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17β-ESTRADIOL MODULATES CARDIOPROTECTIVE EFFECTS OF NUTRACEUTICAL COMPOUNDS

Presentata da: **Dott.ssa Maria Cristina Barbalace**

Coordinatore Dottorato **Supervisore**

Chiar.ma Prof.ssa Patrizia Hrelia **Chiar.ma Prof.ssa Silvana Hrelia**

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ABSTRACT

Cardiovascular disease rarely manifests in pre-menopausal women meanwhile, the incidence of these pathologies dramatically increases after the menopause suggesting the possibility that sex hormones could have a key role. 17β-estradiol is the main female circulating hormone in the premenopausal period and showed protective effects on the cardiovascular system. Moreover, recent evidences underlie the importance to take into account the gender in clinical studies as it can influence the response to cardiovascular medications. Therefore, we hypothesize that sex hormones can also influence the cardioprotective effects of nutraceutical compounds, such as sulforaphane, isothiocyanate present in Brassica vegetables. This study was designed to investigate the protective effects of sulforaphane in presence of 17β-estradiol against H_2O_2-induced oxidative damage in cardiomyocytes. 17β-estradiol enhanced sulforaphane cardioprotection against H_2O_2-induced cell death with respect to 17β-estradiol or sulforaphane alone, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide and lactate dehydrogenase assays. Moreover, 17β-estradiol boosted sulforaphane antioxidant activity, reducing intracellular reactive oxygen species and 8-hydroxy-2′-deoxyguanosine levels and increasing the expression of phase II enzymes. The observed effects seem to be not mediated by estrogen receptor α and β, as we used specific antagonists. Otherwise, ERK1/2 and Akt signaling pathways seem to be involved, as the treatment with specific inhibitors reduced the protective effect of sulforaphane/17β-estradiol co-treatment. Furthermore, estrogen receptor β and G protein-coupled receptor 30 seem to contribute to Akt activation, as using receptor specific agonists sulforaphane-induced Akt phosphorylation was enhanced. The activation of Akt kinase is also involved in the activation of Nrf2 transcription factor elicited by sulforaphane/17β-estradiol co-treatment, as treated cells with Akt-inhibitor, the co-treatment-induced Nrf2 activation was prevented. Our results demonstrated, for the first time, that estrogen could enhance sulforaphane protective effects, suggesting that nutraceutical efficacy might be modulated by sex hormones.

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1. CARDIOVASCULAR DISEASE AND SEX-GENDER DIFFERENCES

Nowadays, cardiovascular disease (CVD) still remain the most common cause of death worldwide, with 4 million people dying only across the European continent, every year [1]. Although they have always been perceived as pathologies regarding essentially males, it is now clear that a sex-gender component is involved in the incidence and death from cardiovascular events. Furthermore, the development of coronary artery disease occurs ten year later in women than men [2]. Indeed, the protection from cardiovascular events during fertile period in women dramatically decrease after menopause, so that the major cause of death in women after 65 years of age is CVD [3, 4]. Many aspects of CVD are similar in males and females but obviously they differ in the anatomy and physiology of the cardiovascular system and some differences in risk profile, symptoms, age of onset and response to medical treatments have been evidenced [5].

These differences are due in part to the biological differences between men and women, usually defined as sex differences, meanwhile the individual interaction with the society and environment results in gender differences. These two concepts are separated but intertwined in medicine, because they interact and become tangled together (Fig. 1.1) [6].

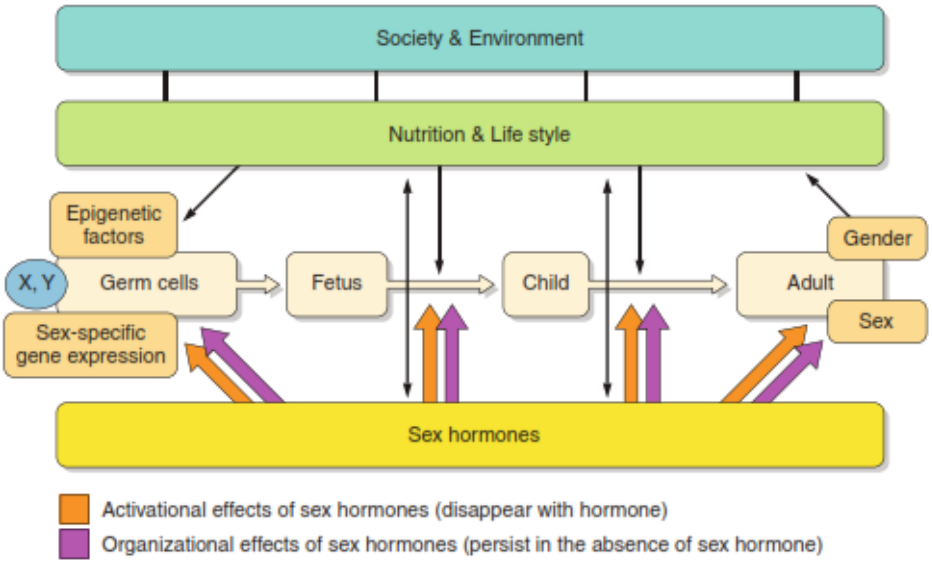


Figure 1.1: ***Schematic representation of the interaction between sex and gender*** [7]

In fact in this scenario it's also clear that sex hormones can influence behavior and lifestyle [6]. However, studies analyzing the impact of sex and gender in health and disease are still inadequate. Despite it has been cleared that there are also sex-gender-related differences in the pharmacokinetics (i.e. how the drugs are absorbed, distributed, biotransformed and excreted) and pharmacodynamics (Fig. 1.2) (i.e. how the drug interacts with the site of action), a real clinical relevance of these differences is moderate or remains uncertain due to underrepresentation of women in clinical trials [2].

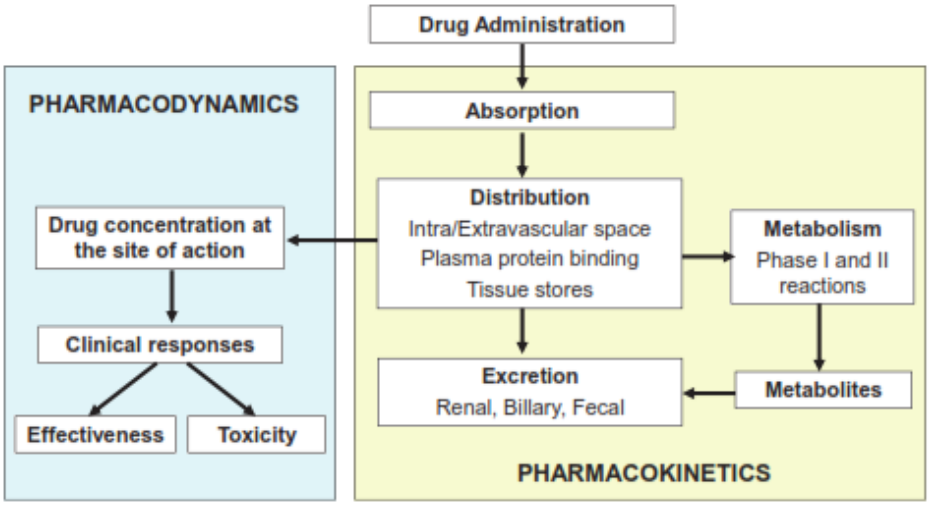


Figure 1.2: ***Schematic representation of a drug pharmacokinetics and pharmacodinamics*** [2].

So the current guidelines on CVD are based on evidences mostly obtained in middle-aged men, implying low appropriateness for therapeutic interventions in women [2].

1.1 Ischemic Heart Disease (IHD)

Ischemic heart disease develops differently in men and women. Men are prone to occlusive artery disease linked to a more pronounced and severe atherosclerosis in their coronary arteries, which they develop earlier than women, with the consequence of precocious myocardial infarction [7]. On the other side, women experience myocardial ischemia, which is due to an imbalance between oxygen supply and the demand of the myocardium, for pathological vasoreactivity [8]. Commonly, perfusion problems in women arise from spasm and endothelial dysfunction, or from microvascular dysfunction.

The reason why pre-menopausal women develop less atherosclerosis than men is poorly investigated. It has been suggested that it could be related both to a better lipid profile than men and to a protective effect mediated by sex hormones. Women with hormonal disorders experience atherosclerosis early than healthy women [7].

Acute mortality after myocardial infarction (MI) is major in younger women than age-matched men [9]. In general, different studies conducted worldwide evidenced that women have a higher mortality rate than men for MI, and women die more from cardiac rupture at acute MI [9].

Takotsubo syndrome is an IHD manifestation which affects predominantly women, and appears as an acute coronary syndrome, though the exact etiopathology is unclear. Probably, the lowering in estrogen levels, which occurs in post-menopausal period, makes heart more sensible to catecholamines activity [7]. However, this syndrome often manifests in pre-menopausal women after a marked acute psychological or physical stress, other cause of increased in catecholamines activity on heart. Most of the patients recover a normal ejection capacity, despite the recent epidemiological data showed a mortality of 8% per year [10].

1.2 Hypertension

The prevalence in hypertension related to gender is different if we compare young or old population. Whereas there are more young males with hypertension, the situation totally capsizes in the old population where the percentage of women with hypertension is double than men [11]. Moreover, at older ages, women are more prone to develop vascular and myocardial harshness than men, and frequently they present aortic harshness, reflecting a systolic hypertension[12]. Nevertheless, hypertensive women maintain major left ventricular ejection fraction than men [13].

1.3 Pressure Overload

The adaptation of female heart to pressure overload is different from male. During progression of aortic stenosis more often women preserve myocardial contractility

and ejection fraction than men, and this could be due to a minor induction of collagen remodeling [14]. In a study from Petrov et al. [15] in patients undergoing aortic valve replacement, the same percentages of women and men had a superior diameter of left ventricle than reference values, but after surgery, this superiority persisted in major percentage in men than in women. Also hypertrophy reversed more in women than in men. All these effects are connected to a different collagen I, collagen III and matrix metalloproteinase 2 gene expression, so that less fibrosis prior surgery can promote a faster regression [15]. Overall, the studies demonstrated sex-specific pattern in myocardial remodeling.

1.4 Exercise-induced Cardiac Hypertrophy

Few data have been collected about endurance-induced hypertrophy, and so far a different cardiac adaptation was described. Women and men undergoing identical training program experience different metabolic adaptation [16]. In particular, women had more body fat reduction in 6 months of training whereas men had a reduction only after 12 months. Moreover, women had similar or greater increase in left ventricular mass as men after 3 months, but not further increase going on with training, instead the greater increase in men left ventricular mass was at month 12. Cardiac hypertrophy stimuli in women is controlled by phosphatidylinositol 3-kinase/Akt and/or β-catenin pathways. In pre-menopausal period women exhibit greater Akt cardiac activity with anti-hypertrophic predominant effect [17]. Hypertrophy in men could be partially explained as an effect of testosterone, that is known to increase with physical activity [18]. However more data are needed in this context.

1.5 Heart Failure

Heart failure (HF) is a chronic, progressive condition in which the heart muscle is unable to pump enough blood for the body needs in terms of blood and oxygen. In Western societies HF affects more than 10% of people over 70 years, and derives from different pathophysiological conditions [7]. Different forms of HF exist, with reduced ejection fraction (typical of men), and with preserved ejection fraction (affecting predominantly women) [19]. Generally, the clinical outcomes for both syndromes are better for women, with a different adaptation of the heart with respect to men [20]. Women develop less ventricular dilatation than men, though they present smaller and stiffer ventricles probably due to the different composition in fibrous tissue, but maybe also related to different calcium handling which comports variable relaxation kinetics [21].

1.6 17β-estradiol and cardioprotection

Estrogens belong to steroid hormone family which includes also glucocorticoids, mineralcorticoids, androgens and progesterone. All steroid hormones derive from cholesterol and they share the same key chemical structure (cyclopentane-perhydro-phenanthrenic) (Fig. 1.3). The synthesis of sex hormones starts early during embryonic development. They are mainly synthesized in gonads, but exist also an extragonadal synthesis in cardiomyocytes, bone cells and neurons. Sex hormones act with many mechanisms, as they belong to the vast family of endogenous signaling molecules, which modulate different cellular processes via gene and protein regulation. The main female circulating hormone is 17β-estradiol (E2), major product of ovary secretion, which owns the strongest potency and has the capability to bind all subtypes of estrogen receptors. Other naturally occurring estrogens are estrone (12 times less potent than E2), produced in both males and females in less extent with

respect to E2, meanwhile estriol (80 times less potent than E2) unlike E2 and estrone is not synthesized in or secreted from the ovaries but it mainly derives from 16α-hydroxylation of the other two estrogens, by cytochrome P450 enzymes in liver.

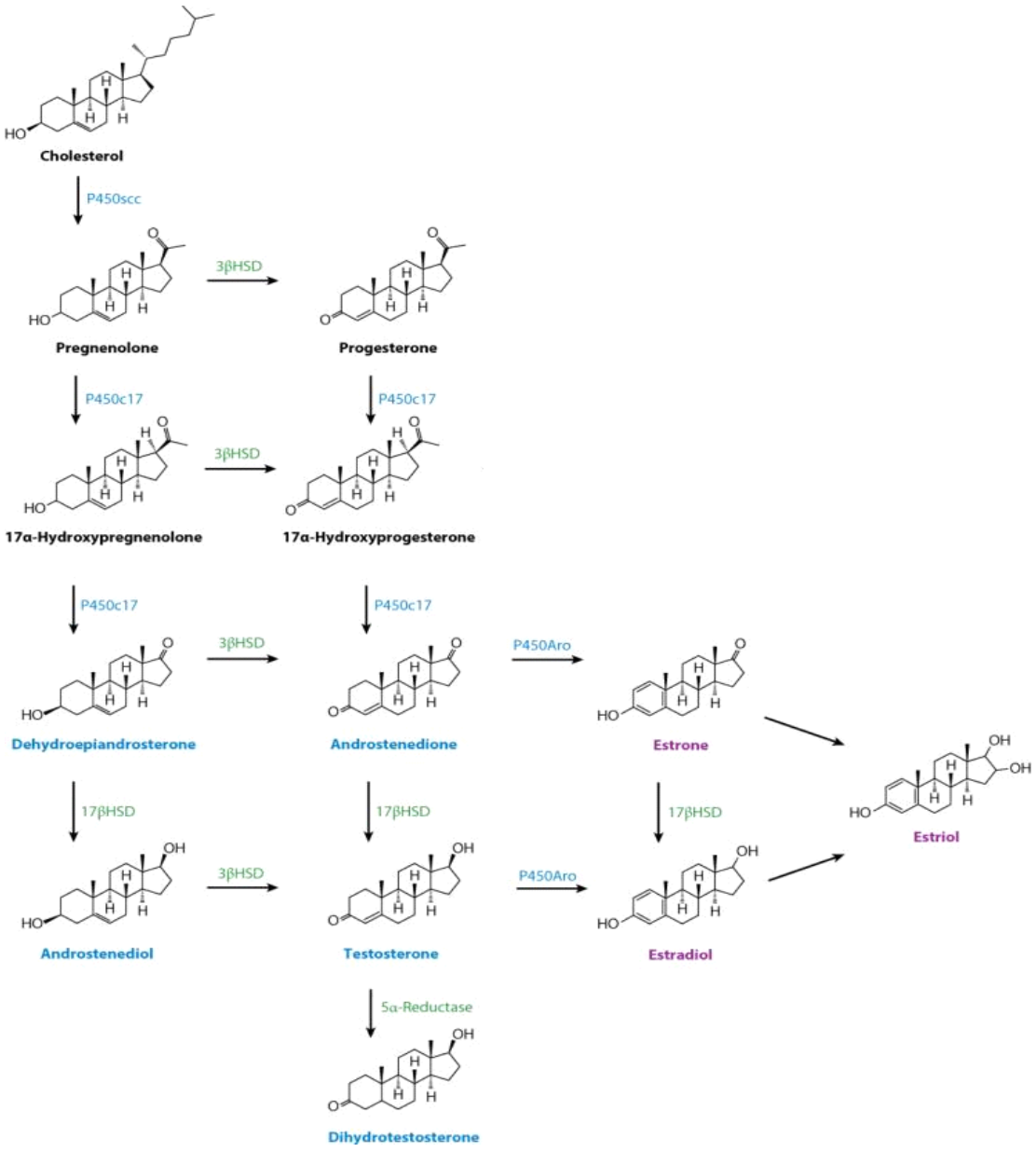


Figure 1.3: ***Biosynthesis of sex hormones*** [22].

As mentioned before, E2 can also be produced locally as a result of the conversion of testosterone by the enzyme aromatase [23]. Aromatase is present in a number of

extragonadal tissues, such as the adipose tissue, bone, brain, heart, and the vasculature in both sexes [24].

It has been proposed that E2 possesses cardioprotective activity and acts through different mechanisms. E2 reduces cardiomyocyte apoptosis in vivo and in vitro through estrogen receptors (ERs) and phosphoinositide-3-kinase (PI3K)/Akt signaling pathways [25, 26]. E2 differentially modulates p38α and β MAPK during hypoxic/oxidative stress, preventing apoptosis and counteracting mitochondrial reactive oxygen species (ROS) generation in cardiomyocytes [27]. In cardiomyocytes, E2 is also able to differently modulate prohypertrophic (class I) and antihypertrophic (class II) histone deacetylase proteins through the binding to ERβ, thus counteracting cardiac hypertrophy [28]. It has also been shown that SIRT1 (class III) functions as an important regulator of E2-mediated cardiomyocyte protection during angiotensin-II-induced heart hypertrophy and injury [29].

In vascular smooth muscle cells, E2 possesses antioxidant activity [30] and up-regulates the expression of genes as endothelial nitric oxide synthase (eNOS), superoxide dismutase (SOD), and down-regulates NADPH oxidase in the cytoplasm [31], thus contributing to its vasoprotective effects.

Moreover, in cardiomyocytes, E2 exposure up-regulates corticotrophin-releasing hormone receptor type 2 expression by interacting with ERα and enhances the protective effect of urocortin against hypoxia/reoxygenation [32]. In the H9C2 myocardial cell line, Hsieh et al. [33] showed that E2 provides cardioprotection through the inhibition of hypoxia induced HIF-1α and downstream BNIP3 and IGFBP3-dependent apoptotic responses. Furthermore, E2 cardioprotective effects were also confirmed in animal models: E2 prevented Fas-dependent and mitochondria-dependent apoptotic pathways, and cardiac hypertrophy induced by ovariectomy, in rat models [34, 35]; E2 reduced infarct size and exerted a protective effect on ischemic myocardium in rabbits, mice, and rats [36-38]; E2 prevented global myocardial ischemia/reperfusion injury in rats [39]. The specificity of these effects was well documented by the reversion of the effect using ER antagonist ICI182780 [40, 41]. The

administration of E2 was also demonstrated to exert antihypertrophic effects in various models of pressure overload. The mechanisms involved are: the inhibition of the expression of prohypertrophic genes and the reduction of left-ventricular hypertrophy [42]; the regulation of two proteins involved in the hypertrophy development, such as atrial natriuretic factor and myosin heavy chain beta [43, 44]; the reduction of systolic dysfunction and fibrosis with the involvement of ERα in 9-weeks administration model [45].

2. ESTROGEN RECEPTORS AND SEX-GENDER DIFFERENCES IN CARDIOVASCULAR SYSTEM

Before 1996, only one estrogen receptor has been discovered and the predominant idea was that all estrogen effects were mediated by this receptor that acted as a ligand-regulated transcription factor. Now we refer to this receptor as ERα. Over the past 20 years the knowledge about estrogen signaling has grown, so that now three different receptors have been characterized: ERα, ERβ and G protein-coupled receptor 30 (GPR30), which can act through traditional transcription regulation as well as by non-genomic pathways.

2.1 Chemical structure and localization of Estrogen receptors

ERα and ERβ belong to the superfamily of nuclear steroid hormone receptors. Like all steroid receptors they have: an amino (NH_2)-terminal region, A/B domain, which contains constitutive AF-1 (activation function 1) that acts on target gene transcription in a ligand-independent way; a zinc finger-based DNA-binding domain (DBD) named as C region, which contains regions that mediate dimerization; a hinge domain (D region) which connects C region to E region and it is the binding site for heat shock protein 90; a carboxy (COOH)-terminal ligand-binding domain (LBD) (E region), which contains the ligand-dependent activation function (AF-2) [7] (Fig. 2.1).
They have been identified in several cardiovascular cells from male and female individuals, such as cardiomyocytes, cardiac fibroblasts, vascular smooth muscle cells and vascular endothelium [46-49].
Interestingly in human heart, ERα mRNA levels are similar in men and women, meanwhile ERβ mRNA levels are more abundant in men than in women [50].

A part of the classical localizations at the plasma membrane and nucleus, both ERs have also been detected in mitochondria [51].

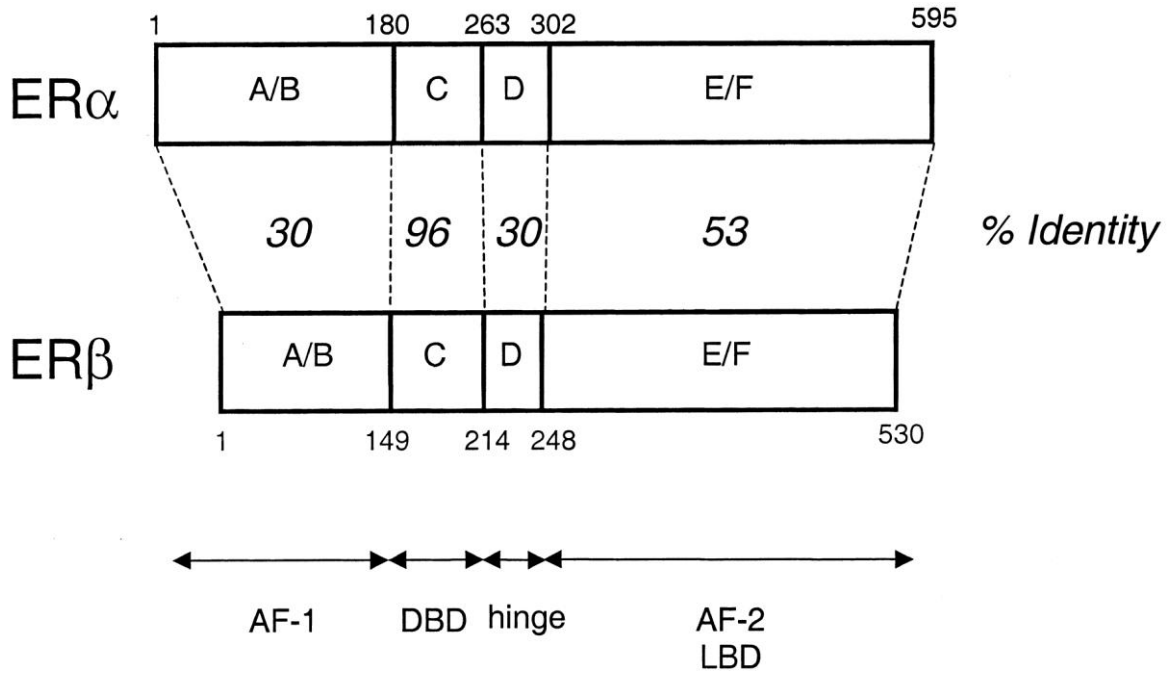


Figure 2.1. ***Domain organization and sequence homology of human ERα and ERβ*** [52]**.**

Only in the last decade, GPR30 emerged as an important mediator of non-genomic estrogen actions. It is a G-protein coupled receptor, so belongs to the largest known class of membrane receptors (Fig. 2.2). The most important characteristic of G-protein coupled receptors is the presence of seven transmembrane alpha helices, and as the name implies they interact with G-proteins. G-proteins are specialized proteins with the ability to bind guanosine diphosphate (GDP) and guanosine triphosphate (GTP), and all those that associate with G-protein coupled receptors are heterotrimeric, meaning they have three different subunits (α-, β-, and γ-subunit).

GPR30 protein is structurally different from classical ERs but it possesses the same binding characteristics [53].

The expression of GPR30 has been identified in multiple tissues, such as ovary, uterine endometrium, brain, kidney, adrenal, breast, heart and endothelium [54-56].

Instead, regarding GPR30 cellular location there is some debate, because some research groups found it localized in the endoplasmic reticulum and the Golgi apparatus but not in plasma membrane [57], meanwhile other reported GPR30 to localize in plasma membrane [58, 59]. Cheng et al. [60], showed a constitutive endocytosis for GPR30, which results in a half-life of 30 minutes, demonstrating its presence also in endosomes and endoplasmic reticulum.

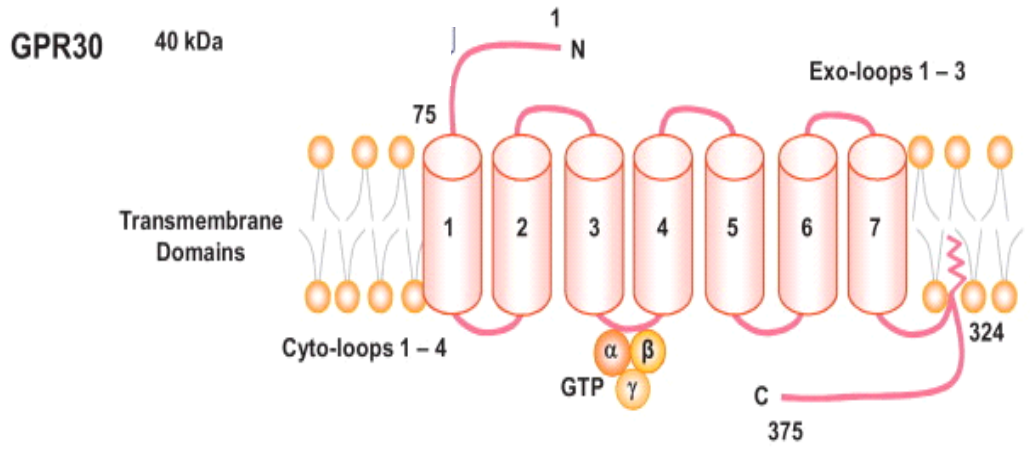


Figure 2.2. ***Structure of GPR30*** *(modified from [61]).*

The GPR30 rapid recycle from plasma membrane through endosomes formation could explain why data on receptor localization are not clear. Therefore, the localization of GPR30 appears to be heterogeneous, but so far data for functionality at endoplasmic reticulum level are uncertain.

2.2 Estrogen genomic effects

E2 activating ERs can regulate gene expression through different pathways: 1) direct binding to DNA; 2) indirect binding to DNA through other transcription factors; 3) ligand-indipendent binding [62] (Fig. 2.3).

In the classical mechanism, E2 binds ER facilitating its dissociation from cytoplasmic chaperones and promoting its translocation to the nucleus and its dimerization, thus facilitating the binding to ERE sequence (estrogen response element) in the DNA and mediating the regulation of gene expression. In this case coactivators and corepressors can activate or inhibit gene expression. E2 can also regulate gene expression via the mediation of ER-tethering with transcription factors, such as activator protein-1 and specificity protein-1, so acting via non-ERE pathways [63]; alternatively, growth factors and E2 can activate kinase signaling pathways leading to the phosphorylation of a specific serine site on ERs [64], significant for the induction of the trascription via ligand-indipendent or ERE-pathway.

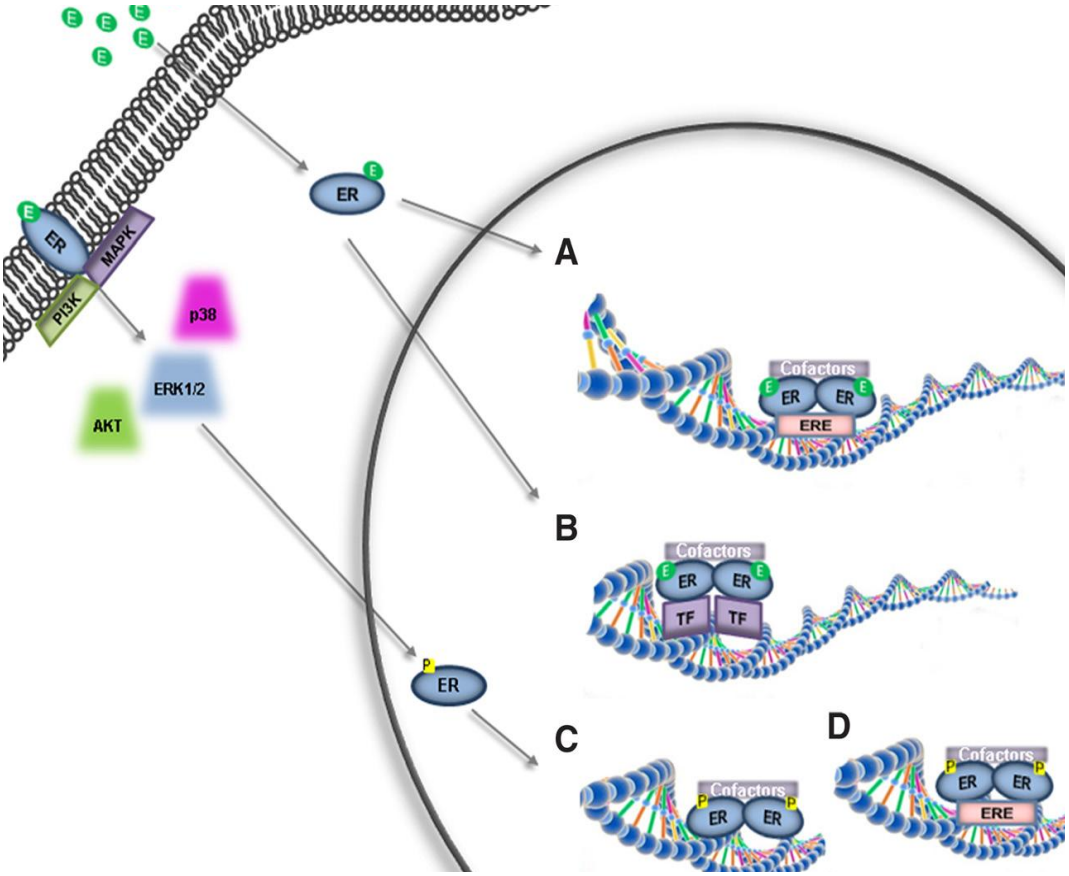


Figure 2.3. ***Genomic ER signaling*** [62].

2.2.1 Distinct roles for ERα and ERβ genomic effects, who wins?

The analysis of ERs genomic effects is really difficult for several reasons. First of all, there are variations in ERα and ERβ protein levels that are temporal and tissue-related, moreover their levels and also coactivators and corepressors levels can vary during ageing, diseases, and with sex, so modifying E2 effects.

In wild type mice aorta O'Lone et al. [65], examining aorta whole vessels after long-term exposure to E2, showed that ERα was sufficient and necessary for the induction of some genes, meanwhile for another group of genes both receptors ERα and ERβ act in concert to stimulate gene expression. The same study revealed the involvement of ERβ in down-regulating some genes, including several nuclear-encoded

mitochondrial genes, supporting its role in a gene active repression and not simply an opposite effect to ERα.

Nikolic et al. examined the effects of 2 h heart perfusion with a selective ERβ agonist, revealing a cardioprotective effect mediated by the up-regulation of different genes. These different results could be due to the diverse exposition times and cells (aorta or ventricular myocytes) [66].

Studies explored the roles of both ERs after myocardial infarction, using unique models of cardiac-specific ER overexpression (OE) in mice. ERα-OE protects the heart from ischemic damage, enhancing neovascularization of peri-infarct area and inducing less fibrotic genes [67]. ERβ-OE mice showed improved survival after myocardial infarction, likely for a better sustenance of Ca^{2+} cycling and attenuated cardiac fibrosis [68].

Also *in vitro* models have been explored for ERs differential genomic regulation. Endothelial nitric oxide synthase (NOS), the enzyme primarily responsible for the generation of nitric oxide (NO) in the vascular endothelium [69], was up-regulated by ERα in endothelial cells [70], whereas ERβ was responsible for the induction in cardiac muscle [71].

As mentioned also before, both ERs can act in concert to regulate gene expression. In endothelial cells, they contrast the over-generation of reactive oxygen species (ROS) from mitochondria, up-regulating superoxide dismutase 2 (SOD2), with the result of a lower mitochondrial dysfunction and vascular damage [72].

2.3 Estrogen non-genomic signaling

The first report of a non-genomic response by estrogen has been showed in the 1960s by Pietras and Szego [73]. They demonstrated a rapid response on cAMP concentration in endometrial cells, within minutes from E2 exposure.

It is now well characterized that physiological concentrations of E2 can induce cellular rapid responses, which do not involve protein synthesis or gene transcription, and that are controlled by receptors localized on plasma membrane [74, 75] (Fig. 2.4).

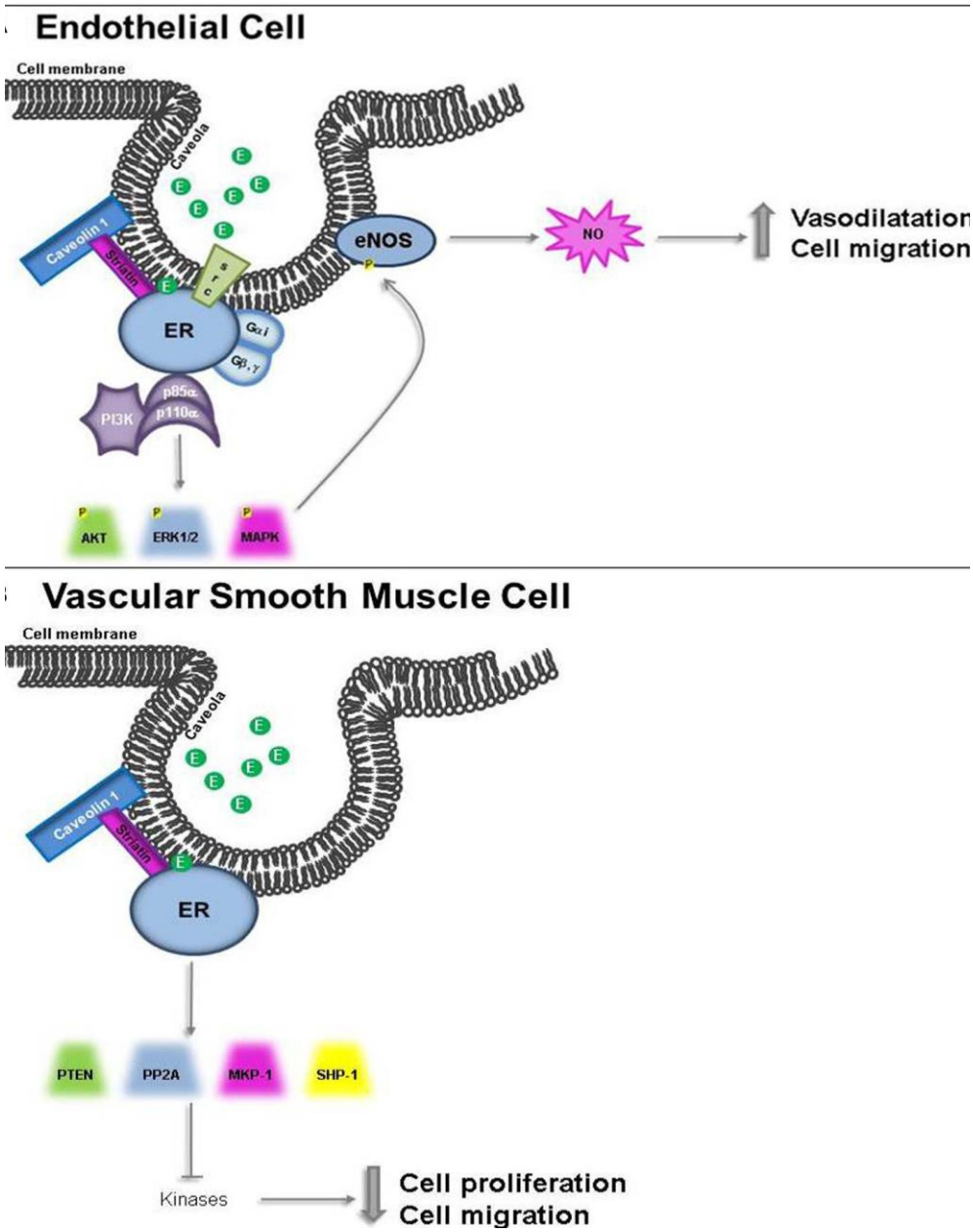


Figure 2.4. ***Non genomic ER signaling*** [62].

