ELSEVIER

European Journal of Pharmacology 408 (2000) 137–141

ejp

www.elsevier.nl/locate/ejphar

Short communication

Inhibition of inducible nitric oxide synthase gene expression by indomethacin or ibuprofen in β-amyloid protein-stimulated J774 cells

Osamu Ogawa [a], Hiroyuki Umegaki [a,*], Daigo Sumi [a], Toshio Hayashi [a], Akira Nakamura [a], Navin Kumar Thakur [a], Juri Yoshimura [a], Hidetoshi Endo [b], Akihisa Iguchi [a]

[a] *Department of Geriatrics, Graduate School of Medicine, Nagoya University, 65 Tsuruma-Cho, Showa-Ku, Nagoya, Aichi, 466-8550, Japan*
[b] *Department of Geriatrics, Chubu National Hospital 36-3 Gengo, Obu, Aichi, 474-8511, Japan*

Received 20 July 2000; received in revised form 6 September 2000; accepted 14 September 2000

Abstract

Recent studies show that a mononuclear phagocyte lineage, including microglia, plays a possible role in the pathogenesis of Alzheimer's disease through nitric oxide (NO)-mediated neurotoxicity. Epidemiological studies show that nonsteroidal anti-inflammatory drugs (NSAIDs) have a protective effect against Alzheimer's disease. Based on these observations, it has been hypothesized that an anti-Alzheimer's disease effect of NSAIDs could result from the inhibition of NO synthesis. We report here that indomethacin or ibuprofen dose-dependently reduce β-amyloid protein and interferon-γ-induced NO production, accompanied by an inhibition of inducible nitric oxide synthase mRNA expression in J774 cells, a murine macrophage cell line. Aspirin, however, does not produce such an effect, suggesting that the cyclooxygenases pathway is not involved in the inhibitory effects of NSAIDs on β-amyloid protein and interferon-γ-induced NO production in J774 cells.

Keywords: Alzheimer's disease; Microglia; NSAIDs; Aspirin; Interferon-γ

1. Introduction

Alzheimer's disease is characterized by progressive cognitive impairment that is a consequence of extensive neuronal loss. One of the principal pathological features of Alzheimer's disease is numerous senile plaques containing extracellular deposits of insoluble, aggregated β-amyloid protein, which is a peptide of 40–43 amino acids (Selkoe, 1991; Terry, 1994). Early studies focused primarily on the direct neuronal effects of β-amyloid protein, but considerable evidence now points to immune and inflammatory factors as possible contributing elements in the pathogenesis of Alzheimer's disease. Elevated levels of inflammatory cytokines and the presence of a number of acute-phase products have been detected in the Alzheimer's disease brain (McGeer and McGeer, 1997). Clinical data supporting an etiologic role for inflammation and immune responsiveness in the pathogenesis of Alzheimer's disease have come from several epidemiological studies (McGeer et al., 1996; Stewart et al., 1997). Patients receiving long-term nonsteroidal anti-inflammatory drugs (NSAIDs) medication for chronic, immune-based conditions unrelated to Alzheimer's disease have been found to exhibit a significantly reduced incidence of Alzheimer's disease compared to the general population. Furthermore, microglial cells in a reactive state are known to be closely associated with senile plaques (Itagaki et al., 1990), and β-amyloid protein has been shown to activate rodent microglial cultures, resulting in morphological changes and an increased production of several pro-inflammatory cytokines and nitrogen intermediates (Araujo and Cotman, 1992; Goodwin et al., 1995; Meda et al., 1995). In the rodent, microglial cells express inducible nitric oxide synthase (iNOS) and release nitric oxide (NO) when stimulated with β-amyloid protein alone or in combination with interferon-γ and excessive NO has been linked with neuronal cell injury (Araujo and Cotman, 1992; Li et al., 1996). Human microglia has also been reported to express iNOS (Colasanti et al., 1995; Ding et al., 1997), suggesting a possible role for NO produced by microglia in the pathogenesis of Alzheimer's

* Corresponding author. Tel.: +81-52-744-2364; fax: +81-52-744-2371.
E-mail address: umegaki@tsuru.med.nagoya-u.ac.jp (H. Umegaki).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00721-4

disease. Based on these observations, a hypothesis that the anti-Alzheimer's disease effects of NSAIDs could result from the inhibition of NO synthesis has been raised. In this study, we examined the effects of three kinds of NSAIDs on the production of NO induced by β-amyloid protein and interferon-γ in J774 cells, a murine macrophage cell line.

