Nitric Oxide 44 (2015) 61–70

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Antipyretic analgesic drugs have different mechanisms for regulation of the expression of inducible nitric oxide synthase in hepatocytes and macrophages

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ARTICLE INFO

Article history:
Received 7 October 2014
Revised 14 November 2014
Available online 8 December 2014

Keywords:
Hepatocyte
Macrophage
Nitric oxide
Inducible nitric oxide synthase
NSAID
Acetaminophen
NF-κB
Antisense transcript

ABSTRACT

Antipyretic analgesic drugs (including non-steroidal anti-inflammatory drugs) inhibit cyclooxygenase-2 and inducible nitric oxide synthase (iNOS), resulting in decreases of the proinflammatory mediators prostaglandin E_2 and nitric oxide (NO), respectively. Both mediators are regulated by nuclear factor-kappa B (NF-κB), a key transcription factor in inflammation. Few reports have compared the efficacy and potency of anti-inflammatory drugs as NO inhibitors. In our study, we examined the effects of four popular antipyretic analgesic drugs on NO production induced in hepatocytes and macrophages. Mouse RAW264.7 macrophages treated with bacterial lipopolysaccharide showed the highest efficacy with regard to NO production; aspirin, loxoprofen, ibuprofen, and acetaminophen dose-dependently suppressed NO induction. Ibuprofen showed the highest potency in suppressing the induced production of NO. In rat hepatocytes, all the drugs inhibited interleukin 1β-induced NO production and ibuprofen and loxoprofen inhibited NO induction effectively. Unexpectedly, the potency of NO suppression of each drug in hepatocytes did not always correlate with that observed in RAW264.7 cells. Microarray analyses of mRNA expression in hepatocytes revealed that the effects of the four antipyretic analgesic drugs modulated the NF-κB signaling pathway in a similar manner to the regulation of the expression of genes associated with inflammation, including the *iNOS* gene. However, the affected signal-transducing molecules in the NF-κB pathway were different for each drug. Therefore, antipyretic analgesic drugs may decrease NO production by modulating the NF-κB pathway in different ways, which could confer different efficacies and potencies with regard to their anti-inflammatory effects.

1. Introduction

The proinflammatory mediator nitric oxide (NO) is synthesized by inducible nitric oxide synthase (iNOS; also known as NOS2) in macrophages and hepatocytes [1,2]. If infection occurs, bacterial endotoxins such as lipopolysaccharides (LPS) stimulate macrophages to produce NO, whereas the proinflammatory cytokine interleukin (IL)-1β stimulates hepatocytes to produce NO. Aspirin is a non-steroidal anti-inflammatory drug (NSAID) that has been used as an antipyretic analgesic drug to treat pain and inflammation [3,4]. NSAIDs inhibit the activity of cyclooxygenase 2 (COX-2, also known as prostaglandin-endoperoxide synthase 2). COX-2 is a key enzyme in the biosynthesis of prostaglandins (PGs) and leads to reduction of the proinflammatory mediator PGE_2 [4]. Loxoprofen is a phenylpropionate NSAID that is more selective to COX-2 than aspirin and ibuprofen (another phenylpropionate derivative) [4,5] (Fig. 1). Aspirin and sodium salicylate suppress LPS-induced iNOS expression and NO production in RAW264.7 macrophages [7]. Sodium salicylate suppresses IL-1β-induced NO production in rat hepatocytes [2,8]. Acetaminophen is the most popular antipyretic analgesic drug used in children, although it is not classified as a NSAID because

Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; IL, interleukin; NSAID, non-steroidal anti-inflammatory drug; COX, cyclooxygenase; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor α; NF-κB, nuclear factor κB; IC_{50}, half-maximal inhibitory concentration; PCR, polymerase chain reaction; RT, reverse transcription; asRNA, antisense transcript; IL1R1, interleukin 1 receptor, type I; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; TRAF6, tumor necrosis factor receptor-associated factor 6; IκB, inhibitor of nuclear factor κB; IKK, IκB kinase.

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http://dx.doi.org/10.1016/j.niox.2014.12.001
1089-8603/

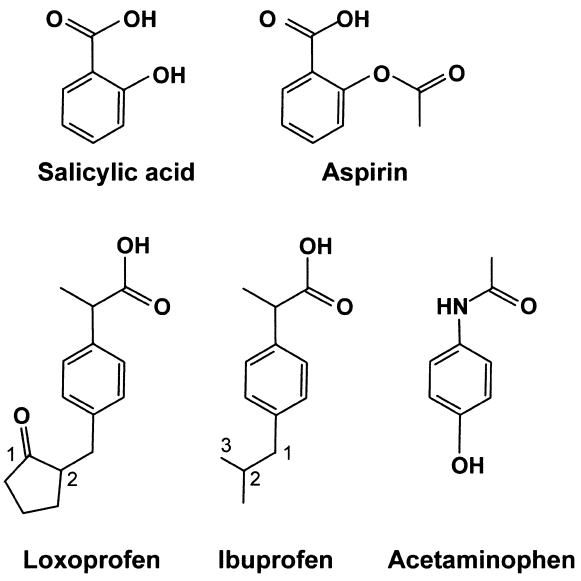

Fig. 1. Structures of antipyretic analgesic drugs. Structures of salicylate derivatives (i.e., salicylic acid and aspirin [acetylsalicylic acid]), phenylpropionate derivatives (i.e., loxoprofen and ibuprofen) and acetaminophen ([*N*-(4-hydroxyphenyl)acetamide]) are depicted. Loxoprofen and ibuprofen have diastereoisomers due to asymmetric carbon atoms. The C-1 carbon of loxoprofen is converted to an active metabolite (*trans*-alcohol form) in the liver [5]. Ibuprofen is metabolized by hydroxylation at carbons (C-1 to C-3) in the liver to metabolites including hydroxyibuprofen [6].

of its low anti-inflammatory effect [9]. Acetaminophen also suppresses LPS-induced iNOS expression and NO production in RAW264.7 cells [10].

