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Ammonia inhibits the C-type natriuretic peptide-dependent cyclic GMP synthesis and calcium accumulation in a rat brain endothelial cell line

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Abstract

Recently we reported a decrease of C-type natriuretic peptide (CNP)-dependent, natriuretic peptide receptor 2 (NPR2)-mediated cyclic GMP (cGMP) synthesis in a non-neuronal compartment of cerebral cortical slices of hyperammonemic rats [Zielińska, M., Fresko, I., Konopacka, A., Felipo, V., Albrecht, J., 2007. Hyperammonemia inhibits the natriuretic peptide receptor 2 (NPR2)-mediated cyclic GMP synthesis in the astrocytic compartment of rat cerebral cortex slices. Neurotoxicology 28, 1260–1263]. Here we accounted for the possible involvement of cerebral capillary endothelial cells in this response by measuring the effect of ammonia on the CNP-mediated cGMP formation and intracellular calcium ($[Ca^{2+}]_i$) accumulation in a rat cerebral endothelial cell line (RBE-4). We first established that stimulation of cGMP synthesis in RBE-4 cells was coupled to protein kinase G (PKG)-mediated Ca^{2+} influx from the medium which was inhibited by an L-type channel blocker nimodipine. Ammonia treatment (1 h, 5 mM NH_4Cl) evoked a substantial decrease of CNP-stimulated cGMP synthesis which was related to a decreased binding of CNP to NPR2 receptors, and depressed the CNP-dependent $[Ca^{2+}]_i$ accumulation in these cells. Ammonia also abolished the CNP-dependent Ca^{2+} accumulation in the absence of Na^+. In cells incubated with ammonia in the absence of Ca^{2+} a slight CNP-dependent increase of $[Ca^{2+}]_i$ was observed, most likely representing Ca^{2+} release from intracellular stores. Depression of CNP-dependent cGMP-mediated $[Ca^{2+}]_i$ accumulation may contribute to cerebral vascular endothelial dysfunction associated with hyperammonemia or hepatic encephalopathy.

Keywords: Ammonia; RBE-4 cells; C-type natriuretic peptide; cGMP; Calcium influx

1. Introduction

Ammonia, a primary pathogenic factor in hepatic encephalopathy (HE) affects a wide range of subcellular and cellular targets in the central nervous system (CNS) (Albrecht and Jones, 1999; Hazell and Butterworth, 1999; Norenberg, 2003; Felipo, 2006). Recent evidence suggests an involvement of alterations of cGMP synthesis in the brain in ammonia neurotoxicity. Acute increase of brain ammonia is associated with enhanced accumulation of cGMP, which results from overstimulation of soluble guanylyl cyclase (sGC) due to NMDA-dependent activation of nitric oxide (NO) synthesis (Hermenegildo et al., 2000; Hilgier et al., 2004). Prolonged ammonia exposure leads to depletion of brain cGMP which is held responsible for some cognitive symptoms retreating upon prevention of cGMP degradation (Erceg et al., 2005). This depletion has likewise been attributed to alterations in the NO-dependent cGMP synthesis: impaired NMDA receptor activation and/or depletion/dysfunction of soluble guanylate cyclase (sGC) (Monfort et al., 2007; Rodrigo et al., 2007). However, cGMP in the brain is also synthesized following interaction of natriuretic peptides (NPs): atrial natriuretic peptide (ANP); brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), with natriuretic peptide receptors (NPR), the membrane-bound (particulate) guanylate cyclases (Pandey, 2005). Of the 3 NPs, CNP is the one that predominates in the brain and acts in an autocrine manner (Kone, 2001). CNP also shows the highest affinity to NPR2 (Koller et al., 1991; Braunewell et al., 2001), which in turn is the prevailing NPR isoform in the CNS (Lucas et al., 2000; Pandey, 2005).

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Recently we observed that acute hyperammonemia leads to a decrease of CNP-dependent cGMP synthesis in the cerebral cortical slices (Zielińska et al., 2007). Interestingly, this inhibitory effect was confined to brain compartments that are sensitive to inhibition by fluoroacetate; this could be represented either by astrocytes (Goncalves et al., 1995) and/or endothelial cells of the cerebral blood vessels (Rist et al., 1996). We focused first on the cerebral endothelial cells because of their well-documented high activity of CNP-dependent cGMP synthesis (Vigne et al., 1994; Tao et al., 1995). In the present study we characterized CNP-mediated cGMP production in an immortalized rat brain endothelial cell line, RBE-4, and analyzed the effect of ammonia on the CNP/NPR2/cGMP pathway. In light of a recent observation by Zolle et al. (2000), that BNP acting at NPR, affects in a complex way Ca^{2+} fluxes in a peripheral endothelial cell line, we hypothesized that cGMP synthesized by NPR2 following stimulation by CNP, and acting *via* PKG may affect $[Ca^{2+}]_i$ accumulation in RBE-4 cells. We therefore studied the effect of CNP added in the presence or absence of a PKG inhibitor on intracellular $[Ca^{2+}]_i$ in control- and ammonia-treated RBE-4 cells using confocal microscopy of Fluo-3-loaded cells. We made a preliminary attempt to identify the routes of Ca^{2+} entry and/or exit in these cells and to see how they are affected by ammonia.

