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Research Report

Coumestrol has neuroprotective effects before and after global cerebral ischemia in female rats

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ABSTRACT

Global ischemia arising during cardiac arrest or cardiac surgery causes highly selective, delayed death of hippocampal CA1 neurons. Phytoestrogens are naturally occurring plant-derived compounds that are present in the human diet and are considered selective estrogen receptor (ER) modulators. The phytoestrogen coumestrol is a potent isoflavonoid, with binding affinities for both ER-α and ER-β that are comparable to those of 17b-estradiol. The present study examined the hypothesis that coumestrol protects hippocampal neurons in ovariectomized rats in a model of cerebral global ischemia. Ovariectomized rats were subjected to global ischemia (10 min) or sham surgery and received a single intracerebroventricular or peripheral infusion of 20 μg of coumestrol, 20 μg of estradiol or vehicle 1 h before ischemia or 0 h, 3 h, 6 h or 24 h after reperfusion. Estradiol and coumestrol afforded significant neuroprotection in all times of administration, with the exception of estradiol given 24 h after the ischemic insult. Animals received icv infusion of the broad-spectrum ER antagonist ICI 182,780 (50 μg) or vehicle into the lateral ventricle just before the E2 or coumestrol administration. The ER antagonist abolished estradiol protection, consistent with a role of classical ERs. In contrast, ICI 182,780 effected only partial reversal of the neuroprotective actions of coumestrol, suggesting that other cellular mediators in addition to classical ERs may be important. Additional research is needed to determine the molecular targets mediating the neuroprotective action of coumestrol and the therapeutic potential of this phytoestrogen in the mature nervous system.

1. Introduction

Stroke is the third leading cause of death in industrialized countries (Lewis et al., 2008) and the most frequent cause of permanent disability in adults worldwide (Donnan et al., 2008). Three months following a stroke, 15–30% of stroke survivors are permanently disabled and 20% require institutional care. Deficits can include partial paralysis, difficulties

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with memory, thinking, language, and movements. In the western world, over 70% of individuals experiencing a stroke are over 65 years of age. Since life expectancy continues to grow, the absolute number of individuals with stroke will further increase in the future (Lakhan et al., 2009). Transient global ischemia arises as a consequence of cardiac arrest and causes selective, delayed death of hippocampal CA1 neurons in humans and can produce serious neurobiological sequellae of which cognitive deficits are most prominent (Lo et al., 2003; Moskowitz et al., 2010; Tanaka et al., 2000; Merchenthaler et al., 2003; Etgen et al., 2010).

Over the last decade, data from many studies support the idea that estrogens provide neuroprotective effects in a variety of focal and global ischemia models (Lebesgue et al., 2009; Merchenthaler et al., 2003; Garcia-Segura et al., 2001; Toran-Allerand, 2004; Shughrue and Merchenthaler, 2003). The potent feminizing hormone, 17 beta-estradiol (E2), is neuroprotective in a host of cell and animal models of stroke and neurodegenerative diseases. The discovery that 17 alpha-estradiol, an isomer of E2, is equally as neuroprotective as E2 yet is >200-fold less active as a hormone, has permitted development of novel, more potent analogs where neuroprotection is independent of hormonal potency (Simpkins and Dykens, 2008). For example, a single dose of estradiol administered immediately after reperfusion (acute estradiol) ameliorates global ischemia-induced neuronal death and cognitive deficits (Jover-Mengual et al., 2010; Gulinello et al., 2006). Moreover, a single injection of 17 β-estradiol administered to ovariectomized rats 2–4 day before ischemia also protects hippocampal neurons against ischemic damage via activation of CREB (Raval et al., 2009). At physiological concentrations it intervenes in apoptotic death cascades and ameliorates neuronal death in experimental models of focal and global ischemia (Brown et al., 2009; Gill et al., 2002; Lebesgue et al., 2009). The cellular targets that mediate estradiol protection of hippocampal neurons in global ischemia are, however, unclear (Miller et al., 2005; Etgen et al., 2010; Strom et al., 2009; Brown et al., 2009; Suzuki et al., 2009; Yang et al., 2003; Barrera-Ocampo et al., 2008; Alonso de Leciñana and Egido, 2006).