2.3.1 ERα and ERβ signaling

ERs localize at plasma membrane for a ≈ 5%-10% of their total amount [76]. Unfortunately, the distribution of membrane-bound ERs in the cardiovascular tissues is poorly investigated, but both ERα and ERβ have been found in vascular endothelial cells [77]. In the other tissues, the relative distribution of ERα and ERβ at the plasma membrane is different among cell type; in breast cancer cells ERβ is more abundant than ERα [78], and the opposite is true for the reproductive cells [79].

Post-translational modifications are found to be involved in the binding of ERs to plasma membrane, such as palmitoylation that increases the association of the receptors to plasma membrane [80, 81].

Moreover, several studies identified different sites on ERα, important for its localization to the plasma membrane. Ser522 is required for the interaction between ERα and caveolin-1, facilitating the receptor binding to caveolae [80, 82], but the mutation of this residue does not block the traslocation of ERα to the membrane as reported by Razandi et al., suggesting that Ser522 is not the only residue involved in the membrane association [82]. Palmitoylation of Cys447 has been reported to be crucial for membrane localization of ERα [82, 83]. Therefore, the group of Levin identified in human and mouse cells an amino acid motif in estrogen binding domain of both ERα and ERβ, as involved in the membrane traslocation, and Cys447 residue is part of this motif [84].

The palmitoylation can happen only on ER monomer, so if dimerization occurs, such as after estrogen binding, the receptors available for palmitoylation are less and in turn less receptors translocate to the membrane [62]. Another mechanism involved in the trafficking to plasma membrane is the binding between heat shock protein 27 and palmitoylation site on ERα monomer [82].

2.3.1.1 Cardiomyocytes

Unfortunately, only few studies have been conducted to examine the role of ERs non-genomic pathways in cardiomyocytes.

Short-term treatment with E2 is able to improve heart function after I/R injury, and this effect is reversed in the presence of NOS inhibitor, suggesting the contribution of NO production via ER activation in this process [85]. Other studies demonstrated that non-genomic estrogen signaling by ERβ can act on cardiac hypertrophy [43, 86]. Moreover, the involvement of this receptor has been elucidated by another study, which found that the prevention on angiotensin-induced hypertrophy elicited by E2 was abolished in ERβ knockout mice [87]. The effects on cardiomyocyte hypertrophy were due to sequestering of transcription factors in the cytoplasm which prevents target gene activation.

2.3.1.2 Endothelial cells

E2 can stimulate eNOS phosphorylation in endothelial cells (EC) activating membrane-localized ERs [88]. Endothelial eNOS activation is a main pathway in the regulation of vascular relaxation, EC proliferation and migration. Endothelium health is really important in the cardiovascular system, indeed endothelial dysfunction is responsible for an increased cardiovascular risk [89]. E2 is able to activate eNOS by several signaling pathways. After E2 binding at membrane level, ERα forms a complex with the regulatory subunit of PI3K, p85α, and with c-Src at the SH2 domain [90]. This complex leads to the activation of protein kinase B (Akt), extracellular-signal-regulated kinases 1/2 (ERK1/2) and also phosphorylation and activation of eNOS [91]. In agreement, the pre-treatment with E2 in mice exposed to I/R injury, increased eNOS activation and decreased leukocyte accumulation at vascular level, and the protective effect was abolished using PI3K or eNOS inhibitors [74].

Moreover, ERα can interact with G proteins Gαi and Gβγ, leading to eNOS activation and stimulation of cell migration [92].

In addition to the effects on eNOS, physiological concentrations of E2 (10^{-9} mol/L) can modulate intracellular calcium homeostasis through non-genomic pathways. E2 was able to cause a rapid increase in intracellular calcium levels in both human and rat EC, and in this last cell type, the effect was blocked using the ER antagonist ICI 182,780 [93, 94].

2.3.1.3 Vascular Smooth Muscle Cells

It has been reported that E2 can rapidly inhibit vascular smooth muscle cells (VSMCs) proliferation [95, 96]. The proliferation of VSMCs is implicated in cardiovascular disease, particularly in atherosclerosis [62]. The activation of ERα through E2 treatment induces the activity and the expression of different phosphatases, such as protein phosphatase 2A, MAP kinase phosphatase-1 and so on. Therefore, stimulating these phosphatases which lead to the dephosphorylation of kinases, E2 induces a stop in cell migration and growth [95, 97, 98]. Karas et al. [99] evidenced the role of E2 on blocking VSMCs proliferation, thanks to an enhanced activity of phosphatase 2A on phosphorylated Akt. This outcome was controlled by the complex formed between ERα and phosphatase 2A. In the same study, the researchers evidenced that in cells derived from a transgenic mouse line, which overexpressed a peptide that disturb the trafficking of ERs to plasma membrane, E2 did not influence cell proliferation. Data from microarray analysis confirm that disturbing ERs non-genomic signaling alters transcriptional response to E2 treatment. The genes involved are those associated with vascular function, so underling the importance of short term signaling on vascular health [100].

2.3.2 GPR30 signaling

The expression of GPR30 is ubiquitous, so it is not surprising that its activation has been connected to multiple biological activities, such as production and secretion of IL-10 in immune system [101]; cardiovascular and cancer cell growth and death [102, 103]; effects on depression disorders [104].

The signaling cascade initiated by GPR30 involves the activation of a stimulatory G protein and the subsequently formation of cAMP after activation of adenyl cyclase. Interestingly, E2 is able to trigger this rapid intracellular response, whereas estrone and estriol are inactive. Moreover, many other signaling pathways have been linked to GPR30 activation, as epidermal growth factor receptor (EGFR) [105], PI3K [106], and ERK pathways [107].

Of note, these pathways are the same widely studied as non-genomic pathways mediated by ERα and ERβ [91].

2.3.2.1 Vascular effects

Several reports aimed to investigate the vascular effects mediated by GPR30.

Surprisingly the largely used ERs antagonist ICI 182,780 did not attenuate the estrogen-induced vasodilatation in arteries [108, 109], and the genomic deletion of ERα and ERβ did not reverse the cardiac responses mediated by E2 [110]. Indeed, although ICI 182,780 is a well known ERα and ERβ antagonist, it acts as an agonist for GPR30. So these discoveries suggest that GPR30 might regulate the vasodilatatory effect of E2. To deepen this aspect multiple studies were conducted using the selective GPR30 agonist G-1, so confirming the vasodilatative effect of GPR30 in both human and non-human arteries, and *in vitro, in vivo* models [111-113]. In particular, in *in vivo* models, researchers evidenced a reduction of blood pressure also in

normotensive rats with no response in animals knockout for GPR30 [113], and the total vasorelaxation effect after G-1 treatment was about 30% to 40% [114].

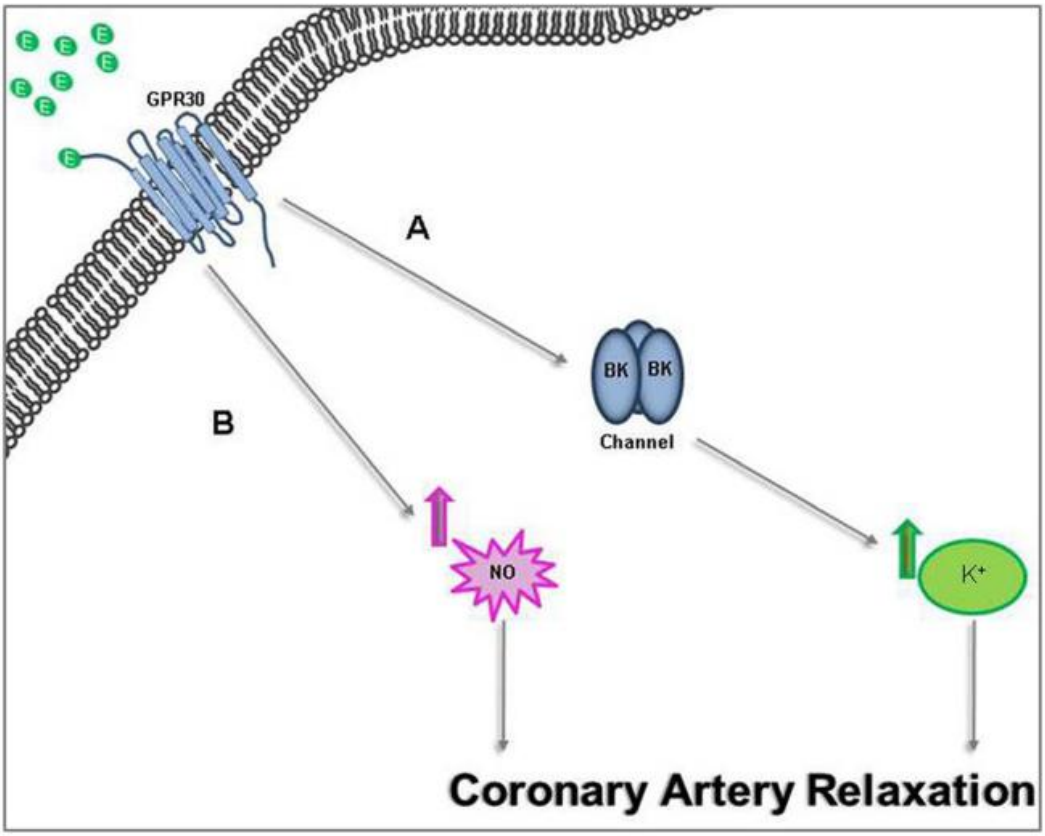


Figure 2.5. ***GPR30 effect on vasculature via endothelium-dependent and endothelium-independent mechanisms*** [62].

The GPR30 effects on vasculature involved mechanisms endothelium-dependent and endothelium-independent (Fig. 2.5). It has been demonstrated that E2-mediated GPR30 activation leads to an increase in NO production [108], so supposing its implication. But several studies also demonstrated a residual effect in endothelium-prived vessels after G-1 treatment [112, 115]. G-1-induced relaxation in coronary smooth muscle is supposed to be mediated by a large conductance calcium-activated potassium channel. Indeed, it has been evidenced an inhibition in G1-induced vasorelaxation, only with the channel block and not when NOS production was

inhibited[115]. Vascular remodeling can occur as response to acute or chronic (vascular injury, and atherosclerosis) stress factors and includes adaptive and maladaptive responses (hypertension and vascular stenosis) [116]. It is well-known that E2 is able to decrease proliferation of VSMC after injury [62]. Different findings support the role of GPR30 in inhibiting the inflammatory response associated with atherosclerosis [117] and proliferation [118], meanwhile it stimulates apoptosis [119], and mitigates the expression of adhesion molecule mediated by TNF [120]. G-1 agonist also demonstrated to be able to reduce DNA synthesis and proliferation in different models of endothelial cells interfering with the cell cycle [121].

A role of GPR30 in atheroprotection has also been evidenced. The deletion of GPR30 gene led to increase in blood pressure, low-density lipoprotein (LDL) cholesterol levels, and the presence of inflammation markers [117].

The mechanisms insight this protection are related to cholesterol metabolism. Indeed GPR30 activation has been linked to the up-regulation of LDL receptors at hepatic level, increasing the clearance of circulating LDL cholesterol; and on the other hand GPR30 activation reduces LDL receptor degradation, through the inhibition of proprotein convertase subtilisin/kexin type-9 (PCSK9), which is the major mechanism for the receptor degradation [122].

2.3.2.2 Cardiac effects

E2 modulation of cardiac calcium intracellular levels was reported to be ERα and ERβ-independent. As confirmation to this, researchers used cells derived from ERα and ERβ knockout mice, and the E2-mediated calcium levels alterations were not changed [110]. The development of a GPR30 knockout model confirmed the role of this receptor in the modulation of calcium influx [113]. Using G-1 agonist a protective effect against I/R injury in rat hearts was also evidenced, preserving cardiac contractility, reducing infarct size, and the levels of inflammatory markers (TNF, IL-1β, IL-6), and

also inhibiting the opening of mitochondria permeability transition pore after the injury, so saving cardiac cells from the death [103, 123-125]. The molecular pathway involved in cardiac protection seems to be PI3K signaling, because using a PI3K inhibitor reverses G-1 protective effects [103].

2.3.3 Sex-gender differences

More and more studies aimed to investigate the role of ERs and GPR30 in different pathological conditions and consequently to observe the presence of sex-differences. For this purpose animal models with specific genetic deletion of these receptors have been used.

The genetic deletion of ERβ (BERKO) in conditions of pressure overload evidenced a detrimental consequence for both males and females but for different mechanisms [126]. The presence of ERβ in females reduced fibrosis, cardiomyocytes hypertrophy and cell apoptosis, meanwhile in males it promotes fibrosis but again limits cardiomyocytes hypertrophy and cell apoptosis [126].

The greater importance of ERβ in females was also confirmed in an I/R model, where BERKO females showed a greater degree of injury [127]. Similarly, in another study E2 treatment resulted in a smaller infarct size in a model of genetic deletion for ERα (ERKO) in respect to BERKO [128].

In a model of exercise-induced hypertrophy ERβ had a role as modulator for sex-differences [129]. The cardiac response to exercise in females is modulated by the regulation of PKB and MAPK signaling pathways, such as protein synthesis and mitochondrial adaptation via ERβ [129]. Moreover, the alteration in circulating free fatty acids with an augmented adipose tissue lipolysis has been reported. Therefore, sex differences in exercise-induced hypertrophy are also related to changes in cardiac metabolism, shifting to a greater use of fatty acids in females[130].

Only female BERKO mice with a condition of chronic MI showed a prolonged ventricular repolarization with a reduction in automaticity [131].

All these considerations promote the hypothesis of a role for ERβ in mediating a mitigated response to several cardiac injuries in females.

Interestingly, from other studies emerged the role of ERα in mediating E2-protection. For example, in I/R injury ERα functionality at endothelial level seems to have an important role in the E2-induced prevention of endothelial dysfunction [132]. Studies utilizing ERKO animals evidenced worse cardiac damages after I/R injury in the group with the deletion [133].

On the other side, also GPR30 cardiovascular effects could be influenced by sex and gender. In particular, regarding vasodilatative effects, the genetic deletion of GPR30 in a mouse model, comported the increase in blood pressure in females [134]. Moreover, the endothelial-dependent GPR30 effects were also observed to be gender-influenced, because in the presence of a NOS inhibitor vasodilatation was reversed only in vessels from pregnant women, and partially in those from postmenopausal women, meanwhile no effects were demonstrated on vessels from men [135].

The importance of GPR30 in the regulation of blood pressure was also pointed out thanks to the identification of a common hypofunctional missense variant of GPR30 namely P16L [136, 137]. Individuals carrying the hypofunctional variant showed higher blood pressure, as observed in a population of normotensive, especially in premenopausal women [136]. Furthermore, the probability to carry this genetic variant was twice in women (mainly postmenopausal) with resistant hypertension [136].

As mentioned in previous paragraph, the deletion of GPR30 gene leads to increase in blood pressure, low-density lipoprotein (LDL) cholesterol levels, and the presence of inflammation markers [117]. All these consequences were more pronounced in females than in males [117]. And in two different cohort populations, women but not men, which present the hypofunctional receptor variant, showed significantly higher LDL and total circulating cholesterol levels [122].

In conclusion, the results provided from the literature suggest that both ERs are important and participate to the cardioprotection by E2.

Moreover, all observations lead to support the role of GPR30 activation especially in the regulation of blood pressure and atherosclerotic risk factors.

3. OXIDATIVE STRESS AND CARDIOVASCULAR DISEASE

3.1 Oxidative stress

Oxidative stress is a condition of disequilibrium between oxidative species, mainly reactive oxygen species (ROS), and the endogenous antioxidant defenses (Fig 3.1). This condition refers to overall alterations that can occur at tissue, cellular and biological macromolecule level. ROS and other reactive species are a natural by-product of different biochemical processes and adequate quantities are essential for the homeostasis maintenance and for cell signaling. In this regard, the 'redox window' hypothesis has been postulated: adequate ROS production is needed for physiological cellular functions, but an excess of ROS levels can contribute to the development of pathological conditions [138]. The alteration of the normal redox state can have damaging effects, with the consequence of damage to proteins, lipids and nucleic acids leading to different pathological states such as atherosclerosis, CVD, aging, diabetes and cancer.

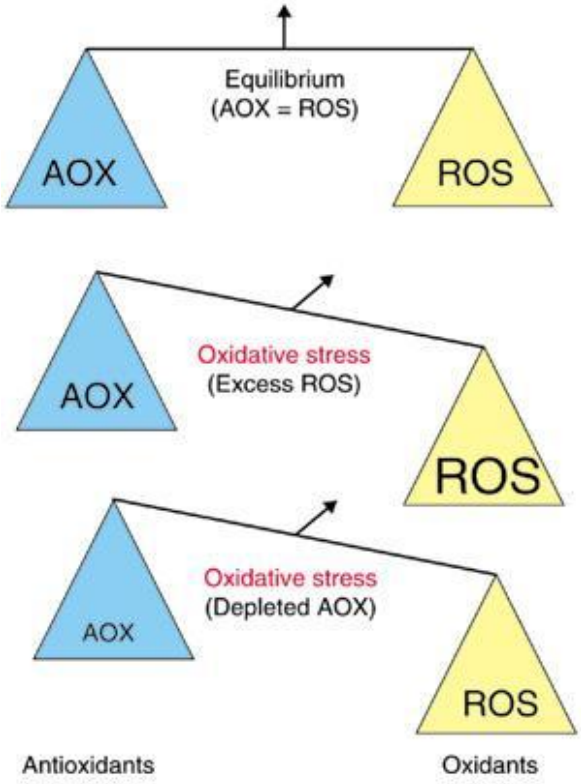


Figure 3.1. ***The balance between antioxidants and oxidants determines oxidative stress*** *[modified from [139]].*

3.2 Free radicals and ROS

Free radical is a chemical specie having one or more unpaired electrons on one atomic or molecular orbital. The free radicals are characterized by an extremely high reactivity and instability, as they tend to catch the electron they miss from other molecules. They trigger chain reactions leading to shutdown of starting radical and/or to the generation of a new radical. The free radicals most involved in cellular processes are the ROS [140, 141]. Sources of ROS production are many and include NADPH oxidase, uncoupled nitric oxide synthases, xanthine oxidase and mitochondria [142] (Fig 3.2).

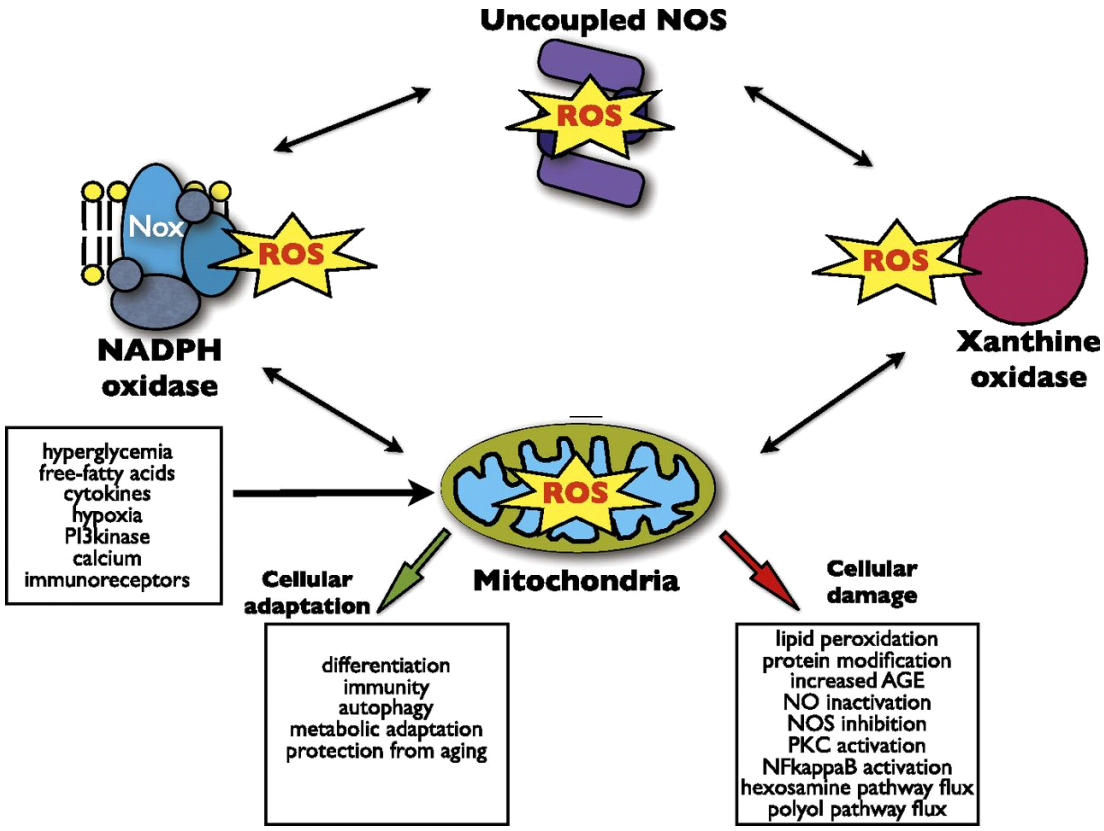


Figure 3.2. ***Representation of the main ROS sources*** *[modified from [143]].*

Our body produces different kinds of ROS, such as superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet}$) free radicals, and hydrogen peroxide (H_2O_2) which is a non-free radical.

H_2O_2 is relatively stable but with strong oxidative properties [144] and its production can be mediated by superoxide dismutation and peroxidase enzymatic reactions in peroxisomes [145]. It represents the main ROS involved in cellular signaling, as it acts activating several cellular signaling pathways as secondary messenger [146]. This is due to its longer half-life in respect to other free radicals and ability to permeate through cellular membranes [147].

The production of H_2O_2 is particularly increased in inflammatory conditions [146, 148]. In addition to the oxidative stress condition which comports alteration of cellular functionality, apoptosis or necrosis, ROS can also cause post-translational

modifications involving important cellular proteins and signaling pathways in the heart [149-151].

3.3 Enzymes involved in ROS production

3.3.1 NADPH oxidase

NAD(P)H oxidase (Nox) is an enzyme catalyzing $O_2^{\bullet-}$ or H_2O_2 release by reduction of molecular oxygen using as electron donor NAD(P)H, in various intracellular and extracellular compartments [152]. Several isoforms of Nox were described in various cardiovascular cells such as enodthelial cells and VSMC. In particular Nox1 and Nox2 isoforms represent an important source of ROS at vascular level in different pathological conditions, as hypertension, diabetes and atherosclerosis [152]. However generation of ROS through Nox enzymes have also a physiological role in various processes, including immune system reactivity and redox-dependent signaling pathways. The activation of Nox enzyme needs of the assembly of multiple protein components in the cell membrane [153] (Fig. 3.3).

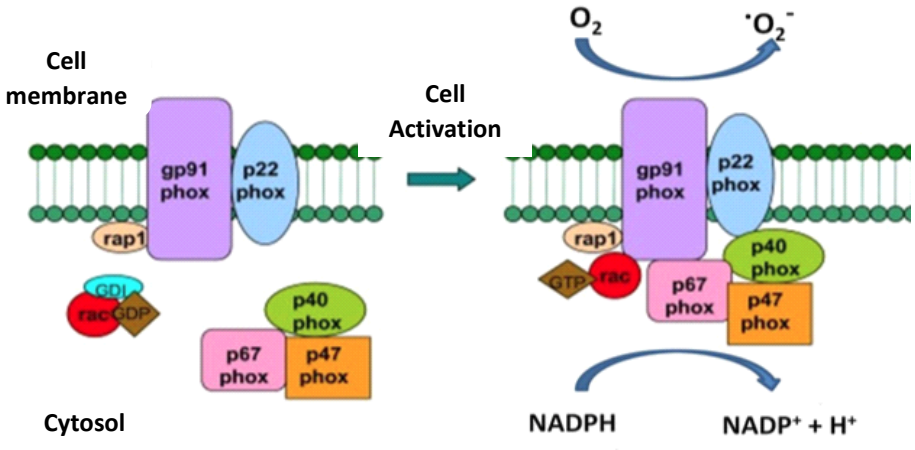


Figure 3.3. ***Schematic representation of NADPH oxidase activation*** *[modified from [153]]*

3.3.2 Xanthine oxidase

Xanthine oxido-reductase system is involved in purine catabolism, it oxidizes hypoxanthine to xanthine, and then xanthine to uric acid [154]. The system consists of two interconvertible forms, the liver synthesizes xanthine dehydrogenase that under pathophysiological conditions, such as ischemia and reperfusion, undergoes proteolysis releasing xanthine oxidase (XO) [154]. XO can associate with glycosaminoglycans present in endothelial cells and localizes with them [155]. As for Nox enzymes, XO catalyzes the reduction of molecular oxygen to superoxide and hydrogen peroxide. Circulating levels of XO were increased in animal models of hypercholesterolemia and using a XO inhibitor as oxypurinol the superoxide levels were reduced leading to an improved endothelial function [155, 156].

3.3.3 Mitochondrial respiratory chain enzymes

Complex I and III are responsible for the production of a substantial amount of superoxide in mitochondrial electron transport chain. Superoxide is released into the mitochondrial matrix by complex I so reversing the electron flow from complex II. Complex III releases superoxide into the mitochondrial intermembrane space. Mitochondria are cellular organelles really sensitive to oxidative damage. Increased ROS production slows their activity and promotes further ROS release [157]. Moreover, mito-ROS can subsequently activate other ROS sources [158]. Increased mito-ROS levels can lead to the release of apoptotic agents [159].

3.3.4 Endothelial nitric oxide synthase (eNOS)

eNOS enzyme has a key function in the endothelium for its production of NO which induces vasodilatation, prevents atherogenesis and inhibits platelet aggregation and adhesion [160]. Similarly to the other NOS isoforms, eNOS switches electrons from NADPH, via the flavins flavin adenine dinucleotide and flavin mononucleotide in the carboxy-terminal reductase domain, to the heme in the amino-terminal oxygenase domain. Here, the substrate L-arginine is oxidized to L-citrulline and NO [160] (Fig. 3.4). Inadequate amount of NO production can lead to endothelial dysfunction. Oxidative stress is markedly implicated in endothelial dysfuction, because the excess of superoxide rapidly inactives NO forming peroxynitrite ($ONOO^-$). The essential NOS cofactor (6R-)5,6,7,8-tetrahydrobiopterin (BH_4) is highly sensitive to oxidation by ONOO(-) [161]. All this lead to eNOS impairment and it is no longer able to produce NO but rather it become a source of superoxide [160]. eNOS uncoupled has been found in various animal models and in patients with endothelial dysfunction [160].

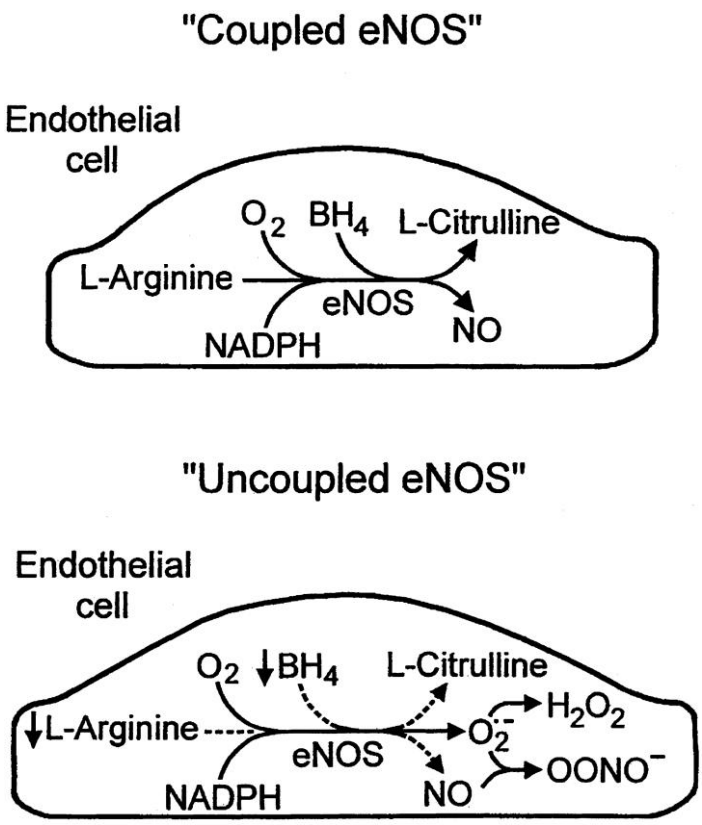


Figure 3.4. ***Schematic representation of eNOS-mediated NO synthesis*** [162].

3.4 Antioxidant defenses

As mentioned before ROS are extremely reactive and can induce damages in all cellular districts. They are able to modify and inactivate proteins and oxidize membrane phospholipids (lipid peroxidation) altering the composition of plasma membrane. In particular, H_2O_2 which is able to cross the membrane, can interact with the DNA inducing mutations. In addition, it has been demonstrated the direct correlation between oxidative stress and inflammation, as ROS can activate NF-kB, a key factor for inflammation [163]. NF-kB is a transcription factor which regulates the expression of many proteins involved in inflammatory processes (cytokines, adhesion molecules, inflammatory enzymes and several receptors) [164].

Our organism is able to protect cells from the overproduction of ROS by different antioxidant defense mechanisms. The defense systems could be: enzymatic and non-

enzymatic, in the first case, the radical removal happens with a catalytic mechanism, while the non-enzymatic defenses have heterogeneous working mechanisms, they can bound and sequestrate pro-oxidant molecules, or they can act as radical scavenger; intracellular and extracellular depending on whether the site of their action is in or out of the cell. The defense systems can be classified as follows:

- enzymatic antioxidants like SOD, CAT, GPx, GR, GST, NQO1, HO-1 and TRXr;
- intracellular non-enzymatic antioxidants: coenzyme Q and Glutathione (GSH);
- extracellular non-enzymatic antioxidants:
    - metal chelating agents: albumin, uric acid, metallothionein, ceruloplasmin, transferrin;
    - non-enzymatic scavengers: ascorbic acid, tocopherol, carotenoid, and polyphenols.

3.4.1 Superoxide dismutase (SOD)

SOD is an enzyme belonging to the oxidoreductase class and catalyzes the reaction where superoxide is converted to H_2O_2 [165]:

$$2\ O_2^{\bullet -} + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

For its catalytic activity SOD exploits metallic ions, mainly manganese (Mn), copper (Cu) and zinc (Zn). Several isoforms of this enzyme exist which are distinguishable for their localization and cofactor (metallic ion). SOD1 localized in the cytoplasm and is known as Cu/Zn SOD, mitochondrial SOD2 is known as MnSOD and SOD3 is the extracellular form [165].

3.4.2 Catalase (CAT)

CAT catalyzes the reduction of H_2O_2 to H_2O and O_2 with the follow reaction [166]:

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

CAT is localized in peroxisomes, citoplasm, and mitochondria. Its activity is reduced when low levels of H_2O_2 are present meanwhile it tends to increase with increasing H_2O_2 levels [167]. Cytosolic or mitochondrial CAT overexpression showed protective effects against oxidative injury [168].

3.2.3 Glutathione Peroxidase (GPx), Glutathione Reductase (GR) and Glutathione-S-transferase (GST)

GPx is together with CAT a key enzyme for H_2O_2 detoxification. It is a selenium-dependent enzyme and uses GSH as electron donor for H_2O_2 or hydroperoxides reduction [167, 169]. During the reaction GSH is oxidized (GSSG) with the formation of a disulfur bridge between two molecules of GSH :

$$H_2O_2 + 2GSH \xrightarrow{GPx} 2H_2O + GSSG$$

$$2GSH + LOOH \xrightarrow{GPx} GSSG + LOH + H_2O$$

GSSG can be reduced by GR with a mechanism NAPH-dependent that led to the reduction of GSSG to GSH and to the oxidation of NADPH to $NADP^+$:

$$GSSG + NADPH \xrightarrow{GR} 2GSH + NADP^+$$

GSTs are a class of enzymes which catalyzes the conjugation of GSH to electrophilic compounds :

$$GSH + Xenobiotic \xrightarrow{GST} GSX +$$

The conjugation with GSH is also the first step of the mercapturic acid pathway, maybe the main detoxification process. Several cytosolic isoforms of GST have been described and grouped in four classes on the basis of their substrate specificity and amino acid sequences: α, μ, π, τ.

3.4.4 GSH

GSH is the most abundant cellular non-enzymatic antioxidant. It is a tripeptide, synthesized at hepatic level, consisting of glutammic acid, L-lysine and L-glycine, and represents the main cellular thiol. GSH synthesis starts from the formation of a γ-dipeptide by γ-glutamilcystein synthetase, after this the dipeptide bounds glycin by γ-glutathione synthetase [170].

At cellular level, glutathione can be reduced (GSH) or oxidized (GSSG), contributing to the maintenance of optimal redox state.
GSH is particularly concentrated in organs which are more exposed to toxics, as liver and kidney.
GSH, GPx and GR represent a key system for ROS detoxification thanks to continue re-cycling of GSH [166, 171].

3.4.5 Thioredoxin Reductase (TRXr), NAD(P)H-quinone oxidoreductase (NQO1), Heme Oxigenase 1 (HO-1)

Thioredoxin/Thioredoxin Reductase constitutes another important system for H_2O_2 detoxification. In particular TRXr acts using NADH to restore the oxidized thioredoxin in the reduced form. The reduced form of thioredoxin is needed for the reduction of H_2O_2 to H_2O by thioredoxin peroxidase [172].

NQO1 is a flavoprotein that catalyzes the reduction of quinone or other similar xenobiotics in a two steps reaction with the involvement of two electrons. The enzyme uses as cofactor NADH or NADPH indistinctly. The reaction mechanism has been named as 'ping pong'. In particular, NQO1 reduces quinone to hydroquinone in a two step reaction with the transfer of two electrons, so avoiding the formation of a reactive intermediary semiquinone [173], which could reacts with O_2 leading to ROS formation [174].

HO system consists of different isoforms and HO-1 is the inducible form in oxidative stress condition [144]. These enzyme family has a key role in the heme catabolism. The enzyme cleaves heme ring and converts it to biliverdin using NADH or NADPH. The biliverdin is then converted into bilirubin by biliverdin reductase. Moreover bilirubin, the final product of heme catabolism showed antioxidant capacities.

3.4.6 Modulation of the endogenous defense system by Nrf2/ARE pathway

The nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2)/ antioxidant responsive element (ARE) pathway is the main regulator of cytoprotective responses to oxidative and electrophilic stress [175, 176]. Nrf2, under basal condition, has a short half life within 7-15 minutes because it is rapidly degradated [177, 178].

In normal (basal) conditions Nrf2/ARE pathway is repressed by the sequestration of Nrf2 in the cytoplasm where it is continuously degradated. In cytoplasm Nrf2 binds the cysteine-rich Kelch-like ECH associated protein 1 (Keap1), which represents its major negative regulator and that forms a RING E3-ubiquitin ligase with Cullin (Cul)3/Rbx1, so targeting the transcription factor for ubiquitinantion and proteasomal degradation. During oxidative stress or in response to electrophiles compounds, redox modification of cysteine residues in Keap1 protein happens, the Nrf2 ubiquitination is prevented, and the transcription factor is accumulated into nucleus activating transcription of nearly 500 genes, encoding for antioxidants, phase II enzymes and anti-inflammatory proteins (Fig. 3.5). On the other hand, activation of intracellular kinases, such as MAPK, PI3K or PKC, can in turn phosphorylate Nrf2, influencing cytosol-nucleus trafficking or Nrf2-nuclear stability [179].

