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Analysis of the mechanism of antinociceptive action of niga-ichigoside F_1 obtained from *Rubus imperialis* (Rosaceae)

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Abstract

We have previously verified that niga-ichigoside F_1 (NI), a triterpene isolated from *Rubus imperialis*, exhibits significant and potent antinociceptive action when evaluated in some pharmacological models of pain in mice. This effect was confirmed in other experimental models and also the mechanism of action has been evaluated. The antinociception caused by NI (60 mg kg^{-1}) in both phases of the formalin test was significantly attenuated by intraperitoneal injection of mice with haloperidol (a dopaminergic antagonist, 0.20 mg kg^{-1}) and L-arginine (precursor of nitric oxide, 600 mg kg^{-1}). Regarding the cholinergic system, atropine (a cholinergic antagonist 60 mg kg^{-1}) reverted only the second phase. The effect of NI was not affected by treatment of mice with yohimbine (an alpha2-adrenoceptor antagonist, 0.15 mg kg^{-1}). The same pharmacological profile was observed for the administration of naloxone (an opioid receptor antagonist, 1 mg kg^{-1}). On the other hand, intraperitoneal injection caused dose-related and significant effects against glutamate- and capsaicin-induced pain, respectively. In conclusion, the marked antinociception of NI appears to be related to the dopaminergic, cholinergic, glutamatergic, tachykininergic and oxinitrergic systems, supporting the ethnomedical use of *Rubus imperialis* (Rosaceae).

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Introduction

The genus *Rubus* consists of many species that are used in various countries around the world to treat different diseases, particularly diabetes (Niero et al 1999; Patel et al 2004). Chemical and pharmacological studies have confirmed that some of these plants produce active principles that exert hypoglycaemic activity, antibacterial effects against Gram-positive bacteria, anti-allergic rhinitis and anti-asthmatic properties. In addition, many plants are used traditionally as a uterine relaxant, for the treatment of diarrohea and similar enteric disorders, and as an astringent (Novaes et al 2001; Emendorfer et al 2005).

Rubus imperialis Chum. Schl. (Rosaceae) grows in abundance in Southern Brazil, where it is known as amora-branca, amora-do-mato or amora-brava. It is frequently used in popular medicine for the treatment of diabetes (Cirilo 1993; Lemus 1999). Previous studies carried out by our research group demonstrated that the potent antinociceptive effect of this plant is related to the presence of a triterpene named niga-ichigoside F_1 (NI, Figure 1). This compound showed potent antinociceptive activity when analysed in both models of nociception: writhing and formalin tests (Niero et al 1999). This plant also showed cytotoxic property established by *Artemia salina* (Kanegusuku et al 2002).

In view of the previous promising antinociceptive effects shown by NI, we have extended these studies to investigate other models of nociception in mice, as well as evaluating the possible mechanism of action.

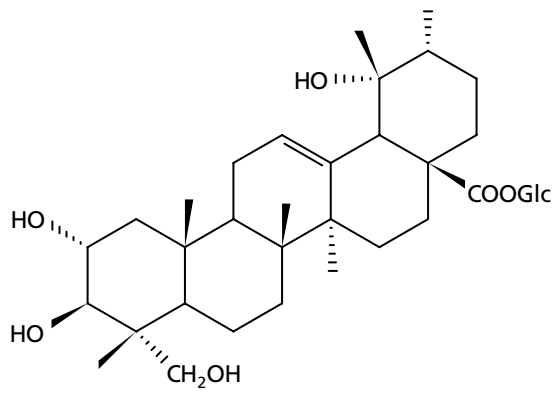

Figure 1 Chemical structure of niga-ichigoside F_1 (NI).

Materials and Methods

Plant material

R. imperialis was collected in Florianopolis, Brazil, in June 1999 and identified by Dr Ademir Reis (Department of Botany, UFSC). A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí-SC-Brazil) under number V.C. Filho 012.

Extraction and isolation

The ethyl acetate fraction was prepared as described previously by Niero et al (1999). This fraction (1.4 g) was chromatographed on a silica gel column and eluted with $CHCl_3$–MeOH with increasing polarity. Similar fractions, which showed a positive reaction with anisaldehyde sulfuric reagent, were combined and rechromatographed as previously described, giving 150 mg of pure colourless solid. The purity was examined by thin-layer chromatography (TLC) using Merck silica gel pre-coated aluminium plates, 200 μm layer thickness, with several solvent systems of different polarity. The compound was identified as Niga-ichigoside F_1 (NI) on the basis of spectral data and direct comparison with an authentic sample (Durham et al 1994; Niero et al 1999).

