



HAL
open science

Direct Antioxidant and Protective Effect of Estradiol on Isolated Mitochondria

Consuelo Borrás, Juan Gambini, Raúl López-Grueso, Federico V. Pallardó,
Jose Viña

► **To cite this version:**

Consuelo Borrás, Juan Gambini, Raúl López-Grueso, Federico V. Pallardó, Jose Viña. Direct Antioxidant and Protective Effect of Estradiol on Isolated Mitochondria. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2009, 1802 (1), pp.205. 10.1016/j.bbadis.2009.09.007 . hal-00543009

HAL Id: hal-00543009

<https://hal.science/hal-00543009>

Submitted on 5 Dec 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Direct Antioxidant and Protective Effect of Estradiol on Isolated Mitochondria

Consuelo Borrás, Juan Gambini, Raúl López-Gruoso, Federico V. Pallardó, Jose Viña

PII: S0925-4439(09)00218-X
DOI: doi:[10.1016/j.bbadis.2009.09.007](https://doi.org/10.1016/j.bbadis.2009.09.007)
Reference: BBADIS 63010

To appear in: *BBA - Molecular Basis of Disease*

Received date: 20 March 2009
Revised date: 2 September 2009
Accepted date: 8 September 2009



Please cite this article as: Consuelo Borrás, Juan Gambini, Raúl López-Gruoso, Federico V. Pallardó, Jose Viña, Direct Antioxidant and Protective Effect of Estradiol on Isolated Mitochondria, *BBA - Molecular Basis of Disease* (2009), doi:[10.1016/j.bbadis.2009.09.007](https://doi.org/10.1016/j.bbadis.2009.09.007)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**DIRECT ANTIOXIDANT AND PROTECTIVE EFFECT OF ESTRADIOL ON
ISOLATED MITOCHONDRIA**

Consuelo Borrás*, Juan Gambini*, Raúl López-Grueso, Federico V. Pallardó^a, and Jose Viña

Department of Physiology, Faculty of Medicine, University of Valencia, Valencia, 46010 Spain. ^a Department of Physiology, Faculty of Medicine, University of Valencia, Valencia, 46010 Spain/CIBERER

*These authors have contributed equally to the work

Corresponding author:

Dr. Jose Vina,

Department of Physiology, Faculty of Medicine, University of Valencia

Avenida Blasco Ibáñez 17, 46010 Valencia, Spain.

Tel. +34963864650

Fax +34963864642

E-mail: jose.vina@uv.es

Key words: estrogenic compounds, oxidative stress, genistein, cytochrome c, mitochondria, antioxidant.

Summary

Estrogens have antioxidant properties which are due to their ability to bind to estrogen receptors and to up-regulate the expression of antioxidant enzymes via intracellular signalling pathways. Mitochondria are key organelles in the development of age-associated cellular damage. Recently, estrogen receptors were identified in mitochondria. The aim of this paper was to test whether estradiol directly affects mitochondria by preventing oxidative stress and protecting frail mitochondria.

Incubation with estradiol at normal intracellular concentrations prevents the formation of reactive oxygen species by mitochondria in a saturable manner. Moreover, estradiol protects mitochondrial integrity as indicated by an increase in mitochondrial membrane potential. It also prevents the apoptogenic leakage of cytochrome c from mitochondria and as a result the mitochondrial content of this cytochrome c is maintained high. Thus, estradiol prevents the onset of the mitochondrial pathway of apoptosis by a direct effect on the organelle. Genistein, a phytoestrogen present at high concentration in soy, mimics the protective effect of estradiol by both decreasing the rate of formation of reactive oxygen species and preventing the release of cytochrome c from mitochondria.

Introduction

The role of mitochondria as key organelles involved in ageing was proposed by Miquel [1]. This concept was experimentally confirmed in many laboratories including our own. Some years ago, work from our laboratory [2] and that of Bruce Ames [3] showed that mitochondria are frail in cells of old animals and that they decay with age. Moreover we found that oxidation of mitochondrial glutathione correlates with an increase in the amount of 8-oxoguanosine, an oxidized form of guanosine which is mutagenic. The role of mitochondria in aging has been highlighted in recent years [4]. Mitochondria are both sources and targets of damage by free radicals. These highly reactive molecules are generated in mitochondria and because of their very high reactivity they cause damage to mitochondrial structures.

Further proof of the role of mitochondria in aging came from studies on the different longevity between genders. Females in many species (including humans) live longer than males. We showed that oxidant production by mitochondria from males is much higher than from females [5]. This is due to the action of estrogens which up-regulate nuclear gene expression of antioxidant enzymes which are directed to mitochondria [5-7]. Moreover, we showed that the antioxidant action of estrogens in vivo is due, not to their chemical phenolic structure, but rather to their interaction with estrogen receptors in cells which eventually lead to the activation of mitogen activated protein kinases (MAPK) and nuclear factor kappa B (NF κ B) [8, 9].

However, proof of a direct effect of estrogens on mitochondria was lacking. Experimental evidence shows, however, that other hormones, such as melatonin act directly on these organelles [10]; moreover estrogen receptors are present in mitochondria [11]. We hypothesized that estrogens (and phytoestrogens), in addition to

their well-established protective effects mediated by the up-regulation of antioxidant genes.

The aim of this work was to test if estrogens protect mitochondria in a direct fashion i.e. independently of nuclear or cytosolic interactions.

Material and methods

Animals

Male Wistar rats between 4 and 6 months of age were used. Animals were housed at constant temperature and humidity and with a 12 h light / 12 h dark cycle. They were fed on a standard laboratory diet (containing 590 g carbohydrates, 30 g lipids, and 160 g protein per kilogram of diet) and tap water ad libitum.

