UNIVERSITÁ DEGLI STUDI ROMA TRE

Facoltà di Scienze Matematiche, Fisiche e Naturali
Dipartimento di Biologia
Scuola Dottorale in Biologia
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THE ENDOCRINE DISRUPTORS: EFFECTS AND ACTION MECHANISMS ON ESTROGEN-INDUCED CELL FUNCTIONS

GLI INTERFERENTI ENDOCRINI: EFFETTI E MECCANISMI D'AZIONE NELLE FUNZIONI CELLULARI ESTROGENO-DIPENDENTI

Ph.D. Student: Dr. Pamela Bulzomi

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Supervisor: Prof. Maria Marino

Coordinatore sezione BASU: Prof. Paolo Visca

To my family,
for its never ending support and patience

To Alessandro,
For standing by me, always encouraging me
"through thick and thin fate"

To Maria,
For patiently and constantly guiding me,
conveying her passion for research to me

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SUMMARY

Besides E2, ERs bind a wide variety of compounds with remarkable structural and chemical diversity (Ascenzi et al., 2006) collected into the class of Endocrine Disruptor (EDs). EDs are defined as "exogenous substances that cause adverse health effects in an intact organism or in its progeny, consequent to changes in endocrine function". Even if present in minute amounts (part per trillion) EDs could interfere with the synthesis, secretion, transport, metabolism, binding, action, or elimination of natural hormones responsible for homeostasis maintenance, reproduction, and developmental processes (Colborn etal.,1993). Animals, including humans, are especially sensitive to EDs at the early stages of development but some effects exerted at these stages may be expressed only in the adult life or even in subsequent generations (Rhind, 2009). Among EDs, several synthetic chemicals have been described to induce several degenerative disease. For example Bisphenol A (BPA) has been demonstrated to promote the development of endometriosis (Signorile et al., 2010) and of various cancer type (e.g. breast, endometrial and prostate cancer) because of its estrogen mimetic activity (Bolli et al., 2008; Ricupito et al., 2009). On the contrary, natural compounds such as flavonoids show a protective effect against various degenerative phenomena (i.e. cardiovascular disease, osteoporosis, several cancer type) (Cassidy et al., 2000; Dang and Lowik, 2005; Keinan- Boker et al., 2004). To date, it's not clear if the discrepant effects between synthetic and natural compounds depend on ED interaction with ERs, or on other mechanisms whose occur independently from ED binding to ERs. Furthermore, since the most of the studies on EDs, particularly flavonoids beneficial effects, were conducted in the absence of E2, the physiological relevance of these findings is not clear. Moreover, since human beings intake daily about 500 g of different chemicals, which exhibit endocrine effects *in vivo* and *in vitro* (Marino and Galluzzo, 2006), mammalians are exposed to several EDs, resulting in systemic circulation of ED and flavonoid mixtures in the body. Although in the last years, some evidence has become available to show the combined effects of EDs (Kortenkamp et al., 2008), the research on the effects of "dietary" and synthetic mixtures, at relevant human levels, remain inconclusive.

Aim of this project is to assess the effects of natural chemicals (i.e. flavonoids) alone or in mixture with the endogenous hormone (E2) or with other EDs (i.e. BPA) on E2-dependent cell functions (i.e. cell proliferation/apoptosis balance, cellular differentiation and oxidative stress)

evaluating their effects, action mechanisms, and the putative involvement of ERα and ERβ.

Our data demonstrated that the well known growth inhibition and cell death effects of the flavonol quercetin, one of the most frequently studied and ubiquitous bioactive flavonoid, are not related to its high-concentration requiring (5×10^{-5}M) antioxidant activity but to quercetin ability to activate, in a very small amount (i.e., 10^{-6}M), a pro-apoptotic cascade mainly modulating both ER activities. Particularly quercetin is able to bind both ERs, and in turn to act as an E2-mimetic, leading cancer cell to apoptotic death, in tissue expressing ERβ, such as colon (Galluzzo et al., 2007), or to antagonize E2 proliferative effect in tissue expressing ERα, such as breast cancer (Bulzomi et al., 2010). Quercetin underlying action mechanism requires ER activity modulation: in the ERβ presence quercetin activates the same E2 pathways (both genomic and extranuclear signal), whereas in presence of ERα, quercetin only allows ERα direct transcriptional activity, impairing ERα-mediated rapid signals important for ERα-induced cell proliferation. Thus, at nutritionally relevant concentration, quercetin antiproliferative activities depends on ER activity modulation, rather than its antioxidant activity. Our data also demonstrate that flavonoids, such as the flavanone Naringenin, preserve their ability to induce apoptosis in ERα-expressing cancer cells also in the presence of E2. Nar is one of the best absorbed flavonoids in the human gastrointestinal tract, and the peak of plasma aglycone Naringenin ranges from 0.7 to 14.8×10^{-6} M (Erlund et al., 2001; Bugianesi et al., 2002; Manach et al., 2004). According to the reported plasma concentration the obtained data demonstrate that 10^{-6} M Nar, although didn't affect E2-induced direct transcriptional activity of ERα, reverts the proliferative effect of E2 impairing ERα-mediated rapid signals and inducing different proapoptotic signal transduction pathways in ERα-expressing cancer cells. As a whole, the assays with nutritionally relevant concentration of Nar, against a background of physiological level of E2 allowed us to elucidate Naringenin disrupting action mechanism giving a physiological meaning to the antiproliferative activity of this compound. The importance of flavonoid chemoprotective properties is strongly supported also by the data obtained on flavonoid and man-made ED mixtures, whose indicate that the small amount of Nar recovered in human plasma (Manach et al., 2004) is sufficient to counteract BPA cancer promoting effect. Our data indicate that BPA is, like Nar, a double sided action mechanism compound, which promotes tumor incidence in breast and other target organs that predominantly express ERα but inhibits the E2

protective effects in the ERβ-expressing colon cell. These two divergent aspects could act synergistically by increasing the E2-disrupting potential of this widespread environmental polluter. Our data, demonstrating Nar ability to revert BPA estrogenic activity in ERα expressing cancer cells and to preserve its antiproliferative activity in ERβ-expressing cancer cells, strongly support the theory of the cell fate as the resulting balance of the ED-activated pathway, highlighting the importance of investigating the chemopreventive effect of flavonoids. Since E2 effects go beyond cell proliferation, and ERα and ERβ are coexpressed in several tissue, we assessed the impact of the Nar on E2 protective effect in non cancerous cells when both receptor isoforms are present. The data obtained on E2 and Nar mixture in skeletal muscle myoblasts, expressing both ERs, allow us to affirm that ERα and ERβ mediate different E2 effect in skeletal muscle. ERα-activated rapid signals are essential for E2-induced skeletal muscle differentiation, while ERβ-activated pathways are the only involved in the E2-protective effect from ROS-induced oxidative stress. Nar ability to specifically affect only E2-induced differentiation raise the existence of a gender-related susceptibilities to flavonoids in the different physiological stages of life.

As a whole, these data enlarge our knowledge of the mechanisms underlying the (anti)estrogenicity of dietary compounds pointing to rapid mechanisms as the most susceptible target of endocrine disruptors. In fact nor Naringenin or Quercetin modify direct ERE-containing promoter transcription, but decoupling ERα from rapid signals, drive cells to different destiny, highlighting the importance of investigating flavonoid effect in different stages of life that could be characterized by a different level and role of this receptor isoform.

Since E2 effects depend on the balance of the relative expression of ER isoform and on the balance of the signals originated by each isoform, ED actions are more complex than originally considered, since different ligands induce ERs to assume different conformations responsible for specific signaling pathway activation.

RIASSUNTO

Oltre ad E2, i recettori degli estrogeni (ER) legano una vasta gamma di molecole, con spiccate differenze chimiche e strutturali (Ascenzi et al., 2006), raggruppate nella classe degli Interferenti Endocrini (IE). IE sono definiti come "sostanze esogene che causano effetti avversi alla salute negli interi organismi o nella progenie, in seguito ad alterazioni delle funzioni endocrine". Sebbene presenti in parti per trilione, IE possono interferire con la sintesi, la secrezione, il trasporto, il metabolismo, il legame, l'azione o l'eliminazione degli ormoni responsabili del mantenimento dell'omeostasi, della riproduzione e dei processi di sviluppo (Colborn et al.,1993). Gli animali sono particolarmente sensibili agli IE nelle prime fasi dello sviluppo, sebbene alcuni effetti possono manifestarsi nella vita adulta o nelle generazioni successive (Rhind, 2009). Tra gli IE, diverse molecole di origine sintetica sono state dimostrati essere in grado di indurre l'insorgenza di diverse patologie. Il Bisfenolo A (BPA), ad esempio, è stato dimostrato promuovere l'insorgenza dell'endometriosi (Signorile et al., 2010) e di diversi tipi di cancro (e.g. cancro al seno, alla prostata e all'endometrio) a causa della sua attività estrogeno mimetica (Bolli et al., 2008; Ricupito et al., 2009). D'altro canto, composti di origine naturale, quali i flavonoidi, hanno mostrato avere un effetto protettivo contro diversi fenomeni degenerativi (i.e. patologie cardiovascolari, osteoporosi, cancro) (Cassidy et al., 2000; Dang and Lowik, 2005; Keinan- Boker et al., 2004). Ad oggi, non è chiaro se le discrepanze tra gli effetti descritti per i composti di origine sintetica e naturale dipendano dall'interazione degli IE con gli ER, o da meccanismi indipendenti da questo. Inoltre, poiché la maggior parte degli studi sugli IE, in particolare sugli effetti benefici dei flavonoidi, sono stati condotti in assenza di E2, il significato fisiologico di queste scoperte non è chiaro. L'uomo assume quotidianamente circa 500 g di diversi composti dimostrati avere attività ormone-simile sia *in vitro* che *in vivo* (Marino e Galluzzo, 2006), risultando esposto a più IE contemporaneamente e conseguentemente a miscele di composti naturali e sintetici. Sebbene siano stati recentemente evidenziati alcuni degli effetti combinati di diversi IE, gli effetti di miscele costituite da molecole di origine alimentare e sintetica sono tuttora ignoti. Scopo di questo progetto è quello di valutare gli effetti di composti naturali (i.e. flavonoidi), da soli o in miscela con l'ormone endogeno (E2) o con altri IE (BPA), sulle funzioni cellulari regolate da E2 (i.e. bilancio tra proliferazione e morte cellulare,

differenziamento e stress ossidativo) evidenziando gli effetti, i meccanismi d'azione, e l'eventuale coinvolgimento di ERα e ERβ.

I nostri dati dimostrano che il noto effetto di inibizione della crescita e induzione dell'apoptosi del flavonolo quercetina, uno dei flavonoidi bioattivi più comune e più studiato, non è dovuto alla sua attività antiossidante, che si estrinseca ad elevate concentrazioni (5×10^{-5}M), ma alla capacità della quercetina di attivare a basse concentrazioni (i.e., 10^{-6}M) una cascata apoptotica modulando l'attività di entrambi gli ER. In particolare, la quercetina è in grado di legare entrambi gli ER e di agire come un estrogeno-mimetico, portando le cellule ad apoptosi, nei tessuti che esprimono ERβ, quale il colon (Galluzzo et al., 2007), o di antagonizzare l'effetto proliferativo di E2 nei tessuti esprimenti ERα, quale il seno (Bulzomi et al., 2010). Il meccanismo di azione della quercetina prevede la modulazione delle attività degli ER: in presenza di ERβ, la quercetina attiva le stesse vie di segnale di E2 (sia genomiche che extranucleari), mentre in presenza di ERα, la quercetina preserva la sola capacità trascrizionale di ERα, bloccando i segnali rapidi attivati da questo recettore, importanti per la proliferazione cellulare. Ne consegue che a concentrazioni nutrizionalmente rilevanti, l'effetto antiproliferativo della quercetina dipende dalla modulazione delle attività degli ER piuttosto che dalle sue proprietà antiossidanti. I nostri dati hanno inoltre dimostrato che i flavonoidi, quale il flavanone Naringenina, mantengono la capacità di attivare l'apoptosi in cellule di cancro esprimenti ERα anche in presenza di E2. La Naringenina è uno dei flavonoidi meglio assorbiti nel tratto gastrointestinale umano, e il picco plasmatico di Naringenina aglicone va da 0.7 a 14.8×10^{-6} M (Erlund et al., 2001; Bugianesi et al., 2002; Manach et al., 2004). In accordo con le concentrazioni plasmatiche riportate, i dati ottenuti in cellule esprimenti ERα mostrano che 10^{-6} M Nar, sebbene non influenzi l'attività trascrizionale diretta di ERα, reverte l'effetto proliferativo di E2 bloccando l'attivazione dei segnali rapidi mediati da questo recettore e attivando vie di segnale coinvolte nell'apoptosi. Nel complesso, gli esperimenti condotti in presenza di concentrazioni fisiologiche di E2 e concentrazioni nutrizionalmente rilevanti di Nar ci hanno permesso di elucidare il meccanismo di interferenza della Nar dando un significato fisiologico alla proprietà antiproliferativa di questo flavonoide. L'importanza delle proprietà chemoprotettive dei flavonoidi sono supportate dai dati ottenuti utilizzando miscele di flavonoidi e IE di origine sintetica, i quali indicano come le piccole quantità di Nar ritrovate nel plasma (Manach et al., 2004) siano sufficienti per contrastare l'effetto

cancerogenico del BPA. I nostri dati infatti mostrano come il BPA sia, al pari di Nar, un composto dalla duplice azione, in grado di promuovere l'insorgenza di cancro al seno e in altri organi bersaglio che esprimono prevalentemente ERα e di bloccare l'effetto protettivo di E2 nei tessuti esprimenti ERβ, quali il colon. Questi aspetti divergenti possono agire in maniera sinergica incrementando il potenziale effetto di interferente di questo contaminante ambientale. I nostri dati, dimostrando che Nar reverte l'attività estrogenica del BPA nelle cellule esperimenti ERα ma che mantiene la sua attività antiproliferativa nelle cellule esprimenti ERβ anche in presenza di BPA, supportano fortemente la teoria del destino cellulare come risultato del bilanciamento delle vie di segnale attivate dai diversi IE, supportando l'importanza degli studi sugli effetti antiproliferativi dei flavonoidi. Poiché l'effetto di E2 va ben oltre la regolazione della proliferazione cellulare, e gli ER sono co-espressi in diversi tessuti, abbiamo valutato l'impatto della Nar in cellule non cancerose esperimenti entrambe le isoforme di ER. I dati ottenuti stimolando mioblasti di ratto, esperimenti entrambi gli ER, ci ha permesso di individuare distinti ruoli per ERα e ERβ nel muscolo scheletrico. Mentre le vie di segnale rapide attivate da ERα sono fondamentali per l'induzione del differenziamento mediato da E2, i segnali rapidi attivati da ERβ sono gli unici coinvolti nell'effetto protettivo di E2 dallo stress ossidativo indotto dai ROS. La capacità della Nar di interferire specificatamente esclusivamente con il differenziamento indotto da E2 pone l'attenzione sulla possibile esistenza di una suscettibilità correlata al genere ai flavonoidi nei diversi stati fisiologici della vita.

Nel complesso, i dati ottenuti ampliano la nostra conoscenza sui meccanismi alla base dell' (anti)estrogenicità dei composti di origine alimentare evidenziando come i meccanismi di azione rapidi siano i bersagli più suscettibili agli interferenti endocrini. Infatti né la Naringenina né la quercetina modificano la trascrizione diretta di promotori contenenti la sequenza ERE, ma, disaccoppiando le attività rapide di ERα da quella trascrizionale diretta, porta le cellule verso destini diversi. Ciò evidenzia l'importanza di studiare gli effetti dei flavonoidi nei diversi stati della vita i quali possono essere caratterizzati da differenti livelli proteici e distinti ruoli di questa isoforma di recettore. Poiché gli effetti di E2 dipendono dal rapporto tra i livelli di espressioni degli ER e dal bilanciamento dei segnali attivati da ciascuna isoforma, gli effetti degli IE risultano molto più complessi di quelli originariamente considerati, dal momento che diversi leganti inducono gli ER ad assumere conformazioni diverse, responsabili dell'attivazione di specifiche vie di segnale.

