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Neuroscience Letters 368 (2004) 116–120
Neuroscience Letters
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Spinal cholinergic involvement after treatment with aspirin and paracetamol in rats

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Received 7 June 2004; received in revised form 28 June 2004; accepted 29 June 2004

Abstract

Aspirin and paracetamol have been shown to suppress non-inflammatory pain conditions like thermal, visceral and mechanical pain in mice and rats. The non-inflammatory antinociception appears to be mediated by central receptor mechanisms, such as the cholinergic system. In this study, we tested the hypothesis that the non-inflammatory antinociception of aspirin and paracetamol could be mediated by an increase of intraspinal acetylcholine release. Microdialysis probes were placed intraspinally in anesthetized rats for acetylcholine sampling. Subcutaneously administered aspirin 100 and 300 mg/kg increased, while paracetamol 300 mg/kg decreased intraspinal acetylcholine release. Intraspinal drug administration did not affect acetylcholine release. Our results suggest that an increased intraspinal acetylcholine release could be involved in part of the non-inflammatory pain suppression by aspirin, but not by paracetamol.

Classification terms: Sensory systems; Pain modulation; Pharmacology

Keywords: Aspirin; Paracetamol; Acetylcholine; Pain; Spinal cord; Microdialysis

Aspirin (acetylsalicylic acid) is a well known non steroid anti-inflammatory drug (NSAID) with analgesic and antipyretic effects, acting mainly via an inhibition of cyclooxygenases (COX), resulting in a reduced production of prostaglandins at the site of inflammation [24]. Paracetamol (acetaminophen) is also an analgesic drug with antipyretic effects, but with less pronounced anti-inflammatory effects. The precise mechanisms of action of paracetamol are still not fully understood, although it has been known for more than a century [8]. Little evidence for peripheral mechanisms of paracetamol has been found, and it has therefore been suggested that it might act by inhibition of COX in the central nervous system as well as inhibition of central nitric oxide synthase [6].

Although aspirin and paracetamol mainly are used as oral analgesics against inflammatory pain, several studies has implied a role for these drugs also in acute non-inflammatory pain conditions in rats and mice, as seen in tail-flick and hot-plate tests (thermal pain), writhing test (visceral pain) and colorectal distension test (mechanical pain) [5,16]. What mechanisms that contribute to the non-inflammatory antinociception produced by NSAIDs and paracetamol are far from fully understood. However, it has been suggested that these drugs might affect central antinociceptive receptor mechanisms such as the adrenergic [19], serotonergic [9], opioid [21] and cholinergic [17,20] systems.

The cholinergic receptor system has been suggested as an important compound in antinociceptive mechanisms, since systemic as well intrathecal administration of muscarinic and nicotinic agonists produce antinociception in several species [10,12,15,23]. The muscarinic antinociception produced at the spinal level appears to be related to an increased release of acetylcholine, whereas a decrease in acetylcholine results in a decrease of pain threshold [2]. Several other studies have demonstrated changes in spinal acetylcholine release after administration of nicotinic and adrenergic antinociceptive

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doi:10.1016/j.neulet.2004.06.070

agents [3,13,14], as well as after systemic lidocaine treatment [1], which further strengthens the hypothesis that acetylcholine is involved in spinal antinociception.

It has been shown that atropine is able to reverse antinociception produced by several NSAIDs including paracetamol in the tail-flick test in mice [20]. This antinociception was suggested to be mediated via an increased release of spinal acetylcholine. This is a reasonable thought, considering the role of acetylcholine in antinociception as mentioned above. However, this has never been shown. Therefore we designed this study to test the hypothesis that systemically and intraspinally administered aspirin and paracetamol produce antinociception in non-inflammatory pain conditions in the same manner as cholinergic agonists, by increasing the intraspinal release of acetylcholine.

