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Molecular mechanisms mediating the neuroprotective role of the selective estrogen receptor modulator, bazedoxifene, in acute ischemic stroke: A comparative study with 17β-estradiol

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ABSTRACT

As the knowledge on the estrogenic system in the brain grows, the possibilities to modulate it in order to afford further neuroprotection in brain damaging disorders so do it. We have previously demonstrated the ability of the selective estrogen receptor modulator, bazedoxifene (BZA), to reduce experimental ischemic brain damage. The present study has been designed to gain insight into the molecular mechanisms involved in such a neuroprotective action by investigating: 1) stroke-induced apoptotic cell death; 2) expression of estrogen receptors (ER) ERα, ERβ and the G-protein coupled estrogen receptor (GPER); and 3) modulation of MAPK/ERK1/2 and PI3K/Akt signaling pathways. For comparison, a parallel study was done with 17β-estradiol (E2)-treated animals. Male Wistar rats subject to transient right middle cerebral artery occlusion (tMCAO, intraluminal thread technique, 60 min), were distributed in vehicle-, BZA- (20.7 ± 2.1 ng/mL in plasma) and E2- (45.6 ± 7.8 pg/mL in plasma) treated groups. At 24 h from the onset of tMCAO, RT-PCR, Western blot and histochemical analysis were performed on brain tissue samples. Ischemia-reperfusion *per se* increased apoptosis as assessed by both caspase-3 activity and TUNEL-positive cell counts, which were reversed by both BZA and E2. ERα and ERβ expression, but not that of GPER, was reduced by the ischemic insult. BZA and E2 had different effects: while BZA increased both ERα and ERβ expression, E2 increased ERα expression but did not change that of ERβ. Both MAPK/ERK1/2 and PI3K/Akt pathways were stimulated under ischemic conditions. While BZA strongly reduced the increased p-ERK1/2 levels, E2 did not. Neither BZA nor E2 modified ischemia-induced increase in p-Akt levels. These results show that modulation of ERα and ERβ expression, as well as of the ERK1/2 signaling pathway accounts, at least in part, for the inhibitory effect of BZA on the stroke-induced apoptotic cell death. This lends mechanistic support to the consideration of BZA as a potential neuroprotective drug in acute ischemic stroke treatment.

1. Introduction

Acute ischemic stroke (AIS) still remains as a leading cause of death, permanent disability, and dementia worldwide. Major advances in AIS treatment came from vascular approaches to dissolve (systemic "recombinant tissue-type plasminogen activator", rt-PA) or remove (endovascular thrombectomy) the occluding clot. Unfortunately, only a minority of patients are eligible for such treatments. Moreover, the treatments can be futile (or harmful) if it does not reperfuse adequately in the brain microvasculature. On the other hand, neuroprotection,

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which refers either to minimizing the harmful effect of ischemia at the level of the neuron or, from the pragmatic point of view of patients and physicians, to keeping brain damage under the threshold of symptom manifestation, has not saved the gap between bench and bedside. However, if neuroprotectors are to be effective in slowing down cell death, this may be very useful in prolonging the therapeutic window and, therefore, the management of AIS (see Chamorro et al. [1]; for a recent review). In this context, the estrogenic system arises as a potentially useful target.

Simpkins et al. [2] provided the first evidence that estrogens (specifically 17β-estradiol, E2) exerted neuroprotective effects in the now widely used rodent model of AIS, namely the middle cerebral artery occlusion (MCAO) model. The authors suggested that estrogens may be a useful therapy to protect neurons against the damaging effects of stroke. As recently reviewed by the same group, after more than two decades of intense investigation, the neuroprotective role of E2 in experimental models of AIS is well-established [3,4]. However, exogenous estrogenic treatments for nervous system disorders including stroke seem to be far from being established as a reliable therapy. This comes mainly from the articles in the Women's Health Initiative trial, with stroke or cognition as outcomes of interest, concluding that E2, alone or in combination with progestin, resulted in approximately 50% excess risk of ischemic stroke and in 76% excess risk of dementia in women 65 years or older [5]. This, along with other undesirable effects, has shifted the focus onto other ligands of the estrogen receptors (ER), especially the "selective estrogen receptor modulators" (SERMs).

Synthetic SERMs are an ever-growing family of compounds aimed at preventing/treating diseases derived from estrogen deprivation (ovariectomized or naturally postmenopausal women). The aim is to mimic the beneficial role of E2, while avoiding its detrimental effects. Ideally a SERM should demonstrate agonistic (protective) effects in bone (osteoporosis), brain (cognitive status) and cardiovascular system (vasomotor symptoms, coronary heart disease, *etc.*), and neutral or antagonistic (safe) effects in breast and uterine tissues (cancer) [6,7]. Kimelberg et al. [8] first reported the ability of the first-generation SERM tamoxifen to reduce infarct volume in the MCAO model, an observation that was subsequently confirmed in the same model [9–13], as well as in models of hypoxic-ischemic brain injury both in the whole animal [14] and in cultured neural tissues [15–17]. Some results in the same direction have been reported for the other "classical" second-generation SERM, raloxifene, in neural cultures [18–20].

Bazedoxifene acetate (BZA) is the first of the third-generation SERMs approved for the treatment of postmenopausal women at risk for, or presenting with, osteoporosis in Europe and Japan [21]. Due to its favorable preclinical effects, BZA has been selected to combine with conjugated estrogens (CE) resulting in CE/BZA as a new progestin-free hormone therapy option for alleviating estrogen deficiency symptoms in postmenopausal women [22]. As for neural tissue, BZA had been reported to prevent neuronal loss in the hippocampus of rats exposed to excitotoxic kainic acid [23], and to decrease the inflammatory response of astrocytes exposed to lipopolysaccharide [24]. We have recently demonstrated that the neuroprotective role of BZA also extends to experimental AIS [25]. The present study has been designed to gain insight into the mechanisms involved in such a neuroprotective action by investigating: 1) stroke-induced apoptotic cell death; 2) expression of ERα, ERβ and GPER; and 3) regulation of MAPK/ERK1/2 and PI3K/Akt signaling pathways. For comparative purposes, a parallel study was done with E2-treated animals.

2. Material and methods

2.1. Animals and ethical issues

Ninety-seven male Wistar rats (300–350 g, Charles River, Barcelona, Spain) were housed under standard environmental conditions, and fed natural-ingredient soy- and alfalfa-free phytoestrogen-reduced diet with water *ad libitum*. Experiments were conducted in compliance with the legislation on protection of animals used for scientific purposes in Spain (RD 53/2013) and the EU (Directive 2010/63/EU). Protocols were approved by the Animal Experimentation Ethics Committee from IIS La Fe.