1. BACKGROUND

1.1 Endocrine disruptors

Global concerns have been raised in recent years over the potential adverse effects that may result from exposure to chemicals that have the potential to interfere with the endocrine system (WHO, 2002). Discovered in the early 1900s, when pig farmers in the USA complained of fertility problems in swine herds fed on mouldy grain (McNutt et al.,1928), and picked out in the 1940s by reports of infertility in sheep grazing on certain clovers in Western Australia (Marino and Mita, 2007) it has become evident that many chemicals present in the environment can interfere with both human and wildlife health mimicking, antagonizing or altering the physiological actions of endogenous hormones, mainly, sex steroid hormones. These substances present in the environment, are now classified as endocrine disruptors (EDs), defined as "exogenous substances that cause adverse health effects in an intact organism or in its progeny, consequent to changes in endocrine function" (European commission DGXII, 1996). Although present in minute amounts (part per trillion), EDs could interfere with the synthesis, secretion, transport, metabolism, binding, action, or elimination of natural hormones responsible for homeostasis maintenance, reproduction, and developmental processes (Diamanti-Kandarakis et al., 2009).

Currently more than 100 chemicals have been identified as EDs. About half of these compounds are substituted with halogen groups, mostly chlorine and bromine, and within this heterogeneous group of molecules we find: (a) synthetic chemicals used in industry, agriculture, and consumer products (e.g. polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins, plasticizers as phthalates bisphenol A (BPA), pesticides as methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT) and fungicides, like vinclozolin (b) synthetic chemicals used as pharmaceutical drugs (e.g. diethylstilbestrol, DES), and (c) natural chemicals found in human and animal food (e.g. flavonoids) (Marino and Mita, 2007, Diamanti-Kandarakis et al., 2009). EDs have long environmental half-life resulting in a continue increase of their global concentration in the environment and can be detected and may concentrate at great distances from where they are produced, used or released (Mita and Marino, 2007). As a consequence, some EDs are detectable in so-called "pristine" environments at remote distances from the site they were produced, used, or released due to water and air currents and via migratory animals that spend part of their life in a contaminated area, to become incorporated into the

food chain in an otherwise uncontaminated region. As these substances do not decay easily, they may not be metabolized, or they may be metabolized or broken down into more toxic compounds than the parent molecule; even substances that were banned decades ago remain in high levels in the environment, and they can be detected as part of the body burden of virtually every tested individual animal or human (Porte et al., 2006; Calafat and Needham, 2007). On the contrary, other EDs may not be as persistent but are so widespread in their use that there is prevalent human exposure (Diamanti-Kandarakis et al., 2009). The sources of exposure to EDs are diverse and vary widely around the world. Humans and animals can be exposed involuntarily to EDs by drinking contaminated polluted water, breathing contaminated air, ingesting food, or, contacting contaminated soil or even in the workplace (Diamanti-Kandarakis et al., 2009). Exposure can be the result of uptake through skin, gills or lungs, through food and drink, and through maternal blood (eg. foetus) or milk (eg. neonate) (Rhind, 2009). In general, persistent endocrine disruptors have low water solubility and extremely high lipid solubility, leading to their bioaccumulation in adipose tissue (Rhind, 2009).

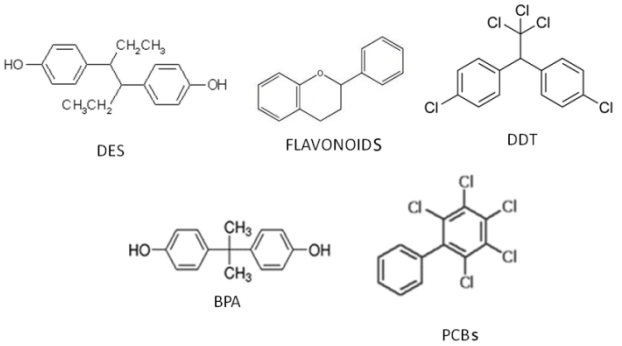


Figure 1.1: Chemical structure of common EDs. Chemical structure of synthetic chemicals used in industry, agriculture, and consumer products (BPA, DDT, PCB), as pharmaceutical drugs (DES) and basic structure of natural compounds (flavonoids). DES: diethylstilbestrol, DDT: dichlorodiphenyltrichloroethane, BPA: bisphenol A , PCBs: polychlorinated biphenyls.

EDs can exert both short and long term effect in many physiological states. The precise nature of these effects will depend on the extent to which they are taken up by the organisms and then on the extent to which they are

degraded, excreted or metabolized; each of these factors in turn, will depend on species, sex, age and compound class (Rhind, 2009). Animals, including humans, are especially sensitive to EDs at the early stages of development but some effects exerted at these stages may be expressed only in the adult life or even in subsequent generations (Rhind, 2009). Since the 1930s an increase in the frequency of development abnormalities of the male reproductive tract, particularly cryptorchidism and hypospadias, as well as a decline of sperm quality, have been reported (WHO, 2002). Developmental exposure to pesticides or to PCBs produce alterations in the dopaminergic system and has been linked to neurodegenerative disorders, including Parkinson's disease (Jones and Miller, 2008). Several concerns have also been raised about the influence of EDs on the timing of puberty and on onset of several pathologies. For example Bisphenol A (BPA) has been demonstrated to promote the development of endometriosis (Signorile et al., 2010) and of various cancer type (e.g. breast, endometrial and prostate cancer) because of its estrogen mimetic activity (Bolli et al., 2008; Ricupito et al., 2009).

From a physiological perspective, an endocrine-disrupting substance is a compound, either natural or synthetic, which, through environmental or during inappropriate developmental exposures, alters the hormonal and homeostatic systems that enable the organism to communicate with and to respond to its environment (Diamanti-Kandarakis et al., 2009). Several historical examples of toxic pills or contamination show a direct causal relationship between an unique chemical and the manifestation of an endocrine or reproductive dysfunction, due to the alteration of the *milieu interieur* (le Maire, et al., 2010). However, these types of single exposure are not representative of more common persistent exposures to a broad mix of chemicals and contaminants (le Maire, et al., 2010). The basic tenet of toxicology from Ames and Gold (2000) that ‘‘dose alone determines the poison’’ is too limited for EDs because both the timing of exposure and the dose can dictate not only the effect, but also whether the effects are adverse versus beneficial, or permanent versus transient (Hotchkiss et al., 2008). The so called “genotropic” effects of EDs appear at concentrations well below that at which they are toxic in the conventional sense (Wetherill et al., 2007). In fact, EDs may exert nontraditional dose-response curves, such as inverted-U or U-shaped curves (vom Saal, 2007) typical of hormone actions. As a consequence, EDs can induce cellular and molecular alterations of endocrine function at low dosage levels producing a cascade of effects that could be more potent effects than higher doses. This concept have been known for hormone and neurotransmitter actions, but only in the past decade it has begun to be appreciated for EDs. The properties of these

substances are particularly well suited for study by endocrinologists because they so often activate or antagonize hormone receptors. There is no endocrine system that is immune to EDs (Fig.1.2). However, because of the shared structures and properties of the chemicals and the similarities of the receptors (Thornton, 2001) and enzymes involved in the synthesis, release, and degradation of hormones, the most caught hormones are the sex steroid hormones (Diamanti-Kandarakis et al., 2009).

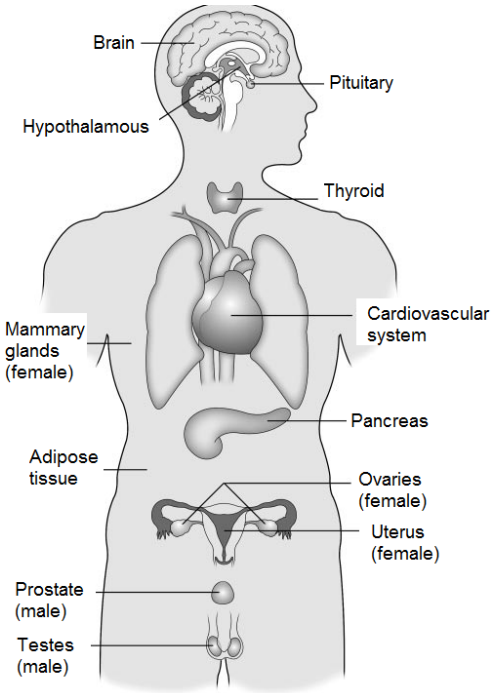


Figure 1.2: Endocrine systems targeted by EDs. Model of hormone-sensitive physiological systems vulnerable to EDs (Diamanti-Kandarakis et al., 2009).

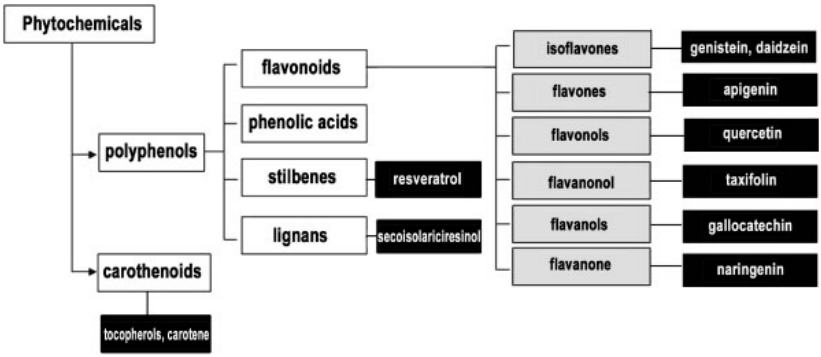
1.1.1 Flavonoids, a particular class of EDs

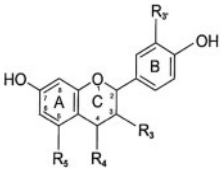
A particular class of EDs is composed by natural polyphenols. The biological activity of these compounds, particularly flavonoids, drew the attention of many researchers for their ability to prevent several degenerative disease in humans. Flavonoids have a long history in science.

Often referred to as weak estrogens, they were chemically synthesized before the ring structure of the mammalian steroids was determined in the 1920's and 1930's (Barnes, 2004). They re-emerged from obscurity in the 1940's as the anti-estrogenic principle in red clover that caused infertility in sheep in Western Australia (Bennetts et al., 1946). This adverse effect of flavonoids, caused by interfering in some way with sex hormone actions, placed these substances in the class of EDs (Jacobs and Lewis, 2002). Primarily recognized as the pigments responsible for the many shades of yellow, orange, and red in flowers (Timberlake and Henry, 1986; , Brouillard and Cheminat, 1988) and as part of plant defense mechanism against stresses of different origins (Birt et al., 2001) at the present more than 4000 flavonoids have been identified in edible plants (Timberlake and Henry, 1986; Manach et al., 2004) and are consumed regularly with the human diet (Timberlake and Henry, 1986).

These low molecular weight substances are phenylbenzo-pyrones (phenylchromones), possessing an assortment of structures based on a common three-ring nucleus (Middleton et al., 2000) in which primary substituents (eg, hydroxyl, methoxyl, or glycosyl groups) can be further substituted (e.g., additionally glycosylated or acylated) sometimes yielding highly complex structures (Cheynier, 2005) (Fig. 1.3). Flavonoids, have been categorized into 6 subclasses as a function of the type of heterocycle involved: flavonols, flavones, flavanols, flavanonols, flavanones, and isoflavones (Fig 1.3) (Birt et al., 2001; Manach et al., 2004). The role played by flavonoids as EDs have more recently been confirmed *in vivo*. Numerous effects in both male and female rats exposed to genistein from gestational day 7 into adulthood through placental transfer, lactational exposure and ingestion were observed including hyperplasia of mammary glands in both sexes, aberrant or delayed spermatogenesis, histological changes in the vagina and ovary, mineralization of renal tubules in males, modulation of natural killer cell activity, myelotoxicity, neuroendocrine changes associated with behavioural outcomes, and sexually dimorphic brain development (Flynn et al., 2000; Delclos et al., 2001; Guo et al., 2005; Doerge et al., 2006). However, for the past 10–15 years scientific evidence has indicated that adult human diets rich in flavonoids lead to significantly decreased serum concentrations of total cholesterol, low-density lipoproteins (LDL) and triglycerides (Kirk et al., 1998; Ricketts et al., 2005), as well as a reduced incidence of cardiovascular diseases (Hertog et al., 1997; Cassidy et al., 2000), and osteoporosis (Dang and Lowik, 2005). These effects, recognized as estrogen-mimetic effects, are currently being explored to prevent osteoporosis (Mikkola and Clarkson, 2002; Dang and Lowik, 2005), the risk of coronary artery disease (Middleton et al. 2000;

Kris- Etherton et al., 2002), and the vasomotor flushing related to estrogen deficiency in women during menopause (Mikkola and Clarkson, 2002; Fitzpatrick, 2003).





Class	Representative flavonoids	Substitutions (R) 3	4	5	3'
Flavanone	Naringen	H	=O	OH	H
Flavanol	Catechin	OH	H	OH	OH
Flavone	Apigenin	H	=O	OH	H
Flavonol	Quercetin	H	=O	OH	OH
Flavanonol	Taxifolin	OH	=O	OH	OH
Isoflavone	Daidzein	OH	=O	H	H
	Genistein	C_6H_6	=O	OH	H

Figure 1.3: Schematic model and chemical structure of flavonoids. Subdivision of bioactive compounds from plants present in foods (top panel). The white, grey, and black boxes are representative of phytochemical families, flavonoid classes, and demonstrative compounds, respectively. General structure and numbering pattern for common food flavonoids (bottom panel) (Marino and Galluzzo, 2008).

The need to develop new estrogen-mimicking agents derives from the necessity of producing their desired beneficial effects without the accompanying adverse side effects of estrogen treatment (Fitzpatrick, 2003). In fact, estrogens are tumor promoting agents known to increase the risk of breast and uterine cancer in women taking estrogen replacement therapy (Castagnetta et al., 2004; Yager and Davidson, 2006). On the contrary, Asian women (large isoflanoid consumers) and vegetarians have a lower than average breast cancer risk (Limer and Speirs, 2004). In addition, flavonoids have been shown to induce responses consistent with the protective effects of fruit and vegetable rich diets against cancer in both in vitro test systems and small animal models (Hollman et al., 1996; Gamet-Payrastre et al., 1999; Birt et al., 2001; Brownson et al., 2002; Keinan-Boker et al., 2004). The anticancer effect of nutritional flavonoids could represent other anti-estrogenic effects ascribed to these compounds. As a result of all these potentially beneficial effects, a huge number of preparations are now commercially available on the market as health food products. As dietary supplements they are obtainable as plant extracts or mixtures, containing varying amounts of isolated or concentrate flavonoids in bakery, dairy, infant formulas (Tomar and Shiao, 2008). The commercial success of these supplements is evident and the consumption of these compounds in Western countries is increasing even though their mechanisms of action is not well understood.

Several mechanisms, such as inhibition or modulation of different kinases or antioxidant activities, have been proposed for flavonoids actions (Marino and Bulzomi, 2009). However, these pathways require high flavonoid concentration (>50 μM) (Marino and Bulzomi, 2009). At concentrations more physiologically achievable in the plasma (from 10^{-7} M to 10^{-5}M) after the consumption of meals rich in flavonoids (Manach et al., 2004), these compounds are thought to function by regulating estrogen receptor (ERs) activity (Birt et al., 2001; Totta et al., 2004) leading to estrogenic or antiestrogenic effects (Totta et al., 2004; Galluzzo and Marino 2006; Galluzzo et al., 2008; Marino and Bulzomi, 2009). At present the relative importance of each of these pathways at physiological level and their putative cross-talk, as well as the correlation among the proposed mechanism and clinical significance in nutritionally relevant flavonoid concentration remain to be established.

