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Combined estradiol and progesterone: a potentially valuable therapeutic treatment for human ischemic strokeOlmos Alonso A.¹

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SUMMARY

Introduction: Ischemic stroke leads adult's mortality and long-term disability. Thus, new therapeutic approaches are needed. Estradiol (E2) and progesterone (P4) protect a variety of neuronal cells under stroke-like conditions *in vitro*; and the rodent brain against injury *in vivo* when administered prior to the ischemic insult, namely middle cerebral artery occlusion (MCAO). But, their therapeutic value after MCAO has been hardly studied. Less is known when coadministered under this paradigm. E2 and P4 also protect the neuronal cell death *in vitro* from ischemic-like injury, by reducing apoptotic cell death and enhancing cell survival signals. Here, we assessed the effect of combined E2 and P4 treatment in rats after permanent MCAO (pMCAO), which best mimics human ischemic stroke, analyzing the phosphatidylinositol 3-kinase (PI3-K)/Akt/glycogen synthase kinase 3 (GSK3)/ β -catenin signal pathway and the activation status of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in the infarct core (i.e., the ipsilateral frontoparietal cortex, ipsi-Ctx). **Materials and Methods:** Age-matched male Wistar rats ($n=48$) underwent pMCAO or sham operation and received either vehicle (ethanol 1% in saline) or combined E2 and P4 (0.04 mg/kg and 4 mg/kg, respectively) treatment at 6, 24, and 48 hrs after surgery. All animals were sacrificed 6 hrs after the last treatment dose and ipsi-Ctx homogenates were analyzed by immunoblotting. **Results:** pMCAO downregulated the PI3-K/Akt/GSK3/ β -catenin survival pathway and activated the proapoptotic protein SAPK/JNK in the ipsi-Ctx, effects which were reversed by E2 and P4 coadministration. **Conclusions:** Our data show that combined E2 and P4 treatment exerts a neuroprotective effect against brain injury when administered after the ischemic insult, which is mediated by modification of the activity of PI3-K/Akt/GSK3/ β -catenin signal pathway and that of SAPK/JNK. Hence, we provide experimental evidence that combined E2 and P4 comprise a potentially valuable therapeutic treatment for human ischemic stroke.

KEY WORDS: Estradiol, Progesterone, pMCAO, Ischemic stroke, Brain, Neuroprotection, Frontoparietal cortex, Immunoblotting, Akt, SAPK/JNK.

Combinación de estradiol y progesterona: un tratamiento potencialmente valioso para el ictus isquémico en humanos

RESUMEN

Introducción: El ictus isquémico es una principal causa de muerte y discapacidad a largo plazo en adultos. Por tanto, es necesario desarrollar nuevas terapias. El estradiol (E2) y la progesterona (P4) protegen a distintas neuronas bajo condiciones que simulan el ictus isquémico *in vitro*; así como el cerebro de roedores cuando se administran antes del daño isquémico *in vivo*, en concreto antes de la oclusión de la arteria cerebral media (MCAO). Sin embargo, su valor terapéutico tras la MCAO ha sido poco estudiado. Menos se sabe aún acerca de su efecto cuando se coadministran en esta circunstancia. Además, el E2 y la P4 previenen la muerte neuronal por daño isquémico *in vitro*, disminuyendo la apoptosis y aumentando las señales de supervivencia. En este estudio, evaluamos en ratas el efecto del tratamiento combinado de E2 y P4 tras la MCAO permanente (pMCAO), ya que es el modelo que mejor simula el ictus isquémico humano, analizando la vía de señalización fosfatidilinositol 3-quinasa (PI3-K)/Akt/glicógeno sintasa quinasa 3 (GSK3)/ β -catenina y la activación de la proteína quinasa activada por estrés/quinasa c-Jun N-terminal (SAPK/JNK) en el núcleo del infarto (es decir, el cortex ipsilateral frontoparietal, ipsi-Ctx). **Materiales y Métodos:** Ratas Wistar macho de edad similar ($n=48$) se sometieron a pMCAO u operación sham y recibieron vehículo (etanol 1% en salino) o tratamiento combinado de E2 y P4 (0.04 mg/kg y 4 mg/kg, respectivamente) a las 6, 24, y 48 h de la cirugía. Todos los animales se sacrificaron 6 h después de la última dosis y los lisados de ipsi-Ctx se analizaron por inmunoblot. **Resultados:** La pMCAO disminuyó la actividad de la vía de supervivencia PI3-K/Akt/GSK3/ β -catenina y activó la proteína proapoptótica SAPK/JNK en el ipsi-Ctx, efectos que fueron revertidos por la coadministración de E2 y P4. **Conclusiones:** Nuestros datos muestran que el tratamiento combinado de E2 y P4 ejerce un efecto neuroprotector en el cerebro cuando se administra tras el daño isquémico, que está mediado por la modificación de la actividad de la vía PI3-K/Akt/GSK3/ β -catenina y de la de SAPK/JNK. Por tanto, ofrecemos la prueba experimental de que la combinación de E2 y P4 es una terapia potencialmente valiosa para el tratamiento del ictus isquémico en humanos.

