to repetitive electrical stimulation (wind-up) observed after the administration of the three compounds. Either the control curves or the number of spikes recorded with the first stimulus were very similar in the three experimental groups before drug administration.

As in responses to noxious mechanical stimulation, the administration of equivalent doses of vehicle caused a nondose-dependent and nonsignificant enhancement of the wind-up curve with a maximum increment of $22 \pm 12\%$ with the equivalent amount used for the dose of 6.4 μmol kg^{-1}. Equivalent doses of 3.2 and 12.8 μmol kg^{-1} induced an increase of 14 ± 6 and $18 \pm 8\%$, respectively. Neither *S*-ketoprofen nor

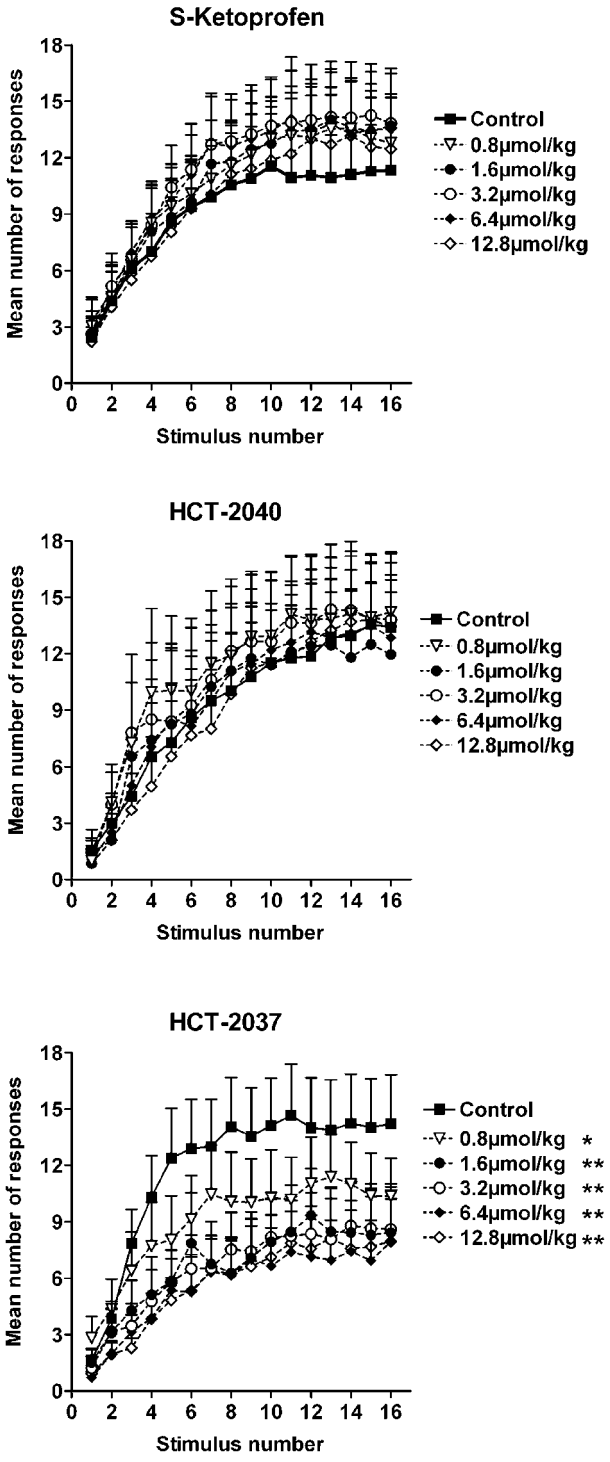

Figure 2 Effect of the NSAIDs on SMU wind-up in normal anesthetized rats. The figure shows the lack of reduction of wind-up observed after the administration of *S*-ketoprofen and HCT-2040, and the intense reduction of responses observed after the administration of HCT-2037. Statistical significance was calculated with the one-way ANOVA with Dunnett's post-test (*$P<0.05$; **$P<0.01$, with respect to control curve).

