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Effects of cyclooxygenase inhibitor pretreatment on nitric oxide production, nNOS and iNOS expression in rat cerebellum

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1 The therapeutic effect of nonsteroidal anti-inflammatory drugs (NSAIDs) is thought to be due mainly to its inhibition of cyclooxygenase (COX) enzymes, but there is a growing body of research that now demonstrates a variety of NSAIDs effects on cellular signal transduction pathways other than those involving prostaglandins.

2 Nitric oxide (NO) as a free radical and an agent that gives rise to highly toxic oxidants (peroxynitrile, nitric dioxide, nitron ion), becomes a cause of neuronal damage and death in some brain lesions such as Parkinson and Alzheimer disease, and Huntington's chorea.

3 In the present study, the *in vivo* effect of three NSAIDs (lysine clonixinate (LC), indomethacine (INDO) and meloxicam (MELO)) on NO production and nitric oxide synthase expression in rat cerebellar slices was analysed. Rats were treated with (a) saline, (b) lipopolysaccharide (LPS) (5 mg kg^{-1}, i.p.), (c) saline in combination with different doses of NSAIDs and (d) LPS in combination with different doses of NSAIDs and then killed 6 h after treatment.

4 NO synthesis, evaluated by Bred and Snyder technique, was increased by LPS. This augmentation was inhibited by coadministration of the three NSAIDs assayed. None of the NSAIDs tested was able to modify control NO synthesis.

5 Expression of iNOS and neural NOS (nNOS) was detected by Western blotting in control and LPS-treated rats. LC and INDO, but not MELO, were able to inhibit the expression of these enzymes.

6 Therefore, reduction of iNOS and nNOS levels in cerebellum may explain, in part, the anti-inflammatory effect of these NSAIDs and may also have importance in the prevention of NO-mediated neuronal injury.

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Abbreviations: COX, cyclooxygenase; INDO, indomethacine; iNOS, inducible NOS; LC, lysine clonixinate; LPS, lipopolysaccharide; MELO, meloxicam; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neural NOS; NSAIDs, nonsteroidal anti-inflammatory drugs

Introduction

Recent studies have suggested that nonsteroidal anti-inflammatory drugs (NSAIDS) have a variety of actions in addition to their well-studied ability to inhibit prostaglandin synthesis via cyclooxygenases (COXs). Nitric oxide (NO) has been proven to be an intra- and extracellular mediator of cell functions (Marletta, 1994). NO produced by the constitutive isoforms of nitric oxide synthase (NOS) is a key regulator of homeostasis, whereas production of NO by the inducible NOS (iNOS) plays an important role in inflammation, host defence response and tissue repair.

An increase in iNOS activity implies an increase in NO production, and may also imply an increase in superoxide generation. The ensuing peroxynitrite generation may actually be more deleterious than NO and superoxide separately. The cellular toxic effects of iNOS-derivated NO and peroxynitrite can contribute to the detrimental effects of endotoxin on tissue functions.

It has been shown that some NSAIDs inhibited iNOS expression in rat alveolar macrophages activated with lipopolyscaccharide (LPS). Some studies (Aeberhard, 1995; Stratman, 1997) demonstrated that ibuprofen reduced iNOS activity, iNOS protein level and iNOS mRNA levels in primary cerebellar glial cells. We have shown that lysine clonixinate (LC), but not indomethacin (INDO), inhibits the expression of LPS-induced iNOS in rat lung (Franchi, 2001). The above actions of NSAIDs have significant implications, since inhibition of NOS induction may prevent increases in NO concentrations that play a role in several pathogeneses.

The present study was undertaken in order to investigate the *in vivo* effects of different doses of LC, INDO and meloxicam (MELO) on NOS activity and iNOS and nNOS expression in control and LPS-treated rat cerebellum.

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Methods

Adult male Wistar rats weighing 200–300 mg were used. Each experiment was done twice and we used eight animals for each treatment. Rats were injected with saline (0.4 ml kg^{-1}, control group), different doses of NSAIDs (i.p.), LPS (5 mg kg^{-1}, i.p.), LPS in combination with different doses of NSAIDs (i.p.) and killed (6 h) after treatment. Cerebella were removed for NO production measurement or Western blot analysis.

The experimental procedures reported here were approved by the Animal Care Committee of the Center of Experimental Pharmacology and Botany of the National Research Council (CEFYBO-CONICET) and carried out in accord with the Declaration of Helsinki.

Conversion of L-*(14C)-arginine to* L-*(14C)-citrulline*

NOS activity was measured following the conversion of L-(^{14}C)-arginine into L-(^{14}C)-citrulline release according to Bredt and Snyder (1989). Cerebella were weighed, homogenized (Ultra Turrax, T25 basic, IKA Labortechnik) in HEPES (HEPES 20 mM, L-valine 25 mM, $CaCl_2$ 0.45 mM, DTT 100 mM, pH 7.4); then homogenates were incubated at 37°C in HEPES buffer containing [^{14}C] L-arginine 0.3 μCi ml^{-1} and NADPH 0.5 mM. After 15 min of incubation, samples were centrifuged for 10 min at 3000 × *g* and then applied to a 1 ml DOWEX AG50W-X8 column (Na + form) and L-(^{14}C)-citrulline was eluted in 3 ml of water. The radioactivity was measured by liquid scintillation counting.