Drugs

The drugs used were formaldehyde, acetic acid, morphine, naloxone (Merck AG, Darmstadt, Germany), N^w-nitro-L-arginine (L-NOARG), L-arginine, D-arginine, glutamic acid, capsaicin, haloperidol, yohimbine, clonidine, apomorphine, atropine (Sigma Chemical Co., St Louis, MO) and Tween 80 (Merck AG, Darmstadt, Germany). All other reagents used were of a high grade of purity. Niga-ichigoside F_1 was dissolved in Tween 80 and diluted just before use in 0.9% NaCl. The final concentration of Tween 80 did not exceed 5% and did not have any effect itself. Due to the limited amount of this compound and based on previous experiments, we selected a dose of 60 mg kg^{-1} to investigate the action mechanism of NI.

Animals

Swiss mice, 25–35 g, housed at 22 ± 2°C under a 12-h light–dark cycle and with free access to food and water, were acclimatized to the laboratory for at least 1 h before testing. For each experiment, one group of mice was used. The experiments reported on here were carried out in accordance with the current ethical and care guidelines for the care of laboratory animals and the investigation of experimental pain in conscious animals (Zimmermann 1983). The experiments were approves by the local ethics committee of this Institution (113/2005-03 UNIVALI). The number of mice (6–8 per group) and intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

Formalin-induced nociception

The procedure used was essentially similar to that described previously (Hunskaar & Hole 1987). Mice from the same strain were used to analyse the first and second phases of formalin-induced pain, and 20 μL of 2.5% formalin solution (0.92% formaldehyde), made up in a phosphate-buffered solution (NaCl 137.0 mM, KCl 2.7 mM and phosphate buffer 10 mM), which was injected intraplantarly in the ventral surface of the right hind paw of the mice. After injection, the time spent licking the injected paw was timed with a chronometer and considered as indicative of pain. The initial nociceptive scores normally peaked 5 min after formalin injection (first phase) and 15–30 min after formalin injection (second phase), representing the neurogenic and inflammatory pain, respectively. In an attempt to investigate the possible action mechanism of NI (60 mg kg^{-1}, i.p.) we used the formalin test with the agonist and antagonist drugs of the systems analysed. All the antagonists (naloxone, yohimbine, haloperidol, L-arginine and atropine) were administered 15 min before the respective agonists (morphine, clonidine, apomorphine and L-NOARG), compound or vehicle.

Glutamate-induced nociception

The procedure used was similar to that described previously Beirith et al (2002). The mice were observed individually for 15 min after glutamate injection (30 μmol/paw). The amount of time spent licking the injected paw was timed using a chronometer and considered indicative of pain. The mice were treated with compound (10–60 mg kg^{-1}, i.p.) 30 min before the glutamate injection. The control group received a similar volume of vehicle (10 mL kg^{-1} i.p.) used to dilute the compound.

Capsaicin-induced licking

The method used was similar to that described previously by Sakurada (1992). Following the adaptation period, 20 μL of capsaicin (1.6 μg/paw prepared in phosphate-buffered solution) was injected under the skin of the dorsal surface of the right hind paw. The mice were pre-treated with compound (10–100 mg kg^{-1}, i.p.) 30 min before injection of the irritant. The controls received a similar volume of the vehicle (10 mL kg^{-1}, i.p.) used to dilute the drugs. After this process, pairs of mice

were placed in different glass cylinders for 5 min following the capsaicin injection. The amount of time spent licking the injected paw was timed using a chronometer and was considered as indicative of nociception.

Statistical analysis

The results are represented as a mean ± s.e.m., except the ID_{50} (the dose of extract that reduced response by 50% relative to the control value) which is presented as geometric mean accompanied by the respective 95% confidence limits. The ID_{50} was determined by linear regression GraphPad software (GraphPad Software, San Diego, CA). Statistical significance between groups was calculated by means of analysis of variance followed by Newman–Keuls' multiple comparison tests. $P < 0.05$ was considered as indicative of significance.

