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NCX-701 (nitroparacetamol) is an effective antinociceptive agent in rat withdrawal reflexes and wind-up

[1]E. Alfonso Romero-Sandoval, [1]Javier Mazario, [2]David Howat & *,[1]Juan F. Herrero

[1]Departamento de Fisiología, Facultad de Medicina, Universidad de Alcalá, Madrid, Spain and [2]NicOx S.A., Sophia-Antipolis, France

1 Non-steroidal anti-inflammatory drugs (NSAIDs) are effective anti-inflammatory and analgesic drugs although they also induce unwanted side effects due to the inhibition of the physiological effects regulated by prostaglandins. This has led to the search for new compounds with fewer side effects, such as the nitro-NSAIDs (NO-NSAIDs). Paracetamol is an analgesic drug devoid of some of the side effect of the NSAIDs but without anti-inflammatory activity. NCX-701 is a nitric oxide releasing version of paracetamol with anti-inflammatory and analgesic properties.

2 We have tested, in the single motor unit technique, the antinociceptive actions of intravenous cumulative doses of NCX-701 vs paracetamol, studying their antinociceptive effects in responses to noxious mechanical and electrical stimulation (wind-up).

3 Paracetamol did not induce any significant effect at the doses tested (maximum of 480 μmol kg^{-1}, 72.5 mg kg^{-1}). NCX-701 however was very effective in reducing responses to noxious mechanical stimulation ($32\pm10\%$ of control response) and wind-up (ED_{50} of 147 ± 1 μmol kg^{-1}, 41.5 ± 0.3 mg kg^{-1}). The inhibition was not reversed by 1 mg kg^{-1} of the opioid antagonist naloxone. In control experiments performed with either the vehicle or the NO donor NOC-18, no significant changes were observed in the nociceptive responses studied.

4 We conclude that NCX-701 is a very effective non-opioid antinociceptive agent in normal animals and its action is located mainly at central areas. The antinociceptive effect was not due solely to the release of NO.

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Abbreviations: ANOVA, analysis of variance; CNS, central nervous system; COX, cyclo-oxygenase; DMSO, dimethyl sulphoxide; ED_{50}, effective dose 50; MAP, mean arterial pressure; MED, minimum effective dose; NMDA, N-Methyl-D-aspartate; NO, nitric oxide; NOC-18 (DETA NONOate), (Z)-1-[2(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; NO-NSAID, nitro non-steroidal anti-inflammatory drug; NSAID, non-steroidal anti-inflammatory drug; PGE_2, prostaglandin E_2; SMU, single motor unit

Introduction

Unwanted side effects induced by non-steroidal anti-inflammatory drugs (NSAIDs) led to the search for new molecules with effective anti-inflammatory and analgesic actions but devoid of the unsafe effects. Nitro-NSAIDs (NO-NSAIDs) are a group of new NSAIDs developed as a result of that strategy (Del Soldato *et al*., 1999). Since nitric oxide (NO) has cytoprotective properties in the gastric mucosa similar to those mediated by prostaglandins, some classic NSAIDs (naproxen, diclofenac, flurbiprofen and aspirin for example) have been modified to include in their structure NO donor molecules, in an attempt to counterbalance the gastrointestinal damage and other side effects induced by the action of the NSAIDs. As a result, NO-NSAIDs seem to be safer to the grastrointestinal mucosa than are classic NSAIDs (Wallace *et al*., 1999), more effective anti-inflammatory drugs (Fiorucci, 2001) and more potent analgesic than their parent compounds (Al-Swayeh *et al*., 2000). This type of 'latest generation' NSAIDs might open new perspectives in the treatment of chronic pain, especially in those patients at risk of developing severe complications. In the present study we have studied the analgesic effects of a new nitric oxide releasing version of paracetamol NCX-701, using the single motor unit (SMU) technique which has been shown to be a good model to test analgesics in acute experiments (Mazario *et al*., 2001). We have compared the analgesic actions of NCX-701 vs paracetamol and have also studied their effectiveness in reducing responses to noxious mechanical (natural) and electrical stimulation (wind-up), and therefore compared peripheral vs central actions of these drugs (Herrero *et al*., 2000). Preliminary results have been published elsewhere in abstract format (Romero-Sandoval *et al*., 2001).