2. Materials and methods

2.1. Materials

β-amyloid protein (1–40) (Quality Controlled Biochemicals, Hopkinton, MA) was dissolved to 1 mg/ml with phosphate-buffered saline, and incubated at 37°C for 48 h in a 5% CO_2-supplemented atmosphere for β-amyloid protein (1–40) aggregation. Aliquots were stored at −20°C until use. Recombinant rat interferon-γ was purchased from Genzyme (Cambridge, MA). Dulbecco's Modified Eagle's Medium (DMEM) without phenol red was obtained from GIBCO. BRL (Grand Island, NY). Indomethacin, ibuprofen, and aspirin were obtained from Sigma (St Louis, MO). All chemicals were of analytical grade.

2.2. Cell culture

The murine macrophage cell line J774 was maintained in DMEM supplemented with 10% fetal bovine serum, 100

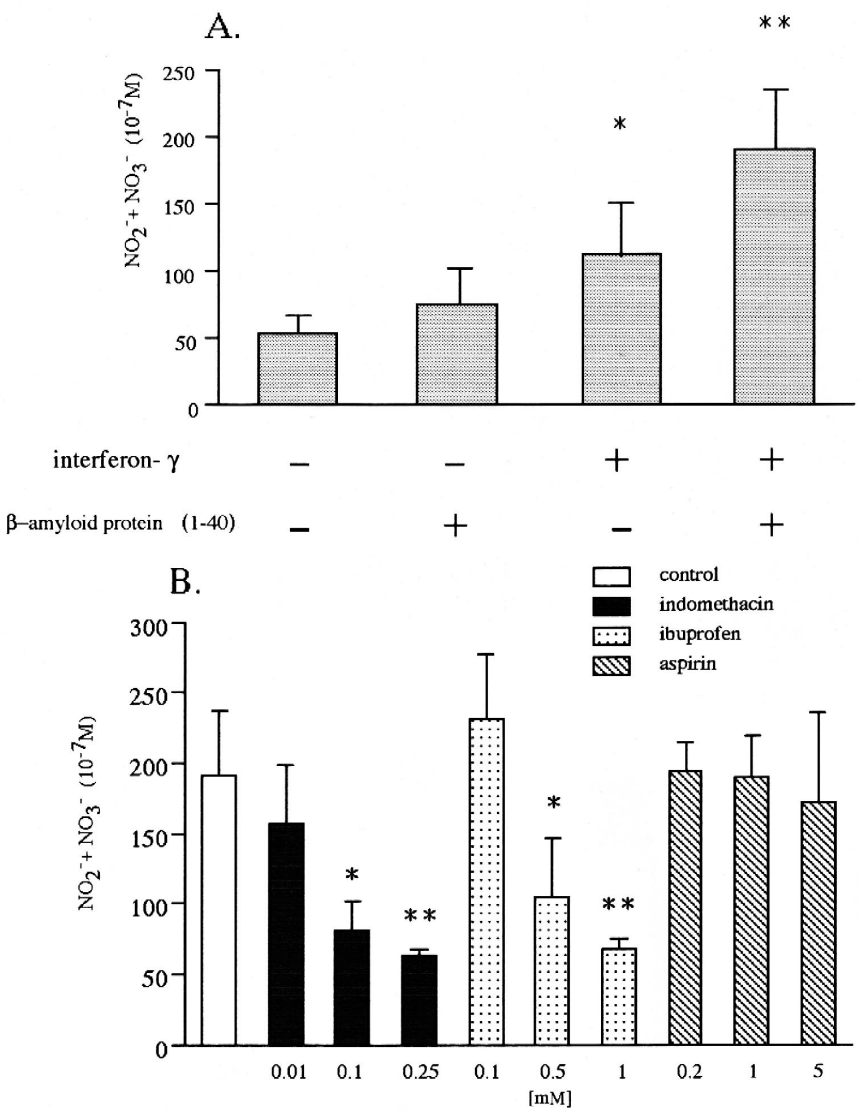

Fig. 1. (A) Effects of β-amyloid protein (1–40) and interferon-γ on NO_x production in J774 cells. J774 cells (1×10^5 cell/100 μl) were incubated with β-amyloid protein (1–40) (33.5 μM) and/or interferon-γ (500 U/ml). The concentrations of NO_x in the culture medium were determined after a 48-h incubation. $^{*}P < 0.0008$ compared to control; $^{**}P < 0.03$ compared to interferon-γ alone. (B) Effects of indomethacin, ibuprofen or aspirin on enhanced NO_x production induced by β-amyloid protein (1–40) and interferon-γ in J774 cells. J774 cells (1×10^5 cell/100 μl) were treated with the indicated compounds and immediately activated by β-amyloid protein (1–40) (33.5 μM) plus interferon-γ (500 U/ml). The concentrations of NO_x in the culture medium were determined after a 48-h incubation. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to corresponding control values (absence of NSAIDs). Data shown are the mean ± S.E.M. from three to six independent experiments performed in duplicate.

