EFFECTS OF ESTRONE ON QUISQUALATE-INDUCED TOXICITY IN PRIMARY CULTURES OF RAT CORTICAL NEURONS

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Estrogens exert protective effects against neurotoxic changes induced by over-activation of ionotrophic glutamate receptors, whereas little is known about their interaction with changes mediated by metabotropic glutamate receptors. We evaluated effects of estrone on quisqualate (QA)-induced toxicity in neuronal cell cultures on 7 and 12 day in vitro (DIV). Twenty four hour exposure to QA (150 µM and 300 µM) significantly decreased cell survival in 7 day old cultures, but the 12 day old cultures were more resistant to its toxicity. DNQX (10 µM), an AMPA/kainate receptor antagonist, partly attenuated the toxic effects of QA, whereas LY 367 385 (100 µM), a selective mGluR1α antagonist, completely reversed the above effect. QA did not activate, but suppressed spontaneous caspase-3-like activity. Estrone (100 nM and 500 nM) attenuated QA-mediated neurotoxic effects independently of estrogen receptors, as indicated with ICI 182, 780 and without affecting the caspase-3-like activity. At early stage of development in vitro (7 DIV) toxic effects of QA were more profound and mediated mainly by metabotropic glutamate receptors of group I, whereas later (12 DIV) they were mediated mostly by ionotropic AMPA/kainate receptors. The toxic effects of QA were partly accompanied by anti-apoptotic action against spontaneous caspase-3-like activity, possibly due to modulation of neuronal plasticity.

Key words: excitotoxicity, estrogen, caspase-3, primary neuronal cultures

INTRODUCTION

Estrogens exert protective effects against neurotoxic changes induced by over-activation of ionotrophic glutamatergic receptors, whereas little is known about their interaction with changes mediated by metabotropic receptors. Being...
involved in modulation of synaptic transmission and neuronal excitability, metabotropic glutamate receptors (mGluRs) play an important role in the modulation of many physiological and pathological processes, including nociception, seizures, epilepsy, ischemia, and neurodegenerative diseases such as Huntington’s disease, amyotrophic lateral sclerosis, and Alzheimer’s disease.

Estrogen implication in excitotoxic processes may include the effects on calcium homeostasis through the interaction with L-type calcium channels as well as with excitatory amino acid receptors. A distinct estrogen-binding site has been found and characterized as being coupled to AMPA/kainate receptors through cAMP-dependent phosphorylation. Moreover, in the presence of 17β-estradiol a rapid and reversible inhibition of NMDA-induced current and rise in intracellular free Ca^{2+} has been observed (1).

Although metabotropic glutamate receptors of group I are potential targets for neuroprotective drugs, their role in neurodegeneration is still controversial. It can be argued that any pharmacological blockade of these receptors is neuroprotective either in acute or chronic central nervous system disorders. It has been found, however, that while antagonists of mGluRI are neuroprotective (2, 3), agonists either attenuate or amplify excitotoxic neuronal death. Recently, Catania et al. (4) provided the first evidence that the endogenous activation of group-I metabotropic glutamate receptors is required for differentiation and survival of cerebellar Purkinje cells. There is a consistent body of evidence that mGluR₃ support cell survival. Copani et al. (5) have suggested that a decline in their expression gives access to apoptosis of cerebellar granule cells. Otherwise, mGluR₁ are thought to be neurotoxic, i.e. the excessive stimulation of these receptors results in cell death.

The neuronal cell death cascades may be counteracted by estrogens which appear to limit the activation of the caspase cascade, suppress oxyradicals, and stabilize calcium homeostasis and mitochondrial function (6-9). Apart from mitochondrial pathway, estrogens appear also to be involved in death receptor-mediated apoptosis affecting cytokine signalling components, such as nitric oxide (NO) synthesis and activation of transcription factor NFκB (10, 11).