*Author for correspondence at: Departamento de Fisiología, Campus Universitario, Universidad de Alcalá, Alcalá de Henares, 28871 Madrid, Spain; E-mail: juanf.herrero@uah.es

Methods

Animals, preparatory surgery and groups of experiments

The experiments were performed in 21 male Wistar rats weighing 235–350 g divided into two groups: animals treated

with paracetamol ($n=6$) and animals treated with nitro-paracetamol (NCX-701, $n=9$). Another two groups of control experiments ($n=3$ each) were performed to test the possible effect of the vehicle used and the actions of the NO donor, NOC-18 (DETA NONOate, Alexis), with similar NO release rate to that of NCX-701 (see Results). The SMU technique has been described in detail elsewhere (Herrero & Headley, 1991; Solano & Herrero, 1997). The preparatory surgery consisted in the cannulation of the trachea, one carotid artery to register the blood pressure, and two superficial jugular veins, and was performed under halothane anaesthesia (5% in oxygen for induction and 2–3% for maintenance). After the surgery, halothane was discontinued and the anaesthesia continued with alpha-chloralose (Sigma, 50 mg kg^{-1} initial dose and 30 mg kg^{-1} h^{-1} by perfusion pump for maintenance in a rate of 1 ml h^{-1}, diluted in saline). The right hind limb was fixed in inframaximal extension in a Perspex block using plaster of Paris (Figure 1). The core temperature was maintained at 37 ± 0.5°C by means of a feed-back controlled heating blanket and the blood pressure was continuously monitored. The preparation was left to rest for at least an 1 h before the experiment started.

Stimulus presentation and recording systems

Single motor units (SMUs) were recorded by means of bipolar tungsten electrodes inserted percutaneously into muscles of the right hind limb, and were activated by noxious mechanical and electrical stimuli applied in 3 min cycles. Figure 1 illustrates the preparation and shows an original recording of a SMU during two cycles of stimulation. Paracetamol or NCX-701 were tested only when the responses observed with either stimulus were stable. Noxious mechanical stimulation was applied over an area of 14 mm^2 using a computer controlled pincher, and a force of 200 mN over the threshold intensity, threshold being the minimum pressure required to evoke a constant firing rate for at least 10 s of stimulation. The electrical stimulation was applied using two 0.2 mm needles inserted percutaneously in the most sensitive area of the cutaneous receptive field, with 16 pulses of 2 ms width, 1 Hz and an intensity of twice the threshold current for long latency responses (C-fibre responses, Herrero & Cervero, 1996a). Only units with a stable firing rate and summation of responses to constant intensity repetitive electrical stimulation (wind-up) were selected for the experiments (Figure 1; Herrero *et al*., 2000). Figure 1 shows an original recording of a single unit with a constant firing rate after noxious mechanical stimulation and a progressive increase in responses to high intensity electrical stimulation.

Drugs and collection and analysis of data

Paracetamol and NCX-701 (kindly supplied by NICOX S.A.) were dissolved in DMSO (Sigma) and polyethylene glycol (1 : 1) (Panreac) in a concentration of 50 mM, diluted in saline and administered intravenously in cumulative log2 regime.