2.2. Transient focal cerebral ischemia

Transient right middle cerebral artery occlusion (tMCAO) was performed by following the intraluminal suture procedure as originally described [26], and adapted to our experimental setup [27]. This includes continuous monitorization under anesthesia of cerebrocortical laser-Doppler flow (cortical perfusion, CP), arterial blood presure (ABP) and core temperature (T), and discontinuous measurement of pH, PaO_2, $PaCO_2$ and glucose at the three stages during surgery: pre-ischemia (basal), ischemia and reperfusion. MCAO was maintained for 60 min, after which reperfusion was monitorized for 30 min. Twenty four hours after the ischemic insult, the rats were euthanized to obtain brain samples according to specific requirements for each determination.

2.3. Experimental groups

Thirty-seven rats were excluded from the study according to the following criteria: 1) CP did not drop after filament gliding (no ischemia, n = 12); 2) CP did not recover after filament withdrawal (no reperfusion, n = 9); 3) no brain infarction in spite of a right ischemia-reperfusion pattern (n = 6); and 4) death before the 24 h time limit (n = 10).

Three MCAO groups were established after exclusions: vehicle-(dimethyl sulfoxide, n = 20), BZA- (n = 23) and E2- (n = 17) treated rats. BZA was from Axon Medchem, Groningen, The Netherlands; and E2 was from Sigma-Aldrich, Madrid, Spain. Treatments were applied 1 h before ischemia in such a way that optimal plasma concentrations were reached 4 h later, and were sustained until the end of the procedure [25]. The BZA-treated rats received a single dose (s.c.) of 1 mg/kg BZA, followed by implantation (i.p.) of an osmotic pump delivering 3 mg/kg/day BZA. The E2-treated rats received a single dose (s.c.) of 30 μg/kg E2, followed by implantation (i.p.) of an osmotic pump (Alzet model 2ML1 with a pumping rate of 10 μL/h, Durect Corp., Cupertino, CA, USA) delivering 100 μg/kg/day E2. Plasma concentrations of BZA and E2 at 4 h were 20.7 ± 2.1 ng/mL and 45.6 ± 7.8 pg/mL, respectively. Since both BZA and E2 were dissolved in 100% dimethyl sulfoxide (1 mg/mL stock), animals in all three experimental groups received the same amount of dimethyl sulfoxide: 1 mL/kg as the initial bolus, plus 10 μL/h during 24 h (osmotic pump).

2.4. TUNEL detection of DNA cleavage and immunolabeling

Rats under anesthesia were perfused transcardially with 4% paraformaldehyde in phosphate buffer (0.2 M, pH 7.4). Brains were removed, postfixed (overnight at 4 °C), cryoprotected (30% sucrose, 48 h), OCT embedded, flash frozen and cut into 40 μm sections in the coronal plane (0.2 to −1.8 mm from bregma). Free-floating sections were blocked in 10% normal serum, 5% bovine serum albumin and 0.01% saponin in PBS (1 h at room temperature). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed using the *In Situ* Cell Death Detection Kit, TMR red, as per the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). After the TUNEL reaction, the sections were processed for immunolabeling with anti-Estrogen Receptor Alpha (ERα) rabbit polyclonal antibody (1:400, Abcam, Cambridge, UK), anti-Estrogen Receptor Beta (ERβ) rabbit polyclonal antibody (1:250, Thermo Scientific, Waltham, MA, USA) or anti-GPER rabbit polyclonal antibody (1:200, Abcam) overnight at 4 °C, followed by incubation with anti-rabbit DyLight 488 (1:500; Vector Laboratories, Burlingame, CA, USA). Nuclei were stained using DAPI according to the manufacturer's

protocol (1:5000, Molecular Probes, Eugene, OR, USA). Images were viewed with a fluorescence microscope (LEICA DM 4500B, Leica Microsystems, Barcelona, Spain) equipped with an image analysis system (LEICA DFC 300 FX camera with LEICA application suite V4). For quantification of TUNEL reactivity, the fluorescence of the images within three fields of the cortical region and one field of the subcortical region of the ipsilateral hemisphere was analyzed using ImageJ-win32 NIH program. The number of TUNEL-positive cells was expressed as a percentage of the corresponding DAPI- stained nuclei.

2.5. Caspase activity assay

Rats were euthanized by intracardiac 5 mEq KCl under anesthesia. Brains were removed, frozen and cut into 18 μm sections in the coronal plane (0.2 to −1.8 mm from bregma) to perform the caspase activity assay by using the APO LOGIX™ carboxyfluorescein (FAM) caspase detection kit (Cell Technology, Minneapolis, MN, USA). Briefly, brain sections were labeled with 5 μM FAM-DEVD-FMK (1 h, 37 °C), washed three times with 1× working dilution wash buffer, fixed and cover slipped with ProLong (Molecular Probes). Image viewing, acquisition and analysis were as above described. For quantitation of caspase-3 activity, the fluorescence of the images, within three fields of the ipsilateral cortex of each animal, was analyzed using ImageJ-win32 NIH program, expressed as a percentage of the field area and averaged for each hemisphere.

2.6. Real-time reverse transcription-polymerase chain reaction analysis

Rats were euthanized by intracardiac 5 mEq KCl under anesthesia and a 2 mm-thick brain coronal section (0.2 to −1.8 mm from bregma) was obtained. Ipsilateral and contralateral hemispheres were separated and immersed in RNA-later solution (Ambion, Foster City, CA, USA). Tissue was homogenized and total RNA was isolated using the TRIZOL reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Quantitative polymerase chain reaction (PCR) was performed on a thermal cycler (IQ™5 Multicolor Real-Time PCR Detection System, Bio-Rad, CA, USA), using PowerSYBR Green PCR Master Mix (Applied Biosystems, Paisley, UK) and the following run profile: 10 min at 95 °C, 40 cycles of melting (15 s at 95 °C), annealing (30 s at 62 °C), and extension (30 s at 72 °C). The threshold cycle (C_T) was determined, and the relative gene expression was calculated with the Livak comparative C_T method [28], using ribosomal protein, large, P0 (*Rplp0*) as housekeeping gene. The following gene-specific primers (5′-3′) (Sigma-Aldrich) were used: *Esr1* F: TACGAAGTGGGCATGATGAA, *Esr1* R: GGCGGGGCTATTCTTCTTAG; *Esr2* F: CTCACGTCAGGCACATCAGT, *Esr2* R: GGTTCTGGGAGCTCTCTTTG; *Gper* F: GCTGCAACTACTCCAGCACA, *Gper* R: TGTTCAGAGAGGTCCCCAGT; *Rplp0* F: CAGCAGGTGTTTGACAATGG, *Rplp0* R: CCCTCTAGGAAGCGAGTGTG.

