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Effects of estradiol and genistein on the insulin signaling pathway in the cerebral cortex of aged female rats

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ABSTRACT

Menopause leads to a decrease in estrogen production that increases central insulin resistance, contributing to the development of neurodegenerative diseases. We have evaluated the influence of aging and estradiol or genistein treatments on some key stages of the insulin signaling pathway in the cerebral cortex. Young and aged female Wistar rats were ovariectomized and treated acutely with 17β-estradiol (1.4 μg/kg body weight), two doses of genistein (10 or 40 mg/kg body weight), or vehicle. The cortical expression of several key insulin signaling pathway components was analyzed by western blotting. Our results showed an age-related deterioration in the interactions between the regulatory subunit of phosphatidylinositol 3-kinase (p85α) and the activated form of insulin receptor substrate 1 (p-IRS1$_{tyr612}$), as well as between p85α and the 46 kDa isoform of the estrogen receptor α (ERα46). Moreover, aging also decreased the translocation of glucose transporter-4 (GLUT4) to the plasma membrane. 17β-Estradiol but not genistein reduced the negative impact of aging on central insulin sensitivity by favoring this GLUT4 translocation, and therefore could be neuroprotective against the associated neurodegenerative diseases. However, protein kinase B (Akt) activation by genistein suggests that other possible mechanisms are involved in the neuroprotective effects of this phytoestrogen during the aging process.

1. Introduction

Aging affects all tissues and organs, including the brain. In fact, the prevalence of neurodegenerative diseases is higher in postmenopausal women than in young women (Rasgon and Jarvik, 2004), suggesting that the decline of ovarian function plays a key role in the negative consequences of aging on brain function (Gonzalez et al., 2008). Moreover, an age-related increase of insulin resistance has been found in both humans and rats (Alonso et al., 2006; Reaven and Reaven, 1985), with the decrease of ovarian hormones being one of the main causes in females (Moran et al., 2013). For a long time, the brain has been thought to be an insulin-insensitive organ. However, insulin and insulin receptors (IR) have been found throughout the brain (Havrankova et al., 1978), suggesting a role for insulin in brain physiology and pathophysiology (Correia et al., 2011). Like in peripheral tissues, insulin signaling is mediated by protein–protein interactions initiated at the IR. IR auto-phosphorylation leads to the subsequent phosphorylation of several proteins belonging to the insulin intracellular signaling pathway, initiated in the insulin receptor substrate (IRS) family, of which insulin receptor substrate 1 (IRS1) is the best characterized (Alonso et al., 2008). IRS1 acts as a docking molecule for the regulatory subunit of phosphatidylinositol 3-kinase (p85α), triggering the activation of the catalytic subunit of phosphatidylinositol 3-kinase (p110α), which is responsible for the subsequent activation of protein kinase B (Akt). Akt activation occurs through the phosphorylation of the serine 473 residue, and the activated form is implicated in the translocation of the insulin-sensitive glucose transporter-4 (GLUT4) from intracellular pools to the plasma membrane to facilitate glucose uptake into cells (Gonzalez et al., 2008; Moreno et al., 2010).

A number of studies have reported the neuroprotective role of estrogens (Alonso et al., 2008; Moran et al., 2013; Zhang et al., 2014), which, among other ways, could be mediated through the activation of the phosphatidylinositol-3 kinase (PI3K) signaling pathway (Gonzalez