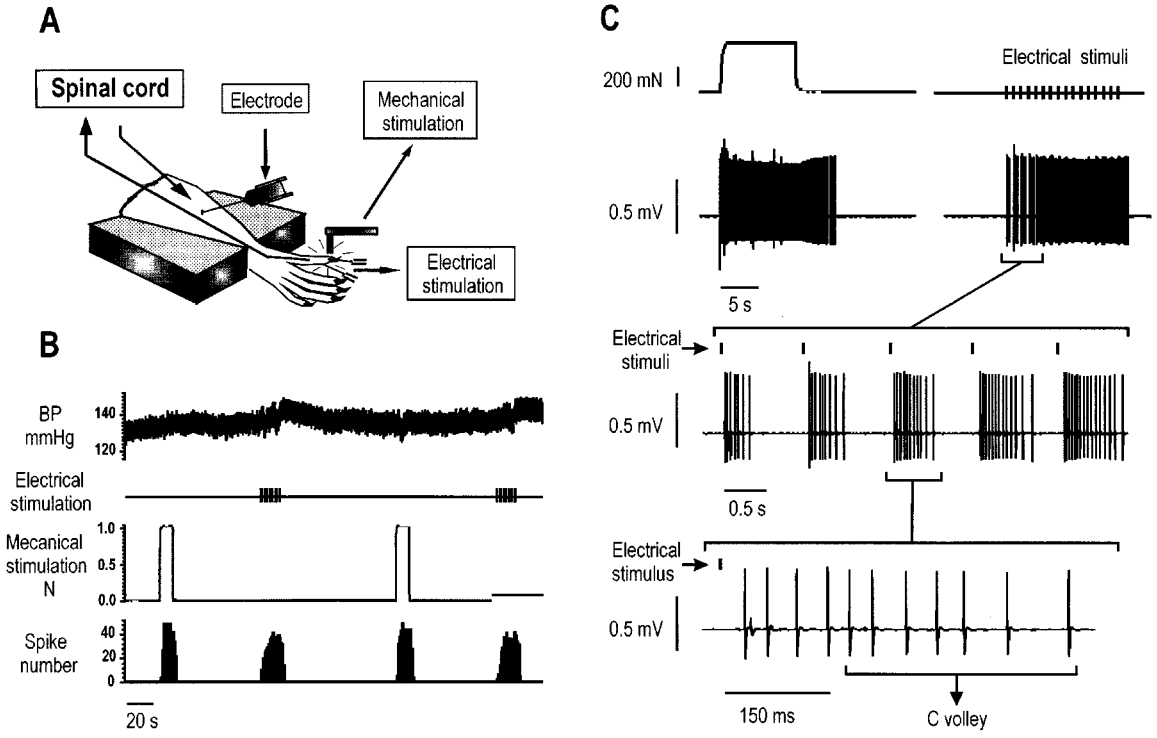

Figure 1 Methods and protocol of stimulation. (A) Single motor units were recorded by means of bipolar tungsten electrodes inserted percutaneously in muscles of the hind limb. (B) Responses were elicited in three min cycles alternating 10 s noxious mechanical stimulation (200 mN above response threshold) and high intensity repetitive electrical stimulation (16 pulses, 1 Hz, 2 ms width, twice times threshold intensity for C-fibre activation). Blood pressure was continuously monitored. (C) Repetitive electrical stimulation induced in all cases a progressive increment of the number of responses (wind-up). These responses were studied as the total number of spikes recorded between 150 and 650 ms after each stimulus.

The initial dose used was 15 μmol kg^{-1} and the highest dose used was 480 μmol kg^{-1}. Each dose was administered every seven cycles of stimulation (21 min). In three experiments, the effect of NCX-701 was challenged with 1 mg kg^{-1} of the opioid antagonist naloxone (Sigma), administered 27 min after the last dose tested. The animals were killed at the end of the experiments with an overdose of sodium pentobarbitone (Euta Lender, Normon). The number of spikes counted in the last two cycles between each dose were averaged and the mean compared to the control response, control being the mean of the three responses previous to the administration of the first dose. The data from the electrical stimulation were analysed by counting the number of spikes evoked between 150 and 650 ms after each stimulus (C-fibre responses, Herrero & Cervero, 1996a).

The collection of data and stimulation protocols were performed by computer using commercial software (CED, UK; Spike 2). Statistical analyses were performed using the one-way analysis of variance (ANOVA) with *post-hoc* Dunnett's test for comparisons between responses observed after the administration of each dose and control. Comparison between responses of paracetamol and NCX-701 was performed using the non-parametric Mann-Whitney *U*-test. Statistical comparisons were performed using commercial software (GraphPad-Prism and GraphPad-Instat for Windows). The data are presented as the mean$\pm$s.e.m. All the experiments performed in this study were carried out in accordance with European Union legislation regarding the use of animals for experimental protocols.

Results

Effects of paracetamol and NCX-701 on responses to noxious mechanical stimulation

Figure 2 shows the effect induced by cumulative doses of paracetamol and NCX-701 in original SMU recordings. Pooled data on responses to noxious mechanical stimulation are represented in Figure 3. The administration of paracetamol did not induce any significant change in responses to noxious mechanical stimulation at the doses tested (117$\pm$5% control with the maximal dose used of 480 μmol kg^{-1}, 72.5 mg kg^{-1}, $n=6$). NCX-701, however, induced a dose-dependent depression of these responses with a minimum significant effective dose (MED) of 120 μmol kg^{-1} ($P<0.05$). The maximum effect observed was 32$\pm$10% of control ($P<0.01$, $n=9$), and the ED_{50} was of 147$\pm$1.1 μmol kg^{-1} (41.5$\pm$0.3 mg kg^{-1}). The number of spikes evoked by the noxious mechanical stimulation used was very similar in all experiments, with a firing rate of around 30 Hz (24–32 Hz). The effect of the drugs was not significantly different when comparison between different responses was studied. To check whether or not this effect was mediated by opioid receptors, the antinociceptive effect of NCX-701 was challenged in three experiments with the non-selective opioid antagonist naloxone at a dose of 1 mg kg^{-1} (Figure 3). No recovery of the effect induced by NCX-701 was observed in any of the tests performed (13$\pm$6% control response, $P<0.01$, $n=3$).