2.7. Western blot analysis

Rats were euthanized by intracardiac 5 mEq KCl under anesthesia, a 2 mm-thick brain coronal section (0.2 to −1.8 mm from bregma) was obtained, and ipsilateral and contralateral hemispheres separated. Tissue was homogenized in lysis buffer (ProteoJetTM Mammalian Cell Lysis Reagent, Fermentas, Burlington, ON, Canada) containing protease and phosphatase inhibitor cocktails (1%, Sigma-Aldrich). Protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). Aliquots of protein (40 μg) were dissolved in NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA) under reducing conditions, loaded on 4–12% Bis-Tris gels (Invitrogen), subjected to SDS-PAGE and electrotransferred onto to 0.2 μm nitrocellulose membranes for immunolabeling using the following primary antibodies: anti-Estrogen Receptor Alpha (ERα) rabbit polyclonal antibody (1:500; Abcam); anti-Estrogen Receptor Beta (ERβ) rabbit polyclonal antibody (1:250; Thermo Scientific); anti-GPER rabbit polyclonal antibody (1:500; Abcam); anti-cleaved caspase-3 affinity purified rabbit polyclonal antibody (1:500, Cell Signaling Technology, Inc., Beverly, MA, USA); anti-phospho MAPK (p-ERK1/2) rabbit monoclonal antibody (1:2000, Cell Signaling Technology); anti-MAPK1/2 (ERK1/2) rabbit polyclonal antibody (1:5000, Millipore, Temecula, CA, USA); anti-phospho Akt (p-Akt) rabbit monoclonal antibody (1:500; Cell Signaling Technology); anti-Akt rabbit monoclonal antibody (1:1000, Millipore); and anti-β-actin mouse monoclonal antibody (1:10000; Sigma-Aldrich). Secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000, Rad, Hercules, CA, USA), and goat anti-mouse IgG (1:2000, Bio-Rad). After reaction, membranes were treated with enhanced chemiluminescence reagents (ECL, Amersham, Buckinghamshire, UK) and imaged using ChemiDoc XRS imaging system (Bio-Rad). Membranes were re-probed with anti-β-actin antibody as a loading control. Bands on Western blots were analyzed using Scion Image Beta 4.0.3 software. Band densities for p-ERK1 and p-ERK2, or p-Akt were normalized to the corresponding band densities for total ERK1 and ERK2, or Akt, respectively. Band densities for caspase-3, ERα, ERβ or GPER were normalized to the corresponding band densities for β-actin.

2.8. Statistical analysis

The results were expressed as mean ± SEM. Data analysis was performed using GraphPad Instat 3.06 software. Statistical comparisons were assessed by analysis of variance (ANOVA) followed by post-hoc Student–Newman–Keuls (SNK) multiple comparison test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Apoptosis inhibition is a mechanism of BZA-induced neuroprotection in transient focal cerebral ischemia

TUNEL-positive cells were undetectable in the contralateral hemisphere of rats subjected to tMCAO. By contrast, ischemia-reperfusion induced the presence of TUNEL-positive cells in the ipsilateral hemisphere of vehicle-, BZA- and E2-treated groups (Fig. 1A). However, ipsilateral hemisphere from BZA-treated animals showed significantly lower TUNEL-positive cell counts than those from vehicle-treated animals, while E2 showed a reduction tendency in TUNEL-positive cell counts (Fig. 1B). Further statistical analysis showed that the reductions in TUNEL-positive cell counts elicited by both BZA and E2 were significant in cortical (Fig. 1C) but not in subcortical (Fig. 1D) regions.

To directly measure apoptosis executioner caspase-3-like functional activity, brain sections were labeled with FAM-DEVD-FMK. Caspase-3 activity was undetectable in the contralateral hemisphere of brains after tMCAO. By contrast, ischemia-reperfusion induced the appearance of caspase-3 activity in the ipsilateral hemisphere of the brain sections from vehicle-, BZA- and E2-treated groups (Fig. 2A). However, marked differences among groups were statistically significant as caspase-3 activity was strongly inhibited in the ipsilateral hemisphere by both BZA and E2 (Fig. 2B). Accordingly, Western blot studies showed significantly higher levels of activated caspase-3 p17/19 form in the ipsilateral hemisphere from the vehicle-treated group when compared to the contralateral hemisphere. Both BZA and E2 inhibited such an increase. No significant difference was found between BZA- and E2-treated groups (Fig. 2C and D).

3.2. BZA modulates ERα and ERβ expression, but not that of GPER, after ischemia-reperfusion

Quantitative PCR analysis showed that the ischemia-reperfusion episode significantly decreased ERα mRNA expression in the ipsilateral