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PALABRAS CLAVE: Estradiol, Progesterona, pMCAO, Ictus isquémico, Cerebro, Neuroprotección, Cortex frontoparietal, Inmunoblot, Akt, SAPK/JNK.

INTRODUCTION

Ischemic stroke is one of the leading causes of death and long-term disability in adults from industrialized countries¹. Currently, only one FDA-approved therapy to treat this condition exists, the thrombolytic tissue plasminogen activator (tPA); which effects are constrained by therapeutic limitations². Thus, novel therapeutic strategies that represent an alternative to tPA are needed.

Estradiol (E2) and progesterone (P4) play potent neurotrophic and neuroprotective roles in the brain^{3,4}. E2 and P4 have been shown to regulate dendritic spine density in hippocampal cells both *in vitro*⁵, and *in vivo*⁶. Also, a large body of evidence indicates that E2 and P4 protect against brain injury and several neurodegenerative conditions⁷. Clinical studies have shown that E2 reduces the incidence and progression of Alzheimer's disease and Parkinson's disease^{8,9}, and ameliorates aging-related cognitive decline¹⁰. E2 has been further implicated as a neuroprotective factor in animal models of epilepsy¹¹, and multiple sclerosis¹². Likewise, P4 has been proven beneficial after experimental traumatic brain injury¹³, diabetic neuropathies¹⁴, and spinal cord injury¹⁵. In addition, both E2 and P4 are believed to reduce the risk or severity of stroke^{16,17}. Premenopausal women have indeed a lower risk of stroke relative to men of the same age¹⁸; but the incidence in women increases after menopause¹⁹. Similarly, female animals sustain less brain injury from middle

cerebral artery occlusion (MCAO) than age-matched males, with loss of protection after ovariectomy²⁰. E2 and P4 have also been reported to exert protection *in vitro* under stroke-like conditions in hippocampal, cortical, and cerebellar cells²¹⁻²³. Moreover, previous studies have proved that E2 reduces cortical, but not striatal, infarct size in rodent models of ischemic stroke when administered prior to transient²⁰, or permanent^{24,25} MCAO. P4 pretreatment has been shown to exert similar neuroprotective effects²⁶⁻²⁸. However, few studies to date have assessed the therapeutic value of both steroids after MCAO^{29,30}. Less is known about their protective effects when coadministered under this paradigm. Recently, combined steroid treatment (E2 and P4) but not the application of single steroids abrogated cell death of cortical neurons by stroke-like injury *in vitro*³¹. Also, the combined treatment with both steroids but not the individual application of E2 and P4 counteracted the process of demyelination in an experimental multiple sclerosis animal model³²; suggesting both steroids attain their fullest protective effect only in concert. Hence, evaluate whether combined E2 and P4 treatment can protect against brain injury when administered after the ischemic insult is essential.