HCT-2040 significantly reduced the SMU wind-up at the tested dosing. The administration of HCT-2037, however, caused a dose-dependent reduction of wind-up. The effect was significant when compared to the control curve from doses of

0.4 μmol kg^{-1} (0.14 mg kg^{-1}, $P<0.05$ vs control and vs vehicle) and the maximum effect observed was of 63±17% of control ($P<0.01$ vs control and vs vehicle) with the highest dose used. No recovery of the effect was observed 45 min after the administration of the drug.

Blood pressure The administration of the drugs induced in all cases a slight increment of the mean arterial pressure (MAP) but this resulted to be neither dose-dependent nor significant in any of the cases. *S*-ketoprofen enhanced MAP in a nondose-dependent manner with a maximum of 107±4% observed with the dose of 0.2 μmol kg^{-1}. The enhancements observed with HCT-2040 and HCT-2037 were respectively of 132±7% with 0.1 μmol kg^{-1} and of 134±9% with 0.2 μmol kg^{-1}.

Behavioral experiments

Since we observed a clear increase in potency and effectiveness of HCT-2037 when compared to either *S*-ketoprofen or HCT-2040, we wondered if this difference might also be observed in a situation of sensitization due to inflammation and if the effect was somehow altered by the presence of anesthesia. We also wondered if HCT-2037 was active p.o., as this is the normal way of administering NSAIDs in patients. We therefore decided to perform some behavioral experiments in anesthesia-free animals testing the effectiveness of HCT-2037 in comparison with that of *S*-ketoprofen.

Paw inflammation The volume of the paw before the administration of carrageenan was of 148±8 μl in animals treated with HCT-2037 and of 144±6 μl in animals treated with *S*-ketoprofen. The administration of carrageenan induced a significant increment of the paw volume in both experimental groups: 247±7 μl ($P<0.01$ vs precarrageenan value) and 237±6 μl ($P<0.01$ vs precarrageenan value), respectively. The administration of HCT-2037 or of *S*-ketoprofen did not reduced the level of inflammation at the doses and timing studied: 220±10 μl ($P<0.01$ vs precarrageenan value) in the group of animals treated with HCT-2037 and 215±7 μl ($P<0.01$ vs precarrageenan value) in animals treated with *S*-ketoprofen. No changes were observed in the paw volume throughout the experiments. The administration of saline in the contralateral paw did not induce any significant increase in the volume of the paw: 91±6 and 101±3% of control volume, respectively.

Inflammatory sensitization to mechanical and thermal stimuli Intensity–response curves were obtained before and after the administration of carrageenan in the paw, and 30 min after the oral treatment with each of the doses of the NSAIDs studied. The administration of carrageenan induced a significant shift of the curve to the left in the three groups of animals studied (Figure 3, $P<0.01$ in all cases). The administration of the vehicle used did not significantly modify any of the responses to noxious mechanical stimulation (Figure 3). The oral administration of cumulative doses of *S*-ketoprofen induced a partial recovery of the curve towards control values, although the curve obtained with the highest dose studied (8 μmol kg^{-1}) was still significantly different ($P<0.01$) of the baseline. However, the administration of 8 μmol kg^{-1} of HCT-2037 caused a total recovery of the curve

