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Estrogen Receptor Alpha as a Key Target of Red Wine Polyphenols Action on the Endothelium

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Abstract

Background: A greater reduction in cardiovascular risk and vascular protection associated with diet rich in polyphenols are generally accepted; however, the molecular targets for polyphenols effects remain unknown. Meanwhile evidences in the literature have enlightened, not only structural similarities between estrogens and polyphenols known as phytoestrogens, but also in their vascular effects. We hypothesized that alpha isoform of estrogen receptor (ER α) could be involved in the transduction of the vascular benefits of polyphenols.

Methodology/Principal Findings: Here, we used ER α deficient mice to show that endothelium-dependent vasorelaxation induced either by red wine polyphenol extract, ProvinolsTM, or delphinidin, an anthocyanin that possesses similar pharmacological profile, is mediated by ER α . Indeed, ProvinolsTM, delphinidin and ER α agonists, 17-beta-estradiol and PPT, are able to induce endothelial vasodilatation in aorta from ER α Wild-Type but not from Knock-Out mice, by activation of nitric oxide (NO) pathway in endothelial cells. Besides, silencing the effects of ER α completely prevented the effects of ProvinolsTM and delphinidin to activate NO pathway (Src, ERK 1/2, eNOS, caveolin-1) leading to NO production. Furthermore, direct interaction between delphinidin and ER α activator site is demonstrated using both binding assay and docking. Most interestingly, the ability of short term oral administration of ProvinolsTM to decrease response to serotonin and to enhance sensitivity of the endothelium-dependent relaxation to acetylcholine, associated with concomitant increased NO production and decreased superoxide anions, was completely blunted in ER α deficient mice.

Conclusions/Significance: This study provides evidence that red wine polyphenols, especially delphinidin, exert their endothelial benefits *via* ER α activation. It is a major breakthrough bringing new insights of the potential therapeutic of polyphenols against cardiovascular pathologies.

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Introduction

Epidemiological studies have enlightened that women have lower cardiovascular risk than men, and this protection progressively disappears after menopause. These studies (protection in premenopausal women) suggest and experimental studies (prevention of atheroma development in animals) demonstrate a major atheroprotective action of 17- β -estradiol (E₂) [1,2]. E₂ actions are essentially mediated by two molecular targets: estrogen receptor alpha (ER α) and beta (ER β), but the former appears to mediate most of the actions of E₂ on the cardiovascular system [1,2]. Endothelium represents a well recognized target of E₂, which elicits several beneficial actions as increased NO production [1–3] subsequent to activation of endothelial NO synthase (eNOS) *via* a G-protein [4], and ERK and phosphatidylinositol-3-kinase pathways.

Epidemiological studies reported a greater reduction in cardiovascular risk and greater vascular protection associated with

diet rich in polyphenols, including those from red wine [5]. We have previously shown that ProvinolsTM, a polyphenolic extract from red wine, and delphinidin, an anthocyanin pharmacologically active and found in the total extract, induce an increase of endothelial NO production leading to endothelium-dependent relaxation [6,7], even in pathophysiological contexts as hypertension, metabolic syndrome or stroke [8–10], and restore endothelial function [11]. Although intracellular pathways involved in the endothelial effects of polyphenols are partially described (increase of intracellular calcium, activation of tyrosine kinases, for instance) [7], the molecular targets of these polyphenols remain unknown. It has to keep in mind that numerous molecules contained in red wine polyphenols including resveratrol might act on synergistic ways, in addition to their antioxidant properties, by acting as agonists of sirtuin in order to increase life span and to silence metabolic and physiological disturbances often associated with endothelial NO dysfunction [12,13].

Evidences in the literature have enlightened, not only structural similarities between estrogens and polyphenols known as phytoestrogens, but also in their vascular effects with regard to endothelial NO production. Indeed, it has been reported that the phytoestrogen genistein produces acute NO-dependent dilation of human forearm vasculature with similar potency to E₂ [14]. Also, genistein induces a late but sustained activation of the eNOS system *in vitro* [15]. Moreover, chronic administration of genistein improves endothelial dysfunction in spontaneously hypertensive rats that involves eNOS, caveolin and calmodulin expression and NADPH oxidase activity [16].

Red wine polyphenols do not contain genistein and no direct evidence for the nature of the receptor triggering the effect of red wine polyphenols receptors in endothelial cells has been demonstrated. Nevertheless, the aim of this work was to investigate the hypothesis that ER α is one of the targets involved in the vasculoprotective effects of ProvinolsTM and delphinidin. For this, we first studied the endothelium-dependent relaxation to polyphenols in aortas from both ER α Knock-Out (KO) and Wild Type (WT) mice, and then we analyzed molecular pathways associated with NO production in endothelial cells stimulated by ProvinolsTM and delphinidin by silencing ER α activity or expression either with pharmacological inhibitor or siRNA, respectively. We also studied binding assay and molecular modelling of interaction between delphinidin and ER α . Finally, we tested the physiological relevance of our findings *in vivo* by testing the effect of short term oral treatment of ER α KO and WT mice with ProvinolsTM with respect to endothelial NO response.