E2 and P4 inhibit cell death as a response to a variety of neuronal stimuli, including excitotoxic amino acids, oxidative stress, and β -amyloid toxicity³³⁻³⁵. Also, numerous studies have demonstrated that E2 and P4 inhibit cell death caused by stroke-like ischemic injury, by decreasing the extent of apoptotic cell death and enhancing cell survival signals^{22,23,36}. The phosphatidylinositol 3-kinase (PI3-K)/Akt signal pathway inhibits apoptosis and supports cell survival³⁷. In the presence of survival factors, activated Akt phosphorylates proapoptotic proteins, such as, Bad, forkhead transcription factor (FKHR), and glycogen synthase kinase 3 (GSK3), leading to suppression of their apoptotic activity, thereby promoting cell survival³⁸⁻⁴⁰. GSK3 is one of the downstream targets of Akt, and its activity is inhibited upon phosphorylation³⁹. Phosphorylation of GSK3 by Akt leads to inhibition of caspase-3 activation and thus attenuation of cell death, and reduces apoptosis⁴¹. Further, inhibited GSK3 promotes the stabilization of β -catenin, a transcription factor playing key roles in cell survival⁴². However, in the survival factors withdrawal, GSK3 induces apoptosis through activation of caspase-3 and phosphorylation of β -catenin, which is degraded⁴¹. E2 and P4 have been reported to simultaneously activate the prosurvival Akt pathway in cortical explants³⁶. Activation of Akt by both steroids has been further associated with increase neuronal survival³⁶. Moreover, activation of the PI3-K/Akt pathway in cultured cerebellar cells has been shown to prevent neuronal cell death by suppressing stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) activation⁴³, which is related to apoptosis⁴⁴. Although alteration in the PI3-K/Akt signal pathway and in SAPK/JNK expression and/or activation is assumed to contribute to ischemic injury^{45,46}, it remains unclear whether cotreatment with E2 and P4 can modify these changes after the ischemic insult and ameliorate the damage in the *in vivo* brain.

Since most cases of human ischemic stroke are caused by permanent MCAO (pMCAO)⁴⁷, we tested the impact of combined E2 and P4 treatment in rats after pMCAO, analyzing the PI3-K/Akt/GSK3/ β -catenin signal pathway and the activation status of

Figure 1. Bar graph showing the mean neuroscore of each experimental group for the modified Yrjänheikki neurobehavioral test attained 6 hrs after surgery. 6 correspond to the lowest neurological involvement and 0 to the highest in the 7-point grading scale. A significant and consistent reduction in the neuroscore was observed in pMCAO-treated animals compared to sham-operated animals. Data ($n=24$ per group) are expressed as mean $\pm$ SEM. ** $p<0.001$ by *t*-test.

SAPK/JNK in the infarct core (i.e., the ipsilateral frontoparietal cortex, ipsi-Ctx). Our results herein indicate that pMCAO down-regulated the PI3-K/Akt/GSK3 β -catenin survival pathway and activated the proapoptotic protein SAPK/JNK in the ipsi-Ctx, effects which were reversed by E2 and P4 coadministration.

MATERIALS AND METHODS

Animals

Age-matched male Wistar rats (2-3 months, 250-310 g, $n=48$; CBMSO, UAM-CSIC, Madrid, Spain) were used throughout this study. Animals were housed in a light controlled room under a 12-hrs light-dark cycle and kept at a temperature of 25°C. Animals were given unrestricted access to food and water. All experimental and animal care procedures were in accordance with the appropriate national legislation (decree 1201/2005; BOE no.252) and the Council of European Communities guidelines (86/609/CEE).

Permanent middle cerebral artery occlusion

pMCAO was carried out as previously described⁴⁸, with slight modifications. Briefly, rats were anesthetized by intraperitoneal injection of ketamine (75 mg/kg) and medetomidine (250 μ g/kg), and subcutaneously injected atropine (100 μ g/kg). Rectal temperature was monitored and maintained at $37.0\pm0.5^\circ\text{C}$ during the surgical procedure by a Homeothermic Blanket System (Harvard Apparatus, Holliston, MA, USA). The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a midline neck incision and ligated with a 6/0 polypropylene suture (Premilene®; B/Braun Aesculap AG, Tuttlingen, Germany). The occipital artery and the pterygopalatine artery were isolated and cauterized. A poly-L-lysine (0.1%, weight volume⁻¹)-coated 4/0 monofilament polyamide suture (Dafilon®; B/Braun Aesculap AG) with a heat-blunted tip was inserted through the ICA via the ECA lumen and advanced until mild resistance was felt (approximately 20 mm beyond the CCA bifurcation) at the point where the monofilament occludes the middle cerebral artery, thus blocking blood flow primarily to the ipsi-Ctx and striatum⁴⁹. The suture was secured in place until sacrifice. Sham-operated animals underwent identical surgical procedure but no suture was inserted.