U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37°C with 5% CO_2. For the experiments, J774 cells were plated in 96-well microtiter plates at a density of 1×10^5 cells/well or 6-cm diameter culture dishes at a density of 1×10^7 cells/dish with DMEM without serum. To stimulate the cells, J774 cells were cultured with β-amyloid protein (1–40) (33.5 μM) and/or interferon-γ (500 U/ml). To explore the effects of NSAIDs, J774 cells were cultured with β-amyloid protein (1–40) (33.5 μM) and interferon-γ (500 U/ml) in the presence or absence of indomethacin, ibuprofen or aspirin. Cells were cultured at 37°C in a humidified 5% CO_2-supplemented atmosphere for 6 and 48 h for semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) and the measurement of nitrite and nitrate (NO_x), respectively.

2.3. Measurement of nitrite and nitrate

NO_x in supernatants was measured with an automated NO detector high-pressure liquid chromatography system (ENO10: EICOM, Kyoto, Japan), as previously reported (Hayashi et al., 1998). In brief, NO_x was separated from the samples by a reverse-phase separation column. Nitrate was reduced to nitrite in a reduction column. The absorbance of the product dye formed by nitrite and a Griess reagent at 540 nm was measured by a spectrophotometer.

2.4. Determination of iNOS mRNA levels

We quantitated iNOS mRNA as copies using semi-quantitative RT–PCR methods (Harrison and Ohara, 1995). Total RNA was extracted using TRIZOL reagent (GIBCO. BRL, Grand Island, NY) following the manufacturer's protocol, and was quantitated by spectrophotometer. Semi-quantitative RT–PCR was performed using the RNA PCR Kit Ver 2.1 (Takara Shuzo, Otsu, Japan). Two primers were designed from the reported rat iNOS cDNA (Gen Bank, Accession number; U03699). Primer 1; 5′-GCCTC-CCTCTGGAAAGA-3′, primer 2; 5′TCCATGCAGA-CAACCTT-3′. PCR samples were electrophoresed through a 3.5% agarose gel. The iNOS band intensities were corrected with β-actin band intensities.

2.5. Statistical analysis

Results are expressed as the means ± S.E.M. of independent experiments, and statistical analysis was performed using the Student's *t*-test.