NOS activity was determined from the difference between L-(^{14}C)-citrulline produced from the control samples and samples containing 1 mM EGTA and 2 mM L-NAME. Enzyme activity is reported in pmol 10 min^{-1} mg^{-1} wet weight.

Western blot analysis

The cerebellum obtained as described in 'animals' was homogenized with an Ultra-Turrax homogenizer in 20 mM Tris-Buffer (pH = 7.4), containing 0.25 sucrose, EDTA (1 mM), PMSF (100 μg ml^{-1}), aprotinin (10 μg ml^{-1}), leupeptin (10 μg ml^{-1}) and soyabean trypsin inhibitor (10 μg ml^{-1}). After centrifugation at 7800 × *g* during 10 min, the supernatants were collected and kept at −70°C until Western blotting assay was performed. Each point represents pooled material from four animals and the experiment was repeated three times. We are showing a typical experiment. Protein (70 μg) was loaded in each lane. Positive control aliquots were also loaded. We used mouse macrophage lysate for iNOS and rat pituitary lysate for neural NOS (nNOS) (Transduction Laboratories, Lexington, KY, U.S.A.). Samples were run on a 7.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred (overnight at 4°C, voltages: 40 V) to a nitrocellulose membrane (Pharmacia, Sweden). The blots were incubated with a rabbit antiserum against iNOS and nNOS for 2 h at room temperature. All the primary antibodies were obtained from Transduction Laboratories (Lexington, KY, U.S.A.) and were used at final dilutions 1 : 1000 in the blocking buffer. The blots were washed with wash buffer (10 mM Tris, 100 mM NaCl, 0.1% v/v Tween-20, pH: 7.5) followed by an alkaline phosphatase-conjugated anti-rabbit IgG as the secondary antibody and developed with 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt (BCIP) and NitroBlue Tetrazolium (NBT). Molecular weight markers (BIO-RAD HERCULES, CA, USA) identified the protein bands. Pictures of the membranes were taken and they were densitometrically scanned and analysed using a Dekmate III scanner and Sigma Gel software package (Sigma Chemical Co, St Louis, Mi, U.S.A.). In each lane, the concentration of protein loaded was measured by the Bradford method (Bradford, 1976).

Drugs and chemicals

LPS from *Escherichia coli* Serotype = 0.55, INDO and citrulline were purchased from Sigma Chemical Co. (St Louis, MI, U.S.A.); L-(^{14}C)-arginine was obtained from Amersham Corp. (Arlington Heights, II, U.S.A.). AG50WX-8 resin was obtained from Bio-Rad. LC was obtained from Roemmers SAICF (Argentina). MELO was purchased from Boehringer Ingelheim (Argentina). The Western blot reagents were obtained from Bio-Rad. Antibodies to the inducible and neuronal isoforms of NOS were purchased from Transduction Laboratory. All other chemicals were analytical grade.

Statistical analysis

Statistical significance was tested by ANOVA and Student–Newman–Keuls multiple comparison and data were expressed as means ± s.e.m. Differences between means were considered significant at $P < 0.05$.

Results

In vivo *effects of NSAIDS on NOS activity in cerebellar preparations of LPS-treated rats*

Our first experiments sought to assess the ability of various NSAIDs, administered *in vivo*, to modify the NO synthesis of control and LPS-treated rats. Doses chosen were the ones used in clinical therapy.

Table 1 shows NO production in cerebellar preparations obtained from control rats and LPS-treated rats. Rats treated with LPS, 6 h before killing, show clear and significantly increased NOS activity ($P < 0.001$).

LC, injected at different clinical doses, in control animals did not affect basal NOS activity. The effect of the administration of different doses of LC, injected simultaneously with LPS 6 h before killing, on the cerebellar LPS-stimulated NO

Table 1 NO production from rat cerebellar preparations obtained from control and LPS-treated animals

	Control	*LPS*
	179 + 12	270 + 18[a]
LC 4 mg kg^{-1}	176 + 9	245 + 16
LC 10 mg kg^{-1}	162 + 9	224 + 12[b]
LC 20 mg kg^{-1}	169 + 12	218 + 18[b]
LC 40 mg kg^{-1}	165 + 11	214 + 13[b]

Rats were treated with LPS (5 mg kg^{-1}) 6 h before killing. Different doses of LC (4, 10, 20, 40 mg kg^{-1}) were injected simultaneously with saline or LPS. Each column indicates the mean ± s.e.m. of $n = 8$ animals. [a]$P < 0.001$ vs control; [b]$P < 0.01$ vs LPS.

production is also shown in Table 1. LC 4 mg kg^{-1} did not modify the LPS-induced NO increase, but at 10, 20 and 40 mg kg^{-1}, it blocked LPS-induced augmentation. *In vivo* administration of 2.5 or 5 mg kg^{-1} of INDO (Table 2) failed to modify control NOS activity and LPS-induced NO synthesis, but the upper dose (10 mg kg^{-1}) suppressed the effect of LPS ($P<0.05$).