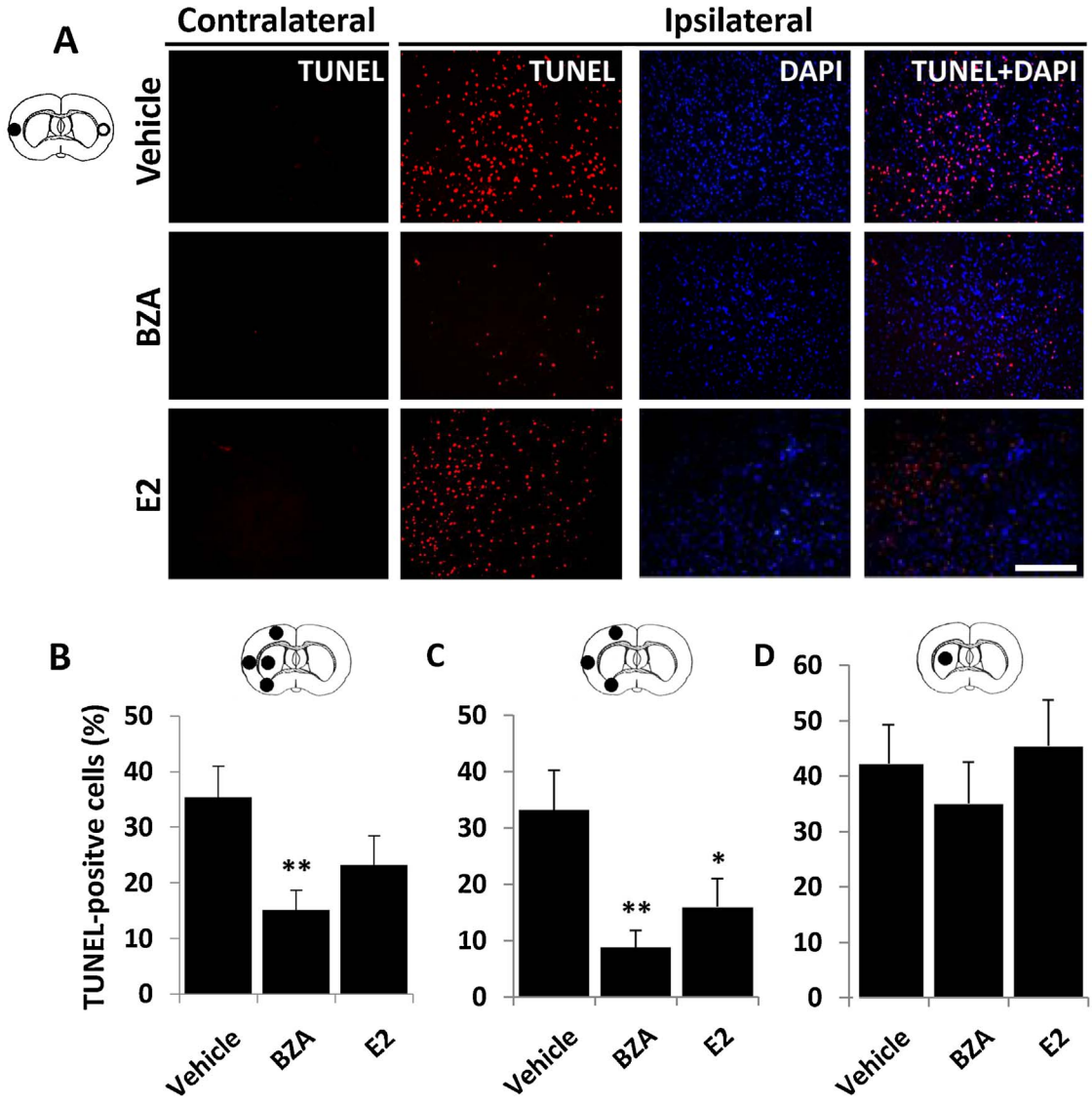

Fig. 1. Bazedoxifene and 17β-estradiol inhibit apoptosis after ischemia-reperfusion: TUNEL. A) Lack of TUNEL-labeling in a cortex field of the contralateral hemisphere (○), and double-labeling (TUNEL, DAPI and merged images) in a cortex field of the ipsilateral hemisphere (●) of representative brain sections. Scale bar, 25 μm. B) TUNEL-positive cell counts expressed as a percentage of the corresponding DAPI-stained nuclei in four selected fields of the entire ipsilateral hemisphere (●), C) three fields of the cortical region (●), and D) a field of the subcortical region (●). Significantly different from vehicle-treated group (*$p < 0.05$; **$p < 0.01$). Data are mean ± SEM of 4–7 animals per group.
BZA = bazedoxifene. E2 = 17β-estradiol.

hemisphere, when compared with the contralateral hemisphere in the vehicle-treated group. As for the effect of the estrogenic compounds, both BZA and E2 significantly counteracted the ischemia-induced downregulation of ERα mRNA expression in the ipsilateral hemisphere (Fig. 3A). Western blot analysis showed that the ischemia-reperfusion also induced a significant decrease in ERα protein expression of the ipsilateral hemisphere, with respect to the contralateral hemisphere in the vehicle-treated group. Both BZA and E2 significantly reversed the ischemia-induced decrease in ERα protein expression of the ipsilateral hemisphere (Fig. 3B and C). To determine the localization of ischemia-induced changes in expression of ERs, triple labeling of each ER subtype plus TUNEL and DAPI was carried out. Labeling of ERs, TUNEL and DAPI was positive in the ipsilateral hemisphere, while in the contralateral hemisphere the labeling was also positive except for the TUNEL method, as expected. With regard to ERα, immunoreactivity was detected predominantly in the nucleus, as mainly colocalized with DAPI stain, and there was not an overlap between ERα immunoreactivity and TUNEL reactivity in the ipsilateral cortex (Fig. 3D).

The ischemia-reperfusion episode significantly decreased ERβ mRNA expression in the ipsilateral hemisphere, when compared with the contralateral hemisphere in the vehicle-treated group. As for the effect of the estrogenic compounds, BZA significantly counteracted the ischemia-induced downregulation of ERβ mRNA expression, while E2 was without significant effect (Fig. 4A). Ischemia-reperfusion did not significantly modify ERβ protein expression in the ipsilateral hemisphere, when compared with the contralateral hemisphere in the vehicle-treated group. ERβ protein expression showed an upregulation tendency in the ipsilateral hemisphere that reached statistical significance in the BZA-treated group. In contrast, E2 had no effect on ERβ protein expression (Fig. 4B and C). ERβ immunoreactivity was detected predominantly in the nucleus, colocalized with DAPI stain, and there was not an overlap between ERβ immunoreactivity and TUNEL reactivity in the ipsilateral cortex (Fig. 4D).

After the ischemia-reperfusion episode, GPER mRNA expression was not significantly altered in the ipsilateral hemisphere, when compared with the contralateral hemisphere in the vehicle-treated group. As for the effect of the estrogenic compounds, neither BZA nor E2 had a significant effect on GPER mRNA expression in the ipsilateral hemi-