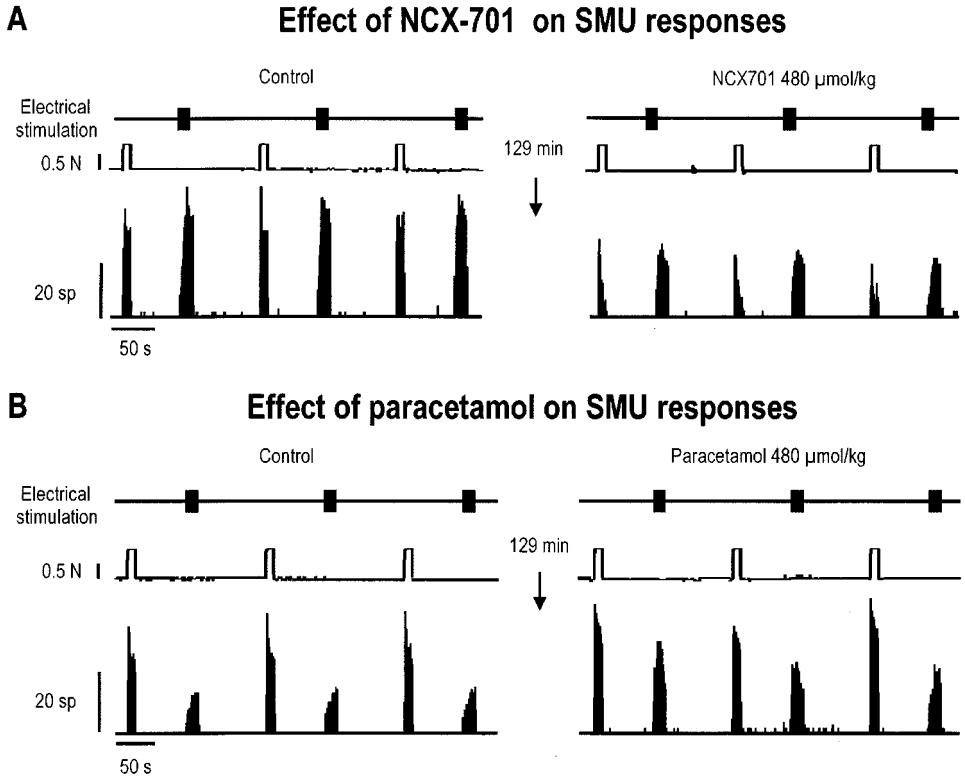

Figure 2 Original recordings of two different single motor units previous to and after the administration of NCX-701 (A) and paracetamol (B). NCX-701 induced a potent dose-dependent reduction of responses to either noxious mechanical stimulation or repetitive electrical stimulation. Paracetamol however did not induce any significant reduction of responses to either type of stimulation.

Figure 4 illustrates the responses observed after the administration of equivalent doses of the vehicle used to dissolve either paracetamol or NCX-701. The administration of the vehicle did not induce any significant reduction of responses to noxious mechanical stimulation. In fact, the responses were increased up to a maximum of $140.7 \pm 18\%$ ($n=3$) of control response with the dose of 120 μmol kg^{-1}.

