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Short communication

Mouse hepatocytes lacking mGlu5 metabotropic glutamate receptors are less sensitive to hypoxic damage

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Abstract

Endogenous activation of type-5 metabotropic glutamate receptors contributes to the development of hypoxia-induced liver cell injury. We have strengthened this hypothesis using glutamate mGlu5 receptor knockout mice. Hepatocytes isolated from knockout mice were less sensitive to hypoxic cell damage than hepatocytes from wild-type mice as assessed by lactate dehydrogenase release and formation of reactive oxygen species. The mGlu5 receptor antagonist, 2-methyl-6-(phenylethynyl)pyridine (MPEP) also protect hepatocytes against hypoxic damage.

Keywords: mGlu5 knockout, mouse; Hypoxia; Reactive oxygen species

1. Introduction

Receptors for glutamate, are currently classified into two major categories: ionotropic glutamate receptors (*N*-methyl-D-aspartic acid-NMDA-, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-AMPA-, and kainate receptors), which form membrane ion channels, and metabotropic glutamate receptors (subtypes: mGlu1 to −8), which are coupled to GTP binding proteins (Nakanishi, 1992). Hepatocytes specifically express the glutamate mGlu5 receptor, which is coupled to polyphosphoinositide hydrolysis (Sureda et al., 1997). Pharmacological blockade of this receptor protects isolated hepatocytes against hypoxic damage (Storto et al., 2000), and attenuates acetominophen hepatotoxicity in mice (Storto et al., 2003). The availability of glutamate mGlu5 receptor knockout mice (Lu et al., 1997) allows a more direct investigation of liver glutamate mGlu5 receptors. We now report that hypoxic damage is delayed and attenuated in hepatocytes isolated from knockout mice.

2. Materials and methods

2.1. Materials

MPEP was purchased from Tocris Cookson (Bristol, UK). All other chemicals were purchased from Sigma (Milano, Italy).

2.2. Animals

mGlu5 knockout mice were obtained from the Jackson Laboratories (Bar Harbor, ME).

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2.3. Hepatocyte isolation and treatments

Hepatocytes were isolated from adult wild type and mGlu5 knockout mice by collagenase perfusion of the liver, as described by Moldeus et al. (1978). Hypoxia was induced by blowing nitrogen into hermetically sealed vials containing the cell suspension, whereas control vials were exposed to an oxygen-containing atmosphere. All vials were saturated with nitrogen at the same time, and were kept hermetically closed throughout the duration of the experiment (from 0 to 90 min). When indicated, hepatocytes were incubated with MPEP starting 30 min prior to the exposure to hypoxia.

2.3.1. Measurement of cell viability, reactive oxygen species formation

Cell viability was monitored by measuring the activity of lactate dehydrogenase released into the medium, as described by Bergmeyer (1965). Maximal lactate dehydrogenase release was determined after exposing the cells to 10% of triton-X-100 (50 μl into 1 ml of cell suspension). Reactive oxygen species formation was monitored as follows. Hepatocytes were preloaded with the cell permeant probe dichlorodihydrofluorescein diacetate at concentrations of 5 μM for 15 min prior to the onset of hypoxia. At the indicated times, aliquots containing 10^6 cells were withdrawn and centrifuged at 2000×*g* for 30 s. Cell pellets were resuspended in 0.5 ml distilled water and immediately frozen. Samples were quickly thawed and 200 μl samples were transferred to a 96-well plate for fluorescence measurements. Fluorescence values were measured on a CytoFluor 4000 (excitation 485 nm; emission 530 nm).