Phytoestrogens are estrogen-like molecules found in many plants. They have the ability to selectively bind classical estrogen receptors (ERs) to regulate gene expression mediated by estrogen response elements (Zhao et al., 2002). Phytoestrogens have been investigated intensively in recent years because of their potential protective effects against many diseases (Lephart et al., 2000). They not only bind to ERs but also exert potent antioxidant activity. It is increasingly clear that physiologically attainable doses of isoflavones, which can behave as phytoestrogens, may mimic some of the neuroprotective effects of estrogens. Some phytoestrogens exhibit some estrogen agonist-like properties (Stahl et al., 1998 and Mäkelä et al., 1995). Zhao et al., 2002 reported a significant reduction in glutamate-induced lactate dehydrogenase release and subsequently neuroprotection by phytoestrogens such as genistein, daidzein, daidzin, equol and formonoetin in cultured hippocampal neurons. A high soy diet reduces stroke injury in female and male rats, and the soy isoflavone genistein is neuroprotective in a mouse cerebral ischemia model (Donzelli et al., 2010). Moreover, dietary intake of phytoestrogens can improve outcomes after focal (Lovekamp-Swan et al., 2007; Burguete et al., 2006) and global ischemia in rats (Liang et al., 2008). However, the mechanisms underlying protection from ischemic injury remain unclear (Schreihofer and Redmond, 2009).

Among the hundreds of molecules that fall under this classification, the coumestan phytoestrogen coumestrol (derived from sprouting plants like alfalfa), has gained prominence

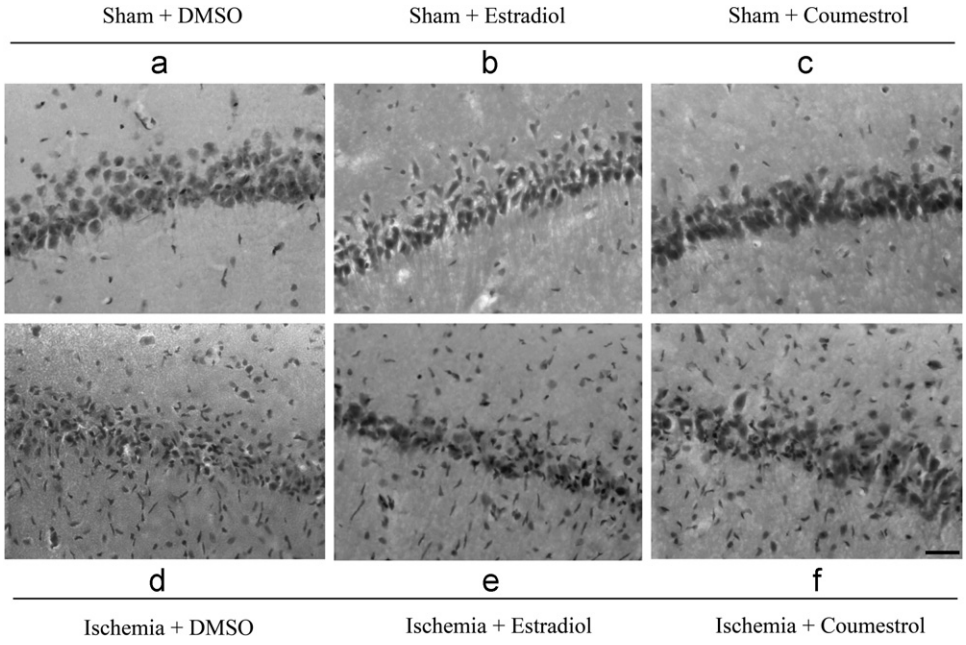

Fig. 1 – Photomicrographs (40X) of the hippocampal CA1 region of female rats with or without 10-min global ischemia 7 day after reperfusion. Rats that underwent global ischemia had significantly fewer surviving neurons than the sham-operated groups ($p<0,01$). Estradiol and Coumestrol treatment afforded robust neuroprotection. Scale bar = 100 μm.

because it is the most potent isoflavonoid, with binding affinities for both ER-α and ER-β that are comparable to those of 17 β-estradiol (Whitten et al., 2002). As ERs are expressed in several regions of the brain that are vulnerable to ischemia-induced neuronal death, we sought to determine whether the coumestrol has neuroprotective actions in CA1 hippocampal cells and whether its effects are mediated through classical ERs. If so, it could be a potential therapeutic treatment for global brain ischemia.

