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Red wine interferes with oestrogen signalling in rat hippocampus

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ABSTRACT

Oestrogens have neuroprotective properties, resulting in memory and learning preservation. Red wine (RW) has been linked to neuroprotection, but mechanisms are largely unknown. The aim of this work was to test the effect of RW or 13% ethanol solution consumption on the expression of aromatase and estrogen receptors (ER) in the rat hippocampus. Beverages were supplied to male Wistar rats and after 8 weeks of treatment animals were euthanised, hippocampus was removed, aromatase expression assessed by western blotting and aromatase and ER transcription determined by RT-PCR. The effects of treatments on hippocampal aromatase activity were also determined, as well as the effect of several red wine polyphenols in hippocampal homogenates from untreated animals. Aromatase transcription was increased by ethanol (to 158 ± 7%) but only significantly by RW (to 180 ± 9%). No difference was found in ERα expression among groups, whereas RW significantly decreased ERβ expression (to 63 ± 10%). Resveratrol, quercetin, myricetin and kaempferol had no effect on aromatase activity and catechin (300 μM), epicatechin (200 μM), procyanidin extract (200 mg/L) and fractioned procyanidins (FI and FII; 200 mg/L) significantly decreased aromatase activity. The contribution of procyanidins in wine to the effect observed in aromatase was investigated in animals treated for the same period with these compounds (200 mg/L), although no effect was seen in aromatase activity, mRNA or protein levels, meaning that this group of compounds had little contribution, if any, to the effects observed. Nevertheless, the increase in aromatase expression induced by RW may corroborate the neuroprotective ability attributed to this beverage. Alterations in the relative abundance of ER expression may also play an important role in the protection.

1. Introduction

Predictions based on epidemiological data state that in the next 50 years the occurrence of neurodegenerative diseases associated with dementia will overcome that of cancer, with more than 33% of all women and 20% of men being affected. This makes the knowledge of strategies to treat or decrease the likelihood of developing such a disease undoubtly urgent [1]. Neurosteroids are gaining attention in this field as they are increasingly being pointed out as having neuroprotective abilities. Indeed, several neurosteroids exert important neuronal functions on the central and peripheral nervous systems and some of these effects are related to neuronal survival. At the same time, it is known that their production declines with age [1]. For this reason, the ability to locally produce steroids in response to changes in the surrounding environment may be crucial for their effects on the nervous system.

Among neurosteroids, the neurological effects of oestrogens are being highlighted. Apart from their functions in the CNS formation and in the regulation of reproductive functions, oestrogens do also play a role in higher cognitive functions, regulation of fine motor skills and pain and seem to be related to protection against some pathological alterations that occur in Alzheimer's disease [2]. Also during aging, the CNS seems to respond to oestrogen-induced changes that have been demonstrated both *in vitro* and *in vivo*.

The CNS is able to produce oestrogens as some brain regions express aromatase, the enzyme required for the last catalytical step in oestrogen production from androgenic precursors [3]. Although this capacity seems to be restricted to neurones, aromatase expression can also be induced in glial cells, contributing to oestrogen synthesis after injury to serve as a compensatory mechanism [3]. At least some of the effects of oestrogens are mediated through their action in oestrogen receptors α (ERα) and ERβ, expressed in hippocampal neurones [4].

Aromatase activity, and therefore local oestrogen availability, can be modulated by food components, namely by polyphenols, a

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wide group of molecules ubiquitously found in plant-based foods. Indeed, it has been shown that polyphenols from soy, wine, tea and beer regulate aromatase activity or expression in several different tissues [5–8]. Actually, there are some recent reports on the protective actions of moderate red wine (RW) intake for cognitive functions, but the most frequent explanation for this association is the protection from oxidative stress and no connection with aromatase or oestrogen signalling modulation has been sought [9,10]. For this reason, we aimed at testing the effects of prolonged RW drinking on oestrogen availability and on oestrogen receptor expression in rat hippocampus. The effects of RW ingestion were compared to ethanol-drinking and control animals and the contribution of one of the most abundant groups of polyphenols present in red wine, the procyanidins, for putative effects of the beverage has been investigated.

2. Materials and methods

2.1. Animals and treatments

Thirty-five male Wistar rats (Harlan Iberica, Barcelona) with mean body weight of 219.1 ± 1.6 g, were maintained under standard temperature and light conditions (20–22 °C, 12 h light/dark cycles). All animal procedures were in conformity with European Union guidelines. The animals were divided into four experimental groups treated with different beverages: (1) water (control group, $n = 10$); (2) red wine (RW, $n = 10$) with 13% ethanol (from Douro region, Portugal); (3) 13% ethanol (EtOH, v/v, $n = 10$); or (4) procyanidin solution (PA; 200 mg/L, $n = 5$). The beverages were supplied to the animals *ad libitum* in dark bottles. The body weight of the animals was recorded weekly and the ingestion of beverage and rat chow was monitored. Fresh beverages were supplied every other day. After 8 weeks of treatment the animals were anesthetised with sodium pentobarbital (60 mg/kg of body weight, i.p.) and perfused with ice-cold saline. The brains were removed from the skulls and the hippocampus carefully dissected from both hemispheres, frozen in liquid nitrogen and stored at −80 °C.