2.3.2. Western blot analysis of mGlu5 receptors

Cell pellets were washed two times with phosphate buffered saline (PBS), resuspended and sonicated at 4 °C in ice-cold sodium-dodecyl sulfate (SDS)-lysis buffer containing 1 mM phenyl methyl sulfonyl fluoride (PMSF), pH 7.4; the cerebral cortex dissected from the brain was homogenized at 4 °C in ice-cold lysis buffer with a motor-driven Teflon-glass homogenizer (1700 rev./min). Five microliters from cellular and tissue were used for protein determinations; 70 μg of proteins were resuspended in SDS-bromophenol blue reducing buffer with 40 mM dithiothreitol and used for protein identification. Western blot analyses were carried out using 8% SDS polyacrylamide gels run on a minigel apparatus (Biorad, Mini Protean II Cell); gels were electroblotted on ImmunBlot polyvinylidene difluoride Membrane (Biorad, Italy) for 1 h using a semi-dry electroblotting system (Biorad, Trans-blot system SD), and filters were blocked overnigth in Tween-tris-buffered saline (TTBS) buffer (100 mM Tris–HCl; 0.9% NaCl, 0.1% Tween 20, pH 7.4) containing 5% non-fat dry milk. Blots were then incubated for 1 h at room temperature with primary polyclonal antibodies (1 μg/ml) which recognize a specific carboxy-terminal epitope of mGluRs (Upstate Biotecnology, DBA, Italy). Blots were washed three times with TTBS buffer and then incubated for 1 h with secondary antibodies (peroxidase-coupled anti mouse, Amersham)

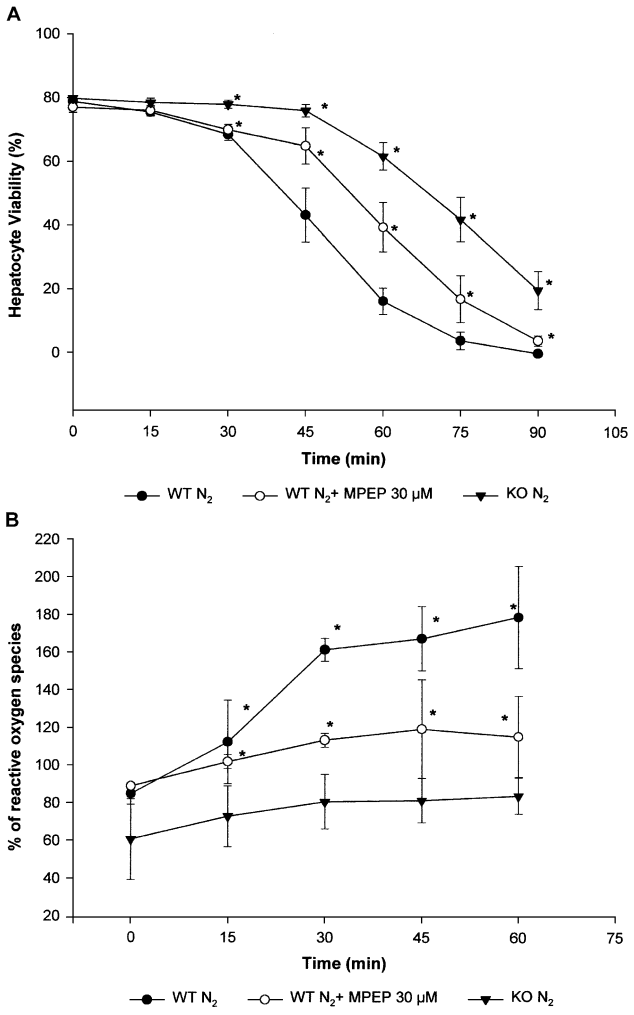

Fig. 1. (A) Hepatocytes isolated from the liver of knockout mice are protected against hypoxic damage. N_2=hypoxia; WT=hepatocytes from wild-type mice; KO=hepatocytes from mGlu5 receptor knockout mice. Hypoxia was delivered at time=0. MPEP was applied 30 min prior to the onset of hypoxia. Note that hepatocytes from mGlu5 knockout mice are more protected than hepatocytes from wild-type mice treated with MPEP. Data refers to lactate dehydrogenase release and are calculated as % of cell viability. Values are means±S.E.M. of four determinations from four individual hepatocyte preparations. *P<0.01 (One-way ANOVA+Fisher's PLSD) vs. the respective WT N_2 values. There was no difference among the three groups when viability was measured under normoxic conditions. (B) Hypoxia-induced reactive oxygen species formation is prevented in hepatocytes isolated from mGlu5 knockout mice. N_2=hypoxia; WT=hepatocytes from wild-type mice; KO=hepatocytes from mGlu5 receptor knockout mice. Hypoxia was delivered at time=0. MPEP was applied 30 min prior to the onset of hypoxia. Data are expressed as per cent of reactive oxygen species formation with respect to WT hepatocytes incubated under normoxic conditions. Values are means±S.E.M. of four determinations from four individual hepatocyte preparations. *P<0.01 (One-way ANOVA+Fisher's PLSD) vs. the respective WT N_2 values. There was no significant difference among the three groups when reactive oxygen species formation was measured under normoxic conditions, although a trend to a reduction was observed in hepatocytes from mGlu5 receptor knockout mice.