2. Materials and methods

2.1. RBE-4 cell culture

Cells were cultured on the αMEM/Ham's F10 medium with glutamax (Gibco) containing 10% of heat-inactivated FCS, bFGF and geneticine. Four days old cultures were subjected to further analyses.

2.2. Treatments

Standard Krebs Medium (SKM) contained 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, aerated with 95% O_2 and 5% CO_2 at pH 7.4. Calcium-free medium was devoid of Ca^{2+} ions and supplemented with 0.5 mM EGTA. In sodium-free media, NaCl was replaced with equimolar LiCl or *N*-methyl-D-glucamine (NMDG). Additionally, sodium-free media contained 20 mM HEPES.

Cells were treated with 5 mM ammonium chloride (ammonia) for 1 h. CNP (Pennisula Labolatories) was added at a final concentration of 1 μM, for times indicated. A PKG inhibitor, 50 μM Rp-8-pCPT-cGMPS (Biolog) and L-type calcium channel inhibitor, 100 nM nimodipine (Sigma) were added 20 and 40 min before the onset of scanning, respectively.

2.3. cGMP assay

cGMP was determined with cGMP Enzymeimmunoassay Biotrak (EIA) System (Amersham Biosciences) according to the manufacturer's protocol. A non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxantine (IBMX) (Sigma) at 0.3 mM concentration was added 3 min before CNP treatment.

2.4. Measurement of $[Ca^{2+}]_i$ with confocal microscopy

RBE4 cells were loaded with 25 μM Fluo-3 AM fluorescent probe (Invitrogen) in Krebs buffer containing 2.5 mM Ca^{2+} according to manufacturer's protocol. After that cells were washed 3 times with Krebs buffer and after 30 min observed under confocal microscope (Zeiss LSM 510, software package v. 2.8) at 485 nm excitation and 538 emission wavelengths. Cells were scanned every 30 s. Results are expressed relative to the basal value of optical intensity and at least 40 cells were measured in each experiment.

2.5. Membrane binding of biotinylated CNP

CNP binding to NPR2 receptors was measured using biotinylated CNP and an ELISA-system. Confluent RBE-4 cell cultures were incubated for 20 s, 1, 5 or 10 min with biotinylated CNP (Phoenix Pharmaceuticals, Inc.) at 0.2 μM concentration. Then cells were fixed with 2% ice-cold paraformaldehyde in phosphate buffered saline (PBS), for 10 min and washed four times with PBS. Receptor-bound biotinylated CNP located on the cell surface was detected by incubation with streptavidin-coupled horseradish peroxidase (Zymed Laboratories) for 45 min. After triple washing with PBS, the cells were incubated with peroxidase substrate, ABTS (Zymed Laboratories) at 37 °C for 30 min. The reaction was stopped by addition of an equal volume of 0.01% sodium azide in 0.1 M citric acid and absorbance of the supernatant was measured at 415 nm in Bio-Rad Model 680 Microplate Reader.

2.6. Statistical analysis

Statistical analysis was performed using one-way analysis of variance followed by the Dunnet's comparison test or using the two-tailed Student's test.

3. Results

3.1. CNP-dependent cGMP accumulation

CNP (1 μM) added in the presence of 0.5 mM concentration of non-specific phosphodiesterase inhibitor IBMX caused a 6-fold stimulation of cGMP production at 15 and 45 min of incubation and a 13-fold at 30 min of incubation with RBE-4 cells (Fig. 1). Ammonia treatment significantly reduced CNP-dependent cGMP production as measured at 45 min of incubation, but did not affect basal cGMP levels.

3.2. Receptor binding of biotinylated CNP

Ammonia decreased the binding of biotinylated CNP to RBE-4 cells at all the incubation times ranging from 20 s to 10 min (Fig. 2).

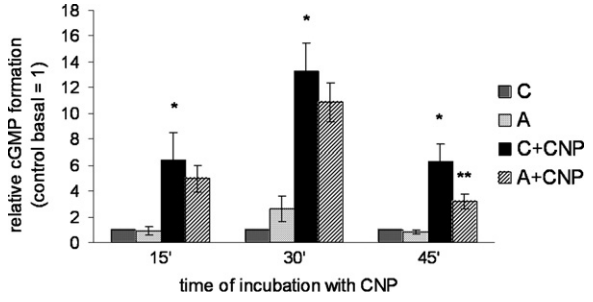

Fig. 1. Basal and CNP-stimulated cGMP production in control (C) and 5 mM ammonium chloride (A) pretreated cells at 15, 30 and 45 min of incubation. Results are mean ± S.E.M. from 3 independent experiments. $*p < 0.05$ vs. C; $**p < 0.05$ vs. C + CNP (Dunnet's test). cGMP production in absolute values in control samples: 1.03 ± 0.55 pmoles/mg protein for 15 min of incubation; 1.1 ± 0.5 pmoles/mg protein for 30 min of incubation; 2.3 ± 0.36 pmoles/mg protein for 45 min of incubation.