Available online at www.sciencedirect.com

SCIENCE DIRECT

Brain Research 1037 (2005) 200–203

BRAIN RESEARCH

www.elsevier.com/locate/brainres

Short communication

Xanthine oxidase, nitric oxide synthase and phospholipase A_2 produce reactive oxygen species via mitochondria

Basavaraju G. Sanganahalli, Preeti G. Joshi, Nanda B. Joshi*

Department of Biophysics, National Institute of Mental Health and Neuro Sciences, Bangalore-560029, India

Accepted 1 January 2005

Abstract

The formation of reactive oxygen species (ROS) has been suggested to be associated with excitotoxicity but the involvement of cytoplasmic enzymes in ROS formation is not clearly known. In the present study, we examined the role of xanthine oxidase (XO), nitric oxide synthase (NOS) and phospholipase A_2 (PLA_2) in glutamate-induced oxidative stress in rat cortical slices. Glutamate-induced ROS formation and mitochondrial depolarization were measured in rat cortical slices in presence of allopurinol, L-NAME and 4-bromophenacylbromide, the specific inhibitors of XO, NOS and PLA_2, respectively. Upon stimulation of slices with glutamate, a significant increase in ROS formation and mitochondrial depolarization was observed. However, pretreatment of slices with allopurinol, L-NAME and 4-bromophenacylbromide inhibited the glutamate-induced ROS formation and mitochondrial depolarization. The glutamate-induced ROS formation was dependent on the concentration of these inhibitors and also on the duration of the treatment. Allopurinol was found to be less effective as compared to L-NAME and 4-bromophenacylbromide. The combined treatment of slices with these enzyme inhibitors showed further inhibition in ROS formation and mitochondrial depolarization. The inhibition in ROS formation as well as mitochondrial depolarization by allopurinol, L-NAME and 4-bromophenacylbromide clearly suggests that the activation of XO, NOS and PLA_2 by calcium during glutamate receptor stimulation may release some chemicals which depolarize mitochondria resulting in ROS formation.

Theme: Neurotransmitters, modulators, transporters and receptors
Topic: Excitatory amino acids-excitotoxicity

Keywords: Neurodegeneration; Mitochondria; Calcium regulation; Reactive oxygen species

Excitatory amino acid neurotransmitter, glutamate, is implicated in a variety of acute and chronic neurological disorders, such as ischemia, stroke, trauma, Alzheimer's disease, Huntington's disease and amyotropic lateral sclerosis [1,6,10,11,15,17,23]. It is widely believed that activation of glutamate receptor causes a large influx of Ca^{2+} [1,5,12], but the immediate calcium-dependent events responsible for cell death remain to be elucidated. Several workers have reported the ROS formation in glutamate-induced cell death [7,9,13,19,20,22]. Cell culture models and in vivo experiments indicate that ROS such as superoxide anion, hydroxyl radical and hydrogen peroxide, etc. are produced during neuronal cell death, but exact source of ROS production is not yet clearly known. Several studies in cultured neurons have suggested that mitochondria are the likely source of ROS production during glutamate exposure [16,18,22]. On the other hand, xanthine oxidase (XO), nitric oxide synthase (NOS) and phospholipase A_2 (PLA_2) have also been shown to produce ROS upon increased $[Ca^{2+}]_i$ [2,4,8,14]. However, the exact role of these enzymes in glutamate-induced ROS production in relation to mitochondria remains unclear. The present study was carried out to evaluate the involvement of these enzymes in glutamate-mediated ROS production. The glutamate-induced ROS production was measured in rat

* Corresponding author. Fax: +91 80 656 4830.
E-mail address: nbjoshi@nimhans.kar.nic.in (N.B. Joshi).

0006-8993/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2005.01.013

cortical slices in presence of allopurinol, L-NAME and 4-bromophenacylbromide, the specific inhibitors of XO, NOS and PLA_2, respectively.

Sprague–Dawley rats aged 80 ± 5 days were used and the animal experiments were carried out in accordance with the guidelines laid down by the institute ethics committee for the care and use of animals for experimental work. The cortical slices were prepared from frontal cortex region as described previously [12,13]. After decapitation, the whole brain was removed and placed in ice cold Krebs–Hensleit buffer containing 124 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 23 mM $NaHCO_3$, 3 mM HEPES and 10 mM D-glucose and was equilibrated with 95% O_2/5% CO_2 (pH 7.4). A hand held blade slicer consisting of two blades separated by a spacer of 350 μm thickness, was used to obtain one slice from each cortical surface. The slices were incubated for 60 min in fresh medium, pH 7.4 at 37 °C.