In all cases, animals were treated in accordance with the principles for the good care of laboratory animals.

Isolation of mitochondria

After the animals were sacrificed by cervical dislocation, their livers and brains were quickly removed. Liver mitochondria were obtained by differential centrifugation, as described by Rickwood et al [12]. Briefly, minced liver fragments were homogenized, centrifuged at 1000g for 10'. This was repeated three times to remove cell debris. Supernatants were then centrifuged at 10000g for 10' to purify mitochondria. Synaptic and non-synaptic mitochondria were isolated following the method of Lai and Clark [13]. This is based on differential centrifugations using different concentrations of ficoll to separate synaptosomes from non-synaptic fraction.

Mitochondrial incubation conditions

Isolated mitochondria from males were incubated at 37°C in a buffer solution containing 0.3 M sucrose, 1 mM EGTA, 5 mM MOPS, 14.7 µM BSA, 5 mM KH₂PO₄, pH 7.4, for 30 minutes, one, two or four hours.

Depending on the experiment, we added 0.02 - 2000 nM estradiol, 0.2 nM estradiol-BSA, 0.5 µM genistein, or 1 µM cyclosporin A (CsA) to the incubation buffer.

Concentrations were chosen following previously published data: briefly, estradiol was incubated at normal intracellular concentration [8] and genistein at nutritionally relevant concentration (i.e. that found in the blood of far Eastern population) [8, 14, 15]

Peroxide production

The rate of peroxide production was determined in isolated mitochondria using a modification of the method described by Barja de Quiroga [16] and according to [5]. Briefly, mitochondria were incubated at 37°C with 10 mM succinate or 5 mM pyruvate plus 2.5 mM malate in 2 ml of phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 5 mM KH₂PO₄, 3 mM MgCl₂, 145 mM KCL, 30 mM Hepes, 0.1 mM homovanílico acid, and 6 U/ml horseradish peroxidase. The incubation was stopped at 5, 10, and 15 min with 1 ml of cold 2 M glycine buffer containing 50 mM EDTA and 2.2 M NaOH. The fluorescence of supernatants was measured using 312 nm as excitation wavelength and 420 nm as emission wavelength. The rate of peroxide production was calculated using a standard curve of H₂O₂.

Mitochondrial membrane potential

Mitochondrial membrane potential was determined by flow cytometry. We used an EPICS ELITE cell sorter (Coulter Electronics, Hialeah, FL). Fluorochromes were from Lambda Fluoreszenz Technologie GmbH (Graz, Austria). Fluorochromes were excited with an argon laser tuned at 488 nm. Samples were acquired for 10,000 individual

mitochondria. Mitochondrial membrane potential was determined using the fluorescent dye rhodamine 123(Rh123) at 525 ± 5 nm fluorescence emission [17].

Cytochrome c determination

Cytochrome c levels were determined by Western blotting in cytosolic and in mitochondrial fractions. Immediately after incubation for one hour with estradiol or genistein, aliquots of cell lysate (40 μ g) were boiled for 10 min to inactivate proteases and phosphatases, electrophoresed in SDS–12.5% polyacrylamide gels and blotted onto an Immun-Blot PVDF Membrane (BIO-RAD, USA). Membranes were incubated with primary antibody against cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Thereafter, the blots were washed three times for 5 m each with a wash buffer (PBS containing 0.2% Tween 20) at room temperature and then further incubated for 1 h with a secondary horseradish peroxidase (HRP)-linked anti-mouse IgG antibody (Cell Signaling Technologies, IZASA, Barcelona, Spain). The blots were again washed and developed using the LumiGLO® reagent as specified (Cell Signaling Technologies IZASA, Barcelona, Spain). Autoradiographic signals were assessed using a Fujifilm scanning densitometer (Fujifilm LAS-1000 Plus, Fuji Madrid, Spain).

Statistics

Results are expressed as mean \pm SD. Statistical analysis was performed by the least-significant difference test which consists of two steps: First an analysis of variance was performed. The null hypothesis was accepted for all numbers of those set in which F was non-significant at the level of $P \leq 0.05$. Second, the sets of data in which F was significant were examined by the modified t-test using $p \leq 0.05$ as the critical limit.

Results

Estradiol decreases peroxide production and increases membrane potential in isolated mitochondria.

We have previously reported that the peroxide production by isolated mitochondria is significantly lower in females than in males [5]. In fact, we have found that females produce as low as 50% less peroxide than males when incubated in the presence of 10 mM succinate.

Moreover, we have studied the different rates of peroxide production in synaptic and non-synaptic mitochondria from brain. Figure 1 shows that synaptic mitochondria (mainly from neurons) produce many more peroxides than non-synaptic ones (mainly from glia). In this tissue, i.e. brain, it is also apparent that males produce approximately twice the amount of peroxides when compared with females. This is, however, completely prevented by ovariectomy. In fact, ovariectomised females produce as much or even more peroxide than males. We would like to point out that synaptic mitochondria come from tissues which are mainly post-mitotic whereas non-synaptic ones come from cells which still have the capacity to divide. Neurons may divide *in vivo* and this has been recently shown including the fact that neuronal stem cells which are produced in the hippocampus may migrate, at least in the rat, to the olfactory bulb [18]. However, this is only a tiny proportion of the total amount of neurons in brain of the adult animal which are predominantly post-mitotic. Another interesting point is that ovariectomy causes a very significant upshot of the production of peroxides. We previously observed [5] that this is due to the fact that oestrogens up-regulate antioxidant genes such as superoxide dismutase or glutathione peroxidase.