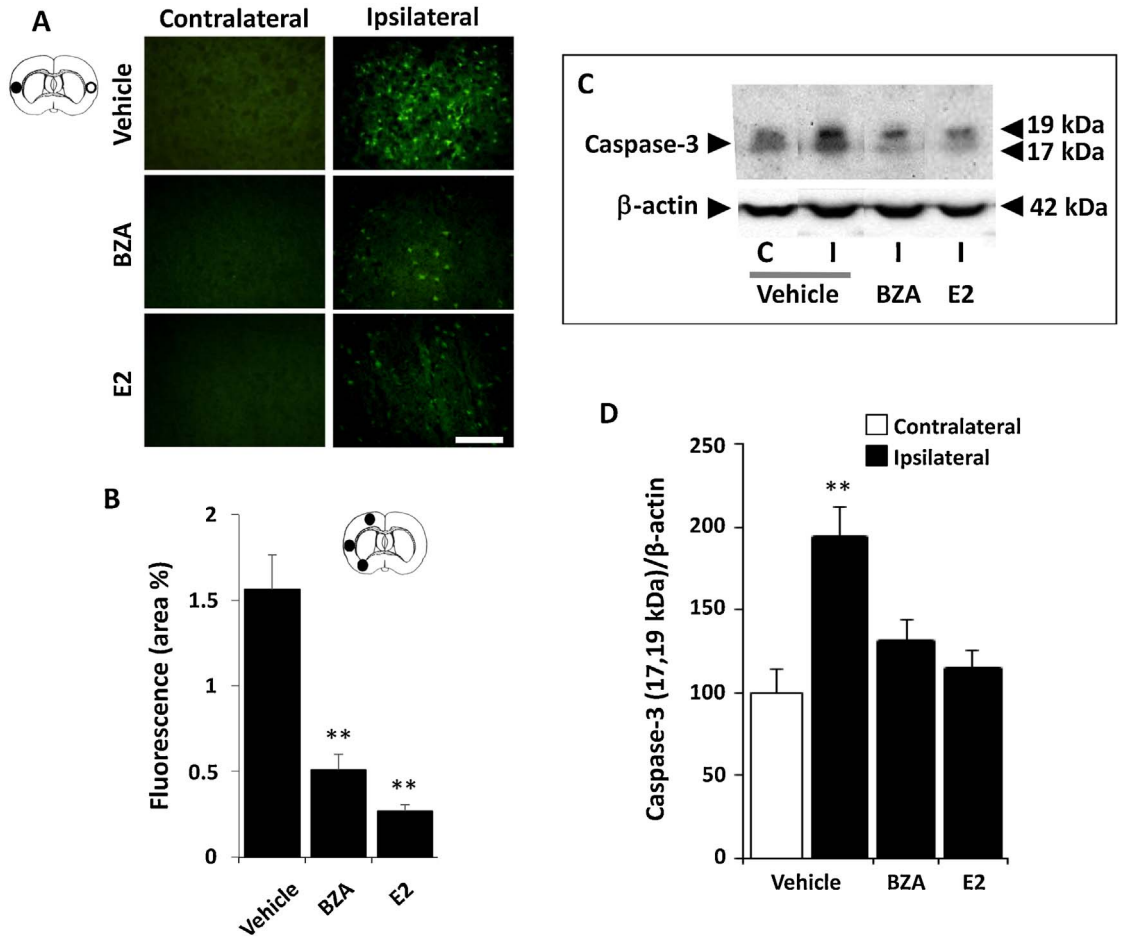

Fig. 2. Bazedoxifene and 17β-estradiol inhibit apoptosis after ischemia-reperfusion: caspase-3. A) FAM-DEVD-FMK labeling in a cortex field of the contralateral hemisphere (○) and the ipsilateral hemisphere (●) of representative brain sections. Scale bar, 25 μm. B) Fluorescence quantification within three fields of the ipsilateral cortex (●), expressed as a percentage of the field area. Significantly different from ipsilateral vehicle-treated group (**p < 0.01). C) Representative Western blots, and D) relative abundance of activated caspase-3 p17/19 form in whole-cell lysates of contralateral (C) and ipsilateral (I) hemispheres. Significantly different from contralateral vehicle-treated group (**p < 0.01). Data are mean ± SEM of 4–7 animals per group.
BZA = bazedoxifene. E2 = 17β-estradiol.

sphere (Fig. 5A). Similarly, the ischemia-reperfusion did not significantly modify GPER protein expression in the ipsilateral hemisphere, when compared with the contralateral hemisphere in the vehicle-treated group. Neither BZA nor E2 had a significant effect on GPER protein expression in the ipsilateral hemisphere (Fig. 5B and C). GPER immunoreactivity was shown mainly in the cytoplasmic/membrane region, and did not overlap with TUNEL reactivity in the ipsilateral cortex (Fig. 5D).

3.3. BZA influences MAPK/ERK but not PI3K/Akt signaling after ischemia-reperfusion

When compared with the contralateral hemisphere, ischemia-reperfusion significantly increased phosphorylation of ERK1 and ERK2 in the ipsilateral hemisphere of vehicle-treated group (Fig. 6). Such increases in p-ERK1/2 levels were not accompanied by a rise in total ERK protein level (Fig. 6A). BZA but not E2 significantly attenuated the ischemia-induced endogenous phosphorylation of ERK1 in the ipsilateral hemisphere (Fig. 6A and B). While p-ERK2 levels were significantly reduced in the ipsilateral hemisphere in animals treated with BZA, there was no significant change in animals treated with E2 (Fig. 6A and C).

As for the PI3K/Akt pathway, ischemia-reperfusion significantly increased phosphorylation of Akt in the ipsilateral hemisphere when compared with the contralateral hemisphere of vehicle-treated group. Such an increase was not altered by either BZA or E2 treatments (Fig. 7).

4. Discussion

The current study represents a step forward in understanding how BZA, a third-generation SERM particularly indicated in the treatment of osteoporosis in postmenopausal women, could protect the brain against an ischemic insult. We had previously demonstrated that BZA reduced infarct volume in the tMCAO stroke model, acting mainly in the cortical region [25]. In the present study we show, for the first time, that BZA inhibits stroke-induced apoptotic neuronal death and modulates both ERα and ERβ expression as well as the downstream ERK1/2 signaling pathway; which could account, in part, for its neuroprotective effect.

Limiting the expansion of the ischemic core by preventing apoptotic cell death in the surrounding penumbra is a major goal in stroke neuroprotection. Neuronal apoptosis can occur through both the intrinsic (mitochondrial) and the extrinsic (death receptors) pathways to activate caspase-3 and lead to DNA fragmentation, two apoptosis hallmarks. A two-step process is needed for the caspase-3 activation. First, the inactive zymogen (caspase-3 proform) is cleaved by upstream caspases to form the intermediate p19/12 complex. The second step involves autocatalytic processing to generate the fully activated p17/12 form of caspase-3 [29]. To study caspase-3 activation we carried out: (1) Western blot analysis using an antibody that detects endogenous