1.1.1.1 Flavonoid bioavalability

Plant flavonoid metabolism and composition are highly variable both qualitatively and quantitatively; some of the compounds are ubiquitous, whereas others are restricted to specific families or species (e.g., isoflavones

in legumes). In most cases, foods contain complex mixtures of polyphenols, which are often poorly characterized. Several factors affect the flavonoid content of plants, including ripeness at the time of harvest, environmental factors, processing, and storage (Manach et al., 2004; Cheynier, 2005). It is quite well established that once eaten, flavonoids enter a complex pathway of bio-transformation so that, the molecular forms reaching the peripheral circulation and tissues to be excreted are usually different from those present in foods (Manach et al., 2004). Ring scission occurs under the influence of intestinal microorganisms, which also account for the subsequent demethylation and dehydroxylation of the resulting phenolic acids (cinnamic acid derivatives and simple phenols). Intestinal bacteria also possess glycosidases capable of cleaving sugar residues from flavonoid glycosides. Such glycosidases do not appear to exist in mammalian tissues. Flavonoids can undergo oxidation and reduction reactions, as well as methylation, glucuronidation, and sulfation in animal species (Middleton et al., 2000). During the course of absorption, polyphenols are conjugated in the small intestine and later in the liver (Hollman and Katan, 1999; Birt et al., 2001; Manach et al., 2004). These glucuronide and sulphate conjugates are more readily transported in the blood and excreted in bile or urine than are the parent aglycones. The spectrum of conjugation products may be species- and gender-dependent and these metabolites are not necessarily biologically inert (Manach et al., 2004). The solubility, the metabolic fate of compounds, due to endogenous and exogenous biotransformation, and their interaction with other dietary components determine flavonoid bioavailability (Hendrich et al., 1998) and effects. Bioavailability differs greatly from one polyphenol to another, so that the most abundant polyphenols in our diet are not necessarily those leading to the highest concentrations of active metabolites in target tissues. The metabolites present in blood, resulting from digestive and hepatic activity usually differ from the native compounds. The plasma concentrations of total flavonoid metabolites ranged from 0 to 4×10^{-6}M with an intake of 50 mg aglycone equivalents, and the relative urinary excretion ranged from 0.3% to 43% of the ingested dose, depending on the polyphenol. Gallic acid and isoflavones are the most well-absorbed polyphenols, followed by catechins, flavanones, and quercetin glucosides. The least well-absorbed polyphenols are the proanthocyanidins, the galloylated tea catechins, and the anthocyanins (Manach et al., 2004). As a consequence, the maximum concentration of flavonoids reached in the circulation ranged from 10^{-7} M to 10^{-5}M (Manach et al., 2004).

Among the huge number of flavonoids, the most studied and well characterized are the flavonol quercetin and the soy isoflavones, such as

genistein or daidzein. Flavonols, especially quercetin, have been extensively studied, mainly because they are widely distributed in dietary plants and because of its excellent antioxidant activity (Hanasaki et al., 1994; Haenen and Bast, 1999; Terao, 2009). However, quercetin content in the diet is generally quite low (Justesen et al., 1997; Hertog et al., 1993; Pietta et al., 1996; Sampson et al., 2002; Manach et al., 2004) and it is not present in plants as an aglycone thus occurs only in conjugated forms (*i.e.* glycosides). As a consequence, baseline quercetin aglycon concentrations, were generally from 5×10^{-8} M to 8×10^{-8}M and values were even lower when a low-polyphenol diet was given to the volunteers before a test meal (Noorozi et al., 2000; Erlund et al., 2002). Quercetin aglycon maximum plasma concentrations reached to 0.6×10^{-6}M-1.5 10^{-6}M after 28 days of supplementation with high doses of quercetin (from 0.8 to 1 g/d) (Conquer et al., 1998; Moon et al., 2000). Thus, quercetin plasma concentration appear to be too slow to exert any of the *in vitro* described effects .

Isoflavones are the most well-absorbed polyphenols (Manach et al., 2004), but these compounds are provided only by soybean-derived products, thus are typical of an Asiatic diet. Furthermore, in some cases isoflavone metabolite (*i.e.* equol) has been shown to be more active than its precursor (*i.e.* daidzein) in many *in vitro* studies and in animal models (Setchell et al., 2002). Since a great interindividual variability in the capacity to produce equol exist and only 30-40% of the Western population are "equol producer" (Manach et al., 2004), only "equol producers" seems to be susceptible to this compound action. The biological activity of flavonoid metabolites received scarce attention and only few paper reported the effects of such compounds (Totta et al., 2005). Furthermore, to date, no clear correlations between dietary habits or microflora composition and the capacity to produce flavonoid metabolite have been reported (Manach et al., 2004). Intriguingly, flavanones, representing a small group of compounds present in plants mainly in glycoside form, have been demonstrated to be more rapidly absorbed as aglycones. In an elegant paper Bugianesi et al. showed that peak plasma concentrations (Cmax, 10^{-7}M) of naringenin aglycone (Nar), a 4',5,7-trihydroxyflavanone widely present in citrus fruits and skin tomato, was reached as early as 2 h after the ingestion of tomato paste (Bugianesi et al., 2002). Furthermore Nar Cmax of 0.6×10^{-6}M and 6×10^{-6}M, were reached in the plasma of volunteers after the ingestion of orange juice and grapefruit juice, respectively (Erlund et al., 2001). Even though Nar represents one of the most widely present flavonoid in Mediterranean diet, regularly consumed in the meal, it is one of the less studied flavonoids. Since at concentration achievable in the plasma, Naringenin has been demonstrated to exert cholesterol-lowering properties

by inhibiting cholesteryl ester synthesis (Borradaile et al., 1999), to act as a modulator of immune system (Nahmias et al., 2008) and to exhibit anti-estrogenic activity (Jacob and Kaul, 1973; Miksicek, R. J. 1993; Ruh et al., 1995; Totta et al., 2004; Virgili et al., 2004; Galluzzo et al., 2008), that may be responsible for the decreased incidence of breast cancer in western women consuming a large amount of phytoestrogens (Adlercreutz et al., 1992) major studies are necessary in order to investigate the chemopreventive and protective effect of this compound.

Thus, in thesis, we focused our attention on Naringenin underlying action mechanisms in modulating E2-dependent cellular effect.

1.2 Estrogens

Estrogens and in particular 17β-estradiol (E2) the most potent estrogen in humans, regulate a widespread of physiological functions (Ascenzi et al., 2006). Just to mention some of them E2 regulates the development of the secondary sexual features, bone turnover inhibition, vasodilatation and relaxation of vascular smooth muscle (Mendelsohn, 2000) white adipose metabolism and location in females. In addition, protective effect of E2 against colon cancer growth (Galluzzo et al., 2007), neurodegenerative diseases (Deroo and Korak, 2006), atherosclerosis (Ascenzi et al., 2006), as well as in skeletal muscle mass maintenance (Galluzzo et al., 2009) have been reported. Furthermore, estrogens, derived from testosterone conversion to estradiol by aromatase, are also fundamental for the masculinization of developing male brain, for prostate growth, for male bone mineralization, and for male fertility (Gorski, 1985; Revelli et a., 1998; Simerly, 1998; Simerly, 2002; Christian et al., 2000; Clarke and Khosla, 2009; Ulubaev et al., 2009).

1.2.1 Estrogen Receptors

The biological actions of E2 are mediated by two estrogen receptor isoforms (ERα and ERβ) (Ascenzi et al., 2006). ERα and ERβ (NR3A1 and NR3A2, respectively) are the products of separate genes (*ESR1* and *ESR2*, respectively) present on distinct chromosomes (locus 6q25.1 and locus 14q23-24.1, respectively) (Gosden et al., 1986; Enmark et al., 1997; Luisi et al., 2006; Zhou et al., 2006) functioning as ligand-activated transcription factors (O'Malley, 2005). ERs, like all the members of the nuclear receptor super-family, are modular proteins sharing common regions, named A/B, C, D, and E/F, as well as a high sequence homology (Fig. 1.4). These regions participate in the formation of independent but interacting functional domains. The *N*-terminal domain (A/B region) is involved in both inter-molecular and intra-molecular interactions as well as in the activation of

gene transcription. The DNA binding domain (DBD, C region) allows ER to dimerize and to bind to the specific estrogen response element (ERE) sequence on DNA through its two "zinc finger" structures. The hinge domain (D region) has a role in receptor dimerization and in binding to chaperone heat-shock proteins (Hsp). The ligand binding domain (LBD, E/F region, *C*-terminal) comprises the estrogen-binding domain and works, synergistically with the *N*-terminal domain in the regulation of gene transcription (Mosselman et al., 1996; Nilsson et al., 2001; Claessens and Gewirth, 2004; Kumar et al., 2004, Ascenzi et al., 2006). ERs contain two regions called activation functions (AFs) important for ligand-dependent transcriptional activity (Fig. 1.4) (Mosselman et al., 1996; Nilsson et al., 2001; Claessens and Gewirth, 2004; Kumar et al., 2004). AF-1 and AF-2 regions of ERs, interacting with a number of trancription co-activators, can independently activate transcription but in most cases, they synergize with one another in a promoter- and cell-context specific manner (McEwan, 2004). AF-1 could be activated even in a ligand-independent manner, depending on the phosphorylation status of ERs. In particular, the Ser118 residue in the AF-1 region of ERα, as well as residues Ser106 and Ser124 in the AF-1 region of ERβ, are the phosphorylation sites essential for the ligand-independent activation of ERs through the Ras-mitogen activated protein kinase (MAPK) signaling cascade (Ortì et al., 1992; Lannigan, 2003; Ascenzi et al., 2006).

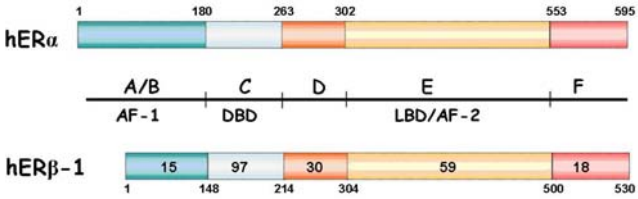


Figure 1.4: A schematic structural comparison of human ERα and ERβ functional domains. Receptor domains are illustrated with different colored boxes, and the approximate size of each domain is indicated. The A/B domain contains the ligand-independent transcriptional-activation function AF-1, the C domain represents the DNA-binding-domain (DBD), the D domain corresponds to the hinge region, and the E/F domain contains the hormone-binding domain (LBD) and the hormone-dependent transcriptional-activation function AF-2, the dimerization domain, and part of the nuclear localization region.The number inside each box of ERβ refers to the percentage of amino acid identity.

Recent progress in studies on genomic and cDNA sequences has accelerated the identification of gene splice variants in the NR super-family. Numerous mRNA splice variants exist for both ERs and the best-characterized splice variants are ERα46 and ERβcx, which are frequently co-expressed with their wild-type counterparts. The exact function and potential role of these and other ERs splice variants in physiology and human disease remain to be elucidated (Herynk and Fuqua, 2004; Marino et al., 2006; Ascenzi et al., 2006).

1.2.2 ER distribution

Both ERs are widely distributed throughout the body, displaying distinct or overlapping expression patterns in a variety of tissues (Couse and Korach, 1999; Pettersson and Gustafsson, 2001). In particular, ERα mRNA is highly expressed in epididymis, testis, ovary, kidney, and adrenal. Moderate amounts of ERα are also present in the prostate gland, bladder, liver, and thymus. The highest amounts of ERβ mRNA were detected in the prostate gland, brain, ovary, gastrointestinal tract and bladder, hematopoietic and central nervous systems. ERα and ERβ are, however, coexpressed in a number of tissues including the mammary gland, epididymis, thyroid, adrenal, bone, and certain regions of the brain. Although both ER subtypes may be expressed in the same tissue, they might not be expressed in the same cell type. In the rat ovary, ERβ is the predominant ER in the granulosa cells, whereas ERα is largely present in the thecal and interstitial cells (Hiroi et al., 1999; Sar and Welsch, 1999; Nilsson et al., 2001). Furthermore, a switch in ER expressions during development has been reported (Brandenberger et al., 1997; Nishihara et al., 2000; Deroo and Korach, 2006; Ascenzi et al., 2006). Nonetheless, ERα and ERβ proteins have been simultaneously detected in many cell types including neurons and thymocytes (Greco et al., 2001; Mor et al., 2001), and these as well as other cell types that coexpress both ER subtypes are targets for potential interplay between the two receptors. However it has been demonstrated that when coexpressed with ERα, ERβ appears to act as a dominant negative regulator of estrogen signaling causing a concentration dependent reduction in ERα-mediated transcriptional and rapid activities (Pettersson et al., 2000; Liu et al., 2002; Matthews and Gustafsson, 2003) even if other underlying mechanisms cannot be excluded.

1.2.3 ER mechanisms of action

The mechanisms underlying ERα and ERβ action are complex pathways that involve two distinct types of signaling which lead to protein kinase activation (rapid membrane-initiated mechanism) and direct or

indirect transcription of target genes (nuclear mechanism) (Fig. 1.5). All these pathways synergize each other to determine the overall effects of E2.

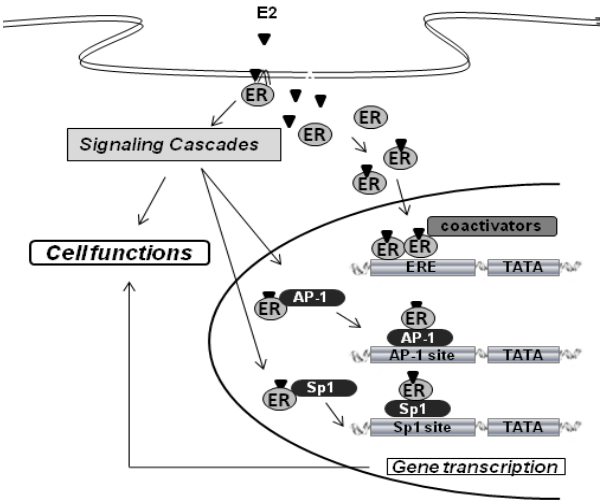


Figure 1.5: Schematic rappresentation of E2:ER complex mechanisms of action. Upon E2 binding, activated ER can dimerize and bind to ERE sequence on DNA, and/or activate signaling cascade important also for ER indirect transcriptional activity, depending on ER interactions with Sp1 and AP-1 factors. E2: 17β-estradiol; ER: Estrogen receptor; AP-1: activating factor-1; Sp1: stimulating factor-1.

In the nuclear mechanism of action, estrogens diffuse into the cell membrane and bind to ERs causing ERs to dissociate from heat shock proteins, dimerize and traslocate into the nucleus. The nuclear ERα- or ERβ-E2 complex directly binds DNA through the ERE (estrogen responsive element) sequences or indirectly through protein-protein interactions with activator protein-1 (AP-1) or stimulating protein (Sp1), resulting in recruitment of coregulatory proteins (coactivators or corepressors) to the promoter, increased or decreased mRNA levels, protein synthesis, and physiological responses (Ascenzi et al, 2006; Deroo and Korach, 2006) (Fig.1.5). A large subset of coregulatory proteins (e.g., steroid receptor coactivator-1, 2, and 3) helps the hormone-receptor complex in the

recruitment of histone acetyltransferases and methyltransferases which, in turn, possess chromatin-remodeling ability and tether activated receptors to the basal transcriptional machinery (Smith and O'Malley, 2004). Both ERα and ERβ regulate gene transcription through this classical mechanism involving ERE, even if ERβ seems to be a weaker transactivator (Cowley and Parker, 1999). AF-1 activity of ERβ is weak compared with that of ERα on ERE, whereas their AF-2 activities are similar (Cowley and Parker, 1999). Consequently, when both AF-1 and AF-2 functions are active in a particular cell and/or on a particular promoter, the activity of ERα greatly exceeds that of ERβ, whereas ERα and ERβ activities are similar when only AF-2 is required (McInerney et al, 1998; Cowley and Parker, 1999; Ascenzi et al, 2006). It has been postulated that differences in the ERα and ERβ activities are due to differences in the ability of the receptors to interact with coregulatory proteins, because of the low amino acid identity in A/B domain of ERs (Fig. 1.4) (Smith and O'Malley, 2004; Ascenzi et al, 2006).