Thus, oestrogens not only protect against oxidative stress in hepatic mitochondria but also in brain (synaptic and non-synaptic) mitochondria. There must be common mechanisms for the protective action of oestrogens in two very different tissues such as

liver or brain and we have shown that this is due to the up-regulation of the antioxidant genes as stated above.

The question that remained open was whether estradiol acted only via interaction with conventional receptors or also via a direct effect in mitochondria. To test this, we incubated mitochondria from males with various concentrations of estradiol and found that peroxide production is significantly lower when mitochondria are incubated in the presence of 0.2 nM or above. Moreover, this effect is saturable. Figure 2 shows that concentrations of 0.2 nM or above (up to 2000 nM) decrease peroxide production to levels of approximately 30% of the controls, but no more. The fact that this effect shows a saturation curve indicates that estradiol may act via interaction with specific receptors present in mitochondria.

As we have shown above, hepatic and brain mitochondria have quite a number of common mechanisms in terms of the control of their peroxide production. Thus, after studying the direct protective effect of estradiol in hepatic mitochondria, we turned to brain mitochondria. And indeed, we found that incubation of mitochondria isolated from brains of young male rats, in the presence of 0.2 nM estradiol caused a very significant decrease in peroxide production. We found that this production fell from 0.145 ± 0.023 to 0.032 ± 0.009 ($p < 0.05$). This is in fact $\frac{1}{4}$ of the rate we found in controls. Thus, the direct protective effect of estradiol on isolated mitochondria is more pronounced in brain than in liver. As we saw in previous studies in which we determined the rate of peroxide production in brain and liver, we find now that basal peroxide production by mitochondria from brain is more than twice that of liver [5]. Again, since brain is a post-mitotic tissue, it is likely that even in the relatively young animal; mitochondria are more damaged than those of liver. The basal mechanisms

controlling peroxide production in brain and liver may remain the same but the actual rate of production is much higher in brain than in liver.

This lower peroxide production coincides with an increased coupling of the mitochondrial respiratory chain and oxidative phosphorylation as indicated by the mitochondrial membrane potential, which is 45% higher in mitochondria incubated with estradiol (0.02 nM) than in controls ($p < 0.05$).

Adding estrogens or phytoestrogens to mitochondria is one of the most simplistic ways to study the protection against radical production by the organelle.

However, the importance of the direct effect of estrogenic compounds on mitochondria relies also on being able to observe these protective effects in cells and eventually in whole animals. Correlation between cellular protection studies such as those reported here must correlate with *in vivo* studies. In fact, we observe that feeding animals with a *Ginkgo biloba* extract (EGB791, which is a standard diet extract from *Ginkgo biloba*) offers a significant protection against oxidative stress in mitochondria and even mitochondrial DNA damage. Table 1 shows that the rate of peroxide generation by old animals is significantly higher than that of young ones, but animals treated with EGB761 show peroxide production rates very close to those of controls. Not only does peroxide production increase with ageing and is prevented by administration of *Ginkgo* extract but also the actual multigenic damage to DNA as determined by measuring the levels of 8-oxo-deoxy-guanosine reflects significant protection by *Ginkgo biloba*. Table 1 shows that mitochondrial DNA from old animals is significantly more oxidised than that of controls and indeed feeding with *Ginkgo biloba* protects against such damage.

Estradiol prevents loss release of cytochrome c from mitochondria

A critical function of mitochondria is the control of cell viability via the apoptotic process. In fact, the release of cytochrome c is a key sub-cellular event to unleash

cellular apoptosis. Moreover, increased mitochondrial levels of cytochrome c may act as antioxidant because by increasing the flow of electrons through the respiratory chain, a lower rate of oxidant production may be expected.

Figure 3, Panel A shows that estradiol, either free or bound to bovine serum albumin, significantly increases the amount of cytochrome c present in mitochondria after one hour incubation. The effect is as marked as when mitochondria are incubated with cyclosporine A. Consequently, the release of cytochrome c to the supernatant is decreased when mitochondria are incubated with estradiol (either free or bound to bovine serum albumin) as shown in Figure 3, Panel B. Thus, it appears that estradiol prevents the release of cytochrome c thus having two functions, one being to prevent the onset of apoptosis and the other being to prevent, or at least decrease, oxidant production by mitochondria.

Genistein decreases oxidant production and cytochrome release from isolated mitochondria

Genistein is a soy isoflavone which binds to estrogen receptors (especially ER β). We have recently shown that it may mimic many of the favourable effects of estradiol when incubated with cells (either alone, [19], or in the presence of amyloid beta peptide, [20]). Therefore, we tested whether genistein could mimic the effects of estradiol on peroxide production by mitochondria as well as on cytochrome c release from the organelle.

Figure 4 shows that 0.5 μ M of genistein, when incubated with isolated mitochondria, causes a lowering in the rate of peroxide production that is similar to that of estradiol.

In a similar fashion, 0.5 μ M genistein prevents cytochrome c release from mitochondria.

Figure 5, Panel A shows that the levels of cytochrome c are higher in mitochondria

incubated with genistein than in controls and conversely the levels of cytochrome c in the incubation medium are lower than those in controls (see Figure 5, Panel B).

These effects are essentially comparable to those of estradiol and therefore our results indicate that genistein is as effective as estradiol to protect isolated mitochondria.