Results

The role of ER α in the endothelium-dependent relaxation to ProvinolsTM and to delphinidin was evaluated by using vessels

taken from ER α WT and KO mice. First, we tested the ability of ER α agonists such as E₂, which acts on both ER α and ER β isoforms, and 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), which is specific for ER α , to activate the endothelium. This was demonstrated by the capacity of the two ER α agonists to induce relaxation in aortas from ER α WT but not from KO mice in the presence of functional endothelium only (Figure 1A and 1B). The concentration of E₂ to elicit maximal relaxation was in accordance with that reported by Li *et al* [17] in the same vessels. As previously described by our group, red wine polyphenols and delphinidin are able to induce endothelium-dependent relaxation in mice aortas. Interestingly, the vasorelaxant effect of these two polyphenols was found in aortas from ER α WT mice (Figure 1C and 1D), but was completely abolished when ER α is deleted. In ER α deficient mice, a slight contraction to ProvinolsTM and delphinidin were even detected (Figure 1C and 1D).

These data suggest the involvement of ER α in the endothelium-dependent relaxation in response to the two polyphenols used. Then, we assessed if the endothelium-dependent relaxation evoked by ER α stimulation is due to an increase in NO production. For this we stimulated the human endothelial cell line, EaHy 926, either with ProvinolsTM or delphinidin for 10 minutes in presence or in absence of Fulvestrant, an ER α pharmacological antagonist, or with a siRNA directed against ER α . ProvinolsTM and delphinidin were used at maximally active concentration to induce relaxation in the rat aortic rings and to increase cytosolic calcium in endothelial cells, as previously described [7,18]. Both ProvinolsTM and delphinidin were able to induce an increase in NO production. However, when ER α was silenced either by Fulvestrant or siRNA, the increase of NO production induced by ProvinolsTM and delphinidin was completely prevented (Figure 2A). As a positive control for ER α activation-induced

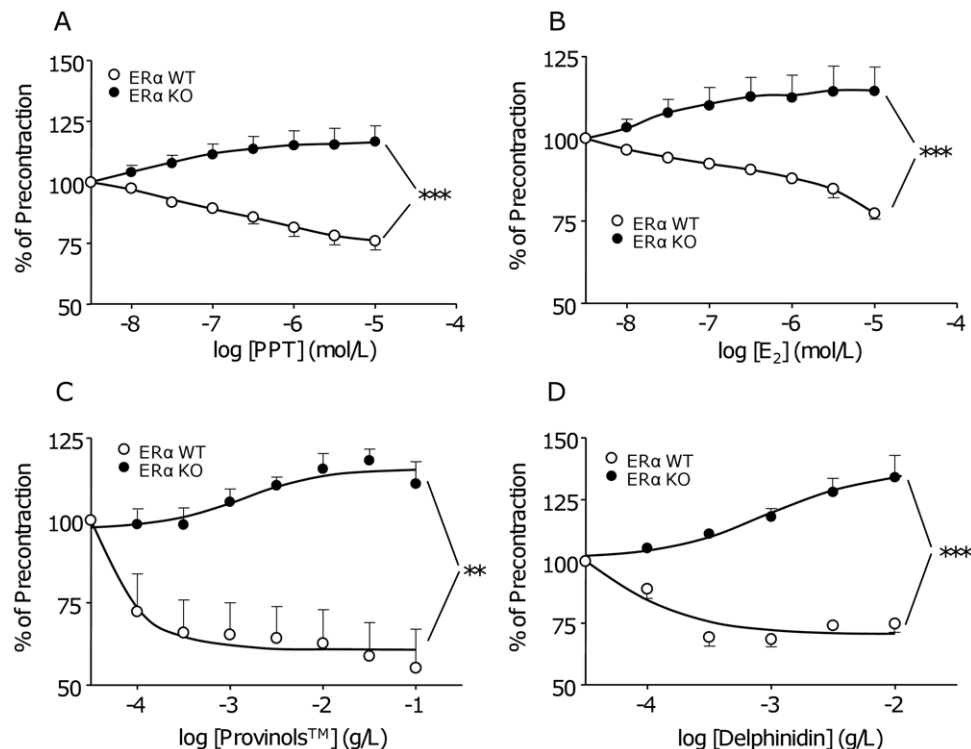


Figure 1. ER α mediates endothelium-dependent relaxation induced by agonists, ProvinolsTM and delphinidin. Concentration-effect curves to increasing concentrations of PPT (A), 17- β estradiol (E₂) (B), ProvinolsTM (C) or delphinidin (D) in aortic rings, with functional endothelium precontracted with U46619, taken from ER α Wild Type (open circles) and Knock-Out (filled circles) mice (n = 5–6). **P < 0.01, ***P < 0.001. doi:10.1371/journal.pone.0008554.g001