Only a fraction of the known mammalian EREs reflects the consensus palindromic element ERE (GGTCAnnnTGACC), initially described based on the ERE in the Xenopus laevis vitellogenin A2 promoter (Klein-Hitpass et al., 1986; Ponglikitmongkol et al., 1990). Thus, many target genes contain response elements that bear little similarity to consensus EREs and affects the affinity that a given receptor isoform has for binding DNA (Loven et al., 2001). Even if ERα and ERβ have similar effects on ERE-mediated gene transcription, only the complex E2:ERα activates promoters lacking any ERE-like sequences and requiring a second DNA-binding transcription factor (e.g., Sp1 and AP-1) to mediate ER association with the DNA (O'Lone et al, 2004). E2 binding to ERβ does not result in the formation of a transcriptionally active complex at a promoter containing Sp1 elements (Saville et al, 2000) and inhibits AP-1-mediated promoter activity (Paech et al, 1997). As an example ERα and ERβ, in the presence of E2, oppose each other's function in the regulation of the cyclin D1 promoter (Liu et al., 2002). Deletion of AP-1 and Sp1 responsive element motifs in the cyclin D1 gene promoter resulted in attenuation of promoter responsiveness to E2 (Marino et al, 2002, 2003). Unlike ERα, E2-bound ERβ did not activate cyclin D1 expression (Acconcia et al, 2005a), important for the progression of cells through the G1 phase of the cell cycle, and blocks ERα-E2-mediated induction when both receptor isoforms are present (Matthew and Gustafsson, 2003). Consequently, these differences in transcriptional activity between the ERα and ERβ may account for the major differences in their tissue specific biological actions.

The 'genomic action' of steroid hormones occurs after a time-lag of at least 2 hours after E2 stimulation and explains some hormone functions in

physiological and pathological situations (Farach-Carson and Davis, 2003; Marino et al., 2005). A physiological dose of E2 was reported to increase the uterine cAMP level in ovariectomized rats within 15 seconds (Szego and Davis, 1967), and only seconds are requested for an E2-induced increase of intracellular calcium level in granulose cells (Morley et al., 1992) and to increase inositol trisphosphate (IP_3) production in the liver and in liver derived HepG2 cells (Marino et al., 1998; Marino et al., 2001a).

These effects are too rapid to be accounted for genomic action(s). It is interesting to note that the cell membrane impermeable E2-bovine serum albumin conjugate mimics the E2 effects in activating rapid signal transduction pathways (Marino et al., 2002; Levin, 2005). Furthermore these events are insensitive to inhibitors of transcription (*e.g.*, actinomycin D) and translation (*e.g.*, cycloheximide) (Losel et al., 2003), and due to the short time required for the activation they have been termed "rapid or non-genomic". Actually the term "non-genomic" is not adequate when referring to rapid changes that may also initiate new gene transcription (Farach-Carson and Davis, 2003; Kampa and Castanas, 2006) and the term extranuclear is now referred. These E2-induced rapid effects have been attributed in most cells to a population of ERs present on the plasma membranes. Debate continues over whether structural changes target nuclear ERs in separate pools localizing them to the membrane (Chambliss, et al., 2000; Acconcia and Kumar, 2005; Marino et al., 2005; Kampa and Castanas, 2006), or whether membrane ER represents a novel receptor (Ahola et al., 2002; Filardo et al., 2002; Ropero et al., 2002; Toran-Allerand et al., 2002; Thomas et al., 2005; Vivacqua et al., 2006). Besides these data, much evidence favors the idea that the membrane-localized ER is the same protein as the nuclear-localized receptor (Pappas et al., 1995; Norfleet et al., 1999; Razandi et al., 1999; Marino et al., 2002, 2003) and that ERα and ERβ must be considered a population of protein(s) which localization in the cell is able to dynamically change, shuttling from membrane to cytosol and to the nucleus, depending on ligand binding (Razandi et al., 1999; Dan et al., 2003; Marino et al., 2005; Leclercq et al., 2006). Current evidence indicates that a small population of ERα and ERβ localize at the plasma membrane exists within caveolar rafts. It is at the plasma membrane that E2-liganded ER associates with the scaffolding protein caveolin-1 and a variety of signal transduction cascade activation occurs. ERs do not contain a *trans*-membrane domain (Björnström and Sjöberg, 2005; Ascenzi et al., 2006), thus the ability of ERα and ERβ to associate with the plasma membrane could be due to its association with membrane proteins and/or by post-translational addition of lipids to ERα (Acconcia et al., 2005b; Levin, 2005). Recently it has been demonstrated that ERα undergoes to *S*-

palmitoylation on a cysteine residue (Cys447) present in the LBD which allows receptor anchoring to plasma membrane, association to caveolin-1, and which accounts for the ability of E2 to activate different signaling pathways (Acconcia et al., 2005a). The Cys399 residue present in the LBD of ERβ is also subjected to *S*-palmitoylation (Galluzzo et al., 2007) indicating that a similar mechanism also works for ERβ localization to the plasma membrane and association to caveolin-1 (Marino and Ascenzi, 2008). E2-induced reversible S-palmitoylation of ERα and ERβ could account for the coexistence of both membrane-bound and soluble isoforms of ERα and ERβ (Marino and Ascenzi, 2006; Galluzzo et al., 2007). S-palmitoylation is necessary for E2-induced rapid events as demonstrated by the loss of signaling cascade activation in human cancer cells treated with physiological concentration of E2 in presence of the palmitoyl-acyl-transferase inhibitor or transfected with the ERα Cys447Ala mutant (Acconcia et al., 2005b, Pietras et al., 2005; Pedram et al., 2007). Various signaling pathways are activated upon E2 binding to membrane ERs. These rapid events may be classified into four main signaling cascade: phospholipase C (PLC)/protein kinase C (PKCs) (Morley et al., 1992; Marino et al., 1998, 2001a, 2001b; Picotto et al., 1999; Perret et al., 2001; Incerpi et al., 2003), Ras/Raf/MAPK (Marino et al., 2002; Watter et al., 1997; Russel et al., 2000; Dos Santos et al., 2002; Migliaccio et al., 2002; Tanaka et al., 2003; Klinge et al., 2005; Woo et al., 2005), phosphatidyl inositol 3 kinase (PI3K)/AKT (Castoria et al., 1999, 2001; Simoncini et al., 2000; Marino et al., 2003; Björnström and Sjöberg , 2005; Levin, 2005; Acconcia et al., 2005a; Marino et al., 2005; Chambliss et al., 2005), and cAMP/protein kinase A (PKA) (Gu and Moss, 1996; Farhat et al., 1996; Picotto et al., 1996; Chen et al., 1998; Malyala et al., 2005). These pathways present numerous interactions with several other pathways. The ERα:E2 complex interacts with the IGF-1 receptor, leading to IGF-1 receptor activation and hence to MAPK signaling pathway activation (Kahlert et al., 2000). In addition, the ERα:E2 complex activates the EGF receptor by a mechanism that involves activation of guanine nucleotide exchange proteins (G-proteins), Src, and matrix metalloproteinases, leading to an increase in extracellular regulated kinases (ERK) and PI3K/AKT activities (Dos Santos et al., 2002; Driggers and Segars, 2002; Improta-Brears et al., 1999; Razandi et al., 2003; Zhang et al., 2004; Kupzig et al., 2005). AKT and PKC could modulate the MAPK pathway through Raf phosphorylation (Chambliss et al., 2000, 2005; Marino et al., 2005; Kim and Bender, 2005). It has been demonstrated that a sub-population of ERβ transfected into Chinese Hamster ovary cells is capable of stimulating IP_3 production, ERK/MAPK activation, and c-JNK phosphorylation (Razandi et al, 1999).

Geraldes and coworkers reported that E2 reduces ERK activity through ERβ stimulation in porcine smooth muscle cells (Geraldes et al, 2003). Recently, E2:ERβ complex has been demonstrated to rapidly induces a persistent membrane-initiated activation of p38/MAPK in ERβ-trasnfected cells and DLD-1 colon cancer cells, endogenously expressing a great amount of ERβ (Acconcia et al., 2005; Galluzzo et al., 2007; Caiazza eta l., 2007). Also E2:ERα complex increased p38/MAPK phosphorylation, however E2:ERα dependent p38 activation is transient (Acconcia et al., 2005). In fact E2:ERα complex, activating ERK and AKT pathways, suppress the activity of the apoptosis signal regulating kinase 1(ASK1), one of the upstream activators of p38. Particularly, E2 induces ASK1 phosphorylation at Ser83 via ERα-AKT cascade (Kim et al., 2001; Yuan et al., 2003; Du et al., 2004; Mabuchi et al. 2004). Thus, the ability of the ERα-E2 complex to activate rapidly ERK and AKT avoids the persistent p38 activation.

The physiological significance of these ER-dependent rapid pathways is quite clarified, at least for some E2 target tissues. E2 actions on proliferation have been assumed to be exclusively mediated by ERα-induced rapid membrane-starting actions (e.g., PI3K/AKT and ERK/MAPK pathway) (Marino et al., 2005; Ascenzi et al., 2006). E2 treatment of mammary-derived MCF-7 cells triggers the association of ERα with Src and p85α leading to DNA synthesis (Castoria et al., 2001). In HepG2 cells multiple and parallel membrane starting pathways are rapidly activated by the ERα-E2 complex (Marino et al., 1998, 2002, 2003) and the blockade of PLC/PKC, ERK, and PI3K/AKT pathways completely prevents the E2-induced DNA synthesis (Marino et al., 2002, 2003). ERK/MAPK and PI3K/AKT pathways, rapidly activated by the ERα-E2 complex, also have a critical role in E2 action as a survival agent. In fact, these pathways enhance the expression of the anti-apoptotic protein Bcl-2, block the activation of the p38/MAPK, reduce the pro-apoptotic caspase-3 activation, and promote G1-to-S phase transition *via* the enhancement of the cyclin D1 expression (Marino et al., 2002, 2003; Acconcia et al., 2005a). E2 affects neural functions, both in male and in female brain, in part by inducing such rapid responses (Farach-Carson and Davis, 2003; Losel et al., 2003). In the skeleton, ERα-dependent Src/Shc/ERK pathway transmits survival signals and prolongs the life span of osteoblasts (Kousteni et al., 2003). At the same time, E2 delivers a pro-apoptotic signal to bone-resorbing osteoclasts, shortening their life span (Kousteni et al., 2002; Manolagas et al., 2002; Kousteni et al., 2003). In the liver, rapid E2-induced signals (i.e., PLC/PKC) are strongly linked to the increased expression of the LDL receptor which leads to a decreased level of LDL-cholesterol in the plasma (Marino et al., 2001b; Distefano et al., 2002). E2-activated PI3K/AKT

pathway is responsible for E2-induced survival signals (Acconcia et al., 2005b) and for activation of endothelial nitric oxide synthase (eNOS), which is at the root of E2 vascular protection in ischemia/reperfusion injury *in vivo* (Simoncini et al., 2000; Chambliss and Shaul, 2002). ERα-dependent PI3K/AKT activation is also essential for E2-induced skeletal myoblast differentiation (Galluzzo et al., 2009).

Collectively these evidences demonstrate that the integration of the E2:ER "genomic action" together with the ability of membrane starting pathways to signal through multiple cascades are at the root of estrogen pleiotropic effects.

1.3 Flavonoid-dependent modulation of ER activities.

Given the wide spectrum of function regulated by E2:ERs and the reported flavonoid estrogen-like or estrogen antagonistic activities it is necessary to understand the mechanism underlying flavonoid-dependent modulation of ER action. A plethora of papers, supported by epidemiological and experimental data, indicates the ability of flavonoids to bind to ER isoforms leading to estrogen mimetic or anti-estrogenic effects (Kuiper et al., 1998; Bolli et al., 2008; Marino and Bulzomi, 2009). From a biochemical point of view, all effective ER ligands require at least one E2 A ring-like phenolic hydroxyl group and a second E2 D ring-like hydroxyl group separated by a rigid hydrophobic linker region (Pike et al., 1999, 2000, 2001; Kumar et al., 2004; McDonnell, 2004; Ascenzi et al., 2006). Each molecule of this wide spectrum of compounds, binds to ERs with different affinity inducing the repositioning of LBD of ERα, which result in different ER conformations that may favor or impair co-activators recruitment and, in turn, receptor transcriptional activity (Kuiper et al., 1997, 1998; Ascenzi et al., 2006). Several studies indicate the ability of flavonoids to bind both ER isoforms maintaining the ER gene transcriptional ability (Kuiper et al., 1997,Totta et al., 2004, Virgili et al., 2004). Nevertheless several epidemiological and experimental data show that flavonoid effects can be both estrogen mimetic and antiestrogenic. Several groups have demonstrated that flavonoid affinity to ERs is lower than E2 (Kuiper et al., 1997). Competition binding studies confirm that nutritional molecules (e.g., genistein, coumestrol, daidzein, and equol) show a distinct preference for ERβ (Kuiper et al., 1997; Mueller et al., 2004; Escande et al., 2006), although the prenylated chalcone occurring in hops, 8-prenylnaringenin, has been found to be a potent ERα agonist, but a weak agonist of ERβ in E2 competition assays (Stevens and Page, 2004). Phytochemicals as the isoflavonoids daidzein and genistein, the flavanone naringenin, and the flavonol quercetin increase the activity of ERE-

luciferase reporter gene construct in cells expressing ERα or ERβ (Mueller, 2002; Totta et al., 2004; Virgili et al., 2004; Totta et al., 2005), but impair ERα interaction with Sp1 and AP-1 (Paech et al., 1997; Liu et al., 2002; Virgili et al., 2004). Cluster analysis of DNA microarray in MCF-7 cells show a very similar profiles between estrogen responding genes and 10 μM genistein (Terasaka et al., 2004) while the expression of only five genes is affected by daidzein with respect to E2 in TM4 Sertoli cells. These five genes were related to cell signaling, cell proliferation, and apoptosis, suggesting a possible correlation with the inhibition of cell viability reported after treatment with daidzein (Adachi et al., 2005). As a whole, even though E2 effects are the final outcome of the integration of genomic and rapid signals, studies on flavonoid ability to act as an estrogen mimetic or an antiestrogen have been mainly focused on their ability to activate the transcription of ERE promoter containing genes (Routledge et al., 2000; Bramlett et al., 2001; Mueller, 2002; Mueller et al., 2004; Virgili et al., 2004; Totta et al., 2004; Totta et al., 2005).

