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Short communication

Paracetamol inhibits nitric oxide synthesis in murine spinal cord slices

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Abstract

Paracetamol is an effective analgesic but its mechanism of action is unclear. We investigated the effect of paracetamol and the analgesic adjuvant caffeine on the activity of NO synthase in mouse spinal cord and cerebellar slices *in vitro*, by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline. Paracetamol (100 μM) had no effect on NO synthase activity in cerebellum, but in the spinal cord both paracetamol (100 μM) and caffeine (30 μM) attenuated glutamate (5 mM)-induced [^{3}H]citrulline production and in combination they abolished it. In conclusion paracetamol inhibits spinal cord NO synthesis and this may be related to its analgesic effects.
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Keywords: Paracetamol; Caffeine; NO; Spinal cord; Mice

1. Introduction

Paracetamol has been used as an over-the-counter analgesic for several years, although its mechanism of action is still not completely understood (Prescott, 2001; Simmons et al., 2004; Kis et al., 2005). It has been suggested (Bjorkman, 1994) that inhibition of nitric oxide (NO) release is involved in the antinociceptive effect of paracetamol. NO has been suggested to have various roles in the mediation of pain, and inhibition of its formation can have different effects dependent on the pain stimulus. Inhibition of NO synthesis has an antinociceptive effect when pain stems from a chemical or thermal insult. For example administration of the NO synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) blocks thermal hyperalgesia and the nociceptive response to formalin in rats and mice, and this effect is reversed by the administration of the NO precursor L-arginine (Kitto et al., 1992; Malmberg and Yaksh, 1993; Yamamoto and Shimoyama, 1995; Sakurada et al., 2001). Nociception induced by acetic acid in the writhing test in mice, has also been found to be inhibited by L-NAME (Duarte and Ferreira, 2000). In addition, both formalin injection and administration of the glutamate receptor agonist *N*-methyl-D-aspartate (NMDA) have been shown to increase the release of NO metabolites in the periphery, and this release was suppressed by the administration of the NO synthase inhibitor N^G-monomethyl-L-arginine or the NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid (Omote et al., 2000). It has therefore been suggested that the NO contributes to hyperalgesia by activating the spinal NO/cyclic GMP pathway and that this release of NO is mediated via the activation of the NMDA receptor in the periphery (Omote et al., 2000).

The non-selective inhibitor L^G-nitro-L-arginine and the nNOS selective inhibitor 7-nitroindazole administered intraperitoneally both potentiated the antinociceptive action of paracetamol in rats subjected to the paw pressure, whereas the iNOS inhibitor L-N^6 (1-iminoethyl)lysine had no effect (Bujalska and Gumulka, 2001). Central administration of L-NAME also potentiated the antinociceptive effect of paracetamol and it was concluded that nNOS may be involved in both the peripheral and the central action of paracetamol (Bujalska, 2003). The inhibition by paracetamol of NMDA or substance P-induced hyperalgesia was attenuated by the administration of the NO synthase substrate L-arginine, indicating that paracetamol may inhibit the NMDA-NO pathway (Bjorkman, 1994).

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These findings suggest that the analgesic action of paracetamol could be partially mediated through the inhibition of NO synthesis in the spinal cord. The aim of this study was therefore to determine directly whether paracetamol inhibited NOS in the spinal cord. We have shown antinociceptive effects of paracetamol in both spinal and supraspinal pain tests in the mouse (Godfrey et al., 2006) and also binding of [^{3}H] paracetamol to mouse spinal cord (Godfrey et al., 2005), so we chose the mouse as our experimental model for these studies. We also looked at the effect on NO synthesis of caffeine alone and in combination with paracetamol, because of the widespread use of paracetamol/caffeine combinations as over-the-counter analgesics.

2. Materials and methods

CD1 male mice (10 day old, 6 per experiment) were killed by decapitation and the brains and spinal cords rapidly removed. Adult mice were not used in these studies because in pilot experiments we were unable to detect NOS activity in response to drug treatment. The cerebella and spinal cord were dissected free, washed in magnesium-free, ice-cold Krebs buffer (composition (mM) NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.0 and $CaCl_2$, 2.0, continuously gassed with 95% O_2/5% CO_2) and immediately cross-chopped (0.4 mm × 0.4 mm) on a McIlwain tissue chopper. Tissue slices were suspended in 250 ml buffer, and incubated at 37 °C for 15 min. The buffer was then decanted off and replaced with fresh buffer and incubated at 37 °C for a further 60 min to allow the slices to equilibrate. The slices were then allowed to settle under gravity, the buffer decanted off and fresh buffer added. This washing step was carried out twice. The slices were then allowed to settle and 50 μl of packed slices were transferred to 5 ml polypropylene tubes containing 200 μl Krebs buffer and 30 μl buffer (drug vehicle) or test substance (final concentrations: 100 μM L-NAME, 100 μM paracetamol, 30 μM caffeine or 100 μM paracetamol +30 μM caffeine, total vol. 280 μl). Tubes were gassed, capped and incubated at 37 °C for 15 min. Glutamate (5 mM) plus glycine (10 μM) or an equal vol. of buffer were then added to the relevant tubes prior to the addition of [^{3}H]L-arginine (3 μCi/ml, 20 μl) giving a final vol. of 330 μl. The tubes were then gassed and capped and incubated at 37 °C for a further 15 min.