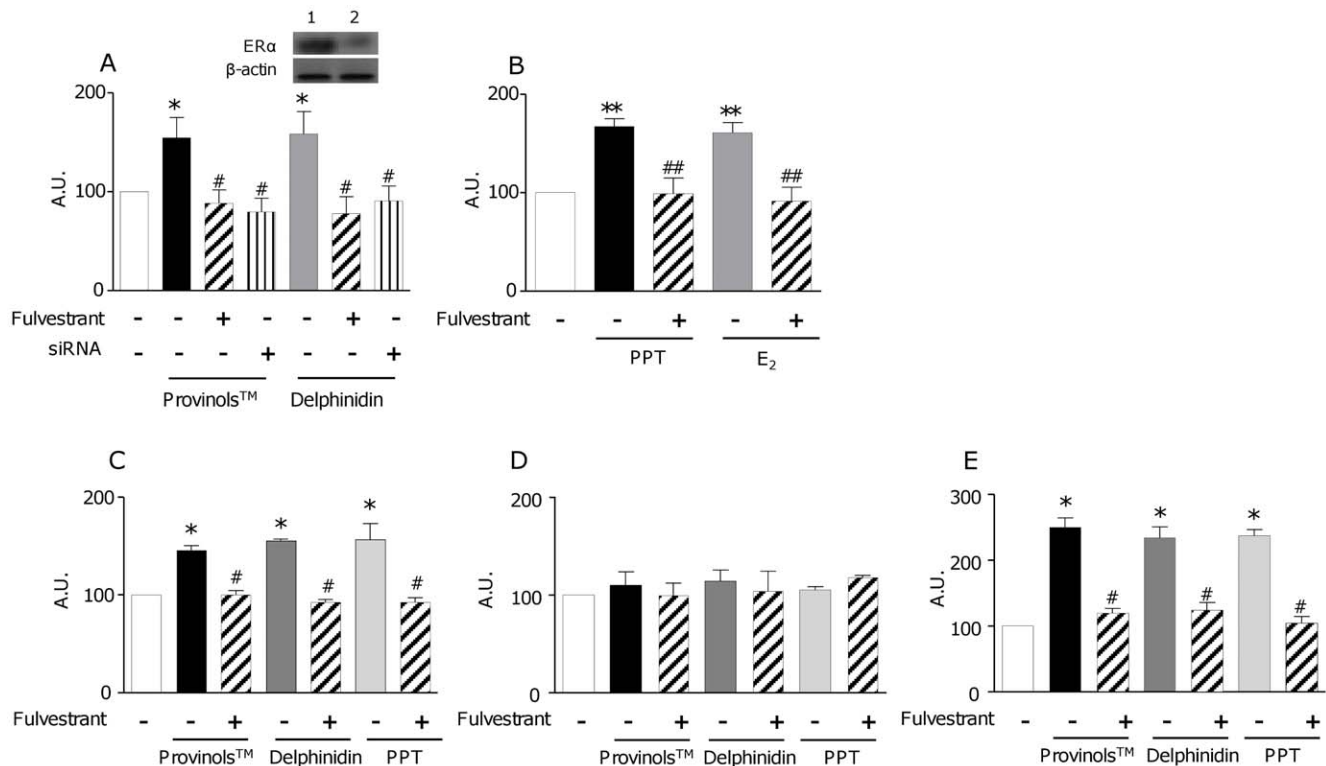


Figure 2. ER α activation induces endothelial NO release. NO production induced by ProvinolsTM, delphinidin, PPT and 17- β estradiol (E₂) in endothelial cells and consequences of ER α pathway inhibition with either Fulvestrant or siRNA directed against ER α (siRNA). EaHy 926 cells (A and B) or aortic endothelial cells from either ER α Wild-Type (WT) mice (C), ER α Knock-Out (KO) mice (D) or from Swiss mice (E) were treated for 10 minutes with ProvinolsTM (10⁻² g/L), delphinidin (10⁻² g/L), PPT (10⁻⁵ M) or E₂ (10⁻⁵ M) and this in the presence or in the absence of either Fulvestrant (30 nM) (hatched bars) or after knocking down the receptor with siRNA (siRNA) (striped bars). Then NO production was assessed by electronic paramagnetic resonance. In panel A, insert shows Western blot for ER α in the control (lane 1) or ER α siRNA (lane 2)-treated EaHy endothelial cells. * P <0.05, ** P <0.01, *** P <0.001 vs non treated; # P <0.05, ## P <0.01 vs ProvinolsTM, delphinidin (A, C and D), PPT or E₂ alone (B and D), (n=4–6). doi:10.1371/journal.pone.0008554.g002