Neurobehavioral testing

A 7-point neuroscore test modified from Yrjänheikki *et al.*⁵⁰, which records neurological deficits and behavioral disturbances caused by MCAO in rats, was used to corroborate the induction of pMCAO. The neurobehavioral test was conducted in all rats before and 6 hrs after surgery, just before administration of the first treatment dose. The 7-point grading scale (6 being the lowest neurological involvement and 0 the highest) was as follows: 6, normal extension of both forelimbs towards the floor when lifted; 5, consistent flexion of the forelimb contralateral to the injured hemisphere; 4, consistently reduced resistance to lateral

push towards the paretic side; 3, circling towards the paretic side if pulled and lifted by the tail; 2, circling towards the paretic side if pulled by the tail; 1, circling spontaneously towards the paretic side; 0, no spontaneous motion. Other neurobehavioral abnormalities not included in the grading scale (e.g., alteration in balance, sensorial perception, proprioception, and/or reflex response) were also recorded.

Drug administration

Animals were randomly assigned to receive either vehicle (ethanol 1% in saline) or combined E2 and P4 (0.04 mg/kg and 4 mg/kg, ref.E8875-250MG and P7556-100MG, respectively; Sigma-Aldrich, St. Louis, MO, USA) treatment. Animals were grouped as follows ($n=12$ per group): sham-operated rats treated with vehicle (Sham+Veh), sham-operated rats cotreated with E2 and P4 (Sham+E2/P4), ischemic rats treated with vehicle (Is-c+Veh), and ischemic rats cotreated with E2 and P4 (Is-c+E2/P4). Drugs were administered by a single intraperitoneal injection of 500 μ l at 6, 24, and 48 hrs after surgery. All animals were sacrificed 6 hrs after the last treatment dose.

Immunoblotting

Immunoblotting was performed according to standard method^{25,28}. In brief, animals were decapitated and brains removed. The ipsi-Ctx were dissected and frozen at -80°C until later analysis. Tissue samples were placed on ice and homogenized in lysis buffer [20 mM HEPES (pH 7.4), 100 mM NaCl, 100 mM NaF, 1% Triton® X-100, 1 mM Na₃VO₄, 5 mM EDTA, and the Complete™ Protease Inhibitor Cocktail (Roche Diagnostics, Barcelona, Spain)]. Tissue lysates were clarified by centrifugation for 15 min at $14,000\times g$ and the supernatant was saved for protein analysis and immunoblotting. Total protein concentration was determined by using the DC™ Protein Assay (BioRad, Hercules, CA, USA) and bovine serum albumin standards. Equal amounts of proteins were fractioned by 8% SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose membranes (Protran®; Whatman, Dassel, Germany), at 30 V overnight. Membranes were blocked in a 5% solution of nonfat dried milk in phosphate buffered saline-0.2% Tween® 20 (PBST) for 1 hr, and incubated at 4°C overnight with the following monoclonal primary antibodies diluted in 10% fetal bovine serum (Gibco®; Invitrogen, Carlsbad, CA, USA)-PBST: anti-Akt (1:1,000, no.9272; Cell Signaling Technology, Beverly, MA, USA), anti-phospho-Akt (Ser473, 1:500, no.9271; Cell Signaling Technology), anti-phospho-Akt (Thr308, 1:1,000, no.2965; Cell Signaling Technology), anti-GSK3 α/β (1:1,000, no.44-610; Invitrogen), anti-phospho-GSK3 α/β (Ser21/9, 1:1,000, no.9331; Cell Signaling Technology), anti- β -catenin (1:800, no.610153; BD Transduction Laboratories, Lexington, KY, USA), anti-SAPK/JNK (1:1,000, no.9252; Cell Signaling Technology), anti-phospho-SAPK/JNK (Thr183/Tyr185, 1:1,000, no.9251; Cell Signaling Technology), anti- β -tubulin (1:1,000, no.T4026; Sigma-Aldrich), and anti- β -actin (1:1,000, no.A5441; Sigma-Aldrich). After overnight incubation, blots were washed

three times with PBST and incubated at room temperature for 1 hr with the appropriate horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,000, no.32430; Thermo Scientific, Rockford, IL, USA) or goat anti-rabbit IgG (1:5,000, no.sc-2004; Santa Cruz Biotechnology, Santa Cruz, CA, USA) secondary antibodies, and washed again. Detection was carried out using the Enhanced Chemiluminescence Detection System (ECLTM; Amersham, Little Chalfont, Buckinghamshire, UK) followed by autoradiography on CurixTM RP2 Plus films (Agfa-Gevaert, Mortsel, Belgium). Band intensity was analyzed by an Imaging Densitometer (model GS-700TM; BioRad) and Quantity One[®] software v.4.6.3 (BioRad). The results were the mean of 3-5 independent experiments.