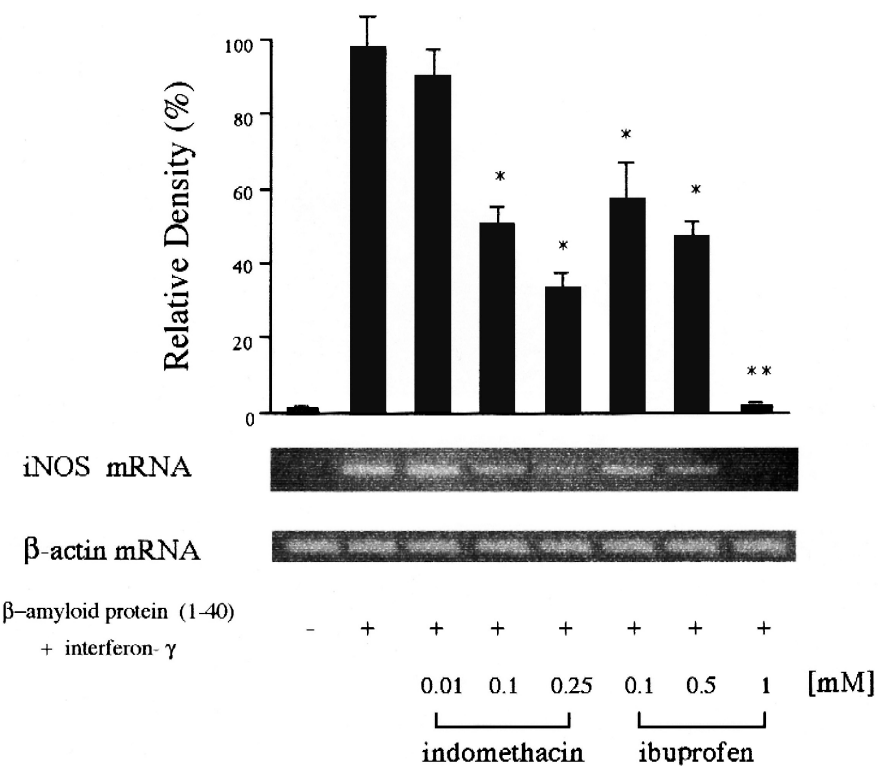

Fig. 2. Effects of NSAIDs on the expression of iNOS mRNA induced by β-amyloid protein (1–40) and interferon-γ in J774 cells. Semi-quantitative RT–PCR method was used for determination of iNOS mRNA. J774 cells (1×10^7 cell/2 ml) were treated with indomethacin or ibuprofen and immediately activated by β-amyloid protein (1–40) (33.5 μM) and interferon-γ (500 U/ml). After a 6-h incubation, total RNA was extracted and subjected to semi-quantitative RT–PCR. Agarose gels show the PCR products amplified with iNOS primers. The top panel of the figure shows the densitometric scanning data from the amplified iNOS mRNA normalized against β-actin controls. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to corresponding control values (absence of NSAIDs).

3. Results

3.1. Effects of NSAIDs on β-amyloid protein-stimulated nitric oxide production

Incubation of the cells with β-amyloid protein (1–40) (33.5 μM) and interferon-γ (500 U/ml) resulted in a synergistic increase in NO_x concentrations in culture medium accompanied by an increase in iNOS mRNA levels (Figs. 1A and 2). This increase in NO_x concentrations in the culture medium was inhibited by indomethacin or ibuprofen but not aspirin in a dose-dependent manner (Fig. 1B). The effects of indomethacin or ibuprofen were observed at concentrations that were not toxic to the cells, as assessed by lactate dehydrogenase release into the culture medium (data not shown).

3.2. Effects of NSAIDs on β-amyloid protein-stimulated nitric oxide synthase gene expression

To investigate whether indomethacin or ibuprofen influences the expression of iNOS mRNA, total RNA for semi-quantitative RT–PCR was prepared from J774 cells stimulated with β-amyloid protein (1–40) (33.5 μM) and interferon-γ (500 U/ml) in the presence of indomethacin or ibuprofen. The iNOS mRNA was not detected from unstimulated cells, indicating that the basal level of iNOS mRNA was very low in J774 cells. After the exposure of cells to both β-amyloid protein (1–40) (33.5 μM) and interferon-γ (500 U/ml), iNOS mRNA was readily detected. The total iNOS mRNA steady-state level in cells cultured with indomethacin or ibuprofen in the presence of inducers decreased in a dose-dependent manner compared to those in cells stimulated only with the inducers (Fig. 2).