Results

The results of this study extend the initial investigations carried out in our laboratories and demonstrate that niga-ichigoside F_1 (NI) also presents a significant antinociceptive effect when analysed in other models of pain in mice. Table 1 shows that NI exhibits antinociceptive effect against capsaicin and glutamate-induced pain. In the capsaicin test, the compound studied (10–100 mg kg^{-1}, i.p.) produced a dose-dependent effect with an ID_{50} value of 87.3 (42.4–109.7) mg kg^{-1} and inhibition of 55.81 ± 4%. NI was about 10-fold more potent against the pain induced by glutamate, with ID_{50} and inhibition values of 7.9 (3.4–18.5) mg kg^{-1} and 81.69 ± 4%, respectively. We also verify that the analgesic effect of the compound lasted for up to 4 h after its intraperitoneal administration in the formalin test (results not shown). Another aim of our work was to analyse the mechanisms by which NI could be promoting its antinociceptive effects. The evaluation of the opioid system showed that prior treatment of mice with naloxone (a non-selective antagonist of opioid receptors) did not reverse the antinociceptive effect of NI, under the same conditions in which naloxone almost completely reverted the effect caused by morphine (Figure 2). Figure 3 shows the results of prior treatment of the mice with atropine (a non-selective antagonist of cholinergic receptors). This treatment also reversed the antinociceptive effect of NI and suggests the involvement of the opioid system. The evaluation of the adrenergic system can be seen in Figure 4. Treatment of the mice with yohimbine (antagonist at α_2-adrenergic receptors) 15 min before the administration of NI under conditions in which yohimbine almost completely reverted the antinociception caused by clonidine (agonist α_2-adrenergic), did not revert the effect caused by the compound. In Figure 5 we showed that intraperitoneal injection of L-arginine (a precursor of nitric oxide synthase), almost completely reverted the antinociceptive action caused by injection of L-NOARG (75mg kg^{-1}, i.p., 30 min before), as well as the antinociceptive effect of NI. We also evaluated the involvement of the dopaminergic system and Figure 6 shows that treatment of mice with haloperidol (a non-selective antagonist of dopaminergic receptors), under conditions in which this drug almost completely reverted the antinociception caused by apomorphine (a potent dopaminergic agonist drug), reverted the antinociceptive effect caused by NI.

Table 1 Inhibition by NI on capsaicin and glutamate-induced pain in mice

Treatment	Route	ID_{50} (mg kg^{-1})	Inhibition (%)
Capsaicin test			
NI	i.p.	87.3 (42.4–109.7)[a]	55.81 ± 4
Diclofenac[b]	i.p.	47.4 (34.5–65.4)	72.00 ± 7
Glutamate test			
NI	i.p.	7.9 (3.4–18.5)[a]	81.69 ± 4

[a]95% confidence limits. [b]Data from Santos (2005).

Discussion

We have reported that niga-ichigoside F_1 (NI) obtained from *Rubus imperialis* inhibited, in a dose-dependent manner, the nociception induced by acetic acid. When compared with well-known NSAIDs, aspirin and paracetamol, it was about 28-fold more potent (Niero et al 1999). We selected the formalin test as an experimental model for studying the mechanism of the antinociceptive action of NI. It has been demonstrated that intraplantar injection of formalin in rodents produces significant increases in spinal levels of different mediators, such as excitatory amino acids, PGE_2, nitric oxide and tachykinin among other peptides (Tjølsen et al 1992; Santos et al 2005). Furthermore, systemic spinal and supraspinal administration of tachykinin receptor antagonists, NOS inhibitors, NMDA receptor antagonists, opioids, α_2-adrenoceptor agonist and NSAIDs, were all found to be effective in antagonizing formalin-induced nociception (Santos & Calixto 1997; Sawamura et al 1999). The evaluation of the opioid system showed that the antinociceptive effect caused by NI might not be through the activation this system because these results are not in accordance with those of Choi et al (2003). We showed that in the hot-plate test NI increased the latency to jumping response at 10 and 30 mg kg^{-1} treatment without affecting the mouse's ability to detect pain threshold (licking response) of a thermal origin. Morphine (10 mg kg^{-1}) also exerted a significant effect in this response. Thus, the authors suggested that this compound may exhibit central and peripheral antinociceptive properties.

Anti-migraine drugs, such as sumatripan, were antagonized by the muscarinic antagonist atropine and the acetylcholine-depletor hemicolinium, in several animal models (Ghelardini et al 1996). Our results showed that atropine, given systemically, reverted the analgesic effect caused by NI only in the second phase of the dolorous process, thus suggesting that the antinociception depended on the cholinergic system. Umeda et al (1997) demonstrated that nonsynaptic release of noradrenaline from rat spinal cord slices is modulated via presynaptic α_2-adrenergic auto receptors. It was observed that the α_2-adrenoreceptor antagonist clonidine inhibited this pathway, whereas yohimbine (a non subtype

