Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 562 (2007) 68–71
www.elsevier.com/locate/ejphar

Short communication

Paracetamol inhibits nitric oxide synthesis in murine spinal cord slices

Lisa Godfrey [a], Ian Bailey [a], Nick J. Toms [b], Geoffrey D. Clarke [c], Ian Kitchen [a], Susanna M.O. Hourani [a,*]

[a] *Pharmacology Group, School of Biomedical and Molecular Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK*
[b] *Institute of Biomedical and Clinical Science, Peninsula Medical School, University of Exeter, St Lukes Campus, Heavitree Road, Exeter EX1 2LU, UK*
[c] *European Institute of Health and Medical Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK*

Received 22 May 2006; received in revised form 10 January 2007; accepted 25 January 2007
Available online 8 February 2007

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.

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).

* Corresponding author. Tel.: +44 1483 689707; fax: +44 1483 686401.
E-mail address: s.hourani@surrey.ac.uk (S.M.O. Hourani).

0014-2999/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2007.01.075

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

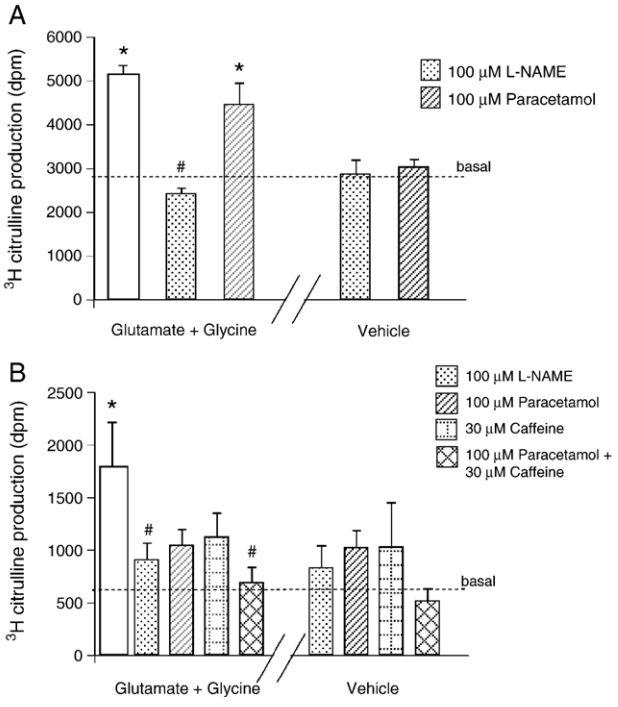

Fig. 1. The effects of paracetamol (100 μM), caffeine (30 μM) or L-NAME (100 μM) on glutamate-induced [^{3}H]citrulline production as a measure of NO synthase activity in A) cerebellum slices (n = 6) and B) spinal cord slices (n = 3–7) of 10 day old mice. Results on the left hand sides of the figures 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).

not alter the basal production of [^{3}H]citrulline and had no effect on the glutamate/glycine-induced increase (Fig. 1A).

3.2. NO synthase activity in spinal cord slices

The basal production of [^{3}H]citrulline in spinal cord slices from 10 day old mice was 625±107 dpm (n=7). L-NAME (100 μM), paracetamol (100 μM) and caffeine (30 μM) alone or in combination did not alter the basal production of [^{3}H] citrulline. Glutamate (5 mM) plus glycine (10 μM) produced a significant increase in production of [^{3}H]citrulline, which was significantly attenuated by preincubation with L-NAME (100 μM). In the presence of either paracetamol (100 μM) or caffeine (30 μM) there was no significant stimulation of [^{3}H] citrulline production by glutamate (5 mM) plus glycine (10 μM), showing an attenuation by these drugs of glutamate/glycine-induced NO synthesis. There was a further reduction in [^{3}H]citrulline production when the drugs were added together, and a significant difference between glutamate plus glycine alone and glutamate plus glycine in the presence of a combination of paracetamol (100 μM) and caffeine (30 μM) (Fig. 1B).