Table 3 shows the effect of different doses of MELO, injected simultaneously with saline or LPS 6 h before killing, on the cerebellar NOS activity. The lower dose of MELO (2.5 mg kg^{-1}) was not able to modify control or LPS-induced NOS activity. However, the dose of 5 ($P<0.01$) and 10 mg kg^{-1} ($P<0.05$) attenuated NO production from LPS-treated animals and the upper dose also diminished control NO synthesis ($P<0.05$).

In vivo *effects of NSAIDS on nNOS expression in cerebellar preparations of LPS-treated rats*

The next experiments were designed to determine if NSAIDs administrated *in vivo* modify nNOS expression.

Jesko *et al*. (2003) demonstrated that constitutive NOS isoforms, especially nNOS, make up for approximately 60% of NOS activity in the adult brain of Wistar rats. Given the importance of cerebellar nNOS, we analysed (by Western Blot) nNOS protein in cerebellar homogenates from animals treated with LPS with and without NSAIDs.

A band at 155 kDa corresponding to the size of nNOS was found to be expressed at detectable levels in cerebellar homogenates of control animals. nNOS protein expression was augmented by the *in vivo* administration of LPS (Figure 1). LC, in all doses tested, decreased nNOS protein expression (LPS induced), and in the upper doses (10, 20 and 40 mg kg^{-1}), it also diminished nNOS expression compared with the samples from control animals.

INDO, in the three doses studied, blocked the augmentation of nNOS protein expression due to LPS (Figure 2), but it did not decrease nNOS expression under control levels.

MELO treatment failed to modify nNOS protein expression of cerebellar homogenates from LPS-treated animals (Figure 3).

Table 2 NO production from rat cerebellar preparations obtained from control and LPS-treated animals

	Control	*LPS*
	155 + 4	237 + 4[a]
INDO 2.5 mg kg^{-1}	147 + 5	275 + 14
INDO 5 mg kg^{-1}	149 + 7	221 + 3
INDO 10 mg kg^{-1}	152 + 10	162 + 9[b]

Rats were treated with LPS (5 mg kg^{-1}) 6 h before killing. Different doses of INDO (2.5, 5 and 10 mg kg^{-1}) were injected simultaneously with saline or LPS. Each column indicates the mean ± s.e.m. of $n=8$ animals. [a]$P<0.05$ vs control; [b]$P<0.05$ vs LPS.

Table 3 NO production from rat cerebellar preparations obtained from control and LPS-treated animals

	Control	*LPS*
	178 + 28	335 + 28[a]
MELO 2.5 mg kg^{-1}	173 + 12	286 + 34
MELO 5 mg kg^{-1}	169 + 12	194 + 25[b]
MELO 10 mg kg^{-1}	154 + 11	240 + 17[b]

Rats were treated with LPS (5 mg kg^{-1}) 6 h before killing. Different doses of MELO (2.5, 5 and 10 mg kg^{-1}) were injected simultaneously with saline or LPS. Each column indicates the mean ± s.e.m. of $n=8$ animals. [a]$P<0.01$ vs control; [b]$P<0.01$ vs LPS; [c]$P<0.05$ vs LPS.

In vivo *effects of NSAIDS on iNOS expression in cerebellar preparations of LPS-treated rats*

The literature describes that LPS induces the expression of iNOS (Franchi *et al*., 2001; Aeberhard, 1995) in different tissues; so we decided to study in cerebellar preparations whether iNOS expression was augmented, and if so, whether NSAIDS were able to modify it. This was determined by Western blot analysis.

The monoclonal antibody to iNOS reacted with the band corresponding to the 130 kDa, which showed that iNOS protein expression was augmented by the administration of LPS (Figure 4). We also observed that the administration of LC in all doses assayed not only inhibited the augmentation of iNOS expression due to LPS, but also decreased it under control levels.

In Figure 5 we show that the coadministration of INDO, in all doses studied, with LPS suppresses the enhancement of iNOS expression even under control level. MELO was not able to modify iNOS expression of LPS-treated rats in any of the doses assayed (Figure 6).

Discussion

It is becoming clear that the first mode of NSAIDs action, COX inhibition, is a significant, but not the only, effect of NSAIDs (Aeberhard, 1995; Heneka, 2000; Franchi, 2001). A growing body of research now demonstrates a variety of NSAIDs effects on cellular signal transduction pathways other than those involving prostaglandins.

This study shows the *in vivo* action of LC, INDO and MELO on NO production and iNOS and nNOS expression in control and LPS-treated rats. When the control animals were treated *in vivo* with the three NSAIDs assayed, no differences compared to the baseline were detected in NOS enzymatic activity. These findings are in concordance with our previous work where we have demonstrated that neither LC (40 mg kg^{-1}) nor INDO (10 mg kg^{-1}) was able to modify basal NOS activity in rat lungs (Franchi *et al*., 2001) and with Mohn (2001), who showed that MELO *in vivo* did not modify basal NO production in the hypothalamus of the rat.