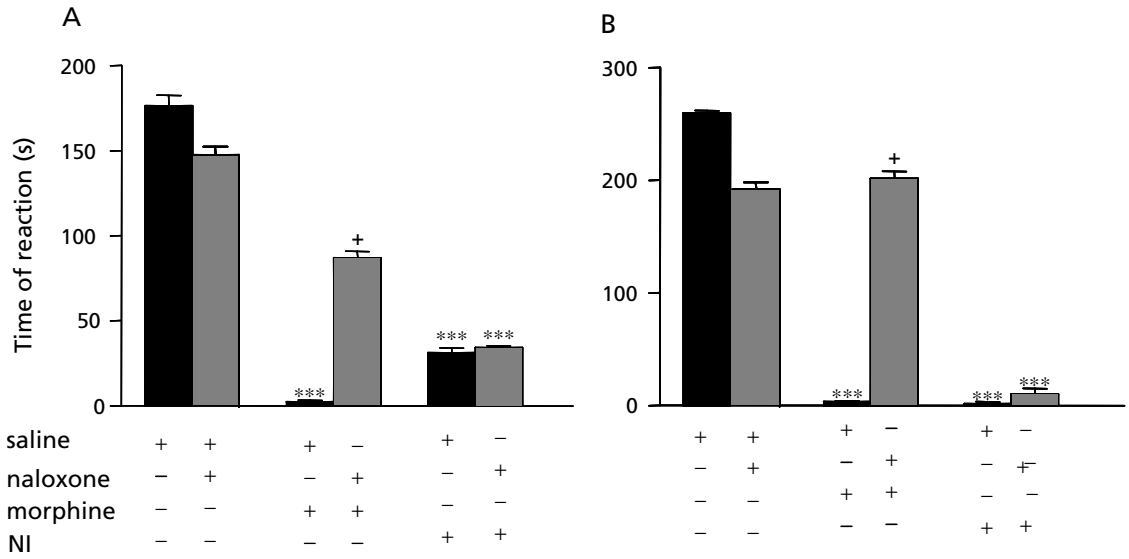

Figure 2 Effect of pre-treatment with naloxone 1 mg kg^{-1}, intraperitoneally (grey column), on the antinociceptive action caused by morphine (5 mg kg^{-1}, s.c.) and NI (60 mg kg^{-1}, i.p.) on formalin-induced nociception in mice. The black columns represent the control (mice treated with saline). A. The first phase (0–5 min). B. The second phase (15–30 min). ***$P < 0.001$, compared with the control; $^{+}P < 0.05$, compared with naloxone plus agonists (compound or morphine).

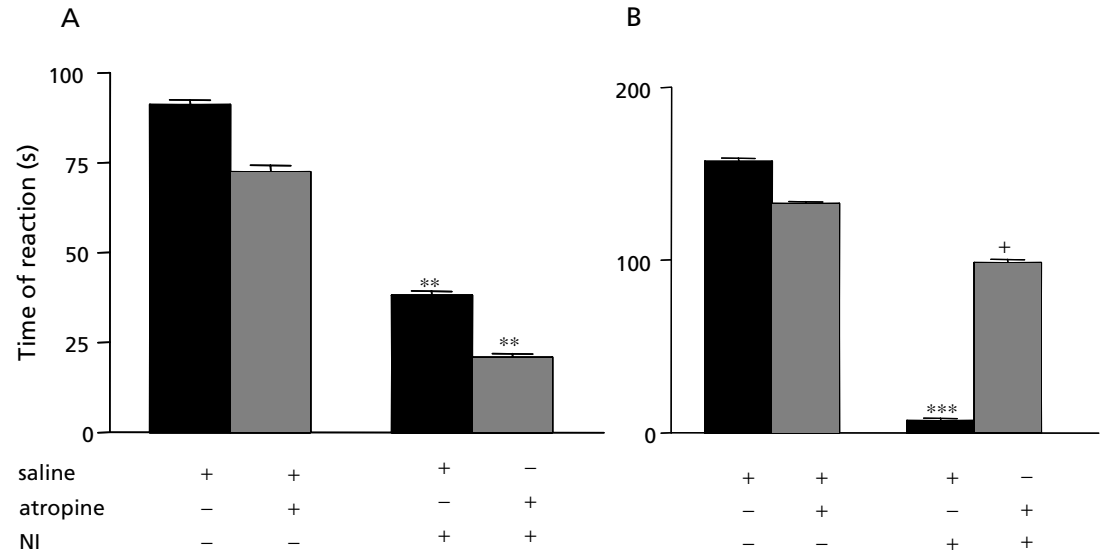

Figure 3 Effect of pre-treatment of mice with atropine 60 mg kg^{-1}, intraperitoneally (grey column), on the antinociceptive action by NI 60 mg kg^{-1}, intraperitoneally, on formalin-induced nociception in mice. The black columns represent the control (mice treated with saline). A. The first phase (0–5 min). B. The second phase (15–30 min). **$P < 0.01$; ***$P < 0.001$, compared with the control; $^{+}P < 0.05$, compared with atropine plus agonists (compound).

selective α_2 antagonist) enhanced the release of noradrenaline in response to neuronal stimulation (Vizi et al 1986). It is possible that the α_2-adrenoceptor agonist exerts antinociceptive effects, at least partially, through a presynaptic modulation of primary afferent fibres that convey the nociceptive messages to the spinal cord (Guyenet et al 1994). In this study, yohimbine failed to reverse the antinociception of NI, suggesting that it is not related to the action of α_2-adrenergic receptors. Moreover, prior treatment with the antagonist increased the potency of NI.