All experiments were conducted after approval of the Animal Ethics Committee in Uppsala, Sweden. Forty-one male outbred Sprague–Dawley rats (B&K Universal, Sollentuna, Sweden), weighing approximately 330–450 g, were used in all experiments. The animals were housed in groups of 4–5 rats in Makrolon® size IV cages (59 cm × 38 cm × 20 cm). They had free access to food (R36, Ewos, Vadstena, Sweden) and tap water at all times. The animals were kept in rooms with a temperature of 20 ± 2 °C and a relative humidity of 50%. The air was changed approximately 15 times per hour. Diurnal rhythm was regulated with a 12-h light:12-h dark cycle with lights on 6.00 a.m.–6.00 p.m. The animals were acclimatised after delivery for one week before they were used.

The drugs aspirin (acetylsalicylic acid), paracetamol (acetaminophen), neostigmine bromide, acetylcholine chloride and choline chloride were purchased from Sigma-Aldrich Sweden AB, Stockholm, Sweden. Isoflurane was purchased from Abbot Scandinavia, Kista, Sweden. The salts NaCl, $CaCl_2$, KCl, and Na_2HPO_4 were purchased from VWR International AB, Stockholm, Sweden. Spinal microdialysis probes were purchased from Marsil Enterprises, San Diego, CA, USA.

For each experiment, anesthesia was induced with 5% isoflurane in 100% oxygen. The rat was intubated and connected to a Harvard® ventilator (Harvard Apparatus Inc., South Natic, MA, USA) and placed on a heated pad to maintain core body temperature at 37.5 °C. During surgery, isoflurane was kept at about 2.7% and during microdialysis sampling, isoflurane was maintained at 1.3%. The end-tidal pCO_2 was kept at 4.0 kPa.

For insertion of the microdialysis probe, a midline incision was made at the back of the skull. The neck muscles were dissected to expose the *cisterna magna*. The *dura* and *pia mater* were cut and a spinal microdialysis probe was inserted in the dorsal part of the spinal cord so that the tip was approximately at the C5 level, as described in previous reports [1–3,11]. The dialysis membrane was constructed by a hollow fiber with an outer diameter of 300 μm, and with a cut-off at a molecular weight of 11 kDa. The membrane was bent to form a U-shaped loop with a length of 12 mm. The probe was perfused (2.5 μl/min) with Ringer's solution (147 mM NaCl, 2.4 mM dihydrous $CaCl_2$, 4.0 mM KCl), containing 10 μM of the acetylcholine esterase inhibitor neostigmine. The presence of neostigmine is necessary to prevent degradation of acetylcholine, and thereby make detection of the transmitter possible [4,11,22]. After insertion of the microdialysis probe, the rats rested for 40 min before spinal microdialysis sampling.

Acetylcholine (nM/10 min dialysis sample) was quantified on-line by high performance liquid chromatography (HPLC) with electrochemical detection (Antech, Leyden, The Netherlands). The mobile phase was 50 mM Na_2HPO_4 (pH 9.0), which enabled detection of acetylcholine at 4.25 min and choline after 5.5 min. The dialysis probe recovery of acetylcholine was determined in vitro both before and after each experiment to ensure that all microdialysis measures accurately reflected the spinal acetylcholine release and were not confounded by intra-experimental probe damage. A standard calibration curve ranging from 50 nM to 1 μM acetylcholine was established before each experiment from two samples of each concentration. Five intraspinal sampling periods were used to calculate baseline release of acetylcholine from which the percent change of release of acetylcholine was calculated.

Aspirin and paracetamol were dissolved in saline heated to approximately 50–55 °C under rapid mixture. Once dissolved, the temperature of the solutions was allowed to decrease to body temperature, and drugs were injected subcutaneously in doses of 30, 100 and 300 mg/kg in volumes of 10 ml/kg. The doses were based on studies by Choi et al. [7], that investigated antinociceptive profiles of aspirin and paracetamol in several pain models, and by Pinardi et al. [20]. The injections were performed so that the expected effects could be seen on the first sampling period after baseline release. Control animals went through the same surgical procedure as the treated animals, but were injected with 10 ml/kg saline or hydrochloric acid (pH 2.5). The latter served as control against any possible effects of the acidosis evoked by treatment with 300 mg/kg aspirin. The control solutions were heated and injected at body temperature as described above. The effects of the drugs on intraspinal acetylcholine release were studied during 10 sampling periods (100 min) after injection. No animal was treated with more than one injection of a drug.

