Journal of Ethnopharmacology 127 (2010) 625–630

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Evaluation of guggulipid and nimesulide on production of inflammatory mediators and GFAP expression in LPS stimulated rat astrocytoma, cell line (C6)

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ARTICLE INFO

Article history:
Received 18 May 2009
Received in revised form
10 November 2009
Accepted 5 December 2009
Available online 16 December 2009

Keywords:
Neuroinflammation
Rat astrocytoma cell line (C6)
Lipo-polysaccharide
Guggulipid
Nimesulide

ABSTRACT

Aim of the study: The present study was designed to investigate effect of guggulipid, a drug developed by CDRI and nimesulide on LPS stimulated neuroinflammatory changes in rat astrocytoma cell line (C6).
Materials and methods: Rat astrocytoma cells (C6) were stimulated with LPS (10 μg/ml) alone and in combinations with different concentrations of guggulipid or nimesulide for 24 h of incubation. Nitrite release in culture supernatant, ROS in cells, expressions of COX-2, GFAP and TNF-α in cell lysate were estimated.
Results: LPS (10 μg/ml) stimulated C6 cells to release nitrite, ROS generation, up regulated COX-2 and GFAP expressions at protein level and TNF-α at mRNA level. Both guggulipid and nimesulide significantly attenuated nitrite release, ROS generation and also down regulated expressions of COX-2, GFAP and TNF-α. Guggulipid and nimesulide per se did not have any significant effect on C6 cells.
Conclusion: Results demonstrate the anti-inflammatory effect of guggulipid comparable to nimesulide which suggest potential use of guggulipid in neuroinflammation associated conditions in CNS disorders.
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1. Introduction

Neuroinflammation is an integrated part of neurodegenerative diseases and other CNS disorders (Minagar et al., 2002). Lipopolysacharide (LPS) stimulated glia serves as a good *in vitro* model for mimicking the neuroinflammatory conditions (Rampe et al., 2004). Therefore the inflammatory mediators production may be the potential target(s) for neuroinflammatory therapeutics.

Guggulipid is a standardized guggul ethyl acetate extract (drug developed by CDRI as anti-hyperlipidemic agent) from the resin of *Commiphora wighitii* (Pratap et al., 2005). Guggulu has been used in traditional ayurvedic medicine for the treatment of epilepsy, ulcers, obesity, rheumatoid arthritis, and atherosclerosis since 600 BC (Sahni et al., 2005). Recently guggulipid has been shown as a potent memory enhancer and antioxidant in the streptozotocin induced mouse model of dementia (Saxena et al., 2007). Guggulsterones, the active components of guggulipid inhibit NF-kB activation supporting its anti-inflammatory activity (Shishodia and Aggarwal, 2004). Nimesulide, a standardized, anti-inflammatory drug has shown beneficial effect in rat model of ischemia (Candelario-Jalil et al., 2005). However, effect of guggulipid and nimesulide on brain cells has not been studied.

Therefore the present study was undertaken to investigate the effects of guggulipid and nimesulide on neuroinflammatory events associated to the production of reactive oxygen species (ROS) generation, NO release, MDA production, intracellular GSH level, COX-2, GFAP and proinflammatory cytokine TNF-α expressions in LPS stimulated rat astrocytoma cell line, C6.

2. Materials and methods

2.1. Materials

Primary antibodies, rabbit polyclonal anti-COX-2, anti-β-actin, secondary got anti-rabbit HRP-conjugated antibodies were purchased from Santa Cruz Biotechnology (USA). RT-PCR (5-prime two steps) kit was purchased from Eppendorf (India), and primers specific for TNF-α, GAPDH were purchased from Metabion International AG (Germany). Guggulipid was obtained from medicinal chemistry division of Central Drug Research Institute (CDRI), Lucknow, India. Rabbit polyclonal anti-GFAP and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