Statistical analysis

All data were expressed as mean±standard error of the mean (SEM). Differences between groups were evaluated by *t*-test. *p*<0.05 was considered statistically significant.

RESULTS

Neurological impairment 6 hrs after pMCAO

To assess whether pMCAO was induced properly, we examined the neurobehavioral status of all rats before and 6 hrs after surgery using a 7-point neuroscore test, as described in Materials and Methods. Prior to surgery, all rats attained the fullest neuroscore (data not shown); however, 6 hrs after surgery pMCAO-treated animals had a significant and consistent lower neuroscore (3.2 ± 0.3) than the sham-operated animals (5.6 ± 0.3 , *p*<0.001; fig. 1), confirming their neurological impairment. Thus, these data reveal that ischemic injury actually occurred.

Combined E2 and P4 treatment modifies the activity of PI3-K/Akt/GSK3/β-catenin signal pathway and that of SAPK/JNK in the ipsi-Ctx 54 hrs after pMCAO

To determine the effect of combined E2 and P4 treatment on PI3-K/Akt/GSK3/β-catenin signal pathway after pMCAO, sham-operated and ischemic animals received either vehicle (ethanol 1% in saline) or combined E2 and P4 (0.04 mg/kg and 4 mg/kg, respectively) treatment at 6, 24, and 48 hrs after surgery. All animals were sacrificed 6 hrs after the last treatment dose and ipsi-Ctx tissue lysates were then analyzed by immunoblotting. No changes in total Akt and GSK3α/β levels were found between experimental groups in the ipsi-Ctx 54 hrs after surgery (fig. 2a,d); however, immunoblot showed that pMCAO markedly decreased Akt activity, as measured by its phosphorylation levels at both Ser473 ($64.8 \pm 8.0\%$ lower in Isc+Veh vs. Sham+Veh group, *p*<0.0001) and Thr308 ($45.0 \pm 11.8\%$ lower in Isc+Veh vs. Sham+Veh group, *p*<0.05), and combined E2 and P4 treatment significantly reversed injury-induced downregulation of Akt activity ($24.0 \pm 11.2\%$ and $24.8 \pm 14.2\%$ higher in Isc+E2/P4 vs. Isc+Veh group for phospho-Akt Ser473 and Thr308 levels, re-

spectively, *p*<0.05 in each case; fig. 2b,c). Also, E2 and P4 coadministration significantly reversed pMCAO-induced decline of GSK3α/β phosphorylation levels at Ser21/9 ($60.7 \pm 14.1\%$ lesser in Isc+Veh vs. Sham+Veh group, and $41.6 \pm 10.1\%$ greater in Isc+E2/P4 vs. Isc+Veh group, *p*<0.001 and *p*<0.05, respectively; fig. 2e) and total β-catenin levels ($19.2 \pm 7.6\%$ lesser in Isc+Veh vs. Sham+Veh group, and $25.4 \pm 6.0\%$ greater in Isc+E2/P4 vs. Isc+Veh group, *p*<0.05 in each case; fig. 2f).

Given the changes in PI3-K/Akt/GSK3/β-catenin pathway caused by E2 and P4 coadministration, we then evaluated the impact of both steroids on the activation status of SAPK/JNK after pMCAO. To address this, ipsi-Ctx tissue lysates from sham-operated and ischemic animals treated with either vehicle (ethanol 1% in saline) or combined E2 and P4 (0.04 mg/kg and 4 mg/kg, respectively) at 6, 24, and 48 hrs after surgery were also analyzed by immunoblotting 6 hrs after the last treatment dose, when all animals were sacrificed. While no changes in total SAPK/JNK levels were detected between experimental groups in the ipsi-Ctx 54 hrs after surgery (fig. 3a), immunoblot showed that pMCAO markedly increased SAPK/JNK activity, as measured by its phosphorylation levels at Thr183/Tyr185 ($39.2 \pm 5.6\%$ higher in Isc+Veh vs. Sham+Veh group, *p*<0.05), and combined E2 and P4 treatment significantly reversed injury-induced upregulation of SAPK/JNK activity ($51.1 \pm 9.4\%$ lower in Isc+E2/P4 vs. Isc+Veh group, *p*<0.05; fig. 3b). Together, all these findings indicate that pMCAO downregulated the PI3-K/Akt/GSK3/β-catenin survival pathway and activated the proapoptotic protein SAPK/JNK in the ipsi-Ctx, effects which were reversed by E2 and P4 coadministration.