The effect of aspirin and paracetamol was also studied after intraspinal administration. In these cases, drugs were dissolved in Ringer's solution in concentrations of 10 μM, 100 μM and 1 mM, and administered directly into the spinal cord via the microdialysis probe. Six animals were used for these experiments, three for aspirin and three for paracetamol. Each animal was used for analysis of all three concentrations of each drug. No hydrochloric acid control was performed, since neither of the drugs affected intraspinal acetylcholine release.

The intraspinal microdialysis data was analyzed using analysis of variance and general linear model (SPSS version

11.5). Tukey's post hoc test was used to determine statistical differences at each time point between groups of animals treated with aspirin and control animals. General linear model with repeated measures was used to determine statistical differences between groups of animals treated with paracetamol and control animals. P values <0.05 were considered significant.

The in vitro probe recovery averaged 28%. Pre- and postexperimental probe recoveries were compared by t-test. For all data presented, there were no statistically significant changes in probe recovery in any single experiment. The average basal release of acetylcholine for all experiments was 127 ± 19 nM (mean ± S.E.M., number of experiments [n] = 36). Basal release for each group of animal is presented in the figure legends.

Subcutaneous administration of aspirin 100 and 300 mg/kg both resulted in an approximately 20% increase of intraspinal acetylcholine release 10 min after injection (Fig. 1). The increase was significantly different from saline control as well as from the hydrochloric acid control. Interestingly, aspirin in a dose of 100 mg/kg also produced a significant increase 60 min after injection, in comparison to hydrochloric acid control. Aspirin 30 mg/kg did not alter the release of intraspinal acetylcholine. A slight increase of end-tidal pCO_2 could be seen after injection of 300 mg/kg aspirin as well as after injection of hydrochloric acid, indicating acidosis. The change in pCO_2 was adjusted back to 4.0 kPa by increasing the respiratory rate. No changes in pCO_2 could be seen after 30 or 100 mg/kg aspirin.

Subcutaneous administration of paracetamol 300 mg/kg significantly reduced the intraspinal acetylcholine release, compared to saline control. The reduction lasted throughout the whole experiment, i.e. during 100 min, and reached a maximal effect at about −25% (Fig. 2). Paracetamol 30 and 100 mg/kg did not alter the release of intraspinal acetylcholine.

The average body temperature at the time for injection was 37.4 ± 0.04 °C (mean ± S.E.M., n = 36). No significant changes in body temperature after injection, compared to control, were detected.

Beside the above described experiments, the effects of 10 μM, 100 μM and 1 mM intraspinally administered aspirin and paracetamol respectively, were studied. Each concentration of a drug was studied three times, and no changes in intraspinal acetylcholine release were observed.

The present study demonstrates that subcutaneous aspirin in doses of 100 and 300 mg/kg produces a small but significant increase of intraspinal acetylcholine 10 min after administration, whereas subcutaneous paracetamol in a dose of 300 mg/kg significantly decreases acetylcholine release for at least 100 min after injection, compared to control injections. Intraspinal administration of aspirin and paracetamol had no effect on intraspinal acetylcholine release. The increase produced by aspirin indicates that part of the antinociception produced by this drug to some extent could be mediated via the spinal cholinergic receptor system. A previous study has demonstrated that an intraspinal acetylcholine increase of approximately 25% is sufficient to produce analgesia, as seen after intravenous treatment with oxotremorine [2]. However, since aspirin was incapable of affecting the acetylcholine release after intraspinal administration, it is evident that the acetylcholine increasing effect after systemic treatment is not mediated by an action at cholinergic receptors at the spinal level, which is the case with oxotremorine. Thus, the effect after systemic aspirin is probably due to an action at the supraspinal level, affecting descending pathways which in turn stimulates acetylcholine release in the spinal cord. However, since the ACh increasing effect is very