In order to study the acute effect of wine polyphenols on aromatase activity, 12 control rats were used with mean body weight of 404.5 ± 13.7 g. Animals were anaesthetised with sodium pentobarbital (60 mg/kg of body weight, i.p.), perfused with ice-cold saline and the hippocampus was removed and homogenised.

2.2. RNA and protein extraction

RNA and protein extraction from hippocampal homogenates was performed using Tripure Isolation Reagent (Roche, Indianapolis, USA). Extracted DNA-free RNA was dissolved in DEPC-treated water and quantified by measuring optical density at 260 and 280 nm (ratio was always 1.8 or higher). Proteins were dissolved in 1% SDS solution and quantified by bicinchoninic acid protein assay (Pierce). Samples were stored at −80 °C until analyses.

2.3. RT-PCR

Five micrograms of RNA were used as template for cDNA production through the incubation with reverse transcriptase (Reverase, Bioron GmbH) for 1 h at 45 °C, in 10 μM random hexamers, 0.375 mM per dNTP, 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris–HCl, pH 8.3, 10 mM dithiothreitol, and 40 U RNAse inhibitor (RNAseOUT™, Gibco BRL), followed by 10 min at 95 °C to inactivate the enzyme. Samples were incubated for 30 min at 37 °C with 0.1 mg/mL RNAse A (Sigma). PCR amplification was performed in the presence of 2.3 mM of $MgCl_2$, 0.5 mM of each primer, 0.2 mM dNTPs and 2 U of Taq DNA polymerase (DFS-Taq DNA polymerase, Bioron GmbH) and 4 μL of RT product, in a final volume of 50 μL. Simultaneous amplification of target proteins and the invariant housekeeping gene GAPDH was performed. For CYP19 amplification, samples were denaturated at 95 °C for 5 min, followed by 30 cycles denaturation at 95 °C for 90 s, annealing at 56 °C for 2 min and elongation at 72 °C for 4 min with a final elongation at 72 °C for 5 min. PCR amplification of ERs started with denaturation at 97 °C for 5 min, 30 cycles of denaturation at 96 °C for 1 min, annealing at 58 °C for 90 s and elongation at 72 °C for 90 s with final elongation at 72 °C for 15 min. Primers were purchased to Metabion International (Martinsried). CYP19 primer set sequences were 5′-ATT TCC ACA ATG GGG CTG TCC-3′ and 5′-ATT TCC ACA ATG GGG CTG TCC-3′. Primers for ERα were 5′-AAT TCT GAC AAT CGA CGC CAG-3′ and 5′-GTG CTT CAA CAT TCT CCC TCC TC-3′ and for ERβ 5′-TTC CCG GCA GCA CCA GTA ACC-3′ and 5′-TCC CTC TTT GCG TTT GGA CTA-3′. GAPDH primer set sequences were 5′-ACT GGC GTC TTC ACC ACC AT-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′. The predicted sizes of the PCR products were (in bp): 271 (CYP19), 344 (ERα), 241 (ERβ) and 682 (GAPDH). PCR products were visualised on a 1.6% agarose gel with ethidium bromide staining. The expression of aromatase, ERα and ERβ was analysed using Gel Pro Analyser® (Media Cybernetics) to determine optical density and the intensity of the bands corresponding to aromatase, ERα and ERβ was normalised to the expression of GAPDH of each sample and compared.

2.4. Western blotting

After separation of 4 μg of hippocampal proteins on 10% SDS–PAGE, proteins were transferred to a nitrocellulose membrane. Membranes were incubated for 1 h with the antibodies dissolved in 5% non-fat dried milk solution in PBS with 0.05% of Tween 20. Anti-aromatase polyclonal antibody (1:200, Santa Cruz Biotechnologies) and HRP-conjugated polyclonal antibody (1:1000) were used followed by chemiluminescent detection. β-Actin primary antibody (1:1000, Lab Vision Corporation) hybridization proceeded by secondary antibody was carried out by the same procedure. Band intensity was determined using Gel Pro Analyser® (Media Cybernetics), aromatase being normalized to β-actin expression.

2.5. Measurement of aromatase activity

Aromatase activity was measured according to the method described by Roselli et al. [11] with slight modifications. Measurements were performed in hippocampal homogenates of rats treated for 8 weeks as described above or in hippocampal homogenates from untreated rats to determine acute aromatase modulation by red wine polyphenols.