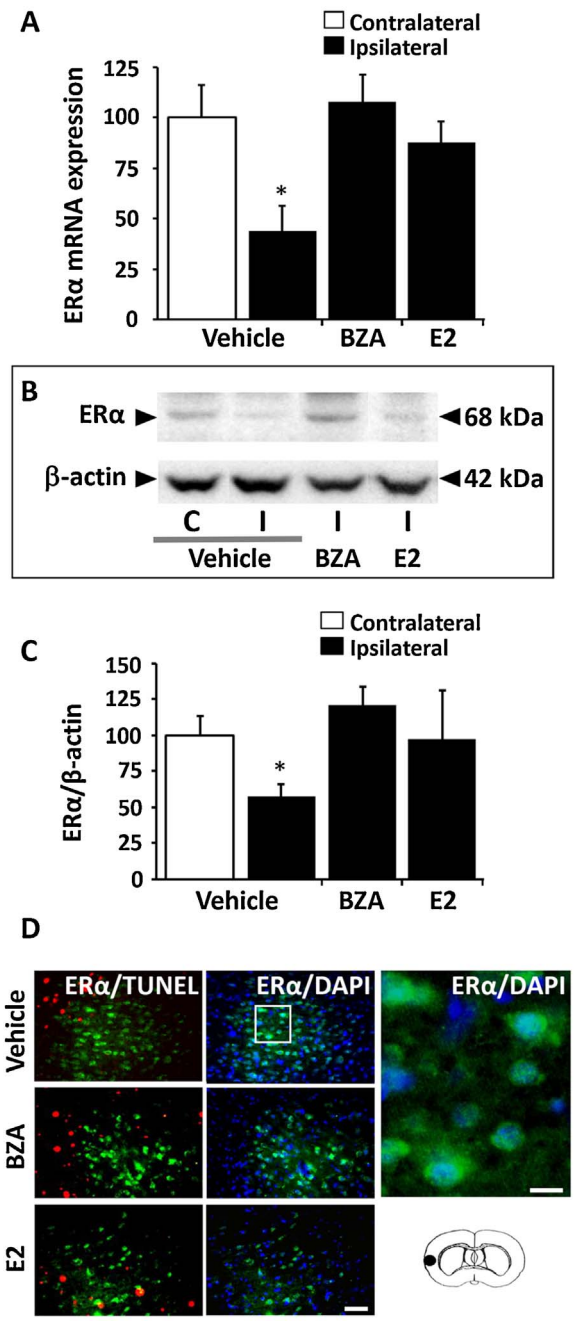

Fig. 3. Bazedoxifene and 17β-estradiol reverse the ischemia-reperfusion-induced down-regulation of estrogen receptor α mRNA and protein expression. A) Relative expression of estrogen receptor α (ERα) mRNA in contralateral and ipsilateral hemispheres. B) Representative Western blots, and C) relative abundance of ERα protein in whole-cell lysates of contralateral (C) and ipsilateral (I) hemispheres. Significantly different from contralateral vehicle-treated group (*$p < 0.05$). Data are mean ± SEM of 4–6 animals per group. D) Merged images of ERα immunolabeling (green) and TUNEL reaction (red) or DAPI staining (blue) in a cortex field of the ipsilateral hemisphere (●) of representative brain sections. Scale bar, 50 μm. An inset of vehicle-treated ERα + DAPI merged image is shown at higher magnification. Scale bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
BZA = bazedoxifene. E2 = 17β-estradiol.

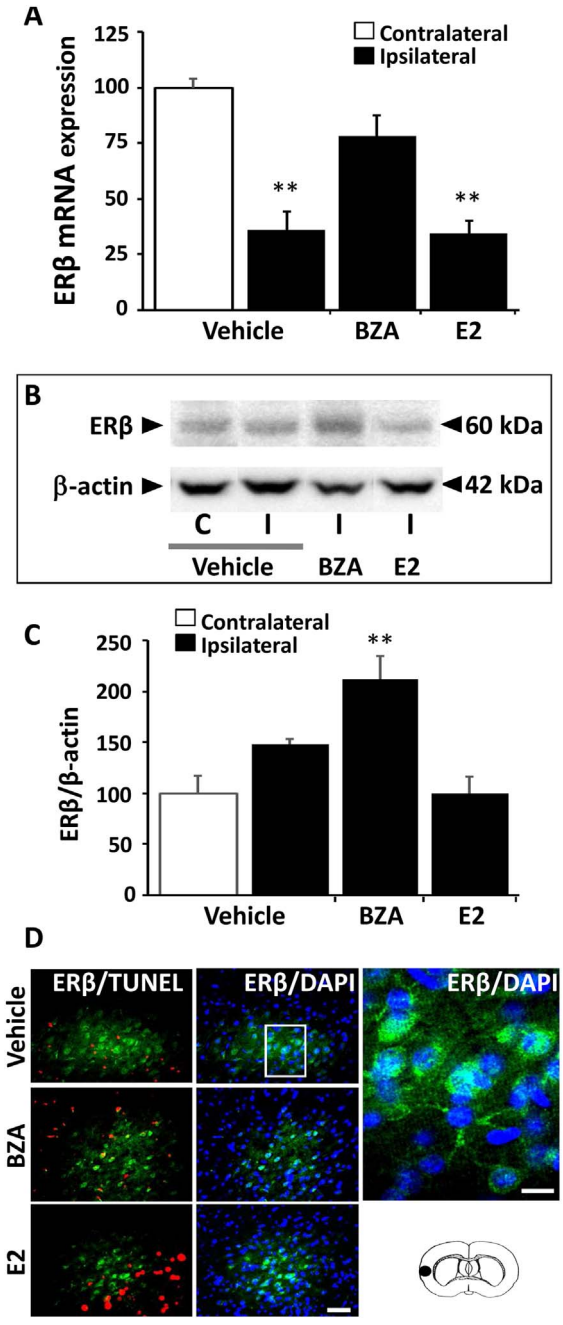

Fig. 4. Bazedoxifene, but not 17β-estradiol, reverses the ischemia-reperfusion-induced downregulation of estrogen receptor β mRNA expression, and upregulates that of estrogen receptor β protein. A) Relative expression of estrogen receptor β (ERβ) mRNA in contralateral and ipsilateral hemispheres. B) Representative Western blots, and C) relative abundance of ERβ protein in whole-cell lysates of contralateral (C) and ipsilateral (I) hemispheres. Significantly different from contralateral vehicle-treated group (**$p < 0.01$). Data are mean ± SEM of 4–6 animals per group. D) Merged images of ERβ immunolabeling (green) and TUNEL reaction (red) or DAPI staining (blue) in a cortex field of the ipsilateral hemisphere (●) of representative brain sections. Scale bar, 50 μm. An inset of vehicle-treated ERβ + DAPI merged image is shown at higher magnification. Scale bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
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levels of the large fragments of both intermediate (19 kDa) and fully activated (17 kDa) caspase-3, and (2) caspase activity assay by FAM-DEVD-FMK labeling in brain sections. Our results demonstrate that BZA exerts an anti-apoptotic effect by strongly inhibiting the caspase-3 activity and lowering the TUNEL-positive cell counts. This occurred in cortical but not subcortical regions of the ischemic hemisphere, in line with our previous results on infarct volume [25]. The same neuroprotection pattern was shown for E2 (our internal control) since, when properly compared, the effects of BZA and those of E2 were of the same magnitude. The anti-apoptotic effect of E2 on ischemia-induced brain damage has been described in many studies by using different experimental paradigms: 1) MCAO model [30–32]; 2) global cerebral