The assay was terminated by the addition of 750 μl ice-cold Krebs buffer containing 4 mM EDTA/5 mM L-arginine, and then centrifuged (4000 ×*g* at 4 °C for 5 min). The pellet was then resuspended in 1 ml of ice-cold 1 M trichloroacetic acid, the samples were centrifuged (4000 ×*g* at 4 °C for 5 min) and the supernatant was removed. Trichloroacetic acid was extracted three times with 2 ml of water-saturated diethyl ether and then neutralized with 2 ml of 20 mM HEPES buffer (pH 6.0). The neutralized samples were applied to 2 ml columns of Dowex AG50WX-8 (Na^+ form), eluted with 2 ml of distilled water and [^{3}H]citrulline in the eluate was quantified by liquid scintillation spectroscopy.

Results were expressed as disintegrations per minute (dpm) of [^{3}H]citrulline (mean ± S.E.M). Statistical analysis was performed by one-way ANOVA (for the factor treatment), followed by Scheffe's post hoc test.

[^{3}H]Arginine (40 Ci/mmol) was purchased from Tocris Cookson Ltd (Bristol, UK). Dowex AG50WX-8 and HEPES were supplied by Biorad Laboratories Ltd (Hertfordshire, UK) and Calbiochem Merck Biosciences (Nottingham, UK) respectively. L-arginine, EDTA, L-NAME, L-glutamate, paracetamol and caffeine were obtained from Sigma Aldrich (Dorset, UK). NaOH, glucose, and glycine were obtained from VWR International (Lutterworth, UK). All other chemicals were purchased from Fisher Scientific (Loughborough, UK). All drugs were made up in magnesium-free Krebs bicarbonate buffer.

3. Results

3.1. NO synthase activity in cerebellar slices

The basal production of [^{3}H]citrulline in cerebella from 10 day old mice was 2768 ± 110 dpm (n = 5). Glutamate (5 mM) plus glycine (10 μM) significantly increased the production of [^{3}H]citrulline, which was abolished by preincubation with L-NAME (100 μM). L-NAME (100 μM) alone did not alter the basal production of [^{3}H]citrulline. Paracetamol (100 μM) did

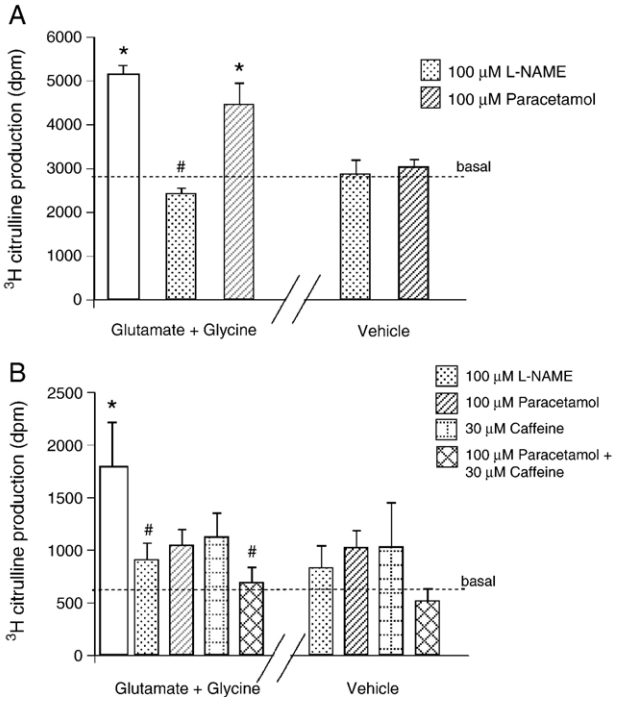

Fig. 1. The effects of paracetamol (100 μM), caffeine (30 μM) or L-NAME (100 μM) on glutamate-induced [^{3}H]citrulline production as a measure of NO synthase activity in A) cerebellum slices (n = 6) and B) spinal cord slices (n = 3–7) of 10 day old mice. Results on the left hand sides of the figures show [^{3}H] citrulline production in the presence of glutamate (5 mM) plus glycine (10 μM), while results on the right hand sides show [^{3}H]citrulline production in the presence of vehicle alone. Dotted lines show the basal production of [^{3}H] citrulline in the absence of any drugs. Data are the means ± S.E.M. * Basal vs treatment; # treatment vs glutamate plus glycine (P < 0.05, ANOVA, Scheffe's post hoc test).

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