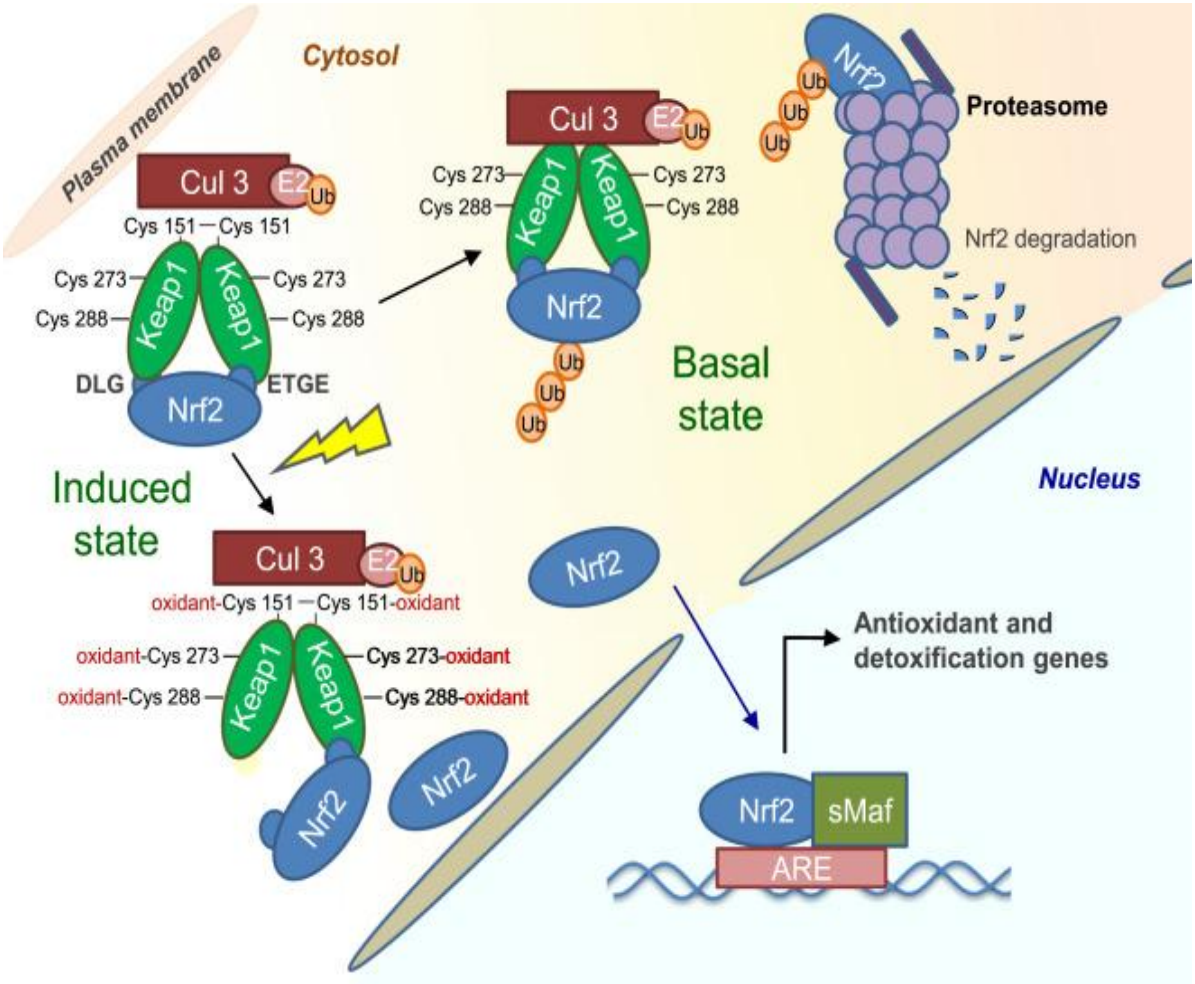


Figure 3.5. ***Nrf2/ARE pathway in the basal and induced state*** [180].

Moreover, using FLIM-FRET-based system the researchers were able to study the dynamism of the Keap1-Nrf2 interaction in live cells [181]. Data from this study revealed that in basal state Keap1-Nrf2 complex exists in two distinct conformations: an 'open conformation' in which only the EDGE (high affinity) motif bounds Keap1, and the 'closed conformation' where both EDGE and DLG motifs bound Keap1 dimer. Deeping the dynamism behind what emerged is that in the basal state Nrf2 protein first binds the free Keap1 through EDGE motif (open conformation) [181, 182], after this the closed conformation is formed through the binding between Nrf2 DLG motif and the other member of Keap1 dimer. Nrf2 in closed conformation is located in the orientation favoring ubiquitination by Keap1-dependet E3-ligase [183, 184], so

Nrf2 is released and degraded from proteasoma, and the free Keap1 can again bound a new Nrf2.

Nrf2 is a protein that consist of 605 aa and possesses seven functional domains, denominated as Neh1-7 [185]. Neh1 has the bZip DNA binding and heterodimerization domain, and thanks to this domain Nrf2 can form the heterodimer with small Maf transcription factor. The heterodimer Nrf2/Maf is responsible for binding to ARE sequence, localized on the promoter of cytoprotective genes. Neh2 domain is functional for Nrf2 binding to Keap1 protein, containing the motifs through which monomeric Nrf2 binds the dimeric Keap1, named 'DLG' and 'ETGE' motif [181]. Neh3 domain binds to a Nrf2 co-activator, the chromo-ATPase/helicase DNA binding protein family member CHD6 [186]. Neh4 and Neh5 domains synergize to bind CBP, another Nrf2 co-activator [187]. Other negative regulatory domains are Neh6, which mediates Keap1-indipendent Nrf2 degradation [188], and Neh7 that is responsible for Nrf2 interaction with retinoid X receptor alpha [189].

On the other side, Keap1 is a 624 aa protein containing [181]:

a N-terminal region; a BTB domain that allow Keap1 to do a homodimer and to interact with Cul3; an intervening region, the cysteine-rich domain; the Kelch domain useful for Nrf2 binding; and the C-terminal region.

Regarding the Nrf2 export from the nucleus, the phosphorylation of Tyr568 by the kinase FYN, which is in turn activated by glycogen synthase kinase 3β (GSK-3β) lead to a strong binding with the nuclear exporting protein CRM1 [188]. Another mechanism is the binding between Nrf2 and the E3 ubiquitin ligase β-TrCP (βtransducing repeat-containing protein) via the Nrf2 Neh6 domain, which can be phosphorylated by GSK-3β, leading to Cullin 1 (Cul1)-dependent ubiquitination and degradation of Nrf2 via a Keap1-independent way [177, 188]. In turn, GSK3β can be inhibited through phosphorylation, which can be catalyzed by Akt, ERK and PKC kinases.

3.3 Oxidative stress and CVD

Several are the cardiovascular sources of ROS. Level and activity of xanthine oxygenase are increased in heart failure as well as that of NAD(P)H oxidase [154, 190].

The implication of ROS in the pathogenesis of CVD is particularly prominent in endothelial dysfunction, where the vascular microenvironment is altered. Vascular endothelium plays an active role in several mechanisms, such as regulation of vascular tone, inflammation, thrombosis, platelet activity and atherosclerosis [154].

Substances like NO, endothelins, and prostacyclins are crucial in the maintenance of endothelium tone [191]. The reduction in NO bioavailability leads to endothelial dysfunction promoting platelet adhesion and aggregation.[154].

Several mechanisms are implicated in the reduction of NO levels, including increased degradation, altered functionality of eNOS, reduced expression of eNOS [192]. Furthermore ROS levels influences NO bioavailability, because ROS can oxidize NO to $ONOO^-$ and tetrahydrobiopterin to dihydrobiopterin, so leading to eNOS dysfunction and further ROS release [191]. As proof of NO importance for endothelial health, the up-regulation of hydrobiopterin and as consequence, NO amount, has been shown to improve endothelial function and decrease superoxide production [193].

Vascular oxidative stress has also been linked to hypertension in several animal models [194-196]. Patients with essential hypertension, renovascular hypertension, malignant hypertension and pre-eclampsia showed an increased ROS yield [197-199]. ROS production has been evidenced in different types of vascular cells as endothelial, adventitial and vascular smooth muscle cells, mainly derived from NAD(P)H oxidase activity[200]. The effects of some pharmaceutical agents (for example angiotensin 1 receptor blockers) acting on cardiovascular system have been attributed to their direct inhibitory activity on NAD(P)H oxidase enzyme. In addition, the outputs of more common antihypertensive agents (ACE-inhibitors, Ca^{2+} channel blockers, β-adrenergic blockers ecc.) could be related to their capacity to diminish vascular oxidative stress [201-203].

As mentioned before oxidative stress leads to endothelial dysfunction so affecting CVD incidence. In this context, ROS can induce a direct injury modifying cellular components, altering key proteins for contraction and diminishing NO availability [204]. During myocardial ischemia, the amount of ROS produced in the reoxygenation step is the crucial cause of the reperfusion injury, and they can damage directly cellular constituents or indirectly through the induction of local inflammation [205]. Moreover ROS can influence and alter several cellular pathways so modifying cellular function [206]. For example, H_2O_2 has been shown to activate the protein kinase Akt in VSMC, leading to hypertrophy [207]; in cardiomyocytes H/R induces activation of p38 and JNK pathways, involved in apoptotic cellular death [208].

3.4 Sex-gender differences in oxidative stress

The importance to deepen potential sex-gender differences in oxidative stress starts from the assumption that oxidative stress is implicated in many diseases with incidence, prevalence, symptoms, severity and outcomes that differ in males and females. Unfortunately, few studies have investigated the differences in oxidative stress or antioxidant defenses due to sex-gender.

Biomarkers for oxidative stress were reported to be higher in young men than in young women [209]. Likewise using vascular cells derived from men or women, the susceptibility to oxidative stress was greater in those from men [210]. Data from clinical and experimental studies evidenced a more pronounced antioxidant capability in females than males [211].

Differences in expression and activities of antioxidant enzymes were analyzed in males and females. Regarding SOD activity, one study showed a higher activity in female hearts but other investigations did not show uniform results, however it has been proposed that the differences depend on the tissue [209, 212-214]. In animals, SOD

levels decreased after castration so implicating a role for sex hormones in the regulation of SOD activity [212].

Catalase activity was found to be similar in both sexes as analyzed in different tissues [213, 215], so suggesting that H_2O_2 detoxification from catalase is not affected by gender or sex hormones.

Several studies confirmed a minor activity for GPx enzyme in females [209, 212, 213], and no significant changes in males and females after castration [212].

On the other side analyses of the NADPH-oxidase levels, guilty of the great production of superoxide, showed discrepancies between sexes.

Different studies were consistent in showing a higher expression of Nox1 and Nox4 in males than females, which can explain the differences in superoxide levels between sexes [216-218]. At the same time, no differences were evidenced for Nox2 or the three isoforms of SOD (SOD1, SOD2, SOD3) [216, 217, 219]. It seems that the greater basal oxidative stress seen in men could be mainly due to a higher superoxide production rather than a minor rate of detoxification.

Apart from this, the major differences in antioxidant properties between males and females could be attributed to the antioxidant action of E2. It can act as scavenger of free radicals thanks to the presence of a phenolic hydroxyl group [212]. Moreover E2 also demonstrated to increase the gene expression of SOD2 via MAP-kinase signaling pathway [220]. Oxidative stress level was also analyzed in pathological conditions.

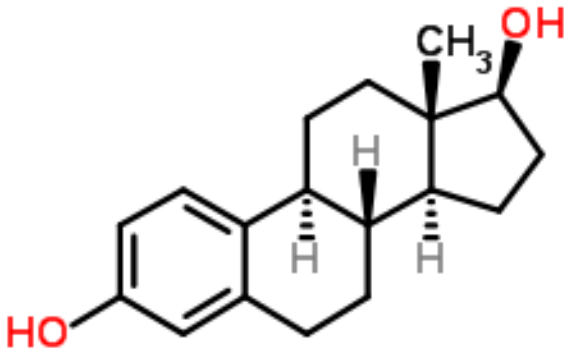


Figure 3.6. ***Chemical structure of 17β-estradiol***

In spontaneously hypertensive rats, males had higher superoxide levels than females due to a higher NADPH-oxidase activity, and, in line with this, males demonstrated lower levels of NO because it was degraded by superoxide [221]. A study on coronary artery disease comparing men and post-menopausal women showed that the post-menopausal group women had oxidative stress level almost three times higher than men [222]. Furthermore, it has been shown that ovariectomyzed females have an increase in NADPH-oxidase activity and treatment with E2 led to normal levels [216]. This suggests a role for the hormone in mediating NADPH-oxidase activity.

In conclusion, what emerged from these studies is that sex-gender differences in susceptibility to oxidative stress are principally due to a higher production of reactive species rather than a low antioxidant levels, and E2 seem to have a role in the orchestration of cellular redox balancing.

4.SULFORAPHANE AND CARDIOVASCULAR DISEASE

4.1 Glucosinolates

In the XIX century the first glucosinolate has been isolated from *Sinapis alba* [223]. Later more than 120 different glucosinolates were described, mainly derived from plants belonging to *Brassicaceae* [224].

The family of *Brassicaceae* includes more than 350 genera and over 3000 species. Common edible plants from this family are: broccoli, brussels sprouts, cabbage, capers, cauliflower, mustard, radishes.

The pungent taste of these vegetables is due to the reaction products of myrosinase enzyme.

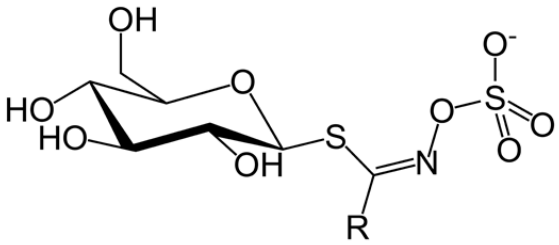


Figure 4.1. ***Glucosinolate chemical structure.***

The glucosinolates are chemically a β-thioglucoside N-hydroxysulfate (Fig. 4.1) [225]. Their basic structure includes a solphonated oxime, a β-D-thioglucose group, and a variable amino acidic side chain R, which can derive from methionine, phenyalanine, tryptophan or branched amino acids [226]. In plants, the glucosinolates are associated, but physically separated, to β-thiogluosidase enzymes, known also as myrosinases [227].

Substrate and enzyme come in contact after plant cells injury (e.g. by chewing and cutting) and the consequence is a rapid hydrolysis with the release of glucose and instable aglycones, these last spontaneously rearrange into reactive compounds (Fig.4.2). In particular, all biological activities can be attributed to the glucosinolate hydrolysis products, and among these isothiocyanates are the most studied. Humans do not have myrosinase enzymes but glucosinolates can be converted into isothiocyanates thanks to gut microbiota. Reduction or abnormalities in the gastrointestinal microbiota reduce the bioavailability of the isothiocyanates [228].

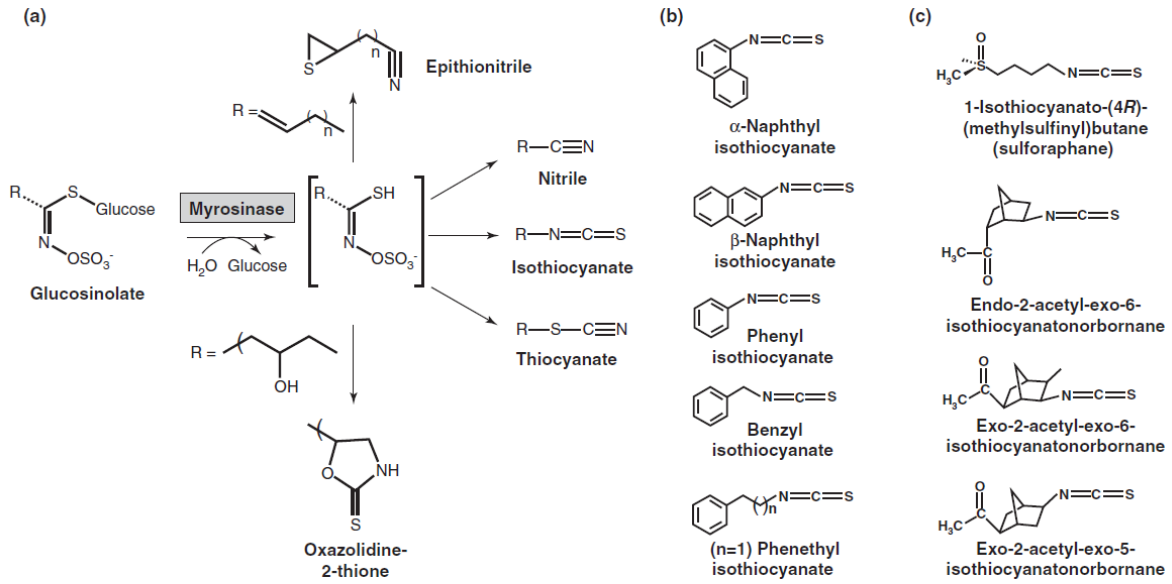


Figure 4.2. ***Myrosinase reaction products.*** *(a) Glucosinolate hydrolisis products. Depending on reaction conditions and glucosinolate side chain (R), a range of products can be formed. (b) Chemical structure of aromatic isothiocyanates with anticarcinogenic activity. (c) Chemical structure of isothiocyanate sulforaphane and its synthetic norbonilic derivates.*

4.2 Sulforaphane

Sulforaphane (SF) (1-isothiocyanate-(4R)-(methylsulfinyl) butane) [229] derives from the hydrolysis of glucoraphanin, the main glucosinolate presents in broccoli (Fig 4.3) [230, 231]. It is the most studied and well described isothiocyanate present in *Brassicaceae* [232].

In particular, SF induces phase II enzymes [233], demonstrating efficacy in a range of cell lines. This group of enzymes, also known as drug-metabolizing enzyme, are responsible for detoxifying electrophiles and oxidants. Examples of enzymes belonging to this group are GST, GR and NQO1. Moreover, the Nrf2/ARE pathway has been demonstrated and widely studied as principal mechanism for SF bioactivities, which in turn up-regulate antioxidant and phase II enzymes expression [233].

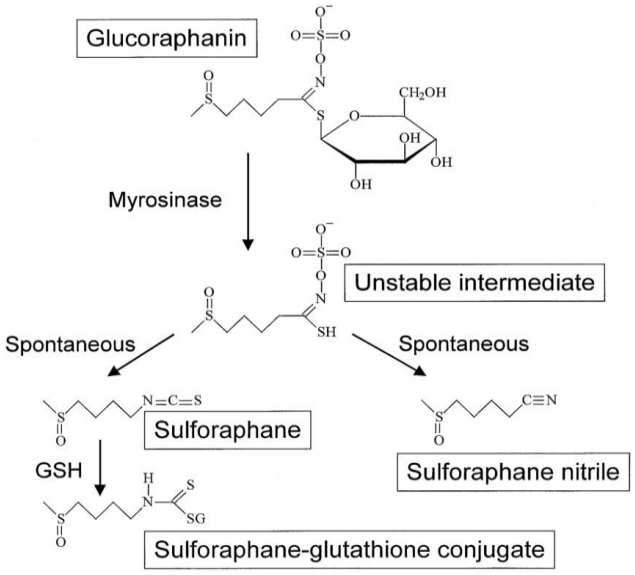


Figure 4.3. ***Schematic reaction between glucoraphanin and myrosinase.*** *Modified from* [234]

4.3 Sulforaphane bioavailability

The isothiocyanates in humans are metabolized through the mercapturic acid pathway. In the human studies, the first critical step was the identification of analytical instruments able to quantify and identify the isothiocyanates and their metabolites. All together they can be quantified by 1,2 benezenedithiol-based cyclocondensation [235] or individually by liquid chromatography coupled with mass spectrometry [236], and these instruments were used in pharmacokinetics studies. A study that compared a high dose (52 µmol) versus a low dose (16 µmol) of SF [237] showed a rapid increase in SF and its metabolite plasmatic concentrations, with the maximum peak at 1.5 h and 2 h for low and high dose, respectively. The AUC (area under the curve) and maximum concentration (C_{max}) resulted to be dose-dependent, with the C_{max} of 2.2 ± 0.8 µM and 7.3 ± 2.9 µM for low and high dose, respectively. Shapiro et al. carried out a phase I clinical study to evaluate security, tolerance, and pharmacokinetics of broccoli sprout extracts containing glucosinolates or isothiocyanates. The study demonstrated absence of toxicity and high variability in glucosinolate metabolism. The participants were divided into four groups: placebo; 25 µmol of glucosinolates; 100 µmol of glucosinolates; 25 µmol of isothiocyanates. The analysis of 32 different markers, including those for thyroid (TSH, T3, T4) and hepatic functionality (transaminases), did not evidence systemic adverse effects. The dithiocarbamates total excretion was (expressed as dose fraction) 17.8 ± 8.6% e 11.7 ± 19.6% for low and high dose of glucosinolates, respectively, finding a high interindividual variation. In the group who received isothiocyanates, the total excretion was 70.6 ± 2.0%, with few interindividual variation [228].

These and other studies [238-240] clearly point out that the intake of glucosinolates, rather than isothiocyanates, is associated with lower bioavailability, slower excretion and more interindividual variation. The most likely cause in interindividual variations is the interconversion of the glucosinolates to isothiocyanates by gut microbiota, as also evidenced by Li et al., supporting the importance of gut microbiota in the degree of

glucosinolates hydrolysis [241]. Atwell et al. suggested that another factor influencing SF absorption could be the food matrix and meal composition. Whole broccoli sprouts contain minerals, nutrients and phytochemicals that could enhance SF transport through cellular membranes. Therefore, raw sprouts have more fiber, slowing gut transit and increasing the contact time with gut epithelium surface [239].

4.4 Nrf2/ARE pathway and sulforaphane

4.4.1 Sulforaphane direct effects

The central carbon in the isothiocyanate group of SF is electrophilic and can react with the cysteine residues present in Keap1 protein. It has been evidenced that SF is able to directly interact with critical Keap1 cysteine residues, blocking Nrf2 ubiquitination and degradation and increasing Nrf2 translocation to the nucleus [242].
In particular, C151 has been implicated as one of the site responsible for SF effects. Indeed, when C151 was mutated to a Ser, the induction of Nrf2 by SF was repressed [243]. C151 residue involvement was also confirmed by other groups using a biotin-switch technique and again mutagenesis experiments [244]. Studies with mass-spectrometry approach evidenced that, depending on the experimental conditions, besides C151 other residues can be involved in SF-Keap1 interaction [245, 246]. Baird et al., with their FILM-FRET findings, hypothesized that inducers such as SF tend to stabilize Nrf2 in the closed conformation [181]. In particular, SF interaction with cysteine residues, that leads to a conformational change of Keap1-Nrf2 complex, may alter the positioning of Nrf2 in respect to the ubiquitination machinery so that it can no longer be ubiquitinated and targeted for proteosomal degradation. However, in this conformation Nrf2 is not released from Keap1, so acting as a suicide substrate to inactivate Keap1 and new free Keap1 is not regenerated. The newly synthesized Nrf2 will not find free Keap1 for its cytoplasmic sequestration, so it can translocate to the nucleus and activate gene transcription. This hypothesis can also explain how in

absence of new translation, using a translation inhibitor, also in presence of inducers, Nrf2 did not induce cytoprotective genes expression [247-249].

4.4.2 Sulforaphane indirect effects

Different kinases can modulate Nrf2/ARE pathway, including MAPK, PKC and PI3K [250]. SF demonstrated to activate a variety of intracellular kinases, which in turn phosphorylate Nrf2, influencing cytosol-nucleus trafficking or Nrf2-nuclear stability [250, 251]. Indeed, Kensler et al. demonstrated that phosphorylation of Nrf2 at serina 40 can potentiate its activation [252].

Leoncini et al. deepened the mechanism involved in SF effects using neonatal rat cardiomyocytes [253]. The results evidenced SF-mediated increasing in Akt and ERK 1/2 phosphorylation, which are kinases implicated in cardiac proliferation and survival. These kinases in turn augmented Nrf2 activation and ARE binding. The use of specific kinase-inhibitors showed the involvement of PI3K/Akt pathway in SF-mediated cytoprotective genes induction via Nrf2/ARE pathway.

4.4.3 Induction of long-term effects

As mentioned before in this chapter, the ingestion of broccoli or SF leads to a peak in plasmatic concentration after few hours, with a rapid clearance from the body. However, several studies evidenced that SF can induce long-term protective effects against oxidative injury. In an astrocyte cellular model, a 4 h treatment with SF triggered an increase in mRNAs of HO-1 and NQO1 up to 24 h later, with the levels of the corresponding proteins elevated up to 48 h [254]. Bai et al. treated diabetic mice with SF for three months and showed as the treatment was able to reduce the incidence of diabetic cardiomyopathy at the end of the treatment and also after six

months [255]. Angeloni et al. also showed as SF treatment can cause long-term protection against oxidative stress, reducing the levels of intracellular ROS and DNA fragmentation and increasing the expression of cytoprotective enzymes in neonatal rat cardiomyocytes [256].

4.5 Sulforaphane in Hypertension

As mentioned in the previous chapter, oxidative stress has been demonstrated to play a key role in the etiopathogenesis of hypertension.

In an animal model of spontaneously hypertensive rats, diet containing broccoli sprouts high in glucoraphanin was able to decrease oxidative stress. Moreover, SF, the main glucoraphanin derivative, demonstrated to improve blood pressure [257]. In vascular smooth muscle cells derived from hypertensive rats, SF-treatment (0.05-1 μM) reduced oxidative injury up-regulating phase II enzyme, increasing basal level of reduced glutathione. These changes have been correlated with an improved endothelial relaxation and blood pressure [258, 259].

4.6 Sulforaphane in Atherosclerosis

Atherosclerosis represents an important risk factor for CVD. It is usually associated with a chronic inflammation status in the arterial wall. Inflammation status increases the expression of cell adhesion molecules as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), both associated with an increase in VSMC proliferation. In several studies SF-treated VSMC (1-5 μg/mL) showed reduced mRNA and protein levels of cell adhesion molecules, after exposure to an inflammatory cytokine as TNF-α, and so suppressing inflammation within the atherosclerotic lesion [260-262].

It is well known that an important factor for the development of atherosclerosis is the plasmatic level of cholesterol and in particular its distribution between HDL and LDL cholesterol. Endothelial lipase is an enzyme belonging to the triacylglycerol family and it is responsible for the reduction in HDL levels. Two different studies carried out in HUVEC cells, indicated SF as a powerful agent in contrasting cholesterol level alterations at endothelial site. In particular SF was able to reduce the increase TNF-induced of endothelial lipase [263], and to counteract oxidative injury induced by oxidized-LDL, increasing the expression of HO-1 and GSH content [264].

Platelet aggregation plays a role in the incidence of atherosclerosis, and the potential effects of SF were investigated. SF demonstrated to inhibit human platelet aggregation in a dose-dependent manner preventing the activation of PI3K-Akt pathway [265, 266]. SF showed to clearly decrease glycoprotein IIb/IIIa activation and thromboxane A2 formation [266].

4.6 Sulforaphane in cardiac Ischemia/Reperfusion injury

I/R injury is well known to be associated with the pathology of important CVD such as stroke or heart attack [267]. The damage that can occur in the two phases, ischemia and reperfusion, is often irreversible for cells or tissues. However, a third phase of this pathological process exists, which is the post-reperfusion phase when tissue remodeling and adaptation occur. Increased ROS levels seem to play a key role in all these phases [267]. Cardiomyocytes treated with SF (0.1-5 μM) and then subjected to I/R injury showed improved cellular viability and Bcl2/Bax ratio associated with increased SIRT1 expression and reduced cellular apoptotic death and expression of endoplasmic reticulum stress-related apoptosis proteins. SF-containing broccoli provided cardioprotective effects against I/R injury, ameliorating post-ischemic ventricular function and reducing the myocardial infarct size, in rats [229, 268]. These effects were associated with higher Nrf2 activity. Regarding cardiac remodeling, SF

demonstrated to reduce fibrosis and to induce a favorable redox environment (increased HO-1) switching the kinase balance toward pro-survival kinases (ERK 1/2 and Akt) so mitigating the progression of cardiac remodeling after the infarction.

4.7 Sulforaphane protection from Diabetes complications

Diabetes pathology reached an epidemic characteristic worldwide. Diabetic patients are prone to develop CVD and the diabetic complications involve micro- and macrovascular impairment. The most common diabetic complications include retinopathy, neuropathy, nephropathy, diabetic cardiomyopathy. A chronic high-glucose status is linked with an increased production of ROS and all contribute to oxidative and inflammatory damage, increasing the possibility to develop a diabetes complication.

In diabetic nephropathy, which results in premature death associated with CVD and kidney collapse, SF helped to counteract the pathological deficits thanks to its antioxidant and antinflammatory activities, with a dual behavior on Nrf2 and NF-kB [269]. In experimental model of streptomycin-induced nephropathy, SF ameliorated renal function and metabolic indices, but only in animals with functional Nrf2/ARE pathway, indicating a key role of this pathway [270]. Streptomycin-induced diabetic type I was associated with increased ROS and inflammation which led to fibrotic response after 6 months of diabetes. In this model SF completely reversed diabetes-induced changes [271].

Diabetic cardiomyopathy is the complication which account the most deaths for diabetes [272]. Researchers investigated whether SF can prevent cardiomyopathy in different diabetic models. In db/db type II diabetic mice, administration of SF (0.5 mg/kg) or a high dose of broccoli sprout extracts (equivalent to 1 mg/kg) prevented hypertrophy, cardiac dysfunction and fibrosis diabetes-induced, and the effects were associated with an improved Nrf2 activity [273]. Also in a model of high-fat diet-

induced type II diabetes mellitus, SF demonstrated to prevent lipid accumulation, significantly improving cardiac function, fibrosis, oxidative stress and inflammation [274].

4.8 Conclusion

Oxidative stress plays a pivotal role in contributing to the development of cardiac pathologies. SF is an isothiocyanate found in edible brassica vegetables, which demonstrated to act as an indirect antioxidant. The main mechanism related to SF-induced protective effects is the activation of Nrf2/ARE pathway and its downstream target genes. Bioavailability studies evidenced that its absorption can be influenced by dietary form, i.e. fresh broccoli sprouts (myrosinase-active) showed the highest bioavailability rather than broccoli with inactive myrosinase (as example broccoli overly cooked). Regarding the amount of SF that should be achieved for CVD prevention, it has been demonstrated that 5 µM SF protects cardiomyocytes from oxidative damage and that 2.8 µmol/kg of SF prevents CVD in diabetic mice. If we applied the conversion formula between animals and humans [275], a man with body weight of 70 kg is supposed to consume a SF dose of 19.6 µmol per day, i.e. 12 g per day of fresh broccoli. This is much lower than the dosage used in studies for cancer prevention (150 µmol per day) which showed no toxic effects [236, 276].

In conclusion, diet habits containing Brassicaceae as source of SF could be helpful in the prevention of CVD. The amount of SF which showed cardioprotective effects is easily achievable. However, it could be opportune to perform clinical trials to clarify the effects and the optimal dose for SF in the prevention of CVD.

5. AIM

Cardiovascular disease continues to be the most common cause of death worldwide [1]. Several studies demonstrate as women with myocardial infarction have a better prognosis than men [277-279], but this difference is reversed after menopause [280]. Furthermore, the development of coronary artery disease occurs ten year later in women than men [2]. Thus, it has been suggested that sexual steroid hormones can play a role in the protection from CVD in women, so implying a sex-gender dependent effect. 17β-estradiol (E2) is the principal female estrogen, major product of ovary secretion, which owns the strongest potency and has the capability to bind all subtypes of estrogen receptors. It has been demonstrated that E2 provides protection on bone density, central nervous and cardiovascular system [281]. Regarding cardioprotection, in several studies it has been investigated the potential role of E2 in cardiac cell survival against different insults. E2 reduces cardiomyocytes apoptosis in vivo and in vitro through estrogen receptors and phosphoinositide-3-kinase (PI3K)/Akt signaling pathways [25, 26]. In cardiomyocytes, E2 is also able to differently modulate prohypertrophic (class I) and antihypertrophic (class II) histone deacetylase proteins through the binding to ERβ, thus counteracting cardiac hypertrophy [28]. Short-term treatment with E2 has shown to be able to improve heart function after I/R injury, and using a NOS inhibitor this effect was reversed, so suggesting the contribution of NO production via ER activation in this process [85].

It has also been shown that sex-gender can influence the pharmacokinetics and pharmacodynamics of a drug [2], and as a consequence it can influence the response to cardiovascular medications [4, 282, 283]. In this context, we hypothesized that sex hormones could also differently influence the preventive/protective effects of nutraceutical compounds in males and females. To the best of our knowledge no studies have been carried out to test this hypothesis.

Among nutraceutical compounds, sulforaphane has been highly investigated for its anticancer activity [284]. Sulforaphane is a dietary isothiocynate, mainly found in brassica vegetables, produced after the breakdown of its precursor glucoraphanin. Recently it has been reported that SF has got cardioprotective activity in different in vitro and in vivo models [285-287]. The main mechanism involved in SF protective effects is the activation of Nrf2/ARE signaling pathway [253] and consequently the up-regulation of the antioxidant defense system. SF elicits many other biological activities beyond the antioxidant effects, as anticancer, anti-inflammatory, antiglycative and neuroprotective properties [284, 288-291].

Aim of this PhD thesis was to explore the modulatory effect of E2 on the cardioprotective activity of SF against oxidative stress in primary cultures of rat cardiomyocytes against oxidative stress by analyzing antioxidant/survival pathways and investigating the involvement of ERs and GPR30.

Part of this thesis was conducted in the Institute of Gender in Medicine-Charité-Universitätsmedizin, in Professor Vera Regitz-Zagrosek's laboratory in Berlin.

As experimental model we used primary cultures of rat cardiomyocytes and rat cardiomyoblast cell line. Cells were pre-treated with SF (0.1-5 μM) and/or E2 (10-500 nM) and oxidative stress was induced exposing cells to 100 μM of H_2O_2. We evaluated cell viability by MTT and LDH assays, oxidative stress injury by DCFH-DA assay and immunofluorescence staining, glutathione levels by MCB assay, antioxidant/phase II enzymes, Nrf2 and ERs gene expression by RT-PCR, Nrf2 activity by ELISA-based kit and Western Blotting, and the phosphorylation of ERK 1/2 and Akt kinases by Western Blotting.

6. MATHERIALS AND METHODS

6.1 Chemicals

PhosSTOP was purchased from Roche Diagnostics (Mannheim, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide (MTT), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), H_2O_2, dimethyl sulfoxide (DMSO), monochlorobimane (MCB), phosphate-buffered saline (PBS), bovine serum albumine (BSA), Dulbecco's modified Eagle's medium (DMEM) F-12, DMEM, fetal calf serum, horse serum, Charcoal-stripped fetal calf serum, gentamicin, sodium pyruvate, inositol, amphotericin B, collagenase IA, 17β-estradiol (E2), LY294002 (LY), PD98059 (PD), 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H–pyrazoledihydrochloride hydrate (MPP), 2-phenyl-3-(4-hydroxyphenyl)5,7-bis(trifluoromethyl)-pyrazolo[1,5-a]pyrimidine,4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a] pyrimidin-3-yl] phenol (PHTPP), sodium fluoride, sodium pyrophosphate, sodium orthovanadate, methylglyoxal (MG) and all other chemicals of the highest analytical grade were purchased from Sigma Chemical (St. Louis, MO, USA), unless otherwise stated. 4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and (±)-1-[(3aR*,4S*,9bS*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]- ethanone (G1) were purchased from TOCRIS (Wiesbaden-Nordenstadt, DE). KB5 were kindly provided from Novartis (Cambridge, MA, USA). E2 was dissolved in DMSO at a concentration of 10 mmol/L and kept at –20 °C until use. D,L-sulforaphane (SF) (LKT Lab., Minneapolis, Minn., U.S.A.) was dissolved in DMSO at a concentration of 10 mmol/L and kept at –20 °C until use.