In these experiments, we have demonstrated the inhibitory effect of three NSAIDs (LC, MELO and INDO) on LPS-stimulated NO synthesis of cerebellar slices. These data are in agreement with Aeberhard *et al*. (1995), who reported that INDO inhibited expression of iNOS in LPS-induced rat alveolar macrophages. They suggested that the mechanism of NOS inhibition was due to a pretranslational control on enzyme expression and not to direct inhibition of enzymatic activity. It has also been shown that ibuprofen reduced iNOS

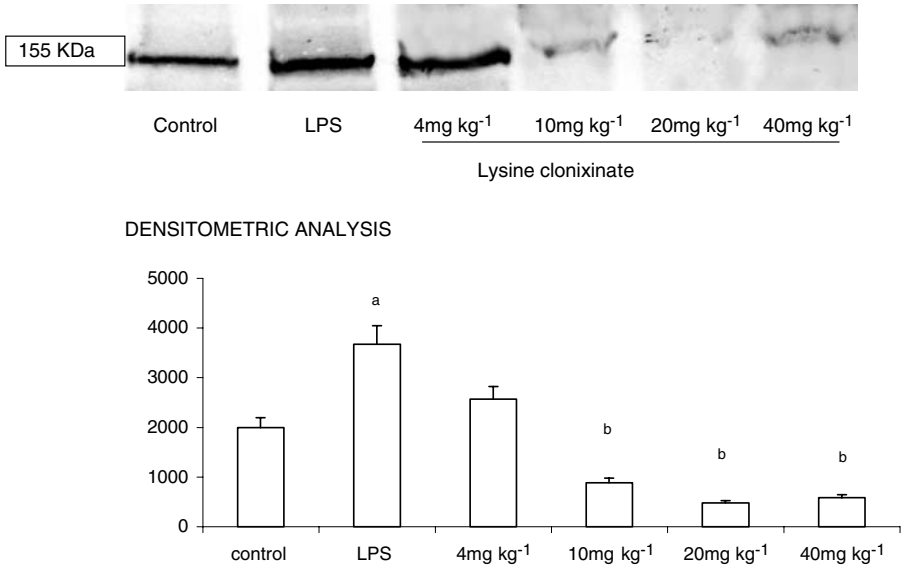

Figure 1 Western blotting: Using a monoclonal antibody against the nNOS form of rat cerebellular preparations obtained from control and LPS-treated animals. Rats were treated with LPS ($5\,mg\,kg^{-1}$) 6 h before killing. Different doses of LC (4, 10, 20 and $40\,mg\,kg^{-1}$) were injected simultaneously with LPS. Each point represents the mean of three determinations using different pooled material from four animals. Rat pituitary lysate was used as an nNOS positive control (Transduction Laboratories, Lexington, KY, U.S.A.). Densitometric analysis was performed with the Sigma Gel programme. (a) $P<0.001$ vs control, (b) $P<0.001$ vs control and vs LPS.

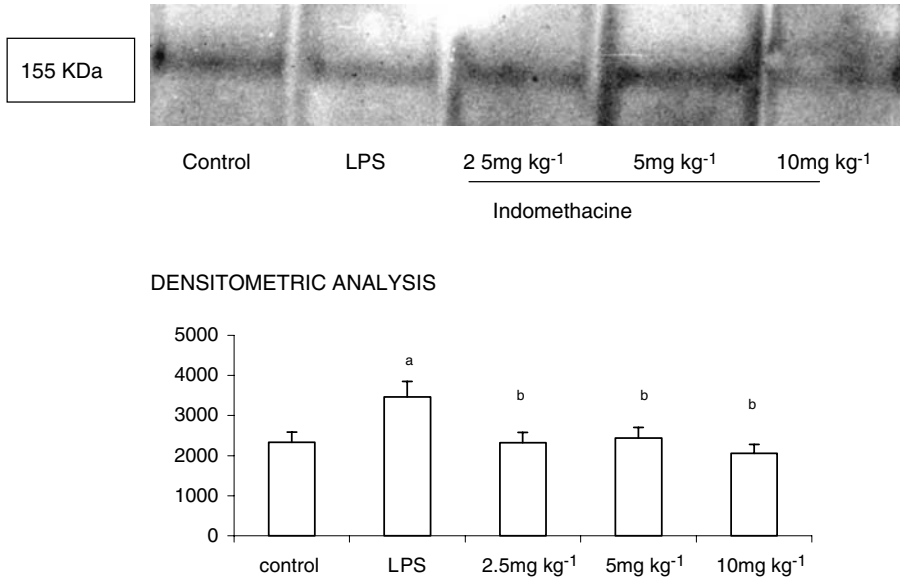

Figure 2 Western blotting: using a monoclonal antibody against nNOS form from rat cerebellar preparations obtained from control and LPS-treated animals. Rats were treated with LPS ($5\,mg\,kg^{-1}$), 6 h before killing. Different doses of INDO (2.5, 5 and $10\,mg\,kg^{-1}$) were injected simultaneously with LPS. Each point represents the mean of three determinations using a different pool of four animals material in each experiment. Rat pituitary lysate was used as nNOS positive control (Transduction Laboratories, Lexington, KY, U.S.A.). Densitometric analysis was done with Sigma Gel. (a) $P<0.05$ vs control, (b) $P<0.05$ vs LPS.

activity, iNOS protein level and iNOS mRNA levels in primary cerebellar glial cells (Stratman, 1997).