NO production, we used PPT and E₂. Both agonists were able to enhance NO production, and this effect was abolished when ER α was antagonized by Fulvestrant (Figure 2B). As EaHy 926 are derived from human umbilical vein endothelial cells, we extracted and cultured endothelial cells from aortas taken either from ovariectomized ER α WT or KO mice, or from ovariectomized Swiss mice, using the method described by Kobayashi *et al.* [19]. Cells were then stimulated with ProvinolsTM, delphinidin and PPT, in absence or in presence of Fulvestrant, using the same protocol as for EaHy 926. Interestingly, in aortic endothelial cells taken either from ER α WT mice or Swiss mice, ProvinolsTM, delphinidin and PPT were all able to induce NO production (Figure 2C and 2E). The ability of the three compounds to stimulate NO release was completely blunted in the aortic endothelial cells extracted from ER α KO mice (Figure 2D) or in aortic endothelial cells taken from ER α WT mice and Swiss mice in the presence of Fulvestrant (Figure 2C and 2E).

Recently, an increase of NO production *via* the interaction of a molecular pathway involving the phosphorylation of Src, ERK1/2 and eNOS on the Ser1177 has been reported with regard to resveratrol [20]. NO pathway was then investigated in order to decipher the molecular mechanisms underlying ER α -associated NO increase and the subsequent vasodilatation induced either by ProvinolsTM, delphinidin or by PPT and E₂. We also analysed caveolin-1 expression, a protein that segregates the inactive form of eNOS on the cellular membrane and modulates eNOS activity. We demonstrated that both ProvinolsTM and delphinidin

increased the phosphorylation of Src, ERK1/2 and eNOS Ser 1177, as well as of caveolin-1, in human endothelial cells. Moreover, when ER α was blocked or silenced, the activation of this pathway was completely blunted (Figure 3A to 3D). In addition, the same effects were observed after PPT or E₂ treatment in the sense that the two ER agonists increased phosphorylation of Src, ERK1/2, eNOS and caveolin-1. Furthermore, when ER α was antagonized with Fulvestrant, neither PPT nor E₂ induced phosphorylation of these enzymes (Figure 3E to 3H), suggesting the involvement of an ER α -dependent mechanism. Even if it has already been shown that both ProvinolsTM and delphinidin are able to induce NO production in endothelial cells [18], and that estrogens are also able to increase NO production *via* ER α [21], here we demonstrate for the first time the direct link between the ability of ProvinolsTM and delphinidin to stimulate NO pathways leading to endothelial NO production through ER α activation.

To verify the direct interaction of red wine polyphenols with ER α and to exclude the implication of other factors, binding assay between delphinidin and ER α was performed. Binding assay showed that delphinidin exerts 73% of specific inhibition against E₂ on ER α (Figure 4D). Furthermore, we performed a docking study of delphinidin on ER α . The predicted binding mode of the ligand-binding domain on ER α is relatively similar to that observed in the X-ray structure of the ER α with E₂ (Figure 4A and 4B). The three aromatic rings undergo significant apolar contacts with hydrophobic residues located at the centre of the binding site (Leu349, Ala350, Leu384, Leu387, Met388, Leu391,