6.2 Cell cultures and treatments

Primary cultures of neonatal Sprague-Dawley rat cardiomyocytes were prepared as previously described [292]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996) and approved by the Ethics Committee of our institution. Briefly, rats were sacrificed and the ventricles were placed in a special medium (946 mg /100 mL Ham-F10, 93 mM KH_2PO_4, 38.5 mM Na_2HPO_4, 14.2 mM $NaHCO_3$ and 0.5 mM inositol) and finely minced. The special medium was then replaced with the trypsinization medium obtained by adding trypsin 10x (10% v/v) and collagenase IA (5% v/v) to the special medium (85%). Subsequently, tissue fragments were resuspended in complete medium (DMEM F-12 supplemented with 10% (v/v) FBS, 10% (v/v) HS, 1mM sodium pyruvate, 0.1 mg/mL gentamicin and 2.5 µg/mL amphotericin B, in a ratio of 1 mL medium / 100 mg of initial tissue. The cell suspension were "pre-plated", after filtration, in T75 flasks. After the steps of "pre-plating", the cells were counted with a hemocytometer and plated at a concentration of $5x10^5$ cells / mL and incubated at 37 °C, 5% CO_2, 95% humidity.

Cells were treated, with different concentration (0.1-5 µM) of SF and with different concentration (10-500 nM) of E2 or co-treated with SF and E2 for 24 h. The control group were treated with an equivalent volume of the vehicle alone. Oxidative stress was induced by 100 µM H_2O_2 for 30 min or by 0.5 mM MG for 24 h.

Rat embryonic cardiomyoblast-derived H9c2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, glucose content 5.5 mM) supplemented with 10% FBS, 2.5 mM glucose, 21.4 mM bicarbonate and a combination of penicillin and streptomycin (1%) in a humidified 5% CO_2 atmosphere at 37 °C. For all experiments, cells were plated at an appropriate density according to the experimental design. Before treatments, cells were starved with 3% charcoal stripped FBS in phenol-red free media. Cells were treated with 0.5 µM SF and with 10 nM E2 or co-treated with

SF and E2 for different times (30 min, 2 h). For experiments with ER agonists, cell were treated with G1 (100 or 1000 nM), PPT (10or 100 nM) and KB5 (10 or 100 nM). The control group was treated with an equivalent volume of the vehicle alone.

6.3 Cell viability and Lactate Dehydrogenase Activity Assays

Cell viability was evaluated by measuring MTT reduction, as previously reported (2). Briefly, at the end of each experiment, 0.5 mg/ml MTT were added and incubated for 1 h at 37°C. After incubation, MTT solution was removed, 200 µl DMSO were added, and the absorbance was measured at λ=595 nm using a microplate spectrophotometer (VICTOR3 V Multilabel Counter; Perkin-Elmer, Wellesley, MA, USA). Lactate dehydrogenase (LDH) activity was evaluated in the culture medium using the Lactate Dehydrogenase Activity Assay Kit (SIGMA) according to the manufacturer's instructions. Data are reported as percentage of control cells.

6.4 Immunofluorescence Staining

Cells were seeded on glass coverslips, and at the end of each experiment, cardiomyocytes were washed twice with PBS, fixed with 3% paraformaldehyde, washed with 0.1 M glycine in PBS and permeabilized in 70% ice cold ethanol. After fixing, the cells were incubated with anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) (StressMarq Biosciences, Victoria, CA, USA) or anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Subsequently, the samples were washed with 1% BSA in PBS and incubated with CY3- or FITC-conjugated secondary antibodies for 1 h at room temperature. DAPI was used for labeling nuclei. Preparations were embedded in Mowiol, and images were acquired using an Axio Scope A1 microscope (Zeiss, Oberkochen, Germany).

6.5 Intracellular ROS production assay

Intracellular ROS levels were evaluated using the fluorescent probe DCFH-DA, as previously reported [293]. At the end of each experiment, cardiomyocytes were incubated with 10 μg/ml DCFH-DA in PBS for 30 min. After removal of DCFH-DA, cells were exposed to 100 μM H_2O_2 in PBS for 30 min. Cell fluorescence was measured using 485-nm excitation and 535-nm emission with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, Perkin-Elmer). Data are reported as percentage of H_2O_2-treated cells.

6.6 Reduced Glutathione levels

Glutathione (GSH) levels were determined with a fluorimetric assay as previously reported [289]. Briefly, at the end of each experiment, culture medium was removed, and cardiomyocytes were washed and incubated for 30 min at 37°C in fresh PBS containing 50 μM MCB. After incubation, fluorescence was measured at 355 nm (excitation) and 460 nm (emission) with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, Perkin-Elmer). Data are reported as percentage of control.

6.7 Western Blotting

Preparation of nuclear and cytoplasmic fractions was performed according to the method of Bahia et al. [294]. Briefly, cells were washed with ice-cold PBS and lysed on ice using a buffer composed of 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 2.5 mM EDTA, 2 mM EGTA and 0.05% NP_4O containing mammalian protease inhibitors. The lysates were centrifuged at 1000 g for 5 min at 4°C and at 2000 g for further minute. The supernatant containing the cytoplasmic fraction was taken off and

the remaining pellet was resuspended in 100 µL of a buffer comprising: 5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 300 mM NaCl and 26% v/v glycerol. Samples were homogenized and centrifuged at 15 000 g for 20 min. The supernatant containing the nuclear fraction was retained. For evaluation of protein kinases phosphorylation, after treatments, cells were collected and homogenized in radioimmunoprecipitation assay (RIPA) buffer with a mammalian protease inhibitor mixture and PhosSTOP.

Samples were boiled for 5 min prior to separation on 4%–20% MiniPROTEAN TGX Precast Protein Gels (BIO-RAD, Hercules, CA, USA). The proteins were transferred to a nitrocellulose membrane (Hybond-C; GE Healthcare, Buckinghamshire, UK) in Tris-glycine buffer at 110 V for 90 min. Membranes were then incubated in a blocking buffer containing 5% (w/v) skimmed milk and incubated with anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-Akt, anti-Akt (Cell Signaling Technology, Beverly, MA) and anti-Nrf2, anti-lamin A (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin (SIGMA), as internal normalizers, overnight at 4°C on a three-dimensional rocking table. The results were visualized by chemiluminescence using Clarity Western ECL reagent according to the manufacturer's protocol (BIO-RAD). Semiquantitative analysis of specific immuno-labeled bands was performed using a ImageLabTM 5.2 Software (Bio-Rad, Hercules, CA).

6.8 Analysis of the Nrf2-ARE binding activity

After cellular treatments, nuclear extracts were prepared using a Nuclear Extraction Kit (Active Motif, Carlsbad, Calif., U.S.A.) according to the manufacturer's instructions. Nrf2 binding activity to ARE was measured in nuclear extracts using a TransAM™ Nrf2 Kit (Active Motif, Carlsbad, Calif., U.S.A.) following the manufacturer's recommended protocol. Briefly, nuclear extracts were added to a 96 well plate coated with double-strand oligodeoxynucleotides containing the ARE

consensus binding site (5′-GTCACAGTGACTCAGCAGAATCTG-3′). A primary antibody against Nrf2 (1: 1000), added to each well and incubated for 1 h, was detected by secondary horseradish peroxidase (HRP)-conjugated antibody, using a colorimetric substrate. The signal was recorded at 450 nm using a microplate reader xMarkTM (Bio-Rad Lab., Hercules, Calif., U.S.A.), and Nrf2-ARE binding was evaluated as optical density units at 450 nm and reported as fold increase in comparison to controls.

6.9 RNA extraction

After the treatments, total RNA was extracted using Absolutely RNA Miniprep Kit (Agilent Technologies, Palo Alto, CA), following the manufacturer's protocol. The yield and purity of the RNA were measured using NanoVue Spectrophotometer (GE Heathcare). The integrity of the RNA was determined using an Agilent 2100 BioAnalyzer (Agilent, Willmington, DE).

6.10 Analysis of mRNA expression by RT-PCR

mRNA was reverse transcribed into cDNA starting from 1 µg of total RNA using iScriptTM cDNA Synthesis Kit (BIO-RAD), following manufacturer's protocol. The subsequent PCR was performed in a total volume of 20 µL containing 5 µL (25 ng) of cDNA, 10 µL (1x) SsoAdvancedTM Universal SYBR Green Supermix (BIO-RAD), and 1 µL (500 nM) of each primer (SIGMA). The primers used are as follows. CAT 5' CAAGTTCCATTACAAGACTGAC (Forward) 3' TTAAATGGGAAGGTTTCTGC (Reverse), NQO1 5' TAGCTGAACAGAAAAAGCTG (Forward) 3' GTCTTCTTATTCTGGAAAGGAC (Reverse), SOD1 5' AATGTGTCCATTGAAGATCG (Forward) 3'

CACATAGGGAATGTTTATTGGG (Reverse), SOD3 5' AGGAATCCTTCACACCTATG (Forward) 3' GTCCTCAGAGTAAAAGGAGAG (Reverse), HO-1 5' CCTGGTTCAAGATACTACCTC (Forward) 3' ACATGAGACAGAGTTCACAG (Reverse), β-actin 5′AAGACCTCTATGCCAACAC3′ (forward) 5′TGATCTTCATGGTGCTAGG3′ (reverse) and β2-microglobulin 5′ACTGGTCTTTCTACATCCTG3′ (forward) 5′AGATGATTCAGAGCTCCATAG3′ (reverse) from Sigma. Another set of primers was purchased from Qiagen (Milano, Italy) and corresponded to the following catalog numbers: GPX-1, PPR45366A; GR, PPR46891B; GSTa3, PPR44866A; Trx, PPR51711A; GSTp2, PPR52644B GSTm1: PPR42787B. β-Actin and β2-microglobulin were used as reference genes. The cDNA amplification was started by activating the polymerase for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. A melt curve was run to ensure quality control and the generation of a single product. Normalized expression levels were calculated relative to control cells according to the $2^{-\Delta\Delta CT}$ method [295].

6.11 Statistical analysis

Each experiment was performed at least three times, and all values are represented as means ± SEM. One-way analysis of variance (ANOVA) was used to compare differences among groups followed by Bonferroni's test (Prism 5; GraphPad Software, San Diego, CA, USA). Values of $p<0.5$ were considered statistically significant.

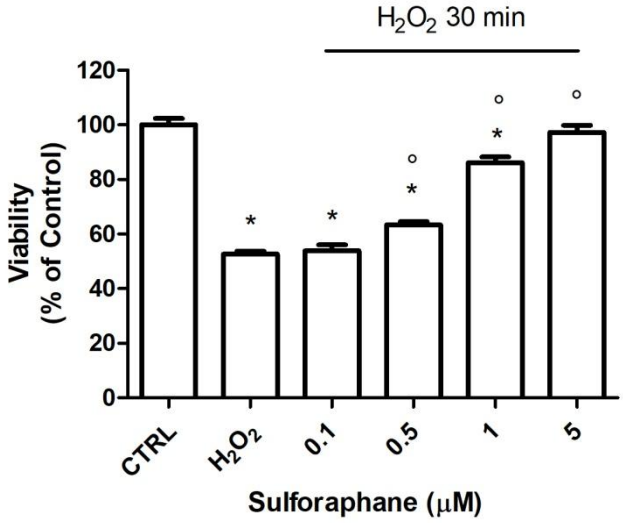
7. RESULTS

7.1 E2-enhancement of SF protective effects against oxidative stress

First aim of this project was to evaluate the potential protective effect of E2 and SF against oxidative injury. Cells were treated with increasing concentrations of E2 (10-500 nM) and SF (0.1-5 µM) and after 24 h cells were exposed to 100 µM H_2O_2 for 30 minutes (Fig. 7.1) and the cellular viability measured by MTT assay. 100 µM H_2O_2 for 30-min has been choosen as in previous studies it demonstrated to reduce cell viability of 50% in rat cardiomyocytes [293, 296]. The pre-treatment with 0.5 µM, 1 µM and 5 µM SF induced protection against oxidative damage, increasing cell viability in respect to H_2O_2-treated cells (Fig. 7.1 A). In particular, 5 µM SF was able to maintain cell viability to level comparable to control cells, meanwhile 0.1 µM SF did not protect from oxidative injury. Differently, the pre-treatment with E2 did not show any protective effect against H_2O_2-induced damage (Fig. 7.1 B).

To explore the possible effect of E2 on SF protective activity against oxidative stress we chosen the highest SF concentration that did not protect from oxidative stress (0.1 µM) and the lowest SF concentration that showed efficacy against oxidative damage (0.5 µM) (Fig. 7.1 A). Noteworthy, these concentrations are easily achievable in plasma after broccoli intake [239, 297]. Regarding E2 concentration, no concentration tested showed protective effects, so we decided to use physiological concentrations of the hormone (10 nM, 50 nM) [298, 299]. Cardiomyocytes were treated with SF (with 0.1 and 0.5 µM) in the absence or presence of E2 (10 and 50 nM). After 24 h cells were exposed to peroxide for 30 min and cell viability was evaluated by MTT assay (Fig. 7.2 A) and LDH release (Fig. 7.2 B). The co-treatments with 0.5 µM SF and E2 was able to increase cell viability with respect to H_2O_2-treated cells. On the contrary, 0.1 µM SF in the presence of E2 did not increase cell viability in respect to peroxide treated cells.

A



B

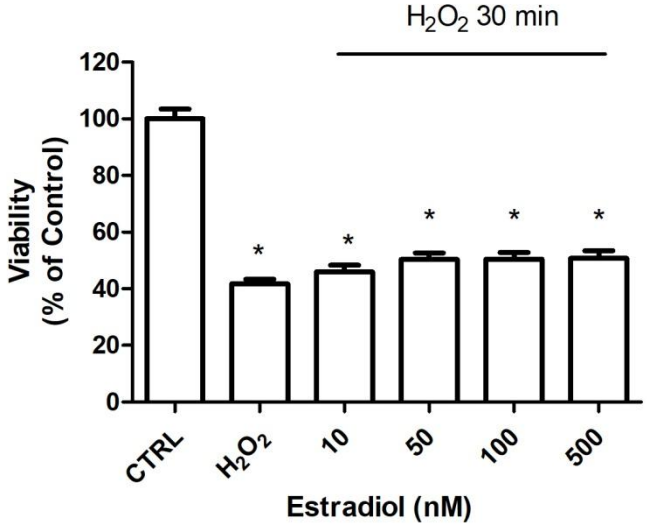


Figure 7.1. ***Effect of increasing SF and E2 concentrations against H_2O_2-induced oxidative stress in cardiomyocytes.*** *Cells were treated with SF (0.1-5 μM) (A) and E2 (10-500 nM) (B) for 24h and exposed to 100 μM H_2O_2 for 30 minutes. Cell viability was measured by MTT assay as reported in Materials and Methods. Each bar represents means±SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p<0.05 vs CTRL; °p<0.05 vs H_2O_2.*

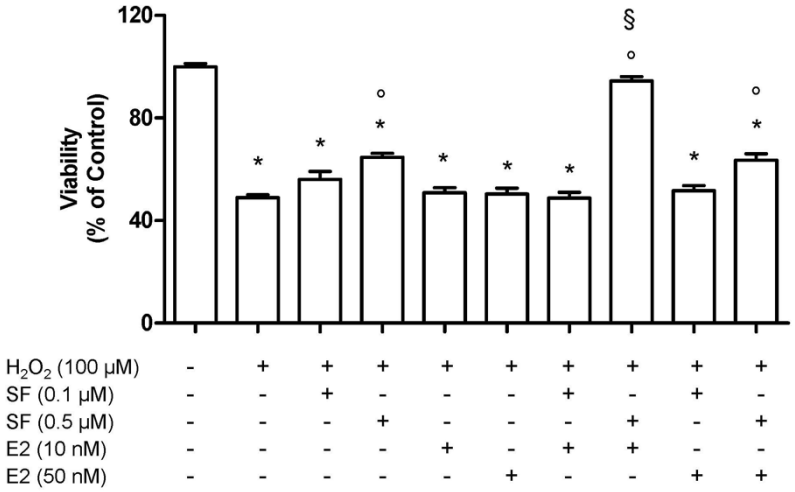
The co-treatment with 0.5 μM SF and 10 nM E2 was the most powerful to mitigate oxidative damage as it significantly increased cell viability with respect to both H_2O_2- and 0.5-μM-SF-treated cells. Moreover, this co-treatment maintained cell viability to level comparable to control cells, suggesting that E2 strongly enhances SF protective effect against oxidative stress (Fig. 7.2 A). These results were confirmed by LDH release assay. The co-treatment with 0.5 μM SF and 10 nM E2 significantly reduced LDH release in respect to both H_2O_2- and 0.5-μM-SF-treated cells (Fig. 7.2 B).

To investigate whether E2 can boost SF protective effects also against other oxidative injuries, cardiomyocytes were pre-treated with 0.5 μM SF in absence or presence of 10 nM E2 and then exposed to 0.5 mM Methylglyoxal (MG) for 24 h (Fig. 7.3).

MG is a dicarbonyl compound, by-product of glycolysis, and its ability to cause oxidative stress has been extensively reported [300, 301]. In a previous paper this MG concentration was able to induce oxidative damage in cardiomyocytes [302]. The treatment with 0.5 μM SF significantly increased cell viability in respect to MG treated cells as measured by MTT assay (Fig. 7.3 A); on the contrary, E2 had no effect against MG-induced injury. In agreement with the data on the protection against H_2O_2, SF and E2 co-treatment significantly increased cell viability in respect to both MG- and SF-treated cells, suggesting that E2 modulates SF ability to counteract also MG-induced oxidative damage. These results were confirmed by LDH release assay. The co-treatment significantly reduced LDH release with respect to both MG and SF-treated cells (Fig. 7.3 B).

As the co-treatment with 0.5 μM SF and 10 nM E2 was the most effective in counteracting oxidative stress, we chose these concentrations for the subsequent experiments. To deepen the mechanisms behind E2 ability to enhance SF cardioprotection against H_2O_2-induced injury, we investigated the potential modulatory effect of E2 on SF capability to reduce intracellular ROS levels in cardiomyocytes by the DCFH-DA assay. Cells were pre-treated with 0.5 μM SF in the absence/presence of 10 nM E2 and then exposed to 100 μM H_2O_2 for 30 min (Fig. 7.4).

A



B

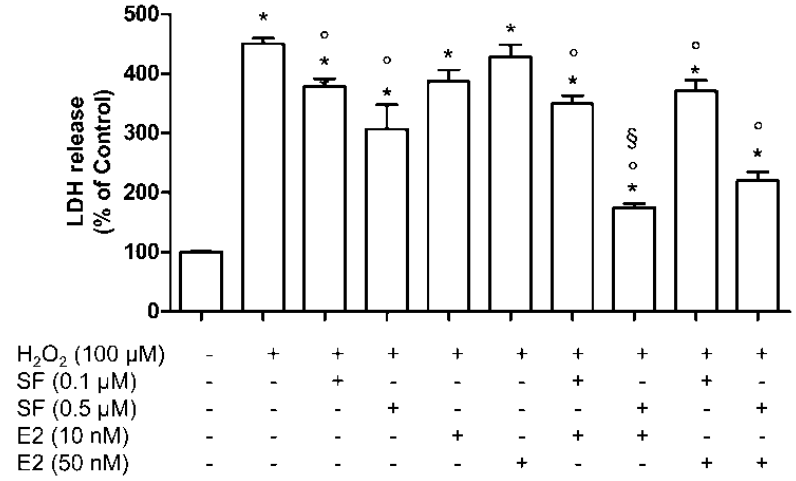


Figure 7.2. ***Effect of E2 on SF protective activity against H_2O_2-induced injury in cardiomyocytes.*** *Cells were treated with SF (0.1 – 0.5 µM) in the absence/presence of E2 (10 – 50 nM). (A) Cell viability was measured by MTT assay as reported in Materials and Methods. (B) Cell damage was measured by LDH activity in the culture medium as reported in Materials and Methods. Each bar represents means ± SEM of at least 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p <0.05 with respect to control; °p< 0.05 with respect to H_2O_2; §p< 0.05 with respect to SF 0.5 µM.*

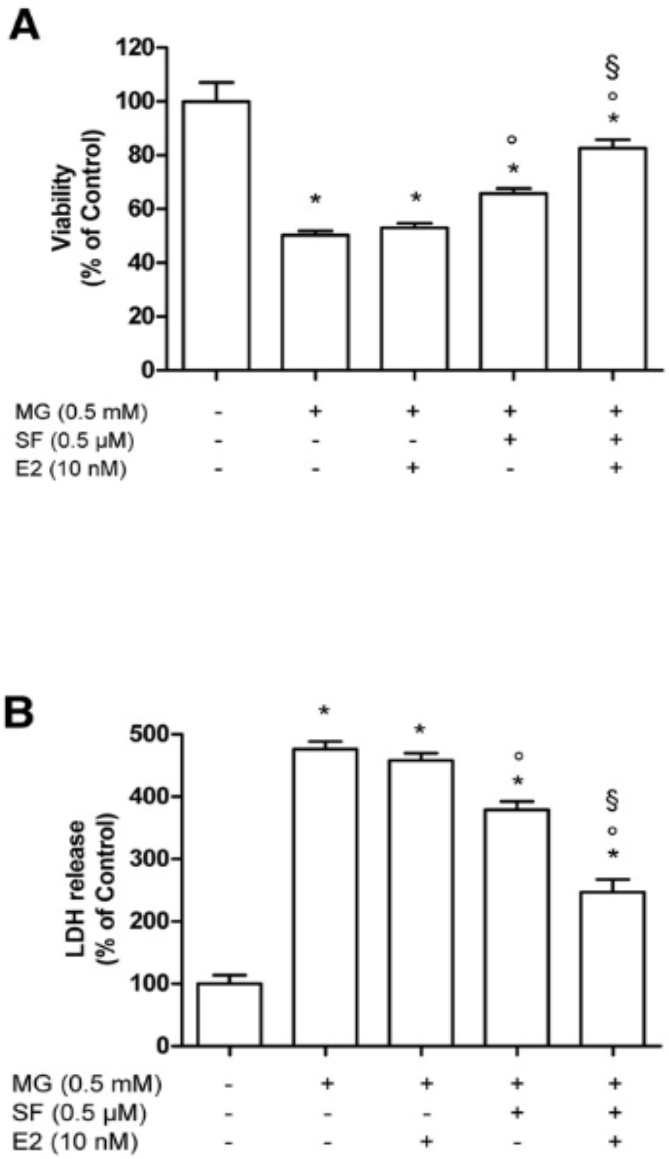


Figure 7.3. ***Effect of E2 on SF protective activity against MG-induced injury in cardiomyocytes.*** *Cells were treated with 0.5 µM SF in the absence/presence of 10 nM E2. (A) Cell viability was measured by MTT assay as reported in Materials and Methods. (B) Cell damage was measured by LDH activity in the culture medium as reported in Materials and Methods. Each bar represents means ± SEM of at least 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p <0.05 with respect to control; °p< 0.05 with respect to H_2O_2; §p< 0.05 with respect to SF 0.5 µM+H_2O_2.*

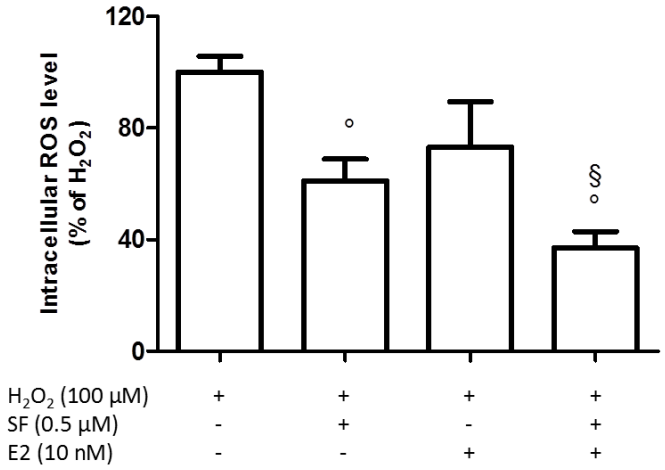


Figure 7.4. ***Effect of E2 on SF capability to reduce intracellular ROS levels in cardiomyocytes.*** *Cells were pre-treated with 0.5 µM SF in absence/presence of 10 nM E2. Intracellular ROS levels were measured with the ROS-sensitive probe DCFH-DA as reported in Material and Methods. Data are expressed as percentage in respect to H_2O_2-treated cells. Each bar represents means ± SEM of at least 4 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. °p< 0.05 with respect to H_2O_2; §p< 0.05 with respect to SF 0.5 µM.*

SF treatment significantly reduced intracellular ROS production with respect to H_2O_2; meanwhile, E2 did not significantly modify ROS levels compared to H_2O_2. Interestingly, the co-treatment was the most effective in reducing ROS levels in respect to SF alone, in agreement with viability data.

To validate the boosting effect on SF antioxidant ability elicited by E2 in cardiomyocytes, we evaluated the formation of 8-OHdG, a marker of oxidative damage to DNA (Fig. 7.5). The cells were plated in coverslips, treated with 0.5 µM SF in absence/presence of 10 nM E2 and then exposed to 100 µM H_2O_2 for 30 min. The coverslips were then fixed and stained with anti-8-OHdG antibody, as reported in Materials and Methods. Positive staining for 8-OHdG was nearly noticeable in the cytoplasm or nucleus of control and SF and/or E2-treated cardiomyocytes not exposed to peroxide. As expected, H_2O_2 triggered a strong positive staining for 8-OHdG, meanwhile the pre-treatment with SF and/or E2 mitigated the positive

staining for 8-OHdG. In agreement with the data on viability, the co-treatment was the most effective in reducing the positive staining.

As GSH is the main endogenous antioxidant involved in the maintenance of cell redox status, we investigated the effect of SF and E2 co-treatment on intracellular GSH levels by the MCB assay. Cells were pre-treated with 0.5 μM SF in the absence/presence of 10 nM E2 and then exposed to 100 μM H_2O_2 for 30 min (Fig. 7.6). Peroxide significantly decreased GSH levels with respect to control cells. E2 was not able to restore GSH levels, meanwhile, both SF alone and the co-treatment were able to significantly increase the amount of GSH with respect to H_2O_2, and to maintain it to a level comparable to control cells.

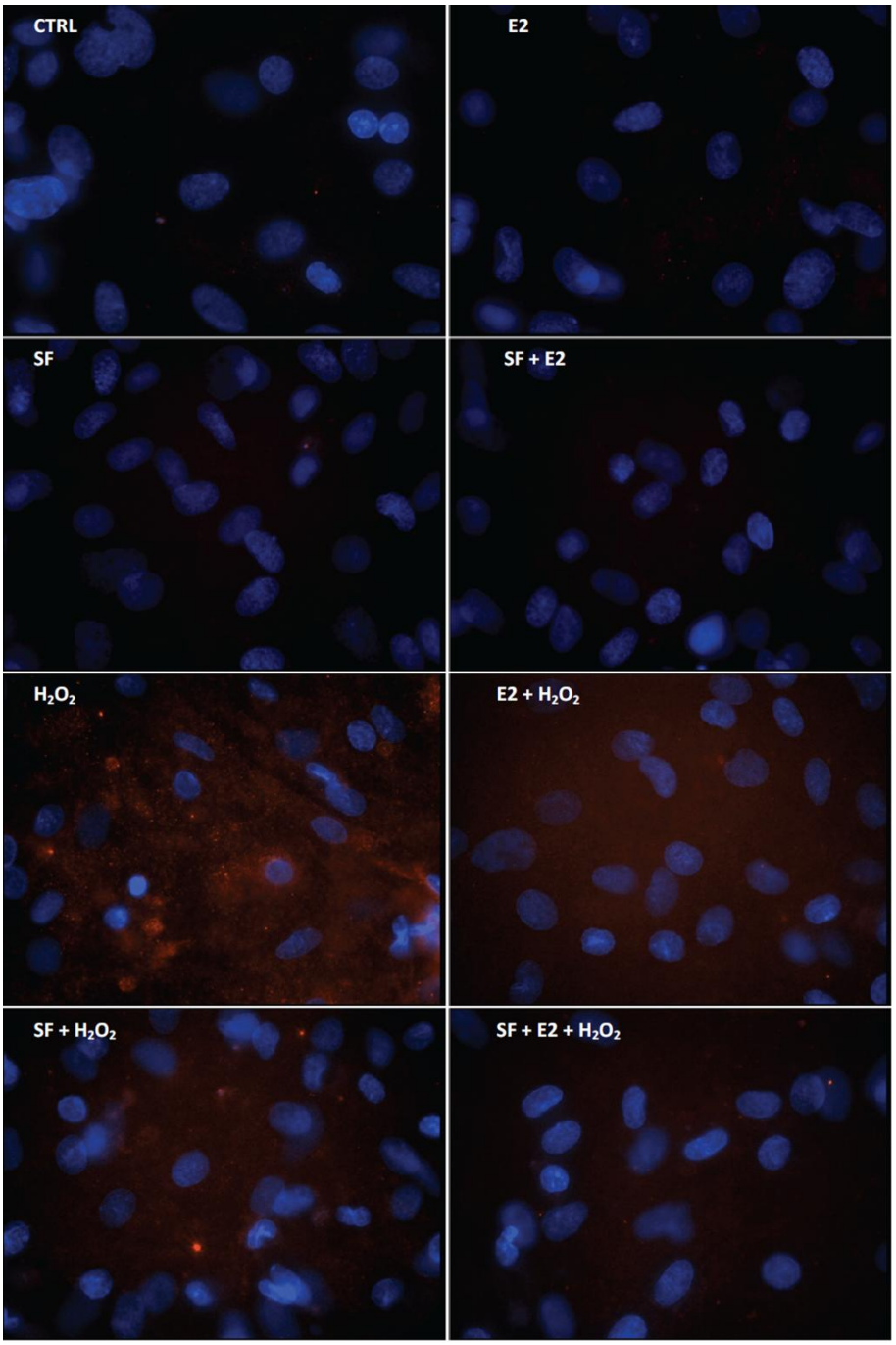


Figure 7.5. ***Effect of E2 on SF capability to contrast oxidative DNA injury in cardiomyocytes.*** *Cells were pre-treated with 0.5 µM SF in absence/presence of 10 nM E2 and then exposed to H_2O_2 for 30 min. Intracellular oxidative DNA damage was detected using an immunofluorescence staining with anti-8-OHdG antibody as reported in Material and Methods. Images were acquired with a 100x objective. 8-OHdG: red fluorescence (Cy3); Nuclei: blue fluorescence (DAPI).*

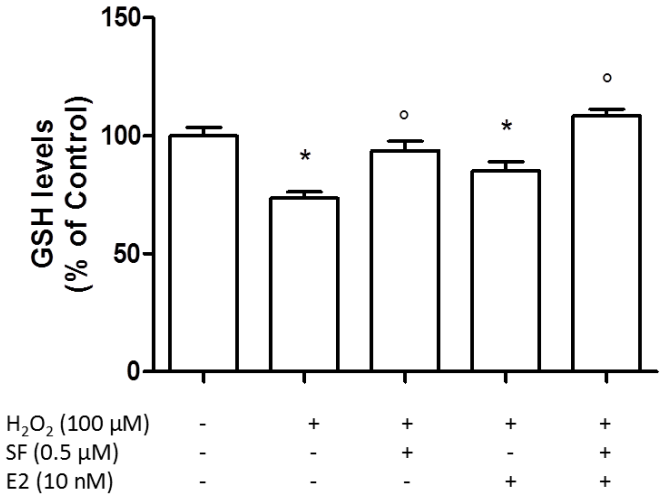


Figure 7.6. ***Effect of E2 and SF on GSH levels in cardiomyocytes.*** *Cells were pre-treated with 0.5 µM SF in absence/presence of 10 nM E2 and then exposed to H_2O_2 for 30 min. GSH levels were assessed using the fluorescence probe MCB as reported in Material and Methods. Each bar represents the mean ± SD of 4 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test. *p < 0.05 with respect to control; °p < 0.05 with respect to H_2O_2.*

7.2 Effect of E2 and SF on Nrf2 and phase II enzymes

It has been extensively reported that SF is a strong phase II enzyme inducer [256], so we next examinated the potential modulatory effect of E2 on SF ability to up-regulate phase II enzymes and the related transcription factor, Nrf2. In particular, we investigated the expression of GSTM1, GSTp2, GSTa3, HO-1, GR, CAT, NQO1, TrxR1, SOD1, SOD3, GPX and Nrf2. Cells were treated with 0.5 µM SF in the absence/presence of 10 nM E2, and after 24 h the RNA has been extracted, reverse transcribed into cDNA and mRNA levels analyzed by RT-PCR.

As reported in Fig. 7.7, SF alone significantly up-regulated GSTM1, GSTp2, GSTa3, GR, CAT, NQO1 and TrxR1 with respect to control cells, meanwhile it did not modulate the expression of HO-1 and Nrf2. On the contrary, the co-treatment was able to significantly up-regulate the expression of Nrf2 and all the enzymes reported

in Fig. 7.7. In particular, in the co-treated cells the expression of GSTp2, HO-1, GR, NQO1 and TrxR1 was higher with respect to SF-treated cardiomyocytes, suggesting that E2 boosts the upregulation of phase II enzymes induced by SF. On the other side, E2 did not influence the expression of any considered gene. Regarding SOD1, SOD3 and GPX, their mRNA levels were not influenced by any treatments (Fig. 7.8).