In our previous experiments we observed non-genomic effects of estrogens, which attenuated the NMDA-induced effects such as an elevated LDH-release and caspase-3 activity, and reduced expression of anti-apoptotic protein - Bcl-2 (12, 13). Both estradiol and its low estrogenic metabolite, estriol, inhibited EAA (excitatory amino acid)-induced neuronal damage with similar potency, suggesting that metabolites of estrogens with low hormonal activity may serve as endogenous neuroprotective agents. Therefore, in the present study we tested hypothesis whether estrone, which naturally occurs in abundance in human brain (14) and has low affinity to estrogen receptors, may affect quisqualate (QA)-induced changes mediated by mGluRI and AMPA/kainate receptors in primary cultures of rat cortical neurons on different stages of development in vitro. We measured the activity of caspase-3-like proteases, since the activation of these
enzymes is the most common phenomenon preceding degradation of genomic DNA and apoptotic cell death. Furthermore, caspase-3 like proteases appeared to cleave directly the GluR1 subunit of the AMPA glutamate receptor, thus modulating neuronal plasticity (15). The measurement of intracellular esterase activity allowed us to assess cell survival being a consequence of either apoptotic or necrotic cell death.

MATERIAL AND METHODS

Cell culture

Neocortical tissue for primary cultures originated from Wistar rat embryos at 17-18 days of gestation and was cultivated essentially as described by Brewer (16), and Junghans and Kappler (17). Pregnant females were anaesthetised with CO₂ vapour, killed by cervical dislocation and subjected to Cesarean section in order to remove foetal brains. Animal care followed official governmental guidelines. Dissected cortices were minced into small pieces, then digested with trypsin (0.025%; Sigma), triturated in the presence of soybean trypsin inhibitor (5200 BAEE units per ml; Sigma) and DNase I (170 Kunitz units per ml; Sigma), and finally centrifuged in gradient of 5% bovine serum albumin. Cells were suspended in estrogen-free neurobasal medium supplemented with B27 (Gibco) and plated at a density of 2 x 10⁵ cells per square centimeter onto poly-L-lysine (0.01 mg per ml; Sigma) coated multi-well plates (Nunc). The cultures were maintained at 37°C in a humidified atmosphere containing a 5% CO₂ and cultivated for 7 or 12 days prior to experimentation.

Treatment with quisqualate and estrone

In order to evoke toxic effects, including possibly apoptotic cell death, primary neuronal cultures were exposed to quisqualate (QA; 150 µM, 300 µM; Tocris) for 6 h, 13 h, and 24 h. DNQX (6,7-Dinitroquinoxaline-2,3-dione; 10 µM; Tocris), an AMPA/kainate receptor antagonist, and LY 367 385 ((S)-(+) α-Amino-4-carboxy-2-methylbenzeneacetic acid; 100 µM; Tocris), a metabotropic glutamate receptor 1α antagonist, were employed to verify specificity of QA-effects. DNQX and LY 367 385 were dissolved in DMSO, which was present in cultures in a final concentration of 0.1 %.

Estrone (Sigma) was introduced to cortical cell cultures in a concentration of 100 nM or 500 nM for the same period of time as QA. An involvement of estrogen receptors in estrone effects was verified with estrogen receptor antagonist - ICI 182, 780 (1 µM; Tocris). Estrone was dissolved in ethanol, which final concentration in culture media was 0.1%. ICI was dissolved like glutamate receptor antagonists.

Calcein AM test

Cell viability was assessed by measuring intracellular esterase activity, which was determined after 24 h treatment with neurotoxic (QA) or potentially neuroprotective compound (estrone). In order to remove esterase activity, which might be present in growth media, cells were washed with Locke's buffer. The cells cultured in 96-well plates were then incubated with 2 µM calcein AM (Molecular Probes) in Locke's buffer, at RT for 30-60 min. The fluorescence of calcein was excited at 485 nm and its emission was measured at 530 nm (Fluoroscan Ascent, Labsystem). Background fluorescence readings (cell-free control) were subtracted from all values prior to calculation of results. The data were normalized to the fluorescence in vehicle-treated cells and expressed as a percentage of control fluorescence from 8-10 separate platings±SEM.
The accuracy of calcein test was validated by measuring the activity of lactate dehydrogenase (LDH) released into the extracellular medium in pilot experiments.