4. Discussion

The mechanism of NO synthesis and its inhibition has previously been studied in the cerebellum of 10 day old rat pups (Bredt and Snyder, 1989; Garthwaite et al., 1989; Toms and Roberts, 1994) as high levels of nNOS are observed in this tissue at this age. NOS activity in the cord has not been previously measured using the stoichiometric conversion of [^{3}H]arginine to [^{3}H]citrulline, so the cerebellum was also used as a brain model of NOS activity to validate the method. The concentration of paracetamol (100 μM) was chosen to reflect peak plasma concentrations following administration of 10–20 mg/kg paracetamol *in vivo*, equivalent to a dose of less that 1 g of paracetamol in man and therefore within the therapeutic range (Prescott, 2001). The concentration of caffeine (30 μM) was chosen to be within the range selective for adenosine receptor blockade and equivalent to a dose of around 7 mg/kg, a dose at the high end of those which could be achieved in man (Fredholm et al., 1996). In each case these doses are similar to those that we have previously used in *in vivo* studies in mice (10–200 mg/kg paracetamol, 10 mg/kg caffeine (Godfrey et al., 2006). While previous studies have reported that caffeine enhances the antinociceptive effect of paracetamol in mice, rats and humans (Laska et al., 1983; Granados-Soto et al., 1993; Engelhardt et al., 1997), we found instead that caffeine can inhibit the antinociceptive effects of paracetamol in mice (Godfrey et al., 2006). This discrepancy may reflect the fact that caffeine is a non-selective adenosine receptor antagonist and that blockade of adenosine A_1 and A_{2A} receptors may have opposing effects on pain pathways.

In agreement with Bredt and Snyder (1989), glutamate (5 mM) plus glycine (10 μM) produced a robust increase (85% increase relative to control) in NOS activity in cerebellar slices from 10 day old mice. Preincubation with L-NAME (100 μM) completely abolished the glutamate/glycine-induced stimulation showing that this method was sensitive enough to detect a possible reduction in NO production by paracetamol. However, paracetamol failed to influence significantly either basal or glutamate/glycine-induced NOS activity in neonatal mouse cerebellar slices. This is in agreement with a brief report by Raffa (2002) who found no evidence that paracetamol (0.01–10 mM) directly inhibited nNOS in the rat cerebellum.

Basal production of NO was significantly stimulated (185% increase relative to control) in the presence of glutamate (5 mM)/glycine (10 μM) in spinal cord slices from 10 day old mice. This stimulation was also significantly attenuated by L-NAME as in the cerebellum. In contrast to the cerebellum, paracetamol (100 μM) attenuated the glutamate/glycine-induced synthesis of NO, but had no effect on the basal release of NO. The reason for the different effect of paracetamol on NO production in the two sites is unclear. Caffeine (30 μM) also had no effect on basal production but attenuated the glutamate/glycine-induced stimulation. In the rat hippocampus adenosine A_1 receptor stimulation has been shown to inhibit both basal and NMDA-induced synthesis of NO, (Bhardwaj et al., 1995), so adenosine A_1 receptor antagonism might be expected to potentiate NO synthesis. However, in our experiments caffeine attenuated NO production, suggesting instead an action via adenosine A_2 receptor antagonism.

When paracetamol (100 μM) and caffeine (30 μM) together were preincubated with spinal cord slices they significantly inhibited the glutamate/glycine-induced stimulation to a greater extent than the paracetamol or caffeine alone. This enhancement by caffeine of the effects of paracetamol is similar to the reported enhancement by caffeine of the analgesic effects of paracetamol (Laska et al., 1983; Granados-Soto et al., 1993; Engelhardt et al., 1997), although from the results presented here it cannot be concluded that the inhibition of NO synthesis is responsible for these analgesic effects. However, our direct demonstration of the inhibition of spinal cord NO synthesis by paracetamol supports the suggestion made by Bjorkman (1994) on the basis of behavioural studies that the analgesic effect of paracetamol is partly through the spinal inhibition of hyperalgesia-induced NO release.

In conclusion, in the mouse cerebellum and spinal cord glutamate/glycine induced the generation of NO. Paracetamol and caffeine either alone or in combination did not affect the basal synthesis of NO but attenuated glutamate/glycine-induced NO synthesis in the spinal cord but not the cerebellum. It is also apparent that caffeine enhanced the inhibitory effect of paracetamol on NO production in the spinal cord.

References

Bhardwaj, A., Northington, F.J., Koehler, R.C., Stiefel, T., Hanley, D.F., Traystman, R.J., 1995. Adenosine modulates *N*-methyl-D-aspartate-stimulated hippocampal nitric oxide production in vivo. Stroke 26, 1627–1633.

Bjorkman, R., 1994. Acetaminophen blocks spinal hyperalgesia induced by NMDA and substance P. Pain 57, 259–264.

Bredt, D.S., Snyder, S.H., 1989. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc. Natl. Acad. Sci. U. S. A. 86, 9030–9033.