2. Results

Ovarectomized female rats were subjected to global ischemia or sham operation and recovered from an icv infusion of estradiol, coumestrol in vehicle or vehicle alone in different times. Global ischemia induced extensive death of pyramidal cells in the CA1 subfield of hippocampus accessed at 7 day post-ischemia ($p<0.01$ vs. sham) (Fig. 1d). Estradiol did not detectably alter the appearance or number of CA1 neurons in sham-operated rats (Fig. 1b), but greatly reduced the ischemia-induced neuronal loss ($p<0.01$ vs. ischemia), (Fig. 1e). As expected, coumestrol did not detectably alter the appearance or number of CA1 neurons in sham-operated rats (Fig. 1c), and also greatly reduced the ischemia-induced neuronal loss ($p<0.01$ vs. ischemia) (Fig. 1f). There were no significative difference between the estradiol and coumestrol groups at 1 h before, 0 h, 3 h and 6 h after ischemia-induced neuronal loss, but at 24 h, the statistical analysis detected a significative difference between these two groups ($p<0.01$ vs. ischemia) (Fig. 2), providing a clear evidence of neuroprotection promoted by coumestrol. The ER antagonist ICI 182,780, when administered at 0 h after surgery, did not detectably alter the number or appearance of surviving neurons in sham-operated rats or vehicle-treated animals subjected to ischemia, but totally abrogated the neuroprotective action of estradiol in the hippocampal CA1 layer ($p<0.01$ vs. estradiol alone) and partially blocked the neuroprotection afforded by coumestrol at 0 h post-ischemia ($p<0.01$ vs. coumestrol alone). Moreover, the statistical comparison showed a significative difference between the ischemic groups coumestrol and estradiol ($p<0.01$) indicating that whereas the antagonist ICI 182,780 reverses the estradiol neuroprotection, it was not totally able to reverse the neuroprotective actions of coumestrol, thus providing strong evidence that this compound is more effective in promoting neuronal survival than estradiol itself (Fig. 3). To access if coumestrol administration could be neuroprotective when administered peripherally as well we injected a single dose of 20 μg/kg intracardiaclly one hour before the global ischemia. The peripheral administration of coumestrol strongly prevented the delayed neuronal death after global ischemia (Fig. 4). Global ischemia induced extensive death of pyramidal cells in the CA1 subfield of hippocampus accessed at 7 day post-ischemia ($p<0.01$ vs. sham) (Fig. 5). We did not detect any changes in the number of cells in the CA1 subfield in sham-operated rats in comparison with the coumestrol sham-operated rats (Fig. 4). The statistical comparison showed a significative difference between the ischemic group and coumestrol ($p<0.01$) indicating that coumestrol was able to afford robust neuroprotection in the ischemic rats ($p<0.01$ vs. ischemia) (Fig. 5).