DISCUSSION

There is broad evidence from clinical and experimental data that E2 and P4 protect against brain injury and several neurodegenerative disorders⁷, including ischemic stroke^{16,17}. It is well-known that stroke risk is lower in premenopausal women than in men of the same age¹⁸; but after menopause, the incidence of stroke in women rapidly increases¹⁹. Likewise, it has been shown that female animals sustain less brain injury from MCAO than age-matched males, and that this advantage is abolished with ovariectomy²⁰. Previous reports have also demonstrated that E2 and P4 exert potent neuroprotective effects in hippocampal, cortical, and cerebellar cells under stroke-like conditions *in vitro*²¹⁻²³; and protect the rodent brain against ischemic damage by reducing cortical, but not striatal, infarct size when administered prior to transient^{20,27,28}, or permanent²⁴⁻²⁶ MCAO. However, the therapeutic value of both steroids after MCAO has been barely studied to date^{29,30}; even though it represents a more clinically-relevant therapeutic strategy for stroke treatment in humans. Much less is known about the protective effects of E2 and P4 when coadministered under this paradigm. Both *in vitro*³¹, and *in vivo*³², E2 and P4 have been recently suggested to attain their fullest protective effect acting in concert. Hence, evaluate whether combined E2 and P4 treatment can protect against brain injury when administered after the ischemic insult is essential.

Figure 2. Representative immunoblots of ipsi-Ctx homogenates from sham-operated and ischemic animals treated with either vehicle or combined E2 and P4 showing total Akt (a), phospho-Akt (Ser473; b), phospho-Akt (Thr308; c), total GSK3α/β (d), phospho-GSK3α/β (Ser21/9; e), and total β-catenin (f) levels 54 hrs after surgery. Bar graphs show quantitative analysis of total Akt (a), phospho-Akt (Ser473; b), phospho-Akt (Thr308; c), total GSK3α/β (d), phospho-GSK3α/β (Ser21/9; e), and total β-catenin (f) levels by densitometry in each experimental group. No changes in total Akt and GSK3α/β levels were observed in any of the experimental groups (a,d); however, a marked reduction in Akt activity, as measured by its phosphorylation levels at both Ser473 and Thr308, was observed after pMCAO (Isc+Veh vs. Sham+Veh group); an effect which was significantly reversed by combined E2 and P4 treatment (Isc+E2/P4 group; b,c). Also, the reduction in GSK3 phosphorylation levels at Ser21/9 and total β-catenin levels observed after pMCAO (Isc+Veh vs. Sham+Veh group) was significantly reversed by E2 and P4 coadministration (Isc+E2/P4 group; e,f). Data are expressed as percentage of Sham+Veh group set at 100%, normalized by β-tubulin or β-actin as loading controls. All results (n=12 per group) are the mean±SEM of 3-5 independent experiments. * $p < 0.05$, ** $p < 0.001$, # $p < 0.0001$ by *t*-test. Sham+Veh indicates sham-operated rats treated with vehicle; Sham+E2/P4, sham-operated rats cotreated with E2 and P4; Isc+Veh, ischemic rats treated with vehicle; Isc+E2/P4, ischemic rats cotreated with E2 and P4.

A growing body of evidence from *in vitro* studies have shown that E2 and P4 protect neuronal cells from a variety of cytotoxic insults that typically result in cell death³³⁻³⁵. Moreover, numerous other studies have suggested that E2 and P4 protect the neuronal cell death against stroke-like ischemic injury, by reducing the extent of apoptotic cell death and enhancing cell survival signals^{22,23,36}. The PI3-K/Akt signal pathway is important for inhibiting apoptosis and promoting cell survival³⁷. It has been demonstrated that E2 and P4 can simultaneously activate Akt in cortical explants and increase neuronal survival³⁶. Also, acti-

vation of the PI3-K/Akt pathway in cultured cerebellar cells has been shown to prevent neuronal cell death by suppressing SAPK/JNK activation⁴³, which is related to apoptosis⁴⁴. Although abnormality in the regulation of PI3-K/Akt signal pathway and in SAPK/JNK expression and/or activation is assumed to contribute to ischemic injury^{45,46}, it remains to be determined whether cotreatment with E2 and P4 can modify these changes after the ischemic insult and ameliorate the damage in the *in vivo* brain.