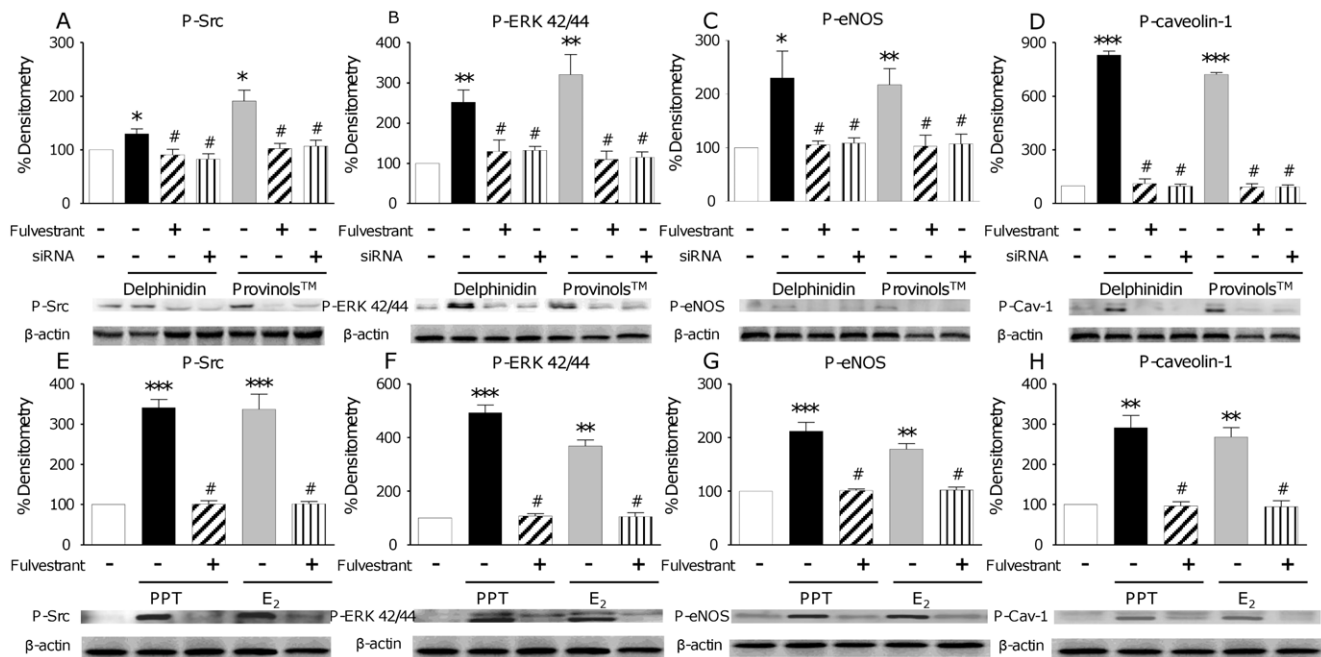


Figure 3. ER α activation induces phosphorylation of NO signaling proteins. Activation of NO pathway induced by ProvinolsTM, delphinidin, PPT and 17- β estradiol (E₂) in endothelial cells and consequences of ER α pathway inhibition with either Fulvestrant or siRNA directed against ER α (siRNA). EaHy 926 cells were treated for 10 minutes with ProvinolsTM (10^{-2} g/L), delphinidin (10^{-2} g/L), PPT (10^{-5} M) or E₂ (10^{-5} M) and this in the presence or in the absence of either Fulvestrant (30 nM) (hatched bars) or after knocking down the receptor with siRNA (siRNA) (striped bars). Then cells were lysed to perform Western Blot analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs non treated; # $P < 0.05$, ## $P < 0.01$ vs ProvinolsTM, delphinidin (A, B, C and D), PPT or E₂ alone (E, F, G and H), (n = 4–6). doi:10.1371/journal.pone.0008554.g003

Phe404, Met421, Ile424, Leu428, Leu525) (Figure 4C). An aromatic edge-to-face interaction is also engaged with the phenyl ring of Phe404, as for E₂. Last, a strong H-bond anchors delphinidin and E₂ to a polar residue (Glu353) at one end of the binding site. A significant difference with the E₂ binding mode is the loss of two H-bonds to Arg394 and His524, which are partly compensated by novel interactions to Leu346 (H-bond to the backbone oxygen atom) and His524 (aromatic interaction). These results indicate that delphinidin, like E₂, interact directly with ER α .

Finally, we show that short term oral administration of ProvinolsTM decreased contraction to serotonin (5-HT) in the presence of functional endothelium (Figure 5A) and enhanced the sensitivity to acetylcholine endothelium-dependent relaxation in aorta from ER α WT mice (Figure 5C) in association with increased NO production (Figure 5E) and reduced superoxide anions in mesenteric arteries (Figure 5F). All of these effects of oral administration of ProvinolsTM were completely blunted in ER α deficient mice (Figure 5B, 5D–5F). These results suggest that ER α triggers the *in vivo* effects of ProvinolsTM and demonstrate for the first time the physiological relevance of this receptor.

Discussion

The present study identifies ER α as the, or at least one of, key receptor transducing vascular effects exerted by red wine polyphenols, particularly delphinidin with respect to NO production. Indeed, E₂ and PPT, as well as ProvinolsTM and delphinidin, are able to activate molecular pathways, involving Src, ERK1/2, eNOS and caveolin-1 phosphorylations, by a mechanism that required ER α activation, with subsequent increase of endothelial NO production and endothelium-dependent vascular relaxation.

Moreover, using a binding assay and a docking, we showed that delphinidin fits on ER α 's activation site. Most importantly, evidence is provided that ER α triggers the *in vivo* effects of ProvinolsTM with respect to improvement in endothelial function given by the concomitant increase in NO and decrease in O₂⁻ superoxide anions releases in vessels. The later demonstrate for the first time the physiological relevance of this receptor in triggering the vascular protection induced by red wine polyphenols.

Red wine contains a wide variety of polyphenols, which derive mainly from grape solids (skin and seeds) and can be divided in two classes, flavonoids and non-flavonoids. Although, the non-flavonoid, resveratrol has been reported to trigger some of the beneficial effects of red wine polyphenols including the activation of sirtuin and NO pathway, we have focused our attention on the flavonoid components of the red wine polyphenols such as delphinidin. Indeed, our previous study looking at the possible active principles that support the endothelial NO-dependent relaxation produced by red wine polyphenols, including ProvinolsTM, demonstrate that anthocyanins and oligomeric-condensed tannins exhibit a pharmacological profile comparable to the original extract, the most potent being delphinidin [6]. Beside, delphinidin has been reported to induce endothelial NO release *via* an increase of cytosolic Ca²⁺ in endothelial cells [7] and protects against endothelial cell apoptosis acting through NO pathway [22].