Discussion

Frailty of mitochondria upon ageing

Ever since the postulation of the mitochondrial theory of ageing by Miquel [1], these organelles have been the subject of close attention by molecular gerontologists. A decade ago, in our laboratory we found that mitochondria are damaged inside cells [2]. Almost simultaneously, Ames' group also found that mitochondria decay within cells upon ageing [3]. A critical question that we raised then and that, to our knowledge has not yet been answered is that mitochondria may be damaged within ageing cells because they are frailer. Both Ames' group and our own, found that peroxide production by mitochondria is increased during ageing [2, 3]. Moreover, we recently attributed the difference in longevity between males and females in many species including humans to a lower peroxide production by mitochondria from females when compared with those of males [6, 21]. We showed that this is due to the fact that estradiol activates cytosolic cell signalling pathways that up-regulate the nuclear expression of antioxidant genes whose protein products are directed towards mitochondria. Manganese superoxide dismutase and glutathione peroxidase are examples of this. However, the question of the possible direct interaction of estrogens with mitochondria had remained unanswered. In this paper we have performed experiments that show that estrogens protect mitochondria in a direct fashion, probably by lowering their frailty, especially in those from old animals.

Role of mitochondria in Alzheimer's disease pathophysiology

Early reports emphasized the role of amyloid plaques in Alzheimer's pathophysiology. However, soon it became apparent that toxic A β peptides could enter neurons and cause cellular damage [22]. Mitochondria were possible targets of A β toxicity. Work from the groups of G. Perry and M. Smith showed that Abnormalities in mitochondrial function occur in Alzheimer's disease [23, 24]. The group of C. Oliveira reported that A β lowers mitochondrial respiration by interfering with the respiratory chain complexes [25]. Moreover, we found that A β causes an increase in radical production by mitochondria which depends on the gender of the animal [26].

Estradiol is an antioxidant in cells because it lowers peroxide production by mitochondria.

Estradiol and other related oestrogenic compounds were postulated to be antioxidants because of the phenolic structure of the A ring. While this may be true in vitro, a simple consideration of the dose in which estradiol acts in vivo rules out the possibility that it may be an antioxidant based strictly on its phenolic chemical properties. For instance, a well known antioxidant such as vitamin E is usually given at doses of approximately 500 mg per day. Estradiol, however, when administered to a patient is given at a dose of 50 micrograms per day. Thus, if estradiol were to be a chemical antioxidant, its antioxidant properties should be 10,000 times more potent than those of vitamin E and this is certainly not the case. Consequently, we postulated that estrogens behave as antioxidants because of their hormonal properties, i.e. due to the binding to estrogen receptors and subsequent action mediated by cell signalling pathways [8]. We now have shown (Fig. 2) that when using highly purified mitochondria, i.e. virtually void of cytosolic components, estradiol lowers the production of oxidants in a saturable manner. This indicates that the hormone must act via a ligand receptor mediated

mechanism. We observe significant reduction in the rate of peroxide production (to approximately 30% of the controls) at concentrations of about 0.2 nM, which is the estimated physiological concentration of estradiol within cells [15]

Estrogen receptors in mitochondria

The presence of estrogen receptors alpha and beta in mitochondria has been recently identified. Pedram et al. [27] identified functional receptors in mitochondria of MCF7 cells. This is a line of cells derived from breast cancers. These authors determined the presence of estrogen receptors in these cancer cells after stimulation with ultraviolet radiation. In fact, estradiol proved protective against ultraviolet irradiation of cells and the authors claimed that this was due to a direct effect of estradiol on mitochondria. The possible protective effect of estradiol mediated by mitochondria was also suggested by Stirone et al. [28]. These authors proposed that nuclear respiratory factor 1 was significantly increased by long-term estrogen treatment in vivo.

However, in this paper, for the first time, we incubate highly purified mitochondria from normal rat tissues, and not from cancer-derived cells, and observe a clear protection against oxidative stress generated by these mitochondria.

Estradiol, mitochondria, and cytochrome c release

Mitochondria are involved in the onset of apoptosis via the so-called intracellular pathway. The first event in this pathway is the release of cytochrome c from the organelle. This has two consequences: the first is that there is more cytochrome c in the cytosol which ultimately leads to the activation of caspases. The second is that there is less cytochrome c in the mitochondrial membranes and therefore the mitochondrial respiratory chain is impaired. We have found that estradiol prevents the release of cytochrome c from mitochondria and also increases the efficiency of the respiratory chain thus increasing the mitochondrial membrane potential and decreasing peroxide

production (see Fig. 2). Thus, a molecular explanation for the prevention of the oxidant production by mitochondria is the prevention of the release of cytochrome c from the mitochondrial membrane.

Hormonal control of the mitochondrial transition pore

The direct inhibition of release of cytochrome c from mitochondria led us to think that it might control the mitochondrial transition pore, which is involved in the release of cytochrome c. In fact, estradiol is as potent as cyclosporine A to prevent the release of cytochrome c from mitochondria.

The effect of estradiol that we are reporting is not the first to be observed in which a lipophilic hormone prevents the opening of the mitochondrial transition pore. Melatonin also inhibits the mitochondria permeability transition pore and this has been proposed as a mechanism responsible for the antiapoptotic effect of the hormone [10].

Thus, we believe we can postulate that estradiol prevents oxidative stress caused by mitochondria not only via pathways involving the cytosolic signalling pathways, but also in a direct fashion. The mechanism that we propose is as follows: estradiol – binds to mitochondrial estrogen receptors – inhibits the opening of the mitochondrial transition pore – prevents the release of cytochrome c from mitochondria. As a result, cytochrome c is maintained in its “normal” location, i.e. within the internal mitochondrial membrane allowing a normal flow through the respiratory pathway and preventing the formation of oxidants by mitochondria.

Needless to say, this mechanism is compatible with the previous we postulated (involving membrane receptors, cytosolic signalling pathways, and nuclear activation of genes) [8]. Both mechanisms may be important to explain the antioxidant properties of estrogens and eventually to help us understand why in many species, estrogens protect cells and explain why females live longer than males.

