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The anti-inflammatory and antinociceptive effects of NF-κB inhibitory guanidine derivative ME10092

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ABSTRACT

The guanidine compound ME10092 (1-(3,4-dimethoxy-2-chlorobenzylideneamino)-guanidine) is known to possess anti-radical and anti-ischemic activity but its molecular targets have not been identified. This study investigated whether ME10092 regulates the nuclear factor kappa B (NF-κB)-mediated signal transduction in vivo.

The effect of ME10092 treatment (1–100 pmol/mouse) on nuclear translocation of NF-κB, activation of expression of inflammatory mediators and production of nitric oxide were measured in the lipopolysaccharide (LPS)-induced brain inflammation model in mice in vivo. The antinociceptive activity of ME10092 was tested in the formalin-induced paw licking test.

ME10092 dose-dependently inhibited LPS-induced nuclear translocation of NF-κB, transcription of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Electron paramagnetic resonance measurements showed that ME10092 inhibited the LPS-induced increase in nitric oxide content in mouse brain tissue in a dose-dependent manner. In the formalin-induced paw licking test, ME10092 (at the dose of 3 mg/kg, p.o. twice daily for eight days) significantly reduced nociceptive response. In conclusion, above results indicate that ME10092 inhibits NF-κB activation and suppresses the up-regulation of inflammatory mediators in experimental models in vivo.

1. Introduction

The guanidine derivative ME10092 (1-(3,4-dimethoxy-2-chlorobenzylideneamino)-guanidine) has shown pharmacological activity in ischemia- and inflammation-related experimental models. Thus, ME10092 has previously demonstrated a strong cardioprotective effect in an experimental rat heart infarction model [1] and in a porcine ischemic heart model [2]. Different pharmacological mechanisms that may underlie the protective effect of ME10092 have been tested. ME10092 was found to bind to α_1- and α_2-adrenoreceptors as well as block adrenaline elicited α_1-adrenergic responses in isolated guinea pig aortas [3]. In both the rat and the baboon, ME10092 decreased blood pressure and heart rate in a dose-dependent manner [3,4]. In addition, it was found that ME10092 possessed a certain anti-oxidative profile as it inhibited the activities of xanthine oxidase, NAD(P)H oxidase and nitric oxide synthase in vivo and in vitro [3].

Together with other melanocortin receptor binding compounds ME10092 was tested for neuroprotective activity in an experimental model of spinal cord injury in rats [5,6]. The anti-inflammatory potential of ME10092 was studied also in a mouse model of contact hypersensitivity [7]. Native melanocortin receptor ligands are known to reduce the main aspects of inflammation through modulation of transcription by redox-sensitive nuclear factor kappa B (NF-κB) [8], which has attracted considerable interest due to its key role in responses to injury, inflammation, and regulation of a multitude of genes. Several of these genes are shown to be activated during reoxygenation after an anoxic period [9–11]. In addition, NF-κB-related signalling pathways have been indicated to play a crucial role in the formation of an immune response, regulation of inflammatory processes, and pain perception [12,13]. The inhibition of NF-κB activation has been used as a promising pharmacological approach in experimental models of inflammatory pain [14–16]. Taking into account the activity of ME10092 in ischemia- and inflammation-related experimental models, in the present study we tested the hypothesis that the anti-inflammatory activity of ME10092 could be mediated through modulation of NF-κB activation. Therefore, we followed the effects of ME10092 throughout the NF-κB signalling pathway in the lipopolysaccharide (LPS)-induced brain inflammation model. The NF-κB signalling pathway was measured by examining activated NF-κB in nuclear extracts, NF-κB-activated pro-inflammatory gene transcription [including tumour necrosis factor-α (TNF-α),

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interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)], and NO production in the forebrain tissues. Since it was shown recently that NF-κB inhibition in glia decreases formalin pain [17], the antinociceptive effect of ME10092 was tested in the formalin-induced paw licking test after per oral (p.o.) administration in mice in vivo. Our data provide evidence that ME10092 inhibits NF-κB activation and thereby suppresses the up-regulation of inflammatory mediators.

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide (LPS, *Escherichia coli* 055:B5), sodium diethyldithiocarbamate (DETC), $FeSO_4$, sodium citrate, bovine serum albumin (BSA), paraformaldehyde, Tris–HCl, NaF, leupeptin, pepstatin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), and TRI reagent were purchased from Sigma (St. Louis, MO, USA). EDTA, $MgCl_2$, NaCl, KCl, KH_2PO_4, $Na_2HPO_4*12H_2O$, o-phenilenediamine dihydrochloride, xylene, ethanol, hydrogen peroxide (H_2O_2) and $CoCl_2$ were purchased from Acros (Belgium). ME10092 was synthesised by Nordic Synthesis, (Karlskoga, Sweden).

2.2. Animals

Male ICR mice (from the Laboratory Animals Breeding Facility, Riga Stradins University, Riga, Latvia) weighing 23–25 g were housed under standard conditions (21–23 °C, 12 h light–dark cycle) with unlimited access to standard food and water. All experimental procedures were carried out in accordance to the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by Ethics Council of Animal Protection at the Veterinary and Food Service, Riga, Latvia.

2.3. Animal treatments

To test the effect of ME10092 on brain inflammation processes, we used an experimental mouse model for acute brain inflammation [18]. The experiment started by intracisternal (i.c.) administration of 10 μg/mouse of LPS with or without different doses of ME10092 (from 1 to 100 pmol/mouse) in a total injection volume of 10 μl. Control animals received i.c. injection of 10 μl saline. Mice were decapitated 6 h after the administration of substances and forebrains were either removed and frozen in liquid nitrogen and stored frozen (−80 °C) until analysed or immediately prepared for determination of NO production.