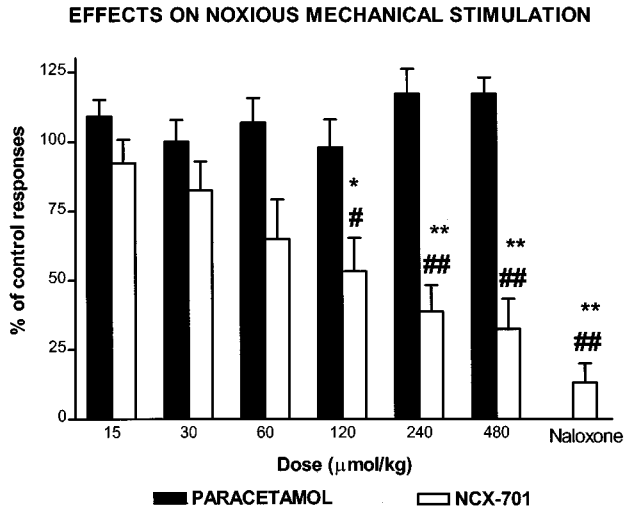

Figure 3 Pooled data of SMU responses evoked by noxious mechanical stimulation after the administration of cumulative doses of paracetamol and NCX-701. The administration of paracetamol did not induce any significant effect. NCX-701, however, induced a dose-dependent reduction of responses, with an ED_{50} of 147 ± 1.1 μmol kg^{-1} (41.5 ± 0.3 mg kg^{-1}). The effect was challenged in three experiments with 1 mg kg^{-1} of the opioid antagonist naloxone but no recovery was observed. (*$P<0.05$; **$P<0.01$, comparison vs control response using the one-way ANOVA, with the *post-hoc* Dunnett test; #$P<0.05$; ##$P<0.01$, comparison between paracetamol and NCX-701 using the non-parametric Mann-Whitney *U*-test.)

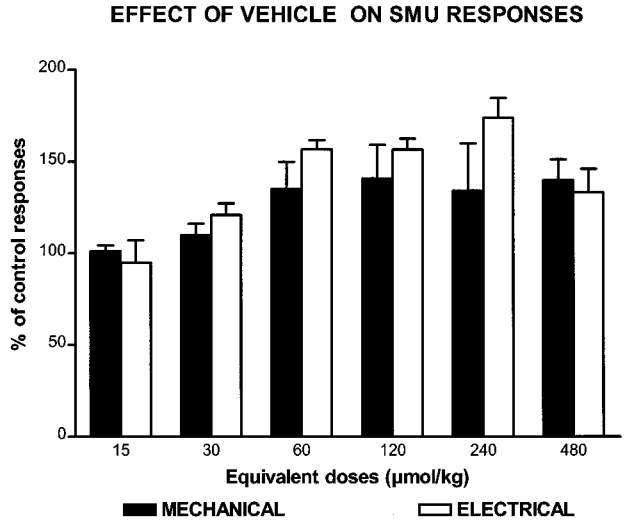

Figure 4 Pooled data of SMU responses evoked by noxious mechanical and electrical stimulation after the administration of equivalent doses of the vehicle used. The administration of the vehicle induced a non dose-dependent and not significant increase of responses to noxious mechanical and electrical stimulation.

This increase in the number of responses was not significant and was not dose-dependent.

Effects of paracetamol and NCX-701 on responses to repetitive electrical stimulation

High intensity electrical stimulation induced an obvious wind-up in all the experiments performed (Figure 5) and the responses elicited with the first pulse were similar in all curves. The administration of cumulative doses of paracetamol did not induce any significant reduction on wind-up (Figure 5), although a non-significant increase of responses evoked with the first pulse was observed. The administration of NCX-701 however induced a very effective reduction of wind-up without changing the number of spikes recorded with the first pulse applied. As in responses to noxious mechanical stimulation, the effect was dose-dependent with a