The hippocampi were homogenised (Thomas Teflon-Glass) in 4 volumes of buffer (10 mM KH_2PO_4, 100 mM KCl, 1 M EDTA, 1 M dithiothreitol and 0.32 M sucrose). Samples of the homogenates (125 μL) were placed in microtubes, preincubated for 5 min at 37 °C and the reaction was initiated by the addition of [^{3}H]-androstenedione (300 nM final concentration). The volume of reaction was 250 μL, in the presence of 1 mM NADPH, at 37 °C for 3 h. The reaction was stopped by adding 750 μL of ice-cold chloroform. After centrifugation at 9000 × *g*, for 1 min, 200 μL of the upper phase was transferred to new microtubes with 200 μL of charcoal (5%)/dextran (0.5%), vortexed and incubated for 10 min at room temperature. Following a new centrifugation at 9000 × *g*, for 5 min, 300 μL of the upper phase were removed and used for liquid scintillation counting. Measurements were performed in triplicate. Hippocampus homogenate proteins were quantified as described by Bradford [12], with bovine serum albumin as standard in order to normalise the activity values for protein content of each sam-

ple. Aromatase activity was expressed as fmol of [^{3}H]-H_2O formed (during the conversion of [^{3}H]-androstenedione to estrone) per mg of protein of the sample per hour of incubation.

To test the acute effect of compounds, the hippocampus from right and left hemispheres from six animals were pooled. The polyphenols tested were resveratrol (5 μM), myricetin (50 μM), quercetin (50 μM), kaempferol (50 μM), (+)-catechin (300 μM) and (−)-epicatechin (200 μM) obtained from Sigma. Whole grape seed procyanidin extracts and fractions containing monomers, dimers and trimers (Fraction I and Fraction II) were also tested (FI or FII, 200 mg/L). The compound to be tested or the vehicle were added to hippocampal homogenates, preincubated and the determination was performed as described above. Two separate experiments were carried out in triplicate. Aromatase activity for acute treatments is shown as the percentage of activity relative to the respective control (incubation with vehicle).

2.6. Procyanidin extract preparation

Grape seeds (500 mg of dry weight) were extracted using a blender (Ultra-Turrax, for 2 min) with 20 mL of ethanol/water/chloroform (1:1:2, v/v) according to the procedure described previously [13,14]. The 50% ethanol aqueous upper layer was separated from the chloroform layer containing chlorophylls, lipids and other undesirable compounds. Ethanol was evaporated using a rotatory evaporator and the resulting aqueous solution containing the polyphenolic compounds was extracted with ethyl acetate followed by precipitation with hexane in order to obtain the procyanidin oligomers. The grape seed extract was fractioned through a TSK Toyopearl HW-40(s) gel column (100 mm × 10 mm i.d., with 0.8 mL/min methanol as eluent) according to the procedure described in the literature [15]. The resulting solids were analysed by HPLC/DAD and laser secondary ionization mass spectrometry. Fraction I was composed of (+)-catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate, monogallate monomers, dimeric procyanidin (B1, B2, B3, B4, B5, B6, B7, B8 and B2-gallate) and trimer C1. Fraction II was composed of dimer B2 esterified with gallic acid, dimer B2-3′-*O*-galate, and trimers C1 and T2.

2.7. Calculations and statistic analysis

Results are expressed as means and standard error of mean. The differences between the groups were evaluated by one-way analysis of variance followed by Bonferroni test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Animals and treatments

Fig. 1 shows that the body weight of all rats increased throughout the 8 weeks of treatment. However, EtOH- or RW-treated rats had a lower weight gain when compared to controls. The amounts of chow, fluid and ethanol intake by the animals are summarised in Table 1. Despite the lower ingestion of chow and fluid by RW and EtOH, energy intake was similar in all groups of rats. Energy ingestion was calculated taking into account the energy supplied by the ethanol contained in RW or in the ethanol solution and from the chow ingested.

3.2. Aromatase, ERα and ERβ expression

RW ingestion for 8 weeks resulted in a significant increase of aromatase expression in hippocampal homogenates as determined by RT-PCR and western blotting. Ethanol treatment did also result in higher aromatase expression in this brain region but the difference did not reach statistical significance. Procyanidin extract treatment did not alter aromatase expression (Fig. 2A and B). Regarding the effect of RW treatment on oestrogen receptor expression, we found no alterations in ERα expression (Fig. 3A), whereas RW significantly decreased ERβ expression in the hippocampus (Fig. 3B). No changes in ER expression were observed after ethanol consumption.

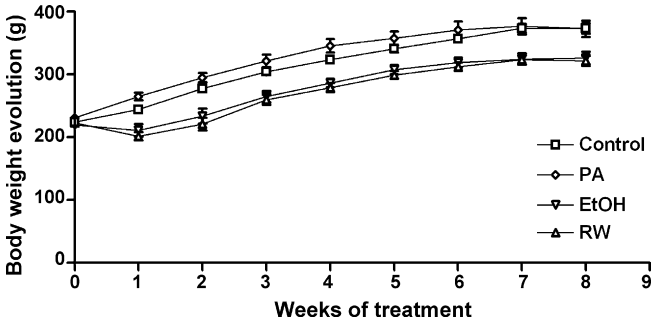

Fig. 1. Body weight evolution of control (C), procyanidin solution (PA), red wine (RW), and 13% ethanol solution (EtOH)-treated animals. Body weights were monitored and recorded weekly throughout the treatment. Results represent mean ± standard error of mean ($n = 5$–10).