As far we know, a little number of studies reported flavonoid effects due to a flavonoid-dependent modulation of ER rapid action mechanisms (Totta et al., 2004; Virgili et al., 2004; Watson et al., 2007a, 2007b). The capability of flavonoids to influence E2 rapid actions in both reproductive and non-reproductive E2-target tissues and how such effects may impact the normal development and physiological properties of cells largely have not been tackled until very recently (Somjen, 2005; Watson, 2005). In fact, scarce information is available on the extranuclear signal transduction pathways activated after the formation of flavonoids:ERα and flavonoids:ERβ complexes. Since ERs do not posses intrinsic effector domains with outcome functions such as chromatin (*i.e.*,, histone) - remodelling, acetyltransferase, or kinase activity, nor it is able to directly interact with and trigger the activation of the basal transcriptional machinery (Ascenzi et al., 2006), the molecular outcomes of ligands-bound ERs depend on the coupling between ligand recognition and the recruitment of partner macromolecules. Thus, it is possible that flavonoids could induce different conformational changes of ERs, also precluding the activation of rapid signaling cascades (Galluzzo et al., 2008). As support of this hypothesis our group have recently demonstrated that both quercetin and Nar hamper ERα-mediated rapid activation of signaling kinases (i.e., ERK/ MAPK and PI3K/AKT) and cyclin D1 transcription only when HeLa cells, devoid of any ER isoforms, were transiently transfected with a human ERα expression vector (Virgili et al., 2004). In particular, Nar, inducing conformational changes in ER, provokes ERα depalmitoylation faster than E2, which results in receptor rapid dissociation from caveolin-1, impairing

ERα binding to molecular adaptor and signaling proteins (e.g., modulator of non genomic actions of the ER, c-Src) involved in the activation of the mitogenic signaling cascades (i.e., ERK/MAPK and PI3K/AKT) (Galluzzo et al., 2008). Moreover, Nar induces the ERα-dependent, but palmitoylation-independent, activation of p38/MAPK, which in turn is responsible for naringenin-mediated antiproliferative effects in cancer cells. Naringenin, decoupling ERα action mechanisms, prevents the activation ERK/MAPK and PI3K/AKT signal transduction pathways thus, drives cells to apoptosis (Galluzzo et al., 2008)**.** On the other hand, Nar does not impair the ERα-mediated transcriptional activity of an ERE-containing promoter (Totta et al., 2004; Virgili et al., 2004). As a whole, this flavanone modulates specific ERα mechanisms and can be considered as 'mechanism-specific ligands of ER' (Totta et al., 2004).

2. AIM

As extensively reported before, besides E2, ERs bind a wide variety of compounds with remarkable structural and chemical diversity (Ascenzi et al., 2006) collected into the class of Endocrine Disruptor (EDs). Among EDs, several synthetic chemicals have been described to induce several degenerative disease (i.e. BPA) whereas natural compounds such as flavonoids show a protective effect against various degenerative phenomena. To date, it's not clear if these discrepant effects depend on ED interaction with ERs, or on other mechanisms whose occur independently from ED binding to ER. Furthermore, since the most of the studies on EDs, particularly flavonoids beneficial effects, were conducted in the absence of E2, the physiological relevance of these findings is not clear. In fact, flavonoid ability to maintain their protective effects against cancer growth even in the presence of E2 is completely unknown. This latter point is particular intriguing in that the final outcome of the exposure to a single or a mixture of these compounds, is strictly dependent on the interaction of the flavonoid-activated and hormone-activated signals (Bulzomi and Marino, 2010). Moreover, human beings intake daily about 500 g of different chemicals, which exhibit endocrine effects *in vivo* and *in vitro* (Marino and Galluzzo, 2007). As a consequence, mammalians are exposed to several EDs, rather than a single compounds resulting in systemic circulation of ED and flavonoid mixtures in the body. As a consequence, although in the last years, some evidence has become available to show the combined effects of EDs (Kortenkamp et al., 2008), the research on the effects of "dietary" and synthetic mixtures, at relevant human levels, remain inconclusive..

Aim of this project is to assess the effects of natural chemicals (i.e. flavonoids) alone or in mixture with the endogenous hormone (E2) or with other EDs (i.e. bisphenol A, BPA) on E2-dependent cell functions (i.e. cell proliferation/apoptosis balance, cellular differentiation and oxidative stress) evaluating their effects, action mechanisms, and the putative involvement of ERα and ERβ.

3. FLAVONOID EFFECTS ON E2-INDUCED REGULATION OF CELL PROLIFERATION

3.1 Introduction

Flavonoids, phenolic compounds widely present in fruits, vegetables, cereals, dry legumes, chocolate, and plant derived beverages, such as tea, coffee, and wine, represent the most abundant "minor components" in the diet (Manach et al., 2004). As reported before, these compounds have been described as health-promoting, disease- preventing dietary components. *In vivo* and *in vitro* experiments support a protective effect of flavonoids to reduce the incidence of certain hormone-responsive cancers (Gamet-Payrastre 1999; Birt et al., 2001; Keinan-Boker et al., 2004; Milner, 2006). Epidemiological data show a lower incidence of breast cancer in Asian countries and in vegetarians (Kris-Etherton et al., 2002). Furthermore, the migrants from Asia to Western countries who lose their traditional diet have a rates of cancer similar to the Western population (Vij and Kumar, 2004; Kamath et al., 1999). In addition, the ability of plant-based food to block the progression of tumors by directly inducing apoptosis has been reported (Béliveau and Gingras, 2007; Kuo, 1996; Choi et al., 2001; Ong et al., 2004; Totta et al., 2004; Virgili et al., 2004; Totta et al., 2005). Moreover, flavonoids are associated with low toxicity, making them good candidates as chemopreventive agents.

The cancer-protective effects of flavonoids have been attributed to a wide variety of mechanisms (Marino and Bulzomi, 2009). In general, any of the beneficial effects observed in epidemiological or intervention studies in association with fruit and vegetables intake have been interpreted on the basis that a "high intake of antioxidant-rich foods is inversely related to cancer risk" (Borek et al., 2004), as a consequence it has been hypothesized that flavonoids might protect against cancer through inhibition of oxidative damage (Hanasaki et al., 1994; Ursini et al., 1994). The theoretical basis of this protection is well known since the chemical structure of flavonoids is compatible with a one electron donor activity. They have been demonstrated to function as antioxidants *in vitro* both in cell cultures and cell free systems by scavenging superoxide anion, singlet oxygen, lipid peroxy radicals, and/or stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species (Hanasaki et al., 1994; Ursini et al., 1994; Birt et al., 2001). However, evidence for *in vivo* antioxidant effects of flavonoids is confusing and equivocal. The maximal flavonoid plasma concentrations achieved, even

after extensive flavonoid intake, are not more than 10^{-6}M (Manach et al., 2004). Many of the products of metabolism, such as methylated and glucuronidated forms, must have decreased antioxidant activity because of the blocking of radical-scavenging phenolic hydroxyl groups (Rechner et al., 2002). Therefore, whether plasma concentrations of flavonoids *in vivo* can be sufficient to exert systemic antioxidant actions is difficult to predict (Halliwell et al., 2005). Furthermore, *in vitro* experiments reported that most of dietary compounds with therapeutic properties act as potent pro-oxidants molecules (Chichirau et al., 2005; Meunier et al., 2005) at high concentrations or in presence of transition metals (Podmore et al., 1998). This pro-oxidant effect increases reactive oxygen species (ROS) production which, acting as second messengers of signaling networks, may induce growth arrest and apoptosis (Filomeni et al., 2007). Although the correlation between the decreased risk of developing several diseases and the antioxidant properties of flavonoids at concentration achievable in the plasma after a meal rich in flavonoid are still confusing and equivocal, the consumption of flavonoid-rich food has been usually considered beneficial for the human health and according to this hypothesis, a huge number of preparations are commercially available on the market in the form of plant extracts or mixtures, containing varying amounts of isolated phytochemicals as dietary supplements and as health food products. The commercial success of these supplements is evident, even though several activities and mechanisms, in part or totally independent from phytochemical participation to the intracellular redox balance, have been also described (Elahi and Matata, 2006; Vina et al., 2006; Galluzzo and Marino, 2006). In fact, a spectrum of cellular effects not directly related to the pro-oxidant/antioxidant capacity has been recently reported widening the perspective of research on the relationship between nutrition and health (Akiyama et al., 1987; Hagiwara et al., 1988; Spencer et al., 2003; Totta et al., 2004, Galluzzo et al., 2008; Kim et al., 2008). Among other the flavonoid ability to bind ERs modulating their activity is particular intriguing, rendering these compounds a particular class of EDs.

We recently reported that the flavanone naringenin and the flavonol quercetin exert anti-proliferative and pro-apoptotic effects altering selectively ERα signaling important for cyclin D1 transcription and cell proliferation (Totta et al., 2004; Virgili et al., 2004; Galluzzo et al., 2008). Contrarily to ERα-containing cancer cells, Nar mimics E2 effects in ERβ-expressing cancer cells, inducing, as well as E2, the activation of p38/MAPK, which leads to the activation of a pro-apoptotic cascade (*i.e.* caspase-3 activation and the poly(ADP-ribose) polymerase, PARP, cleavage) (Galluzzo et al., 2008).

At the present, the contribute of different flavonoid-induced mechanisms (i.e. antioxidant, kinase inhibitor, ER ligand) to their protective effects against cancer growth is completely unknown, as well as, their ability to maintain this effect even in the presence of E2. This latter point is particular intriguing in that the final outcome of the exposure to a single or a mixture of these compounds, is strictly dependent on the interaction of the ED-activated and hormone-activated signals (Bulzomi and Marino, 2010).

Aim of this part of the project is to evaluate the molecular mechanism(s) underlying naringenin and quercetin anticancer properties.

For these this purpose, quercetin and naringenin concentration achievable in the plasma after a meal rich in flavonoids plasma (from 10^{-7}M to 10^{-5}M) (Manach et al., 2004) has been used.

In order to establish the contribution of antioxidant activities, kinases inhibition, and/or ER-dependent mechanism in quercetin-induced anti-proliferative effects, HeLa cells were used as experimental model. This cell line, devoid of any ERs, can be rendered E2-responsive after the transient transfection with the human ERα or ERβ expression vector. Furthermore, naringenin effect in the presence of physiological concentration of E2 (10^{-8}M), in HeLa cancer cells devoid or transiently transfected with the human ERα expression vector will be evaluated.

These experimental models will allow to reveal not only the physiological significance of flavonoid nutritionally relevant concentrations but also the contribution and the putative interaction among the several proposed mechanisms of action in anticancer effects.

3.2 Results

3.2.1 Quercetin effect on HeLa cell growth

3.2.1.1 Quercetin decreases cell number of ERα-transfected HeLa cells

First of all we compared Q and E2 effects in ERα-transfected HeLa cells on well known cell functions modulated by ERα:E2 complex such as promotion of cell growth. Contrarily to E2, which, as expected, increased cell number only in ERα-containing HeLa cells (Fig. 3.1b) from 10^{-9}M to 10^{-7}M E2, Q stimulation decreased the number of ERα-transfected HeLa cells with respect to un-stimulated cells (Fig. 3.1a) in a dose-dependent manner within the range utilized (10^{-9}M-10^{-4}M). On the other hand, empty plasmid-transfected HeLa cells growth was not affected by any concentration of E2 nor Q suggesting that ERα presence is necessary for both E2 and flavonoid effects. Notably, Q stimulation of ERα-transfected

HeLa cell still decreased cell number even in the presence of 10^{-8}M E2 (Fig. 3.1c).

3.2.1.2 Quercetin as pro-oxidant/antioxidant

Quercetin is suggested to substantially empower the endogenous antioxidant shield due to its contribution to the total plasma antioxidant capacity which is 6.24 times higher than the reference antioxidant trolox (Arts et al., 2004), however, emerging studies showed it can also act as a prooxidant and induce H_2O_2 production under certain conditions (Briante et al., 2004; Robaszkiewicz et al., 2007; Jia et al., 2010). In order to determine whether quercetin induces ROS generation HeLa cells, transfected with empty or ERα vectors, were exposed to different concentration of quercetin (10^{-6}M to 5×10^{-5}M) or 6×10^{-4}M of H_2O_2 and changes in dichlorofluorescein (DCF) fluorescence were measured. Either vehicle (Fig. 3.2a) and Q (5×10^{-5}M) (Fig. 3.2c) did not cause any increase in ROS generation after 15 min of treatment in both ERα containing and ERα devoid (data not shown) HeLa cells, whereas H_2O_2 (6×10^{-4}Fig. 3.2b) caused a rapid and marked increase in ROS generation (284 ± 18 % over the control). Similar results were obtained at all tested Q concentrations (10^{-6} M - 5×10^{-5} M data not shown). On the other hand, quercetin prevented, in a dose-response manner, H_2O_2-induced ROS production in both ERα devoid (Fig. 3.2d) and ERα containing (Fig. 3.2e) HeLa cells. In fact, 10^{-6}M quercetin impaired ROS production of about 50%, while 5×10^{-5}M completely blocks H_2O_2- induced ROS production, confirming the potent antioxidant effect of this flavonoid.

3.2.1.3 Effect of quercetin as kinase inhibitor

The flavonoid-induced inhibition of EGF-R, PKC, PI3K and ERK have been described (Hagiwara et al., 1988; Spencer et al., 2003; Kim et al., 2008). Particularly, ERK and AKT play a pivotal role in cell proliferation, differentiation, and survival. It has been suggested that quercetin-induced cell death could be caused by down-regulating these kinases (Kim et al., 2008). To test this possibility, ERα containing and ERα devoid HeLa cells were exposed to 1 to 5×10^{-5}M quercetin for various times and activation of ERK and AKT was evaluated by detecting their phosphorylation forms. In ERα-containing or ERα- devoid HeLa cells quercetin failed to induce ERK and AKT phosphorylation (Fig. 3.3) at any of tested concentration (data not shown). Notably, quercetin did not decrease the basal, constitutive, phosphorylation status of both kinases or the expression level of total ERK and AKT (*i.e.,* phosphorylated and non phosphorylated) (Fig. 3.3).

Previously we reported that the flavanone naringenin, in the presence of ERα, drives the cells out of cell cycle by the pro-apoptotic p38 pathway activation (Totta et al., 2004). Thus, we evaluated the ability of quercetin in modulating this ERα-dependent kinase activation. Quercetin induced a rapid (15 min) and sustained (120 min) increase of p38/MAPK phosphorylation only in ERα-transfected HeLa cells (Fig. 3.4) suggesting that quercetin induces the ERα-dependent activation of a pro-apoptotic cascade.

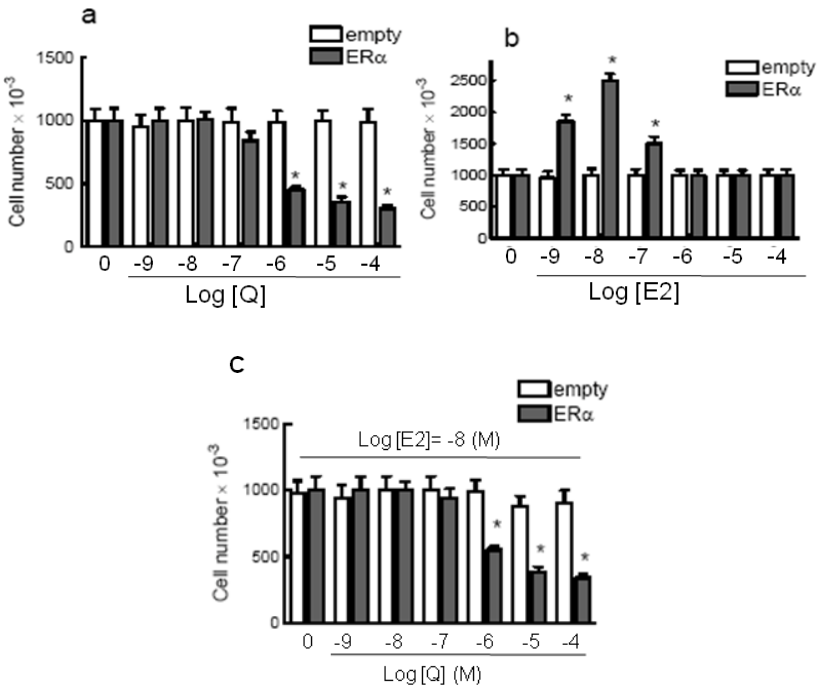


Figure 3.1: Quercetin effect on Hela cell growth. HeLa cells, transfected with empty (open bars) or human ERα (filled bars) expression vectors, were grown for 30 h in the presence of different concentration of quercetin (Q) (a) or 17β-estradiol (E2) (b), or different Q concentrations in the presence of E2(10^{-8}M) (c) . Data are the mean ± S.D. of 4 independent experiments carried out in duplicate. *P<0.001,calculated with Student's t test, was compared with non stimulated control values (0, vehicle).