Brain protection by estrogenic compounds

We report here that mitochondria from brain, particularly synaptic ones, produce many more peroxides than those from liver. In fact, this basic fact correlates well with *in vitro* cellular studies and from *in vivo* experiments. Evidence from the clinic is less clear.

Our report that estradiol protects against production of peroxides by mitochondria, is in keeping with previous studies from many labs including our own that mitochondria are protected from age-associated damage by estrogens (for a review, see [29]) The mechanisms by which estrogens protect against mitochondrial damage are numerous. Many reports out the role of calcium for mitochondrial damage associated with ageing and in fact estradiol seemed to protect against alteration in calcium homeostasis [30]. We report here that estradiol protects against release of cytochrome c by isolated mitochondria. Previously, we reported that estradiol or genistein prevented against cellular damage caused by Alzheimer's A β peptide by preventing activation of p38 [20]. A β peptide causes mitochondrial aggregation in neurons [26] and this has also been shown not only in isolated mitochondria but also within cells [20]. Nevertheless, the evidence that estradiol may prevent or delay the onset of Alzheimer's disease in the clinic is much more controversial. So far, we cannot put forward a clear recommendation as to whether estradiol can be used in the clinic in the prevention of Alzheimer's disease. However, apparently the only clinical study which is showing promising results is one in which administration of *Ginkgo biloba* (which indeed contains high amounts of estrogenic compounds) is administered to patients.

Along these lines we have found that amyloid beta peptide does not increase the rate of oxidant production in mitochondria from young females but it does indeed increase in

brain mitochondria from old ones. Thus, we came with the concept that mitochondria may be a source of signals for toxicity for survival in Alzheimer's disease [31].

Clearly, more studies are required to establish whether and under what conditions, replacement with estrogenic compounds may delay or alleviate the symptoms of Alzheimer's disease.

Genistein mimics the protective effects of estradiol on isolated mitochondria

A major problem facing research of the protective effects of estrogens on biological systems is that the practical applications of these studies are limited by the fact that estrogen replacement therapy presents serious problems in the clinical setting. To begin with, estradiol cannot be administered to males because of its feminising effects. Moreover, its administration to women also presents serious problems. It has been shown to increase the risk of breast cancer [32], and even to increase the risk of cardiovascular disease in the post-menopausal stage [33]. Phytoestrogens, on the contrary, present us with the possibility of treating both men and women. They do not show any significant feminising effects and so far, very limited, if any, effects on cardiovascular disease or on the promotion of cancer have been reported. Interestingly, genistein, which is the major phytoestrogen presence in soy, binds to estrogen receptors, preferentially to estrogen receptor beta. We have previously shown that genistein, after binding to estrogen receptors, activates a number of signalling pathways, particularly the MAPK pathway which leads to the phosphorylation and thus activation of NF κ B. NF κ B when entering the nucleus activates a transcription of a number of longevity-associated genes such as glutathione peroxidase or superoxide dismutase [19]. In this study, we tested whether genistein could mimic the favorable effect of estradiol in terms of their direct protection of mitochondrial integrity and lowering of mitochondrial peroxide production. And indeed this was the case. As shown in Figures 4 and 5,

genistein not only prevents mitochondrial peroxide production, but also the liberation of cytochrome c from the organelle. Therefore, phytoestrogens mimic the action of estrogens and since they do not have their feminising and tumour promotion properties, they constitute an excellent alternative to estradiol to promote protection of mitochondria in a direct fashion.

The possible clinical relevance of genistein and other phytoestrogens to activate longevity-related genes (as we showed in previous work, (see [19, 34]) and to directly protect mitochondria remains to be more closely studied.

ACKNOWLEDGMENTS

We thank Ms. Marilyn Noyes for her help in writing the manuscript.

The author's work was supported by grants BFU2007-65803/BFI and ISCIII2006-RED13-027 from the "Red Temática de investigación cooperativa en envejecimiento y fragilidad (RETICEF) to J.V., grant BFU2005/230 to F.V.P. and by grant GV/2007/263 to C.B.

REFERENCES

- [1] J. Miquel, A.C. Economos, J. Fleming and J.E.J. Johnson, Mitochondrial role in cell aging. *Exp. Gerontol.* 15 (1980) 579-591.
- [2] J. Sastre, F.V. Pallardo, R. Pla, A. Pellin, G. Juan, J.E. O'Connor, J.M. Estrela, J. Miquel and J. Vina, Aging of the liver: age-associated mitochondrial damage in intact hepatocytes *Hepatology* 24 (1996) 1199-205.
- [3] T.M. Hagen, D.L. Yowe, J.C. Bartholomew, C.M. Wehr, K.L. Do, J.Y. Park and B.N. Ames, Mitochondrial decay in hepatocytes from old rats: membrane potential

declines, heterogeneity and oxidants increase Proc Natl Acad Sci U S A 94 (1997) 3064-9.

[4] G.C. Kujoth, A. Hiona, T.D. Pugh, S. Someya, K. Panzer, S.E. Wohlgemuth, T. Hofer, A.Y. Seo, R. Sullivan, W.A. Jobling, J.D. Morrow, H. Van Remmen, J.M. Sedivy, T. Yamasoba, M. Tanokura, R. Weindruch, C. Leeuwenburgh and T.A. Prolla, Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging Science 309 (2005) 481-4.

[5] C. Borras, J. Sastre, D. Garcia-Sala, A. Lloret, F.V. Pallardo and J. Vina, Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males Free Radic Biol Med 34 (2003) 546-52.

[6] J. Sastre, C. Borras, D. Garcia-Sala, A. Lloret, F.V. Pallardo and J. Vina, Mitochondrial damage in aging and apoptosis Ann N Y Acad Sci 959 (2002) 448-51.