**Caspase-3 like activity measurement**

Caspase-3-like activity was measured according to Nicholson et al. (18) in the samples treated for 6 h, 13 h, or 24 h with possibly pro-apoptotic (QA) or anti-apoptotic agents (estrone). After replacing media with buffer composed of 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, and 10 mM DTT cells were incubated with caspase-3 substrate - Ac-DEVD AMC (7-amino-4 methyl coumarin; Sigma) for over 60 min. at 37°C. The amount of fluorescent products was measured with a fluorescent plate reader (Fluoroscan Ascent, Labsystem) at excitation of 355 nm and emission of 600 nm. The data were analysed with Ascent software, normalized to the fluorescence in vehicle-treated cells and expressed as a percent of control fluorescence established from 8-10 separate platings±SEM.

**Identification of apoptotic cells**

Apoptotic cell death was detected by uptake of acridine orange and ethidium bromide, according to Gorman et al. (19). Cells with bright green (early apoptosis) or orange nuclei (late apoptosis) showing condensation of chromatin were identified as dying in apoptotic mode.

**Data analysis**

All data were normalized to the fluorescence in vehicle-treated cells and presented as a percentage of control from 8-10 separate platings±SEM. One-way analysis of variance used to determine overall significance was followed by post hoc Duncan test, with the significant differences marked as follows: *p< 0.05, **p<0.01, and ***p<0.001 (versus control cultures) and *p< 0.05, **p<0.01, and ***p<0.001 (versus QA-treated cultures).

**RESULTS**

I. Effects on cell survival in cortical neurons on 7 DIV and 12 DIV

I.1. Effects of quisqualate on cell survival on 7 DIV

Quisqualate (QA) in concentration of 150 µM and 300 µM suppressed cell survival in primary cultures on 7 DIV by 35% and 33%, respectively, as determined by measuring intracellular esterase activity (Fig. 1). Both DNQX (10 µM) and LY 367 385 (100 µM) inhibited QA effects, however, a statistical significance of the inhibition was reached only in case of LY 367 385.

I.2. Effects of estrone on quisqualate-affected cell survival on 7 DIV

Estrone (100 nM and 500 nM) attenuated the effects of QA increasing cell survival for 40% to over 50%, depending on the concentration (Fig. 2). ICI 182, 780 (1 µM) did not change estrone effects in any treatment.
Fig. 1. Effects of quisqualate (QA; 150 µM and 300 µM) and glutamate receptor antagonists, DNQX (10 µM) and LY 367 385 (100 µM), on intracellular esterase activity in rat cortical neurons in primary cultures on 7 day in vitro (DIV). Cells were treated either with QA alone, QA and DNQX, QA and LY 367 385 or vehicle for 24 h. Each bar represents an average value taken from 8-10 separate platings±SEM. p<0.05 versus control cultures; *p<0.05, and ***p<0.001 versus the cultures exposed to QA.

Fig. 2. Effects of estrone (E; 100 nM and 500 nM) and ICI 182,780 (ICI; 1 µM) on the QA-affected intracellular esterase activity in rat cortical neurons in primary cultures on 7 day in vitro (DIV). Cells were treated either with QA alone, QA and estrone and/or ICI 182,780 or vehicle for 24 h. Each bar represents an average value taken from 8-10 separate platings±SEM. p<0.05 versus control cultures; *p<0.05, and ***p<0.001 versus the cultures exposed to QA.
Fig. 3. Effects of quisqualate (QA; 150 µM and 300 µM) and glutamate receptor antagonists, DNQX (10 µM) and LY 367 385 (100 µM), on intracellular esterase activity in rat cortical neurons in primary cultures on 12 day in vitro (DIV). Cells were treated either with QA alone, QA and DNQX, QA and LY 367 385 or vehicle for 24 h. Each bar represents an average value taken from 8-10 separate platings±SEM. #p<0.05 versus control cultures; *p<0.05 versus the cultures exposed to QA.

Fig. 4. Effects of estrone (E; 100 nM and 500 nM) and ICI 182,780 (ICI; 1 µM) on the QA-affected intracellular esterase activity in rat cortical neurons in primary cultures on 12 day in vitro (DIV). Cells were treated either with QA alone, QA and estrone and/or ICI 182,780 or vehicle for 24 h. Each bar represents an average value taken from 8-10 separate platings±SEM. #p<0.05 versus control cultures; *p<0.05 versus the cultures exposed to QA.
I.3. Effects of quisqualate on cell survival on 12 DIV

QA inhibited effectively cell survival on 12 DIV only in concentration of 300 µM, when it diminished an intracellular esterase activity by 17% below the control (Fig. 3). Both glutamate receptor antagonists inhibited QA effects, however a statistical significance was obtained only with DNQX.