LPS initially was dissolved in sterile PBS (stock) and subsequent dilutions were made in the medium. Nimesulide was dissolved in ethanol and guggulipid was dissolved (0.5%) in methyl cellulose

Abbreviations: LPS, lipo-polysacharide; iROS, intracellular reactive oxygen species; RNS, reactive nitrogen species; TNF-α, tumor necrosis factor-α; COX-2, cyclooxygenase-2; GFAP, glial fibrillary acidic protein; DCF-DA, dichlorofluorescein diacetate; GL, guggulipid; iNOS, inducible nitric oxide synthase; MDA, melondialdehyde; GSH, reduced glutathione; GAPDH, glyceraldehyde phosphate dehydrogenase; Ne, nimesulide.

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doi:10.1016/j.jep.2009.12.012

and subsequent dilutions were made in medium as per the required concentrations.

2.2. Cell culture

Rat astrocytoma cell line (C6) was obtained from National Centre for Cell Sciences, Pune, India, and maintained in CDRI tissue culture facility. C6 cells were cultured in DMEM nutrient mixture medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. Cells were incubated with LPS (10 μg/ml) in the absence or presence of non interruptable concentrations of guggulipid 0.78 μg/ml, 1.56 μg/ml, 3.12 μg/ml, 6.25 μg/ml and nimesulide 0.37 μg/ml, 0.75 μg/ml, 1.5 μg/ml for 24 h. Measurement for nitrite release, ROS generation, MDA formation, intracellular glutathione (GSH) Level, TNF-α mRNA level, COX-2 and GFAP expressions was done in respective groups.

2.3. Nitrite estimation

Nitrite estimations in culture supernatant from different groups were done by the Griess reagent (Martinez et al., 2000). C6 cells were seeded at the cell density of 1×10^4 cells/well in 96 well plates. After the treatment period an equal amount of culture supernatant (100 μl) from treated groups were mixed with equal amount (100 μl) of Griess reagent (1% p-amino-benzene sulfonamide, 0.01% napthylethylenediamide in 2.5% phosphoric acid) and an incubation period of 20 min was given in dark and absorbance was read at 570 nm. Nitrite release is expressed as percent increase from basal.

2.4. Measurement of ROS generation

Generation of intracellular ROS (iROS) measurement was accomplished by fluorescence dye DCF-DA (Peng et al., 2005). C6 cells were seeded at the cell density of 1×10^4 cells/well in the 96 well plates. After treatment period medium was aspirated and DCF-DA 100 μl/well (10 μM final concentrations) in phenol red free HBSS buffer was added to plate. A minimum incubation period of 30 min in dark at 37 °C was given to the cells in CO_2 incubator. Fluorescence was measured at wavelength excitation = 485 nm and emission at = 530 nm, by microplate fluorescence reader (using Cary Eclipse software, VARIAN Optical Spectroscopy Instruments, Australia).

2.5. Melondialdehyde estimation

Melondialdehyde (MDA) estimation in C6 cells was done according to the method of Colado et al. (1997). C6 cells were seeded at the cell density of 4×10^5 cells/well in the 6 well plates and were left for 24 h for the proper attachment. After the treatment period cells were scraped in 100 μl of the sodium phosphate buffer pH 7.0. Cells were lysed by sonication (a pulse of 30 s for each) and then centrifuged at $10{,}000 \times g$ for 5 min. Pellet was discarded and supernatant was collected. In supernatant (100 μl) 60 μl of TCA (trichloroacitic acid) and 30 μl of 5N HCl was added. After 5 min of incubation 30 μl of TBA (thiobarbituric acid) in 1N NaOH was added and then samples were heated at 90 °C. After heating samples were again centrifuged at 10,000 RPM for 10 min. Supernatants were then transferred to a micro plate and absorbance was read at 532 nm. MDA level was expressed as percent increase in basal.

