Pharmacological Reports 67 (2015) 281–288

Contents lists available at ScienceDirect

Pharmacological Reports

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

Original research article

Neuroprotective effect of noscapine on cerebral oxygen–glucose deprivation injury

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ARTICLE INFO

Article history:
Received 9 July 2014
Received in revised form 17 September 2014
Accepted 15 October 2014
Available online 30 October 2014

Keywords:
Noscapine
NO
Oxygen–glucose deprivation
Cortical culture

ABSTRACT

Background: The present study aims to investigate the effect of noscapine (0.5–2.5 μM), an alkaloid from the opium poppy, on primary murine fetal cortical neurons exposed to oxygen–glucose deprivation (OGD), an *in vitro* model of ischemia.
Methods: Cells were transferred to glucose-free DMEM and were exposed to hypoxia in a small anaerobic chamber. Cell viability and nitric oxide production were evaluated by MTT assay and the Griess method, respectively.
Results: The neurotoxicities produced by all three hypoxia durations tested were significantly inhibited by 0.5 μM noscapine. Increasing noscapine concentration up to 2.5 μM produced a concentration-dependent inhibition of neurotoxicity. Pretreatment of cells with MK-801 (10 μM), a non-competitive NMDA antagonist, and nimodipine (10 nM), an L-type Ca^{2+} channel blockers, increased cell viability after 30 min OGD, while the application of NBQX (30 μM), a selective AMPA-kainate receptor antagonist partially attenuated cell injury. Subsequently, cells treated with noscapine in the presence of thapsigargin (1 μM), an inhibitor of endoplasmic reticulum Ca^{2+} ATPases. After 60 min OGD, noscapine could inhibit the cell damage induced by thapsigargin. However, noscapine could not reduce cell damage induced by 240 min OGD in the presence of thapsigargin. Noscapine attenuated nitric oxide (NO) production in cortical neurons after 30 min OGD.
Conclusions: We concluded that noscapine had a neuroprotective effect, which could be due to its interference with multiple targets in the excitotoxicity process. These effects could be mediated partially by a decrease in NO production and the modulation of intracellular calcium levels.

Introduction

Ischemia reperfusion-induced cell injury is a multi-factor, multi-mechanism cascade reaction of damaging events, such as increased release of excitatory amino acids (specifically glutamate), loss of calcium homeostasis, free radical production and ultimately, cell death. These reactions reinforce each other, leading to apoptosis or necrosis [1]. In fact, oxygen and glucose deficits caused by ischemia, result in a massive accumulation of extracellular glutamate [2]. The glutamergic excitation elevates intracellular sodium and calcium, and the high level of intracellular calcium in turn causes mitochondrial dysfunction, protease activation, and accumulation of reactive oxygen species ultimately leading to release of nitric oxide (NO) [3]. Intracellular Ca^{2+} overload activates neuronal nitric oxide synthase (nNOS), resulting in excessive formation of NO and reactive oxygen species (ROS) as well as lipid peroxidation [4]. However, NO and free radicals inhibit both Ca^{2+}-ATPases and the release Ca^{2+} from the endoplasmic reticulum [33].

N-methyl-D-aspartate (NMDA) receptors play a key role in glutamate neurotoxicity. Several studies have reported that Ca^{2+} mediated rapidly triggered excitotoxicity *via* NMDA receptors. In contrast, α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor-triggered excitotoxicity occurs more slowly and requires a long period of activation to induce lethal injury [3]. Although the concentration of intracellular free calcium depends on Ca^{2+} influx through the NMDA receptor, voltage-dependent calcium channels, and Na^{+}/Ca^{2+} exchangers, Ca^{2+}

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http://dx.doi.org/10.1016/j.pharep.2014.10.011
1734-1140/

released from intracellular stores such as the endoplasmic reticulum (ER) also have an important role in glutamate-induced cell injury [5].

Endoplasmic reticulum has been shown to sequester Ca^{2+} from cytosol through sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). In contrast, several stimuli, including inositol 1,4,5-trisphosphate (IP3) can mobilise Ca^{2+} from ER into cytosol [6].

Noscapine is an isoquinoline alkaloid that is derived from the opium poppy *Papaver somniferum* [7]. Unlike most other alkaloids from the opium latex, this drug has no significant analgesic, sedative or euphoric effects generally associated with this group [8]. Noscapine, used routinely as an antitussive drug, has been demonstrated to bind tubulin and cause cell cycle arrest, and thus induce apoptosis in cancer cells both *in vitro* and *in vivo* [9,10]. Many studies demonstrated that noscapine was an efficient agent in the therapy of lymphomas, melanoma, prostate and lung cancers [11]. Ebrahimi et al. [12] while trying to delineate the mechanism of antitussive action of noscapine showed that this alkaloid suppressed the cough response probably by interfering with the actions of bradykinin. As this peptide was thought to be involved in the inflammatory process Mahmoudian et al. [8] suggested its suppression by noscapine could decrease inflammatory damage during ischemia. They went on to show the protective effect of noscapine in an *in vivo* model of ischemic brain injury. Furthermore in a subsequent study, they were able to show that noscapine improved clinical out come and death (from 80% to 20% of subjects) in patient who had suffered stroke [13].