It has been suggested that L-arginine–nitric oxide plays an important role in the modulation of nociception (Ferreira et al 1999). The results obtained confirm these observations and demonstrate that the systemic administration of L-N^{w}-nitro-arginine (L-NOARG, nitric oxide synthase inhibitor) antagonized, in a pronounced manner, both the first and second phases of formalin-induced pain, this effect being selectively reversed by the injection of L-arginine (precursor to nitric oxide). Our results also demonstrate that L-arginine–nitric oxide appears to be involved in the antinociceptive action of NI.

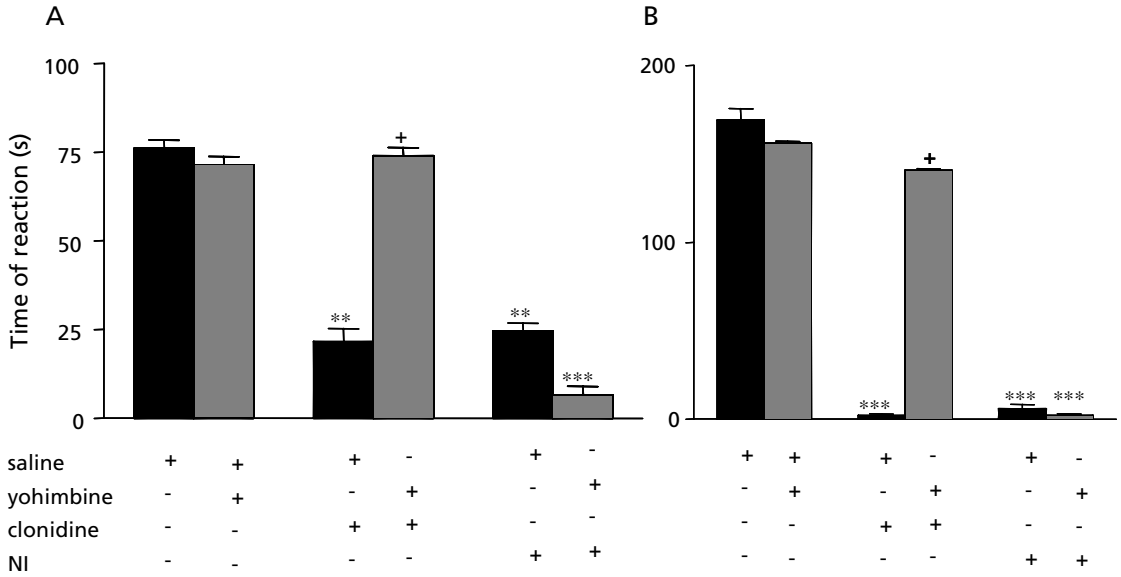

Figure 4 Effect of pre-treatment of mice with yohimbine 0.15 mg kg^{-1}, intraperitoneally (grey column), on the antinociceptive action by clonidine (0.1 mg kg^{-1}, i.p.) and NI 60 mg kg^{-1}, intraperitoneally, on formalin-induced nociception in mice. The black columns represent the control (mice treated with saline). A. The first phase (0–5 min). B. The second phase (15–30 min). $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control; $^{+}P < 0.05$, compared with yohimbine plus agonists (compound or clonidine).