The increase in the expression of nNOS due to LPS stimulation agrees with previous findings of our group demonstrating that LPS was able to induce nNOS levels in rat uterus (Cella *et al*., 2001) and reports of Harada *et al*. (1999), who found that nNOS mRNA expression in the paraventricular nucleus was significantly increased 2 h after LPS injection. We have shown that LC and INDO were able to block LPS induction of nNOS expression. To our best knowledge, it is the first time that it has been reported that a NSAID *in vivo* was able to decrease LPS-induced nNOS protein.

We have also found that two NSAIDs (LC and INDO) inhibit the LPS-stimulated expression of iNOS in cerebellar slices. In coincidence with this finding, aspirin has been reported to inhibit iNOS expression, including iNOS mRNA expression (Aeberhard, 1995) and translation into enzyme

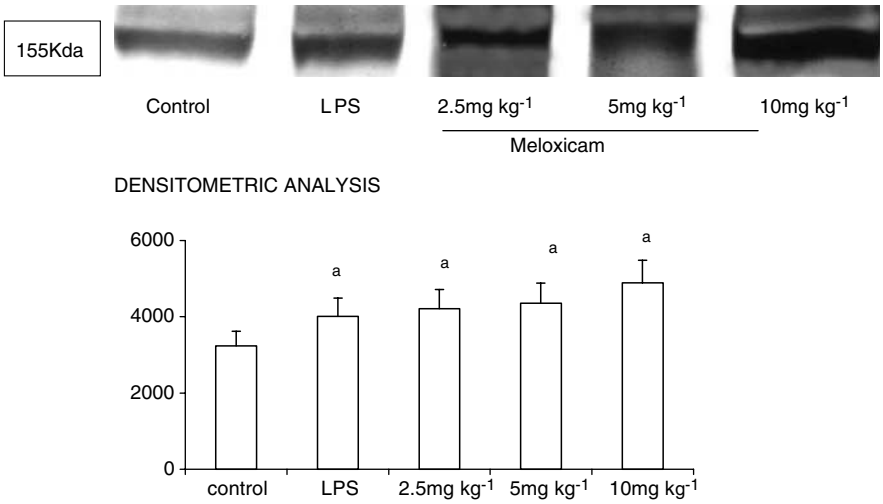

Figure 3 Western blotting: using a monoclonal antibody against nNOS form from rat cerebellar preparations obtained from control and LPS-treated animals. Rats were treated with LPS (5 mg kg^{-1}) 6 h before killing. Different doses of MELO (2.5, 5 and 10 mg kg^{-1}) were injected simultaneously with LPS. Each point represents the mean of three determinations using a different pool of four animals material in each experiment. Rat pituitary lysate was used as nNOS positive control (Transduction Laboratories, Lexington, KY, U.S.A.). Densitometric analysis was done with Sigma Gel. (a) $P<0.05$ vs control.