Abbreviations: p85α, regulatory subunit of phosphatidylinositol 3-kinase; p-IRS1$_{tyr612}$, activating phosphorylation of insulin receptor substrate 1; ER, estrogen receptor; GLUT, glucose transporter; Akt, protein kinase B; IR, insulin receptor; IRS, insulin receptor substrate; p110α, catalytic subunit of phosphatidylinositol 3-kinase; PI3K, phosphatidylinositol-3 kinase; CNS, central nervous system; SERMs, selective estrogen receptor modulators; Y, young; A, aged animals; YE, young animals treated with estradiol; AE, aged animals treated with estradiol; YG1, young animals treated with low doses of genistein; AG1, aged animals treated with low doses of genistein; YG2, young animals treated with high doses of genistein; AG2, aged animals treated with high doses of genistein; YV, young animals treated with vehicle; AV, aged animals treated with vehicle; YC, young control animals; AC, aged control animals; TBS-T, Tris–NaCl-Tween 20 0.1% v/v; p-Akt$_{ser473}$, activating phosphorylation of protein kinase B; HRP, horseradish-peroxidase; ECL, enhanced chemiluminescence; GPR30, G protein-coupled receptor 30; NMDARs, N-methyl-D-aspartate receptors; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GABAAR, γ-aminobutyric acid type A receptor; SIRT1, NAD-dependent deacetylase sirtuin-1.

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et al., 2008). Moreover, estradiol treatment could modify some steps of the insulin signaling pathway (Alonso et al., 2008) as well as glucose utilization in the brain (Cheng et al., 2001; Namba and Sokoloff, 1984). Some findings suggest that the neuroprotective effects of estrogens through activating the PI3K signaling pathway are mediated by estrogen receptors (ERs) (Alonso et al., 2008). There are two known ER subtypes, ERα and ERβ, and two main ERα isoforms produced by alternative splicing, ERα67 and ERα46 (Marin et al., 2003; Moran et al., 2013). The majority of studies carried out in this field have focused on ERα, because it seems to be more strongly implicated in the activation of the PI3K pathway (Alonso et al., 2008). However, recent works have also highlighted the key role of ERβ for the activation of the PI3K pathway in the central nervous system (CNS) (Giddabasappa et al., 2010; Zhao et al., 2011), pointing out that both ER subtypes should be taken into account.

Nevertheless, some estrogenic treatments have been associated with increased risk of several pathologies such as breast cancer (Colditz, 1999; Gapstur et al., 1999), coronary illness or stroke (Alonso and Gonzalez, 2012). There is therefore a growing interest in the study of alternative therapies based on phytoestrogens such as genistein. These compounds are derived from plants such as soybean, alfalfa and clover, and they have structural similarities to 17β-estradiol, which allow them to bind with estrogen receptors leading to estrogenic or antiestrogenic effects (Kuiper et al., 1998). In fact, phytoestrogens are considered as natural selective estrogen receptor modulators (SERMs) (Baker et al., 2000), which are able to act as ER agonists or antagonists depending on the tissue (Beck et al., 2005). As estrogens, phytoestrogens are also able to play a neuroprotective role (Azcoitia et al., 2006), but there is still little knowledge about the mechanisms through which they exert these effects.

Taking into account this background, this study was designed to evaluate how the decrease of ovarian hormones inherent to aging influences some key molecules of the insulin signaling pathway in the cerebral cortex. Moreover, we have checked whether acute treatment with estradiol or genistein can modify these checkpoints, either directly or through interaction between ERs and these proteins, to evaluate their possible role as neuroprotective strategies against central insulin resistance. The cerebral cortex has been chosen for our experiments due to its essential role for cognitive tasks (Conejo et al., 2007; Jo et al., 2007), and because it is a brain region enriched with estrogen receptors (McEwen and Alves, 1999; Montague et al., 2008).

2. Material and methods

2.1. Animals

Fifty virgin female Wistar rats weighing 130–150 g (age 6–8 weeks, young animals, Y) and 500–600 g (age 90–96 weeks, aged animals, A) were supplied by the Central Biotery of the University of Oviedo (Spain). They were kept under standard conditions of temperature (23 ± 2 °C), relative humidity (65 ± 5%) and on an artificial light–dark cycle of 12 h (lights on at 8:00 a.m.). Food and water were available ad libitum. All experimental procedures were approved by a local veterinary committee from the University of Oviedo vivarium and performed following the European Communities Council Directive of 22 September, 2010 (2010/63/UE) and the Spanish legislation (R.D. 53/2013) on care and use of experimental animals.