The major aim of this work was to investigate the hypothesis that ER α is one of the targets involved in the vasculoprotective effects of ProvinolsTM and delphinidin. Firstly, we report the existence of an endothelium-dependent relaxation associated with ER α -stimulation, c-Src/ERK1/2-mediated activation of eNOS, with consequent endothelial NO release *via* a non-genomic mechanism at the same range of concentration than that reported

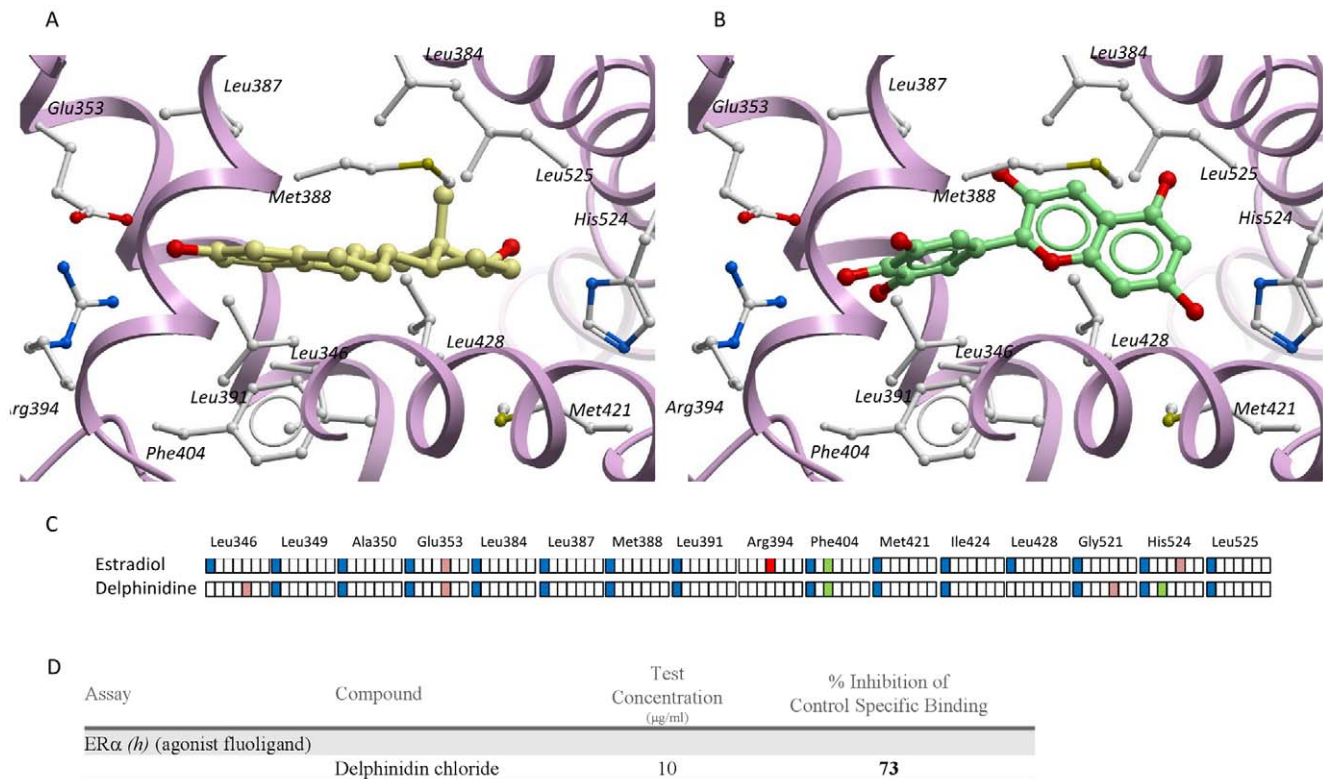


Figure 4. Binding assay and docking show direct interaction between delphinidin and ER α activator site. Binding mode of 17- β estradiol (E_2) (panel A, X-ray structure) and delphinidin (panel B, docking model) to the ligand-binding domain of the ER α (1a52 PDB entry). The receptor backbone is displayed by solid ribbons. Ligand-contacting side chains are displayed by white ball and sticks. Carbon atoms of receptor-bound E_2 and delphinidin are in yellow and green, respectively. In panel C, the ligand-receptor interactions for both compounds are encoded by an interaction fingerprint converting into a 7 bit string the interaction of the ligand with each residue of the binding site using the following color-coding: blue, apolar contact; green: aromatic interaction; red: hydrogen-bond. Eventually, D represents results of a binding assay of delphinidin on ER α . E_2 was used as control agonist for ER α and delphinidin was used at concentration exerting endothelial effects shown before (10^{-2} g/L). doi:10.1371/journal.pone.0008554.g004