4. Discussion

In this study, we demonstrated that the enhanced production of NO_x induced by β-amyloid protein (1–40) and interferon-γ is inhibited by indomethacin or ibuprofen, but not aspirin, in a dose-dependent manner, accompanied by an inhibition of iNOS mRNA expression. Furthermore, indomethacin or ibuprofen at the highest concentrations employed in the study suppress the NO_x production induced β-amyloid protein (1–40) and interferon-γ to baseline levels.

It has been reported that excess amounts of NO_x released from microglia induced by β-amyloid protein and interferon-γ are neurotoxic in the rodent (Araujo and Cotman, 1992; Li et al., 1996). In Alzheimer's disease patients, iNOS has been reported to be highly expressed in the hippocampus (Lee et al., 1999), and the levels of peroxynitrite, a powerful oxidant produced from the reaction of superoxide with NO, or nitrotyrosine, a marker for peroxynitrite, in autopsy brain or cerebrospinal fluid are significantly higher than in control subjects (Tohgi et al., 1999; Smith et al., 1997). Epidemiological study has shown that anti-inflammatory treatment with NSAIDs results in a decreased incidence of Alzheimer's disease and reduces the number of plaque-associated reactive microglia (McGeer et al., 1996; Stewart et al., 1997; Mackenzie and Munoz, 1998). These data suggest that the protective effects of NSAIDs on Alzheimer's disease may result, at least in part, from the inhibition of NO_x production by microglia. Our data shown here support this notion. Interestingly, aspirin did not inhibit the NO_x production induced by β-amyloid protein (1–40) and interferon-γ in this study. In epidemiological studies, the extended use of aspirin has not appeared to be associated with a reduction in the risk of Alzheimer's disease (Stewart et al., 1997). The principal target of NSAIDs action has been thought to be cyclooxygenases, the rate-limiting enzymes responsible for the conversion of arachidonic acid into inflammatory mediators, including prostaglandin E_2. However, the therapeutic benefits of NSAIDs are typically observed at doses much greater than those required to inhibit cyclooxygenases (Jiang et al., 1998), suggesting that there are other targets of NSAIDs action. Recently, it has been recognized that NSAIDs possibly directly regulate gene expression via their interaction with a class of nuclear receptor superfamily members termed peroxisome proliferator-activated receptors (PPAR) (Lehmann et al., 1997). The PPAR-γ isoform is expressed in a Mϕ lineage, where its principal action is to suppress the expression of pro-inflammatory cytokines and other pro-inflammatory products, including iNOS (Petrova et al., 1999). The PPAR-γ isoform has reportedly been activated by indomethacin and ibuprofen (Lehmann et al., 1997). Taken together, the effects of indomethacin or ibuprofen on NO_x production by J774 cells may be mediated via PPAR-γ rather than by the inhibition of cyclooxygenases.

Acknowledgements

This study was supported in part by the grant from Sumitomo Marine Welfare Foundation.

References

Araujo, D.A., Cotman, C.W., 1992. Beta-amyloid stimulates glial cells in vitro to produce growth factors that accumulate in senile plaques in Alzheimer's disease. Brain Res. 569, 141–145.

Colasanti, M., Pucchio, T.D., Persichini, T., Sogos, V., Presta, M., Lauro, G.M., 1995. Inhibition of inducible nitric oxide synthase mRNA expression by basic fibroblast growth factor in human microglial cells. Neurosci. Lett. 195, 45–48.

