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Estrogen suppresses the impact of glucose deprivation on astrocytic calcium levels and signaling independently of the nuclear estrogen receptor

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Glucose deprivation of astrocytes results in an elevation of cytosolic calcium concentration ($[Ca^{2+}]_i$) [Kahlert, S., Reiser, G., 2000. Requirement of glycolytic and mitochondrial energy supply for loading of Ca^{2+} stores and $InsP_3$-mediated Ca^{2+} signaling in rat hippocampus astrocytes. J. Neurosci. Res. 61, 409–420; Silver, I.A., Deas, J., Erecinska, M., 1997. Ion homeostasis in brain cells: differences in intracellular ion responses to energy limitation between cultured neurons and glial cells. Neuroscience 78, 589–601] equivalent to an impairment of astrocytic energy metabolism and function. Superfusion of fura-2 loaded primary cortical astrocytes with glucose-free solution triggered a slow and progressive, 56-fold increase of the $[Ca^{2+}]_i$ from 60 nM up to 3.3 μM within 2 h. Re-addition of glucose led to a rapid drop of $[Ca^{2+}]_i$, yet $[Ca^{2+}]_i$ did not fully recover to the low levels recorded prior to glucose deprivation and, moreover, astrocytic Ca^{2+} signaling was impaired: adenosine 5′-triphosphate (ATP) and uridine 5′-triphosphate (UTP) were no longer able to trigger a transient Ca^{2+} response as recorded in controls. 17β-estradiol protected astrocytes from the glucose deprivation-induced $[Ca^{2+}]_i$ increase and the impaired signaling independently of the nuclear estrogen receptor, as the antiestrogen tamoxifen and the protein synthesis inhibitor cycloheximide did not impede the protective effect of 17β-estradiol.

Keywords: Astrocytes; Glucose deprivation; Calcium; Estrogen

Introduction

Glycolysis has been described as the main process of energy production in astrocytes (Magistretti and Pellerin, 1999). Consequently, glucose deprivation leads to functional disturbances in this brain cell type, similar to disturbances resulting from an increase of intracellular calcium (Duffy and MacVicar, 1996; Juurlink et al., 1996; Kahlert and Reiser, 2000; Silver et al., 1997). Aglycemia is involved in ischemia, but can also occur in vivo following an overdose of insulin in diabetic patients. If aglycemia is profound, brain metabolism may severely be affected resulting in coma or, possibly, permanent brain injury (Auer et al., 1989). In addition, prenatal aglycemia leads to destruction of the white matter. Therefore, the study of the impact of glucose deprivation on astrocytes may help to elucidate mechanisms for preventing negative effects.

Clinical evidence suggests that estrogen acts neuroprotectively against various neurodegenerative disorders including stroke (reviewed in Dhandapani and Brann, 2002a,b; Garcia-Segura et al., 2001). Simpkins et al. (1997), for instance, have shown that pretreatment with 17β-estradiol reduces animal mortality and ischemic area in ovariectomized rats after middle cerebral artery occlusion.

Estrogen exerts its protective effect in response to degenerative diseases or injury through several pathways that can be classified into the following three groups: genomic pathways, non-genomic pathways, and free-radical scavenging pathways. Genomic pathways are mediated by the classical nuclear estrogen receptor, through which estrogen alters expression of estrogen responsive genes that play a role in cell differentiation, death, survival, or general trophic support. The second group of non-genomic pathways is independent of the classically defined nuclear receptors. These involve unidentified receptors in the plasma membrane or the cytoplasm, or hormonal activation of intracellular signaling pathways (Disshon and Dluzen, 1997; Gu and Moss, 1996; Morales et al., 2003; Moss and Gu, 1999; Raap et al., 2000), for instance, alterations of intracellular calcium levels (Beyer and Raab, 1998; Pozzo-Miller et al., 1999). Finally, the third group of non-receptor antioxidant free-radical scavenging pathways is primarily observed when pharmacological, non-physiologically high doses of estrogen in the micromolar range are applied (Behl et al., 1995, 1997; Culmsee et al., 1999; Moosmann and Behl, 1999; Sugishita et al., 2003).

There is substantial evidence that glial cells are targets for estrogens (Garcia-Segura et al., 1996; Jordan, 1999; Langub and

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Watson, 1992; Nichols, 1999). Astrocytes express the nuclear estrogen receptors ERα and ERβ (Azcoitia et al., 1999; Hosli et al., 2000, 2001; Jung-Testas et al., 1992; Langub and Watson, 1992; Santagati et al., 1994). Therefore, they are an essential component of both normal neural function as well as of normal response of neural tissue to injury (Garcia-Segura et al., 1996, 1999b; Jordan, 1999; Melcangi et al., 1999; Mong and McCarthy, 1999).

This work demonstrates that 17β-estradiol is able to protect astrocytes from the impact of glucose deprivation on intracellular calcium levels and impaired neurotransmitter-induced signaling. Evidence obtained so far points to a non-classical rapid estrogen action in astrocytes. The exact mechanism and physiological consequences of this phenomenon are not yet understood but might have a pivotal role for estrogen-mediated protective effects in astrocytes.