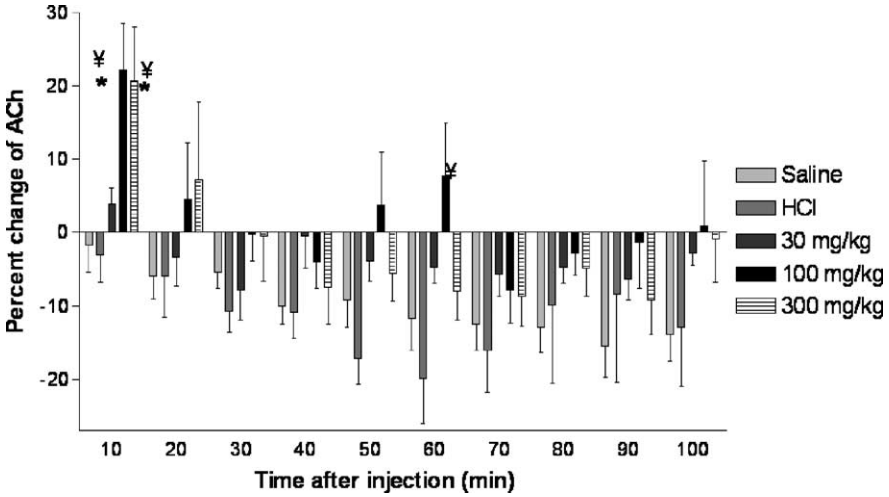

Fig. 1. Effects of subcutaneously administered saline (number of experiments [n] = 6), hydrochloric acid pH 2.5 (n = 4), 30 mg/kg (n = 4), 100 mg/kg (n = 4) and 300 mg/kg (n = 5) aspirin on intraspinal release of acetylcholine. The effects are expressed as percent change from baseline. Basal release of acetylcholine was 120 ± 47 nM, 196 ± 71 nM, 186 ± 109 nM, 134 ± 57 nM and 136 ± 44 nM (mean ± S.E.M.), respectively. $^{*}P < 0.05$ vs. saline control at each time point as determined with Tukey's post hoc test ($F_{(2,12)} = 5.82$). $^{¥}P < 0.05$ vs. hydrochloric acid control at each time point as determined with Tukey's post hoc test ($F_{(3,13)} \geq 4.63$).

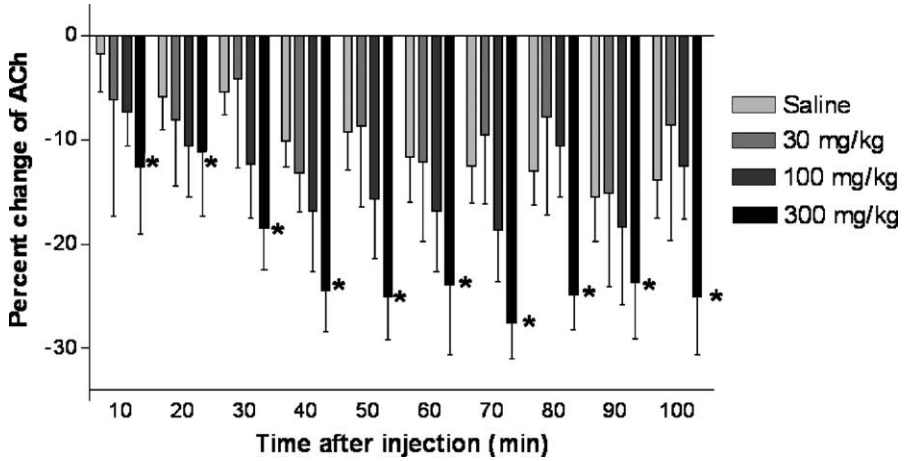

Fig. 2. The effects of subcutaneously administered saline (n = 6), 30 mg/kg (n = 4), 100 mg/kg (n = 4) and 300 mg/kg (n = 5) paracetamol on intraspinal acetylcholine release. The effect is expressed as percent change from baseline. Basal release of acetylcholine was 120 ± 47 nM, 74 ± 26 nM, 57 ± 11 nM and 120 ± 32 nM (mean ± S.E.M.). *P < 0.05 vs. saline control as determined with general linear model with repeated measures, showing that paracetamol 300 mg/kg was significantly different from saline control 10–100 min after injection ($F_{(1)}$ = 6.33).