Bujalska, M., 2003. Effect of cyclooxygenase and NO synthase inhibitors administered centrally on antinociceptive action of acetaminophen (Part II). Pol. J. Pharmacol. 55, 1001–1011.

Bujalska, M., Gumulka, W.S., 2001. Effect of cyclooxygenase and NO synthase inhibitors on antinociceptive action of acetaminophen. Pol. J. Pharmacol. 53, 341–350.

Duarte, I.D., Ferreira, S.H., 2000. L-NAME causes antinociception by stimulation of the arginine-NO-cGMP pathway. Mediat. Inflamm. 9, 25–30.

Engelhardt, G., Mauz, A.B., Pairet, M., 1997. Role of caffeine in combined analgesic drugs from the point of view of experimental pharmacology. Arzneim.-Forsch. 47, 917–927.

Fredholm, B.B., Battig, K., Holmen, J., Nehlig, A., Zvartau, E.E., 1996. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol. Rev. 51, 83–133.

Garthwaite, J., Garthwaite, G., Palmer, R.M., Moncada, S., 1989. NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. Eur. J. Pharmacol. 172, 413–416.

Godfrey, L., Morselli, A., Bennion, P., Clarke, G.D., Hourani, S.M.O., Kitchen, I., 2005. An investigation of binding sites for paracetamol in the mouse brain and spinal cord. Eur. J. Pharmacol. 508, 99–106.

Godfrey, L., Yan, L., Clarke, G.D., Ledent, C., Kitchen, I., Hourani, S.M.O., 2006. Modulation of paracetamol antinociception by caffeine and by selective adenosine A_2 receptor antagonists in mice. Eur. J. Pharmacol. 531, 80–86.

Granados-Soto, V., Lopez-Munoz, F.J., Castaneda-Hernandez, G., Salazar, L.A., Villarreal, J.E., Flores-Murrieta, F.J., 1993. Characterization of the analgesic effect of paracetamol and caffeine combinations in the pain induced functional impairement model in the rat. J. Pharm. Pharmacol. 45, 627–631.

Kis, B., Snipes, J.A., Busija, D.W., 2005. Acetaminophen and the cyclooxygenase-3 puzzle: sorting out facts, fictions and uncertainties. J. Pharmacol. Exp. Ther. 315, 1–7.

Kitto, K.F., Haley, J.E., Wilcox, G.L., 1992. Involvement of nitric oxide in spinally mediated hyperalgesia in the mouse. Neurosci. Lett. 148, 1–5.

Laska, E.M., Sunshine, A., Zighelboim, I., Roure, C., Marrero, I., Wanderling, J., Olson, N., 1983. Effect of caffeine on acetaminophen analgesia. Clin. Pharmacol. Ther. 33, 498–509.

Malmberg, A.B., Yaksh, T.L., 1993. Spinal nitric oxide synthesis inhibition blocks NMDA-induced thermal hyperalgesia and produces antinociception in the formalin test in rats. Pain 54, 291–300.

Omote, K., Kawamata, T., Kawamata, M., Nakayama, Y., Hazama, K., Namiki, A., 2000. Activation of peripheral NMDA-nitric oxide cascade in formalin test. Anesthesiology 93, 173–178.

Prescott, L.F., 2001. Paracetamol (Acetaminophen): A Critical Bibliographic Review, 2nd edition. Taylor and Francis, London.

Raffa, R.B., 2002. Acetaminophen and metabolite: lack of effect on nitric oxide synthase (constitutive or inducible). Headache 42, 237–238.

Sakurada, C., Sugiyama, A., Nakayama, M., Yonezawa, A., Sakurada, S., Tan-No, K., Kisara, K., Sakurada, T., 2001. Antinociceptive effect of spinally injected L-NAME on the acute nociceptive response induced by low concentrations of formalin. Neurochem. Int. 38, 417–423.

Simmons, D.L., Botting, R.M., Hla, T., 2004. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol. Rev. 56, 387–437.

Toms, N.J., Roberts, P.J., 1994. NMDA receptor-mediated stimulation of rat cerebellar nitric oxide formation is modulated by cyclic AMP. Eur. J. Pharmacol. 266, 63–66.

Yamamoto, T., Shimoyama, N., 1995. Role of nitric oxide in the development of thermal hyperesthesia induced by sciatic nerve constriction injury in the rat. Anesthesiology 82, 1266–1273.