I.4. Effects of estrone on quisqualate-affected cell survival on 12 DIV

Estrone attenuated QA-inhibited cell survival only when given in concentration of 100 nM (Fig. 4). No ICI 182, 780 influence on estrone effects was observed.

II. Effects on caspase-3-like activity in cortical neurons on 7 DIV and 12 DIV

II.1. Effects of quisqualate on caspase-3-like activity

Six hour and 14 hour exposure to QA (300 µM) did not increase caspase-3-like activity in cortical neurons on 7 DIV and 12 DIV (Fig. 5). However, in
cultures on 7 DIV 6 h after treatment with 300 µM QA a 26% decrease in spontaneous caspase-3-like activity was observed.

II.2. Effects of estrone on quisqualate-induced caspase-3-like activity

Estrone (100 nM) did not affect QA-inhibited caspase-3-like activity observed on 7 DIV (Fig. 6).

III. Effects on apoptotic condensation of chromatin in cortical neurons on 7 DIV and 12 DIV

III.1. Effects of quisqualate on uptake of acridine orange and ethidium bromide

Twenty four hours after treatment with QA no significant rise (5-7%) in apoptotic body formation was detected, suggesting an involvement of necrotic rather than apoptotic processes in QA toxicity.
DISCUSSION

Estrogen implication in excitotoxic and apoptotic processes has been elucidated only partially. Although estrogens appeared to exert protective effects against neurotoxic changes induced by over-activation of ionotropic glutamatergic receptors, little is known about their interaction with changes mediated by metabotropic receptors.

Quisqualate (QA) triggers its effects by ionotrophic AMPA and metabotropic glutamate receptors of group I. An excessive activation of ionotropic glutamate receptors leads to excitotoxicity, but an activation of group I metabotropic glutamate receptors may either enhance or attenuate excitotoxic neuronal death depending on the experimental conditions. The present study has shown that QA (150 µM and 300 µM) suppresses cell survival in terms of intracellular esterase activity by 35% and 33%, respectively, as observed in primary cultures of rat cortical neurons on 7 DIV (Fig. 1). Similarly, Witt et al. (20) indicated that QA (300 µM for 20 min.) inhibited cortical neuron responsiveness on 7-8 DIV, as defined by the ability of the cells to respond to K⁺-induced depolarization by a transient increase in Ca²⁺-influx. The authors demonstrated that cortical neurons were vulnerable to the QA-induced loss of function (about 55%) which suggested a loss of cell viability likewise in our experiments.

The effects of QA on 7 DIV were inhibited by DNQX (10 µM) and LY 367 385 (100 µM), but a statistical significance of the inhibition was reached only in case of LY 367 385, pointing to metabotropic glutamate receptors as major mediators of QA effects at that stage of neuronal development in vitro (Fig. 1). In contrast, on 12 DIV the neurotoxic effect of QA was significantly blocked by AMPA/kainate ionotropic receptor antagonist, DNQX, suggesting the prevalence of ionotropic glutamate receptors in mediating QA effects at that time in vitro (Fig. 3). Accordingly, Patel et al. (21) demonstrated that in hippocampal neuronal cultures the maximum response of ionotropic glutamate receptors to QA appeared relatively later, i.e. at 14 days. Lee et al. (22) have indicated that neurons increased their sensitivity to excitatory amino acids as a function of time in culture (whole rat brains from 16-18 days gestation). A marked stimulation of poly-PI turnover by AMPA was seen in the cultured neurons on 4 DIV, but not on 17 DIV, suggesting that a distinct EAA receptor sensitive to AMPA is transiently expressed.

QA inhibited cell survival on 12 DIV only in higher, 300 µM, concentration and diminished an esterase activity to a lesser extent, i.e. by 17% below the control (Fig. 3). Previously, Zinkand et al. (23) demonstrated that QA (250-1000 µM for 5 min.) caused 2-4 fold increase in LDH-release, depending on drug concentration, from mixed cortical cultures on 14-18 DIV. Since the authors experimented on glial and neuronal cells cultivated in a presence of serum, we suggest that the enormous QA effect was partly due to undefined concentrations of glutamate in serum, which could additionally sensitise neurons to subsequent
stimuli. Likewise in our study, Bruno et al. (24) indicated that QA (50 µM for 1 min.) was toxic to mixed and pure cultures of cortical neurons on 10-12 DIV amplifying neurodegeneration induced by pulse of NMDA (100 µM for 10 min.).