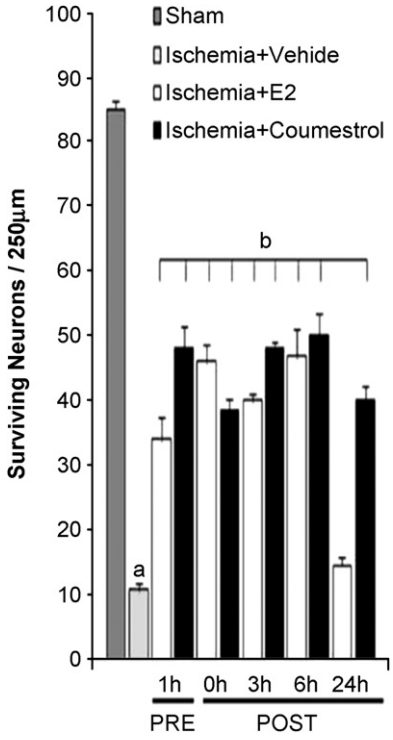

Fig. 2 – Effect of the treatment of coumestrol and estradiol in different times of administration. Ovarectomized female rats were subjected to global ischemia (10 min) or sham surgery and received a single icv infusion of 20 μg of coumestrol or 20 μg of estradiol or vehicle 1 h before ischemia or 0 h, 3 h, 6 h and 24 h after reperfusion. The ischemic groups had less surviving neurons in comparison with the sham groups. Estradiol and coumestrol afforded significant neuroprotection in all times of administration, with the exception of estradiol in 24 h after the ischemic insult. (a) Difference between ischemic and sham groups; (b) difference between ischemic treated groups and vehicle, with the exception of estradiol in 24 h after ischemia. Each bar represents the mean ± standard error of the mean (SEM). ANOVA followed by Duncan's test, $p<0.01$.

3. Discussion

Estradiol and estrogen-like compounds are powerful neuroprotective agents against numerous in vivo and in vitro apoptotic stimuli including experimental stroke (Hurn and Brass, 2003; McCullough and Hurn, 2003; Alonso de Leciñana and Egido, 2006; Gibson et al., 2006). However, the precise mechanisms underlying these protective effects are still under investigation.

It is now well established in the literature that endogenous and exogenous estrogens exert profound neuroprotective effects in animal models of focal and global ischemia and produce their cellular actions by binding the classical estrogens receptors. Thus, estrogens hold great promise as

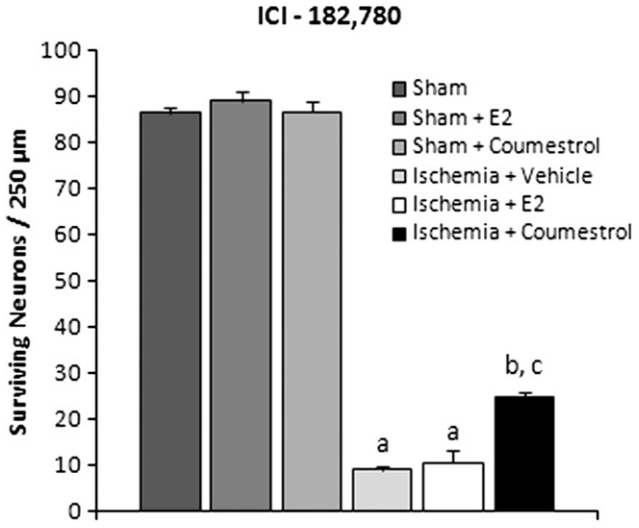

Fig. 3 – Effect of the ER antagonist ICI 182,780 treatment 0 h after ischemia. All groups were treated with estradiol or coumestrol (20 µg icv) and undergone to ischemia or sham surgery. The ER antagonist ICI 182,780 treatment fully abolished the neuroprotection promoted by estradiol but partially abrogated the coumestrol neuroprotection effect. Treatment with the ER antagonist did not affect the number of surviving neurons in any of the other treatment groups; therefore, for the statistical analysis, sham-operated vehicle and ICI 182,780 treated groups were combined. (a) Difference between sham and ischemic groups; (b) difference between the ischemic treated coumestrol and vehicle groups; (c) difference between the ischemic coumestrol and estradiol treated groups. Each bar represents the mean ± standard error of the mean (SEM). ANOVA followed by Duncan's test, $p < 0.01$.