3.2.1.4 Quercetin as pro-apoptotic agent

To verify that the decreased cell number reported in Fig. 3.7 is associated to the quercetin-induced apoptosis the flow cytometry analysis of ERα- containing or ERα-devoid HeLa cells was performed 30 h after flavonoid treatment. The typical plot of transfected-HeLa cell population is illustrated in Fig. 3.12 (Control). The first peak indicates the cell number present in G1 phase of the cell cycle (51.0% ± 7) followed by S phase (15.4% ± 3.5), and by the peak of G2/M phase (19.6% ± 2.9). This trend was unchanged after quercetin stimulation of empty vector-transfected HeLa cells (Fig. 3.4, lane empty). On the other hand, in the presence of ERα the cell number present in sub-G1 region increased 30 h after quercetin stimulation (Fig. 3.5, line ERα) strongly suggesting the presence of DNA fragmentation. This quercetin effect was completely blocked pre-treating cells with the ER inhibitor ICI 182,780 (Fig. 3.5, lane quercetin+ICI). In order to determine whether the quercetin-induced increase of cell population present in the sub-G1 phase was related to the induction of an apoptotic cascade, we analysed the activation of the caspase-3 proform and the cleavage of its substrate PARP, well known markers of apoptosis in several cell types. The 32-kDa proform of caspase-3 was expressed in HeLa cells transfected with either empty or ERα expression vectors (Fig. 3.6a). The 17-kDa band corresponding to the active subunit of caspase-3 was present only in ERα-containing HeLa cells stimulated for 1 to 24 h with quercetin (Fig. 3.6a, b). To confirm that the quercetin-induced appearance of the 17-kDa band was associated with an increase in caspase-3 activity, we analysed the cleavage of the caspase-3 substrate, the DNA repair enzyme PARP. By Western blot analysis, the treatment of ERα-transfected HeLa cells with quercetin resulted in the conversion of 116-kDa PARP into its inactive 85-kDa fragment (Fig. 3.6c, d). This result is consistent with the idea that quercetin specifically activated an apoptotic cascade involving the caspase-3 activation and its downstream substrate PARP only in the presence of ERα. The p38/MAPK pathway involvement in quercetin-induced apoptotic cascade was confirmed by the pre-treatment of transfected cells with the specific p38 inhibitor, SB 203580. This inhibitor completely prevented the quercetin-induced p38 phosphorylation (Fig. 3.7a), caspase-3 activation (Fig. 3.7b) and PARP cleavage (Fig. 3.7c) thus, linking the rapid quercetin-induced p38/MAPK phosphorylation to the activation of apoptotic cascade.

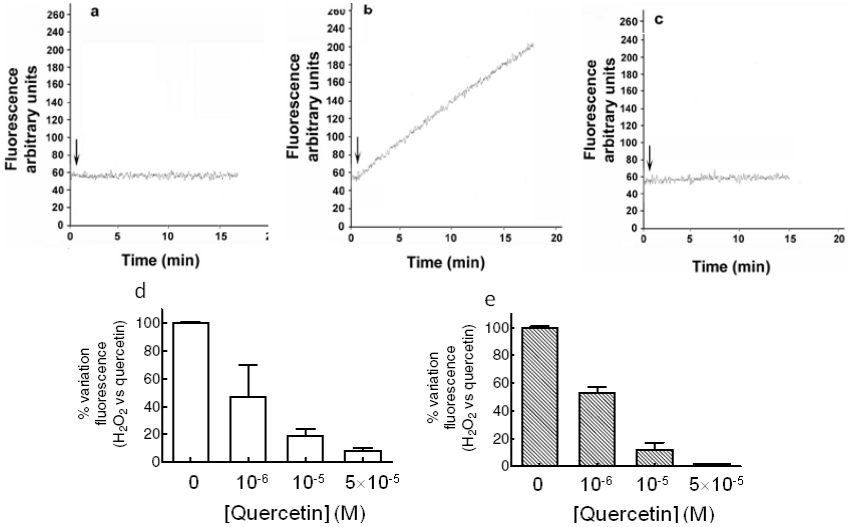


Figure 3.2: Quercetin effect on ROS generation and on H_2O_2-induced ROS production. HeLa cells, transfected with ERα expression vector, were exposed to vehicle (a), or H_2O_2 (6×10^{-4}M) (b), or quercetin (5×10^{-5}M) (c), and changes in DCF fluorescence were measured. Upper panels represent original outputs (arbitrary units) of the registrations captured by the spectrofluorimeter during 15 minutes substance administration. In panels e and f, HeLa cells, transfected with empty (e) or human ERα (f) expression vectors, were pre-treated with different quercetin concentrations before esposition to vehicle (0) or H_2O_2 (6×10^{-4}M). Data, expressed as % of variation between H_2O_2-stimulated fluorescence versus quercetin stimulated fluorescence, are the mean ± S.D. of 3 independent experiments carried out in duplicate.

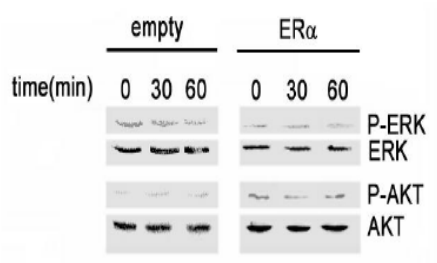


Figure 3.3: Quercetin effect on ERK and AKT activity.
Western blot analysis of phosphorylated and un-phosphorylated ERK, and AKT, were performed, as described in Methods, on HeLa cells transfected with empty or human ERα expression vectors, stimulated with vehicle (0) or30 min or 60 min with quercetin (10^{-6}M). Typical blot chosen among 3 independent experiments.

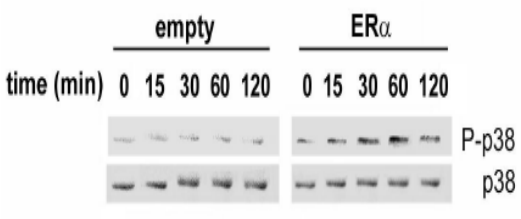


Figure 3.4: Quercetin effect on p38 activity.
Western blot analysis of phosphorylated and unphosphorylated p38 were performed, on HeLa cells transfected with empty or human ERα expression vectors, stimulated with vehicle (0) or quercetin (10^{-6}M) at different times. The data is a typical blot of 3 independent experiments.

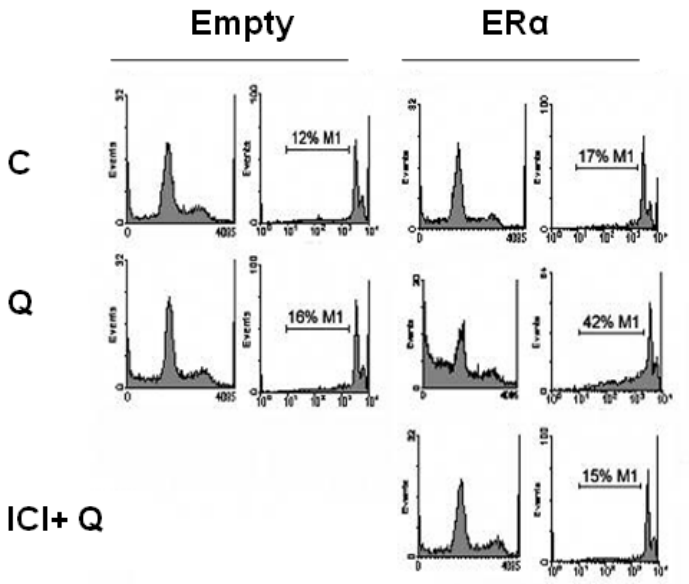


Figure 3.5: Quercetin effect on HeLa transfected cell cycle. Cell cycle distribution (left panels) and distribution of cells present in sub-G1 phase (right panels) was performed by cytofluorimetric analysis of HeLa cells transfected with empty or human ERα expression vectors after 30 h of cell treatment with quercetin (10^{-6}M) in the presence or absence of the ER inhibitor ICI 182,780 (ICI, 10^{-6}M) and compared with cells treated with vehicle (Control).

3.2.1.5 Quercetin decreases ERβ-transfected HeLa cell number

Our next step was to evaluate quercetin effect in ERβ-transfected Hela cells. As well as E2 (10^{-8}M) (Fig.3.8b), Q stimulation decreased the number of ERβ-transfected HeLa cells with respect to un-stimulated cells (Fig. 3.8a) in a dose-dependent manner within the range utilized (10^{-8}M-10^{-4}M).

These data, accordingly to reported quercetin affinity binding constant to ERβ (Kuiper et al., 1997), strongly suggested that quercetin induced reduction of ERβ-transfected HeLa cells was dependent on ERβ-presence.

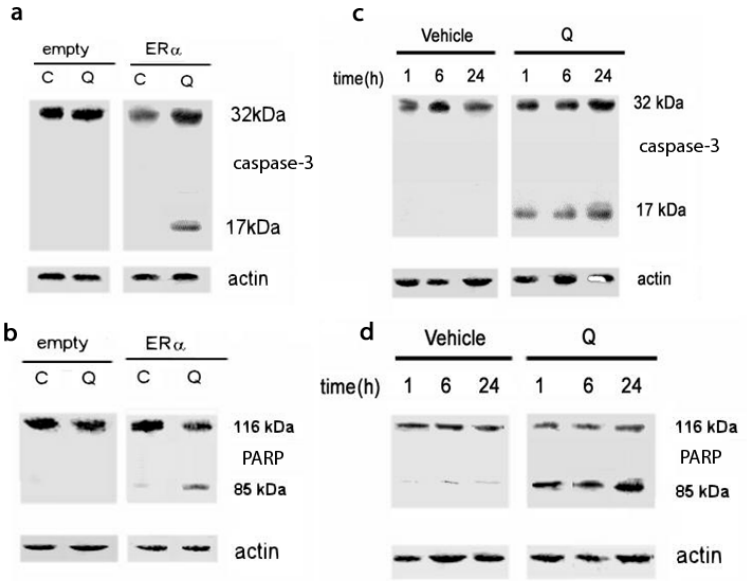


Figure 3.6: Quercetin effect on the activation of a propaoptotic cascade in HeLa transfected cells. Western blot analysis of caspase-3 (a, b) and PARP (c, d) cleavage was performed on HeLa cells transfected with empty or human ERα expression vectors treated for 24 h with vehicle (C) or quercetin (Q, 10^{-6}M) for different times (Panels c and d). Typical blot of 3 independent experiments..

3.2.1.6 Quercetin effect on kinase activation in ERβ-transfected HeLa cells

Since quercetin exerts the same E2 effect on ERβ-transfected HeLa cell growth we decided to evaluate also quercetin effect in comparison with E2, on kinases activation. As well as E2 (10^{-8}M), quercetin (10^{-6}M) rapidly (15min) induces p38 phosphorylation (Fig. 3.9a) but, like E2, is not able to activate ERK and AKT (Fig. 3.9b, c).

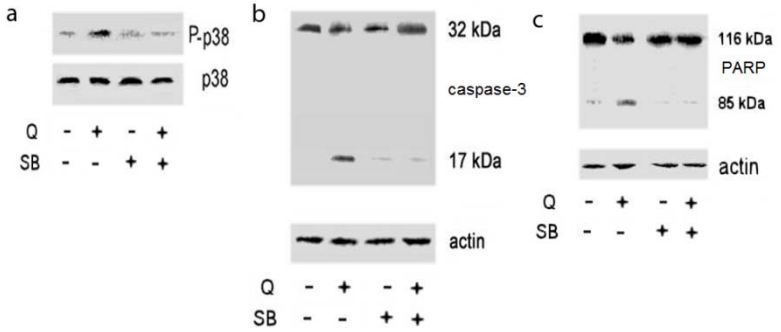


Figure 3.7: Quercetin effect on p38 phosporylation and p38 involvement in apoptosis cascade activation. Effects of quercetin (10^{-6}M) on p38 (a), caspase-3 (b), and PARP (c) activation in ERα-transfected HeLa cells. When indicated 5×10^{-6}M of p38 inhibitor, SB 203580 (SB), was added 15 min before quercetin administration. Typical blot of 3 independent experiments.

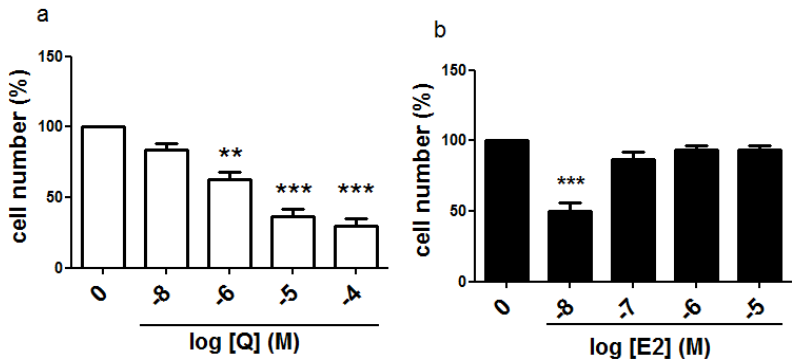


Figure 3.8: Quercetin and 17β estradiol effect on Hela cell growth. ERβ-transfected HeLa cells were grown for 30 h in the presence of different concentrations of quercetin (a) or 17β-estradiol (E2) (b). Data are the mean ± S.D. of 4 independent experiments carried out in duplicate. *P<0.001, calculated with Student's t test, was compared with non stimulated control values (0, vehicle).

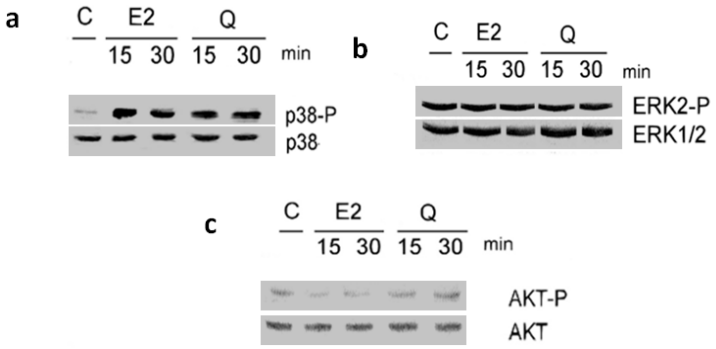


Figure 3.9: Effect of 17β-estradiol (E2) and quercetin on kinases activation. Western blot analyses of p38 (panel a), ERK (panel b), and AKT (panel c) activation after 15 and 30 min of 17β-estradiol (E2,10^{-8} M) and quercetin (Q, 10^{-6} M) stimulation in ERβ-transfected HeLa cells. Typical blot of 3 independent experiments.

3.2.1.7 Quercetin:ERβ complex induced pro-apoptotic cascade activation via p38 pathway

It is well known that E2:ERβ dependent p38 activation has as final outcome the activation of a proapopototic cascade (caspase-3 and its substrate, PARP, cleavage) in cancer cells (Galluzzo et al., 2007). Since the data obtained in ERβ-transfected HeLa cells strongly suggest that quercetin act as an estrogen mimetic in the presence of ERβ we decided to evaluate if the quercetin induced reduction of ERβ-containing HeLa cell number was due to the ERβ-dependent activation of a propapoptotic cascade. In order to verify this hypothesis, a flow cytometry analysis of ERβ-containing HeLa cells was performed 30 h after flavonoid treatment. The typical plot of transfected-HeLa cell population, illustrated in Fig. 3.10 (Control), was unchanged after quercetin stimulation of empty vector-transfected HeLa cells (Fig. 3.10, lane empty). On the other hand, in the presence of ERβ the cell number present in sub-G1 region increased 30 h after quercetin stimulation (Fig. 3.10, line ERβ) strongly suggesting the presence of DNA fragmentation.