In the liver, LPS stimulates the resident macrophages (i.e., Kupffer cells) to produce NO, PGE_2, and the proinflammatory cytokines, such as IL-1β and tumor necrosis factor (TNF)-α [2,11]. Subsequently, IL-1β stimulates the hepatocytes adjacent to Kupffer cells to induce NO and TNF-α [2,12]. Hepatocytes do not express COX-2, thereby producing NO alone. NO possesses antibacterial and antiviral activities, but a high concentration of NO causes cell injury and thus is detrimental to tissues [13]. Expression of iNOS and COX-2 is regulated by a key transcription factor in inflammation: nuclear factor κB (NF-κB) [1,14,15]. NF-κB also regulates the expression of many genes encoding proinflammatory cytokines and chemokines.

Several cell lines express the *iNOS* gene to produce NO and are used as *in vitro* models of injury to assess the anti-inflammatory effects of drugs. Primary cultured rat hepatocytes produce a large amount of NO in response to IL-1β, and NO production and iNOS expression are suppressed when an anti-inflammatory drug is added with IL-1β [2]. In general, the suppression of IL-1β-induced iNOS expression in hepatocytes is correlated with the anti-inflammatory activity of the drug. Conversely, hepatocyte-derived cell lines, such as HepG2 and HuH-7, show very low expression of iNOS because they lose most of the features of hepatocytes during tumorigenesis [15]. The macrophage line RAW264.7 expresses iNOS and COX-2 and is often used to assess the anti-inflammatory effects of a drug by monitoring NO production and/or PGE_2 production [16,17]. Several macrophage lines are available, but the NO levels produced from these cell lines have not been studied thoroughly.

Suppression of NO induction is a property of anti-inflammatory drugs. The NO suppression in hepatocytes and macrophages has been reported for sodium salicylate [8] and dexamethason [18], as well as for several herbal medicines (Japanese *Kampo* medicines) and their constituents [19–22]. In the case of aspirin and sodium salicylate, other targets of their actions are intracellular signaling pathways, including protein kinases and transcription factors (e.g., NF-κB) [23]. Furthermore, few reports have compared the efficacy and potency of antipyretic analgesic drugs in NO suppression or compared their effects on mRNA expression.

In our studies on anti-inflammatory drugs, we noticed that the efficacy and potency of NO production differed with various drugs and cell lines. In the present study, we compared NO production in several macrophage lines to select a cell line suitable for a NO assay. Then, we compared the anti-inflammatory potency of four commonly-used antipyretic analgesic drugs (i.e., aspirin, loxoprofen, ibuprofen, and acetaminophen) in NO production and *iNOS* gene expression in hepatocytes and macrophages. Finally, we analyzed the transcriptomes affected by these drugs, as well as changes in mRNA expression in the NF-κB pathway, and we elucidated the mechanisms of drug actions modulating iNOS expression.

2. Materials and methods

2.1. Chemicals

Aspirin (Wako Pure Chemical Industries, Osaka, Japan), loxoprofen sodium (Kolon Life Science, Inchon, Korea), ibuprofen (Shiratori Pharmaceutical, Chiba, Japan), and acetaminophen (Nacalai Tesque, Kyoto, Japan) were purchased. Loxoprofen and ibuprofen were racemic mixtures due to their asymmetric carbon atoms.

2.2. Macrophage culture

Murine macrophage lines RAW264.7 (RCB0535), PU5-1.8 (RCB0538), and J774 (RCB2652) and the human monocyte line THP-1 (RCB1189) were obtained from the Cell Bank, Riken BioResource Center (Tsukuba, Ibaraki, Japan). Cells ($0.1–0.5 \times 10^6$ cells per dish) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, and 10% heat-inactivated fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C in a humidified incubator containing 5% CO_2. RAW264.7 cells were seeded at 0.2×10^6 cells per 35-mm dish (Falcon Plastic, Oxnard, CA, USA). After 48 h of culture, cells were treated with 1 μg mL^{-1} LPS (*Escherichia coli* O111:B4; Wako Pure Chemicals) and each drug for 24 h.

2.3. Preparation of primary cultured rat hepatocytes

Male Wistar rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). They were housed at 21–23 °C and allowed to acclimatize to their environment before experimentation. Hepatocytes were isolated from the livers of the rats using the collagenase perfusion method [24]. Briefly, dispersed cells were purified, resuspended in Williams' E medium (Sigma–Aldrich), supplemented with newborn calf serum (SAFC Biosciences, Lenexa, KS, USA), and seeded at 1.2×10^6 cells per 35-mm dish. Cells were incubated at 37 °C for 2 h, and the medium was replaced. The purity of the resultant hepatocytes was >99% according to microscopic observation (data not shown). Hepatocytes were incubated at 37 °C overnight and analyzed the next day (day-1). All animal care and experimental procedures were carried out in accordance with the guidelines and laws of the Japanese Government and were approved by the Animal Care Committee of Ritsumeikan University (Biwako-Kusatsu Campus, Kusatsu, Japan). All efforts were made to minimize animal suffering. All surgery was undertaken under anesthesia (sodium pentobarbital).