Materials and methods

Reagents

All chemicals were purchased from Sigma-Aldrich, Taufkirchen, Germany unless otherwise noted. Estradiols, cholesterol, testosterone, tamoxifen, and nifedipine were prepared as stock solutions in ethanol, KB-R7943 as stock solution in DMSO and stored at −20°C for up to 2 weeks.

Cell culture

Primary cultures of cortical astrocytes were prepared from neonatal NMRI mouse brains as described previously (Lyons and Kettenmann, 1998). In brief, brain cortices were surgically isolated and washed twice in ice-cold Hanks balanced salt solution (HBSS, Biochrom, Berlin, Germany) without Ca^{2+} and Mg^{2+}. Subsequently, the tissue was trypsinized in HBSS containing 2.5% trypsin (Biochrom, Berlin, Germany) and 0.5% DNase (Worthington Biochemical Corp., Lakewood, USA) and triturated through a Pasteur pipette to yield single cells. The cell suspension was centrifuged at 800 g for 10 min at 4°C and washed twice in ice-cold HBSS. Cells were re-suspended in Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen, Karlsruhe, Germany) containing 20 mM glucose, 2 mM L-glutamine, 10% heat-inactivated, fetal calf serum (FCS, Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml) and plated on coverslips in culture dishes previously coated with poly-L-lysine (100 μg/ml). The culture dishes were kept in a humidified incubator under 5% CO_2/95% air at 37°C. The medium was changed twice weekly.

Immunocytochemistry

More than 99% of primary astrocytes were immunoreactive to an antibody against glial fibrillary acidic protein (GFAP; Dako, Glostrup, Denmark). Experiments were carried out using non-passaged primary astrocytes after 7 to 14 days in vitro (DIV) after plating the cells.

Cell viability test

Cells were stained with Hoechst 33342 trihydrochloride (final concentration 1 μg/ml; Molecular Probes, Eugene, USA) and Sytox Green nucleic acid stain (final concentration 500 nM; Molecular Probes) to determine the amount of surviving cells. After incubation with these dyes for less than 5 min, cells were washed in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 10 to 30 min at room temperature, and washed with PBS. Coverslips were mounted under Aqua Poly-Mount (Polysciences Inc., Warrington, USA). Labeling with Hoechst 33342 showed the amount of apoptotic cells, whereas staining with Sytox Green revealed the number of dead astrocytes. Cells were counted in four observation fields using a fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany) with a 40× magnification.

Intracellular calcium imaging and measurement of $[Ca^{2+}]_i$

Cultured primary astrocytes on coverslips were loaded with a $[Ca^{2+}]_i$ indicator dye by incubation with 5 μM fura-2 acetoxymethylester (fura-2/AM; Molecular Probes, Eugene, USA; 5 mM stock solution in DMSO) in bath solution composed of (in mM): NaCl 150; KCl 5.4; $CaCl_2$ 2; $MgCl_2$ 1; HEPES/NaOH 10; glucose 10; pH 7.4, at 37°C for 20 min. Following dye loading, cells were incubated in HEPES buffer for additional 20 min to ensure fura-2/AM de-esterification.

Subsequently, coverslips were transferred to the stage of an upright microscope (Axioskop, Zeiss, Oberkochen, Germany). A fluorescence ratio-imaging system (Till Photonics, Martinsried, Germany) was used for excitation and monitoring the emitted fluorescence. Excitation wavelength was switched between 340 nm (F_{340}) and 380 nm (F_{380}) by means of a monochromator (12 nm band width). Using a 12-bit CCD camera, the resultant fluorescence signals were recorded at intervals of 30 s (long-lasting glucose deprivation experiments) or 2 s (short-lasting tests on neurotransmitter action) after passing a dichroic beam splitter and an emission filter at 510 ± 10 nm. Changes in the fluorescence ratio (F_{340}/F_{380}) were registered at room temperature, unless noted otherwise, as a measure of $[Ca^{2+}]_i$ from selected regions of interest covering single cells. Data acquisition and image analysis were performed using Till Vision software (Till Photonics) and standard PC software. The $[Ca^{2+}]_i$ was calculated from the ratio (F_{340}/F_{380}) of single cell fluorescence recorded at 340 and 380 nm excitation wavelengths. Calibrations (conversion of F_{340}/F_{380} values into molar calcium concentrations) were performed as described previously (Grynkiewicz et al., 1985). Basal $[Ca^{2+}]_i$ was determined from the initial ten images of each cell recording. A $[Ca^{2+}]_i$ signal was defined as an increase in F_{340}/F_{380}, and amplitudes (ΔF) were calculated as difference between F_{340}/F_{380} basal and peak values.

Glucose deprivation experiments

In glucose deprivation experiments, primary astrocytes on coverslips were superfused with glucose-containing HEPES bath solution for the first 15 min. Subsequently, the superfusion was switched to a glucose-free bath solution for up to 2 h. When required by experimental protocol, pyruvate, 2-deoxy-D-glucose and other substances were added to the glucose-containing or glucose-free bath solutions. Estradiols, tamoxifen, testosterone, cholesterol, and nifedipine were applied at indicated concentrations from a 10 mM stock solution in ethanol, KB-R7943 from 10 mM stock in DMSO, that was diluted further in the bath solution. Cycloheximide (3 μM) was dissolved in the bath solution from a 3-