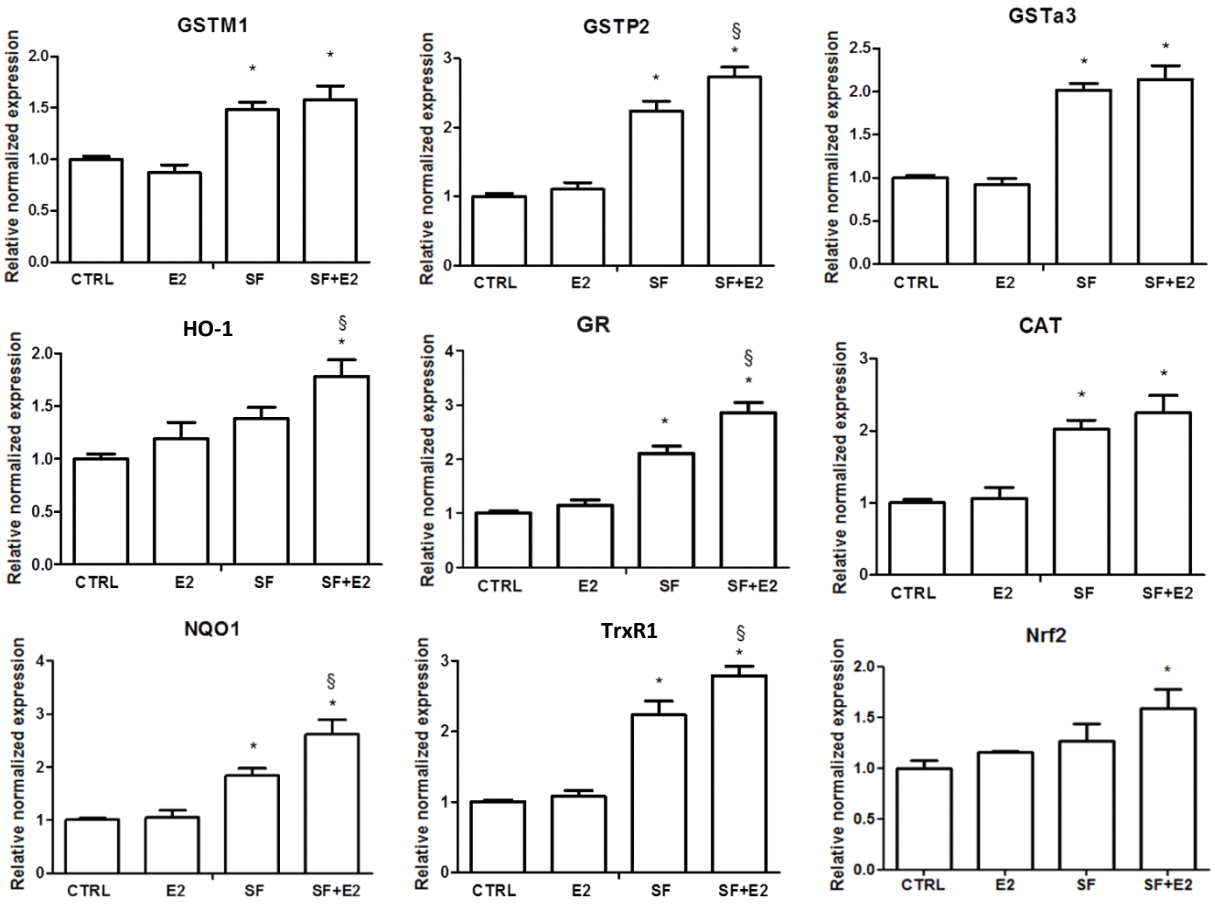


Figure 7.7. ***Effect of E2 and SF on the mRNA level of GSTM1, GSTp2, GSTa3, HO-1, GR, CAT, NQO1, TrxR1, Nrf2 in cardiomyocytes.*** *Cells were treated with 0.5 μM SF in absence/presence of 10 nM E2 for 24 h. Total RNA was isolated and the mRNA level of target genes was quantified using RT-PCR normalized to β-actin and β2-microglobulin housekeeping genes as reported in Materials and Methods. Triplicate reactions were performed for each experiment. Each bar represents the mean ± SEM of 3 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test. * $p < 0.05$ with respect to control; § $p < 0.05$ with respect to SF.*

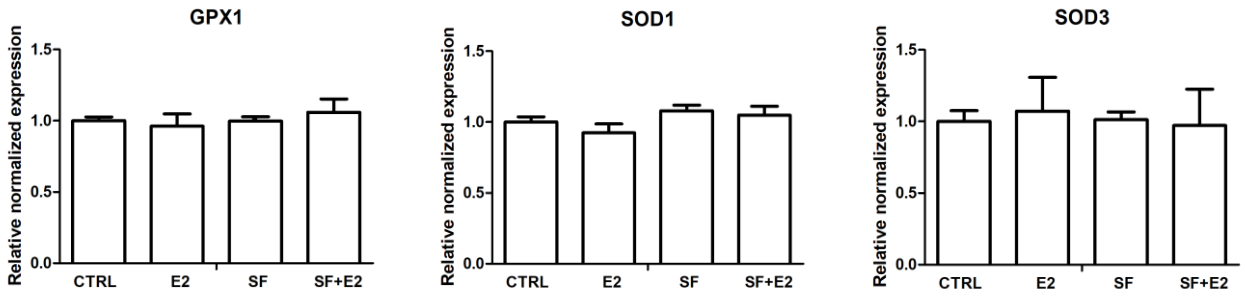


Figure 7.8. ***Effect of E2 and SF on the mRNA level of GPX, SOD1 and SOD3 in cardiomyocytes.*** *Cells were treated with 0.5 μM SF in absence/presence of 10 nM E2 for 24 h. Total RNA was isolated and the mRNA level of target genes was quantified using RT-PCR normalized to β-actin and β2-microglobulin housekeeping genes as reported in Materials and Methods. Triplicate reactions were performed for each experiment. Each bar represents the mean ± SEM of 3 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test.*

To further study the modulation of Nrf2 by SF/E2 co-treatment, we measured it by immunofluorescence staining (Fig. 7.9) and by Western blotting analysis (Fig. 7.10). As reported in Materials and Methods, for immunofluorescence analysis, cells were seeded in coverslips, and at the end of each treatment, cells were fixed and incubated with an anti-Nrf2 specific antibody. As for the RT-PCR data, E2 did not increase total level of Nrf2 protein expression (Fig. 7.9); meanwhile, SF and the co-treatment induced a positive staining for Nrf2 with respect to control cells. Of note, the co-treatment increased Nrf2 protein expression more effectively than SF alone. In order to investigate the translocation of Nrf2 to the nucleus, we treated cells with SF or E2 and then performed immunoblot analysis of the cytosolic and nuclear fractions (Fig. 7.10) using a specific Nrf2 antibody. Data revealed that E2 treatment reduced Nrf2 protein level in the cytosolic fraction without increasing Nrf2 level in the nuclear fraction. On the contrary, both SF and the co-treatment were able to significantly reduce Nrf2 expression in the cytosolic fraction and, at the same time, to significantly increase Nrf2 in the nucleus. In particular, the Nrf2 translocation to the nucleus was higher in co-treated cells.

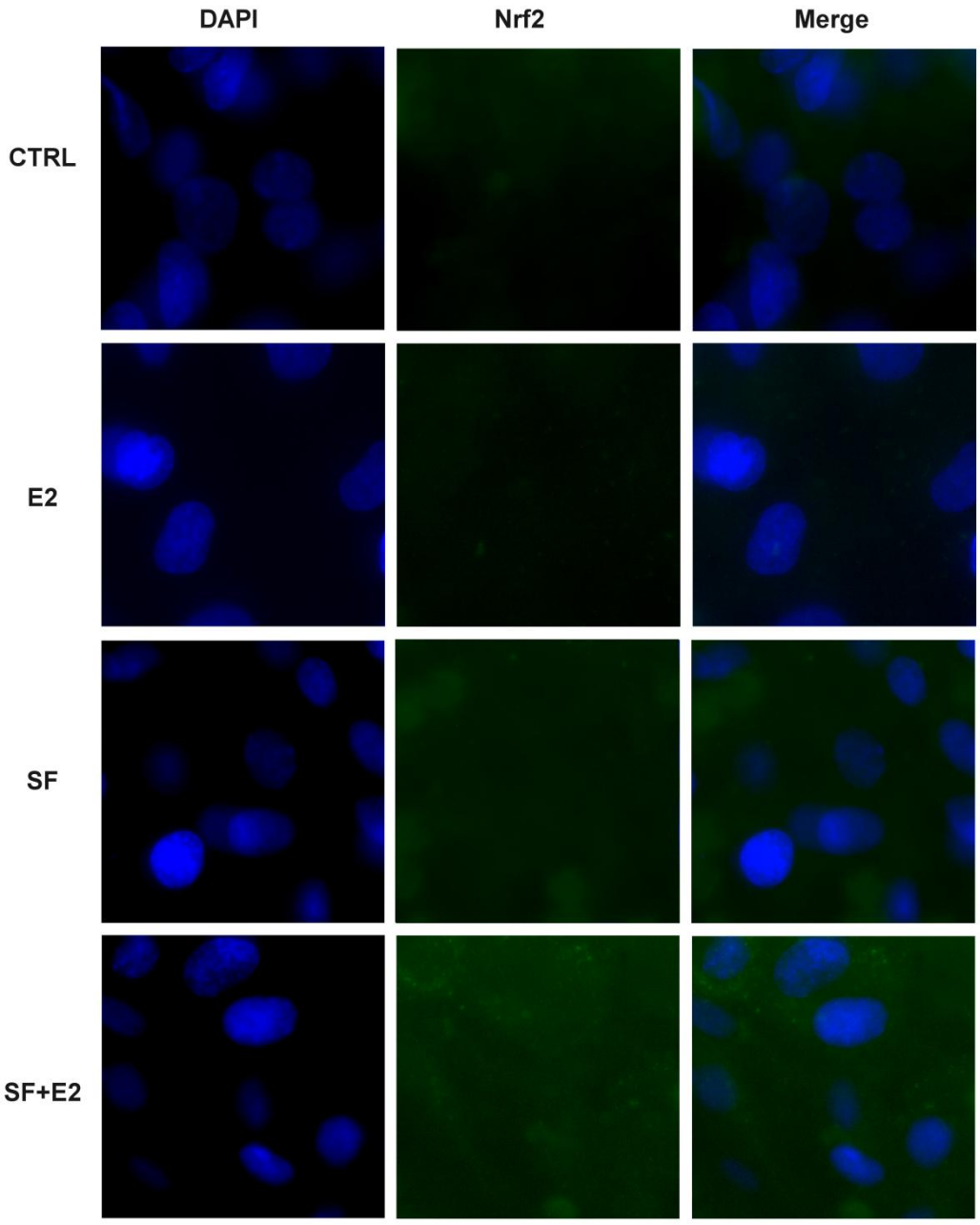


Figure 7.9. ***Effect of E2 and SF on Nrf2 protein expression in cardiomyocytes.*** *Cells were treated with 0.5 µM SF in absence/presence of 10 nM E2 for 24 h. Nrf2 was detected using an immunofluorescence staining with anti-Nrf2 antibody as reported in Material and Methods. Images were acquired with a 100x objective. Nrf2: green fluorescence (FITC); Nuclei: blue fluorescence (DAPI).*

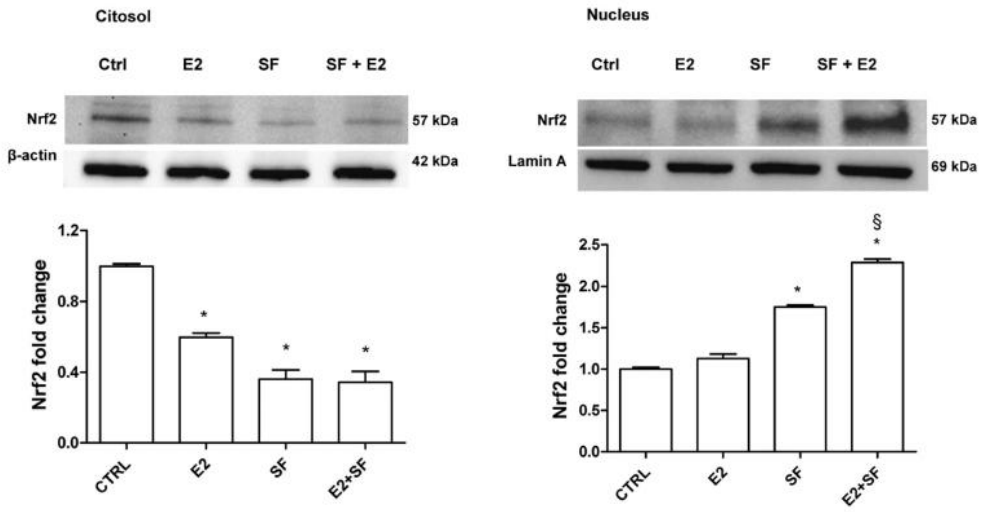


Figure 7.10. ***Effect of E2 and SF on Nrf2 translocation.*** *Cells were treated with 0.5 μM SF in absence/presence of 10 nM E2 for 24 h, and cytosolic and nuclear extracts were immunoblotted with anti-Nrf2 antibody as reported in Materials and Methods. Relative amounts were normalized to the intensity of β-actin (cytosolic fraction) or lamin A (nuclear fraction) and represented as fold increase with respect to control. Data were analyzed by one-way ANOVA followed by Dunnett's test. *p<0.05 with respect to control cells; §p<0.05 with respect to SF.*

7.3. Involvement of ERα and ERβ in SF/E2 co-treatment counteracting H_2O_2-induced damage

E2 mediates its physiological functions through the activation of estrogen receptors. So, we next analyzed the expression of both ERα and ERβ by RT-PCR in neonatal cardiomyocytes treated with 0.5 μM SF in the absence/presence of 10 nM E2 (Fig. 7.11). Both receptors are expressed in the cells, and the treatments did not alter their mRNA levels. To investigate whether ERα or ERβ could influence E2 ability to boost SF protection against H_2O_2, we examined cell viability of cells co-treated with SF and E2 in the absence/presence of specific ERα and ERβ antagonists, MPP and PHTPP, respectively, before oxidative stress induction (Fig. 7.12). Both antagonists, MPP and PHTPP did not modify cell viability with respect to control cells. Interestingly, neither MPP nor PHTPP, reduce the efficacy of SF/E2 co-treatment against H_2O_2-

induced damage, suggesting that ERs are not involved in co-treatment-mediated cardioprotection.

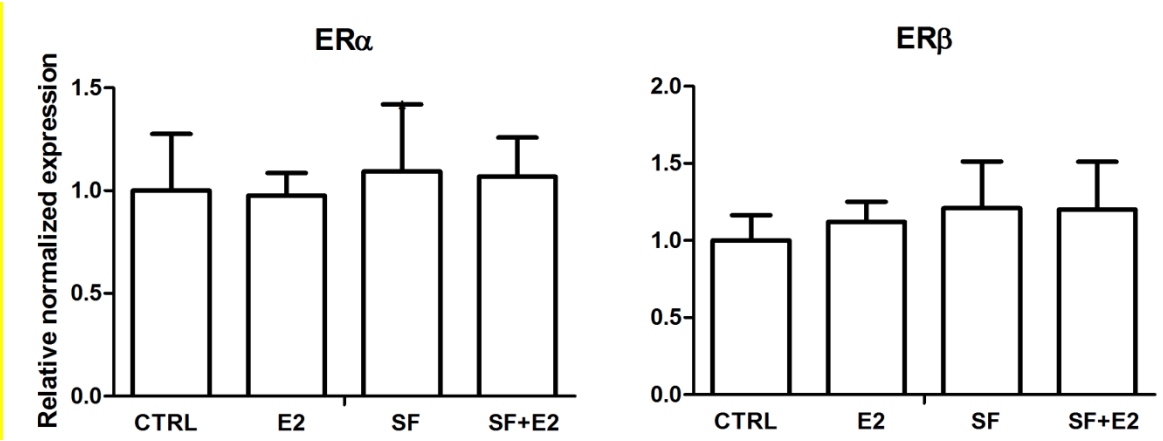


Figure 7.11. ***Effect of E2 and SF on the expression of ERα and ERβ in cardiomyocytes.*** *Cells were treated with 0.5 μM SF in absence/presence of 10 nM E2 for 24 h. Total RNA was isolated and the mRNA level of target genes was quantified using RT-PCR normalized to β-actin and β2-microglobulin housekeeping genes as reported in Materials and Methods. Triplicate reactions were performed for each experiment. Each bar represents the mean ± SEM of 3 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test.*

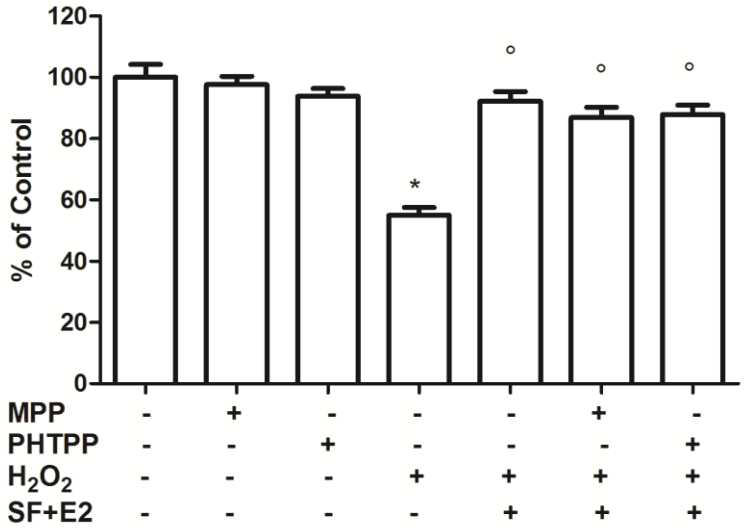


Figure 7.12. ***Effect of ERα and ERβ antagonists on H_2O_2-induced damage in cardiomyocytes.*** *Cells were co-treated with 0.5 μM SF and 10 nM E2 in the absence/presence of 100 nM MPP (ERα antagonist) or 100 nM PHTPP (ERβ antagonist) for 24 h prior to H_2O_2 exposure. Cell viability was measured by MTT assay as reported in Materials and Methods. Each bar represents means ± SEM of at least 4 independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test. *p <0.05 with respect to control; °p< 0.05 with respect to H_2O_2;*

7.4 Effect of E2 and SF on ERK1/2 and Akt signaling

We next evaluated the effect of E2 and SF on two pro-survival signaling pathways in cardiomyocytes [303, 304], ERK1/2 and Akt pathways by Western Blot analysis (Fig. 7.13). Cells were treated with 0.5 μM SF in the absence/presence of 10 nM E2 for different time points (30 min, 2 h and 6 h) and then the phosphorylated- and total-forms of ERK1/2 and Akt kinases were analyzed by immunoblotting, using specific antibodies. ERK1/2 was rapidly activated (phosphorylated) by all treatments, but only SF/E2 co-treatment maintained ERK1/2 activation at 2 and 6 h, suggesting a synergic effect of SF and E2 on the phosphorylation of this MAPK.

Like ERK1/2, Akt was rapidly phosphorylated by SF, E2 and the co-treatment but at 2 h, only E2 and the co-treatment were able to maintain Akt activation. Noteworthy,

with the exception of 6 h time point, SF/E2 co-treatment induced a significantly greater Akt activation than the single treatments.

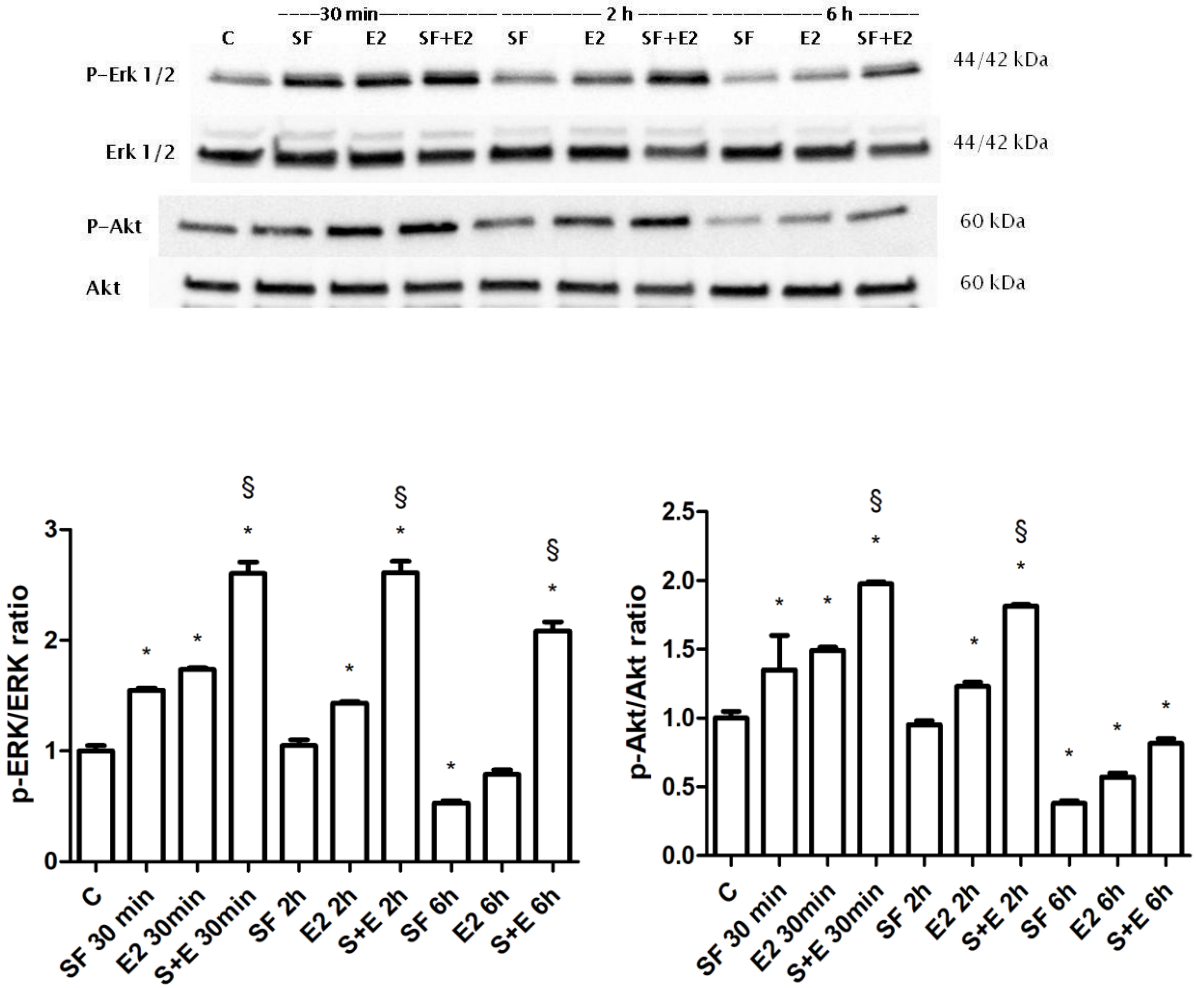


Figure 7.13. ***Modulation of ERK1/2 and Akt by SF/E2 co-treatment in cardiomyocytes.*** *Cells were pre-treated with 0.5 μM SF in the absence/presence of 10 nM E2 for different times (30 min–6 h), and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblotted and probed for total and phosphorylated forms of ERK1/2 and Akt as reported in Materials and Methods. Each bar represents means ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p<0.05 with respect to C; §p<0.05 with respect to SF, E2.*

To characterize the role of these two kinases on the enhanced cardioprotection elicited by SF/E2 co-treatment against oxidative injury, we verified the viability of

cells pre-treated with SF/E2 co-treatment in the absence/presence of specific ERK1/2 and Akt inhibitors, PD and LY, respectively, before the induction of oxidative stress (Fig. 7.14). Interestingly, only when the cells were simultaneously exposed to the two inhibitors, a significant reduction of cell viability was recorded with respect to SF/E2-treated cells.

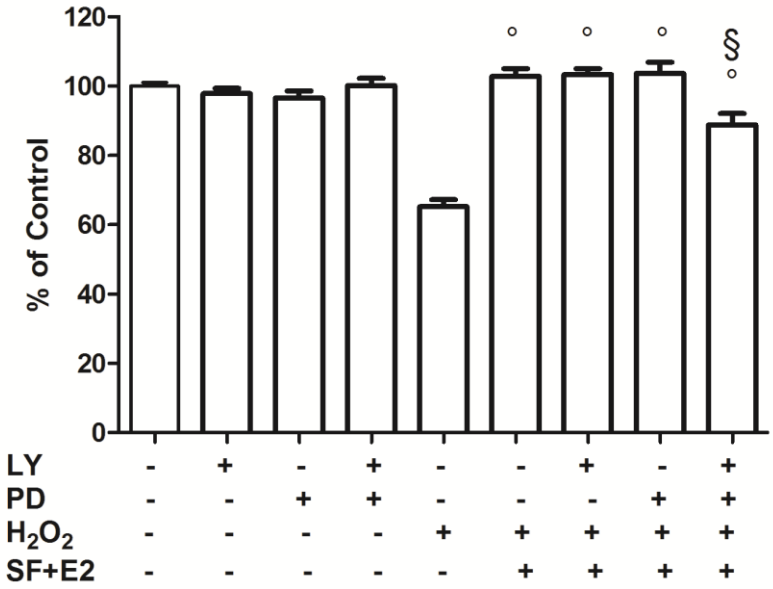


Figure 7.14. ***Effect of Akt and ERK1/2 inhibitors on H_2O_2-induced damage in cardiomyocytes.****. Cells were treated with SF/E2 co-treatment in the absence/presence of 10 μM LY or 10μM PD prior to H_2O_2 exposure. Cell viability was measured by MTT assay as reported in Materials and Methods. Each bar represents means ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. °$p<0.05$ with respect to H_2O_2, §$p<0.05$ with respect to SF/E2 + H_2O_2 cells.*

7.5 Involvement of ERs and GPR30 in the activation of Akt kinase in H9c2 cells

Our next aim was to better characterized the effect of SF/E2 co-treatment on Akt signaling pathway. To this purpose, we used the cardiomyoblast cell line H9c2. To verify that the co-treatment of H9c2 cells elicits the same effect observed in cardiomyocytes on Akt activation, we treated H9c2 cells with 0.5 μM SF in the

absence/presence of 10 nM E2 for different time points (30 min, 2 h) and then the phosphorylated- and total-forms of Akt kinase were analyzed by immunoblotting. Akt was rapidly activated by all treatments at 30 min, with SF/E2 co-treatment most effective that the other treatments (Fig. 7.15). To analyze the possible involvement of estrogen receptors in the activation of Akt induced by SF/E2 co-treatment, we treated H9c2 cells with selective agonists of ERα, ERβ and GPR30 and analyzed the activation of Akt by western blotting.

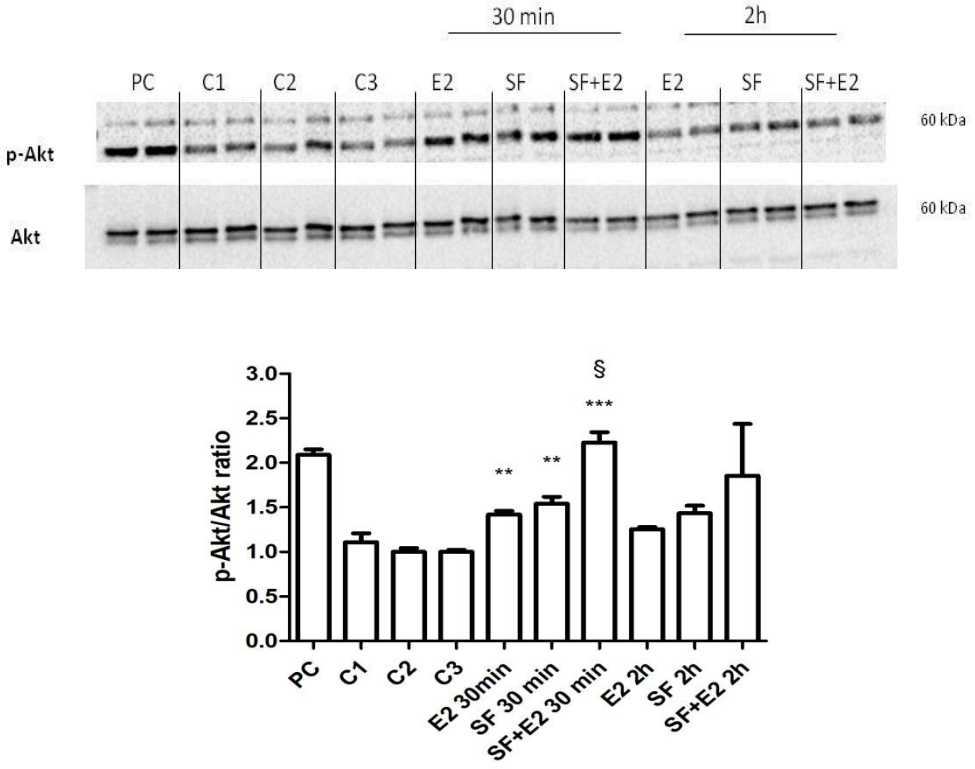


Figure 7.15. ***Modulation of Akt by SF/E2 co-treatment in H9c2 cells****. Cells were treated with 0.5 μM SF in the absence/presence of 10 nM E2 for different times (30 min–2 h), and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblotted and probed for total and phosphorylated forms of Akt (A) as reported in Materials and Methods. We used as positive control (PC) cells starved for 1h in DMEM w/o FBS and then exposed to 10% FBS DMEM for 20 min. C1: DMEM 30 min; C2: DMSO 30 min (as SF+E2 treatment); C3: DMSO 2h (as SF+E2 treatment). Each bar represents means ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p<0.05 with respect to C2, §p<0.05 with respect to SF and E2.*

As illustrated in Fig. 7.16 (A), we used PPT, agonist for ERα, at two different concentrations (10 nM and 100 nM) in absence/presence of 0.5 µM SF. SF and 10 nM PPT alone were able to induce the activation (phosphorylation) of Akt; whereas SF/PPT co-treatments did not activate Akt kinase at any agonist concentration tested. Indeed, rather than enhancing SF-induced Akt activation, PPT inhibited its activation. To test ERβ contribution to Akt activation, we treated cells with 0.5 µM SF in absence/presence of KB5, agonist of ERβ, at two different concentrations (10 nM and 100 nM) (Fig. 7.16 B). SF and 10 nM KB5 alone significantly activated Akt, meanwhile the SF and KB5 co-treatments enhanced Akt activation, suggesting that KB5 agonist could act like E2 in enhancing Akt phosphorylation induced by SF. Finally, we verify GPR30 involvement using G1 agonist. Cells were treated with 0.5 µM SF in absence/presence of G1 at two concentrations (100 nM and 1 µM) (Fig. 7.16 C). SF alone induced Akt phosphorylation, meanwhile G1 alone did not act on Akt phosphorylation. However, in agreement with the data obtained with KB5 agonist, SF treatment in the presence of 1 µM G1 significantly boosted Akt phosphorylation induced by SF. These results suggest that the contribution of E2 on SF/E2 co-treatment effect could be mediated by ERβ and GPR30.

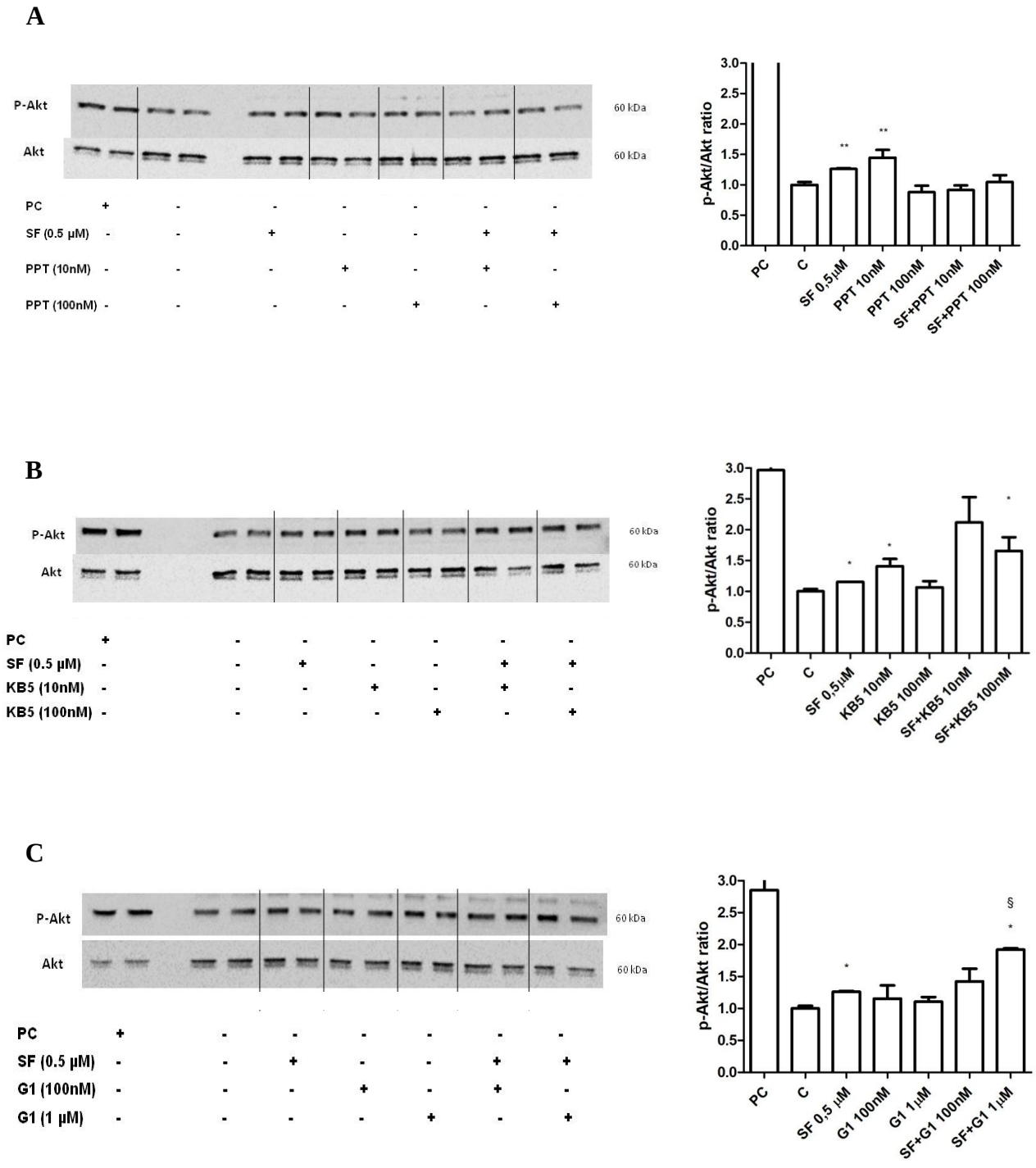


Figure 7.16. ***Involvement of estrogen receptors in the activation of Akt kinase.*** *Cells were treated with 0.5 µM SF in the absence/presence of selective agonists for 30 min, and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblotted and probed for total and phosphorylated forms of Akt as reported in Materials and Methods. (A) PPT is a selective agonist for ERα; (B) KB5 is a selective agonist for ERβ; (C) G1 is a selective agonist for GPR30. Each bar represents means ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p<0.05 with respect to C, §p<0.05 with respect to SF/agonist co-treatment.*

7.6 Nrf2 activation and Akt signaling in H9c2 cells

To further confirm the involvement of Nrf2/ARE pathway in the cardioprotection elicited by SF/E2 co-treatment, we evaluated Nrf2 ARE-binding activity through ELISA-based kit in H9c2 cells. Cells were treated with 0.5 μM SF in the absence/presence of 10 nM E2 and then Nrf2 ARE-binding activity was analyzed in nuclear extracts.

In agreement with the data obtained in primary cardiomyocytes, the co-treatment demonstrated to be the most effective in increasing Nrf2 activity (Fig. 7.17).

Akt kinase is involved in Nrf2 nuclear stabilization. Indeed, Akt activation results in inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3), which mediates Nrf2 export from the nucleus. Therefore, Akt-mediated GSK-3 inhibition prevents Nrf2 proteosomal degradation [305]. For this reason, we used a specific Akt inhibitor to study the involvement of Akt activation by the co-treatment in Nrf2/ARE pathway. To confirm LY ability to inhibit Akt activation we co-treated cells with SF and E2 in the absence/presence of LY before performing western blot analysis (Fig. 7.18). As expected, in presence of LY co-treatment-induced Akt phosphorylation was significantly reduced. So we next verified the effect of the co-treatment in absence/presence of LY on Nrf2 ARE-binding activity by ELISA-based kit (Fig. 7.19). As illustrated in Fig 7.18, SF and E2 co-treatment significantly increased Nrf2 activity, meanwhile, in the presence of LY, the activation of Nrf2 induced by the co-treatment was prevented. These results suggest a key role played by Akt kinase in co-treatment-induced Nrf2 activation.