3.3. Hippocampal aromatase activity

Chronic treatment with red wine resulted in a striking increase in aromatase activity in the hippocampus. Ethanol treatment did also tend to increase aromatase activity, although the difference did not reach statistical significance (Fig. 4). No difference was observed in hippocampal aromatase activity from procyanidin-treated rats when compared to controls.

The effect of some of the most important polyphenols present in RW was tested on aromatase activity from hippocampal homogenates of untreated rats. Concentrations were chosen according to their content in RW as earlier reported [16]. Some of the tested compounds were found to modulate aromatase activity. Resveratrol (5 μM), quercetin (50 μM), myricetin (50 μM) and kaempferol (50 μM) did not significantly interfere with aromatase activity (Fig. 5A) although in their presence aromatase activity tended to be lower than in the samples treated with vehicle (9.85 ± 2.12 fmol/(mg protein h)$^{-1}$). On the other hand, RW 3-flavanols (catechins and procyanidins) either as the whole extract (200 mg/L), procyanidin fractions (FI or FII, 200 mg/L) or isolated (+)-catechin (300 μM) and (−)-epicatechin (200 μM), all decreased aromatase activity when compared to control (9.34 ± 2.27 fmol/(mg protein h)$^{-1}$). The strongest aromatase inhibition was obtained in the presence of (+)-catechin, although the effects of procyanidin extract or of their fractions were also very pronounced (Fig. 5B).

Table 1
Chow, fluid and ethanol intake by control (C), procyanidin solution (PA), red wine (RW) and 13% ethanol solution (EtOH)-treated animals ($n = 5$–10)

	Chow (g/day)	Fluid (mL/day)	Ethanol (mL/(kg b.w. day)$^{-1}$)
C	21.71 ± 0.39	25.33 ± 1.13	–
PA	23.83 ± 0.59	26.16 ± 0.64	–
RW	15.34 ± 0.41*	14.85 ± 0.65*	6.28 ± 0.27
EtOH	15.65 ± 0.30*	16.48 ± 1.36*	6.81 ± 0.56

* $p < 0.05$ *vs.* control.

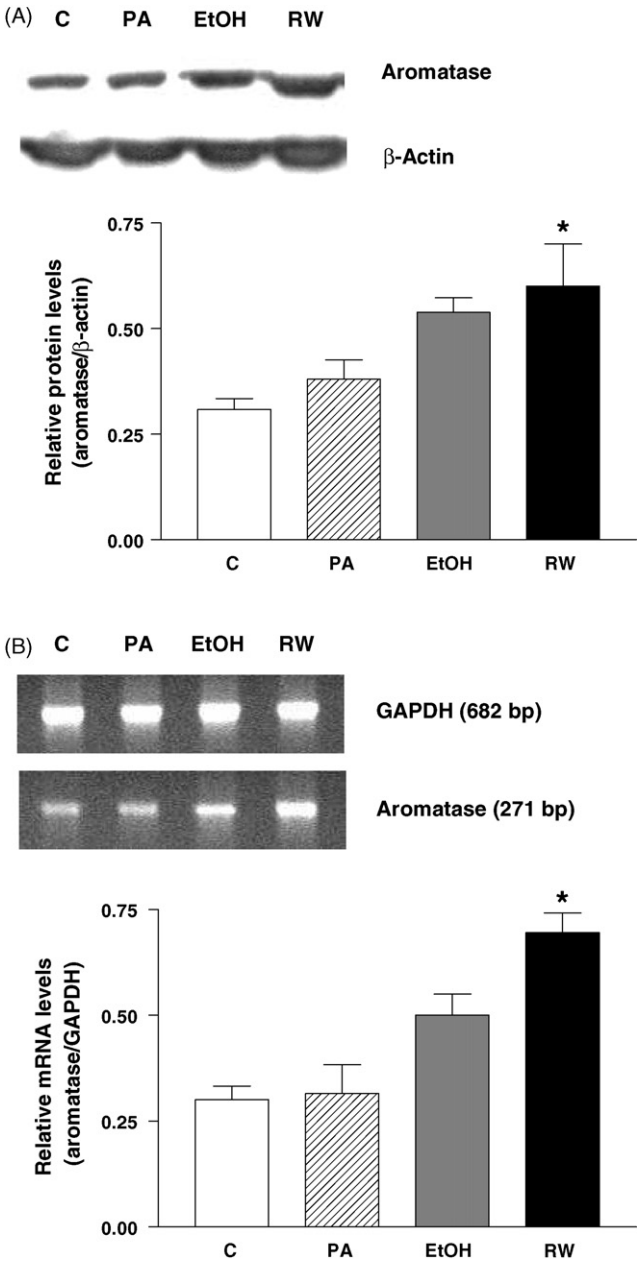

Fig. 2. Relative aromatase expression in the hippocampus of control (C), procyanidin solution (PA), red wine (RW) and 13% ethanol solution (EtOH)-treated rats. At the end of the treatment hippocampus was removed and total RNA and protein were extracted to determine protein expression by western blotting (A) and mRNA levels by RT-PCR (B). Representative western blots or PCR photographs are shown for each group. Aromatase was normalised to β-actin or GAPDH expression. Results represent mean ± standard error of mean ($n = 4$–5). $^*p < 0.05$ *vs.* control.