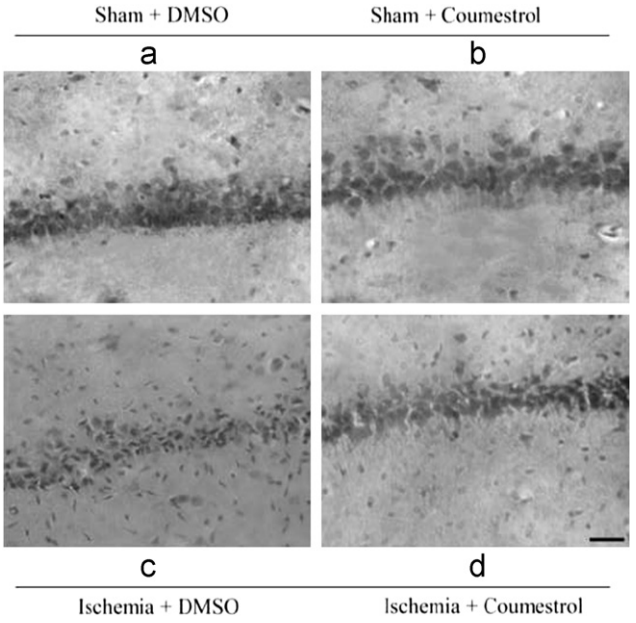

Fig. 4 – Photomicrographs (40X) of the hippocampal CA1 region of female rats with or without 10-min global ischemia 7 day after reperfusion. Rats that underwent global ischemia had significantly fewer surviving neurons than the sham-operated groups ($p < 0.01$). The peripheric pre-treatment of coumestrol strongly protected the CA1 hippocampal layer. Scale bar = 100 µm.

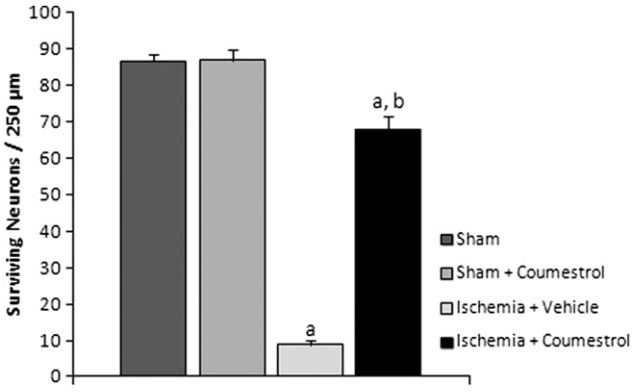

Fig. 5 – Effect of the peripheric administration of coumestrol one hour before global ischemia. Ovarectomized female rats were subjected to global ischemia (10 min) or sham surgery and received a single intracardiac infusion of 20 µg of coumestrol or vehicle 1 h before ischemia. The ischemic groups had less surviving neurons in comparison with the sham groups. Coumestrol afforded significant neuroprotection in (a) difference between ischemic and sham groups; (b) difference between ischemic vehicle group and coumestrol group. Each bar represents the mean ± standard error of the mean (SEM). ANOVA followed by Duncan's test, $p < 0.01$.

potential therapeutic agents in treatment of ischemia (Etgen et al., 2010). Along with phytoestrogens, the coumestan coumestrol, which is present in sprout of soybeans, clover and alfalfa, is another significant phytoestrogens regularly consumed by humans (Belcher and Zsarnovszky, 2001). This compound is known to be the most potent isoflavonoid, with binding affinities for both ERs that are comparable to those of 17 β-estradiol (Whitten et al., 2002).

Our results show that coumestrol, at all time of administrations, injected icv or intracardiaclly, protected neurons against global ischemia-induced CA1 neuronal death, indicating that this compound may work against the cascade of pathological events that lead to neuronal death. Both estradiol and coumestrol were able to promote neuroprotection in a cerebral global ischemia model when administered 1 h before and 0 h, 3 h and 6 h after ischemia. However, estradiol at 24 h after the ischemic event was not effective in preventing massive neuronal death at the hippocampal layer. It is interesting to note that coumestrol, at this same time of administration, was able to prevent the neuronal death promoted by the global ischemia. There are a few reports in the literature showing treatments that are still effective when delayed 24 h after ischemia. The two most cited long term strategies to the treatment of global ischemia is hypothermia (Tooley et al., 2002; Colbourne et al., 2000; Corbett et al., 2000; Colbourne and Corbett, 1994; Valentim et al., 2003) and preconditioning (Zhang et al., 2010; Yoshida et al., 2004; Boche et al., 2003; Dowden and Corbett, 1999). The mechanisms of coumestrol-mediated neuronal protection have not

been completely elucidated, but appear to be via both estrogen receptor and non-receptor actions.