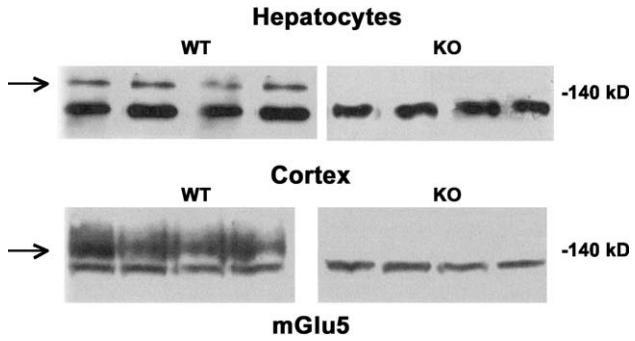

Fig. 2. Western blot analysis of mGlu5 receptors in hepatocytes and cerebral cortex from the wild-type and mGlu5 receptor knock-out mice used for the assessment of hypoxic damage. The arrows indicate the band corresponding to the mGlu5 receptor monomer.

diluted 1:10,000 with TTBS. Immunostaining was revealed by enhanced-chemiluminescence luminosity (Amersham).

3. Results

In isolated hepatocytes from wild-type mice, hypoxia led to time-dependent reduction in cell viability, which became substantial after 30–45 min of exposure. Cell death was nearly maximal after 75 min of hypoxia. Addition of the mGlu5 receptor antagonist, MPEP delayed the development of hepatocyte damage (Fig. 1A). In hepatocytes isolated from mGlu5 knockout mice a substantial reduction in cell viability was observed only after 60–75 min, and cell death was not yet maximal after 90 min of hypoxia (Fig. 1A). These results were paralleled by measurements of reactive oxygen species formation in isolated hepatocytes (Fig. 1B). Western blot analysis carried out in protein extracts from mouse hepatocytes and cerebral cortex (used as a positive control) showed that the upper band corresponded to the mGlu5 receptor monomer, which was absent in the cerebral cortex of knockout mice and in the hepatocytes of knockout mice used for the assessment of cell viability in our experiments (Fig. 2).

4. Discussion

Using mGlu5 knockout mice we have demonstrated that mGlu5 receptors play a permissive role in the development of hypoxic cell damage in isolated hepatocytes. mGlu5 receptors are coupled to polyphosphoinositide hydrolysis and their activation generates oscillatory increases in intracellular calcium concentrations. Although the physiological role of mGlu5 receptors in the liver remains to be identified, it is likely that the release of intracellular calcium generated from receptor activation becomes detrimental under pathological conditions, such as liver hypoxia. The spontaneous activity of mGlu5 receptors or their activation by the glutamate released from hepatocytes (from 2 to 13 μM; Storto et al., 2000) may be responsible for the amplification of hypoxic damage. It is noteworthy that MPEP, i.e. the drug that was proven to be protective against liver damage (Storto et al., 2000, 2003 and present data), behaves as non-competitive antagonist/inverse agonist, i.e. it can inhibit the constitutive activity of mGlu5 receptors (see Schoepp et al., 1999 for a review). It will be interesting to examine whether pharmacological blockade of mGlu5 receptors is beneficial under conditions of hypoxic liver damage, as occurs during severe episodes of hypotension or during organ transplantation.

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