2.2. Experimental design

Rats were subject to an ovariectomy either at the beginning of sexual maturity (young rats, Y), or at the end of reproductive period (aged rats, A). Sexual maturity of young rats was ensured by the presence of normal estrous cycles determined by vaginal smears. On the other hand, aged animals showed persistent diestrous phase instead of repetitive estrous cycles, highlighting the end of the reproductive period. The ovariectomy was carried out through a midline incision under light anesthesia by inhalation of halothane. Both, young and aged rats, were randomly divided into five groups: ovariectomized animals injected with 17β-estradiol (1.4 μg/kg body weight in olive oil/ethanol 3:2 v/v; Sigma Chemical Co., St Louis, MO, USA) (YE and AE), ovariectomized rats treated with two different doses of genistein (G1 = 10 mg/kg body weight; G2 = 40 mg/kg body weight in olive oil/ethanol 3:2 v/v; LC Laboratories, Woburn, MA, USA) (YG1, AG1, YG2, AG2), and ovariectomized animals treated with vehicle (olive oil/ethanol 3:2 v/v) (YV and AV). Furthermore, sham surgery animals were used as control group (YC and AC). This dosing regimen corresponds to 17β-estradiol physiological levels (Alonso et al., 2008; Moran et al., 2013), which are able to improve insulin sensitivity (Alonso et al., 2006), and to be neuroprotective in rats (Alonso et al., 2008). In addition, genistein is able to exert estrogenic actions at the proposed doses (Alonso et al., 2010), but two genistein doses were tested because phytoestrogens activate different cellular mechanisms in a concentration dependent way (Wang and Kurzer, 1997; Zhao et al., 2002).

All experimental treatments began exactly one week after ovariectomy to ensure a uniform period of estrogen depletion before replacement and the recovery from surgical stress. In order to study the acute effects of these treatments, animals were injected 1 h prior to the sacrifice.

2.3. Euglycemic hyperinsulinemic clamp experiment

Animals were anesthetized with sodium pentobarbital (50 mg/kg body weight) after 12 h of fasting. Then, the left saphenous vein was catheterized for insulin and glucose infusion, and clamp experiments were performed as we previously described (Gonzalez et al., 2000). Blood samples from jugular vein and tail were collected in order to determine insulin and glucose levels, respectively. Finally, animals were sacrificed by bleeding and their brains were quickly removed. Data from this experiment have already been published (Alonso et al., 2010), showing that acute treatment with estradiol or both genistein doses improves the insulin sensitivity impaired by both, ovariectomy and aging.

On the other hand, data from this experiment showed an increase in serum insulin levels after finishing clamp experiments compared to serum insulin levels at fasting for all groups (Alonso et al., 2010). In spite of the fact that there is not a general agreement regarding how serum insulin levels could influence brain glucose uptake, some works have found that a physiologic hyperinsulinemia does not change cerebral glucose metabolism compared to basal insulin levels, showing similar data for both conditions in different brain regions, including cortex (Cranston et al., 1998; Hasselbalch et al., 1999). Therefore, we could expect no interferences on our data derived from the euglycemic hyperinsulinemic clamp experiment performed previously of animals' dissection.

2.4. Crude extract preparation, immunoprecipitation, plasma membrane isolation and Western blot analysis

Just after finishing clamp experiments, brains were frozen in isopentane (VWR International S.A.S., Fontenay-sous-Bois, France) and stored at −80 °C. Later, the cerebral cortex was dissected and homogenized in lysis buffer [Tris–HCl 50 Mm (pH 7.5), NaCl 150 mM, Nonidet P-40 1% v/v (Roche Diagnostics, Indianapolis, USA), sodium deoxycholate 0.05% w/v, sodium orthovanadate 1 mM, EDTA 5 mM] at 4 °C. Then, extracts were centrifuged at 3500 rpm at 4 °C for 15 min, and the supernatant was stored at −20 °C for the subsequent protein content determination by the Bradford dye-binding method (Bradford, 1976).

First of all, equal amounts of protein were resolved by 10% SDS–PAGE gel and electro-transferred from the gel to PVDF membranes (Immobilon-P Transfer Membrane, Millipore Corporation, Billerica MA,