by Li *et al.* [17]. Secondly, the most important novel observation is that the endothelium-dependent relaxation to ProvinolsTM and delphinidin found in aortas from ER α WT is completely abolished when ER α is deleted. Interestingly, both compounds elicit contraction in endothelium-denuded arteries taken from ER α deficient mice suggesting that the molecular targets of the two compounds on the smooth muscle are different to ER α (data not shown). Moreover, the ability of the two compounds to enhance the rapid release of NO (10 min) from cultured endothelial cells associated with phosphorylation of Src, ERK1/2, eNOS and caveolin-1 is blunted after silencing ER α either by Fulvestrant or siRNA. Finally, the capacity of ProvinolsTM and delphinidin to increase NO in mouse aortic endothelial cells was not only abolished in the presence of Fulvestrant and most likely when these cells were taken from ER α deficient mice. Altogether, these data demonstrate the direct link between the ability of ProvinolsTM and delphinidin to stimulate NO pathways leading to endothelial NO production through ER α activation. Very recently, it has been reported that the stilbene, resveratrol, rapidly activates MAPK signalling through ER localized in a “signalosome complex” at the plasma membrane and that may couple to G proteins, activate MEK1 and cause the release of Ca^{2+} accounting for NO release in endothelial cells [23]. However, the exact nature of the receptor isoform involved, ER α or ER β , has not been directly assessed in their study although it is now well accepted that ER α is necessary in the response of E_2 on endothelial NO production [2].

In the present study, direct interaction of delphinidin with ER α excluding the implication of other factors is demonstrated by the capacity of this compound to exert 73% of specific inhibition against E_2 on ER α . Furthermore, we perform a docking study of delphinidin on ER α . The predicted binding mode of the ligand-binding domain on ER α is relatively similar to that observed in the X-ray structure of the ER α with E_2 .

Altogether, these data provide evidence that red wine polyphenols and delphinidin in particular, through direct interaction with ER α , activate molecular pathways including Src, ERK1/2, eNOS, leading to endothelial NO production, accounting for vasorelaxation.

Finally, we demonstrate that the ability of oral administration of ProvinolsTM to decrease contraction to serotonin in the presence of functional endothelium and to improve endothelium-dependent relaxation in aorta from ER α WT mice in association with increased NO production and reduced superoxide anions in mesenteric arteries are completely blunted in ER α deficient mice. These data strongly suggest that ER α triggers the *in vivo* effects of ProvinolsTM and demonstrate for the first time the physiological relevance of this receptor. One can advance the hypothesis that ER α might be the or one of the molecular target(s) triggering the beneficial effects of dietary supplementation of ProvinolsTM on obesity-associated alterations with respect to metabolic disturbances and cardiovascular functions recently reported in Zucker fatty (ZF) rats [24]. Further studies should be conducted in order