2.6. Glutathione estimation

Reduced glutathione (GSH) was measured by the method of Anderson (1985). In brief, C6 cells were seeded at the cell density of 4×10^5 cells/well in the 6 well plates. After incubation period cells were scraped in 100 μl of PBS (ice cold). After lysis (by ultrasonication) cell lysate was centrifuged at $10{,}000 \times g$ for 5 min at 4 °C. Cell supernatants were then deproteinised by adding pre-cooled 10% trichloroacetic acid (100 μl) with an incubation period of 1 h at 4 °C. After this samples were further re-centrifuged at $5000 \times g$ for 5 min. Supernatants were collected and pellets were discarded. Supernatants (75 μl) were mixed with 25 μl of the distilled water + 100 μl of buffer [0.25 M of Tris base + 20 mM EDTA] + 50 μl of DTNB (0.1%). After 10 min of incubation absorbance was measured at 412 nm. Results were expressed as relative GSH level.

2.7. Western blotting for COX-2 and GFAP proteins

After incubation in the presence or absence of different stimuli cell lysate were prepared in the 200 μl of the lysis buffer, containing 100 mM NaCl, 50 mM Tris–HCl, 1 mM EDTA, 1 μg/ml aprotenin, 100 μg/ml phenylmethylsulfonyle fluoride (PMSF), 10 μg/ml pepstatin. Cell lysate was then centrifuged at $10{,}000 \times g$ for 5 min at 4 °C. Protein estimation in supernatant was done by Follin Lowry method. Samples were mixed with the 3× loading buffer containing 100 mM Tris–HCl (pH = 6.8), 200 mM dithiothritol (DTT), 4% sodiumdodecyl sulfate (SDS), 0.2% bromophenol blue, 20% glycerol and were boiled for 5 min at 100 °C. Protein 100 μg for COX-2, 50 μg for GFAP were separated on 8–12% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with blocking buffer (5% non fat dry milk, 10 mM Tris pH = 7.5, 100 mM NaCl, and 0.1% tween-20) over night at 4 °C. Membranes were then treated (after washing) with primary anti-COX-2 and anti-GFAP antibodies at 1:1000 dilutions at room temperature for a period of 2 h. A dilution of 1:2000 was used for anti-beta actin primary antibodies. After washing membranes were again treated with HRP-conjugated secondary antibodies in a 1:2000 dilutions for 1 h at room temperature. Blots were then developed by ECL (enhanced chemiluminescence) system provided by the Amersham Biosciences. Densitometry analyses of bands were accomplished by Alpha Image gel documentation system (Alpha Innotech, U.S.A.).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR) for transcriptional analysis of TNF-α, and GAPDH

RNA was isolated by tri-reagent (Sigma–Aldrich). RNA (2 μg) was quantitated spectrophotometrically and was reverse transcribed using oligo-(dT) primers by kit 5-prime (manual master script kit & manual master script RT-PCR system) according to the manufacture's protocol (Eppendorf). Equal amount of cDNA was subjected to subsequent PCR analysis in a total volume of 50 μl containing 0.5 μM of primers specific for TNF-α, and GAPDH (glyceraldehyde phosphate dehydrogenase). Detail of primers is given in Table 1. PCR was performed at following conditions: (1) 5 min at 94 °C, (2) 45 s at 94 °C, 45 s at 60 °C for TNF-α or 68 °C for GAPDH, 45 s at 72 °C for 35 cycles to TNF-α and GAPDH (30 cycles), (3) 10 min at 70 °C. Controls included RNA subjected to the RT-PCR procedure without addition of reverse transcriptase and PCR performed in the absence of cDNA which always yielded negative results.

2.9. Statistical analysis

Mean ± S.E.M. was calculated and the statistical analysis was done by one-way analysis of variance (ANOVA), followed by Newman–Keuls test as post hoc test. GraphPad prism (version-3) was used to perform statistical tests. The value $p < 0.05$ was considered statistically significant.

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