[7] J. Vina, J. Sastre, F.V. Pallardo, J. Gambini and C. Borras, Role of mitochondrial oxidative stress to explain the different longevity between genders: protective effect of estrogens Free Radic Res 40 (2006) 1359-65.

[8] C. Borras, J. Gambini, M.C. Gomez-Cabrera, J. Sastre, F.V. Pallardo, G.E. Mann and J. Vina, 17beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade Aging Cell 4 (2005) 113-8.

[9] J. Vina, C. Borras, M.C. Gomez-Cabrera and W.C. Orr, Part of the series: from dietary antioxidants to regulators in cellular signalling and gene expression. Role of reactive oxygen species and (phyto)estrogens in the modulation of adaptive response to stress Free Radic Res 40 (2006) 111-9.

- [10] S.A. Andrabi, I. Sayeed, D. Siemen, G. Wolf and T.F. Horn, Direct inhibition of the mitochondrial permeability transition pore: a possible mechanism responsible for anti-apoptotic effects of melatonin *Faseb J* 18 (2004) 869-71.
- [11] J.Q. Chen, M. Delannoy, C. Cooke and J.D. Yager, Mitochondrial localization of ERalpha and ERbeta in human MCF7 cells *Am J Physiol Endocrinol Metab* 286 (2004) E1011-22.
- [12] D. Rickwood, M.T. Wilson and V. Darley-Usmar. in (Darley-Usmar, V., Rickwood, D. and Wilson, M.T., eds.) *Mitochondria: a practical approach.*, IRL Press Ltd., Oxford 1987, pp. 1-16.
- [13] J.C. Lai, J.M. Walsh, S.C. Dennis and J.B. Clark, Synaptic and non-synaptic mitochondria from rat brain: isolation and characterization *J Neurochem* 28 (1977) 625-31.
- [14] G.J. Borrás C, Gómez-Cabrera MC, Sastre J, Pallardó FV., . Mann G E, Viña J, Genistein, a soy isoflavone, upregulates expression of antioxidant genes: involvement of estrogen receptors, ERK1/2 and NFkappaB *Faseb J In Press* (2006).
- [15] S.M. Rink, K.J. Yarema, M.S. Solomon, L.A. Paige, B.M. Tadayoni-Rebek, J.M. Essigmann and R.G. Croy, Synthesis and biological activity of DNA damaging agents that form decoy binding sites for the estrogen receptor *Proc Natl Acad Sci U S A* 93 (1996) 15063-8.
- [16] C. Barja de Quiroga, Mitochondrial oxygen radical generation and leak: Sites of production in states 4 and 3, organ specificity and relation to aging and longevity. *J. Bioenergetics Biomembranes* 31 (1999) 347-366.
- [17] L.B. Chen, Mitochondrial membrane potential in living cells *Annu Rev Cell Biol* 4 (1988) 155-81.

- [18] J.M. Garcia-Verdugo, F. Doetsch, H. Wichterle, D.A. Lim and A. Alvarez-Buylla, Architecture and cell types of the adult subventricular zone: in search of the stem cells *J Neurobiol* 36 (1998) 234-48.
- [19] C. Borrás, J. Gambini, M.C. Gomez-Cabrera, J. Sastre, F.V. Pallardo, G.E. Mann and J. Vina, Genistein, a soy isoflavone, up-regulates expression of antioxidant genes: involvement of estrogen receptors, ERK1/2, and NFkappaB *Faseb J* 20 (2006) 2136-8.
- [20] S.L. Valles, C. Borrás, J. Gambini, J. Furriol, A. Ortega, J. Sastre, F.V. Pallardo and J. Vina, Oestradiol or genistein rescues neurons from amyloid beta-induced cell death by inhibiting activation of p38 *Aging Cell* 7 (2008) 112-8.
- [21] J. Vina, C. Borrás, J. Gambini, J. Sastre and F.V. Pallardo, Why females live longer than males? Importance of the upregulation of longevity-associated genes by oestrogenic compounds *FEBS Lett* 579 (2005) 2541-5.
- [22] O. Wirths, G. Multhaup and T.A. Bayer, A modified beta-amyloid hypothesis: intraneuronal accumulation of the beta-amyloid peptide--the first step of a fatal cascade *J Neurochem* 91 (2004) 513-20.
- [23] R. Castellani, K. Hirai, G. Aliev, K.L. Drew, A. Nunomura, A. Takeda, A.D. Cash, M.E. Obrenovich, G. Perry and M.A. Smith, Role of mitochondrial dysfunction in Alzheimer's disease *J Neurosci Res* 70 (2002) 357-60.
- [24] X. Zhu, G. Perry, P.I. Moreira, G. Aliev, A.D. Cash, K. Hirai and M.A. Smith, Mitochondrial abnormalities and oxidative imbalance in Alzheimer disease *J Alzheimers Dis* 9 (2006) 147-53.
- [25] C. Pereira, M.S. Santos and C. Oliveira, Mitochondrial function impairment induced by amyloid beta-peptide on PC12 cells *Neuroreport* 9 (1998) 1749-55.