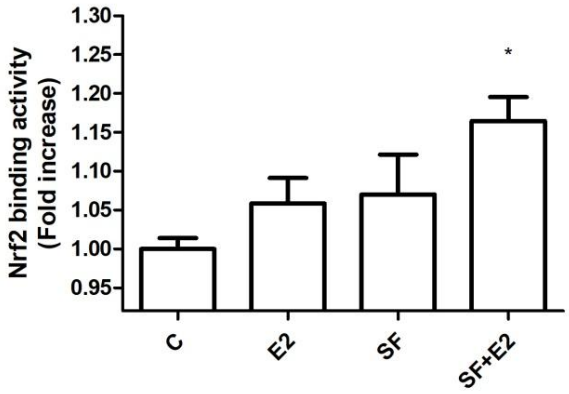


Figure 7.17. ***Effect of E2 and SF on Nrf2 binding activity.*** *Cells were treated with 0,5 μM SF in absence/presence of 10 nM of E2 for 6h. ARE binding activity of Nrf2 was analyzed in nuclear extracts using the ELISA-based kit Trans AM Nrf2 as reported in Materials and Methods. Values represent means ± SEM of 3 independent determinations and are reported as fold increase over controls. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p< 0.05 with respect to C.*

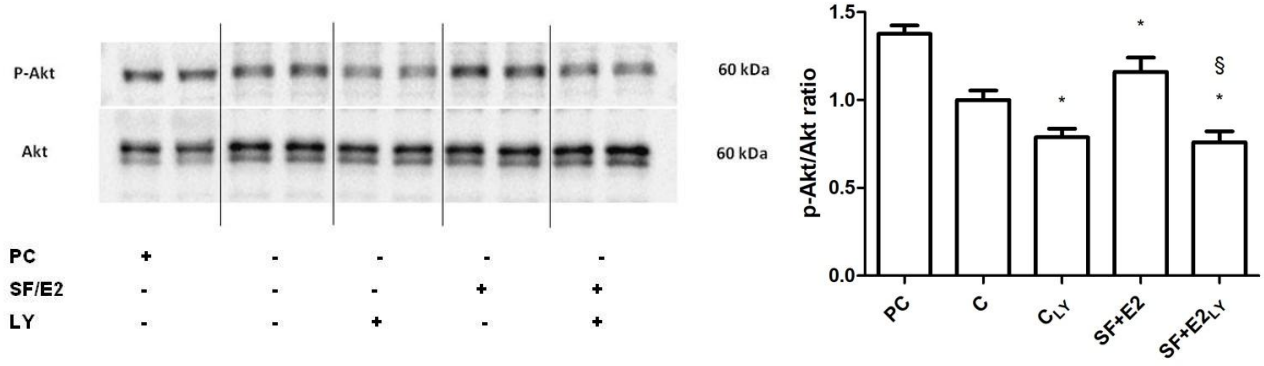


Figure 7.18. ***Effect of SF/E2 co-treatment on Akt phosphorylation in presence of Akt inhibitor.*** *Cell were treated with SF/E2 co-treatment for 30 min in absence/presence of 10 μM LY inhibitor. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblotted and probed for total and phosphorylated forms of Akt as reported in Materials and Methods. *p<0.05 with respect to C; §p<0.05 with respect to SF/E2 co-treatment.*

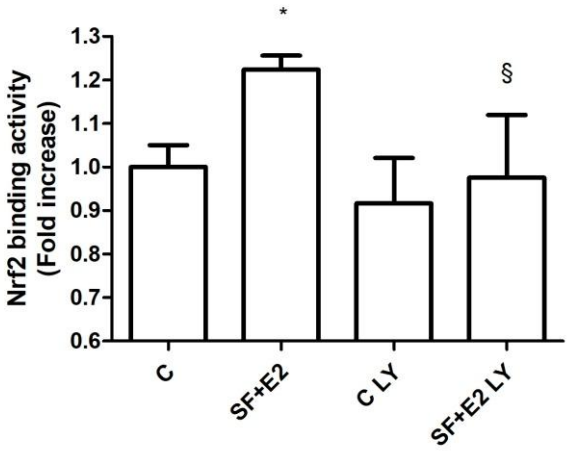


Figure 7.19. ***Effect of co-treatment on Nrf2 binding activity in presence of Akt inhibitor.*** *Cells were treated with the co-treatment for 6 h in absence/presence of 10 μM LY. ARE binding activity of Nrf2 was analyzed in nuclear extracts using the ELISA-based kit Trans AM Nrf2 as reported in Materials and Methods. Values represent means ± SD of 3 independent determinations and are reported as fold increase over controls. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *p < 0.05 with respect to C; §p < 0.05 with respect to SF/E2 co-treatment.*

8. DISCUSSION

In 2010, the prestigious journal Nature, entitled its editorial as 'Putting gender on the agenda', underling that still nowadays, the medications used on women have been prevalently tested on men [306]. Indeed, from Ippocrates to the modern medicine, preclinical and clinical studies including subjects of both sexes are really few. Women enrolled in clinical trials for cardiovascular disease medications represent only the 30%; and the first trial on statins (WOSCOP) did not enroll any woman, as well as that studying aspirin as preventive agent of CVD. The philosophy of drug development needed to change from the paradigm of 'one size fits all' to a new concept of precision medicine. Women and men can be affected by the same pathologies but they show differences in the age of onset, symptoms, risk factors, pathology progression and not less important, they respond differently to the medications. These discrepancies are due to those we refer to as sex-gender differences. Women and men are biologically different at the level of the cells, the organs and the organism [307]. Sex leads to biological differences between males and females, in terms of endocrinology, epigenetics and genetics, while gender refers to individual interaction with the society and environment. Gender concept is specific for humans, while sex differences can be evidenced in animal models and isolated cells. The impact of sex-gender differences is well studied in the field of cardiovascular disease. Nowadays, CVD are still the first cause of death worldwide, and despite the improvement of their prevention, in the elderly CVD represent the first cause of disability [1]. CVD rarely manifest in pre-menopausal women meanwhile, the incidence of these pathologies dramatically increases after the menopause. This phenomenon has been in part correlated to the lost of sexual hormones after menopause, in particular to the lower levels of 17β-estradiol, the main premenopausal circulating form of estrogen [4]. In post-menopausal women, oxidative stress status could represent a helpful biomarker for the risk of cardiovascular disease. Oxidative

stress is a condition of disequilibrium between oxidative species, mainly reactive oxygen species, and the endogenous antioxidant defenses, and it leads to high level of lipid peroxidation, oxidative damage to DNA, mitochondrial dysfunction and altered protein expression. Oxidative stress represents the common characteristic in CVD, as it plays a role in the initiation and progression of these [154, 308, 309]. In physiological conditions, cells are able to counteract the excess of intracellular ROS production through the endogenous antioxidant defense system, which belongs phase II and antioxidant enzymes. SOD, CAT, GST, GR, TrxR1, NQO1, HO-1 are examples of phase II enzymes, which play a key role in the maintenance of intracellular redox state and in the protection of cells from oxidative damages. In particular, Nrf2 transcription factor represents the main regulator of these cytoprotective proteins.

The intriguing possibility to counteract the elevation of ROS levels in CVD through dietetic intervention has been largely deepened, and many investigations reported cardioprotective effects played by nutraceutical compounds with several mechanisms, including the reduction of oxidative stress [310, 311]. However, all studies investigating the cardioprotective role of nutraceutical compounds did not take into account sex-gender differences.

Sulforaphane is a dietary isothiocyanate, plant secondary metabolite, which derives from the hydrolysis of its precursor glucoraphanin, present in brassica vegetables. The main mechanism elicited by SF is the induction of a battery of cytoptotective enzymes through the involvement of Nrf2/ARE pathway [229]. Several studies reported the cardioprotective role of sulforaphane in counteracting oxidative injury [256, 285-287].

As mentioned before, it has been demonstrated that males and females respond differently to cardiovascular medications. These differences were mainly attributed to the actions of E2, so we hypothesize that sex hormones could also differently influence the preventive/protective effects of nutraceutical compounds in males and females, such as those of SF.

Aim of this PhD thesis was to explore the modulatory effect of E2 on the cardioprotective activity of SF in primary cultures of rat cardiomyocytes against oxidative stress by analyzing antioxidant/survival pathways and investigating the involvement of ERs and GPR30.

In this thesis we focused on the effects of E2 on SF protection against oxidative damage in primary cardiomyocytes. And to the best of our knowledge, for the first time we demonstrated that estrogen could modulate the protective activity of a nutraceutical compound. In particular, our data show that E2 can enhance SF capacity to counteract oxidative injury by boosting the up-regulation of antioxidant enzymes and the activation of pro-survival signaling pathways. Our data evidence that E2 significantly enhances SF cardioprotection against oxidative injury. Previous studies demonstrated that 5 µM SF is able to protect against oxidative damage, inducing a panel of key cellular cytoprotective enzymes [253, 256, 302]. Interestingly, in these previous studies, only 5 µM SF led to a total protection of cardiomyocytes against oxidative stress. In this thesis, we used 0.5 µM SF, a 10-fold lower concentration which, in absence of E2, led to a slight protection in cardiomyocytes; meanwhile, in presence of E2, it induced a full protection against oxidative injury. Differently, E2 alone did not protect from H_2O_2-induced oxidative injury. Data from Urata et al. [312] are in accordance with our data, as they showed that 10 nM E2 was not able to contrast oxidative injury elicited by H_2O_2; differently, other researchers observed protective effects with E2 treatment against different damages, in cardiomyoblast cell line. In H9c2 cells, Hsieh et al. [33] demonstrated that 10 nM E2 is able to reduce hypoxia-induced apoptosis. Likewise, Kim et al [27], and Cong et al. [32], using the same E2 concentration on neonatal cardiomyocytes, showed protective effects against H/R-induced apoptosis. The dissimilarity between our data and these results could be attributed to the different injury induced to cells, as H/R leads to a more complex scenario than H_2O_2, involving other mechanisms beside oxidative stress. To further confirm the observation that E2 boosts SF protective effect against oxidative damage,

we evaluated the effect of E2 on SF protection in another oxidative stress model. Therefore, we exposed cardiomyocytes to the alpha-oxoaldehyde MG, which widely demonstrated to induce oxidative stress [300, 301]. According to the data obtained with H_2O_2, E2 demonstrated to enhance SF protection against MG, suggesting that E2 can modulate SF cardioprotection in different oxidative stress models. To deepen the mechanisms behind E2 ability to enhance SF protection against H_2O_2-induced damages, we also evaluated the effect of SF/E2 co-treatment on redox intracellular state, in cardiomyocytes exposed to peroxide. In agreement with the viability data, the SF/E2 co-treatment counteracted, more effectively than SF alone, intracellular ROS release and 8-OHdG formation induced by H_2O_2, suggesting a modulator effect of E2 on SF antioxidant capability. Differently, the recovery action on GSH levels of SF/E2 co-treatment, after peroxide exposure, was comparable to that of SF alone.

In rat cardiomyocytes, 5 µM SF demonstrated to up-regulate several antioxidant and phase II enzymes, so counteracting oxidative stress [253, 256, 302] through the involvement of Nrf2 transcription factor. Genes codifying for phase II enzymes contain in their promoter regions ARE (antioxidant responsive element) sequence, which is recognized from Nrf2 [180]. These enzymes represent key components of the cellular antioxidant defense system and are important for the prevention/protection of the CVD [313]. The importance of Nrf2/ARE signaling pathway has been widely explored in the cardio-prevention of oxidative injury [293, 314], as well as in the protection from heart dysfunction [315-317]. In this project, SF capacity to induce antioxidant enzymes was significantly enhanced by E2; in particular, SF/E2 co-treatment significantly up-regulated the expression of NQO1, TrxR1, GR, GSTp2, HO-1 and Nrf2 with respect to SF alone. Interestingly, E2 alone did not influence the expression level of any tested enzyme. To verify the mechanism behind the induction of phase II enzyme and Nrf2 mRNA levels elicited by SF/E2 co-treatment, we studied the effect of SF and E2 on Nrf2 nuclear translocation. Indeed, Nrf2 is known to induce, once translocated into the nucleus, itself expression beyond that of antioxidant enzymes [248]. The co-treatment significantly enhanced Nrf2 nuclear accumulation with respect to SF alone,

so suggesting that E2 is able to boost the up-regulation of phase II enzymes induced by SF through Nrf2. Interestingly, E2 alone led to a reduction in Nrf2 cytosolic level, which did not correspond to an increase in Nrf2 nuclear level. As E2 did not show to modulate neither antioxidant enzymes expression nor Nrf2 expression with respect to control cells, the reduction in Nrf2 cytosolic level might involve different mechanisms. Recently, novel E3 ubiquitin ligases has been identified that mediate Nrf2 degradation via Keap1-indipendent mechanisms [318]. We can hypothesize that E2 mediates the release of Nrf2 from Keap1 but the free Nrf2 is rapidly degraded before its translocation to the nucleus with a Keap1-indipendent mechanism. Obviously, further investigations are needed to verify this hypothesis.

Our data on E2 effect on Nrf2 activation are not in agreement with the results obtained by Yu et al. [319], as they showed the activation of Nrf2 and the induction of HO-1, SOD1 and GST with 5 μM E2. In our opinion, these discrepancies between our and their results are not surprising, as we used physiological concentration of E2 (10nM), that is one order of magnitude lower than the ones used by Yu et al.

E2 mediates its physiological functions through genomic and non-genomic pathways [320]. The firsts can be mediated by ERα and ERβ, which in the classical mechanism act as transcription factors; whereas non genomic signaling is modulated by membrane-associated ERα and ERβ [321], and by the G protein–coupled receptor named as GPR30, which is the main responsible for estrogen effects acting via non-classic receptor systems [116]. All receptors are expressed in cardiac cells [46, 322] from both male and female rodents [112, 323, 324]. Different authors suggested that E2 cardioprotection against oxidative stress is mediated by ERs. In H9c2 cardiomyoblasts, E2 exerted protection from H_2O_2-mediated injury through a transcriptional modulation mechanism controlled by ERβ [312]. In another study, E2 treatment counteracted, in rat cardiomyocytes, H/R-induced damage with a mechanism mediated by ERα via the up-regulation of corticotrophin-releasing hormone receptor type 2 [32].

In our study, we used specific ERα and ERβ antagonists (MPP and PHTPP, respectively) but we did not observe a reduction in the protective effect elicited by

SF/E2 co-treatment, suggesting that probably E2 enhances SF cardioprotection against oxidative injury without the involvement of ERs.

A battery of protein kinases, including Akt and ERK1/2, showed to exert cardioprotective activity against oxidative stress [325]; so we hypothesized that in the enhanced protection elicited by SF/E2 co-treatment, Akt and ERK1/2 signaling pathways could be involved.

Several studies showed a beneficial role of ERK1/2 pathway in the heart [326]. Once activated, ERK1/2 can phosphorylate many intracellular targets at both cytoplasmic and nuclear level. ERK1/2 cytosolic targets include approximately 70 proteins [327], while in the nucleus it phosphorylates multiple transcription factors, inducing gene expression in the heart [328]. Interestingly, ERK1/2 activation was markedly higher in SF/E2 co-treated cells with respect to single treatments, suggesting a synergic effect of SF and E2 on the phosphorylation of this MAPK.

Akt is a serine/threonine kinase which modulates several aspects of cellular functions, such as growth, survival and metabolism, and its upstream kinase is PI3K [329, 330]. After 30 min, all treatments increase Akt phosphorylation with respect to control cells; meanwhile at 2h, only E2 and the co-treatment maintained Akt activation; nevertheless, the co-treatment greatly activated Akt with respect to SF or E2 alone, so suggesting a potential contribution of E2 in the regulation of this protective kinase. Our data are in agreement with previous observations on E2 capacity to activate Akt signaling pathway in cardiac cells [26, 312]. Moreover, these effects cannot be explained by a simple additive effect of E2 and SF but rather by a synergic action.

To better clarify the role of these two kinases on enhanced cardioprotection of SF induced by E2, we used specific inhibitors of Akt and ERK1/2 phosphorylation (LY and PD, respectively). Of note, only the simultaneously presence of both inhibitors, significantly reduced co-treatment protective effect against oxidative injury. This is not surprising, because these kinases often have the same target protein, and can act in concert to promote cell survival [331]. Examples are forkhead box O (FOXO) and c-

Myc transcription factors BCL2-associated agonist of cell death and GSK3 [332-335]. To better characterize the contribution of E2 on Akt phosphorylation, we used receptor selective agonists. In particular, we selected PPT, KB5 and G1 as selective agonists for ERα, ERβ and GPR30, respectively. The co-treatment with SF and PPT did not activate Akt kinase, so implying no involvement of ERα in SF/E2 co-treatment synergic effect on Akt phosphorylation. Meanwhile, co-treatment with SF and KB5 or G1 agonists enhanced the activation of Akt with respect to single treatments, suggesting a role for these two receptors in the modulation of Akt phosphorylation. Moreover, it has been widely demonstrated the involvement of Akt signaling pathway in the nuclear accumulation and activation of Nrf2 [179, 253]. As expected, the inhibition of Akt phosphorylation by LY led to a reduction of the ARE-binding activity of Nrf2 induced by SF/E2 co-treatment, suggesting, once more, a role for this kinase in the protective effects elicited by SF/E2 co-treatment.

9. CONCLUSION

Our data show that E2 enhances SF protective effects against oxidative damage in cardiomyocytes. In particular, E2 enhanced the expression of antioxidant enzymes induced by SF through the involvement of Nrf2/ARE pathway, and the activation of cardioprotective signaling pathways. Interestingly, our findings reveal that the enhanced protective effects elicited by SF in presence of E2 can only be related to a synergic effect between the bioactive compound and the hormone. Therefore, this study suggests that nutraceutical efficacy might be modulated by sex hormones. Moreover, it provides promising indications for the promotion of a isothiocyanate-rich diet for cardiovascular prevention in women.

In conclusion, the data open new avenues for further researches and strengthen the concept that, similarly to studies on drugs, investigations on bioactive compounds should take into account sex-gender differences.

10. REFERENCES

[1] Townsend, N., Wilson, L., Bhatnagar, P., Wickramasinghe, K., Rayner, M., and Nichols, M. (2016) Cardiovascular disease in Europe: epidemiological update 2016, *Eur Heart J 37*, 3232-3245.

[2] Tamargo, J., Rosano, G., Walther, T., Duarte, J., Niessner, A., Kaski, J. C., Ceconi, C., Drexel, H., Kjeldsen, K., Savarese, G., Torp-Pedersen, C., Atar, D., Lewis, B. S., and Agewall, S. (2017) Gender differences in the effects of cardiovascular drugs, *Eur Heart J Cardiovasc Pharmacother 3*, 163-182.

[3] Garcia, M., Mulvagh, S. L., Merz, C. N., Buring, J. E., and Manson, J. E. (2016) Cardiovascular Disease in Women: Clinical Perspectives, *Circ Res 118*, 1273-1293.

[4] Regitz-Zagrosek, V. (2006) Therapeutic implications of the gender-specific aspects of cardiovascular disease, *Nat Rev Drug Discov 5*, 425-438.

[5] Rosano, G. M., Lewis, B., Agewall, S., Wassmann, S., Vitale, C., Schmidt, H., Drexel, H., Patak, A., Torp-Pedersen, C., Kjeldsen, K. P., and Tamargo, J. (2015) Gender differences in the effect of cardiovascular drugs: a position document of the Working Group on Pharmacology and Drug Therapy of the ESC, *Eur Heart J 36*, 2677-2680.

[6] Franconi, F., Raparelli, V., and Regitz-Zagrosek, V. (2017) Sex and gender landscape in pharmacology, *Pharmacol Res 123*, 93-94.

[7] Regitz-Zagrosek, V., and Kararigas, G. (2017) Mechanistic Pathways of Sex Differences in Cardiovascular Disease, *Physiol Rev 97*, 1-37.

[8] Berger, J. S., Elliott, L., Gallup, D., Roe, M., Granger, C. B., Armstrong, P. W., Simes, R. J., White, H. D., Van de Werf, F., Topol, E. J., Hochman, J. S., Newby, L. K., Harrington, R. A., Califf, R. M., Becker, R. C., and Douglas, P.

S. (2009) Sex differences in mortality following acute coronary syndromes, *JAMA 302*, 874-882.

[9] Vaccarino, V., Parsons, L., Every, N. R., Barron, H. V., and Krumholz, H. M. (1999) Sex-based differences in early mortality after myocardial infarction. National Registry of Myocardial Infarction 2 Participants, *N Engl J Med 341*, 217-225.

[10] Templin, C., Ghadri, J. R., Diekmann, J., Napp, L. C., Bataiosu, D. R., Jaguszewski, M., Cammann, V. L., Sarcon, A., Geyer, V., Neumann, C. A., Seifert, B., Hellermann, J., Schwyzer, M., Eisenhardt, K., Jenewein, J., Franke, J., Katus, H. A., Burgdorf, C., Schunkert, H., Moeller, C., Thiele, H., Bauersachs, J., Tschöpe, C., Schultheiss, H. P., Laney, C. A., Rajan, L., Michels, G., Pfister, R., Ukena, C., Böhm, M., Erbel, R., Cuneo, A., Kuck, K. H., Jacobshagen, C., Hasenfuss, G., Karakas, M., Koenig, W., Rottbauer, W., Said, S. M., Braun-Dullaeus, R. C., Cuculi, F., Banning, A., Fischer, T. A., Vasankari, T., Airaksinen, K. E., Fijalkowski, M., Rynkiewicz, A., Pawlak, M., Opolski, G., Dworakowski, R., MacCarthy, P., Kaiser, C., Osswald, S., Galiuto, L., Crea, F., Dichtl, W., Franz, W. M., Empen, K., Felix, S. B., Delmas, C., Lairez, O., Erne, P., Bax, J. J., Ford, I., Ruschitzka, F., Prasad, A., and Lüscher, T. F. (2015) Clinical Features and Outcomes of Takotsubo (Stress) Cardiomyopathy, *N Engl J Med 373*, 929-938.

[11] Nwankwo, T., Yoon, S. S., Burt, V., and Gu, Q. (2013) Hypertension among adults in the United States: National Health and Nutrition Examination Survey, 2011-2012, *NCHS Data Brief*, 1-8.

[12] Mancusi, C., Gerdts, E., De Simone, G., Abdelhai, Y. M., Lønnebakken, M. T., Boman, K., Wachtell, K., Dahlöf, B., and Devereux, R. B. (2014) Impact of isolated systolic hypertension on normalization of left ventricular structure during antihypertensive treatment (the LIFE study), *Blood Press 23*, 206-212.

[13] Gerdts, E., Okin, P. M., de Simone, G., Cramariuc, D., Wachtell, K., Boman, K., and Devereux, R. B. (2008) Gender differences in left ventricular structure and function during antihypertensive treatment: the Losartan Intervention for Endpoint Reduction in Hypertension Study, *Hypertension 51*, 1109-1114.

[14] Villar, A. V., Llano, M., Cobo, M., Expósito, V., Merino, R., Martín-Durán, R., Hurlé, M. A., and Nistal, J. F. (2009) Gender differences of echocardiographic and gene expression patterns in human pressure overload left ventricular hypertrophy, *J Mol Cell Cardiol 46*, 526-535.

[15] Petrov, G., Regitz-Zagrosek, V., Lehmkuhl, E., Krabatsch, T., Dunkel, A., Dandel, M., Dworatzek, E., Mahmoodzadeh, S., Schubert, C., Becher, E., Hampl, H., and Hetzer, R. (2010) Regression of myocardial hypertrophy after aortic valve replacement: faster in women?, *Circulation 122*, S23-28.

[16] Howden, E. J., Perhonen, M., Peshock, R. M., Zhang, R., Arbab-Zadeh, A., Adams-Huet, B., and Levine, B. D. (2015) Females have a blunted cardiovascular response to one year of intensive supervised endurance training, *J Appl Physiol (1985) 119*, 37-46.

[17] Camper-Kirby, D., Welch, S., Walker, A., Shiraishi, I., Setchell, K. D., Schaefer, E., Kajstura, J., Anversa, P., and Sussman, M. A. (2001) Myocardial Akt activation and gender: increased nuclear activity in females versus males, *Circ Res 88*, 1020-1027.

[18] Vingren, J. L., Kraemer, W. J., Ratamess, N. A., Anderson, J. M., Volek, J. S., and Maresh, C. M. (2010) Testosterone physiology in resistance exercise and training: the up-stream regulatory elements, *Sports Med 40*, 1037-1053.

[19] Cleland, J. G., Swedberg, K., Follath, F., Komajda, M., Cohen-Solal, A., Aguilar, J. C., Dietz, R., Gavazzi, A., Hobbs, R., Korewicki, J., Madeira, H. C., Moiseyev, V. S., Preda, I., van Gilst, W. H., Widimsky, J., Freemantle, N., Eastaugh, J., Mason, J., and Cardiology, S. G. o. D. o. t. W. G. o. H. F. o. t. E. S. o. (2003) The EuroHeart Failure survey programme-- a survey on the quality

of care among patients with heart failure in Europe. Part 1: patient characteristics and diagnosis, *Eur Heart J 24*, 442-463.

[20] Martínez-Sellés, M., Doughty, R. N., Poppe, K., Whalley, G. A., Earle, N., Tribouilloy, C., McMurray, J. J., Swedberg, K., Køber, L., Berry, C., Squire, I., and (MAGGIC), M.-A. G. G. I. C. H. F. (2012) Gender and survival in patients with heart failure: interactions with diabetes and aetiology. Results from the MAGGIC individual patient meta-analysis, *Eur J Heart Fail 14*, 473-479.

[21] Regitz-Zagrosek, V., Brokat, S., and Tschope, C. (2007) Role of gender in heart failure with normal left ventricular ejection fraction, *Prog Cardiovasc Dis 49*, 241-251.

[22] Finco, I., LaPensee, C. R., Krill, K. T., and Hammer, G. D. (2015) Hedgehog signaling and steroidogenesis, *Annu Rev Physiol 77*, 105-129.

[23] Simpson, E. R., Zhao, Y., Agarwal, V. R., Michael, M. D., Bulun, S. E., Hinshelwood, M. M., Graham-Lorence, S., Sun, T., Fisher, C. R., Qin, K., and Mendelson, C. R. (1997) Aromatase expression in health and disease, *Recent Prog Horm Res 52*, 185-213; discussion 213-184.

[24] Stocco, C. (2012) Tissue physiology and pathology of aromatase, *Steroids 77*, 27-35.

[25] Angeloni, C., Teti, G., Barbalace, M. C., Malaguti, M., Falconi, M., and Hrelia, S. (2017) 17β-Estradiol enhances sulforaphane cardioprotection against oxidative stress, *J Nutr Biochem 42*, 26-36.

[26] Patten, R. D., Pourati, I., Aronovitz, M. J., Baur, J., Celestin, F., Chen, X., Michael, A., Haq, S., Nuedling, S., Grohe, C., Force, T., Mendelsohn, M. E., and Karas, R. H. (2004) 17beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/Akt signaling, *Circ Res 95*, 692-699.

[27] Kim, J. K., Pedram, A., Razandi, M., and Levin, E. R. (2006) Estrogen prevents cardiomyocyte apoptosis through inhibition of reactive oxygen species and differential regulation of p38 kinase isoforms, *J Biol Chem 281*, 6760-6767.

[28] Pedram, A., Razandi, M., Narayanan, R., Dalton, J. T., McKinsey, T. A., and Levin, E. R. (2013) Estrogen regulates histone deacetylases to prevent cardiac hypertrophy, *Mol Biol Cell 24*, 3805-3818.

[29] Shen, T., Ding, L., Ruan, Y., Qin, W., Lin, Y., Xi, C., Lu, Y., Dou, L., Zhu, Y., Cao, Y., Man, Y., Bian, Y., Wang, S., Xiao, C., and Li, J. (2014) SIRT1 functions as an important regulator of estrogen-mediated cardiomyocyte protection in angiotensin II-induced heart hypertrophy, *Oxid Med Cell Longev 2014*, 713894.

[30] Strehlow, K., Rotter, S., Wassmann, S., Adam, O., Grohé, C., Laufs, K., Böhm, M., and Nickenig, G. (2003) Modulation of antioxidant enzyme expression and function by estrogen, *Circ Res 93*, 170-177.

[31] Huang, A., and Kaley, G. (2004) Gender-specific regulation of cardiovascular function: estrogen as key player, *Microcirculation 11*, 9-38.

[32] Cong, B., Xu, Y., Sheng, H., Zhu, X., Wang, L., Zhao, W., Tang, Z., Lu, J., and Ni, X. (2014) Cardioprotection of 17β-estradiol against hypoxia/reoxygenation in cardiomyocytes is partly through up-regulation of CRH receptor type 2, *Mol Cell Endocrinol 382*, 17-25.

[33] Hsieh, D. J., Kuo, W. W., Lai, Y. P., Shibu, M. A., Shen, C. Y., Pai, P., Yeh, Y. L., Lin, J. Y., Viswanadha, V. P., and Huang, C. Y. (2015) 17β-Estradiol and/or Estrogen Receptor β Attenuate the Autophagic and Apoptotic Effects Induced by Prolonged Hypoxia Through HIF-1α-Mediated BNIP3 and IGFBP-3 Signaling Blockage, *Cell Physiol Biochem 36*, 274-284.

[34] Liou, C. M., Yang, A. L., Kuo, C. H., Tin, H., Huang, C. Y., and Lee, S. D. (2010) Effects of 17beta-estradiol on cardiac apoptosis in ovariectomized rats, *Cell Biochem Funct 28*, 521-528.

[35] Wu, C. H., Liu, J. Y., Wu, J. P., Hsieh, Y. H., Liu, C. J., Hwang, J. M., Lee, S. D., Chen, L. M., Chang, M. H., Kuo, W. W., Shyu, J. C., Tsai, J. H., and Huang, C. Y. (2005) 17beta-estradiol reduces cardiac hypertrophy mediated through the up-regulation of PI3K/Akt and the suppression of calcineurin/NF-AT3 signaling pathways in rats, *Life Sci 78*, 347-356.

[36] Booth, E. A., Marchesi, M., Knittel, A. K., Kilbourne, E. J., and Lucchesi, B. R. (2007) The pathway-selective estrogen receptor ligand WAY-169916 reduces infarct size after myocardial ischemia and reperfusion by an estrogen receptor dependent mechanism, *J Cardiovasc Pharmacol 49*, 401-407.

[37] Lee, T. M., Lin, M. S., Chou, T. F., Tsai, C. H., and Chang, N. C. (2004) Adjunctive 17beta-estradiol administration reduces infarct size by altered expression of canine myocardial connexin43 protein, *Cardiovasc Res 63*, 109-117.

[38] Hale, S. L., Birnbaum, Y., and Kloner, R. A. (1996) beta-Estradiol, but not alpha-estradiol, reduced myocardial necrosis in rabbits after ischemia and reperfusion, *Am Heart J 132*, 258-262.

[39] Zhai, P., Eurell, T. E., Cotthaus, R., Jeffery, E. H., Bahr, J. M., and Gross, D. R. (2000) Effect of estrogen on global myocardial ischemia-reperfusion injury in female rats, *Am J Physiol Heart Circ Physiol 279*, H2766-2775.

[40] Booth, E. A., Marchesi, M., Kilbourne, E. J., and Lucchesi, B. R. (2003) 17Beta-estradiol as a receptor-mediated cardioprotective agent, *J Pharmacol Exp Ther 307*, 395-401.

[41] Dubey, R. K., Jackson, E. K., Keller, P. J., Imthurn, B., and Rosselli, M. (2001) Estradiol metabolites inhibit endothelin synthesis by an estrogen receptor-independent mechanism, *Hypertension 37*, 640-644.

[42] van Eickels, M., Grohé, C., Cleutjens, J. P., Janssen, B. J., Wellens, H. J., and Doevendans, P. A. (2001) 17beta-estradiol attenuates the development of pressure-overload hypertrophy, *Circulation 104*, 1419-1423.

[43] Babiker, F. A., De Windt, L. J., van Eickels, M., Thijssen, V., Bronsaer, R. J., Grohé, C., van Bilsen, M., and Doevendans, P. A. (2004) 17beta-estradiol antagonizes cardiomyocyte hypertrophy by autocrine/paracrine stimulation of a guanylyl cyclase A receptor-cyclic guanosine monophosphate-dependent protein kinase pathway, *Circulation 109*, 269-276.

[44] Patten, R. D., Pourati, I., Aronovitz, M. J., Alsheikh-Ali, A., Eder, S., Force, T., Mendelsohn, M. E., and Karas, R. H. (2008) 17 Beta-estradiol differentially affects left ventricular and cardiomyocyte hypertrophy following myocardial infarction and pressure overload, *J Card Fail 14*, 245-253.

[45] Westphal, C., Schubert, C., Prelle, K., Penkalla, A., Fliegner, D., Petrov, G., and Regitz-Zagrosek, V. (2012) Effects of estrogen, an ERα agonist and raloxifene on pressure overload induced cardiac hypertrophy, *PLoS One 7*, e50802.

[46] Grohé, C., Kahlert, S., Löbbert, K., Stimpel, M., Karas, R. H., Vetter, H., and Neyses, L. (1997) Cardiac myocytes and fibroblasts contain functional estrogen receptors, *FEBS Lett 416*, 107-112.

[47] Karas, R. H., Patterson, B. L., and Mendelsohn, M. E. (1994) Human vascular smooth muscle cells contain functional estrogen receptor, *Circulation 89*, 1943-1950.

[48] Meyer, R., Linz, K. W., Surges, R., Meinardus, S., Vees, J., Hoffmann, A., Windholz, O., and Grohé, C. (1998) Rapid modulation of L-type calcium

current by acutely applied oestrogens in isolated cardiac myocytes from human, guinea-pig and rat, *Exp Physiol 83*, 305-321.

[49] Venkov, C. D., Rankin, A. B., and Vaughan, D. E. (1996) Identification of authentic estrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormone regulation of endothelial function, *Circulation 94*, 727-733.

[50] Nordmeyer, J., Eder, S., Mahmoodzadeh, S., Martus, P., Fielitz, J., Bass, J., Bethke, N., Zurbrügg, H. R., Pregla, R., Hetzer, R., and Regitz-Zagrosek, V. (2004) Upregulation of myocardial estrogen receptors in human aortic stenosis, *Circulation 110*, 3270-3275.

[51] Chen, J. Q., Delannoy, M., Cooke, C., and Yager, J. D. (2004) Mitochondrial localization of ERalpha and ERbeta in human MCF7 cells, *Am J Physiol Endocrinol Metab 286*, E1011-1022.

[52] Dutertre, M., and Smith, C. L. (2000) Molecular mechanisms of selective estrogen receptor modulator (SERM) action, *J Pharmacol Exp Ther 295*, 431-437.

[53] Thomas, P., Pang, Y., Filardo, E. J., and Dong, J. (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells, *Endocrinology 146*, 624-632.

[54] Haas, E., Meyer, M. R., Schurr, U., Bhattacharya, I., Minotti, R., Nguyen, H. H., Heigl, A., Lachat, M., Genoni, M., and Barton, M. (2007) Differential effects of 17beta-estradiol on function and expression of estrogen receptor alpha, estrogen receptor beta, and GPR30 in arteries and veins of patients with atherosclerosis, *Hypertension 49*, 1358-1363.

[55] Hazell, G. G., Yao, S. T., Roper, J. A., Prossnitz, E. R., O'Carroll, A. M., and Lolait, S. J. (2009) Localisation of GPR30, a novel G protein-coupled

oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues, *J Endocrinol 202*, 223-236.

[56] Wang, C., Prossnitz, E. R., and Roy, S. K. (2007) Expression of G protein-coupled receptor 30 in the hamster ovary: differential regulation by gonadotropins and steroid hormones, *Endocrinology 148*, 4853-4864.

[57] Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., and Prossnitz, E. R. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling, *Science 307*, 1625-1630.

[58] Filardo, E., Quinn, J., Pang, Y., Graeber, C., Shaw, S., Dong, J., and Thomas, P. (2007) Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane, *Endocrinology 148*, 3236-3245.

[59] Funakoshi, T., Yanai, A., Shinoda, K., Kawano, M. M., and Mizukami, Y. (2006) G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane, *Biochem Biophys Res Commun 346*, 904-910.

[60] Cheng, S. B., Quinn, J. A., Graeber, C. T., and Filardo, E. J. (2011) Down-modulation of the G-protein-coupled estrogen receptor, GPER, from the cell surface occurs via a trans-Golgi-proteasome pathway, *J Biol Chem 286*, 22441-22455.

[61] Khalil, R. A. (2013) Estrogen, vascular estrogen receptor and hormone therapy in postmenopausal vascular disease, *Biochem Pharmacol 86*, 1627-1642.

[62] Menazza, S., and Murphy, E. (2016) The Expanding Complexity of Estrogen Receptor Signaling in the Cardiovascular System, *Circ Res 118*, 994-1007.

[63] Burns, K. A., Li, Y., Arao, Y., Petrovich, R. M., and Korach, K. S. (2011) Selective mutations in estrogen receptor alpha D-domain alters nuclear translocation and non-estrogen response element gene regulatory mechanisms, *J Biol Chem 286*, 12640-12649.