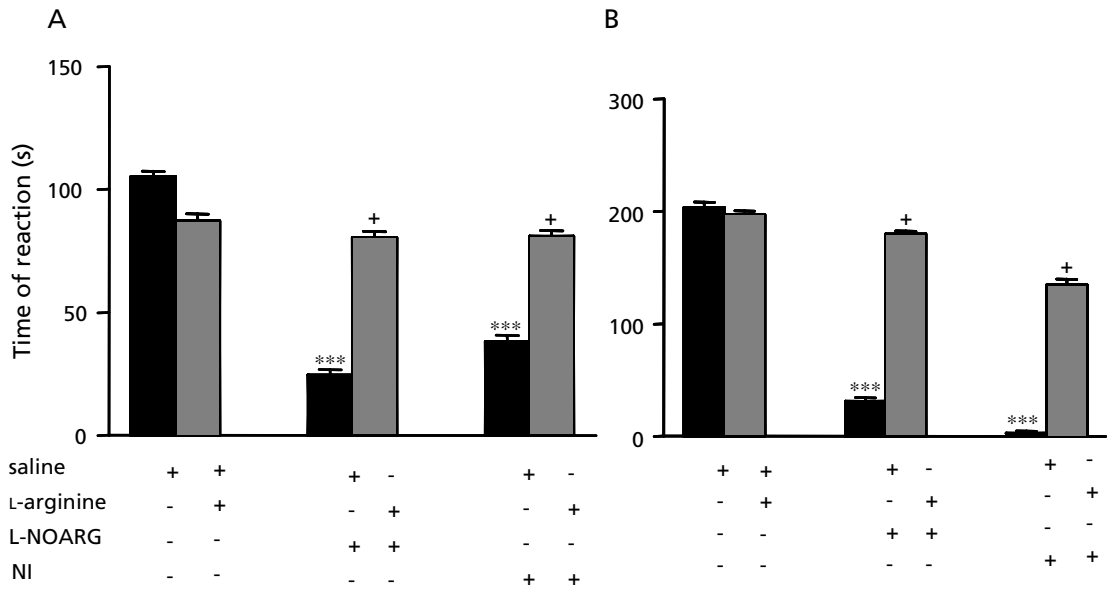

Figure 5 Effect of pre-treatment of mice with L-arginine 600 mg kg–$^{-1}$, intraperitoneally (grey column), on the antinociceptive action by L-NOARG 75 mg kg^{-1}, intraperitoneally, and NI (60 mg kg^{-1}, i.p.) on formalin-induced nociception in mice. The black columns represent the control (mice treated with saline). A. The first phase (0–5 min). B. The second phase (15–30 min). $^{***}P < 0.001$, compared with the control; $^{+}P < 0.05$, compared with L-arginine plus agonists (compound or L-NOARG).

Little is known about the pathophysiological modulation of spinal dopaminergic transmission, although it is known that both inputs and sustained acute noxious stimuli accelerate dopamine turnover in the dorsal horn, suggesting an enhancement in the activity of descending dopaminergic pathways. Dopaminergic mechanisms may play a part in the accompanying antinociception (Millan 1999). In the same way, haloperidol also reversed the antinociception showing that the dopaminergic system appears to be involved with the action mechanism of NI. Sakurada (1993) proposed the capsaicin-induced pain model for the study of compounds that act on pain of neurogenic origin. More recently studies have shown that capsaicin evokes the release of neuropeptides, excitatory amino acids (glutamate and aspartate), nitric oxide

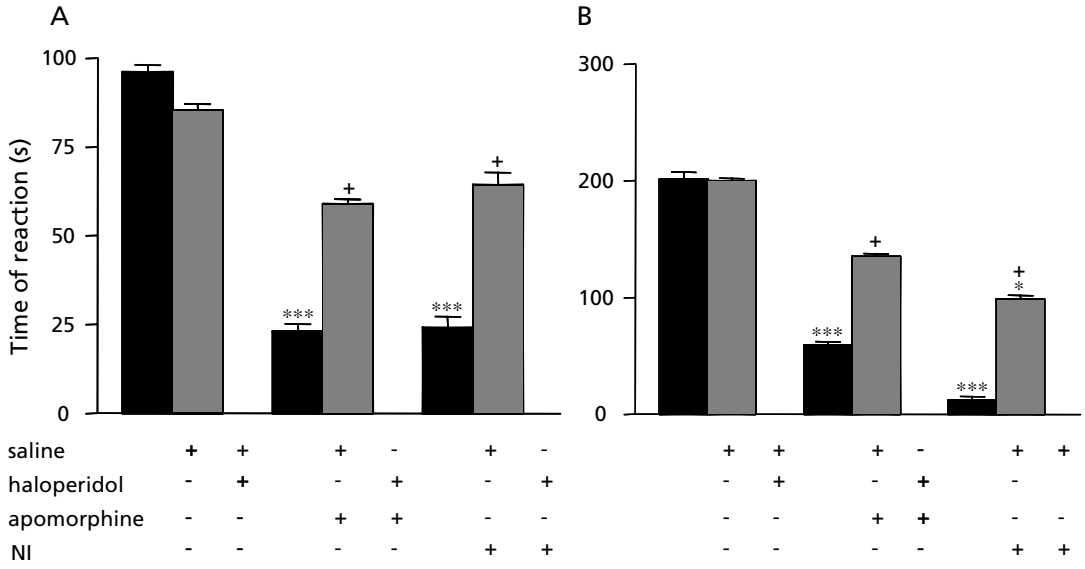

Figure 6 Effect of pre-treatment of mice with haloperidol 0.2 mg kg^{-1}, intraperitoneally (grey column), on the antinociceptive action by apomorphine (1 mg kg^{-1}, i.p.) and NI (60 mg kg^{-1}, i.p.) on formalin-induced nociception in mice. The black columns represent the control (mice treated with saline). A. The first phase (0–5 min). B. The second phase (15–30 min). $^{*}P < 0.05$, compared with the control; $^{+}P < 0.05$, compared with haloperidol plus agonists (compound or apomorphine).