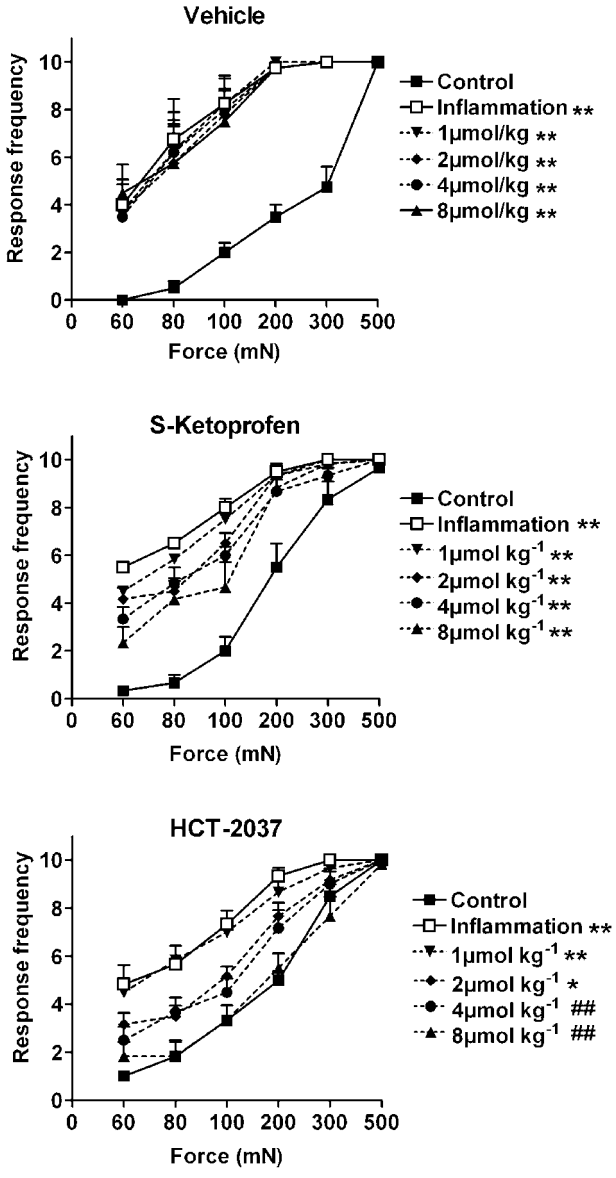

Figure 3 Paw withdrawal frequencies to von Frey filaments following the oral administration of vehicle, *S*-ketoprofen and HCT-2037. The control curve was obtained previous to the induction of inflammation, whereas the rest of the curves were plotted 16 h after carrageenan-induced inflammation and 30 min after the administration of each dose. No recovery of the curve towards the baseline was observed with the vehicle. It was only partial in animals treated with *S*-ketoprofen but it was complete in those treated with equimolar doses of HCT-2037 (*$P<0.05$; **$P<0.01$, comparison vs control response; $^{\#}P<0.05$; $^{\#\#}P<0.01$, comparison vs inflammation curve. Comparison was made with the one-way ANOVA, with the *post hoc* Dunnett test).

and the results were not significantly different of the control curve from doses of 4 μmol kg^{-1}. Also, the curves obtained with 4 and 8 μmol kg^{-1} were significantly different from the carrageenan-induced intensity–response curve.

The data were also analyzed as allodynia, as the increase in the response frequency observed with 60 and 80 mN, than in the noninflamed state do not evoke any nociceptive response, and hyperalgesia, considered as the increase in the number of responses obtained with 100–300 mN of stimulation (Figure 4), that in the normal state evoke a medium number of responses.