[64] Thomas, R. S., Sarwar, N., Phoenix, F., Coombes, R. C., and Ali, S. (2008) Phosphorylation at serines 104 and 106 by Erk1/2 MAPK is important for estrogen receptor-alpha activity, *J Mol Endocrinol 40*, 173-184.

[65] O'Lone, R., Knorr, K., Jaffe, I. Z., Schaffer, M. E., Martini, P. G., Karas, R. H., Bienkowska, J., Mendelsohn, M. E., and Hansen, U. (2007) Estrogen receptors alpha and beta mediate distinct pathways of vascular gene expression, including genes involved in mitochondrial electron transport and generation of reactive oxygen species, *Mol Endocrinol 21*, 1281-1296.

[66] Nikolic, I., Liu, D., Bell, J. A., Collins, J., Steenbergen, C., and Murphy, E. (2007) Treatment with an estrogen receptor-beta-selective agonist is cardioprotective, *J Mol Cell Cardiol 42*, 769-780.

[67] Mahmoodzadeh, S., Leber, J., Zhang, X., Jaisser, F., Messaoudi, S., Morano, I., Furth, P. A., Dworatzek, E., and Regitz-Zagrosek, V. (2014) Cardiomyocyte-specific Estrogen Receptor Alpha Increases Angiogenesis, Lymphangiogenesis and Reduces Fibrosis in the Female Mouse Heart Post-Myocardial Infarction, *J Cell Sci Ther 5*, 153.

[68] Schuster, I., Mahmoodzadeh, S., Dworatzek, E., Jaisser, F., Messaoudi, S., Morano, I., and Regitz-Zagrosek, V. (2016) Cardiomyocyte-specific overexpression of oestrogen receptor β improves survival and cardiac function after myocardial infarction in female and male mice, *Clin Sci (Lond) 130*, 365-376.

[69] Fish, J. E., and Marsden, P. A. (2006) Endothelial nitric oxide synthase: insight into cell-specific gene regulation in the vascular endothelium, *Cell Mol Life Sci 63*, 144-162.

[70] Tan, E., Gurjar, M. V., Sharma, R. V., and Bhalla, R. C. (1999) Estrogen receptor-alpha gene transfer into bovine aortic endothelial cells induces eNOS gene expression and inhibits cell migration, *Cardiovasc Res 43*, 788-797.

[71] Nuedling, S., Karas, R. H., Mendelsohn, M. E., Katzenellenbogen, J. A., Katzenellenbogen, B. S., Meyer, R., Vetter, H., and Grohé, C. (2001) Activation of estrogen receptor beta is a prerequisite for estrogen-dependent upregulation of nitric oxide synthases in neonatal rat cardiac myocytes, *FEBS Lett 502*, 103-108.

[72] Liu, Z., Gou, Y., Zhang, H., Zuo, H., and Yao, D. (2014) Estradiol improves cardiovascular function through up-regulation of SOD2 on vascular wall, *Redox Biol 3*, 88-99.

[73] Pietras, R. J., and Szego, C. M. (1977) Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells, *Nature 265*, 69-72.

[74] Simoncini, T., Mannella, P., Fornari, L., Caruso, A., Varone, G., and Genazzani, A. R. (2004) Genomic and non-genomic effects of estrogens on endothelial cells, *Steroids 69*, 537-542.

[75] Meyer, M. R., Haas, E., and Barton, M. (2006) Gender differences of cardiovascular disease: new perspectives for estrogen receptor signaling, *Hypertension 47*, 1019-1026.

[76] Pedram, A., Razandi, M., and Levin, E. R. (2006) Nature of functional estrogen receptors at the plasma membrane, *Mol Endocrinol 20*, 1996-2009.

[77] Guo, X., Razandi, M., Pedram, A., Kassab, G., and Levin, E. R. (2005) Estrogen induces vascular wall dilation: mediation through kinase signaling to nitric oxide and estrogen receptors alpha and beta, *J Biol Chem 280*, 19704-19710.

[78] Huang, B., Omoto, Y., Iwase, H., Yamashita, H., Toyama, T., Coombes, R. C., Filipovic, A., Warner, M., and Gustafsson, J. (2014) Differential expression of estrogen receptor α, β1, and β2 in lobular and ductal breast cancer, *Proc Natl Acad Sci U S A 111*, 1933-1938.

[79] Irsik, D. L., Carmines, P. K., and Lane, P. H. (2013) Classical estrogen receptors and ERα splice variants in the mouse, *PLoS One 8*, e70926.

[80] Chambliss, K. L., Yuhanna, I. S., Mineo, C., Liu, P., German, Z., Sherman, T. S., Mendelsohn, M. E., Anderson, R. G., and Shaul, P. W. (2000) Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae, *Circ Res 87*, E44-52.

[81] Chambliss, K. L., and Shaul, P. W. (2002) Estrogen modulation of endothelial nitric oxide synthase, *Endocr Rev 23*, 665-686.

[82] Razandi, M., Alton, G., Pedram, A., Ghonshani, S., Webb, P., and Levin, E. R. (2003) Identification of a structural determinant necessary for the localization and function of estrogen receptor alpha at the plasma membrane, *Mol Cell Biol 23*, 1633-1646.

[83] Acconcia, F., Ascenzi, P., Fabozzi, G., Visca, P., and Marino, M. (2004) S-palmitoylation modulates human estrogen receptor-alpha functions, *Biochem Biophys Res Commun 316*, 878-883.

[84] Pedram, A., Razandi, M., Sainson, R. C., Kim, J. K., Hughes, C. C., and Levin, E. R. (2007) A conserved mechanism for steroid receptor translocation to the plasma membrane, *J Biol Chem 282*, 22278-22288.

[85] Fukumoto, T., Tawa, M., Yamashita, N., Ohkita, M., and Matsumura, Y. (2013) Protective effects of 17beta-estradiol on post-ischemic cardiac dysfunction and norepinephrine overflow through the non-genomic estrogen receptor/nitric oxide-mediated pathway in the rat heart, *Eur J Pharmacol 699*, 74-80.

[86] Skavdahl, M., Steenbergen, C., Clark, J., Myers, P., Demianenko, T., Mao, L., Rockman, H. A., Korach, K. S., and Murphy, E. (2005) Estrogen receptor-beta mediates male-female differences in the development of pressure overload hypertrophy, *Am J Physiol Heart Circ Physiol 288*, H469-476.

[87] Pedram, A., Razandi, M., Lubahn, D., Liu, J., Vannan, M., and Levin, E. R. (2008) Estrogen inhibits cardiac hypertrophy: role of estrogen receptor-beta to inhibit calcineurin, *Endocrinology 149*, 3361-3369.

[88] Haynes, M. P., Sinha, D., Russell, K. S., Collinge, M., Fulton, D., Morales-Ruiz, M., Sessa, W. C., and Bender, J. R. (2000) Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells, *Circ Res 87*, 677-682.

[89] Khazaei, M., Moien-Afshari, F., and Laher, I. (2008) Vascular endothelial function in health and diseases, *Pathophysiology 15*, 49-67.

[90] Haynes, M. P., Li, L., Sinha, D., Russell, K. S., Hisamoto, K., Baron, R., Collinge, M., Sessa, W. C., and Bender, J. R. (2003) Src kinase mediates phosphatidylinositol 3-kinase/Akt-dependent rapid endothelial nitric-oxide synthase activation by estrogen, *J Biol Chem 278*, 2118-2123.

[91] Chen, Z., Yuhanna, I. S., Galcheva-Gargova, Z., Karas, R. H., Mendelsohn, M. E., and Shaul, P. W. (1999) Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen, *J Clin Invest 103*, 401-406.

[92] Kumar, P., Wu, Q., Chambliss, K. L., Yuhanna, I. S., Mumby, S. M., Mineo, C., Tall, G. G., and Shaul, P. W. (2007) Direct interactions with G α i and G βγ mediate nongenomic signaling by estrogen receptor α, *Mol Endocrinol 21*, 1370-1380.

[93] Stefano, G. B., Prevot, V., Beauvillain, J. C., Cadet, P., Fimiani, C., Welters, I., Fricchione, G. L., Breton, C., Lassalle, P., Salzet, M., and Bilfinger, T. V. (2000) Cell-surface estrogen receptors mediate calcium-dependent nitric oxide release in human endothelia, *Circulation 101*, 1594-1597.

[94] Rubio-Gayosso, I., Sierra-Ramirez, A., García-Vazquez, A., Martinez-Martinez, A., Muñoz-García, O., Morato, T., and Ceballos-Reyes, G. (2000) 17Beta-estradiol increases intracellular calcium concentration through a short-term and nongenomic mechanism in rat vascular endothelium in culture, *J Cardiovasc Pharmacol 36*, 196-202.

[95] Li, Q. Y., Chen, L., Zhu, Y. H., Zhang, M., Wang, Y. P., and Wang, M. W. (2011) Involvement of estrogen receptor-β in farrerol inhibition of rat thoracic aorta vascular smooth muscle cell proliferation, *Acta Pharmacol Sin 32*, 433-440.

[96] Ortmann, J., Veit, M., Zingg, S., Di Santo, S., Traupe, T., Yang, Z., Völzmann, J., Dubey, R. K., Christen, S., and Baumgartner, I. (2011) Estrogen receptor-α but not -β or GPER inhibits high glucose-induced human VSMC proliferation: potential role of ROS and ERK, *J Clin Endocrinol Metab 96*, 220-228.

[97] Lu, Q., Surks, H. K., Ebling, H., Baur, W. E., Brown, D., Pallas, D. C., and Karas, R. H. (2003) Regulation of estrogen receptor alpha-mediated transcription by a direct interaction with protein phosphatase 2A, *J Biol Chem 278*, 4639-4645.

[98] Yang, L., Wang, Y., Chen, P., Hu, J., Xiong, Y., Feng, D., Liu, H., Zhang, H., Yang, H., and He, J. (2011) Na(+)/H(+) exchanger regulatory factor 1 (NHERF1) is required for the estradiol-dependent increase of phosphatase and tensin homolog (PTEN) protein expression, *Endocrinology 152*, 4537-4549.

[99] Ueda, K., Lu, Q., Baur, W., Aronovitz, M. J., and Karas, R. H. (2013) Rapid estrogen receptor signaling mediates estrogen-induced inhibition of vascular smooth muscle cell proliferation, *Arterioscler Thromb Vasc Biol 33*, 1837-1843.

[100] Bernelot Moens, S. J., Schnitzler, G. R., Nickerson, M., Guo, H., Ueda, K., Lu, Q., Aronovitz, M. J., Nickerson, H., Baur, W. E., Hansen, U., Iyer, L. K., and Karas, R. H. (2012) Rapid estrogen receptor signaling is essential for the protective effects of estrogen against vascular injury, *Circulation 126*, 1993-2004.

[101] Brunsing, R. L., and Prossnitz, E. R. (2011) Induction of interleukin-10 in the T helper type 17 effector population by the G protein coupled estrogen receptor (GPER) agonist G-1, *Immunology 134*, 93-106.

[102] Rigiracciolo, D. C., Scarpelli, A., Lappano, R., Pisano, A., Santolla, M. F., Avino, S., De Marco, P., Bussolati, B., Maggiolini, M., and De Francesco, E. M. (2016) GPER is involved in the stimulatory effects of aldosterone in breast cancer cells and breast tumor-derived endothelial cells, *Oncotarget 7*, 94-111.

[103] Deschamps, A. M., and Murphy, E. (2009) Activation of a novel estrogen receptor, GPER, is cardioprotective in male and female rats, *Am J Physiol Heart Circ Physiol 297*, H1806-1813.

[104] Dennis, M. K., Burai, R., Ramesh, C., Petrie, W. K., Alcon, S. N., Nayak, T. K., Bologa, C. G., Leitao, A., Brailoiu, E., Deliu, E., Dun, N. J., Sklar, L. A., Hathaway, H. J., Arterburn, J. B., Oprea, T. I., and Prossnitz, E. R. (2009) In vivo effects of a GPR30 antagonist, *Nat Chem Biol 5*, 421-427.

[105] Filardo, E. J. (2002) Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer, *J Steroid Biochem Mol Biol 80*, 231-238.

[106] Fujiwara, S., Terai, Y., Kawaguchi, H., Takai, M., Yoo, S., Tanaka, Y., Tanaka, T., Tsunetoh, S., Sasaki, H., Kanemura, M., Tanabe, A., Yamashita, Y., and Ohmichi, M. (2012) GPR30 regulates the EGFR-Akt cascade and predicts lower survival in patients with ovarian cancer, *J Ovarian Res 5*, 35.

[107] Feldman, R. D., and Gros, R. (2011) Unraveling the mechanisms underlying the rapid vascular effects of steroids: sorting out the receptors and the pathways, *Br J Pharmacol 163*, 1163-1169.

[108] Meyer, M. R., Baretella, O., Prossnitz, E. R., and Barton, M. (2010) Dilation of epicardial coronary arteries by the G protein-coupled estrogen receptor agonists G-1 and ICI 182,780, *Pharmacology 86*, 58-64.

[109] Shaw, L., Taggart, M. J., and Austin, C. (2000) Mechanisms of 17 beta-oestradiol induced vasodilatation in isolated pressurized rat small arteries, *Br J Pharmacol 129*, 555-565.

[110] Ullrich, N. D., Krust, A., Collins, P., and MacLeod, K. T. (2008) Genomic deletion of estrogen receptors ERalpha and ERbeta does not alter estrogen-mediated inhibition of Ca2+ influx and contraction in murine cardiomyocytes, *Am J Physiol Heart Circ Physiol 294*, H2421-2427.

[111] Han, G., Ma, H., Chintala, R., Fulton, D. J., Barman, S. A., and White, R. E. (2009) Essential role of the 90-kilodalton heat shock protein in mediating nongenomic estrogen signaling in coronary artery smooth muscle, *J Pharmacol Exp Ther 329*, 850-855.

[112] Lindsey, S. H., Cohen, J. A., Brosnihan, K. B., Gallagher, P. E., and Chappell, M. C. (2009) Chronic treatment with the G protein-coupled receptor 30 agonist G-1 decreases blood pressure in ovariectomized mRen2.Lewis rats, *Endocrinology 150*, 3753-3758.

[113] Haas, E., Bhattacharya, I., Brailoiu, E., Damjanović, M., Brailoiu, G. C., Gao, X., Mueller-Guerre, L., Marjon, N. A., Gut, A., Minotti, R., Meyer, M. R., Amann, K., Ammann, E., Perez-Dominguez, A., Genoni, M., Clegg, D. J., Dun, N. J., Resta, T. C., Prossnitz, E. R., and Barton, M. (2009) Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity, *Circ Res 104*, 288-291.

[114] Broughton, B. R., Miller, A. A., and Sobey, C. G. (2010) Endothelium-dependent relaxation by G protein-coupled receptor 30 agonists in rat carotid arteries, *Am J Physiol Heart Circ Physiol 298*, H1055-1061.

[115] Yu, X., Ma, H., Barman, S. A., Liu, A. T., Sellers, M., Stallone, J. N., Prossnitz, E. R., White, R. E., and Han, G. (2011) Activation of G protein-coupled estrogen receptor induces endothelium-independent relaxation of

coronary artery smooth muscle, *Am J Physiol Endocrinol Metab 301*, E882-888.

[116] Feldman, R. D., and Limbird, L. E. (2017) GPER (GPR30): A Nongenomic Receptor (GPCR) for Steroid Hormones with Implications for Cardiovascular Disease and Cancer, *Annu Rev Pharmacol Toxicol 57*, 567-584.

[117] Meyer, M. R., Fredette, N. C., Howard, T. A., Hu, C., Ramesh, C., Daniel, C., Amann, K., Arterburn, J. B., Barton, M., and Prossnitz, E. R. (2015) Erratum: G Protein-coupled Estrogen Receptor Protects from Atherosclerosis, *Sci Rep 5*, 13510.

[118] Li, F., Yu, X., Szynkarski, C. K., Meng, C., Zhou, B., Barhoumi, R., White, R. E., Heaps, C. L., Stallone, J. N., and Han, G. (2013) Activation of GPER Induces Differentiation and Inhibition of Coronary Artery Smooth Muscle Cell Proliferation, *PLoS One 8*, e64771.

[119] Ding, Q., Gros, R., Limbird, L. E., Chorazyczewski, J., and Feldman, R. D. (2009) Estradiol-mediated ERK phosphorylation and apoptosis in vascular smooth muscle cells requires GPR 30, *Am J Physiol Cell Physiol 297*, C1178-1187.

[120] Chakrabarti, S., and Davidge, S. T. (2016) Analysis of G-Protein Coupled Receptor 30 (GPR30) on Endothelial Inflammation, *Methods Mol Biol 1366*, 503-516.

[121] Holm, A., Baldetorp, B., Olde, B., Leeb-Lundberg, L. M., and Nilsson, B. O. (2011) The GPER1 agonist G-1 attenuates endothelial cell proliferation by inhibiting DNA synthesis and accumulating cells in the S and G2 phases of the cell cycle, *J Vasc Res 48*, 327-335.

[122] Hussain, Y., Ding, Q., Connelly, P. W., Brunt, J. H., Ban, M. R., McIntyre, A. D., Huff, M. W., Gros, R., Hegele, R. A., and Feldman, R. D. (2015) G-protein estrogen receptor as a regulator of low-density lipoprotein cholesterol

metabolism: cellular and population genetic studies, *Arterioscler Thromb Vasc Biol 35*, 213-221.

[123] Weil, B. R., Manukyan, M. C., Herrmann, J. L., Wang, Y., Abarbanell, A. M., Poynter, J. A., and Meldrum, D. R. (2010) Signaling via GPR30 protects the myocardium from ischemia/reperfusion injury, *Surgery 148*, 436-443.

[124] Meyer, M. R., Prossnitz, E. R., and Barton, M. (2011) The G protein-coupled estrogen receptor GPER/GPR30 as a regulator of cardiovascular function, *Vascul Pharmacol 55*, 17-25.

[125] Bopassa, J. C., Eghbali, M., Toro, L., and Stefani, E. (2010) A novel estrogen receptor GPER inhibits mitochondria permeability transition pore opening and protects the heart against ischemia-reperfusion injury, *Am J Physiol Heart Circ Physiol 298*, H16-23.

[126] Fliegner, D., Schubert, C., Penkalla, A., Witt, H., Kararigas, G., Dworatzek, E., Staub, E., Martus, P., Ruiz Noppinger, P., Kintscher, U., Gustafsson, J. A., and Regitz-Zagrosek, V. (2010) Female sex and estrogen receptor-beta attenuate cardiac remodeling and apoptosis in pressure overload, *Am J Physiol Regul Integr Comp Physiol 298*, R1597-1606.

[127] Gabel, S. A., Walker, V. R., London, R. E., Steenbergen, C., Korach, K. S., and Murphy, E. (2005) Estrogen receptor beta mediates gender differences in ischemia/reperfusion injury, *J Mol Cell Cardiol 38*, 289-297.

[128] Babiker, F. A., Lips, D. J., Delvaux, E., Zandberg, P., Janssen, B. J., Prinzen, F., van Eys, G., Grohé, C., and Doevendans, P. A. (2007) Oestrogen modulates cardiac ischaemic remodelling through oestrogen receptor-specific mechanisms, *Acta Physiol (Oxf) 189*, 23-31.

[129] Dworatzek, E., Mahmoodzadeh, S., Schubert, C., Westphal, C., Leber, J., Kusch, A., Kararigas, G., Fliegner, D., Moulin, M., Ventura-Clapier, R., Gustafsson, J. A., Davidson, M. M., Dragun, D., and Regitz-Zagrosek, V.

(2014) Sex differences in exercise-induced physiological myocardial hypertrophy are modulated by oestrogen receptor beta, *Cardiovasc Res 102*, 418-428.

[130] Foryst-Ludwig, A., Kreissl, M. C., Sprang, C., Thalke, B., Böhm, C., Benz, V., Gürgen, D., Dragun, D., Schubert, C., Mai, K., Stawowy, P., Spranger, J., Regitz-Zagrosek, V., Unger, T., and Kintscher, U. (2011) Sex differences in physiological cardiac hypertrophy are associated with exercise-mediated changes in energy substrate availability, *Am J Physiol Heart Circ Physiol 301*, H115-122.

[131] Korte, T., Fuchs, M., Arkudas, A., Geertz, S., Meyer, R., Gardiwal, A., Klein, G., Niehaus, M., Krust, A., Chambon, P., Drexler, H., Fink, K., and Grohé, C. (2005) Female mice lacking estrogen receptor beta display prolonged ventricular repolarization and reduced ventricular automaticity after myocardial infarction, *Circulation 111*, 2282-2290.

[132] Favre, J., Gao, J., Henry, J. P., Remy-Jouet, I., Fourquaux, I., Billon-Gales, A., Thuillez, C., Arnal, J. F., Lenfant, F., and Richard, V. (2010) Endothelial estrogen receptor {alpha} plays an essential role in the coronary and myocardial protective effects of estradiol in ischemia/reperfusion, *Arterioscler Thromb Vasc Biol 30*, 2562-2567.

[133] Zhai, P., Eurell, T. E., Cooke, P. S., Lubahn, D. B., and Gross, D. R. (2000) Myocardial ischemia-reperfusion injury in estrogen receptor-alpha knockout and wild-type mice, *Am J Physiol Heart Circ Physiol 278*, H1640-1647.

[134] Mårtensson, U. E., Salehi, S. A., Windahl, S., Gomez, M. F., Swärd, K., Daszkiewicz-Nilsson, J., Wendt, A., Andersson, N., Hellstrand, P., Grände, P. O., Owman, C., Rosen, C. J., Adamo, M. L., Lundquist, I., Rorsman, P., Nilsson, B. O., Ohlsson, C., Olde, B., and Leeb-Lundberg, L. M. (2009) Deletion of the G protein-coupled receptor 30 impairs glucose tolerance,

reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice, *Endocrinology 150*, 687-698.

[135] Arefin, S., Simoncini, T., Wieland, R., Hammarqvist, F., Spina, S., Goglia, L., and Kublickiene, K. (2014) Vasodilatory effects of the selective GPER agonist G-1 is maximal in arteries of postmenopausal women, *Maturitas 78*, 123-130.

[136] Feldman, R. D., Gros, R., Ding, Q., Hussain, Y., Ban, M. R., McIntyre, A. D., and Hegele, R. A. (2014) A common hypofunctional genetic variant of GPER is associated with increased blood pressure in women, *Br J Clin Pharmacol 78*, 1441-1452.

[137] Giess, M., Lattrich, C., Springwald, A., Goerse, R., Ortmann, O., and Treeck, O. (2010) GPR30 gene polymorphisms are associated with progesterone receptor status and histopathological characteristics of breast cancer patients, *J Steroid Biochem Mol Biol 118*, 7-12.

[138] Devasagayam, T. P., Tilak, J. C., Boloor, K. K., Sane, K. S., Ghaskadbi, S. S., and Lele, R. D. (2004) Free radicals and antioxidants in human health: current status and future prospects, *J Assoc Physicians India 52*, 794-804.

[139] Kelly, F. J. (2003) Oxidative stress: its role in air pollution and adverse health effects, *Occup Environ Med 60*, 612-616.

[140] Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., and Malik, A. B. (2014) Reactive oxygen species in inflammation and tissue injury, *Antioxid Redox Signal 20*, 1126-1167.

[141] Pervaiz, S., Taneja, R., and Ghaffari, S. (2009) Oxidative stress regulation of stem and progenitor cells, *Antioxid Redox Signal 11*, 2777-2789.

[142] Mueller, C. F., Laude, K., McNally, J. S., and Harrison, D. G. (2005) ATVB in focus: redox mechanisms in blood vessels, *Arterioscler Thromb Vasc Biol 25*, 274-278.

[143] Wheatcroft, S. B. (2013) Teaching an old drug new tricks: can paroxetine ease the burden of cardiovascular disease in diabetes?, *Diabetes 62*, 698-700.

[144] Kohen, R., and Nyska, A. (2002) Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification, *Toxicol Pathol 30*, 620-650.

[145] Tiedge, M., Lortz, S., Drinkgern, J., and Lenzen, S. (1997) Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells, *Diabetes 46*, 1733-1742.

[146] Marinho, H. S., Real, C., Cyrne, L., Soares, H., and Antunes, F. (2014) Hydrogen peroxide sensing, signaling and regulation of transcription factors, *Redox Biol 2*, 535-562.

[147] Holmström, K. M., and Finkel, T. (2014) Cellular mechanisms and physiological consequences of redox-dependent signalling, *Nat Rev Mol Cell Biol 15*, 411-421.

[148] Vitetta, L., and Linnane, A. W. (2014) Endocellular regulation by free radicals and hydrogen peroxide: key determinants of the inflammatory response, *Inflammopharmacology 22*, 69-72.

[149] Figtree, G. A., Keyvan Karimi, G., Liu, C. C., and Rasmussen, H. H. (2012) Oxidative regulation of the Na(+)-K(+) pump in the cardiovascular system, *Free Radic Biol Med 53*, 2263-2268.

[150] Rasmussen, H. H., Hamilton, E. J., Liu, C. C., and Figtree, G. A. (2010) Reversible oxidative modification: implications for cardiovascular physiology and pathophysiology, *Trends Cardiovasc Med 20*, 85-90.

[151] Dalle-Donne, I., Colombo, G., Gagliano, N., Colombo, R., Giustarini, D., Rossi, R., and Milzani, A. (2011) S-glutathiolation in life and death decisions of the cell, *Free Radic Res 45*, 3-15.

[152] Drummond, G. R., Selemidis, S., Griendling, K. K., and Sobey, C. G. (2011) Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets, *Nat Rev Drug Discov 10*, 453-471.

[153] Assari, T. (2006) Chronic Granulomatous Disease; fundamental stages in our understanding of CGD, *Med Immunol 5*, 4.

[154] Elahi, M. M., Kong, Y. X., and Matata, B. M. (2009) Oxidative stress as a mediator of cardiovascular disease, *Oxid Med Cell Longev 2*, 259-269.

[155] White, C. R., Darley-Usmar, V., Berrington, W. R., McAdams, M., Gore, J. Z., Thompson, J. A., Parks, D. A., Tarpey, M. M., and Freeman, B. A. (1996) Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits, *Proc Natl Acad Sci U S A 93*, 8745-8749.

[156] Ohara, Y., Peterson, T. E., and Harrison, D. G. (1993) Hypercholesterolemia increases endothelial superoxide anion production, *J Clin Invest 91*, 2546-2551.

[157] Davidson, S. M., and Duchen, M. R. (2007) Endothelial mitochondria: contributing to vascular function and disease, *Circ Res 100*, 1128-1141.

[158] Schulz, E., Wenzel, P., Münzel, T., and Daiber, A. (2014) Mitochondrial redox signaling: Interaction of mitochondrial reactive oxygen species with other sources of oxidative stress, *Antioxid Redox Signal 20*, 308-324.

[159] Zeini, M., López-Fontal, R., Través, P. G., Benito, G., and Hortelano, S. (2007) Differential sensitivity to apoptosis among the cells that contribute to the atherosclerotic disease, *Biochem Biophys Res Commun 363*, 444-450.

[160] Förstermann, U., and Li, H. (2011) Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling, *Br J Pharmacol 164*, 213-223.

[161] Laursen, J. B., Somers, M., Kurz, S., McCann, L., Warnholtz, A., Freeman, B. A., Tarpey, M., Fukai, T., and Harrison, D. G. (2001) Endothelial regulation of

vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin, *Circulation 103*, 1282-1288.

[162] Katusic, Z. S. (2001) Vascular endothelial dysfunction: does tetrahydrobiopterin play a role?, *Am J Physiol Heart Circ Physiol 281*, H981-986.

[163] Pantano, C., Reynaert, N. L., van der Vliet, A., and Janssen-Heininger, Y. M. (2006) Redox-sensitive kinases of the nuclear factor-kappaB signaling pathway, *Antioxid Redox Signal 8*, 1791-1806.

[164] Gessner, D. K., Ringseis, R., and Eder, K. (2017) Potential of plant polyphenols to combat oxidative stress and inflammatory processes in farm animals, *J Anim Physiol Anim Nutr (Berl) 101*, 605-628.

[165] Fukai, T., and Ushio-Fukai, M. (2011) Superoxide dismutases: role in redox signaling, vascular function, and diseases, *Antioxid Redox Signal 15*, 1583-1606.

[166] Eruslanov, E., and Kusmartsev, S. (2010) Identification of ROS using oxidized DCFDA and flow-cytometry, *Methods Mol Biol 594*, 57-72.

[167] Gandhi, S., and Abramov, A. Y. (2012) Mechanism of oxidative stress in neurodegeneration, *Oxid Med Cell Longev 2012*, 428010.

[168] Bai, J., Rodriguez, A. M., Melendez, J. A., and Cederbaum, A. I. (1999) Overexpression of catalase in cytosolic or mitochondrial compartment protects HepG2 cells against oxidative injury, *J Biol Chem 274*, 26217-26224.

[169] Dasuri, K., Zhang, L., and Keller, J. N. (2013) Oxidative stress, neurodegeneration, and the balance of protein degradation and protein synthesis, *Free Radic Biol Med 62*, 170-185.

[170] Lee, M., Cho, T., Jantaratnotai, N., Wang, Y. T., McGeer, E., and McGeer, P. L. (2010) Depletion of GSH in glial cells induces neurotoxicity: relevance to aging and degenerative neurological diseases, *FASEB J 24*, 2533-2545.

[171] Franco, R., Schoneveld, O. J., Pappa, A., and Panayiotidis, M. I. (2007) The central role of glutathione in the pathophysiology of human diseases, *Arch Physiol Biochem 113*, 234-258.

[172] Mustacich, D., and Powis, G. (2000) Thioredoxin reductase, *Biochem J 346 Pt 1*, 1-8.

[173] Dinkova-Kostova, A. T., and Talalay, P. (2010) NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector, *Arch Biochem Biophys 501*, 116-123.

[174] Dinkova-Kostova, A. T., and Talalay, P. (2000) Persuasive evidence that quinone reductase type 1 (DT diaphorase) protects cells against the toxicity of electrophiles and reactive forms of oxygen, *Free Radic Biol Med 29*, 231-240.

[175] Baird, L., and Dinkova-Kostova, A. T. (2011) The cytoprotective role of the Keap1-Nrf2 pathway, *Arch Toxicol 85*, 241-272.

[176] Motohashi, H., and Yamamoto, M. (2004) Nrf2-Keap1 defines a physiologically important stress response mechanism, *Trends Mol Med 10*, 549-557.

[177] McMahon, M., Thomas, N., Itoh, K., Yamamoto, M., and Hayes, J. D. (2004) Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron, *J Biol Chem 279*, 31556-31567.

[178] Nguyen, T., Sherratt, P. J., Huang, H. C., Yang, C. S., and Pickett, C. B. (2003) Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome, *J Biol Chem 278*, 4536-4541.

[179] Keum, Y. S. (2011) Regulation of the Keap1/Nrf2 system by chemopreventive sulforaphane: implications of posttranslational modifications, *Ann N Y Acad Sci 1229*, 184-189.

[180] Espinosa-Diez, C., Miguel, V., Mennerich, D., Kietzmann, T., Sánchez-Pérez, P., Cadenas, S., and Lamas, S. (2015) Antioxidant responses and cellular adjustments to oxidative stress, *Redox Biol 6*, 183-197.

[181] Baird, L., Swift, S., Llères, D., and Dinkova-Kostova, A. T. (2014) Monitoring Keap1-Nrf2 interactions in single live cells, *Biotechnol Adv 32*, 1133-1144.

[182] Fukutomi, T., Takagi, K., Mizushima, T., Ohuchi, N., and Yamamoto, M. (2014) Kinetic, thermodynamic, and structural characterizations of the association between Nrf2-DLGex degron and Keap1, *Mol Cell Biol 34*, 832-846.

[183] McMahon, M., Thomas, N., Itoh, K., Yamamoto, M., and Hayes, J. D. (2006) Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex, *J Biol Chem 281*, 24756-24768.

[184] Tong, K. I., Kobayashi, A., Katsuoka, F., and Yamamoto, M. (2006) Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism, *Biol Chem 387*, 1311-1320.

[185] Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, *Genes Dev 13*, 76-86.

[186] Nioi, P., Nguyen, T., Sherratt, P. J., and Pickett, C. B. (2005) The carboxy-terminal Neh3 domain of Nrf2 is required for transcriptional activation, *Mol Cell Biol 25*, 10895-10906.

[187] Katoh, Y., Itoh, K., Yoshida, E., Miyagishi, M., Fukamizu, A., and Yamamoto, M. (2001) Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription, *Genes Cells 6*, 857-868.

[188] Chowdhry, S., Zhang, Y., McMahon, M., Sutherland, C., Cuadrado, A., and Hayes, J. D. (2013) Nrf2 is controlled by two distinct β-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity, *Oncogene 32*, 3765-3781.

[189] Wang, H., Liu, K., Geng, M., Gao, P., Wu, X., Hai, Y., Li, Y., Luo, L., Hayes, J. D., Wang, X. J., and Tang, X. (2013) RXRα inhibits the NRF2-ARE signaling pathway through a direct interaction with the Neh7 domain of NRF2, *Cancer Res 73*, 3097-3108.

[190] Warnholtz, A., Nickenig, G., Schulz, E., Macharzina, R., Bräsen, J. H., Skatchkov, M., Heitzer, T., Stasch, J. P., Griendling, K. K., Harrison, D. G., Böhm, M., Meinertz, T., and Münzel, T. (1999) Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system, *Circulation 99*, 2027-2033.

[191] Zorio, E., Gilabert-Estellés, J., España, F., Ramón, L. A., Cosín, R., and Estellés, A. (2008) Fibrinolysis: the key to new pathogenetic mechanisms, *Curr Med Chem 15*, 923-929.

[192] Antoniades, C., Shirodaria, C., Warrick, N., Cai, S., de Bono, J., Lee, J., Leeson, P., Neubauer, S., Ratnatunga, C., Pillai, R., Refsum, H., and Channon, K. M. (2006) 5-methyltetrahydrofolate rapidly improves endothelial function and decreases superoxide production in human vessels: effects on vascular tetrahydrobiopterin availability and endothelial nitric oxide synthase coupling, *Circulation 114*, 1193-1201.

[193] Lerman, A., Burnett, J. C., Higano, S. T., McKinley, L. J., and Holmes, D. R. (1998) Long-term L-arginine supplementation improves small-vessel coronary endothelial function in humans, *Circulation 97*, 2123-2128.

[194] Yang, S., Chen, D., Chen, F., Zhao, X., Zhang, Y., Li, Z., Jin, L., Xu, Y., Sanchis, D., and Ye, J. (2017) Deletion of protein kinase B2 preserves cardiac

function by blocking interleukin-6-mediated injury and restores blood pressure during angiotensin II/high-salt-diet-induced hypertension, *J Hypertens*.

[195] Chen, H., Man, R. Y. K., and Leung, S. W. S. (2017) PPARalpha agonists acutely inhibit calcium-independent PLA2 to reduce H2O2-induced contractions in aortae of spontaneously hypertensive rats, *Am J Physiol Heart Circ Physiol*, ajpheart.00314.02017.

[196] Gao, W., Shao, R., Zhang, X., Liu, D., Liu, Y., and Fa, X. (2017) Up-regulation of caveolin-1 by DJ-1 attenuates rat pulmonary arterial hypertension by inhibiting TGFβ/Smad signaling pathway, *Exp Cell Res 361*, 192-198.

[197] Lee, V. M., Quinn, P. A., Jennings, S. C., and Ng, L. L. (2003) Neutrophil activation and production of reactive oxygen species in pre-eclampsia, *J Hypertens 21*, 395-402.

[198] Lip, G. Y., Edmunds, E., Nuttall, S. L., Landray, M. J., Blann, A. D., and Beevers, D. G. (2002) Oxidative stress in malignant and non-malignant phase hypertension, *J Hum Hypertens 16*, 333-336.

[199] Higashi, Y., Sasaki, S., Nakagawa, K., Matsuura, H., Oshima, T., and Chayama, K. (2002) Endothelial function and oxidative stress in renovascular hypertension, *N Engl J Med 346*, 1954-1962.