4. Discussion

Steroid hormones play an important role in the regulation of brain function related to the reproductive physiology and behaviour. Recently, special attention has been given to the effect of oestrogens in synaptic plasticity and their influence in learning and memory processes [17]. Several activities related to neuronal preservation such as the increase in the number of synapses and dendritic spines have been attributed to this class of hormones, particularly in the hippocampus, one of the main brain areas involved in cognition [17–20]. Part of the effect is mediated by oestrogen receptors, ERα or ERβ, which in the presence of their ligand are

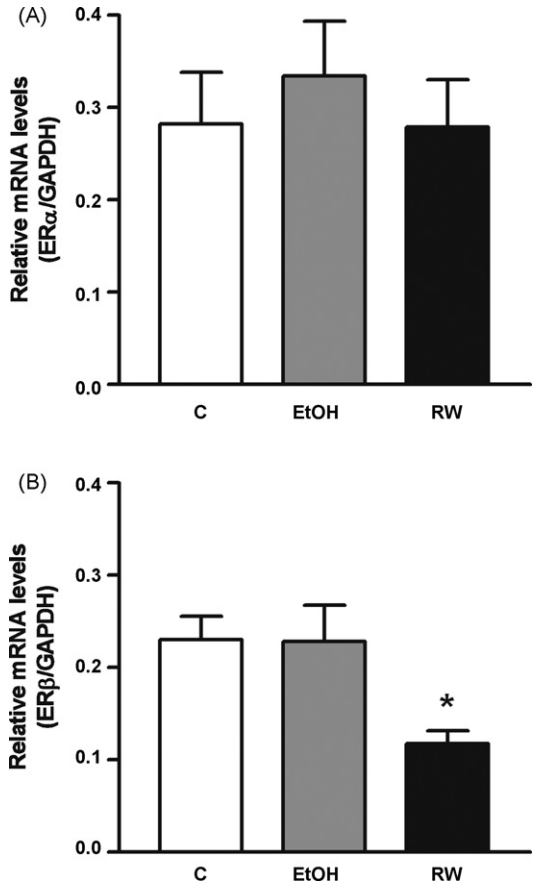

Fig. 3. Relative oestrogen receptor α (A) and oestrogen receptor β (B) expression in the hippocampus of control (C), red wine (RW) and 13% ethanol solution (EtOH)-treated rats. At the end of the treatment hippocampus was removed and total RNA was extracted to determine protein transcription by RT-PCR. ERα and ERβ transcriptions were normalised to GAPDH expression. Results represent mean ± standard error of mean ($n = 4$–5). $^*p < 0.05$ *vs.* control.

translocated to the nucleus to interact with oestrogen response elements, activating or repressing the expression of proteins [21,22].

More evidence of the involvement of oestrogens in cognition is the decline in cognitive performance that occurs after menopause when circulating oestrogen levels are lower, which can be prevented by oestrogen replacement therapies [23]. Another notorious observation corroborating oestrogen involvement in memory is

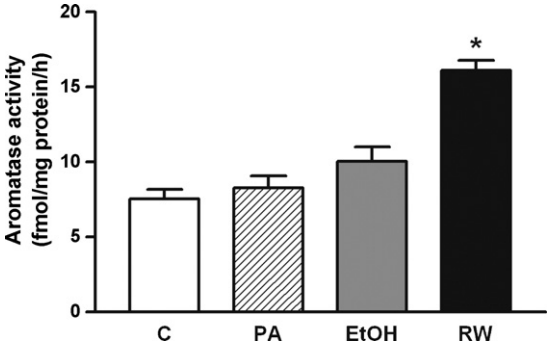

Fig. 4. Aromatase activity in hippocampal homogenates of control (C), procyanidin solution (PA), red wine (RW) and 13% ethanol solution (EtOH)-treated rats. Aromatase activity was quantified by measuring $[^3H]$-H_2O release during the aromatization of $[^3H]$-androstenedione (300 nM) to oestrone, for 3 h at 37 °C. Results represent mean ± standard error of mean measurements performed in triplicate ($n = 5$). $^*p < 0.05$ *vs.* control.