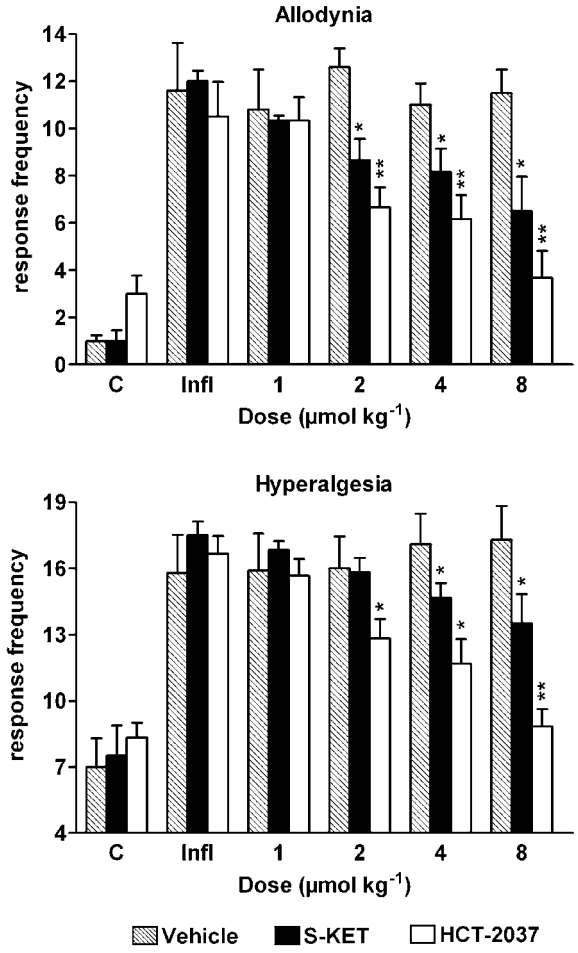

Figure 4 Evolution of allodynia and hyperalgesia in animals treated with vehicle, *S*-ketoprofen and HCT-2037. Allodynia and hyperalgesia to mechanical stimulation were only partially reduced by *S*-ketoprofen. The administration of equimolar doses of HCT-2037 induced a total recovery (*$P<0.05$; **$P<0.01$, comparison vs vehicle using the nonparametric Mann–Whitney *U*-test).

As in the intensity–response curves, allodynia and hyperalgesia were fully reversed by the administration of 8 μmol kg^{-1} of HCT-2037: 91$\pm$12 and 94$\pm$8% reduction, respectively. Only a partial reduction was observed with the equivalent dose of *S*-ketoprofen: 50$\pm$11% reduction of allodynia and 40$\pm$4% reduction of hyperalgesia. The levels of allodynia and hyperalgesia were not changed by the administration of vehicle (Figure 4).

The administration of carrageenan also induced a clear and significant thermal hyperalgesia (Figure 4, $P<0.01$ in the three experimental groups). The administration of *S*-ketoprofen or HCT-2037, but not equivalent doses of vehicle, reversed in a dose-dependent manner (Figure 5) the withdrawal latency to thermal stimulation and full recovery was observed with the dose of 8 μmol kg^{-1}. No significant differences were observed between *S*-ketoprofen and HCT-2037.

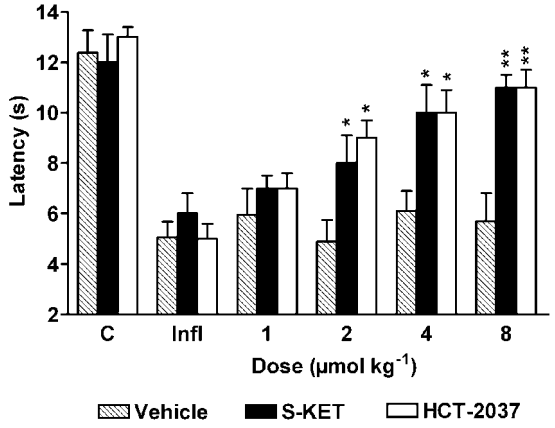

Figure 5 Thermal hyperalgesia observed after carrageenan-induced inflammation and recovery after the oral administration of the NSAIDs. A total recovery was observed after the administration of the cumulative dose of 8 μmol kg^{-1} of either *S*-ketoprofen or HCT-2037. No change was observed with the administration of vehicle (significance and layout as for Figure 4).

Discussion

The results observed in this study indicate that HCT-2037 is more potent and effective as antinociceptive agent than HCT-2040 and than its parent compound *S*-ketoprofen in most of the tests performed. Either *S*-ketoprofen or HCT-2040 were equipotent and equieffective in the reduction of SMU responses to noxious mechanical stimulation, suggesting that the molecular change made in the structure of the drug does not improve its pharmacological profile, at least in the type of studies made. The potency and efficacy of these compounds were similar to that observed in previous experiments using the same technique and experimental protocol with dexketoprofen trometamol (Mazario *et al*., 2001). HCT-2037 was however more potent, with an ID50 of about two-fold lower than that observed with the administration of the other two drugs. Also, the SMU responses to noxious mechanical stimulation were more effectively inhibited by HCT-2037 than by *S*-ketoprofen or HCT-2040. These observations indicate that the molecular structure of HCT-2037 is more active as antinociceptive agent in normal noninflamed animals than the other two compounds.