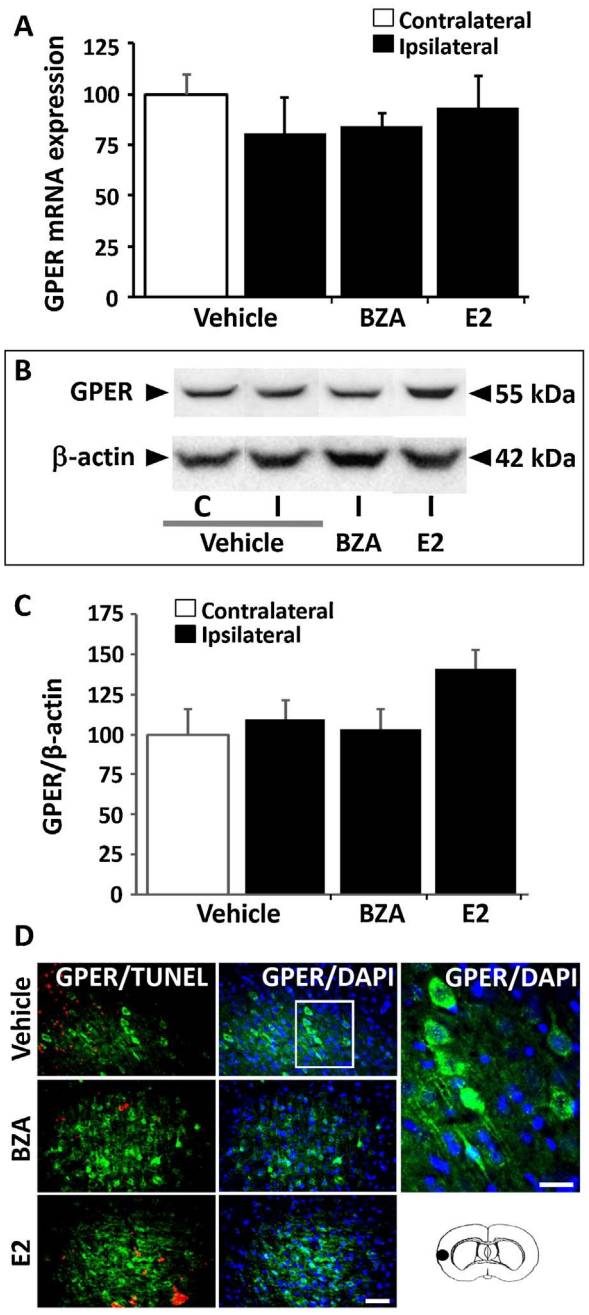

Fig. 5. Neither bazedoxifene nor 17β-estradiol alter the expression of G protein-coupled estrogen receptor mRNA and protein. A) Relative expression of G protein-coupled estrogen receptor (GPER) mRNA in contralateral and ipsilateral hemispheres. B) Representative Western blots, and C) relative abundance of GPER protein in whole-cell lysates of contralateral (C) and ipsilateral (I) hemispheres. Data are mean ± SEM of 4–6 animals per group. D) Merged images of GPER immunolabeling (green) and TUNEL reaction (red) or DAPI staining (blue) in a cortex field of the ipsilateral hemisphere (●) of representative brain sections. Scale bar, 50 μm. An inset of vehicle-treated GPER + DAPI merged image is shown at higher magnification. Scale bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
BZA = bazedoxifene. E2 = 17β-estradiol.

ischemia model [33–35]; and 3) neural culture oxygen-glucose deprivation (OGD) model (namely *in vitro* cerebral ischemia model) [36,31,35,37]. As for SERMs, such a point does not seem to have received much attention. The hypothesis that the neuroprotective mechanism of the SERM, tamoxifen, could be due to its ability to attenuate apoptotic cell death was first suggested and verified by Wakade et al. [11], and subsequently confirmed by Zou et al. [17].

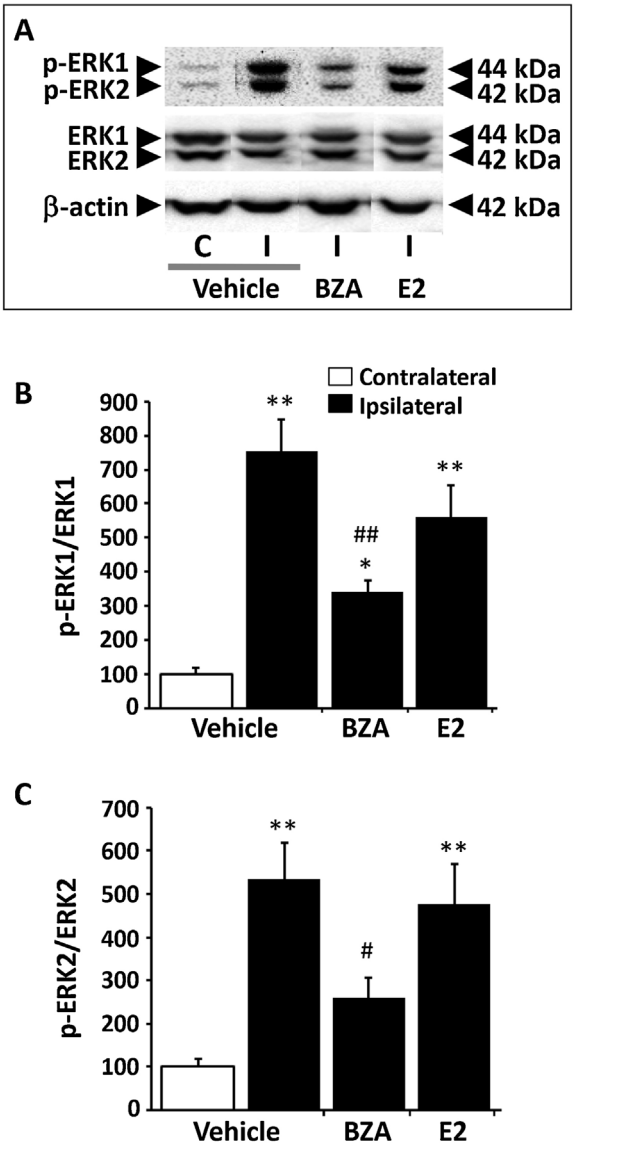

Fig. 6. Bazedoxifene, but not 17β-estradiol, inhibited the ischemia-reperfusion-induced increase in phosphorilated extracellular signal-regulated kinase 1/2 protein expression. A) Representative Western blots, B) relative abundance of phosphorilated extracellular signal-regulated kinase 1 (p-ERK1) and C) relative abundance of p-ERK2 in whole-cell lysates of contralateral (C) and ipsilateral (I) hemispheres. Significantly different from contralateral vehicle-treated group (*$p < 0.05$; **$p < 0.01$). Significantly different from ipsilateral vehicle-treated group ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$). Data are mean ± SEM of 4–7 animals per group.
BZA = bazedoxifene. E2 = 17β-estradiol.

By contrast, the SERM, raloxifene, has been recently reported to have no effect on the hypoxia-induced increase of caspase-3 activity in cultured hippocampal cells [20]. Therefore, our results lend support to the view that, as in the case of E2, SERMs (specifically BZA) are effective anti-apoptotic drugs in stroke.