- [26] A. Lloret, M.C. Badia, N.J. Mora, A. Ortega, F.V. Pallardo, M.D. Alonso, H. Atamna and J. Vina, Gender and age-dependent differences in the mitochondrial apoptogenic pathway in Alzheimer's disease *Free Radic Biol Med* 44 (2008) 2019-25.
- [27] A. Pedram, M. Razandi, D.C. Wallace and E.R. Levin, Functional estrogen receptors in the mitochondria of breast cancer cells *Mol Biol Cell* 17 (2006) 2125-37.
- [28] C. Stirone, S.P. Duckles, D.N. Krause and V. Procaccio, Estrogen increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels *Mol Pharmacol* 68 (2005) 959-65.
- [29] J. Nilsen, S. Chen, R.W. Irwin, S. Iwamoto and R.D. Brinton, Estrogen protects neuronal cells from amyloid beta-induced apoptosis via regulation of mitochondrial proteins and function *BMC Neurosci* 7 (2006) 74.
- [30] J. Qiu, M.A. Bosch, K. Jamali, C. Xue, M.J. Kelly and O.K. Ronnekleiv, Estrogen upregulates T-type calcium channels in the hypothalamus and pituitary *J Neurosci* 26 (2006) 11072-82.
- [31] J. Vina, A. Lloret, S.L. Valles, C. Borrás, M.C. Badia, F.V. Pallardo, J. Sastre and M.D. Alonso, Effect of gender on mitochondrial toxicity of Alzheimer's A β peptide *Antioxid Redox Signal* 9 (2007) 1677-90.
- [32] A. Cassidy, Potential risks and benefits of phytoestrogen-rich diets *Int J Vitam Nutr Res* 73 (2003) 120-6.
- [33] J.E. Rossouw, G.L. Anderson, R.L. Prentice, A.Z. LaCroix, C. Kooperberg, M.L. Stefanick, R.D. Jackson, S.A. Beresford, B.V. Howard, K.C. Johnson, J.M. Kotchen and J. Ockene, Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial *Jama* 288 (2002) 321-33.

[34] K. Mahn, C. Borrás, G.A. Knock, P. Taylor, I.Y. Khan, D. Sugden, L. Poston, J.P. Ward, R.M. Sharpe, J. Vina, P.I. Aaronson and G.E. Mann, Dietary soy isoflavone induced increases in antioxidant and eNOS gene expression lead to improved endothelial function and reduced blood pressure in vivo *Faseb J* 19 (2005) 1755-7.

ACCEPTED MANUSCRIPT

FIGURE LEGENDS**Figure 1.- Effects of ovariectomy and 17 β -estradiol on peroxide production by synaptic and non-synaptic brain mitochondria.**

Mitochondria were incubated with 10 mM succinate or 5 mM pyruvate plus 2.5 mM malate. Values denote the mean \pm S.D. of measurements from n different animals: synaptic mitochondria (n = 3-5) and non-synaptic mitochondria (n = 3-5). The statistical difference is indicated as follows: * P < 0.05 vs control female rats; # P < 0.05 vs ovariectomy.

Figure 2.- Estradiol decreases hydrogen peroxide production by isolated hepatic mitochondria.

The rate of peroxide production was measured in isolated hepatic mitochondria from male Wistar rats (3-5 months-old). Values denote the mean \pm S.D. of measurements from 6-10 different animals. The statistical difference is indicated as follows: * P < 0.05; ** P < 0.01 vs control. E₂ means incubation with 17 β -estradiol at corresponding concentration.

Figure 3.- Estradiol inhibits cytochrome c release from mitochondria

a) The level of cytochrome c was measured in isolated hepatic mitochondria from male Wistar rats (3-5 months-old). Values denote the mean \pm S.D. of measurements from 4 different animals. The statistical difference is indicated as follows: * P < 0.05 vs control. E₂ means incubation with 0.2 nM 17 β -estradiol; E₂-BSA means incubation with 0.2 nM 17 β -estradiol bound to bovine serum albumine; CsA means 1 μ M cyclosporine

A

b) The level of cytochrome c was measured in supernatant of isolated hepatic mitochondria from male Wistar rats (3-5 months-old). Values denote the mean \pm S.D. of measurements from 4 different animals. The statistical difference is indicated as

follows: * $P < 0.05$ vs control. E₂ means incubation with 0.2 nM 17 β -estradiol; E₂-BSA means incubation with 0.2 nM 17 β -estradiol bound to bovine serum albumine; CsA means 1 μ M cyclosporine A

Figure 4.- Genistein is as effective as estradiol in decreasing hydrogen peroxide production by isolated hepatic mitochondria.

The rate of peroxide production was measured in isolated hepatic mitochondria from male Wistar rats (3-5 months-old). Values denote the mean \pm S.D. of measurements from 6-10 different animals. The statistical difference is indicated as follows: * $P < 0.05$; ** $P < 0.01$ vs control. Estradiol means 0.2nM 17 β -estradiol and genistein concentration is 0.5 μ M.

Figure 5.- Genistein is as effective as estradiol in inhibing cytochrome c release from mitochondria.

a) The level of cytochrome c was measured in isolated hepatic mitochondria from male Wistar rats (3-5 months-old). Values denote the mean \pm S.D. of measurements from 4 different animals. The statistical difference is indicated as follows: * $P < 0.05$ or ** $P < 0.01$ vs control. Estradiol means 0.2nM 17 β -estradiol and genistein concentration is 0.5 μ M; 1 μ M CsA means cyclosporine A

b) The level of cytochrome c was measured in supernatant of isolated hepatic mitochondria from male Wistar rats (3-5 months-old). Values denote the mean \pm S.D. of measurements from 4 different animals. The statistical difference is indicated as follows: * $P < 0.05$ vs control. Estradiol means 0.2nM 17 β -estradiol and genistein concentration is 0.5 μ M; 1 μ M CsA means cyclosporine A

TABLES

Table 1.- Prevention of age-associated mitochondrial impairment by treatment with a *Ginkgo biloba* extract.

| | YOUNG | OLD | OLD-TREATED |
|--|-------------|--------------|--------------------------|
| PEROXIDE PRODUCTION (% vs young) | 100 | 116 ± 8* | 105 ± 6 [#] |
| OXO-8-DEOXYGUANOSINE (pmol/μg mtDNA) | 0.58 ± 0.21 | 1.29 ± 0.20* | 0.49 ± 0.13 [#] |