and pro-inflammatory mediators in the periphery, and transmits nociceptive information to the spinal cord (Sakurada et al 2003). Our results shown that NI caused significant effects when administered by the intraperitoneal pathway in different doses. This is another interesting finding because the capsaicin-induced neurogenic paw-licking response was similar to the first phase of the formalin test. Compounds with this action are promising candidates for treatment of neuropathic pain, for which efficacy is difficult (Santos et al 2005).

Finally, we also investigated the glutamatergic system. Several glutamatergic receptors, such $NMDA_R$ and metabotropic glutamate receptors (mGluRs), are known to be involved in the modulation of formalin-induced nociception (Hizue et al 2005). Choi et al (2001) demonstrate that aspirin and paracetamol reduced nociceptive behaviour induced by glutamate administered intrathecally, suggesting that the antinociceptive effect of these drugs occurs not only through selective inhibition of prostaglandin synthetase. In fact, the intraplantar injection of glutamate induced direct stimulation of the nociceptive neurons, causing the liberation of various inflammatory and neuropeptide mediators involved in the transmission of pain (Yashpal et al 2001). This work verified, therefore, that NI could be inhibiting the liberation of inflammatory and neuropeptide mediators involved in the pain process or could be blocking the glutamate receptors.

Conclusion

In summary, the results of this study provide convincing evidence that niga-ichigoside F_1 (NI) exerts a rapid onset, relatively long-lasting and pronounced systemic antinociception against formalin, glutamate and capsaicin models of pain in mice. They also confirm and extend previous investigations carried out in our laboratories relating to the antinociceptive action of NI on acetic acid-induced visceral nociceptive response. Concerning the mechanism of action of NI, the results show that it seems to be related to different systems, such as dopaminergic, L-arginine–nitric-oxide, tachykininergic and glutamatergic, whose pharmacological effects might be mediated centrally by a non-specific mechanism. These findings are of interest because they support, at least partly, the notion that NI is the active principle present in *R. imperialis* and is useful in the development of new analgesic drugs for the management of several types of pain.

References

Beirith, A., Santos, A. R. S, Calixto, J. B. (2002) Mechanism underlying the nociception and paw edema caused by injection of glutamate into the mouse paw. *Brain Res.* **924**: 219–228

Choi, S. S., Lee, J. K., Suh, H. W. (2001) Antinociceptive profiles of aspirin and acetaminophen in formalin, substance P and glutamate pain models. *Brain Res.* **7**: 233–239

Choi, J., Lee, K. T., Ha, J., Yun, S. Y., Ko, C. D., Jung, H. J., Park, H. J. (2003) Antinociceptive and antiinflammatory effects of Niga-ichigoside F1 and 23-hydroxytormentic acid obtained from *Rubus coreanus. Biol. Pharm. Bull.* **26**: 1436–1441

Cirilo, V. K. *Manual de plantas medicinais*. 44. ed. Curitiba: Assessoar, 1993

Durham, D. G., Liu, X., Richards, R. M. (1994) A triterpene from *Rubus pinfaensis. Phytochemistry* **36**: 1469–1472

Emendorfer, F., Emendorfer, F., Bellato, F., Noldin, V. F., Niero, R., Cechinel-Filho, V., Cardozo, A. M. (2005) Evaluation of the relaxant action of some Brazilian medicinal plants in isolated

guinea-pig ileum and rat duodenum. *J. Pharm. Pharmacol*. **1**: 63–68

Ferreira, J., Santos, A. R. S., Calixto, J. B. (1999) The role of systemic, spinal and supraspinal L-arginine-nitric oxide-cGMP pathway in thermal hyperalgesia caused by intrathecal injection of glutamate in mice. *Neuropharmacol*ogy **38**: 835–842

Ghelardini, C., Galeotti, N., Figini, M., Imperato, A., Nicolodi, M., Sicuteri, F., Gessa, G. L., Bartolin, A. (1996) The central cholinergic system has a role in the antinociception induced in rodents and guinea pigs by the antimigraine drug sumatriptan. *J. Pharm. Exp. Ther*. **279**: 884–890