It is also important to note that the effect observed on wind-up was very different. Wind-up is a phenomenon mediated by spinal cord nociceptive neurones and the reduction of this phenomenon indicates an action at levels above peripheral nociceptors (see for review Herrero *et al*., 2000). A reduction of wind-up has been observed with other nonselective COX inhibitors such as metamizol (Mazario & Herrero, 1999) and indomethacin (Willingale *et al*., 1997). Flurbiprofen and the COX-1 inhibitor SC-58560 were also effective in reducing wind-up curves, as well as inhibiting responses to noxious mechanical stimulation in normal animals (Mazario *et al*., 2001). Other NSAIDs, as for instance meloxicam, do not seem to alter the wind-up curves (Laird *et al*., 1997) or require very high doses, as for example the racemic mixture of ketoprofen (Herrero *et al*., 1997).

The doses of *S*-ketoprofen and HCT-2040 used in the present experiments do not seem to be high enough to reduce the wind-up phenomenon, especially when compared to those used with the racemic compound in the latter experiments. As for HCT-2037, however, the doses administered were high enough to induce a very potent reduction of wind-up. This indicates that either HCT-2037 is more effective in the

modulation of the mechanisms involved in the generation of wind-up, or that the mechanism of action is different or, finally, that the penetration of HCT-2037 to the central nervous system, since wind-up is a centrally mediated event, is greater than that of the other two drugs. The latter does not seem to be the case as ketoprofen crosses the blood–brain barrier easily and, after systemic administration, high levels of ketoprofen in the CSF are reached rapidly (Netter *et al*., 1985).

We have observed in similar experiments that the systemic administration of paracetamol do not modify SMU wind-up, however, similar doses of nitro-paracetamol produce a total inhibition of wind-up (Romero-Sandoval *et al*., 2002) and these results were interpreted as the consequence of a different mechanism of action due to the different molecular structure. A similar interpretation has been given to the fact that paracetamol does not show an anti-inflammatory activity whereas its NO-releasing derivative does (Al-Swayeh *et al*., 2000b). In fact, NO-NSAIDs have been suggested to have COX-independent activities (Del Soldato *et al*., 1999; Fiorucci, 2001; Kiss & Vizi, 2001) and this may also be true for HCT-2037. It is therefore possible that the introduction of a NO donor in the molecular structure of *S*-ketoprofen changes its conformation and probably its mechanism of action. Although further experiments are needed to assess this possibility, the results observed in the present study indicate that the antinociceptive actions are centrally mediated and not dependent on an anti-inflammatory action.

The inhibitory effect observed in behavioral experiments support the intense antinociceptive action of HCT-2037. The results observed show that it is effective either in the normal situation or after sensitization due to soft-tissue inflammation. They also indicate that the effect was not secondary to a reduction of paw edema and, therefore, to a reduction of the peripheral nociceptive activity arriving to the spinal cord neurons. This suggests a central mechanism of action of the drugs. These experiments also show that the inhibition of responses observed with the drugs tested are not influenced by the anesthetic used in SMU experiments nor the way of administration. Either *S*-ketoprofen or HCT-2037 were active when administered orally but, also in this occasion, HCT-2037 was more effective than *S*-ketoprofen in responses to noxious mechanical stimulation. The effect observed in thermal hyperalgesia was, however, similar for the two drugs.

Finally, no major changes were observed in blood pressure after systemic administration. Other nonselective COX inhibitors including NO-releasing derivatives induced a minimal effect on the mean arterial pressure, as previously observed in similar experiments (Mazario *et al*., 2001; Romero-Sandoval *et al*., 2002). A small increase in the blood pressure was similar to that observed with the administration of the vehicle and, therefore, probably due to the action of the solvent. In any case, the release of NO is very slow in this type of compounds (see Romero-Sandoval *et al*., 2002, for further discussion) and does not affect much the level of mean arterial pressure when administered systemically.

In conclusion, HCT-2037 is a potent and effective analgesic agent either in normal animals or in animals with soft-tissue inflammation and after intravenous or oral administration. The effect, at least in part, is located at central sites, probably in the spinal cord, since wind-up resulted being strongly inhibited and it is not secondary to a reduction of the level of inflammation. It is therefore concluded that the molecular changes made in the structure of *S*-ketoprofen improve the antinociceptive profile of the compound and this opens new perspectives in a safer use of NSAIDs as analgesic drugs.

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