Figure 1

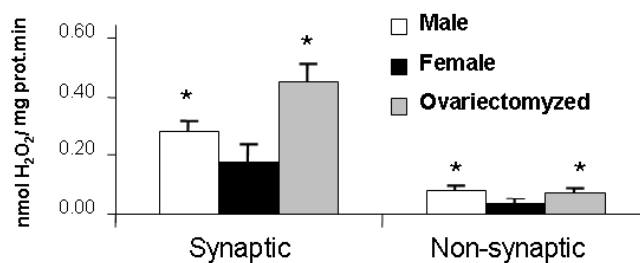


Figure 2

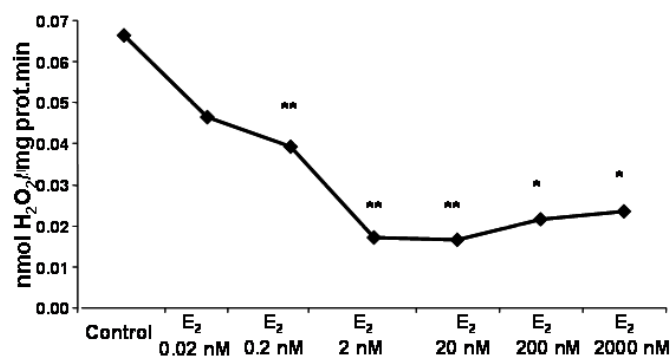


Figure 3

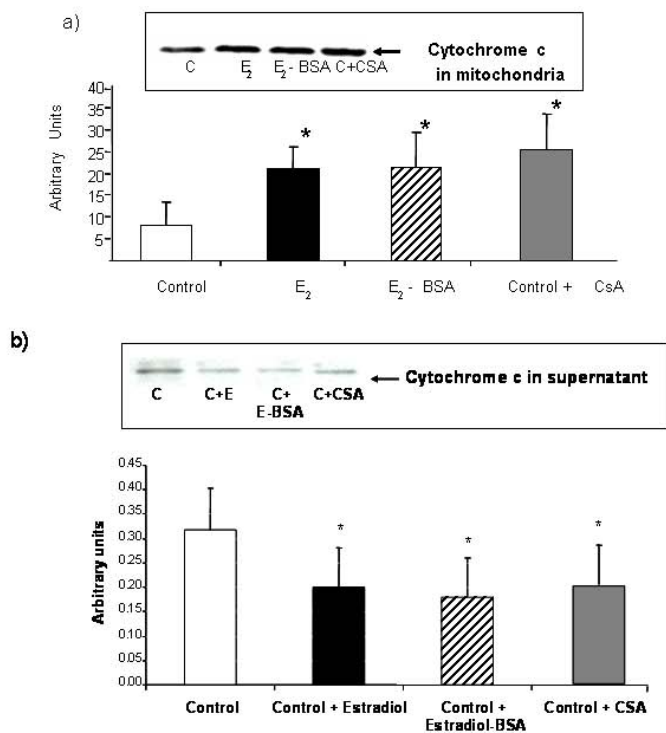
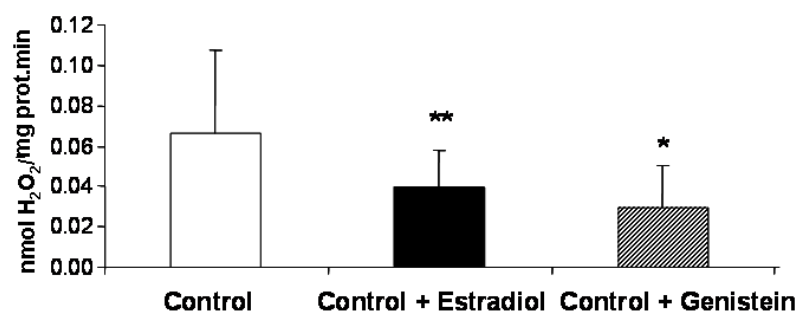
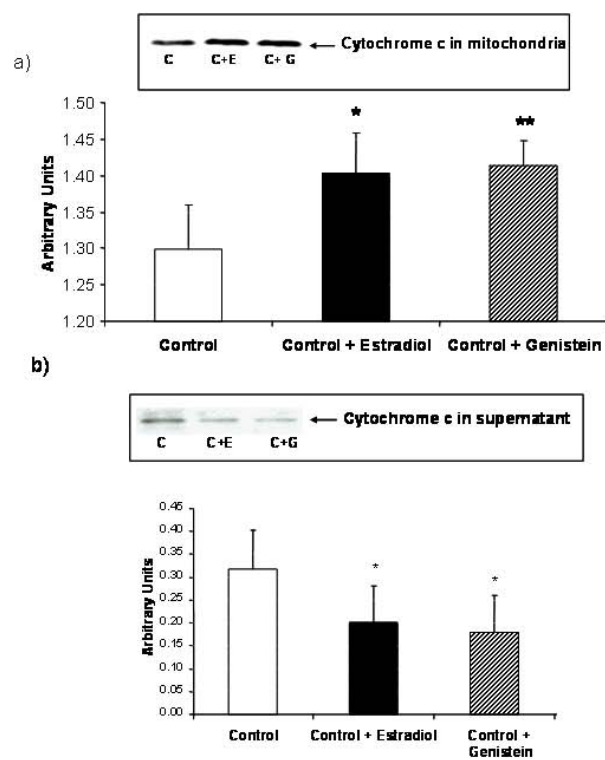


Figure 4



ACCEPT

Figure 5



ACCEPTED