In the present study, we explored the effect of combined E2 and P4 treatment in rats after pMCAO; which more closely

Figure 3. Representative immunoblots of ipsi-Ctx homogenates from sham-operated and ischemic animals treated with either vehicle or combined E2 and P4 showing total SAPK/JNK (a), and phospho-SAPK/JNK (Thr183/Tyr185; b) levels 54 hrs after surgery. Bar graphs show quantitative analysis of total SAPK/JNK (a), and phospho-SAPK/JNK (Thr183/Tyr185; b) levels by densitometry in each experimental group. No changes in total SAPK/JNK levels were observed in any of the experimental groups (a); however, a marked increase in SAPK/JNK activity, as measured by its phosphorylation levels at Thr183/Tyr185, was observed after pMCAO (Isc+Veh vs. Sham+Veh group); an effect which was significantly reversed by combined E2 and P4 treatment (Isc+Veh vs. Isc+E2/P4 group; b). Data are expressed as percentage of Sham+Veh group set at 100%, normalized by β-tubulin or β-actin as loading controls. The results (n=12 per group) are the mean±SEM of 3-5 independent experiments. *p<0.05 by t-test. Sham+Veh indicates sham-operated rats treated with vehicle; Sham+E2/P4, sham-operated rats cotreated with E2 and P4; Isc+Veh, ischemic rats treated with vehicle; Isc+E2/P4, ischemic rats cotreated with E2 and P4.

mimics human ischemic stroke⁴⁷. Our results reveal, for the first time to our knowledge, a potentially important clinical effect of combined E2 and P4 treatment: combined E2 and P4 treatment exerts a neuroprotective effect against brain injury when administered after the ischemic insult, which is mediated by modification of the activity of PI3-K/Akt/GSK3/β-catenin signal pathway and that of SAPK/JNK. Actually, we proved that pMCAO downregulated the PI3-K/Akt/GSK3/β-catenin survival pathway and activated the proapoptotic protein SAPK/JNK in the infarct core (i.e., the ipsi-Ctx), effects which were reversed by E2 and P4 coadministration.

We showed that pMCAO markedly decreased Akt phosphorylation levels at both Ser473 and Thr308, and combined E2 and P4 treatment significantly reversed injury-induced downregulation of Akt. Also, combined E2 and P4 treatment significantly reversed pMCAO-induced decline of GSK3α/β phosphorylation levels at Ser21/9 and total β-catenin levels. Phosphorylation of both Ser473 and Thr308 is required for Akt activity³⁷. Activated Akt phosphorylates a variety of proapoptotic proteins, such as, Bad, FKHR, and GSK3, leading to suppression of their apoptotic activity³⁸⁻⁴⁰. GSK3 is one of the downstream targets of Akt, and phosphorylation of GSK3α at Ser21 and GSK3β at Ser9 inhibits its activity via Akt³⁹. The decrease in Akt phosphorylation levels at Ser473 and Thr308, and in GSK3α/β at

Ser21/9 observed in the ipsi-Ctx after pMCAO, thus correlates with both reduced Akt activity and enhanced GSK3α/β activity; which may have contributed to apoptotic cell death. Indeed, dephosphorylation of GSK3 leads to its activation, which induces apoptosis through activation of caspase-3⁴¹. Further, the concomitant decline in total β-catenin levels seen after pMCAO is in accordance with this notion, since activated GSK3 has been previously suggested to induce apoptosis through phosphorylation of β-catenin, thus marking it for degradation⁴¹; β-catenin is a transcription factor that plays key roles in cell survival⁴². Our results are consistent with those observed by others: downregulation of the PI3-K/Akt signal pathway has been described up to 24 hrs after pMCAO⁵¹⁻⁵⁴. Conversely, in the presence of E2 and P4, we detected an increase in Akt phosphorylation levels at Ser473 and Thr308, and in GSK3α/β at Ser21/9 in the ipsi-Ctx, correlating with both enhanced Akt activity and reduced GSK3α/β activity. This may have contributed to cell survival, because phosphorylation of GSK3 by Akt leads to inhibition of caspase-3 activation and thus attenuation of apoptosis⁴¹. That is also consistent with the concomitant increase in total β-catenin levels observed in the presence of E2 and P4, given that inhibited GSK3 stabilizes β-catenin⁴², thereby promoting cell survival. In support of our data, previous studies have demonstrated that the neuroprotective effect of E2 and P4 against ischemic damage is

mediated through activation of Akt. Indeed, activation of Akt by both steroids diminished the number of apoptotic nuclei and increased neuronal survival in cortical^{36,55}, and hippocampal⁵⁶ explants after an ischemic-like insult. Interestingly, the inhibition of Akt with LY294002 abrogated this protective effect^{36,56}.