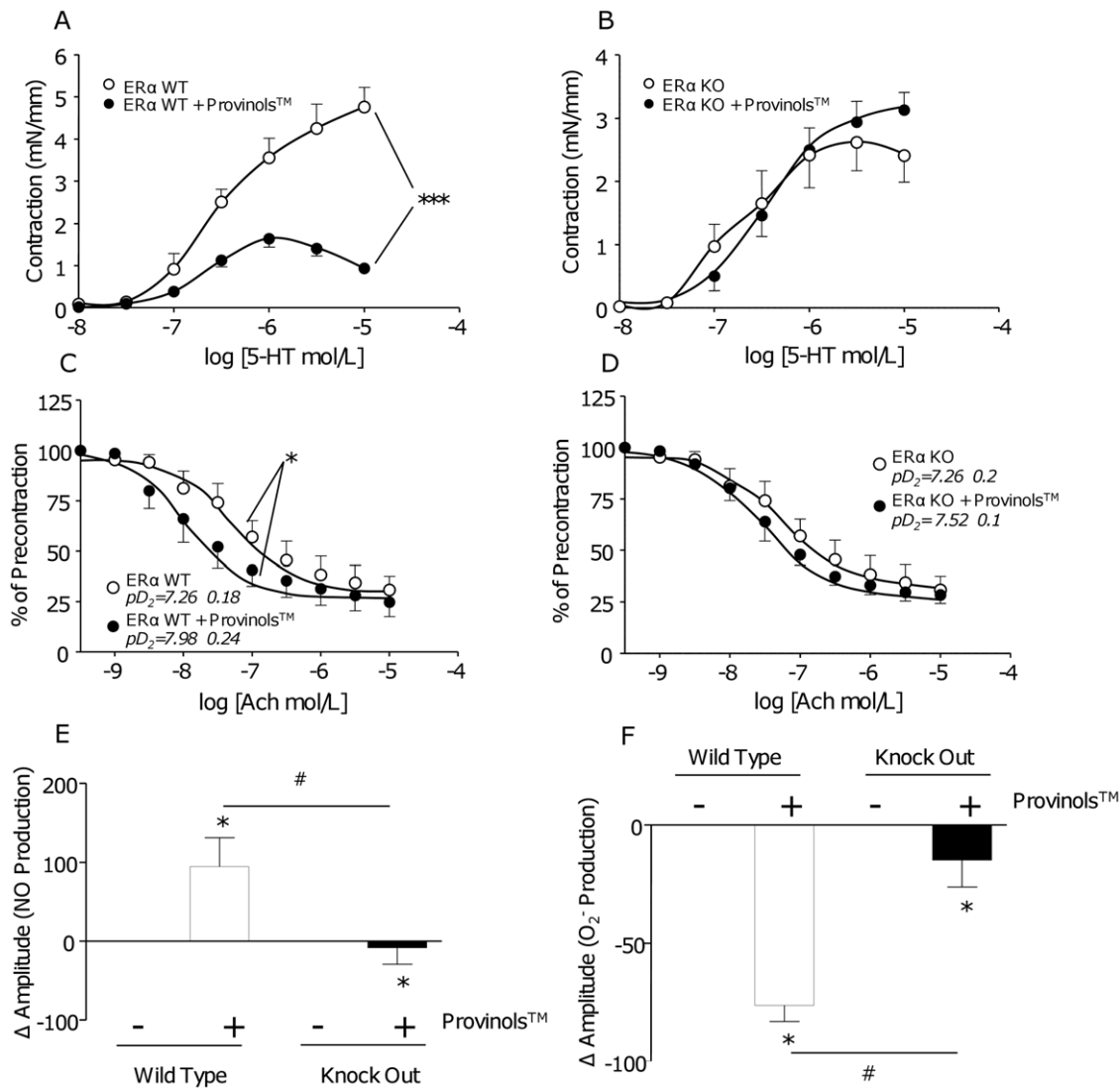


Figure 5. Involvement of ER α in vascular effects induced by an oral treatment with ProvinolsTM. Concentration-effect curves to increasing concentrations of serotonin (5-HT) in aortas, with endothelium, from ER α Wild Type (WT) (A) or Knock-Out (KO) (B) mice treated either with control diet (open circles) or diet containing ProvinolsTM for 2 weeks at 20 mg/kg/day (filled circles, ****P*<0.001, *n*=6). Concentration-effect curves to increasing concentrations of acetylcholine (ACh) in aortas with endothelium, pre-contracted at 80% of the maximal contraction with U46619 from ER α WT (C) or KO (D) mice treated either with control diet (open circles) or diet containing ProvinolsTM for 2 weeks at 20 mg/kg/day (filled circles) (**P*<0.05, *n*=6). Quantification of NO (E) and O₂⁻ production (F) in mesenteric arteries from ER α WT or KO mice receiving either standard diet or diet containing ProvinolsTM (**P*<0.05 vs control diet; #*P*<0.05 KO vs WT; *n*=5). doi:10.1371/journal.pone.0008554.g005

to evaluate the role of other pathways that may be involved in cardiovascular effects induced by red wine polyphenols including ER β or cyclooxygenase pathways.

The findings that vascular protection induced by red wine polyphenols, and in particular by delphinidin, requires ER α activation is a major breakthrough in understanding the therapeutic potential of polyphenols in cardiovascular pathologies. These properties of red wine might explain the prevention of ischemic heart disease [24,25], stroke [10] and metabolic diseases [24], in different experimental models.

Methods

ProvinolsTM was obtained from Société Française des Distilleries (Vallon Pont d'Arc, France) and delphinidin was purchased from Extrasynthèse (Genay, France). The university of Angers

ethical committee approved the present protocol. All animal studies were carried out using approved institutional protocols and were conformed the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Methods for vascular reactivity performed in mice [26,27] and endothelial cells extraction and culture were set up as previously described [19]. Methods for RNA interference and transient transfection to silence ER α were adapted from Agouni *et al.* [26]. NO and O₂⁻ spin trapping and electronic paramagnetic resonance (EPR) studies and Western blotting were conducted as previously described [26]. Binding assay was performed by CEREP (Paris, France) using fluorescence polarization methods in human recombinant S19. Delphinidin was docked on ER α using default settings of the GOLD4.0 program [28]. Additional details of the methods used are provided in the Supplemental Data file (Methods S1).

Supporting Information

Methods S1

Found at: doi:10.1371/journal.pone.0008554.s001 (0.04 MB DOC)

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Author Contributions

Conceived and designed the experiments: MC AT RA. Performed the experiments: MC AT DR. Analyzed the data: MC AT DR RA. Contributed reagents/materials/analysis tools: MC AT DR. Wrote the paper: MC AT MCM JFA RA.