As in other tissues, the actions of E2 in the brain are mediated by the activation of two classical nuclear ER, ERα and ERβ, also associated with plasma membrane, and the membrane-associated non-classical ER, G protein-coupled ER (GPER) [38,3]. BZA binds to both ERα and ERβ, with a slightly higher affinity for ERα [39]. Differential modulation of ERα and ERβ in ischemic brain injury after permanent MCAO in female rats and mice have been previously reported [40,41]. Therefore, we studied the involvement of ERs in the neuroprotective effects of BZA. PCR analysis showed that, when compared with the contralateral (healthy) hemisphere, the ischemia-reperfusion episode decreased both

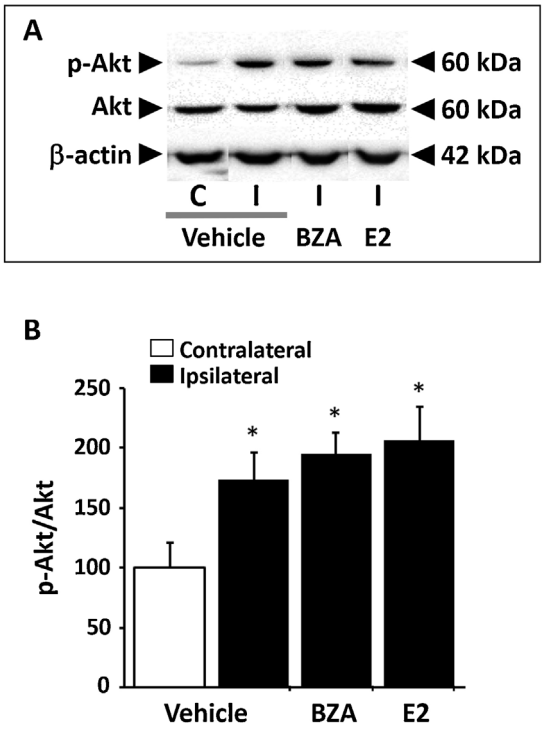

Fig. 7. Neither bazedoxifene nor 17β-estradiol affected the phosphorylated phosphatidylinositol 3-kinase/Akt protein expression previously increased by ischemia-reperfusion. A) Representative Western blots, and B) relative abundance of phosphorylated phosphatidylinositol 3-kinase/Akt (p-Akt) in whole-cell lysates of contralateral (C) and ipsilateral (I) hemispheres. Significantly different from contralateral vehicle-treated group (*$p < 0.05$). Data are mean ± SEM of 4–7 animals per group.
BZA = bazedoxifene. E2 = 17β-estradiol.

ERα and ERβ mRNA expression in the ischemic hemisphere, while GPER mRNA expression was not altered. BZA and E2 showed different effects on such expression profile: while BZA reversed the ischemia-induced downregulation of both ERα and ERβ mRNA expression, E2 reversed the ischemia-induced downregulation of ERα mRNA expression but was without effect on ERβ mRNA expression. Then we compared these results with those obtained by Western blot analysis. Ischemia-reperfusion also induced a decrease in ERα protein expression in the ischemic hemisphere, but did not modify the levels of ERβ and GPER. In accordance with PCR results, BZA reversed the ischemia-induced decrease in ERα protein expression and induced the over-expression of ERβ protein. By contrast, E2 reversed the ischemia-induced downregulation of ERα protein expression but was without effect on ERβ, as seen for mRNA expression. Finally, neither BZA nor E2 induced changes in GPER expression. Several studies in models of neuronal damage have demonstrated that ERs are involved in the antiapoptotic effects of E2 or specific agonists. ERα seems to be particularly relevant [42,43,41,44], although both ERβ [35] and GPER [36,45] have been reported to mediate estrogen-induced antiapoptotic effects as well. Overall, our results demonstrate that the anti-apoptotic effect of BZA is mainly mediated by the modulation of ERα and ERβ (but not GPER) expression in ischemic brain tissue. Finally, regardless of BZA or E2 treatment, the localization studies have shown that ERα and ERβ immunoreactivity was detected predominantly in the nucleus, as mainly colocalized with DAPI stain, while GPER immunoreactivity was shown mainly in the cytoplasmic/membrane region of neuron-like cells. This is in line with the well known distribution of ER [3].

The mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) are important signaling pathways involved in the regulation of some important features of stroke pathophysiology. Therefore they might represent therapeutic targets. The extracellular signal-regulated kinase 1/2 (ERK1/2) is a subfamily of the MAPK signaling pathway implicated, among other major cellular functions, in the regulation of cell apoptosis after stroke [46]. In most studies with the MCAO model, as reviewed by Sawe et al. [47], phosphorylated ERK1/2 (p-ERK1/2) levels reflecting ERK activity have been reported to increase during the reperfusion stage, especially in the penumbra. Our results are in close agreement since ischemia-reperfusion significantly increased p-ERK1/2 levels in the ipsilateral hemisphere. As for the treatments, BZA strongly inhibited the ischemia-induced increases in p-ERK1/2, while E2 was less effective in doing that. Such findings are in close agreement with those of Wakade et al. [11] showing that p-ERK1/2 was elevated significantly after MCAO, and that the SERM, tamoxifen attenuated the elevation of pERK1/2, an effect correlated with reduced infarct size. Moreover, Sun and Nan [46] have recently summarized some studies in which ERK inhibitors (U0126 and PD184161) have shown to decrease infarct volume in the MCAO model. Conceivably, the MAPK/ERK1/2 signaling pathway seems to have a detrimental rather than a beneficial effect in our experimental paradigm, so that its inhibition by BZA is an effective neuroprotective tool. However, the controversy remains as to the nature of the ERK signaling since both anti-apoptotic and pro-apoptotic roles have been described [48].

The main features of the PI3/Akt signaling pathway in cerebral ischemia have been recently updated [49,50]. Opposite results have been reported: ischemia-reperfusion has been shown either to inhibit or to activate the PI3K/Akt pathway. Obviously, the question arises as to whether the PI3K/Akt pathway should be inhibited or activated to avoid apoptosis and promote cell survival. Regardless of the answer to this question, our results clearly demonstrate that phosphorylated Akt (p-Akt) levels are strongly increased after ischemia-reperfusion, and that neither BZA nor E2 affected such activity. Such findings do not correlate with the decreased infarct volume [25] and the decreased apoptotic cell death (present study), and suggest that the antiapoptotic neuroprotective role of BZA and E2 does not develop by modulating the PI3K/Akt pathway.

The study of the pathophysiology of stroke is yielding a never-ending list of targets susceptible to being hit with selective inhibitors/activators to afford neuroprotection. It seems reasonable, therefore, to think that a putative neuroprotector able to influence various instead of only one event is a more suitable approach. As recently pointed out by Engler-Chiurazzi et al. [3], the mechanism behind estrogen's neuroprotective effects is most likely a multifactorial combination of diverse neurobiological and signaling impacts, *e.g.*: cerebral microvasculature and blood-brain barrier, mitochondrial function, anti-inflammatory actions, free-radical scavenging, synaptic and structural plasticity, cholinergic neurotransmitter system, and cellular maintenance and survival. Probably, the same could be attributed to SERMs. In this regard, we have recently characterized the pharmacological profile of BZA in cerebral arteries, which is compatible with a beneficial effect on the cerebrovascular function [51]. In the study of the rest of possible mechanisms mediating the neuroprotective effect of BZA in stroke, the present study shows that this SERM inhibits ischemia-induced apoptotic neuronal death.

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