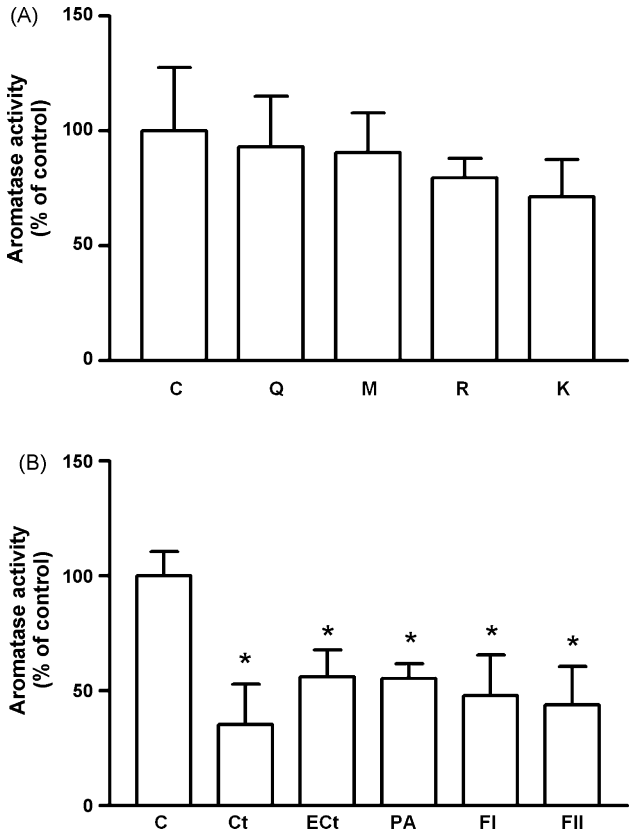

Fig. 5. Aromatase activity in hippocampal homogenates from control rats. Aromatase activity was quantified by measuring [^{3}H]-H_2O release during the aromatization of [^{3}H]-androstenedione (300 nM) to oestrone, for 3 h at 37 °C. (A) C, DMSO (0.1%, v/v); Q, quercetin (50 μM); M, myricetin (50 μM); R, resveratrol (5 μM); K, kaempferol (50 μM). (B) C, ethanol (0.1%, v/v); Ct, catechin (300 μM), ECt, epicatechin (200 μM); PA, procyanidin whole extract (200 mg/L), FI, procyanidin fraction I (200 mg/L); FII, procyanidin fraction II (200 mg/L). Results represent mean ± standard error of mean from two experiments performed in triplicate. $^*p < 0.05$ *vs.* control.

the fluctuation of synapse number in female rats during the oestral cycle. In fact, synapse number was shown to be higher during *pro-oestrus*, when oestrogen levels are higher [24]. Also, aromatase knockouts have cognitive impairments that can be partially reversed by oestrogen administration [25]. Furthermore, it has also been suggested that testosterone, androstenedione and dehydroepiandrosterone have neuroprotective abilities, although some authors have demonstrated that at least part of the protection requires brain aromatase activity, suggesting that their conversion to oestrogen mediates their neuroprotective effects [3].

Several diet components have the ability to modulate aromatase activity as has been shown previously in different experimental settings. However, there is no knowledge about brain aromatase modulation by diet components.

Wine is a complex solution composed of various classes of compounds [16,26]. On the one hand it contains ethanol, which at high dosage has undisputable devastating effects on the CNS [27,28] and on the other hand it is enriched with polyphenols which are being more and more related to neuroprotection and are frequently related with the ability to counteract the deleterious effects of ethanol in the brain [29–32].

There is an increasing number of epidemiological studies showing an association between moderate RW consumption and the amelioration of neurological parameters, the prevention of brain function decline, and dementia, Alzheimer's disease and ischemic stroke incidence reduction [9,10]. Investigators have made an effort to discover mechanisms behind this relationship and several have been suggested. The decrease of lipid peroxidation, free radical scavenging, metal quelation and modulation of inflammatory enzymes are only some [27,31]. Nevertheless, as far as we know, the ability of this beverage to interfere with brain oestrogen signalling had not been described.

In this work we show that after 8 weeks of RW consumption, hippocampal aromatase transcription and protein levels are significantly increased. From this result, it is tempting to speculate that local production of oestrogens, and as a consequence oestrogen-mediated events, are enhanced. This is supported by the results observed in aromatase activity in these rats that revealed an increase in aromatase activity after chronic red wine exposure. Ethanol solution intake did also result in increased aromatase transcription, although not as pronounced as in the case of RW treatment. This remarkable difference between RW and ethanol groups indicates that non-alcoholic components of RW markedly contribute for the effect, increasing oestrogen production and allowing for the occurrence of their CNS effects.