In order to further ascertain whether coumestrol could be a tangible therapeutic strategy against global ischemia injury, we injected intracardiaclly a single dose of 20 μg/kg of coumestrol one hour before the global ischemia. For our surprise, the peripheric administration appears to be even more neuroprotective in comparison with the icv administration (statistical analysis not shown). It is well documented that systemic administration of pharmacological doses of estradiol can be neuroprotective in global ischemia (Lebesgue et al., 2010) and in focal ischemia (Fan et al., 2003). We presume that coumestrol can reach similar brain levels as much as estradiol since both are small molecules that are highly lipophilic therefore, they cross the Blood Brain Barrier and cell membranes easily.

The mechanisms by which coumestrol is acting either icv or peripherally to afford robust neuroprotection remain unclear. Its protective effects appear to be receptor-mediated since its beneficial effect in histological parameter was partially prevented by the broad-spectrum ER antagonist ICI 182,780. ERs play a critical role in the neuroprotective effects of phytoestrogens (Schreihofer and Redmond, 2009). Coumestrol has a relative binding affinity for ER-β approximately equivalent to 17 β-estradiol (Kuiper et al., 1998). Both ERs are expressed in the rodent hippocampus but ER-β is more prevalent regulating hippocampal synaptic plasticity (Mitra et al., 2003) and improving neuronal survival. Increased ER-β immunoreactivity in the post-ischemic monkey hippocampus has also been found (Takahashi et al., 2004).

There are several lines of evidence that ER-β is involved in neuroprotection (Sawada et al., 1998). Comparison of relative binding affinities from various studies indicates that some phytoestrogens appear to have a higher affinity for ER-β than for ER-α and therefore suggests that the ER-mediated effects of phytoestrogens may be mediated through ER-β (Belcher and Zsarnovszky, 2001). However, is still unclear which ER subtype mediates the neuroprotective efficacy of estrogen/phytoestrogen.

The icv and the peripheral administration of coumestrol in different times before and after ischemia and the partial neuroprotection abrogation by the ER antagonist indicate that the neuroprotection afforded by this compound likely involves activation of the classical ERs. However, this not rules out the possibility that other estrogen receptors or pathways of neuronal survival may play a role in coumestrol neuroprotection following ischemic insult. The partial abrogation by the antagonist suggest that it might be another alternative pathway that coumestrol is using to reach neuroprotection to CA1 than just through the ER pathway. Furthermore, some neuroprotective effects of estrogen-like compounds appear to be independent of their ability to bind ERs (Prokai and Simpkins, 2007).

Studies conducted with other phytoestrogens affording neuroprotection in models of cerebral ischemia and other neurodegenerative diseases agree with our findings (Al-Nakkash et al., 2009; Donzelli et al., 2010; Kim et al., 2009; Carswell et al., 2004). Genistein (Kindy, 1993; Donzelli et al., 2010), (-) catechin (Inanami et al., 1998), green tea extracts rich in phytoestrogens (Hong et al., 2001) have been shown to limit brain injury in gerbil model of global cerebral ischemia. In a study conducted by Schreihofer (2005) genistein demonstrated to protect neurons from transient global ischemia injury in rat hippocampus by attenuating oxidative stress, lipid peroxidation, and the signaling cascade leading to apoptotic cell death.

Recent evidence indicates that the production of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxide is increased after cerebral ischemia. Since the rates of oxidative metabolic activities are high and the antioxidants enzyme activities are low in the brain, neurons are vulnerable to ischemic events. In studies about phytoestrogen antioxidant proprieties, coumestrol showed a high hydrogen/electron donation via hydroxyl groups and demonstrated to have an effective antioxidant activity (Mitchell et al., 1998). It is well know that phytoestrogens, acting as antioxidants, can decrease the accumulation of ROS, thereby protecting cell membrane integrity and so promoting neuronal survival (Cai et al., 1997; Mitchell et al., 1998). However, the ROS production after the ischemic insult remains for a very short period in the cell (Thiyagarajan et al., 2004; Golden and Patel, 2009; Kleinschnitz et al., 2010) suggesting that perhaps the neuroprotection seeing after 24 h or even after 6 h afforded by coumestrol administration may be not due its antioxidant proprieties. The mechanism, however, by which coumestrol was neuroprotective against delayed neuronal death has not been fully elucidated. Further studies are necessary to elucidate other molecular targets mediating the action of the coumestrol.