In addition, we showed in our study that pMCAO markedly increased SAPK/JNK phosphorylation levels at Thr183/Tyr185, and combined E2 and P4 treatment significantly reversed injury-induced upregulation of SAPK/JNK. Phosphorylation of SAPK/JNK at Thr183/Tyr185 correlates with its activation in response to different stimuli, such as, environmental stresses and inflammatory cytokines⁵⁷, and is associated with apoptosis⁴⁴. Thus, alternatively to the dysfunction of the prosurvival Akt pathway, the increase in SAPK/JNK phosphorylation levels at Thr183/Tyr185, which suggests activation of SAPK/JNK, may also have contributed to apoptotic cell death in the ipsi-Ctx after pMCAO. Indeed, activated SAPK/JNK phosphorylates antiapoptotic Bcl-2, suppressing its prosurvival function, or the transcription factor c-Jun, which is an important mediator of cell death, apoptosis^{58,59}. This finding accords with previous studies in which the activation of SAPK/JNK throughout different time-points occurred after pMCAO⁶⁰⁻⁶². Likewise, the protective effect of combined E2 and P4 treatment on cell survival through activation of the Akt pathway may be alternatively explained by inhibition of SAPK/JNK, since combined E2 and P4 treatment reduces SAPK/JNK phosphorylation levels at Thr183/Tyr185 and thus its proapoptotic activity. Furthermore, inhibition of SAPK/JNK with D-JNKI1 has been shown to prevent neuronal cell death caused by stroke-like ischemic injury in hippocampal explant cultures⁶³.

As a whole, our data suggest that combined E2 and P4 treatment exerts a prosurvival effect in the brain; which is specifically mediated by activation of the Akt pathway and inhibition of SAPK/JNK after the ischemic insult. However, the exact mechanism underlying this neuroprotective effect is unknown. Typically, upon stimulation with E2 and P4, the estrogen and progesterone receptors (ER and PR, respectively) enhance gene transcription^{3,4}. But, given that total Akt, GSK3 α/β , and SAPK/JNK levels do not changed after pMCAO, in contrast to their phosphorylation levels, it is unlikely that combined E2 and P4 treatment regulates their protein levels through transcription. This rather suggests that kinase activation/deactivation is an important component in E2- and P4-induced neuroprotection after pMCAO and that rapid, *nontranscriptional* mechanisms also contribute to the protective effect of E2 and P4 in the brain. It has indeed been reported that ER α physically interacts with the p85 regulatory subunit of PI3-K⁶⁴. Moreover, it has been recently demonstrated that ER α is linked to PI3-K-associated cytoplasmic signaling in the brain in primary neurons; where E2 can stimulate the interaction between PI3-K and ER α and activate the PI3-K/Akt signal pathway⁶⁵⁻⁶⁸, promoting neuronal survival⁶⁹. Further, previous work by Wang *et al.*⁷⁰ revealed that, in addition to activation of the PI3-K/Akt pathway, the interaction between PI3-K and ER α in response to E2 treatment causes the inhibition of SAPK/JNK by activated Akt, protecting neurons from cell death. Interestingly, this effect was blocked by application of the PI3-K inhibitor LY294002 or the ER α agonist ICI 182,780. But, the protective effect of combined E2 and P4 treat-

ment cannot be solely attributable to E2. Indeed, P4, like E2, has been proven to be equally capable of activate Akt in neurons³⁶. It is also known the effect of P4 on SAPK/JNK and the control of cell death⁷¹. However how P4 binding to PR exerts these effects has not so far been elucidated. Thus, further work is needed to unravel the particular mechanism whereby both steroids acting in concert provide the observed neuroprotective effect in the brain after pMCAO.

CONCLUSIONS

Regardless the precise mechanism involved, here we showed that combined E2 and P4 treatment exerts a neuroprotective effect against brain injury when administered after the ischemic insult, which is mediated by modification of the activity of PI3-K/Akt/GSK3/ β -catenin signal pathway and that of SAPK/JNK. Although our results are limited, thus we provide experimental evidence that combined E2 and P4 comprise a potentially valuable therapeutic treatment for human ischemic stroke.

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