[200] Lassègue, B., and Clempus, R. E. (2003) Vascular NAD(P)H oxidases: specific features, expression, and regulation, *Am J Physiol Regul Integr Comp Physiol 285*, R277-297.

[201] Paravicini, T. M., and Touyz, R. M. (2008) NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities, *Diabetes Care 31 Suppl 2*, S170-180.

[202] Yoshida, J., Yamamoto, K., Mano, T., Sakata, Y., Nishikawa, N., Nishio, M., Ohtani, T., Miwa, T., Hori, M., and Masuyama, T. (2004) AT1 receptor

blocker added to ACE inhibitor provides benefits at advanced stage of hypertensive diastolic heart failure, *Hypertension 43*, 686-691.

[203] Ghiadoni, L., Magagna, A., Versari, D., Kardasz, I., Huang, Y., Taddei, S., and Salvetti, A. (2003) Different effect of antihypertensive drugs on conduit artery endothelial function, *Hypertension 41*, 1281-1286.

[204] Wilson, S. H., Best, P. J., Edwards, W. D., Holmes, D. R., Carlson, P. J., Celermajer, D. S., and Lerman, A. (2002) Nuclear factor-kappaB immunoreactivity is present in human coronary plaque and enhanced in patients with unstable angina pectoris, *Atherosclerosis 160*, 147-153.

[205] Gutteridge, J. M., and Halliwell, B. (2000) Free radicals and antioxidants in the year 2000. A historical look to the future, *Ann N Y Acad Sci 899*, 136-147.

[206] Elkind, M. S. (2006) Inflammation, atherosclerosis, and stroke, *Neurologist 12*, 140-148.

[207] Xu, Q., Konta, T., Nakayama, K., Furusu, A., Moreno-Manzano, V., Lucio-Cazana, J., Ishikawa, Y., Fine, L. G., Yao, J., and Kitamura, M. (2004) Cellular defense against H2O2-induced apoptosis via MAP kinase-MKP-1 pathway, *Free Radic Biol Med 36*, 985-993.

[208] Winyard, P. G., and Blake, D. R. (1997) Antioxidants, redox-regulated transcription factors, and inflammation, *Adv Pharmacol 38*, 403-421.

[209] Ide, T., Tsutsui, H., Ohashi, N., Hayashidani, S., Suematsu, N., Tsuchihashi, M., Tamai, H., and Takeshita, A. (2002) Greater oxidative stress in healthy young men compared with premenopausal women, *Arterioscler Thromb Vasc Biol 22*, 438-442.

[210] Matarrese, P., Colasanti, T., Ascione, B., Margutti, P., Franconi, F., Alessandri, C., Conti, F., Riccieri, V., Rosano, G., Ortona, E., and Malorni, W. (2011) Gender disparity in susceptibility to oxidative stress and apoptosis induced by

autoantibodies specific to RLIP76 in vascular cells, *Antioxid Redox Signal 15*, 2825-2836.

[211] Bhatia, K., Elmarakby, A. A., El-Remessy, A. B., El-Remessey, A., and Sullivan, J. C. (2012) Oxidative stress contributes to sex differences in angiotensin II-mediated hypertension in spontaneously hypertensive rats, *Am J Physiol Regul Integr Comp Physiol 302*, R274-282.

[212] Barp, J., Araújo, A. S., Fernandes, T. R., Rigatto, K. V., Llesuy, S., Belló-Klein, A., and Singal, P. (2002) Myocardial antioxidant and oxidative stress changes due to sex hormones, *Braz J Med Biol Res 35*, 1075-1081.

[213] Chen, Y., Ji, L. L., Liu, T. Y., and Wang, Z. T. (2011) Evaluation of gender-related differences in various oxidative stress enzymes in mice, *Chin J Physiol 54*, 385-390.

[214] Brandes, R. P., and Mügge, A. (1997) Gender differences in the generation of superoxide anions in the rat aorta, *Life Sci 60*, 391-396.

[215] Gómez-Pérez, Y., Gianotti, M., Lladó, I., and Proenza, A. M. (2011) Sex-dependent effects of high-fat-diet feeding on rat pancreas oxidative stress, *Pancreas 40*, 682-688.

[216] Miller, A. A., Drummond, G. R., Mast, A. E., Schmidt, H. H., and Sobey, C. G. (2007) Effect of gender on NADPH-oxidase activity, expression, and function in the cerebral circulation: role of estrogen, *Stroke 38*, 2142-2149.

[217] Zhang, R., Thor, D., Han, X., Anderson, L., and Rahimian, R. (2012) Sex differences in mesenteric endothelial function of streptozotocin-induced diabetic rats: a shift in the relative importance of EDRFs, *Am J Physiol Heart Circ Physiol 303*, H1183-1198.

[218] Wong, P. S., Randall, M. D., and Roberts, R. E. (2015) Sex differences in the role of NADPH oxidases in endothelium-dependent vasorelaxation in porcine isolated coronary arteries, *Vascul Pharmacol 72*, 83-92.

[219] Rodford, J. L., Torrens, C., Siow, R. C., Mann, G. E., Hanson, M. A., and Clough, G. F. (2008) Endothelial dysfunction and reduced antioxidant protection in an animal model of the developmental origins of cardiovascular disease, *J Physiol 586*, 4709-4720.

[220] Vina, J., Gambini, J., Lopez-Grueso, R., Abdelaziz, K. M., Jove, M., and Borras, C. (2011) Females live longer than males: role of oxidative stress, *Curr Pharm Des 17*, 3959-3965.

[221] Dantas, A. P., Franco, M. o. C., Silva-Antonialli, M. M., Tostes, R. C., Fortes, Z. B., Nigro, D., and Carvalho, M. H. (2004) Gender differences in superoxide generation in microvessels of hypertensive rats: role of NAD(P)H-oxidase, *Cardiovasc Res 61*, 22-29.

[222] Vassalle, C., Sciarrino, R., Bianchi, S., Battaglia, D., Mercuri, A., and Maffei, S. (2012) Sex-related differences in association of oxidative stress status with coronary artery disease, *Fertil Steril 97*, 414-419.

[223] Robiquet P.J., B. F. (1831) Sur la semence de moutarde., pp 279-282, J. Pharm. Chim.

[224] Sønderby, I. E., Geu-Flores, F., and Halkier, B. A. (2010) Biosynthesis of glucosinolates--gene discovery and beyond, *Trends Plant Sci 15*, 283-290.

[225] Fahey, J. W., Zalcmann, A. T., and Talalay, P. (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants, *Phytochemistry 56*, 5-51.

[226] Kliebenstein, D. J., Kroymann, J., and Mitchell-Olds, T. (2005) The glucosinolate-myrosinase system in an ecological and evolutionary context, *Curr Opin Plant Biol 8*, 264-271.

[227] Koroleva, O. A., Gibson, T. M., Cramer, R., and Stain, C. (2010) Glucosinolate-accumulating S-cells in Arabidopsis leaves and flower stalks

undergo programmed cell death at early stages of differentiation, *Plant J 64*, 456-469.

[228] Shapiro, T. A., Fahey, J. W., Wade, K. L., Stephenson, K. K., and Talalay, P. (1998) Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables, *Cancer Epidemiol Biomarkers Prev 7*, 1091-1100.

[229] Boddupalli, S., Mein, J. R., Lakkanna, S., and James, D. R. (2012) Induction of phase 2 antioxidant enzymes by broccoli sulforaphane: perspectives in maintaining the antioxidant activity of vitamins a, C, and e, *Front Genet 3*, 7.

[230] Hwang, E. S., and Jeffery, E. H. (2005) Induction of quinone reductase by sulforaphane and sulforaphane N-acetylcysteine conjugate in murine hepatoma cells, *J Med Food 8*, 198-203.

[231] Ghawi, S. K., Methven, L., and Niranjan, K. (2013) The potential to intensify sulforaphane formation in cooked broccoli (Brassica oleracea var. italica) using mustard seeds (Sinapis alba), *Food Chem 138*, 1734-1741.

[232] Fimognari, C., Lenzi, M., and Hrelia, P. (2008) Interaction of the isothiocyanate sulforaphane with drug disposition and metabolism: pharmacological and toxicological implications, *Curr Drug Metab 9*, 668-678.

[233] Guerrero-Beltrán, C. E., Calderón-Oliver, M., Pedraza-Chaverri, J., and Chirino, Y. I. (2012) Protective effect of sulforaphane against oxidative stress: recent advances, *Exp Toxicol Pathol 64*, 503-508.

[234] Herr, I., and Büchler, M. W. (2010) Dietary constituents of broccoli and other cruciferous vegetables: implications for prevention and therapy of cancer, *Cancer Treat Rev 36*, 377-383.

[235] Zhang, Y., Cho, C. G., Posner, G. H., and Talalay, P. (1992) Spectroscopic quantitation of organic isothiocyanates by cyclocondensation with vicinal dithiols, *Anal Biochem 205*, 100-107.

[236] Egner, P. A., Chen, J. G., Wang, J. B., Wu, Y., Sun, Y., Lu, J. H., Zhu, J., Zhang, Y. H., Chen, Y. S., Friesen, M. D., Jacobson, L. P., Muñoz, A., Ng, D., Qian, G. S., Zhu, Y. R., Chen, T. Y., Botting, N. P., Zhang, Q., Fahey, J. W., Talalay, P., Groopman, J. D., and Kensler, T. W. (2011) Bioavailability of Sulforaphane from two broccoli sprout beverages: results of a short-term, cross-over clinical trial in Qidong, China, *Cancer Prev Res (Phila) 4*, 384-395.

[237] Gasper, A. V., Al-Janobi, A., Smith, J. A., Bacon, J. R., Fortun, P., Atherton, C., Taylor, M. A., Hawkey, C. J., Barrett, D. A., and Mithen, R. F. (2005) Glutathione S-transferase M1 polymorphism and metabolism of sulforaphane from standard and high-glucosinolate broccoli, *Am J Clin Nutr 82*, 1283-1291.

[238] Vermeulen, M., Klöpping-Ketelaars, I. W., van den Berg, R., and Vaes, W. H. (2008) Bioavailability and kinetics of sulforaphane in humans after consumption of cooked versus raw broccoli, *J Agric Food Chem 56*, 10505-10509.

[239] Atwell, L. L., Hsu, A., Wong, C. P., Stevens, J. F., Bella, D., Yu, T. W., Pereira, C. B., Löhr, C. V., Christensen, J. M., Dashwood, R. H., Williams, D. E., Shannon, J., and Ho, E. (2015) Absorption and chemopreventive targets of sulforaphane in humans following consumption of broccoli sprouts or a myrosinase-treated broccoli sprout extract, *Mol Nutr Food Res 59*, 424-433.

[240] Fahey, J. W., Wehage, S. L., Holtzclaw, W. D., Kensler, T. W., Egner, P. A., Shapiro, T. A., and Talalay, P. (2012) Protection of humans by plant glucosinolates: efficiency of conversion of glucosinolates to isothiocyanates by the gastrointestinal microflora, *Cancer Prev Res (Phila) 5*, 603-611.

[241] Li, F., Hullar, M. A., Beresford, S. A., and Lampe, J. W. (2011) Variation of glucoraphanin metabolism in vivo and ex vivo by human gut bacteria, *Br J Nutr 106*, 408-416.

[242] Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., and Talalay, P. (2002) Direct evidence that

sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants, *Proc Natl Acad Sci U S A 99*, 11908-11913.

[243] Zhang, D. D., and Hannink, M. (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress, *Mol Cell Biol 23*, 8137-8151.

[244] McMahon, M., Lamont, D. J., Beattie, K. A., and Hayes, J. D. (2010) Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals, *Proc Natl Acad Sci U S A 107*, 18838-18843.

[245] Eggler, A. L., Luo, Y., van Breemen, R. B., and Mesecar, A. D. (2007) Identification of the highly reactive cysteine 151 in the chemopreventive agent-sensor Keap1 protein is method-dependent, *Chem Res Toxicol 20*, 1878-1884.

[246] Hu, C., Eggler, A. L., Mesecar, A. D., and van Breemen, R. B. (2011) Modification of keap1 cysteine residues by sulforaphane, *Chem Res Toxicol 24*, 515-521.

[247] Kobayashi, A., Kang, M. I., Watai, Y., Tong, K. I., Shibata, T., Uchida, K., and Yamamoto, M. (2006) Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1, *Mol Cell Biol 26*, 221-229.

[248] Kwak, M. K., Itoh, K., Yamamoto, M., and Kensler, T. W. (2002) Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter, *Mol Cell Biol 22*, 2883-2892.

[249] Shay, K. P., Michels, A. J., Li, W., Kong, A. N., and Hagen, T. M. (2012) Cap-independent Nrf2 translation is part of a lipoic acid-stimulated detoxification stress response, *Biochim Biophys Acta 1823*, 1102-1109.

[250] Yu, X., and Kensler, T. (2005) Nrf2 as a target for cancer chemoprevention, *Mutat Res 591*, 93-102.

[251] Magesh, S., Chen, Y., and Hu, L. (2012) Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents, *Med Res Rev 32*, 687-726.

[252] Kensler, T. W., Egner, P. A., Agyeman, A. S., Visvanathan, K., Groopman, J. D., Chen, J. G., Chen, T. Y., Fahey, J. W., and Talalay, P. (2013) Keap1-nrf2 signaling: a target for cancer prevention by sulforaphane, *Top Curr Chem 329*, 163-177.

[253] Leoncini, E., Malaguti, M., Angeloni, C., Motori, E., Fabbri, D., and Hrelia, S. (2011) Cruciferous vegetable phytochemical sulforaphane affects phase II enzyme expression and activity in rat cardiomyocytes through modulation of Akt signaling pathway, *J Food Sci 76*, H175-181.

[254] Bergström, P., Andersson, H. C., Gao, Y., Karlsson, J. O., Nodin, C., Anderson, M. F., Nilsson, M., and Hammarsten, O. (2011) Repeated transient sulforaphane stimulation in astrocytes leads to prolonged Nrf2-mediated gene expression and protection from superoxide-induced damage, *Neuropharmacology 60*, 343-353.

[255] Bai, Y., Cui, W., Xin, Y., Miao, X., Barati, M. T., Zhang, C., Chen, Q., Tan, Y., Cui, T., Zheng, Y., and Cai, L. (2013) Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation, *J Mol Cell Cardiol 57*, 82-95.

[256] Angeloni, C., Leoncini, E., Malaguti, M., Angelini, S., Hrelia, P., and Hrelia, S. (2009) Modulation of phase II enzymes by sulforaphane: implications for its cardioprotective potential, *J Agric Food Chem 57*, 5615-5622.

[257] Senanayake, G. V., Banigesh, A., Wu, L., Lee, P., and Juurlink, B. H. (2012) The dietary phase 2 protein inducer sulforaphane can normalize the kidney

epigenome and improve blood pressure in hypertensive rats, *Am J Hypertens 25*, 229-235.

[258] Wu, L., and Juurlink, B. H. (2001) The impaired glutathione system and its up-regulation by sulforaphane in vascular smooth muscle cells from spontaneously hypertensive rats, *J Hypertens 19*, 1819-1825.

[259] Wu, L., Noyan Ashraf, M. H., Facci, M., Wang, R., Paterson, P. G., Ferrie, A., and Juurlink, B. H. (2004) Dietary approach to attenuate oxidative stress, hypertension, and inflammation in the cardiovascular system, *Proc Natl Acad Sci U S A 101*, 7094-7099.

[260] Kim, J. Y., Park, H. J., Um, S. H., Sohn, E. H., Kim, B. O., Moon, E. Y., Rhee, D. K., and Pyo, S. (2012) Sulforaphane suppresses vascular adhesion molecule-1 expression in TNF-α-stimulated mouse vascular smooth muscle cells: involvement of the MAPK, NF-κB and AP-1 signaling pathways, *Vascul Pharmacol 56*, 131-141.

[261] Kwon, J. S., Joung, H., Kim, Y. S., Shim, Y. S., Ahn, Y., Jeong, M. H., and Kee, H. J. (2012) Sulforaphane inhibits restenosis by suppressing inflammation and the proliferation of vascular smooth muscle cells, *Atherosclerosis 225*, 41-49.

[262] Zakkar, M., Van der Heiden, K., Luong, l. A., Chaudhury, H., Cuhlmann, S., Hamdulay, S. S., Krams, R., Edirisinghe, I., Rahman, I., Carlsen, H., Haskard, D. O., Mason, J. C., and Evans, P. C. (2009) Activation of Nrf2 in endothelial cells protects arteries from exhibiting a proinflammatory state, *Arterioscler Thromb Vasc Biol 29*, 1851-1857.

[263] Kivelä, A. M., Mäkinen, P. I., Jyrkkänen, H. K., Mella-Aho, E., Xia, Y., Kansanen, E., Leinonen, H., Verma, I. M., Ylä-Herttuala, S., and Levonen, A. L. (2010) Sulforaphane inhibits endothelial lipase expression through NF-κB in endothelial cells, *Atherosclerosis 213*, 122-128.

[264] Huang, C. S., Lin, A. H., Liu, C. T., Tsai, C. W., Chang, I. S., Chen, H. W., and Lii, C. K. (2013) Isothiocyanates protect against oxidized LDL-induced endothelial dysfunction by upregulating Nrf2-dependent antioxidation and suppressing NFκB activation, *Mol Nutr Food Res 57*, 1918-1930.

[265] Chuang, W. Y., Kung, P. H., Kuo, C. Y., and Wu, C. C. (2013) Sulforaphane prevents human platelet aggregation through inhibiting the phosphatidylinositol 3-kinase/Akt pathway, *Thromb Haemost 109*, 1120-1130.

[266] Oh, C. H., Shin, J. I., Mo, S. J., Yun, S. J., Kim, S. H., and Rhee, Y. H. (2013) Antiplatelet activity of L-sulforaphane by regulation of platelet activation factors, glycoprotein IIb/IIIa and thromboxane A2, *Blood Coagul Fibrinolysis 24*, 498-504.

[267] Kleikers, P. W., Wingler, K., Hermans, J. J., Diebold, I., Altenhöfer, S., Radermacher, K. A., Janssen, B., Görlach, A., and Schmidt, H. H. (2012) NADPH oxidases as a source of oxidative stress and molecular target in ischemia/reperfusion injury, *J Mol Med (Berl) 90*, 1391-1406.

[268] Masutani, H., Otsuki, R., Yamaguchi, Y., Takenaka, M., Kanoh, N., Takatera, K., Kunimoto, Y., and Yodoi, J. (2009) Fragrant unsaturated aldehydes elicit activation of the Keap1/Nrf2 system leading to the upregulation of thioredoxin expression and protection against oxidative stress, *Antioxid Redox Signal 11*, 949-962.

[269] Negi, G., Kumar, A., and Sharma, S. S. (2011) Nrf2 and NF-κB modulation by sulforaphane counteracts multiple manifestations of diabetic neuropathy in rats and high glucose-induced changes, *Curr Neurovasc Res 8*, 294-304.

[270] Zheng, H., Whitman, S. A., Wu, W., Wondrak, G. T., Wong, P. K., Fang, D., and Zhang, D. D. (2011) Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy, *Diabetes 60*, 3055-3066.

[271] Miao, X., Bai, Y., Sun, W., Cui, W., Xin, Y., Wang, Y., Tan, Y., Miao, L., Fu, Y., Su, G., and Cai, L. (2012) Sulforaphane prevention of diabetes-induced aortic damage was associated with the up-regulation of Nrf2 and its down-stream antioxidants, *Nutr Metab (Lond) 9*, 84.

[272] Amos, A. F., McCarty, D. J., and Zimmet, P. (1997) The rising global burden of diabetes and its complications: estimates and projections to the year 2010, *Diabet Med 14 Suppl 5*, S1-85.

[273] Xu, Z., Wang, S., Ji, H., Zhang, Z., Chen, J., Tan, Y., Wintergerst, K., Zheng, Y., Sun, J., and Cai, L. (2016) Broccoli sprout extract prevents diabetic cardiomyopathy via Nrf2 activation in db/db T2DM mice, *Sci Rep 6*, 30252.

[274] Zhang, Z., Wang, S., Zhou, S., Yan, X., Wang, Y., Chen, J., Mellen, N., Kong, M., Gu, J., Tan, Y., Zheng, Y., and Cai, L. (2014) Sulforaphane prevents the development of cardiomyopathy in type 2 diabetic mice probably by reversing oxidative stress-induced inhibition of LKB1/AMPK pathway, *J Mol Cell Cardiol 77*, 42-52.

[275] Blanchard, O. L., and Smoliga, J. M. (2015) Translating dosages from animal models to human clinical trials--revisiting body surface area scaling, *FASEB J 29*, 1629-1634.

[276] Latté, K. P., Appel, K. E., and Lampen, A. (2011) Health benefits and possible risks of broccoli - an overview, *Food Chem Toxicol 49*, 3287-3309.

[277] Ho, K. K., Pinsky, J. L., Kannel, W. B., and Levy, D. (1993) The epidemiology of heart failure: the Framingham Study, *J Am Coll Cardiol 22*, 6A-13A.

[278] Levy, D., Kenchaiah, S., Larson, M. G., Benjamin, E. J., Kupka, M. J., Ho, K. K., Murabito, J. M., and Vasan, R. S. (2002) Long-term trends in the incidence of and survival with heart failure, *N Engl J Med 347*, 1397-1402.

[279] Simon, T., Mary-Krause, M., Funck-Brentano, C., and Jaillon, P. (2001) Sex differences in the prognosis of congestive heart failure: results from the Cardiac Insufficiency Bisoprolol Study (CIBIS II), *Circulation 103*, 375-380.

[280] Mosca, L., Barrett-Connor, E., and Wenger, N. K. (2011) Sex/gender differences in cardiovascular disease prevention: what a difference a decade makes, *Circulation 124*, 2145-2154.

[281] Osborne, C. K., Zhao, H., and Fuqua, S. A. (2000) Selective estrogen receptor modulators: structure, function, and clinical use, *J Clin Oncol 18*, 3172-3186.

[282] Spoletini, I., Vitale, C., Malorni, W., and Rosano, G. M. (2012) Sex differences in drug effects: interaction with sex hormones in adult life, *Handb Exp Pharmacol*, 91-105.

[283] Anderson, G. D. (2005) Sex and racial differences in pharmacological response: where is the evidence? Pharmacogenetics, pharmacokinetics, and pharmacodynamics, *J Womens Health (Larchmt) 14*, 19-29.

[284] Lenzi, M., Fimognari, C., and Hrelia, P. (2014) Sulforaphane as a promising molecule for fighting cancer, *Cancer Treat Res 159*, 207-223.

[285] Bonetto, J. H., Fernandes, R. O., Seolin, B. G., Müller, D. D., Teixeira, R. B., Araujo, A. S., Vassallo, D., Schenkel, P. C., and Belló-Klein, A. (2016) Sulforaphane improves oxidative status without attenuating the inflammatory response or cardiac impairment induced by ischemia-reperfusion in rats, *Can J Physiol Pharmacol 94*, 508-516.

[286] Li, Z., Galli, U., Becker, L. E., Bruns, H., Nickkolgh, A., Hoffmann, K., Karck, M., and Schemmer, P. (2013) Sulforaphane protects hearts from early injury after experimental transplantation, *Ann Transplant 18*, 558-566.

[287] Fernandes, R. O., De Castro, A. L., Bonetto, J. H., Ortiz, V. D., Müller, D. D., Campos-Carraro, C., Barbosa, S., Neves, L. T., Xavier, L. L., Schenkel, P. C., Singal, P., Khaper, N., da Rosa Araujo, A. S., and Belló-Klein, A. (2016)

Sulforaphane effects on postinfarction cardiac remodeling in rats: modulation of redox-sensitive prosurvival and proapoptotic proteins, *J Nutr Biochem 34*, 106-117.

[288] Tarozzi, A., Angeloni, C., Malaguti, M., Morroni, F., Hrelia, S., and Hrelia, P. (2013) Sulforaphane as a potential protective phytochemical against neurodegenerative diseases, *Oxid Med Cell Longev 2013*, 415078.

[289] Angeloni, C., Malaguti, M., Rizzo, B., Barbalace, M. C., Fabbri, D., and Hrelia, S. (2015) Neuroprotective effect of sulforaphane against methylglyoxal cytotoxicity, *Chem Res Toxicol 28*, 1234-1245.

[290] Qi, T., Xu, F., Yan, X., Li, S., and Li, H. (2016) Sulforaphane exerts anti-inflammatory effects against lipopolysaccharide-induced acute lung injury in mice through the Nrf2/ARE pathway, *Int J Mol Med 37*, 182-188.

[291] Vauzour, D., Buonfiglio, M., Corona, G., Chirafisi, J., Vafeiadou, K., Angeloni, C., Hrelia, S., Hrelia, P., and Spencer, J. P. (2010) Sulforaphane protects cortical neurons against 5-S-cysteinyl-dopamine-induced toxicity through the activation of ERK1/2, Nrf-2 and the upregulation of detoxification enzymes, *Mol Nutr Food Res 54*, 532-542.

[292] Angeloni, C., Maraldi, T., Ghelli, A., Rugolo, M., Leoncini, E., Hakim, G., and Hrelia, S. (2007) Green tea modulates alpha(1)-adrenergic stimulated glucose transport in cultured rat cardiomyocytes, *J Agric Food Chem 55*, 7553-7558.

[293] Angeloni, C., Motori, E., Fabbri, D., Malaguti, M., Leoncini, E., Lorenzini, A., and Hrelia, S. (2011) H2O2 preconditioning modulates phase II enzymes through p38 MAPK and PI3K/Akt activation, *Am J Physiol Heart Circ Physiol 300*, H2196-2205.

[294] Bahia, P. K., Rattray, M., and Williams, R. J. (2008) Dietary flavonoid (-)epicatechin stimulates phosphatidylinositol 3-kinase-dependent anti-oxidant

response element activity and up-regulates glutathione in cortical astrocytes, *J Neurochem 106*, 2194-2204.

[295] Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic Acids Res 29*, e45.

[296] Wang, F., He, Q., Sun, Y., Dai, X., and Yang, X. P. (2010) Female adult mouse cardiomyocytes are protected against oxidative stress, *Hypertension 55*, 1172-1178.

[297] Saha, S., Hollands, W., Teucher, B., Needs, P. W., Narbad, A., Ortori, C. A., Barrett, D. A., Rossiter, J. T., Mithen, R. F., and Kroon, P. A. (2012) Isothiocyanate concentrations and interconversion of sulforaphane to erucin in human subjects after consumption of commercial frozen broccoli compared to fresh broccoli, *Mol Nutr Food Res 56*, 1906-1916.

[298] Nagira, K., Sasaoka, T., Wada, T., Fukui, K., Ikubo, M., Hori, S., Tsuneki, H., Saito, S., and Kobayashi, M. (2006) Altered subcellular distribution of estrogen receptor alpha is implicated in estradiol-induced dual regulation of insulin signaling in 3T3-L1 adipocytes, *Endocrinology 147*, 1020-1028.

[299] La Colla, A., Vasconsuelo, A., Milanesi, L., and Pronsato, L. (2017) 17β-Estradiol Protects Skeletal Myoblasts From Apoptosis Through p53, Bcl-2, and FoxO Families, *J Cell Biochem 118*, 104-115.

[300] Kalapos, M. P. (2008) The tandem of free radicals and methylglyoxal, *Chem Biol Interact 171*, 251-271.

[301] Angeloni, C., Zambonin, L., and Hrelia, S. (2014) Role of methylglyoxal in Alzheimer's disease, *Biomed Res Int 2014*, 238485.

[302] Angeloni, C., Turroni, S., Bianchi, L., Fabbri, D., Motori, E., Malaguti, M., Leoncini, E., Maraldi, T., Bini, L., Brigidi, P., and Hrelia, S. (2013) Novel targets of sulforaphane in primary cardiomyocytes identified by proteomic analysis, *PLoS One 8*, e83283.

[303] Kehat, I., and Molkentin, J. D. (2010) Extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in cardiac hypertrophy, *Ann N Y Acad Sci 1188*, 96-102.

[304] Matsui, T., Tao, J., del Monte, F., Lee, K. H., Li, L., Picard, M., Force, T. L., Franke, T. F., Hajjar, R. J., and Rosenzweig, A. (2001) Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo, *Circulation 104*, 330-335.

[305] Hayes, J. D., Chowdhry, S., Dinkova-Kostova, A. T., and Sutherland, C. (2015) Dual regulation of transcription factor Nrf2 by Keap1 and by the combined actions of β-TrCP and GSK-3, *Biochem Soc Trans 43*, 611-620.

[306] (2010) Putting gender on the agenda, *Nature 465*, 665.

[307] Ventura-Clapier, R., Dworatzek, E., Seeland, U., Kararigas, G., Arnal, J. F., Brunelleschi, S., Carpenter, T. C., Erdmann, J., Franconi, F., Giannetta, E., Glezerman, M., Hofmann, S. M., Junien, C., Katai, M., Kublickiene, K., König, I. R., Majdic, G., Malorni, W., Mieth, C., Miller, V. M., Reynolds, R. M., Shimokawa, H., Tannenbaum, C., D'Ursi, A. M., and Regitz-Zagrosek, V. (2017) Sex in basic research: concepts in the cardiovascular field, *Cardiovasc Res 113*, 711-724.

[308] Touyz, R. M., and Briones, A. M. (2011) Reactive oxygen species and vascular biology: implications in human hypertension, *Hypertens Res 34*, 5-14.

[309] Csányi, G., Yao, M., Rodríguez, A. I., Al Ghouleh, I., Sharifi-Sanjani, M., Frazziano, G., Huang, X., Kelley, E. E., Isenberg, J. S., and Pagano, P. J. (2012) Thrombospondin-1 regulates blood flow via CD47 receptor-mediated activation of NADPH oxidase 1, *Arterioscler Thromb Vasc Biol 32*, 2966-2973.

[310] Alissa, E. M., and Ferns, G. A. (2012) Functional foods and nutraceuticals in the primary prevention of cardiovascular diseases, *J Nutr Metab 2012*, 569486.

[311] Malaguti, M., Angeloni, C., and Hrelia, S. (2015) Nutraceutical Bioactive Compounds Promote Healthspan Counteracting Cardiovascular Diseases, *J Am Coll Nutr 34 Suppl 1*, 22-27.

[312] Urata, Y., Ihara, Y., Murata, H., Goto, S., Koji, T., Yodoi, J., Inoue, S., and Kondo, T. (2006) 17Beta-estradiol protects against oxidative stress-induced cell death through the glutathione/glutaredoxin-dependent redox regulation of Akt in myocardiac H9c2 cells, *J Biol Chem 281*, 13092-13102.

[313] Hybertson, B. M., Gao, B., Bose, S. K., and McCord, J. M. (2011) Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation, *Mol Aspects Med 32*, 234-246.

[314] Zhu, H., Itoh, K., Yamamoto, M., Zweier, J. L., and Li, Y. (2005) Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury, *FEBS Lett 579*, 3029-3036.

[315] Harder, B., Jiang, T., Wu, T., Tao, S., Rojo de la Vega, M., Tian, W., Chapman, E., and Zhang, D. D. (2015) Molecular mechanisms of Nrf2 regulation and how these influence chemical modulation for disease intervention, *Biochem Soc Trans 43*, 680-686.

[316] Lu, Z., Xu, X., Hu, X., Zhu, G., Zhang, P., van Deel, E. D., French, J. P., Fassett, J. T., Oury, T. D., Bache, R. J., and Chen, Y. (2008) Extracellular superoxide dismutase deficiency exacerbates pressure overload-induced left ventricular hypertrophy and dysfunction, *Hypertension 51*, 19-25.

[317] Matsushima, S., Kinugawa, S., Ide, T., Matsusaka, H., Inoue, N., Ohta, Y., Yokota, T., Sunagawa, K., and Tsutsui, H. (2006) Overexpression of glutathione peroxidase attenuates myocardial remodeling and preserves diastolic function in diabetic heart, *Am J Physiol Heart Circ Physiol 291*, H2237-2245.

[318] Hu, C. M., Chen, Y. H., Chiang, M. T., and Chau, L. Y. (2004) Heme oxygenase-1 inhibits angiotensin II-induced cardiac hypertrophy in vitro and in vivo, *Circulation 110*, 309-316.

[319] Yu, J., Zhao, Y., Li, B., Sun, L., and Huo, H. (2012) 17β-estradiol regulates the expression of antioxidant enzymes in myocardial cells by increasing Nrf2 translocation, *J Biochem Mol Toxicol 26*, 264-269.

[320] Knowlton, A. A., and Lee, A. R. (2012) Estrogen and the cardiovascular system, *Pharmacol Ther 135*, 54-70.

[321] Mendelsohn, M. E., and Karas, R. H. (2005) Molecular and cellular basis of cardiovascular gender differences, *Science 308*, 1583-1587.

[322] Machuki, J. O., Zhang, H. Y., Harding, S. E., and Sun, H. (2017) Molecular pathways of oestrogen receptors and β-adrenergic receptors in cardiac cells: Recognition of their similarities, interactions and therapeutic value, *Acta Physiol (Oxf)*.

[323] Lizotte, E., Grandy, S. A., Tremblay, A., Allen, B. G., and Fiset, C. (2009) Expression, distribution and regulation of sex steroid hormone receptors in mouse heart, *Cell Physiol Biochem 23*, 75-86.

[324] De Francesco, E. M., Rocca, C., Scavello, F., Amelio, D., Pasqua, T., Rigiracciolo, D. C., Scarpelli, A., Avino, S., Cirillo, F., Amodio, N., Cerra, M. C., Maggiolini, M., and Angelone, T. (2017) Protective Role of GPER Agonist G-1 on Cardiotoxicity Induced by Doxorubicin, *J Cell Physiol 232*, 1640-1649.

[325] Hausenloy, D. J., and Yellon, D. M. (2004) New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway, *Cardiovasc Res 61*, 448-460.

[326] Javadov, S., Jang, S., and Agostini, B. (2014) Crosstalk between mitogen-activated protein kinases and mitochondria in cardiac diseases: therapeutic perspectives, *Pharmacol Ther 144*, 202-225.

[327] Tarone, G., Sbroggiò, M., and Brancaccio, M. (2013) Key role of ERK1/2 molecular scaffolds in heart pathology, *Cell Mol Life Sci 70*, 4047-4054.

[328] MacLellan, W. R., and Schneider, M. D. (2000) Genetic dissection of cardiac growth control pathways, *Annu Rev Physiol 62*, 289-319.

[329] Vadlakonda, L., Dash, A., Pasupuleti, M., Anil Kumar, K., and Reddanna, P. (2013) The Paradox of Akt-mTOR Interactions, *Front Oncol 3*, 165.

[330] Shiojima, I., and Walsh, K. (2006) Regulation of cardiac growth and coronary angiogenesis by the Akt/PKB signaling pathway, *Genes Dev 20*, 3347-3365.

[331] Mendoza, M. C., Er, E. E., and Blenis, J. (2011) The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation, *Trends Biochem Sci 36*, 320-328.

[332] Greer, E. L., and Brunet, A. (2005) FOXO transcription factors at the interface between longevity and tumor suppression, *Oncogene 24*, 7410-7425.

[333] Yang, J. Y., Zong, C. S., Xia, W., Yamaguchi, H., Ding, Q., Xie, X., Lang, J. Y., Lai, C. C., Chang, C. J., Huang, W. C., Huang, H., Kuo, H. P., Lee, D. F., Li, L. Y., Lien, H. C., Cheng, X., Chang, K. J., Hsiao, C. D., Tsai, F. J., Tsai, C. H., Sahin, A. A., Muller, W. J., Mills, G. B., Yu, D., Hortobagyi, G. N., and Hung, M. C. (2008) ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation, *Nat Cell Biol 10*, 138-148.

[334] Biggs, W. H., Meisenhelder, J., Hunter, T., Cavenee, W. K., and Arden, K. C. (1999) Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1, *Proc Natl Acad Sci U S A 96*, 7421-7426.

[335] Manning, B. D., and Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream, *Cell 129*, 1261-1274.