2.4. Preparation of nuclear extracts, ELISA and EMSA analysis

For determination of activated NF-κB, samples of brain tissue were homogenised 1:10 (w/v) at +4 °C in a buffer containing 100 mM Tris–HCl, pH 7.4, 10 mM EDTA, 5 mM $MgCl_2$, 1 mM NaF and protease inhibitors (10 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, and 100 μM PMSF). Purification of nuclei and determination of activated NF-κB (p65 protein) in the nuclear extracts were performed using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (NF-kappaB Transcription Factor Assay kit, Cayman Chemicals Company, USA).

For electrophoretic mobility-shift assay the double-stranded NF-κB oligonucleotide with binding sequence of 5′-AGTTGAGGGGACTTTCC-CAGGC-3′ was purchased from Promega (Madison, WI, U.S.A.) and labelled with [^{32}P]P_i using Redivue [γ-^{32}P]ATP (Amersham Biosciences, Piscataway) and DNA 5′-end labelling system (Promega). The DNA–protein-binding reaction was conducted in a 20 μl reaction mixture including 3 μg of poly(dI/dC) (Sigma), 10 μg of nuclear protein extract, 40000 c.p.m. of ^{32}P-labelled oligonucleotide probe (1 μl) and 4 μl of 5× gel-shift running buffer [50 mM Tris–HCl (pH 8.0), 750 mM KCl, 2.5 mM EDTA, 0.5% Triton-X 100, 62.5% glycerol (v/v), 1 mM DTT]. This mixture was incubated for 30 min at room temperature, and then 2 μl of dye [25 mM Tris–HCl (pH7.5)/50% (v/v) glycerol/0.1% Bromophenol Blue] was added to stop the reaction. The DNA–protein complexes were resolved on a 4% polyacrylamide gel, and the loaded gel was run at 140 V for 2 h in 0.5× Tris/boric acid/EDTA buffer. The gel was then dried and placed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY, U.S.A.) for autoradiography. The film was developed after a 5-min exposure at room temperature.

2.5. Isolation of RNA and quantitative RT-PCR analysis

Total RNA from brain tissues was isolated using TRI reagent (Sigma, USA) according to the manufacturer's protocol. The quality and quantity of extracted total RNA were examined by measuring the absorbance at 260 and 280 nm with a μQuant™ (BioTek) spectrophotometer and by loading 5 μg of total RNA on a denaturing agarose gel.

The first strand cDNA was synthesised using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) following the manufacturer's instructions. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis for TNF-α, IL-1β, COX-2, iNOS and β-actin was performed by mixing synthesised cDNA, adequate primers, and RT^2 qPCR Master Mix (Super Array). Samples were run in Applied Biosystems Prism 7700 according to the manufacturer's protocol. Transcript levels for the constitutive housekeeping gene product, β-actin, were quantitatively measured for each sample. PCR data are reported as the number of transcripts per the number of β-actin mRNA molecules. All samples were analysed twice with and without reverse transcriptase. To avoid genomic DNA contamination, primers were also designed spanning an intron. The primer sequences used in this study were as follows: TNF-α, 5′-GAC CCT CAC ACT CAG ATC ATC TTC T-3′ (forward) and 5′-CCT CCA CTT GGT GGT TTG CT-3′ (reverse); IL-1β, 5′-CTG GTG TGT GAC GTT CCC ATT A-3′ (forward) and 5′-CCG ACA GCA CGA GGC TTT-3′ (reverse); COX-2, 5′-GCT GGC CTG GTA CTC AGT AGG TT-3′ (forward) and 5′-CGA GGC CAC TGA TAC CTA TTG C-3′ (reverse); iNOS 5′-TCC ATA GTT TCC AGA AGC AG-3′ (forward) and 5′-AAC ACC ACT TTC ACC AAG AC-3′ (reverse); β-actin, 5′-GTA TGA CTC CAC TCA CGG CAA A-3′ (forward) and 5′-GGT CTC GCT CCT GGA AGA TG-3′ (reverse). The primers were obtained from Metabion International AG (Germany).

2.6. Determination of NO production

The determination of NO content in mouse brain tissue was carried out by an electron paramagnetic resonance (EPR) method as described by Vanin et al [19]. Thirty minutes before decapitation, the mice received intraperitoneal (i.p.) injections of spin trap reagent (400 mg/kg DETC) and following subcutaneous injection of ferrous citrate (40 mg/kg $FeSO_4$ + 200 mg/kg sodium citrate) prepared directly in the syringe just before use. The spin trap reagents were administered 5.5 h after LPS and ME10092 administration. The forebrains were dissected and immediately frozen in liquid nitrogen. The frozen brain tissues were compacted into plastic tubes to form rods, 20 mm long and 4 mm in diameter, and these rods were then immediately immersed into liquid nitrogen. EPR spectra of the samples were recorded under liquid nitrogen using an EPR spectrometer Radiopan SE/X2544 (Poland) as described previously. The content of NO was computed from the third spectral component (Ix) at $g = 2.031$. The NO concentration (ng/g of frozen tissue) was calculated according to Vanin et al [19].

2.7. Formalin-induced paw licking test

Formalin-induced paw licking test was performed as described previously by Zvejniece et al [20]. Animals received ME10092 (1 and 3 mg/kg) or saline p.o. twice daily for eight days prior to formalin injection. Other groups received ME10092 (1 and 3 mg/kg) or saline i.p. 30 min prior to formalin injection. In brief, mice were gently