Journal of Steroid Biochemistry & Molecular Biology 144 (2014) 294–303

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Tibolone protects T98G cells from glucose deprivation

Marco Ávila Rodriguez [a], Luis Miguel Garcia-Segura [b], Ricardo Cabezas [a], Daniel Torrente [c], Francisco Capani [d], Janneth Gonzalez [a], George E. Barreto [a,*]

[a] Departamento de Nutrición y Bioquímica, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá D.C.,Colombia
[b] Instituto Cajal, CSIC, Avenida Doctor Arce 37, 28002 Madrid, Spain
[c] Department of Physics and Astronomy, The University of Texas at San Antonio, United States
[d] Laboratorio de Citoarquitectura y Plasticidad Neuronal, Instituto de Investigaciones cardiológicas Prof. Dr. Alberto C. Taquini (ININCA), Facultad de Medicina, UBA-CONICET, Marcelo T. de Alvear 2270, C1122AAJ Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 14 April 2014
Received in revised form 23 July 2014
Accepted 24 July 2014
Available online 31 July 2014

Keywords:
Neuroprotection
Estrogenic activity
Metabolic insult
Free radicals

ABSTRACT

The steroidal drug Tibolone is used for the treatment of climacteric symptoms and osteoporosis in post-menopausal women. Although Tibolone has been shown to exert neuroprotective actions after middle cerebral artery occlusion, its specific actions on glial cells have received very little attention. In the present study we have assessed whether Tibolone exerts protective actions in a human astrocyte cell model, the T98G cells, subjected to glucose deprivation. Our findings indicate that Tibolone decreases the effects of glucose deprivation on cell death, nuclear fragmentation, superoxide ion production, mitochondrial membrane potential, cytoplasmic calcium concentration and morphological parameters. These findings suggest that glial cells may participate in the neuroprotective actions of Tibolone in the brain.

1. Introduction

The neuroprotective actions of estradiol have been extensively documented and characterized [1–3]. However, the use of estradiol for neuroprotective therapies is limited by its detrimental peripheral effects [4,5]. Therefore, several laboratories have assessed the neuroprotective actions of molecules that may imitate the neuroprotective actions of estradiol without the detrimental peripheral effects of the hormone, such as selective estrogen receptor modulators (SERMs) and selective tissue estrogenic activity regulators (STEARs) [6,7]. One of such molecules is Tibolone, a steroidal drug with estrogenic, progestogenic and slight androgenic actions [8–12] that is used for the treatment of climacteric symptoms and osteoporosis in post-menopausal women [13–15]. Tibolone is considered a STEAR, since its estrogenic activity depends on its differential metabolism in each tissue, combined with the different affinity of its metabolites for hormone receptors and the different action of its metabolites on the inhibition of steroid sulphatase activity [10,11,16–18].

Brain tissue is a target for Tibolone, which exerts neuroprotective actions, reducing infarct volume, increasing Bcl-2 expression and acting as an anti-apoptotic agent in the brain of adult female Sprague-Dawley rats exposed to middle cerebral artery occlusion [19]. Several studies have explored the neuroprotective mechanisms of Tibolone, showing that the drug activates the Akt/GSK3ß signaling pathway and reduces Tau phosphorylation in the hippocampus and the cerebellum of ovariectomized rats [20] and increases antioxidant activity in primary neuronal cultures [21] and in the brain of adult female Wistar rats [22].

Little is known about the role of glial cells in the neuroprotective actions of Tibolone. However, previous studies have shown that 3-hydroxy-metabolites of Tibolone exert agonistic actions on human astrocytes through the activation of estrogen receptors [23], indicating that astrocytes are a target for Tibolone. Astrocytes play an important role in the maintenance of homeostasis in the central nervous system (CNS), regulating neuronal function and metabolism [24–27] and proper astrocyte function is fundamental for neuronal survival after different brain insults, such as glucose deprivation, traumatic brain injury and ischemia [25,27–33]. Astrocytes are known to participate in the neuroprotective mechanisms of estradiol [34] and the hormone protects primary astrocytes from metabolic insults, including oxygen glucose deprivation [1,35,36]. This protective action of

* Corresponding author. Tel.: +57 1 320 8320 ext. 4096; fax: +57 1 320 8320.
E-mail addresses: gsampaio@javeriana.edu.co, gesbarreto@gmail.com (G.E. Barreto).

http://dx.doi.org/10.1016/j.jsbmb.2014.07.009
0960-0760/

estradiol on astrocytes may contribute to the protective mechanism of the hormone after stroke. Therefore, it is important to determine whether Tibolone, which is used for hormonal therapy in women, exerts similar protective actions on human astrocytes. In the present study, we have assessed whether Tibolone exert protective actions on human T98G cells exposed to glucose deprivation (GD). T98G cells were selected for the experiments due to its biological resemblance with primary astrocytes and its broad use in research as an astrocyte cell model [37–39].

2. Materials and methods

2.1. Cell culture

The T98G cell line was used as an astrocytic cell model system (ATCC CRL-1690). Cells were maintained under exponential growth in Dulbecco's modified Eagle's medium (DMEM) (LONZA, Walkersville, USA), containing 10% fetal bovine serum (LONZA, Walkersville, USA), and 10U penicillin/10 μg streptomycin/25 ng amphotericin (LONZA, Walkersville, USA). Cultures were incubated at 37 °C in a humidified atmosphere containing 5% carbon dioxide. The medium was changed three times a week. Cells were seeded in 96-well plates for death cell measurement, 12-well plates for flow cytometry determinations and 24-well plate for calcium and tetramethyl rhodamine methyl ester (TMRM) fluorescence measurements.