Our laboratory has been interested in clarifying the mechanism of protective effect of noscapine after ischemic insult as it occurs during stroke. As both NO and intracellular calcium overload have been implicated in excitotoxic damage induced by ischemia, it is probable that noscapine may affect one or both of these mediators. This study was undertaken to investigate effects of noscapine on primary murine fetal cortical neurons cell proliferation, *in vitro* and NO generation by these cells under normal or OGD conditions. In order to obtain a better understanding of the mechanistic effects of noscapine, antagonists of receptors known to be important in excitotoxic damage were also used.

Materials and methods

Chemicals and reagents

Noscapine was obtained from Temad-D Pharmaco-Chemical Company (Tehran, Iran). The stock solution was prepared at 1 mol/l in dimethyl sulfoxide (DMSO) and stored at −20 °C. RPMI-1640, glutamine-free Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), horse serum, penicillin–streptomycin, cytosine arabinoside, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), poly-L-lysine, dizocilpine (MK-801), NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), L-NG-nitroarginine methyl ester (L-NAME), thapsigargin and modified Griess reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of the purest grade available from regular commercial sources.

Murine neuronal cell cultures

Primary cultures of cerebral cortex neurons were obtained from mouse embryos (E_{15-18}) as described previously [14,15]. Briefly, timed-pregnant female mice were anaesthetised with chloroform and killed by cervical dislocation. Fetuses were removed and decapitated with small scissors. The skin and skull were removed, the cerebral cortex was isolated and the cortex was cut into small pieces. The tissue was then dissociated by repeated pipetting. The resulting homogenate was centrifuged at 300 × *g* for 5 min at 20 °C and plated on poly-L-lysine-coated 12-well culture dishes at a density of 6×10^4 cells in RPMI-1640 medium supplemented with 10% FBS, 5% horse serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Poly-L-lysine (150,000–300,000 MW) was dissolved in deionised water, and the plate bases were filled with this solution (20 μg/ml) 24 h before the experiment. After 5 min standing at room temperature, the solution was aspirated, and the plates were left to dry in a laminar flow bench overnight. The culture dishes were kept at 37 °C in humidified 95% air and 5% CO_2. After 24–48 h, 10 μM cytosine arabinoside (cytosine 1-β-D-arabinofuranoside) was added to the culture medium to inhibit the proliferation of non-neuronal cells. The culture medium was changed twice weekly, and the neurons were used for experiments after 13–16 days of incubation. All experiments were approved by the Institute of Animal Care Committee at the Iran University of Medical Sciences.

Oxygen–glucose deprivation and drug exposure

Procedures for oxygen–glucose deprivation were performed as described previously [16]. Briefly, the cell cultures were pretreated for 24 h before oxygen–glucose deprivation with 0.5–2.5 μM noscapine dissolved in DMSO. These drug concentrations were chosen based on the results of preliminary experiments to identify nontoxic levels of noscapine (data not shown). The culture medium was replaced with glucose/glutamine-free DMEM, and cells were exposed to hypoxia for 30, 60, and 240 min in a small anaerobic chamber previously filled with 95% (v/v) N_2 and 5% (v/v) CO_2 at 37 °C. To terminate the oxygen–glucose deprivation, the chamber was opened, the medium was replaced with RPMI-1640 containing D-glucose, and the cultures were then placed in an incubator with 5% CO_2 for 24 h recovery. To investigate the effects of various inhibitors on oxygen–glucose deprivation-induced attenuation of cell proliferation, 10 μM MK-801 (a non-competitive antagonist of the NMDA receptor), 30 μM NBQX (an AMPA receptor antagonist), 10 nM nimodipine (an L-type Ca^{2+} channel blockers), 200 μM L-NAME (a NOS inhibitor), and 1 μM thapsigargin (an intracellular calcium depletor) were added to the medium 24 h before oxygen–glucose deprivation. All measurements were made in duplicate, and each experiment was repeated at least three times. Every data point is therefore the mean of at least 6 measurements.

Analysis of neuronal cell viability

Neuronal cell viability was measured using the colorimetric MTT assay, as previously described by Mosmann [17]. Briefly, cells were incubated with 0.5 mg/ml MTT in RPMI at 37 °C under 5% CO_2 for 3 h. The blue formazan reduction product, which is generated by the action of succinate dehydrogenase in living cells on the dye, was dissolved in 100 μL DMSO, and its absorbance was read at 570 nm using a Dynex MMX microplate reader (Dynex, Richfield, MN, USA). The data were expressed as the percentage of viable cells in oxygen–glucose deprivation-exposed plates compared with control normoxic plates.

Measurement of nitrite levels

The level of nitrite as an indicator of NO production in the culture medium was measured using the modified Griess reagent. In brief, after the experiment, the medium in each well was removed and centrifuged at 10,000 × *g* for 10 min at 20 °C. 100 μL of the supernatant was mixed with an equal volume of Griess reagent at room temperature for 10 min, and the absorbance was measured at 540 nm using a microplate reader. The nitrite concentration was determined from a sodium nitrite standard curve. Plates not exposed to oxygen–glucose deprivation were