Guyenet, P. G., Stornetta, R. L., Riley, T., Norton, F. R., Rosin, D. L., Lynch, K. R. (1994) Alpha 2A-Adrenergic receptors are present in lower brainsten catecholaminergic and serotonergic neurons innervating spinal cord. *Brain Res*. **646**: 356–357

Hizue, M., Pang, C. H., Yokoyama, M. (2005) Involvement of N-methyl-D-aspartate-type glutamate receptor epsilon1 and epsilon4 subunits in tonic inflammatory pain and neuropathic pain. *Neuroreport* **17**: 1667–1670

Hunskaar, S., Hole, K. (1987) The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* **30**: 103–114

Kanegusuku, M., Benassi, J. C., Pedrosa, R. C., Yunes, R., Cechinel Filho, V., Maia, A. A., Souza, M. M., Delle Monache, F., Niero, R. (2002) Citotoxic, hypoglycemic activity and phytochemical analyses of *Rubus imperialis* (Rosaceae). *Z. Naturforsch*. **57**: 272–276

Lemus, I. (1999) Hypoglycemic activity of four plants used in Chilean popular medicine. *Phytother. Res*. **13**: 91–94

Millan, M. J. (1999) The induction of pain: an integrative review. *Prog. Neurobiol*. **57**: 1–164

Niero, R., Cechinel Filho, V., Souza, M. M., Montanari, J. L., Yunes, R. A., Delle Monache, F. (1999) Antinociceptive activity of niga-ichigoside F_1 from *Rubus imperialis. J. Nat. Prod*. **62**: 1145–1146

Novaes, A. P., Rossi, C., Poffo, C., Pretti-Júnior, E., Oliveira, A. E., Schlemper,V., Niero, R., Cechinel Filho, V., Bürger, C. (2001) Preliminary evaluation of the hypoglycemic effect of some Brazilian medicinal plants. *Thérapie* **56**: 427–430

Patel, A. V., Rojas-Vera, J., Dacke, C. G, (2004) Therapeutic constituents and actions of *Rubus* species. *Curr. Med. Chem*. **11**: 1501–1512

Sakurada, T. (1992) The capsaicin test in mice for evaluating tachykinin antagonist in the spinal cord. *Neuropharmacol*ogy **31**:1279–1285

Sakurada, T. (1993) Antinociception induced by CP 96345, a non-peptide NK-1 receptor antagonist, in the formalin and capsaicin tests. *Neurosci. Lett*. **151**: 142–145

Sakurada, T., Matsumura, T., Moryama, T, Sakurada, C., Ueno, S. (2003) Differential effects of intraplantar capsazepine and ruthenium red on capsaicin-induced desensitization in mice. *Pharmacol. Biochem. Behav*. **75**: 115–121

Santos, A. R. S., Calixto, J. B. (1997) Further evidence for the involvement of tachykinin receptor subtypes in formalin test and capsaicin models of pain in mice. *Neuropeptides* **31**: 381–389

Santos, A. R., Gadotti, V. M., Oliveira, G. L., Tibola, D., Paszcuk, A. F., Neto, A., Spindola, H. M. De- Souza, M. M., Rodrigues, A. L. Calixto, J. B. (2005) Mechanisms involved in the antinociception caused by agmatine in mice. *Neuropharmacology* **48**: 1021–1034

Sawamura, S., Fujinaga, M., Kingery, W. S., Belanger, N., Davies, M. F., Maze, M. (1999) Opioidergic and adrenergic modulation of formalin evoked spinal c-fos mRNA expression and nocifensive behavior in the rat. *Eur. J. Pharmacol*. **379**: 141–149

Tjølsen, A., Berge, O. G., Hunnskaar, S., Rosland, J. H., Hole, K., (1992) The formalin test: an evaluation of the method. *Pain* **51**: 5–17

Umeda, E., Satoh, T., Nagashima, H., Potter, P. A., Tarkovacs, G., Vizi, E. S. (1997) α_{2A}-subtype of presynaptic α_2-adrenoreceptors modulates the release of [^{3}H]-noradrenalin from rat spinal cord. *Brain Res. Bull*. **42**: 129–132

Vizi, E. S, Hársing, J., Gaal, J., Kapocsi, J., Bernath, S., Somogyi, G. T. (1986) Ch-38083; a selective potent antagonist of α_2 adrenoceptors. *J. Pharmacol. Exp. Ther*. **238**: 701–706

Yashpal, K., Fisher, K., Chabot, J. G. (2001) Differential effects of NMDA and group I mGluR antagonists on both nociception and spinal cord protein kinase C translocation in the formalin test and a model of neuropathic pain in rats. *Pain* **94**: 17–29

Zimmermann, M. (1983) Ethical guidelines for investigations of experimental pain in conscious animals. *Pain*. **16**: 109–110