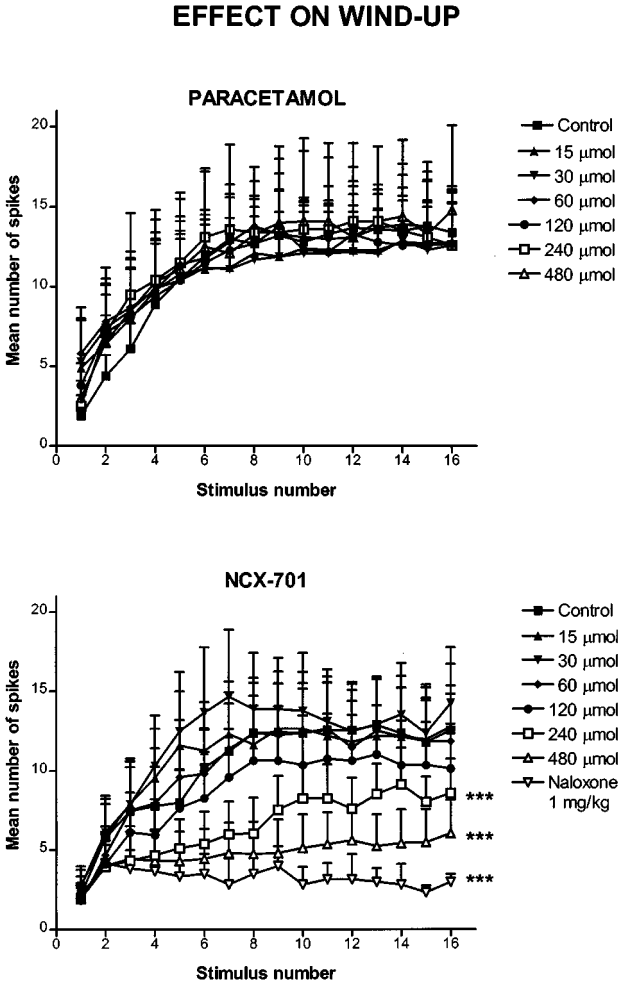

Figure 5 SMU wind-up observed after the administration of cumulative doses of paracetamol and NCX-701. The administration of paracetamol did not induce any significant effect of wind-up responses. NCX-701 induced a dose-dependent reduction of wind-up with a MED of 240 μmol kg^{-1}. As for responses to mechanical stimulation, the effect was not reversed by 1 mg kg^{-1} of the opioid antagonist naloxone. (Statistical comparison between responses observed with each dose and the control response was made using the one-way analysis of variance, ANOVA, with the *post-hoc* Dunnett test, ***$P<0.001$.)

MED of 240 μmol kg^{-1} ($P<0.001$). The administration of 1 mg kg^{-1} of naloxone did not reverse the depression induced by NCX-701 (Figure 5, $P<0.001$).

The administration of equivalent doses of the vehicle used induced an increase in the spikes counted, up to a maximum of $173.8\pm10\%$ ($n=3$) of control response, at a dose of 240 μmol kg^{-1} (Figure 4). This increase was not dose-dependent.

Effects of paracetamol and NCX-701 on blood pressure

Blood pressure was monitored throughout the experiment and, as for responses to noxious mechanical and electrical stimulation, data analysed in the last two cycles of stimulation between each dose were averaged and the mean compared to the control response. Figure 6 shows pooled data for mean arterial pressure (MAP) values observed after the administration of paracetamol and NCX-701. As can be seen, no significant change in MAP was observed after the administration of either drug.

Control experiments performed with NOC-18 (DETA NONOate)

In order to assess whether the antinociceptive effects observed with NCX-701 were only due to the release of NO, three more experiments were performed using the NO donor NOC-18. This compound was chosen since its ability to release NO is very slow (Hrabie *et al*., 1993; Keefer *et al*., 1996) similar to that of NCX-701 (Del Soldato *et al*., 1999; Futter *et al*., 2001). Figure 7 shows the results observed after the administration of equivalent doses of NOC-18 in responses to noxious mechanical and electrical stimulation. A small reduction in the number of spikes was observed either in responses to noxious mechanical stimulation (maximum of $75.1\pm2\%$ of control response with 30 μmol kg^{-1}, $n=3$) or electrical stimulation (maximum of $56.8\pm2\%$ with 480 μmol kg^{-1}, $n=3$). This effect was neither dose-dependent nor statistically significant.

The effect of NOC-18 on blood pressure was also analysed and, as shown in Figure 7, no significant reduction in MAP was observed after the administration of any of the doses studied.

Discussion

The main observation made in this study is that NCX-701 is an effective antinociceptive agent in normal, non-inflamed animals. Paracetamol, like other NSAIDs, is usually not effective in experiments performed in the normal, non-inflamed situation, since the synthesis of prostaglandins is not augmented and therefore there is not a clear target to be modulated. In similar experiments, we have previously observed no effect caused on SMU responses to noxious mechanical and electrical stimulation, for instance, by flunixin (Herrero *et al*., 1996) or flurbiprofen (Mazario *et al*., 2001). An effective analgesia can be observed however if the compound