Fluorescence measurements were performed with SLM 8000C spectrofluorometer as described earlier [12,13]. The dye loaded slice was washed with fresh buffer and subsequently mounted on the baffle for fluorescence measurement. The formation of reactive oxygen species in slices was measured using 2′, 7′ dichlorofluorescein (2′, 7′-DCF). The slices were incubated with 100 μM 2′, 7′-dichlorofluorescein diacetate (2′, 7′-DCFH-DA) in K-H buffer for 30 min at 30 °C. The excitation and emission wavelengths used were 490 nm and 530 nm, respectively. ROS formation was expressed as percent increase in the fluorescence intensity. Mitochondrial membrane potential was measured using rhodamine 123 (Rh123) as described elsewhere [12,13]. Briefly, the slices were incubated with 2 μg/ml Rh123 in K-H buffer for 20 min at 30 °C. Mitochondrial membrane potential changes were measured as the change in the fluorescence intensity of Rh123 at 537 nm using excitation wavelength of 490 nm. An increase in the fluorescence intensity indicates mitochondrial depolarization. In control experiments, the fluorescence intensity of Rh123 was measured for the same duration in slices not treated with glutamate.

Data are presented as mean ± SD of more than five experiments. Statistical comparison of data was performed by Student's *t*-test and the difference was considered significant with $P < 0.05$.

Earlier we have shown that stimulation of brain slices with glutamate induced ROS formation in a Ca^{2+}-dependent manner [13]. As given in Table 1, the specific agonists of ionotropic glutamate receptors NMDA, AMPA and kainite induced ROS formation suggesting that both NMDA and non-NMDA (AMPA/Kainate) receptors are involved in glutamate induced ROS formation. Rat cortical slices were treated with allopurinol, L-NAME and 4-bromophenacylbromide and the ROS formation was measured upon exposure to glutamate. Fig. 1 shows the ROS formation in presence of different concentrations of these inhibitors. The ROS formation was gradually inhibited by increasing concentration of allopurinol, L-NAME and 4-bromophenacylbromide up to 25 μM and no further inhibition was observed at higher concentrations. Fig. 2 depicts the time course of glutamate-induced ROS production in absence and presence of 50 μM allopurinol, L-NAME and 4-bromophenacylbromide. In case of L-NAME and 4-bromophenacylbromide, the inhibition in ROS formation was observed as early as 5 min after glutamate exposure, whereas allopurinol exerted the inhibitory effect after 30 min. At 60 min, allopurinol, L-NAME and 4-bromophenacylbromide inhibited glutamate induced ROS formation by 22%, 56% and 43%, respectively. These results suggest that activation of XO, NOS and PLA_2 enzymes by $[Ca^{2+}]_i$ generates ROS in cortical slices during glutamate exposure.

Table 1
Glutamate induced ROS formation in cortical slices in presence of NMDA, AMPA and kainate

Agonists	DCF fluorescence $(F - F_0) / F_0 \times 100$
NMDA	119.0 ± 4.6
AMPA	30.1 ± 4.5
Kinate	161 ± 11.4

The concentration of agonist used was 600 μM and the measurements were made at 60 min time point. The data represent the mean ± SD of more than five experiments.

It is possible that Ca^{2+}-dependent activation of these enzymes may produce ROS directly or indirectly via mitochondria by perturbing the mitochondrial functions. To explore the second possibility, we studied the effect of allopurinol, L-NAME and 4-bromophenacylbromide on glutamate-induced depolarization of mitochondrial membrane. Pretreatment of slices with 50 μM of allopurinol, L-NAME and 4-bromophenacylbromides reduced the glutamate induced mitochondrial depolarization as shown in Fig. 3. Allopurinol, L-NAME and 4-bromophenacylbromide inhibited glutamate induced mitochondrial depolarization by 20%, 56% and 52%, respectively at 5 min after stimulation and it did not show significant change further. L-NAME was found most effective, whereas allopurinol showed minimum effect. The decrease in glutamate-mediated mitochondrial depolarization in presence of allopurinol, L-NAME and 4-bromophenacylbromide suggests that XO, NOS and PLA_2 are involved in perturbing the mitochondria which subsequently produces ROS.

The data presented here reveal that the pretreatment of slices with the inhibitors of XO, NOS and PLA_2 confers protection against glutamate induced oxidative stress. The decrease in glutamate stimulated ROS formation in presence of allopurinol, L-NAME and 4-bromophenacylbromide indicates that XO, NOS and PLA_2 are involved in the ROS formation. However, suppression of mitochondrial depolarization by these inhibitors suggests that ROS formation is linked with mitochondria. We have earlier shown that the glutamate induced ROS formation was