Beyond chemical antioxidant proprieties, other biochemical mechanisms might also play a role in neuronal survival. It is now clear that estrogens initiate rapid signaling events in neurons by binding to recognition molecules other than the classical receptors ER-α and ER-β. Recent studies reveal the existence of transmembrane receptors capable of responding to steroids with cellular activation. On such receptor, GPR30, is a member of the G protein coupled receptor superfamily and mediates transcription-dependent and independent actions of estrogens and widely expressed in the brain including hippocampus (Filardo et al., 2002; Filardo and Thomas, 2005); Prossnitz et al., 2007, 2008). Estradiol exhibits an affinity for GPR30 similar to ER-α and ER-β (Etgen et al., 2010) and its binding to GPR30 stimulates production of cAMP, mobilization of calcium and activation of growth factor signaling (Prossnitz et al., 2007, 2008; Filardo et al., 2000, 2002). There is strong evidence that GPR30 can act together with intracellular ERs to activate cell signaling pathways to promote neuronal survival after global ischemia (Lebesgue et al., 2009). Therefore this might be an alternative pathway of neuronal survival afforded by coumestrol in cerebral global ischemia. Additional studies are needed to verify the molecular mechanisms involving this receptor and its targets in neuroprotection. Determining whether ERs and/or other membrane estrogen receptors mediate estradiol and coumestrol neuroprotection following global ischemia is of great interest both for the development of therapeutic strategies and for elucidating underlying molecular mechanisms of delayed neuronal death.

In conclusion, with the present study we have demonstrated that coumestrol prevented long-term neuronal death in CA1 hippocampal layer in rats when submitted to 10 min global ischemia. Such findings suggest that this compound interferes with the early and delayed stages of neuronal damage. Furthermore, our study reports the first evidence that an acute administration of coumestrol significantly reduces the delayed neuronal cell death occurring in hippocampus of female rats following a transient global ischemic insult. The mechanisms underlying the neuroprotection exerted by coumestrol seem to involve, at least in part, estrogen receptor activation, antioxidant activity and activation of other membrane receptors that mediate estradiol neuroprotection. Additional studies are needed to determine the molecular targets mediating the neuroprotective action of coumestrol and the effects that this phytoestrogen may have on the mature nervous system.

4. Experimental procedure

4.1. Animals

Female adult Wistar rats (3 months, 170–210 g BW) were obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22±1 °C) colony room, with free access to water. This work was carried out in accordance with the EC directive 86/609/EEC for animal experiments. The study was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil.

4.2. Surgery

Rats weighing between 150 and 250 g at time of surgery were ovariectomized (OVX) by the surgical removal of both ovaries under intraperitoneal (i.p.) ketamine anesthesia (90 mg/kg) and xylazine (10 mg/kg) to eliminate endogenous ovarian steroids (Waynforth and Flecknell, 1992).

4.3. Groups

The animals were randomized into six groups: Vehicle-treated sham and ischemic; coumestrol-treated sham and ischemic; 17 β-estradiol-treated sham and ischemic (used as positive control). For the broad-spectrum ER antagonist ICI 182,780 experiment, the same groups were used ($n=5$ animals/group).

4.4. Global ischemia

One week following the OVX surgery, rats was subjected to transient global ischemia by four vessel occlusion as previously described by Pulsinelli and Brierley (1979). Rats were deeply anesthetized under halothane (4% induction, 1% maintence in 70% N_2O:30% O_2), and the vertebral arteries were irreversibly occluded by electrocoagulation to prevent collateral blood flow to the forebrain during the subsequent occlusion of the common carotid arteries. A silk thread was looped around the carotid arteries to facilitate subsequent occlusion. Twenty-four hours later, the animals were anesthetized again, the wound was reopened and both carotid arteries were occluded with micro arterial clamps for 10 min. The 4 VO model were chosen because it is the most used model that resembles a human cardiac arrest where the blood supply in the brain is almost depleted. The outcomes are neurological damage, loss of memory, convulsions and coma. During clamping, the animals were awake and spontaneously ventilating. During both surgeries, rectal temperature was monitored and maintained at 36.5–37.5 °C with a rectal thermistor and heat lamp until recovery from anesthesia. Sham operated animals were subjected to the same anesthesia and surgical procedures as animals subjected to global ischemia, except the carotid arteries were not occluded (Netto et al., 1993). Animals that failed to show complete loss of the righting reflex and pupillary dilatation (from 2 min after occlusion has initiated until the end of occlusion); animals that exhibited obvious behavioral manifestations (abnormal vocalization when handled, convulsions, hyperactivity etc.) were excluded from the experiment; and animals with loss of greater than 20% of body weight by 3–7 day after ischemia. There were 5 deaths due to respiratory arrest; 11 other rats were excluded from the study because they failed to show neurological signs of ischemia (no loss of consciousness or incomplete dilation of the pupils during occlusion).