transient in comparison to the antinociceptive effect of aspirin, it should be mentioned that the effect of aspirin on spinal ACh release likely is only one out of several mechanisms regulating the antinociceptive effect of aspirin. To fully understand the relationship between the spinal ACh release and non-inflammatory antinociception of aspirin, further investigations are needed.

The observation 60 min after injection that the ACh release is significantly higher after aspirin 100 mg/kg compared to the hydrochloric acid control is somewhat surprising. However, the difference is rather due to that the ACh release is somewhat lower at this time point after hydrochloric acid injection than to that the ACh release increases after aspirin 100 mg/kg. In addition, since the effect was not significantly different from the saline control, one could question the significance of this observation, as aspirin 100 mg/kg did not produce any acidosis and hydrochloric acid in this case in an irrelevant control.

The intraspinal acetylcholine release decreased after systemic administration of 300 mg/kg paracetamol. Since paracetamol did not affect the spinal acetylcholine release after intraspinal administration, it is likely that supraspinal mechanisms are involved. Why paracetamol decreases the spinal acetylcholine release, and what physiological effect this might have, remains uncertain. Any how, this did not correspond to our hypothesis, neither to the suggestions made by Pinardi et al. [20]. It is therefore evident that the antinociceptive effect of paracetamol on non-inflammatory pain conditions is not mediated via an increased release of intraspinal acetylcholine, at least not in rats. According to the hypothesis that a decrease of intraspinal acetylcholine results in a decreased pain threshold [2], paracetamol 300 mg/kg would rather produce hyperalgesia, which obviously is not the case. Therefore, the antinociceptive effects after paracetamol treatment involving cholinergic mechanisms, as seen after atropine pretreatment [20], must be mediated by some system not involving spinal cholinergic receptors. Serotonergic receptors have been shown to be involved in antinociception by paracetamol in the paw pressure test [9]. Serotonergic receptors have also been shown to be involved in antinociception produced by the α_2-adrenergic antagonist yohimbine in the formalin-test. This is interesting, since yohimbine decreases the intraspinal release of acetylcholine with up to 50% [3], and efficiently blocks the antinociceptive effect of the α_2-adrenergic agonist clonidine [18]. This indicates that even though a drug has one effect on one system, it is not necessary that this effect will be the most physiologically important. Furthermore, our results do not rule out the involvement of central mechanisms of paracetamol, such as central COX inhibition or central antinociceptive receptor mechanisms. Previous studies have suggested that the adrenergic [19], serotonergic [9] and opioid [21] systems are important compounds in paracetamol antinociception. Thus, it is likely that paracetamol stimulates descending pathways and that part of its antinociceptive effect is mediated at the spinal level. However, it is clear from the present data that the antinociceptive effect of paracetamol is not produced by the same mechanisms as cholinergic agonists.

In conclusion, systemic aspirin in doses of 100 and 300 mg/kg increases the intraspinal acetylcholine release, while paracetamol 300 mg/kg decreases the same. These effects are suggested to be mediated by supraspinal mechanisms rather than by an action on spinal cholinergic receptors, since intraspinal administration of the drugs did not affect spinal acetylcholine release. The data indicates that part of the antinociceptive effects of aspirin in non-inflammatory pain conditions could be mediated by an increase in intraspinal acetylcholine release. On the other hand, non-inflammatory pain suppression by paracetamol is likely mediated by other receptor systems, overshadowing the decrease of intraspinal acetylcholine release.

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