2.2. Drug treatment

Once the cells were seeded in multi-well plates for the drug treatment procedures, the culture medium was replaced with DMEM supplemented with 1% fetal bovine serum (LONZA, Walkersville, USA) for 12 h. In a first experiment the cells were incubated in DMEM without serum and treated with different concentrations of Tibolone (Sigma–Aldrich, T0827) or vehicle during different time periods in order to determine the optimum time and dose conditions. In the following experiments, a concentration of 10 nM Tibolone for 24 h prior to the metabolic insult (glucose deprivation (GD)) was selected. Tibolone was dissolved in 0.01%, DMSO as stock solution at 5 mM, and further dilutions were made using serum free culture medium. After 24 h of Tibolone treatment, the cells were subjected to GD protocol.

2.3. Glucose deprivation

Glucose deprivation (GD) was performed as previously reported [40,41]. Briefly, before GD exposure, cells were washed three times with glucose-free, balanced salt solution (BSS 0) containing in mM: NaCl, 116; $CaCl_2$, 1.8; $MgSO_4$, ($7{\cdot}H_2O$) 0.8; KCl, 5.4; NaH_2PO_4, 1; $NaHCO_3$, 14.7, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10; pH 7.4. Subsequently, the cells were incubated with BSS 0 and transferred to the incubator. The wash controls cells had their medium changed to BSS 5, which was identical to BSS 0, but supplemented with 5.5 mM glucose.

2.4. Determination of viability and nuclear fragmentation

Viability was tested using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

Assay (Sigma, St Louis, Missouri, USA). Cells were seeded into 96-well plates in DMEM culture medium containing 10% bovine fetal serum at seeding density of 10,000 cells per well, incubated for 2–3 days until they reach confluence. Subsequently, cells were treated according to different experimental paradigms. Viability was assessed and standardized at 6, 12, 24, 48, and 72 h, following injury by adding MTT solution at the final concentration of 5 mg/ml, for 4 h at 37 °C in order to let the formazan crystal formation. Then cells were lysed by adding DMSO. The blue formazan product was evaluated in a plate reader at 595 nm, and then the values were normalized to the value of control cultures without GD, which was considered as 100% survival. Each assay was performed with a minimum of six replicate wells for each condition.

Nuclear fragmentation was determined by Hoechst 33,258 staining. After exposure to GD, cells were washed three times in phosphate-buffered saline and fixed in 4% formaldehyde for 20 min. Subsequently, cells were washed and labeled with Hoechst 33,258 (5 mg/ml; Invitrogen) for 15 min. Cell nuclei were observed and photographed using an inverted fluorescence microscope Nikon Eclipse TS100. The number of cells that showed nuclear fragmentation nuclei was determined in at least eight randomly selected areas (0.03 mm^2) from each experimental group. The experiment was repeated 3 times. Data were expressed as a percentage of nuclear fragmented cells relative to the value in control cultures.

2.5. Determination of reactive oxygen species (ROS)

Reactive oxygen species production was evaluated by flow cytometry and fluorescence microscopy as previously described [42]. Briefly, cells were seeded at a density of 75,000 cells per well into 12-wellplates in DMEM culture medium containing 10% FBS and then were treated according to each experimental paradigm in the next day. To measure the effect of Tibolone on superoxide (O^{2-}) and oxygen peroxide (H_2O_2) production, cells were treated in the dark at 37 °C for 30 min with 10 mM dihydroethidium (DHE; Sigma) or 1 mM 2′,7′-dichlorofluorescein diacetate (DCFDA), respectively. Then cells were washed in PBS and trypsinized (Trypsin/EDTA 500 mg/l:200 mg/L-LONZA, Walkersville, USA) for flow cytometry analysis. Cells were analyzed in a Guava EasyCyte™ (Millipore, Billerica, Massachusetts, USA) cytometer. Each assay was performed with a minimum of six replicates for each condition. The experiment was repeated 3 times.

For DHE fluorescence imaging analysis, cells were seeded at a density of 30,000 cells per well into 24-well plates in DMEM culture medium containing 10% FBS, and subjected to Tibolone treatment. The next day, the cells were subjected to GD and then incubated with DHE for 30 min. Finally the cells were washed with PBS and photographed in a Nikon Eclipse TS100 fluorescence microscope. The images were processed with Image J software, and the mean fluorescence intensity of randomly selected cells was determined as described below.

2.6. Determination of mitochondrial membrane potential

Mitochondrial membrane potential was evaluated using tetramethyl rhodamine methyl ester (TMRM). TMRM is a cell-permeable, cationic fluorescent dye that is sequestered by active mitochondria. After 24 h of GD, cells were loaded in the dark with 500 nM TMRM at 37 °C for 20 min. Thereafter, cells were washed with PBS to eliminate all the unsequestered dye, and photographed as described above. As experimental control, we used the protonophoric uncoupler CCCP (carbonyl cyanide m-chlorophenylhydrazone; Sigma–Aldrich; 10 mM) to dissipate the membrane potential and define the baseline for analysis of mitochondrial potential.

2.7. Cytoplasmic calcium concentration

Cytoplasmic calcium concentration was evaluated using the fluo-4- acetyl ester (Fluo-4-AM). Fluo-4 is an analog of fluo-3 with