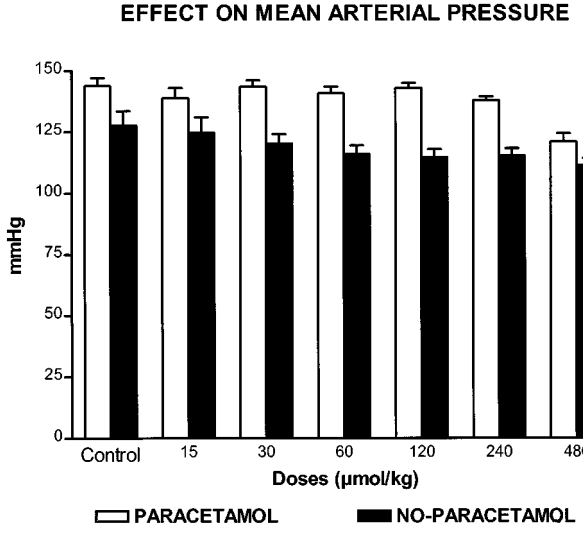

Figure 6 Pooled data of mean arterial pressure values previous to and after the administration of cumulative doses of paracetamol and NCX-701. Neither of the drugs studied induced any significant change in blood pressure (data taken and averaged 18 and 21 min after the administration of each dose).

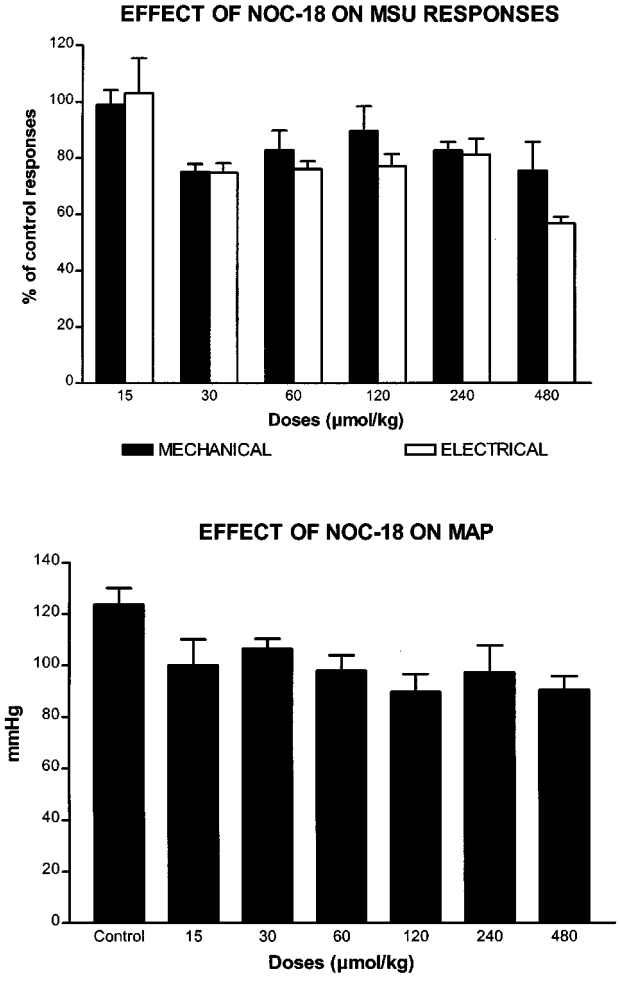

Figure 7 Effect of NOC-18 on mean arterial pressure and SMU responses evoked by noxious mechanical and electrical stimulation. (A) The administration of cumulative doses of the NO donor NOC-18 did not induce any significant change in SMU responses to noxious mechanical and electrical stimulation. (B) NOC-18 did not either modify the mean arterial pressure values in a significant manner.