Since oestrogens interact with specific receptors to produce part of their effects [22] and because the results form ERα and ERβ activation are not always the same, the effect of RW or ethanol solution treatment on hippocampus ER expression was also investigated. Whereas none of the treatments altered ERα transcription, RW induced a decrease in ERβ transcription. We were unable to confirm if the alteration in ERβ transcription was reflected in protein levels of this protein, due to the current lack of availability of appropriate antibodies against ERβ. The consequences of the activation of each one of the ERs by oestrogens and its meaning for oestrogens demonstrated effects is still not very clear. In fact, some studies state that both ERs mediate the protection of brain injury by oestrogens, as in the case of ischemia-induced neuronal death [4]. However, in the case of dendritic spine formation, increased ERβ expression seems unfavourable [24]. It has also been shown that low oestrogen levels are associated with an increase of ERβ RNA and protein and that correction of oestrogen levels for only 1 week is able to reduce ERβ expression in several brain areas, including the hippocampus [24]. This indicates that oestrogens regulate the expression of their own receptors as could be confirmed by our results, i.e., the higher aromatase expression observed in RW-treated animals might have resulted in decreased ERβ in the hippocampus allowing for synaptogenic activity.

Polyphenols may be responsible for aromatase expression modulation. This enzyme belongs to the cytochrome P450 family, which includes xenobiotic-metabolizing enzymes, and polyphenols are known to regulate the expression of cytochrome P450 enzymes through interaction with aryl hydrocarbon nuclear receptors [33]. The acute effect of RW polyphenols on aromatase activity may also play a role, as aromatase inhibition by 3-flavanols could also account for the stimulation of aromatase synthesis as a feedback mechanism. These compounds constitute one of the most abundant groups of polyphenols in red wine and comprise both monomeric compounds (catechin and epicatechin) and oligomeric procyanidins, being likely candidates for aromatase interference. In fact, it has been suggested in other experimental settings that procyanidins were responsible for part of aromatase modulation effects of red wine extracted compounds. However, rats treated for 8 weeks with procyanidin solution in a concentration which has been described for red wines [16] had no alteration of aromatase activity and protein and mRNA levels. The alteration of bioavailability of the compounds when separated from their natural, alcoholic matrix could justify this lack of effect, although we have obtained the same results on aromatase activity and mRNA levels when procyanidins were supplied to the animals in a 13% ethanol solution (results not shown). Thus, it seems likely that another compound or

group of compounds may be more relevant than the 3-flavanols to aromatase modulation and probably also for the inhibition of ERβ expression which is likely to arise from the increase in estradiol production induced by red wine.

Altogether, these results show that oestrogen signalling is altered by prolonged RW consumption, revealed by the increase in aromatase expression and down-regulation of ERβ synthesis. Although ethanol may contribute to the effect, the non-alcoholic components of red wine are of major relevance to this effect. This study contributes to the elucidation of neuroprotective mechanisms of moderate RW ingestion on the CNS and may shed light on the design of new approaches for neurodegenerative disease management.