To determine whether the quercetin-induced increase of cell population present in the sub-G1 phase was related to the induction of an apoptotic cascade, the activation of the caspase-3 proform and the cleavage of its substrate PARP were analysed. The 17-kDa band corresponding to the active subunit of caspase-3 was present only in ERβ-containing HeLa cells stimulated for 24 h with both E2 and quercetin (Fig. 3.11a). To confirm that the quercetin-induced appearance of the 17-kDa band was associated with an increase in caspase-3 activity, we analysed the cleavage of the caspase-3 substrate, the DNA repair enzyme PARP. As expected, the treatment of ERβ-transfected HeLa cells with quercetin resulted in the conversion of 116-kDa PARP into its inactive 85-kDa fragment (Fig. 3.11b). Furthermore, ERβ-transfected cell pre-treatment with the specific p38 inhibitor, SB 203580, completely prevented both E2- and quercetin-induced caspase-3 activation (Fig. 3.11a) and PARP cleavage (Fig. 3.11b), thus confirming p38/MAPK pathway involvement in quercetin-induced apoptotic cascade also in ERβ-transfected cells.

3.2.1.8 Quercetin as modulator of ERα and ERβ transcriptional activity

Our final target was to assess the effects of quercetin, in comparison with E2, on a well known E2:ER complex modulated cell function: estrogen responsive element- (ERE)-containing gene transcription. Figure 3.12 shows E2 (10^{-8}M) and quercetin (10^{-6}M) on pC3 promoter activity. Although at higher concentration, quercetin induced ERE-containing (pC3) promoter to a level comparable to that of E2 in HeLa cells transfected with ERα or ERβ expression vector. No transcriptional activity was present when HeLa cells, transiently transfected with the empty plasmid, were stimulated with E2 or quercetin. However, ERα, unlike ERβ, is able to associate with other transcription factors (e.g., AP-1; Sp1) leading to the transcription of promoter containing AP-1 and Sp1 elements (Ascenzi et al., 2006, Deroo and Korach, 2006). This indirect transcriptional activity requires ERα-mediated non-genomic mechanisms (Marino et al., 2001b).

Unlike E2, quercetin stimulation of ERα-transfected Hela cells prevents the indirect ERα-mediated transcriptional activity of cyclin D1 promoter, important for the progression of cells through the G1 phase of the cell cycle, which occurs through receptor association to other transcription factors (e.g., AP-1) (Fig. 3.12). On the other hand, according to the inability of E2:ERβ complex to form a transcriptionally active complex at a promoter containing Sp1 and AP-1 elements (Paech et al, 1997; Saville et al, 2000; Acconcia et al, 2005a) quercetin binding to ERβ doesn't lead to cyclin D1 expression (Fig. 3.12).

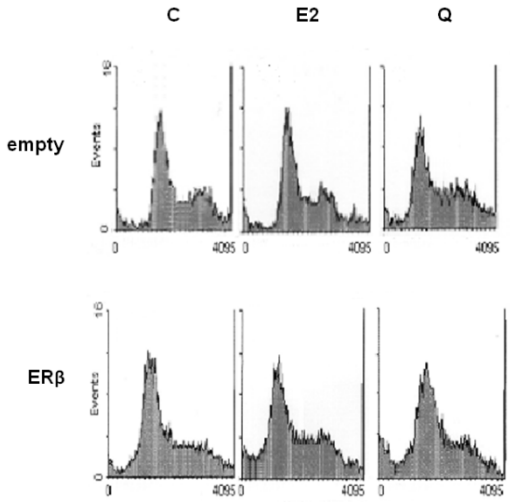


Figure 3.10: Cell cycle distribution of transfected HeLa cells. Cell cycle distribution of empty or ERβ-transfected Hela cells has been performed by cytofluorimetric analysis after 30 h of cell treatment with 17β estradiol (E2, 10^{-8}M) or quercetin (10^{-6}M) and compared with cells treated with vehicle (Control).

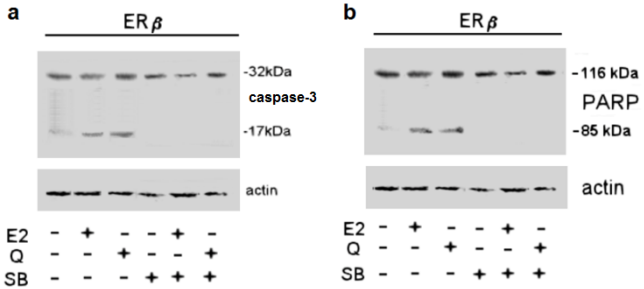


Figure 3.11: Effect of quercetin on propaptotic cascade activation in ERβ –transfected HeLa cells. Western blot analysis of caspase-3 (panel a), and PARP (panel b) activation after 24h of 17β-estradiol (E2, 10^{-8}M) and Quercetin (10^{-6}M) stimulation in ERβ-transfected HeLa cells. When indicated 5 µmol/l of p38 inhibitor, SB 203580 (SB), was added 15 min before E2 or Quercetin administration. Typical blot of 3 independent experiments.

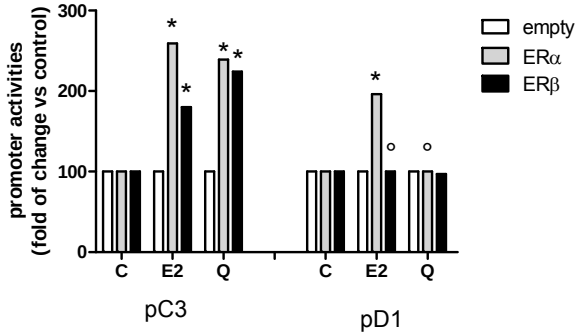


Figure 3.12: Effect of E2 and quercetin on pC3 promoter and cyclin D1 promoter activity. HeLa cells were co-transfected with ERβ expression vector and pC3-luciferase (pC3) or cyclin D1 (pD1) reported plasmids. After 24 h, cells were treated for 24 h with vehicle, 10^{-8} M E2 or 10^{-6} M Q and the expression of the pC3-luciferase reporter gene was evaluated. Data are the mean of six different experiments ±SD. P > 0.001 was calculated with Student *t*-test with respect to samples treated with vehicle (control) (*) or E2 (°).

3.2.2.Effect of Naringenin and E2 coadministration.

3.2.2.1 E2 and Nar Binding to ERa

First of all, the Nar ability to compete with E2 for binding to human recombinant ERα have been assessed. E2 saturation experiments have been performed in the absence and presence of 10^{-6} and 10^{-5} M Nar. E2 binding to ERα is characterized by an intrinsic equilibrium dissociation constant (K_d^{E2}) of (2.0±0.5) 3×10^{-10} M. In the presence of unlabeled Nar, the apparent equilibrium constant for E2 binding to ERα increased to K'_d= (5.2± 0.6) × 10^{-9} M and (3.1±0.4) × 10^{-8} M in the presence of 1.0×10^{-6} and 1.0×10^{-5} M of Nar, respectively (Fig. 3.13). This confirms that Nar binds to ERα with an affinity lower by about three orders of magnitude (K_d^{nar} = 1.4 ± 0.3×10^{-7} M). than that of E2. Furthermore, in the presence of nutritionally relevant Nar concentrations, the molar fraction of E2 bound to ERα decreases.

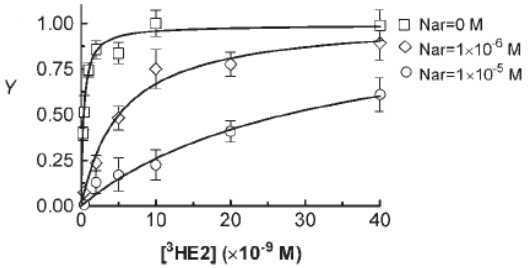


Figure 3.13: Nar effect on 17β-estradiol (E2) binding to human recombinant ERα. (a) Dependence of the intrinsic molar fraction (*Y*) of [^{3}H]E2-bound to ERα from [^{3}H]E2 concentration in the absence (squares) and in the presence of 2 representative Nar concentrations (1.0×10^{-6} M [rhomb] and 1.0×10^{-5} M [circles]). Data are the means 6 SD of five different experiments.

3.2.2.2 ERα Transcriptional Activities

The result of Nar binding to ERα prompted us to evaluate the effect of co-stimulation of E2 and Nar on the ERα activities. We first assessed the ERα-mediated direct gene transcription (i.e. estrogen responsive element (ERE)-dependent). HeLa cells, transiently transfected with ERα or empty vector, and the ERE-containing reporter plasmid (pC3) were incubated with either E2 alone (10^{-8}M) or Nar alone (10^{-6} M) or in the presence of E2 (10^{-8}M) and different Nar concentrations. Nar, alone or with E2, induced the ERE-containing promoter activity to a level comparable with that of E2 alone (Fig. 3.14a). No pC3 promoter activity was present when HeLa cells, transiently transfected with the empty plasmid, were stimulated with different ERα ligands (Fig. 3.14a), thus demonstrating the ERα dependence of this effect. The indirect transcriptional activity of ERα [i.e. through interaction with activator protein-1 (AP-1) or stimulating protein 1 (Sp1) transcription factors] was assessed by transfection with cyclin D1 (pD1) promoter (cfr par.1.2.2).

In fact, cyclin D1 is a well-known E2-responsive gene, even if ERE-like sequence in its promoter has not been detected (17). As expected, cell treatment with E2 resulted in a significant increase in cyclin D1 promoter activity (Fig. 3.14b) comparable with those previously reported (Marino et al., 2001b).

Notably, 10^{-7}M Nar reduced the E2 effect, and higher Nar concentrations (i.e. 10^{-6} M to 10^{-4}M) completely prevented E2-induced pD1 promoter activity (Fig. 3.14b). To determine the ER involvement in the

ligand-induced cyclin D1 promoter activity, experiments were performed also in HeLa cells transfected with the empty plasmids (Fig. 3.14b). Results indicate that no pD1 promoter activity was present when these cells were stimulated with E2 or Nar (Fig. 3.14b).

3.2.2.3 ERα-Dependent Rapid Signals

The E2-induced cyclin D1 promoter activity requires rapid signal transduction pathways. In particular, the rapid (15 min) E2-induced activation of ERK1/2 and PI3K/AKT cascades are fundamental for E2-induced pD1 promoter activity (Castoria et al., 2001; Ascenzi et al., 2006). On the other hand, Nar stimulation induces the rapid and persistent (15 min to 24 h) activation of p38, another component of MAPK family (Totta et al., 2004; Galluzzo et al., 2008). Thus, the ability of E2 to still induce rapid signal kinase cascades even in the presence of 10^{-6} M Nar was evaluated in HeLa cells transfected with the empty vector or with ERα expression vector. No kinase activation was detected in HeLa cells devoid of ERαstimulated with E2 or Nar (data not shown), whereas E2 ability to induce the rapid (15 min) ERK1/2 and AKT activation without any effect on the persistent (24 h) p38 activation has been confirmed in ERα-containing HeLa cells (Fig. 3.15). Remarkably, Nar stimulation prevents E2-induced ERK1/2 and AKT activation and still induces the persistent p38 phosphorylation even in the presence of E2 (Fig. 3.15).

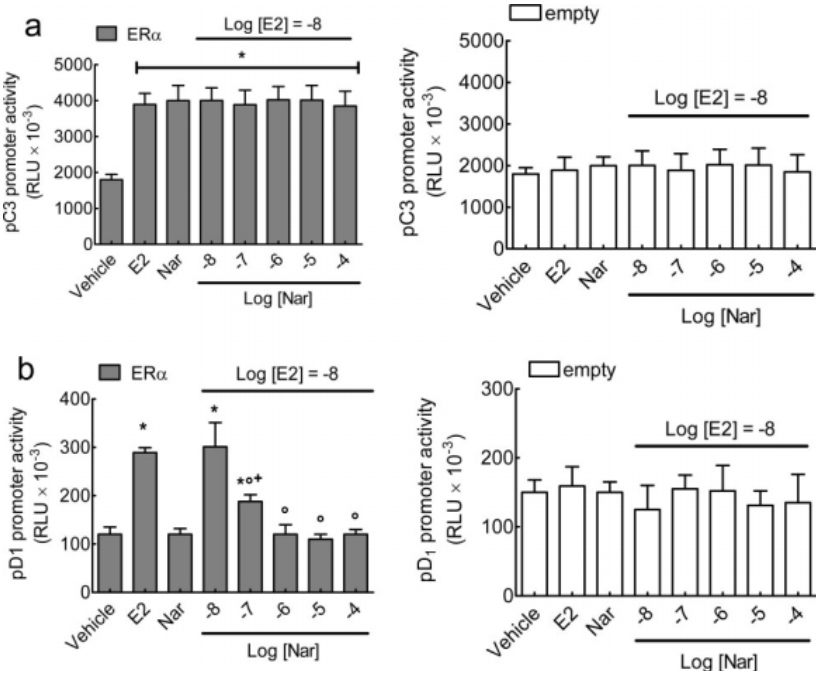


Figure 3.14: Nar effect on E2-induced promoter activities. (a) Luciferase assay detection on HeLa cells cotranfected with ERα (left panel) or empty vector (right panel) and pC3-luciferase construct and then treated 24 h with either vehicle or E2 (10^{-8} M) or Nar (10^{-6} M) or with different Nar concentrations (10^{-8} M to 10^{-4}M) in the presence of 10^{-8}M E2. Data are the means ± S.D. of four independent experiments. *$P < 0.001$, compared with vehicle values, was determined using Student's t test. (b) Luciferase assay detection on HeLa cells co-tranfected with ERα (left panel) or empty vector (right panel) and cyclin D1 (pD1)-luciferase construct and then treated 24 h with either vehicle or E2 (10^{-8} M) or Nar 10^{-6} M) or with different Nar concentrations (10^{-8} M to 10^{-4} M) in the presence of 10^{-8} M E2. Data are the means ± S.D. of four independent experiments. $P < 0.001$, determined by using Student's t test, was compared with vehicle (*) or E2 (°) or Nar (+) values.

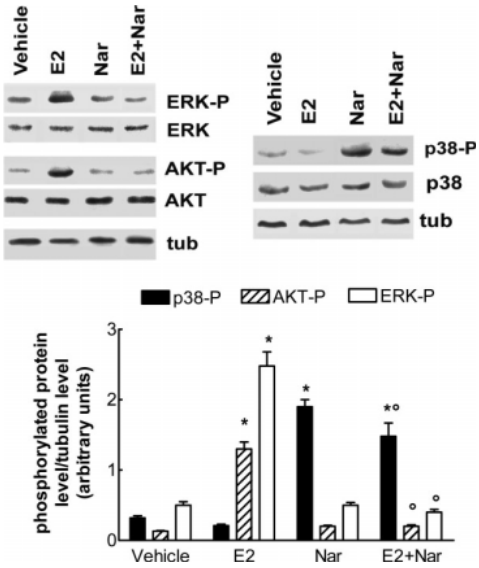


Figure 3.15: Nar effects on E2-induced rapid ERα activities. ERα-transfected HeLa cells were treated with either vehicle or E2 (10^{-8} M) or Nar (10^{-6} M) or with a mixture of Nar (10^{-6} M) + E2 (10^{-8} M). After 15 min (left panel, ERK 2 and AKT) or after 24 h (right panel, p38), the phosphorylation of the kinases was evaluated. The amounts of protein were normalized by comparison with un-phosphorylated ERK1/2 or AKT or p38 and tubulin antibodies. Upper panels show representative Western blots, lower panel shows the densitometric analysis. Data are the means ± S.D. of four independent experiments. $P < 0.001$, determined by using Student's t test, was compared with vehicle (*) or E2 (°) values.