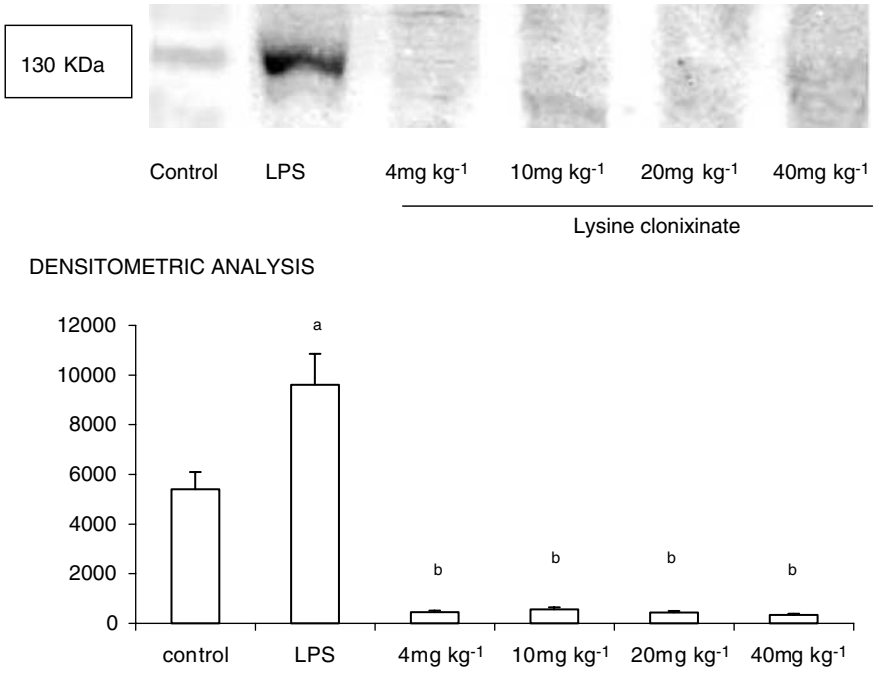

Figure 4 Western blotting: using a monoclonal antibody against iNOS from rat cerebellar preparations obtained from control and LPS-treated animals. Rats were treated with LPS (5 mg kg^{-1}) 6 h before killing. Different doses of LC (4, 10, 20 and 40 mg kg^{-1}) were injected simultaneously with LPS. Each point represents the mean of three determinations using a different pool of four animals material in each experiment. Mouse macrophage lysate was used as positive iNOS control. Densitometric analysis was done with Sigma Gel. (a) $P<0.01$ vs control, (b) $P<0.001$ vs control and vs LPS.

(Known, 1997; Sakitani *et al*., 1997). Aspirin significantly inhibits the IL-1 beta-stimulated expression of iNOS, in ovarian dispersates cultured *in vitro* (Carnovale, 2001).

It is important to emphasize that MELO, a COX-2 inhibitor, was not able to modify nNOS or iNOS expression in any dose assayed, a fact that is in concordance with Sadowski & Steinmeyer (2001), who demonstrated that MELO was not able to reduce IL-1-stimulated mRNA expression and protein synthesis of iNOS by articular chondrocytes. Neither MELO nor INDO, in lower doses, or LC in the lowest dose assayed, changed NO synthesis, although these doses are sufficient to inhibit COX and block prostaglandin synthesis (Engelhardt, 1996). Heneka (2000) have shown that another selective COX-2 inhibitor, NS-398, has not reduced cerebellar iNOS-positive cell staining. These authors (Heneka, 1999) have previously shown that *in vitro* concentrations of NSAIDs sufficient to inhibit COX and block prostaglandin synthesis had no effect on iNOS expression. Similarly, the inhibitory effect of LC and INDO on nNOS and iNOS expression could not be attributed to COX inhibition, since the actions of those

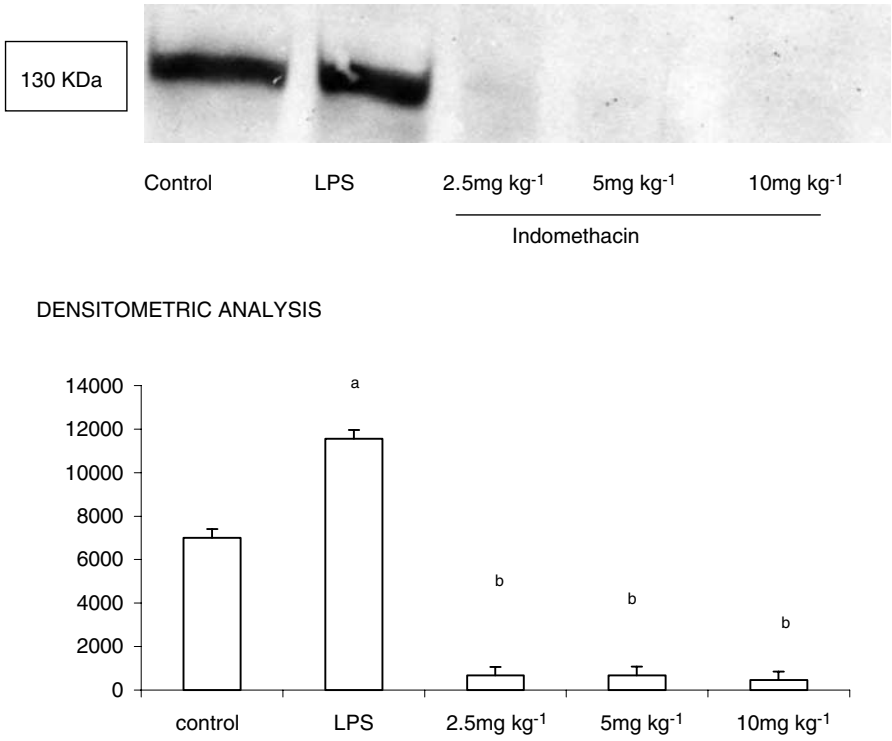

Figure 5 Western blotting: using a monoclonal antibody against iNOS from rat cerebellar preparations obtained from control and LPS-treated animals. Different doses of INDO (2.5, 5 and 10 mg kg^{-1}) were injected simultaneously with LPS. Each point represents the mean of three determinations using a different pool of four animals material in each experiment. Mouse macrophage lysate was used as positive iNOS control. Densitometric analysis was done with Sigma Gel. (a) $P<0.001$ vs control, (b) $P<0.001$ vs control and vs LPS.