crosses the blood-brain barrier easily, and an action upon the central processing of nociceptive information is directly exerted (Mazario & Herrero, 1999; Mazario *et al*., 2001). Paracetamol has been shown to have central antinociceptive effects, but only when administered at doses much higher that those used in the present study (Pelissier *et al*., 1995; Pini *et al*., 1997). In our hands, a central action of paracetamol does not seem very important when studied in the present conditions, since a central effect would be expected to modify the excitability of SMUs and the wind-up phenomenon, at least partially (Herrero *et al*., 2000, see below). NCX-701 however was very effective either in responses to noxious mechanical stimulation or high intensity electrical stimulation. An action at central sites is supported by the strong effect exerted on the wind-up phenomenon. In this phenomenon, repetitive electrical stimulation induces a progressive increase of nociceptive responses from spinal cord neurones (Herrero *et al*., 2000, and references within) and is mediated by NMDA (Davies & Lodge, 1987; Dickenson & Sullivan, 1987) and NK1 receptors (De Felipe *et al*., 1998). A reduction of wind-up implies an inhibitory action of the circuitry involved in its generation, which is located in the central nervous system, at spinal cord level (see Herrero *et al*., 2000 for further discussion), although a modulation of the system by higher levels in the CNS is also possible (Herrero & Cervero, 1996b). The effect of NCX-701 therefore takes place presumably at central sites rather than in the periphery. A second possibility is that NCX-701 induced an increase in the threshold for activation of nociceptive afferents, and therefore a reduction in the amount of input reaching the spinal cord, as described with some opioid agonists (Chapman & Dickenson, 1992). This is not probable since the experiments showed that the number of spikes recorded with the first electrical stimulus was very similar at all doses tested.

The question that remains unanswered is how the NO released by NCX-701 was able to enhance the antinociceptive effects of paracetamol. A possibility is that NO was somehow able to facilitate the entrance of paracetamol into the CNS; this might explain why NCX-701 inhibits wind-up whereas paracetamol does not. Although possible, it does not seem probable, since paracetamol crosses the blood-brain barrier easily (see for instance Ochs *et al*., 1985; Bannwarth *et al*., 1989). Also, 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 NCX-701. In fact, in similar experiments to those performed in the present study, we and others have observed that some NSAIDs, including paracetamol, may induce a potent antinociceptive action by modulating opioid systems (Herrero & Headley, 1996; Pini *et al*., 1997). It is for that reason that we challenged the inhibition of nociceptive responses induced by NCX-701 with high doses of the opioid antagonist naloxone. The dose of naloxone used is several times higher than that needed in similar experiments to reverse a total inhibition of responses induced by morphine (Herrero & Headley, 1991) or to prevent any action of the μ-opioid agonist fentanyl (Thorn *et al*., 1994; Gaitan & Herrero, 2002). Since no reversal of the effect was observed, we conclude that the effect observed with NCX-701 was not due, in our experiments, to an action on opioid receptors.

Another possibility is that NO induced an antinociceptive effect in its own. This, again, does not seem to be the case since the experiments performed with NOC-18 showed no variation in the responses evoked by either noxious mechanical or electrical stimulation. Also, the possibility that a general depression of the system due to a blood pressure fall, induced by a NO-mediated vasodilation, is not likely since the release of NO by NCX-701 is very slow and, similar to that observed with NOC-18, no major changes were observed in blood pressure with the doses studied.

It is therefore possible that the progressive release of NO favoured or promoted an antinociceptive effect of paracetamol (and/or vice versa), perhaps by a different mechanism of action. In fact, although we have not seen any clear effect after the administration of NOC-18, NO donors may induce either pronociception or antinociception, and the most clear effect in spinal cord-mediated withdrawal reflexes seems to be antinociceptive (Sousa & Prado, 2001). This action however appears to be NO concentration-dependent (Kurihara & Yoshioka, 1996; Sousa & Prado, 2001). Also, NO synthesis in the CNS is mediated by the constitutive neuronal isoform of the NO synthase which seems to be positively modulated by the activation of NMDA (Kiss & Vizi, 2001). It has been suggested that NO release, as a consequence of NMDA receptor activation, modulates negatively in turn the release of glutamate, by enhancing the amount of monoamines in the synaptic space (Kiss & Vizi, 2001). On the other hand, PGE2 enhances the release of glutamate and aspartate in the spinal cord (Nishihara *et al*., 1995) and the blockade of COX activity might reduce the levels of PGE_2 and therefore the release of glutamate. If this is true, NCX-701 might negatively modulate the activity of NMDA receptors by a joint action of the NO released and the paracetamol molecule, depressing the release of glutamate through different mechanisms, and causing an effect greater than that observed when administered separately. This possibility would explain the potent effect of NCX-701 observed in the present study in wind-up, a phenomenon mediated by NMDA receptors (see above). Nevertheless, further experiments are needed to assess this possibility.

In conclusion, NCX-701 is a potent and effective analgesic agent in normal animals after intravenous administration. The effect is not due solely to the action of NO and, at least in part, is mediated at central sites, probably the spinal cord, since wind-up was greatly inhibited.

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