Ding, M., Pierre, B.A., Parkinson, J.F., Medberry, P., Wong, J.L., Rogers, N.E., Ignarro, L.J., Merrill, J.E., 1997. Inducible nitric-oxide synthase and nitric oxide production in human fetal astrocytes and microglia. J. Biol. Chem. 272, 11327–11335.

Goodwin, J.L., Uemura, E., Cunnick, J.E., 1995. Microglial release of

nitric oxide by the synergistic action of beta-amyloid and IFN-gamma. Brain Res. 692, 207–214.

Harrison, D.G., Ohara, Y., 1995. Physiologic consequences of increased vascular oxidant stresses in hypercholesterolemia and atherosclerosis: implications for impaired vasomotion. Am. J. Cardiol. 75, 75B–81B.

Hayashi, T., Yamada, K., Esaki, T., Muto, E., Chaudhuri, G., Iguchi, A., 1998. Physiological concentrations of 17β-estradiol inhibit the synthesis of nitric oxide synthase in macrophages via a receptor-mediated system. J. Cardiovasc. Pharmacol. 31, 292–298.

Itagaki, S., McGeer, P.L., Akiyama, H., Zhu, S., Selkoe, D.J., 1990. Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. J. Neuroimmunol. 39, 151–170.

Jiang, C., Ting, A.T., Seed, B., 1998. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 391, 82–86.

Lee, S.C., Zhao, M.L., Hirano, A., Dickson, D.W., 1999. Inducible nitric oxide synthase immunoreactivity in the Alzheimer disease hippocampus: association with Hirano bodies, neurofibrillary tangles, and senile plaques. J. Neuropathol. Exp. Neurol. 58 (11), 1163–1169.

Lehmann, J.M., Lenhard, J.M., Oliver, B.B., Ringold, G.M., Kliewer, S.A., 1997. Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem. 272, 3406–3410.

Li, M., Sunamoto, M., Ohnishi, K., Ichimori, Y., 1996. β-Amyloid protein-dependent nitric oxide production from microglial cells and neurotoxicity. Brain Res. 720, 93–100.

Mackenzie, I.R., Munoz, D.G., 1998. Nonsteroidal anti-inflammatory drug use and Alzheimer-type pathology in aging. Neurology 50, 986–990.

McGeer, E.G., McGeer, P.L., 1997. The role of immune system in neuro-degenerative disorders. Mov. Disord. 12, 855–888.

McGeer, P.L., Schulzer, M., McGeer, E.G., 1996. Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epideomiologic studies. Neurology 47, 425–432.

Meda, T., Cassatella, M.A., Szendrei, G.I., Otovos, L. Jr., Baron, P., Villalba, M., Ferrari, D., Rossi, F., 1995. Activation of microglial cells by β-amyloid protein and interferon-γ. Nature 374, 647–650.

Petrova, T.V., Akama, K.T., Van Eldik, L.J., 1999. Cyclopentenone prostaglandins suppress activation of microglia: down-regulation of inducible nitric-oxide synthase by 15-deoxy-delta12,14-prostaglandin J2. Proc. Natl. Acad. Sci. U. S. A. 96, 4668–4673.

Selkoe, D.J., 1991. The molecular pathology of Alzheimer's disease. Neuron 6, 487–498.

Smith, M.A., Richey Harris, P.L., Sayre, L.M., Beckman, J.S., Perry, G., 1997. Widespread peroxynitrite-mediated damage in Alzheimer's disease. J. Neurosci. 17 (8), 2653–2657.

Stewart, W.F., Kawas, C., Corrada, M., Metter, E.J., 1997. Risk of Alzheimer's disease and duration of NSAID use. Neurology 48, 626–632.

Terry, R.D., 1994. Neuropathological changes in Alzheimer disease. Prog. Brain Res. 101, 383–390.

Tohgi, H., Abe, T., Yamazaki, K., Murata, T., Ishizaki, E., Isobe, C., 1999. Alternations of 3-nitrotyrosine concentration in the cerebrospinal fluid during aging and in patients with Alzheimer's disease. Neurosci. Lett. 269, 52–54.