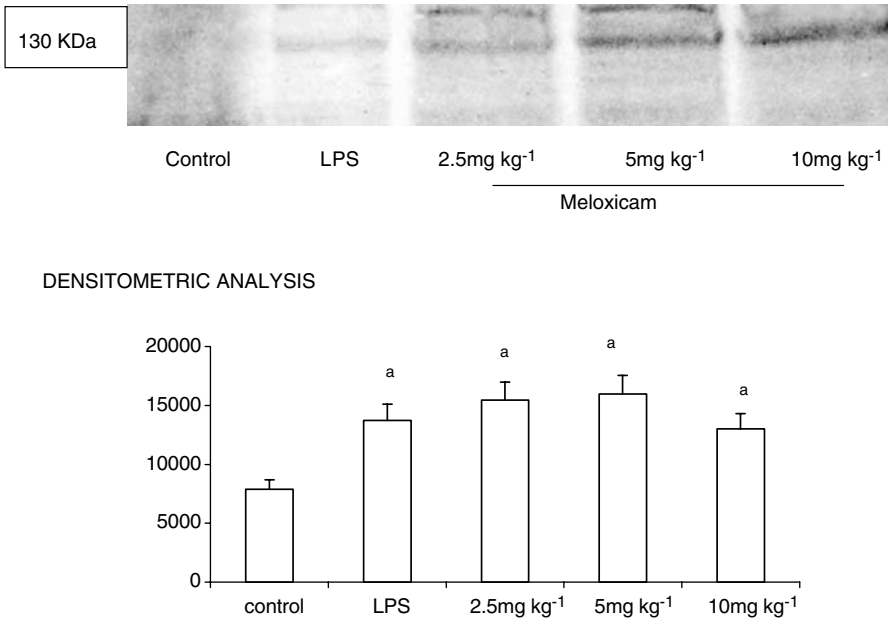

Figure 6 Western blotting: using a monoclonal antibody against iNOS from rat cerebellar preparations obtained from control and LPS-treated animals. MELO was injected simultaneously with LPS. Each point represents the mean of three determinations using a different pool of four animals material in each experiment. Mouse macrophage lysate was used as positive iNOS control. Densitometric analysis was done with Sigma Gel. (a) $P<0.01$ vs control.

NSAIDs were not replicated by MELO, and doses sufficient to inhibit COX and block prostaglandin synthesis were not able to modify NO production (Franchi *et al*., 1999).

This study has proved that the NSAIDs assayed inhibit NO production and two of them (LC and INDO) also inhibit expression of iNOS and nNOS. Therefore, part of the anti-inflammatory action of these NSAIDs could be mediated by the inhibition of NOS expression.

The inhibitor effect of some NSAIDs on iNOS expression may be due, at least in part, to inhibition of NF-κB activation.

For example, Kopp & Ghosh (1994) found that sodium salicylate is capable of inhibiting NF-κB activation. Glibetic *et al*. (2001) showed that NF-κB activation is an early step in the induction of iNOS gene expression by Group B streptococci, and that ibuprofen suppressed GBS-induced iNOS mRNA expression blocking NF-κB activation. Another possible mechanism of NSAIDs to inhibit iNOS expression could be the one proposed by Heneka *et al*. (2000), who suggested that NSAIDs binding to peroxisome proliferator-activated receptor gamma (PPAR) present in Alzhemier brain, prevent iNOS expression and neuronal cell death.

Thorns *et al*. (1998) have shown that nNOS-expressing neurons are highly susceptible to neurodegeneration and nNOS might contribute to the pathogenesis of Alzheimer's disease. Simic *et al*. (2000) suggested that an upregulated production of NO by nNOS from reactive astrocytes may play a key role in the pathogenesis of Alzheimer's disease. These findings and our report of an inhibitory effect of LC and INDO on nNOS expression suggest that, at least in part, these NSAIDs may be of therapeutic value by inhibiting nNOS expression.

In conclusion, the decrease in NO synthesis and NOS isoform expression following *in vivo* treatment with NSAIDs not only represents a novel mechanism of therapeutic action of these NSAIDs in inflammatory diseases, but may also be of importance in the prevention of NO-mediated neuronal injury.

The currently established ideas on the processes of cerebral NO production and on the pathogenetic mechanisms of this agent's cytotoxicity, open up new avenues for various NSAIDs that inhibit NO synthesis as new strategies to reduce the risk of Alzheimer's disease.

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References

AEBERHARD, E.E., HENDERSON, S.A., ARABOLOS, N.S., GRISCAVAGE, J.M., CASTRO, F.E., BARRETT, C.T. & IGNARRO, L.J. (1995). Nonsteroidal anti-inflammatory drugs inhibit expression of the inducible nitric oxide synthase gene. *Biochem. Biophys. Res. Commun.,* **208,** 1053–1059.

BRADFORD, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.,* **72,** 246–254.

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.

CARNOVALE, D.E., FUKUDA, A., UNDERHILL, D.C., LAFFAN, J.J. & BREUEL, K.F. (2001). Aspirin dose dependently inhibits the interleukin-1 beta-stimulated increase in inducible nitric oxide synthase, nitric oxide, and prostaglandin E(2) production in rat ovarian dispersates cultured in vitro. *Fertil. Steril.,* **75,** 778–784.