4.5. Drugs

One hour before ischemia or 0 h, 3 h, 6 h or 24 h after ischemia animals received intracerebroventricular (icv) injections into the right lateral ventricle of 20 μg of coumestrol (Sigma) (diluted in 100% dimethylsufoxide) (DMSO; Sigma), 20 μg of 17 β-estradiol (diluted in 0.9% saline solution containing 10% DMSO) or 50 μg of ICI 182,780 (Sigma), in a volume of 2 μl. Control animals were infused with vehicle (100% DMSO). The dose of 20 μg was chosen based on previous studies with estrogen-like compounds (Azcoitia et al., 1999;Picazo et al., 2003; Callier et al., 2001; Bryant et al., 2005; Toung et al., 2000) with similar proprieties and actions in the central nervous system. Animals also received icv infusion of the broad-spectrum antagonist ICI 182,780 or vehicle into the lateral ventricle. The administration of 50 μg was done 10 min prior to the other drugs administration. For the peripheral administration, a dose of 20 μg/kg of coumestrol was injected intracardiaclly one hour before the ischemic insult. Coumestrol was diluted in 100% dimethylsufoxide (DMSO; sigma) in a volume of 300 μl.

4.6. Intracerebroventricular and peripheral injections

In the first experiment, rats were positioned in a stereotaxic apparatus and icv injections performed under halothane anesthesia either 1 h before ischemia or 0 h, 3 h, 6 h or 24 h after ischemia, The position of the right lateral ventricle was calculated based on the position of bregma: 0.92 mm posterior to bregma, 1.2 mm lateral to bregma, 3.6 mm below the skull surface according to the atlas of Paxinos Ga (1998) and

then coumestrol, 17 β-estradiol, the non-selective ER antagonist ICI 182,780 and/or vehicle was infused with a Hamilton syringe (Fisher scientific, Pittsburgh, PA) in a volume of 2 μl per infusion over 2 min. The injection needle was left in place for an additional 2 min before being withdrawn. For the coumestrol peripheral administration, rats received a single dose of 20 μg diluted in 300 μl of 100% DMSO injected intracardiaclly one hour before the ischemic insult.

4.7. *Histological analysis and hippocampal cell counts*

The impact of transient global ischemia on the survival of hippocampal CA1 pyramidal neurons was examined seven days after ischemia or sham surgery, rats were killed by transcardiac perfusion with 4% paraformaldehyde under deep anesthesia. Brains were rapidly removed. Hematoxiline–Eosine method was used to stain coronal sections of 25 μm collected through the entire dorsal hippocampus. Digital images of every tenth section from each animal (~100 sections per brain) were captured and used to trace the outline of the CA1. Medial, middle and lateral sectors from the CA1 region of the left and right hippocampus were photographed at 40X magnification using a Nikon microscope and digital camera. As previously described by Colbourne and Corbett (1995) a microscope counting grid (250 μm × 250 μm) was positioned a few cells medial from CA2 neurons (lateral sector), at the apex of the CA1 (middle sector) and the upswing of CA1 and the number of viable pyramidal neurons in this 250 μm × 250 μm region of interest was counted. Viable neurons had rounded cell bodies and clearly visible nucleoli. Pyknotic and shrunken neurons were not counted. All cell counts were carried out by an investigator who was blind to the animal's treatment.

5. Statistical analysis

Statistical comparison of the number of surviving CA1 pyramidal neurons among groups was performed using a two-way ANOVA followed by Duncan's multiple range test for post hoc analysis. Differences were considered significant at $p<0.01$.

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