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References

[1] M. Schumacher, S. Weill-Engerer, P. Liere, F. Robert, R.J. Franklin, L.M. Garcia-Segura, J.J. Lambert, W. Mayo, R.C. Melcangi, A. Parducz, U. Suter, C. Carelli, E.E. Baulieu, Y. Akwa, Steroid hormones and neurosteroids in normal and pathological aging of the nervous system, Prog. Neurobiol. 71 (2003) 3–29.
[2] B.S. McEwen, Invited review: estrogens effects on the brain: multiple sites and molecular mechanisms, J. Appl. Physiol. 91 (2001) 2785–2801.
[3] L.M. Garcia-Segura, S. Veiga, A. Sierra, R.C. Melcangi, I. Azcoitia, Aromatase: a neuroprotective enzyme, Prog. Neurobiol. 71 (2003) 31–41.
[4] N.R. Miller, T. Jover, H.W. Cohen, R.S. Zukin, A.M. Etgen, Estrogen can act via estrogen receptor alpha and beta to protect hippocampal neurons against global ischemia-induced cell death, Endocrinology 146 (2005) 3070–3079.
[5] K.M. Edmunds, A.C. Holloway, D.J. Crankshaw, S.K. Agarwal, W.G. Foster, The effects of dietary phytoestrogens on aromatase activity in human endometrial stromal cells, Reprod. Nutr. Dev. 45 (2005) 709–720.
[6] E.T. Eng, D. Williams, U. Mandava, N. Kirma, R.R. Tekmal, S. Chen, Suppression of aromatase (estrogen synthetase) by red wine phytochemicals, Breast Cancer Res. Treat. 67 (2001) 133–146.
[7] R. Monteiro, H. Becker, I. Azevedo, C. Calhau, Effect of hop (Humulus lupulus L.) flavonoids on aromatase (estrogen synthase) activity, J. Agric. Food Chem. 54 (2006) 2938–2943.
[8] K. Satoh, Y. Sakamoto, A. Ogata, F. Nagai, H. Mikuriya, M. Numazawa, K. Yamada, N. Aoki, Inhibition of aromatase activity by green tea extract catechins and their endocrinological effects of oral administration in rats, Food Chem. Toxicol. 40 (2002) 925–933.
[9] S. Bastianetto, Red wine consumption and brain aging, Nutrition 18 (2002) 432–433.
[10] T. Truelsen, D. Thudium, M. Gronbaek, Amount and type of alcohol and risk of dementia: the Copenhagen City Heart Study, Neurology 59 (2002) 1313–1319.
[11] C.E. Roselli, L.E. Horton, J.A. Resko, Time-course and steroid specificity of aromatase induction in rat hypothalamus-preoptic area, Biol. Reprod. 37 (1987) 628–633.
[12] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[13] G. Darné, J. Madero-Tamargo, Mise au point d'une méthode d'extraction des lipides solubles totaux, des glucides solubles totaux et des composés phénoliques solubles totaux des organes de la vigne, Vitis 18 (1979) 221–228.
[14] J. Michaud, P. Lacaze, J. Masquelier, Fractionnement des oligomères flavanoliques du raisin, Bull. Soc. Pharm. Bordeaux 110 (1971) 111–116.
[15] V.A. de Freitas, Y. Glories, G. Bourgeois, C. Vitry, Characterization of oligomeric and polymeric procyanidins from grape seeds by liquid secondary ion mass spectrometry, Phytochemistry 49 (1998) 1435–1441.
[16] J.B. German, R.L. Walzem, The health benefits of wine, Annu. Rev. Nutr. 20 (2000) 561–593.
[17] M.M. Adams, J.H. Morrison, Estrogen the aging hippocampal synapse, Cereb. Cortex 13 (2003) 1271–1275.
[18] O. Kretz, L. Fester, U. Wehrenberg, L. Zhou, S. Brauckmann, S. Zhao, J. Prange-Kiel, T. Naumann, H. Jarry, M. Frotscher, G.M. Rune, Hippocampal synapses depend on hippocampal estrogen synthesis, J. Neurosci. 24 (2004) 5913–5921.
[19] B. McEwen, K. Akama, S. Alves, W.G. Brake, K. Bulloch, S. Lee, C. Li, G. Yuen, T.A. Milner, Tracking the estrogen receptor in neurons: implications for estrogen-induced synapse formation, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 7093–7100.
[20] G.M. Rune, M. Frotscher, Neurosteroid synthesis in the hippocampus: role in synaptic plasticity, Neuroscience 136 (2005) 833–842.
[21] C.J. Saldanha, B.A. Schlinger, P.E. Micevych, T.L. Horvath, Presynaptic *N*-methyl-D-aspartate receptor expression is increased by estrogen in an aromatase-rich area of the songbird hippocampus, J. Comp. Neurol. 469 (2004) 522–534.
[22] T. Simoncini, A.R. Genazzani, Non-genomic actions of sex steroid hormones, Eur. J. Endocrinol. 148 (2003) 281–292.
[23] A. Markou, T. Duka, G.M. Prelevic, Estrogens and brain function, Hormones (Athens) 4 (2005) 9–17.
[24] S. Szymczak, K. Kalita, J. Jaworski, B. Mioduszewska, A. Savonenko, A. Markowska, I. Merchenthaler, L. Kaczmarek, Increased estrogen receptor beta expression correlates with decreased spine formation in the rat hippocampus, Hippocampus 16 (2006) 453–463.
[25] S. Martin, M. Jones, E. Simpson, M. van den Buuse, Impaired spatial reference memory in aromatase-deficient (ArKO) mice, Neuroreport 14 (2003) 1979–1982.
[26] G.J. Soleas, E.P. Diamandis, D.M. Goldberg, Wine as a biological fluid: history, production, and role in disease prevention, J. Clin. Lab. Anal. 11 (1997) 287–313.
[27] A.Y. Sun, G.Y. Sun, Ethanol and oxidative mechanisms in the brain, J. Biomed. Sci. 8 (2001) 37–43.
[28] D. Wu, A.I. Cederbaum, Alcohol, oxidative stress, and free radical damage, Alcohol Res. Health 27 (2003) 277–284.
[29] P.A. da Silva Porto, J.A. Laranjinha, V.A. de Freitas, Antioxidant protection of low density lipoprotein by procyanidins: structure/activity relationships, Biochem. Pharmacol. 66 (2003) 947–954.
[30] K. Ishige, D. Schubert, Y. Sagara, Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms, Free Radic. Biol. Med. 30 (2001) 433–446.
[31] R.J. Nijveldt, E. van Nood, D.E. van Hoorn, P.G. Boelens, K. van Norren, P.A. van Leeuwen, Flavonoids: a review of probable mechanisms of action and potential applications, Am. J. Clin. Nutr. 74 (2001) 418–425.
[32] A. Simonyi, D. Woods, A.Y. Sun, G.Y. Sun, Grape polyphenols inhibit chronic ethanol-induced COX-2 mRNA expression in rat brain, Alcohol Clin. Exp. Res. 26 (2002) 352–357.
[33] P. Hodek, P. Trefil, M. Stiborova, Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450, Chem. Biol. Interact. 139 (2002) 1–21.