Another interesting finding in this study is that estrone (100 nM and 500 nM) attenuates the effects of QA increasing cell survival for 40% to over 50% on 7 DIV, depending on the concentration (Fig. 2). Estrone (100 nM) attenuates also QA-inhibited cell survival on 12 DIV (Fig. 4). Since ICI 182, 780 (1 µM) does not change estrone effects in any treatment, we assume that estrogen neuroprotection is due to rather non-genomic action. Indeed, estrogens have been reported to interact either with ionotropic receptors or with cell membranes and thus interfere with ionic currents or rapidly activate signal transduction pathways. It is also possible that estrogens stabilize mitochondrial function either by scavenging free radicals or by affecting ATP-ase FOF1 (6). Both estradiol isoforms, α and β, can modulate an activity of the brain-derived mitochondrial Na/K ATP-ase. A direct action of estrogens on mitochondrial membranes followed by inhibition of oxygen consumption may also be important in this regard (25).

Since an involvement of QA in apoptotic processes is controversial, we decided to measure the caspase-3-like activity to distinguish possible necrotic and apoptotic effects of this glutamate receptor agonist. Previously, Finniels et al. (26) demonstrated that cortical neurons cultured in the absence of serum and stimulated with quisqualate showed significant degeneration and displayed an apoptotic characteristics, except internucleosomal DNA degradation, after 3-5 days in vitro. This effect was blocked by 1 µM MK-801, indicating that it was mediated by the activation of NMDA receptors. In our study, however, we did not observe pro-apoptotic effects of QA, because the caspase-3-like activity was not elevated in cortical cultures after 6 h and 14 h on 7 DIV and 12 DIV (Fig. 5) and only slight rise in apoptotic body formation was detected (uptake of acridine orange and ethidium bromide after 24 h, data not shown), suggesting an involvement of necrotic rather than apoptotic processes in QA toxicity. Surprisingly, in cultures on 7 DIV 6 h after treatment with 300 µM QA we observed a 26% decrease in spontaneous caspase-3-like activity. Since no such effect appeared on 12 DIV, we assume an involvement of metabotropic rather than ionotropic receptors in transient inhibition of caspase-3-like proteases. Accordingly, Maise and Vincent (27) reported that an activation of only group I mGluRs completely ameliorated the NO-mediated activation of caspase-3 and prevented programmed cell death in rat hippocampal neurons. Moreover, Allen et al. (28) showed that selective activation of group I mGlu receptors exacerbated necrosis induced by oxygen-glucose deprivation, but significantly attenuated apoptotic cell death induced by staurosporine or etoposide.

Assuming that group I mGluR exhibits opposite effects on necrotic and apoptotic neuronal cell death, in the present study cortical neurons died because of over-activation of both types of glutamate receptors, but avoided apoptosis due
to activation of metabotropic glutamate receptors. Accordingly, there is a consistent body of evidence that type 5 of mGluR (mGluR5) supports cell survival and a decline in its expression seems to give access to apoptosis of cerebellar granule cells (5). Otherwise, mGluR1 are thought to be neurotoxic, i.e. the excessive stimulation of these receptors results in cell death, including apoptosis.

Summing up, this study demonstrated that estrone attenuated toxic effects of QA in cortical neurons both on 7 DIV and 12 DIV independently of estrogen receptors and without affecting the caspase-3-like activity. At early stage of development in vitro, on 7 DIV, toxic effects of QA were mediated mainly by metabotropic glutamate receptors of group I, whereas later, on 12 DIV, they were mediated mostly by ionotropic AMPA/kainate receptors. The QA effects were partly accompanied by anti-apoptotic action against spontaneous caspase-3-like activity, possibly due to modulation of neuronal plasticity, because this enzyme may directly cleave the GluR1 subunit of the AMPA glutamate receptor (15). Since estrone triggered its effects via non-genomic mechanisms, it suggests that searching for estrogen analogs exhibiting low affinity to hormone receptors would be important to retain the favourable and minimize the adverse side effects of estrogens.

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