CELLA, M., OGANDO, D., FARINA, M., RIBEIRO, M.L. & FRANCHI, A. (2001). El lipopolisacárido (LPS) aumenta la síntesis de óxido nítrico (NO) vía dos isoformas de la Nosintasa (NOS): nNOS e iNOS en el útero de rata ovariectomizada. *Reproducao Climaterio,* **16,** 15 (Abstract).

ENGELHARDT, G., BÖGEL, R., SCHNITZLER, C.H.R. & UTZMANN, R. (1996). Meloxicam: influence on arachidonic acid metabolism. Part II: *In vivo* findings. *Biochem. Pharmacol.,* **51,** 29–38.

FRANCHI, A.M., DIGIROLAMO, G., DE LOS SANTOS, A.R., MARTÍ, M.L. & GIMENO, M.A.F. (1999). Ex vivo effects of lysine clonixinate on cyclooxygenases in rat lung and stomach preparations. *Inflammopharmacology,* **7,** 401–411.

FRANCHI, A., DIGIROLAMO, G., FARINA, M., DE LOS SANTOS, A.R., MARTÍ, M.L. & GIMENO, M.A.F. (2001). In vivo and in vitro effects of lysine clonixinate on nitric oxide synthase in rat lung preparations treated or not with lipopolysaccharide. *Nitric Oxide,* **5,** 150–157.

GLIBETIC, M., SAMLALSINGH-PARKER, J., RAYKOVA, V., OFENSTEIN, J. & ARANDA, J.V. (2001). Group B streptococci and inducible nitric oxide synthase: modulation by nuclear factor κ B and ibuprofen. *Semin. Perinatol.,* **25,** 65–69.

HARADA, S., IMAKI, T., CHIKADA, N., NARUSE, M. & DEMURA, H. (1999). Distinct distribution and time-course changes in neuronal nitric oxide synthase and inducible NOS in the paraventricular nucleus following lipopolysaccharide injection. *Brain Res.,* **821,** 322–332.

HENEKA, M.T., FEINSTEIN, D.L., GALEA, E., GLEICHMANN, M., WULLNER, U. & KLOCKGETHER, T. (1999). Peroxisome proliferator-activated receptor gamma agonists protect cerebellar granule cells from cytokine-induced apoptotic cell death by inhibition of inducible nitric oxide synthase. *J. Neuroimmunol.,* **100,** 156–168.

HENEKA, M., KLOCKGETHER, T. & FEINSTEIN, D.L. (2000). Peroxisome proliferator-activated receptor-ligands reduce neuronal inducible nitric oxide synthase expression and cell death in-vivo. *J. Neurosci.,* **20,** 6862–6867.

JESKO, H., CHALIMONIUK, M. & STROSZNAJDER, J.B. (2003). Activation of constitutive nitric oxide synthase(s) and absence of inducible isoform in aged rat brain. *Neurochem. Int.,* **42,** 315–322.

KOPP, E. & GHOSH, S. (1994). Inhibition of NF-κB by sodium salicylate and aspirin. *Science,* **265,** 956–959.

KNOWN, G., HILL, J.R., CORBETT, J.A. & MCDANIEL, M.L. (1997). Effects of aspirin on nitric oxide formation and the *novo* synthesis by RINm5F cells and rat islets. *Mol. Pharmacol.,* **52,** 398–405.

MARLETTA, M.A. (1994). Nitric oxide synthase: aspects concerning structure and catalysis. *Cell,* **78,** 927–930.

MOHN, C., LOMNICZI, A., FALETTI, A., SCORTICATI, C., ELVERDIN, J.C., MCCANN, S.M. & RETTORI, V. (2001). Effects of aminoguanidine and meloxicam on nitric oxide and prostaglandin E production induced by lipopolysaccharide in the hypothalamus and anterior pituitary of the rat. *Neuroimmunomodulation,* **9,** 276–285.

SADOWSKI, T. & STEINMEYER, J. (2001). Minocycline inhibits the production of inducible nitric oxide synthase in articular chondrocytes. *J. Rheumatol.,* **28,** 336–340.

SAKITANI, K., KITADE, H., INOUE, K., KAMIYAMA, Y., NISHIZAWA, M., OKUMURA, T. & ITO, S. (1997). The anti-inflammatory drug sodium salicylate inhibits nitric oxide formation induced by scriptional step, in hepatocytes. *Hepatology,* **25,** 416–420.

SIMIC, G., LUCASSEN, P.J., KRSNIK, Z., KRUSLIN, B., KOSTOVIC, I., WINBLAD, B. & BOGDANOVIC, N. (2000). nNOS expression in reactive astrocytes correlates with increased cell death related DNA damage in the hippocampus and entorhinal cortex in Alzheimer's disease. *Exp. Neurol.,* **165,** 12–26.

STRATMAN, N.C., CARTE, D.B. & SETHY, V.H. (1997). Ibuprofen: effect on inducible nitric oxide synthase. *Mol. Brain Res.,* **50,** 107–112.

THORNS, V., HANSEN, L. & MASLIAH, E. (1998). nNOS expression neurons in the entorhinal cortex and hippocampus are affected in patients with Alzheimer's disease. *Exp. Neurol.,* **150,** 14–20.

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