3.2.2.4 ERα-Dependent E2-Induced Cell Proliferation

Cyclin D1 represents the upstream sensor of E2-induced proliferative signals, which, in turn, depends on the rapid activation of upstream E2-induced kinase (Acconcia et al., 2005a). However, in the presence of ERα, Nar prevents cell proliferation inducing a proapoptotic cascade (Totta et al., 2004; Galluzzo et al., 2008). Figure 3.16a confirms that 10^{-6}, 10^{-5}, and 10^{-4} M Nar reduced cell number only in ERα-containing HeLa cells, whereas physiological E2 concentrations (*i.e.,* 10^{-9} and 10^{-8} M) doubled the cell numbers in 24 h. Note that high Nar or E2 concentration (10^{-4}M) reduced cell numbers also in empty vector- transfected HeLa cells, suggesting an ERα-independent cytotoxic effects for both substances (Fig. 3.16a). Intriguingly, Nar stimulation reverted the E2-induced effect on cell proliferation significantly reducing the number of cells in a dose-dependent manner (Fig. 3.16b). Furthermore, 10^{-6}M Nar changed the E2-induced distribution of cell population in the cell cycle phases (Fig. 3.16c), decreasing the number of cells presents in G1 phase and increasing the number of cell present in sub-G1 phase of the cell cycle as follows 15.0 ± 1.3 % (Vehicle), 20.2 ± 0.5% (E2), 42.0 ± 0.7 % (Nar), and 43.4 ± 1.0 % (E2+Nar) (Fig. 3.16b). In line with these results, Nar increased the level of the active caspase-3 (i.e. 17 kDa band, Fig. 3.17a) as demonstrated by the increased level of poly(ADP-ribose)polymerase (PARP) cleavage, a caspase-3 substrate, even in the presence of 10^{-8} M E2 (Fig. 3.17b), thus demonstrating the strong antagonistic effects of this flavanone on E2-induced proliferation.

To avoid any problem due to the receptor overexpression in HeLa cells, the Nar effect on p38 phosphorylation and on the activation of a proapoptotic cascade was performed in parallel in cancer cells that express endogenous ERα (HepG2). These cells, derived from liver, could be one of main targets of flavonoid action after oral administration. Moreover, HepG2 cells represent an E2-dependent proliferative model (Marino et al., 2001b). The level of endogenous ERα was assessed in HepG2 by Western blot analysis, which confirmed the presence of a unique band at 67 kDa corresponding to ERα (data not shown). In HepG2 cells, Nar stimulation, both alone or in the presence of E2, increased p38 phosphorylation, caspase-3 activation, and PARP cleavage (Fig. 3.18), confirming that, also in the presence of endogenous receptor, Nar reverts the E2-dependent proliferative effects as obtained in Hela cells.

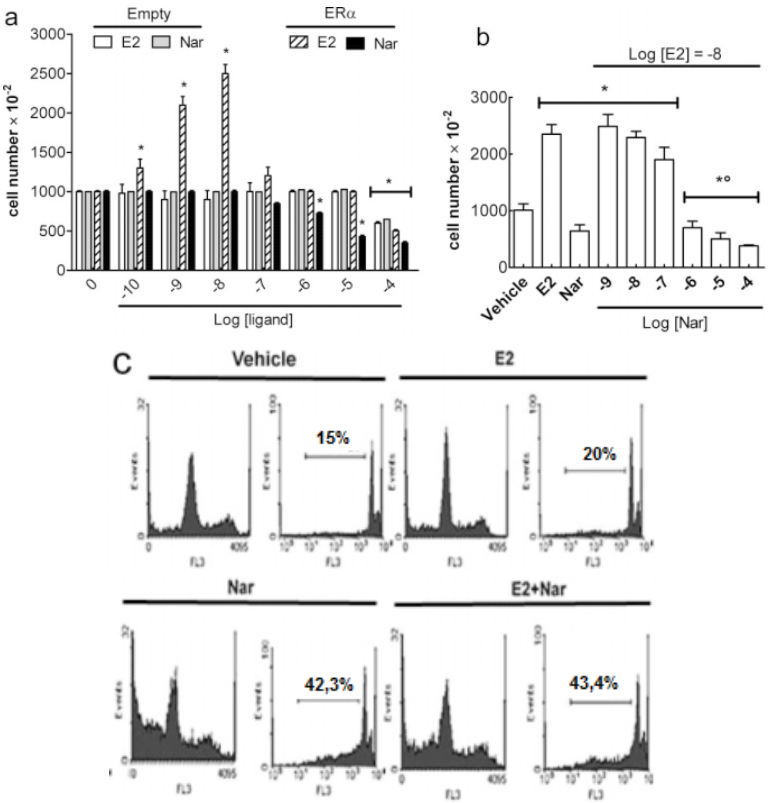


Figure 3.16: Nar effects on E2-induced HeLa cells proliferation. (a) Number of HeLa cells tranfected with ERα or empty vector and treated 24 h with either vehicle (0) or different concentrations of E2 or Nar (10^{-10} M to 10^{-4} M). (b) ERα-tranfected HeLa cells were stimulated for 24 h with either vehicle or E2 (10^{-8} M) or Nar (10^{-6} M) or with different Nar concentrations (10^{-9} M to 10^{-4} M) in the presence of 10^{-8} M E2. Data are the means ± S.D. of five independent experiments. $P < 0.001$, determined by using Student's t test, was compared with vehicle (0, *) or E2 (°) values. (c) Flow cytometric analysis of ERα tranfected HeLa cells after 24 h of treatment with vehicle or E2 (10^{-8} M) or Nar (10^{-6} M) or E2+Nar (10^{-8} M and 10^{-6} M, respectively). The plots indicate a typical cell cycle distribution present in sub-G1, G1, S, and G2/M phases, respectively (left panels) and the percentage of cell present in sub-G1 phases (right panels).

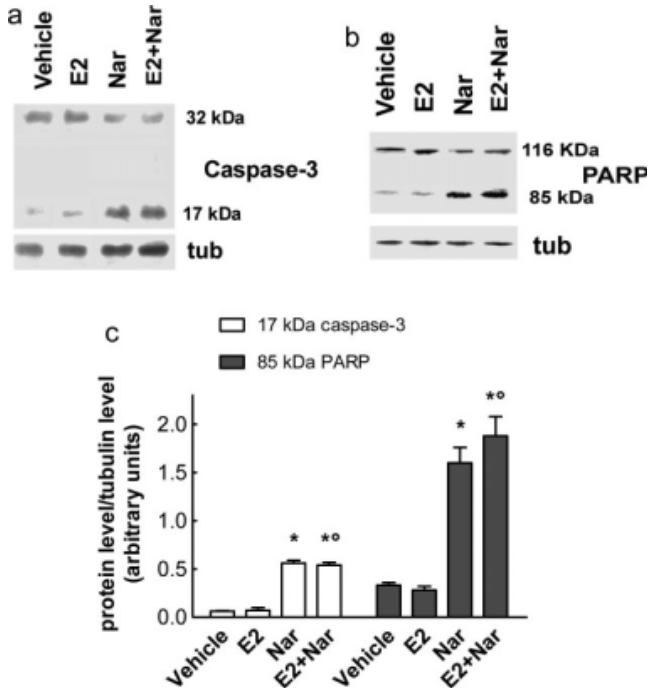


Figure 3.17: Effect of Nar on the induction of proapoptotic proteins in HeLa cells. Analysis of caspase-3 activation (a) and PARP cleavage (b) were performed in HeLa cells transfected with human ERα and treated 24 h with vehicle or 17β-estradiol (E2, 10^{-8} M) or Nar (10^{-6} M) or E2 + Nar (10^{-8} M and 10^{-6} M, respectively). Typical Western blots of three independent experiments are presented in panels a and b. In panel c, the relative densitometric analysis is reported. The data are the mean values ± S.D. $P < 0.001$, determined by using Student's t test, was compared with vehicle (*) or E2 (°) values.

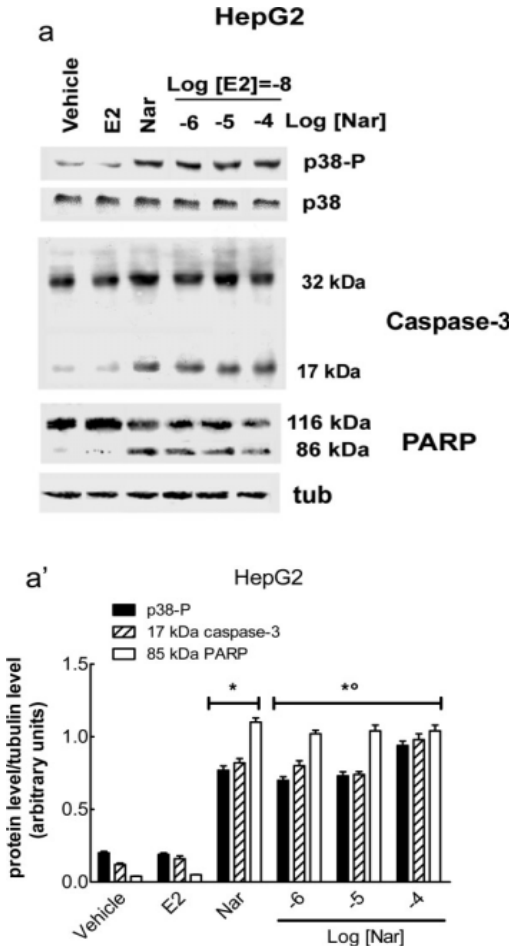


Figure 3.18: Effect of Nar on the induction of proapoptotic proteins in HepG2 cells. Analysis of p38 phosphorylation, caspase-3 activation, and PARP cleavage was performed in HepG2 cells treated 24 h with vehicle or 17β-estradiol (E2,10^{-8} M) or Nar (10^{-6} M) or with different Nar concentrations (10^{-6} M to 10^{-4} M) in the presence of 10^{-8} M E2. Typical Western blots of three independent experiments are presented in panel a. In panel a are presented the relative densitometric analyses. The data are the mean values ± SD. P<0.001, determined by using Student's t test, was compared with vehicle (*) or E2 (°) value.

3.3 Discussion

The present study was undertaken to examine the molecular mechanism(s) underlying the well known growth inhibition and cell death effects of the flavonol quercetin, one of the most frequently studied and ubiquitous bioactive flavonoid, which action mechanisms remains poorly understood. Our results indicated that quercetin did not increase ROS generation in HeLa cells and confirm that quercetin is an excellent antioxidant *in vitro*. In fact, 5×10^{-5}M quercetin prevented the H_2O_2-induced ROS production by 95% even in the absence of ER. However, smallest amount of quercetin (i.e., 10^{-6}M), activates a pro-apoptotic cascade only in ER-transfected HeLa cells. Normally, human quercetin plasma concentrations are in the low nanomolar range (from 5×10^{-8}M to 8×10^{-8}M) (Manach et al., 2005), but upon quercetin supplementation they may increase to the high nanomolar ($1\text{-}2\times10^{-7}$M) or low micromolar range ($0.5\text{-}1.5\times10^{-6}$M) (Boots et al., 2008), whereas the concentration of circulating endogenous antioxidant, ascorbate or urate, has been estimated to be in the range of 1.6×10^{-4}M - 3.8×10^{-4}M for a normal individual (Stevenson et al., 2007). Furthermore, it must be considered that this flavonoid is more efficiently absorbed as glucosides than that in the aglycon form (Manach et al., 2005), thus, quercetin, bioavailability differs among food sources, depending on the type of glycosides they contain (Manach et al., 2005). The data here reported clearly indicate that the presence of ERs is necessary for the antiproliferative, pro-apoptotic effects elicited by flavonoids at nutritional relevant concentrations. Nutritional antioxidant molecules possess distinct action mechanisms, possibly interacting one another, on cell signaling and response. As a result, the antioxidant properties could be considered a simplified approach to the function of molecules of nutritional interest due to the fact that their antioxidant capacities are a chemical property which is not necessarily associated to an equivalent biological function (Galluzzo et al., 2009a) .

Accordingly, the impact of flavonoids and in general of polyphenols as antioxidants has been recently reconsidered and questioned, opening to the evidence that the molecular basis of their activity is much larger than originally considered (Virgili and Marino, 2008).

As a whole, flavonoids do not appear to be present in the circulation at high enough concentrations to contribute significantly to total antioxidant capacity. The increase in plasma total antioxidant capacity from apple consumption (rich in quercetin) has been explained by a ~37% increase in urate concentration, as a consequence of fructose metabolism, with no detectable effect associated with the apple flavonoids (Lotito et al., 2004).

These results, consistent with findings in human promyeloleukemic HL-60 cells (Shen et al., 2003) and glioma cells (Kim et al., 2008), render unlikely that quercetin-induced HeLa cell death is linked to the activation of ROS-dependent signal generation.

Data here reported also demonstrated that quercetin, as well as Naringenin, inhibits cancer cell proliferation by ERα-dependent and ERβ-dependent p38 pathway activation. This occurs at quercetin concentration physiologically achievable in the plasma after the consumption of meals rich in this flavonol. Particularly, quercetin, like Nar, decouples the ERα action mechanisms impairing the ERα ability to activate ERK and PI3K/AKT signal transduction pathways and allowing the sustained ERα-dependent p38 phosphorylation and the downstream caspase-3 activation and PARP cleavage. The activation of the same pathway (p38 and caspase 3 activation) is still present in ERβ-transfected HeLa cells. On the other hand quercetin, as well as Nar, preserve ERα and ERβ transcriptional ability of an ERE containing promoter gene, but impairs ERα ability transcriptional activitiy (i.e. on Cyclin D1)associated with other transcription factors (e.g., AP-1, Sp1) These data completely agree with quercetin inability to activate ERK and AKT kinases, since indirect transcriptional activity requires ERα-mediated non-genomic mechanisms (Marino et al., 2001b).

Finally, since flavonoids bind to ERβ with up to five times higher affinities compared with ERα (Kuiper et al., 1997; Kuiper et al., 1998) they may be able to trigger beneficial responses through their preferential interaction with this ER isoform. As a consequence, the preventive effects elicited by Nar and quercetin on E2-dependent cancers may be enhanced, in ERβ expressing some tissues, through the induction of specific ERβ-dependent proapoptotic signalling (Totta et al., 2004).

These experimental approaches allow us to underlie flavonoids disrupting effect and action mechanism, however, if endogenous hormone are not included in the studies the physiological relevance of these findings is limited. Since the final outcome of the exposure to a single or a mixture of EDs, is strictly dependent on the interaction of the ED-activated and hormone-activated signals, we take in account the contribution of both E2 and Nar.

Several mechanisms, that may even occur independently of ER binding (Sarkar and Li, 2002; Magee and Rowland 2004), have been described for Nar effects, but, as previously said, require high plasma Nar concentrations (*i.e.* 0.8 to 25× 10^{-5} M), which are difficult to obtain by the oral ingestion of food rich in this bioflavonoid. In the best case scenario, only 15% of ingested Nar will get absorbed in the human gastrointestinal tract, and the

peak of plasma naringenin ranges from 0.7 to 14.8 $\times$ 10^{-6} M (Erlund et al., 2001) .

Our previous studies indicate that 10^{-6}M Nar impaired E2-induced proliferative signals interfering with ERα-mediated activation of ERK1/2 and PI3K pathways without affecting ER-direct transcriptional activity (Totta et al., 2004; Galluzzo et al., 2008). On the other hand, Nar induced the ERα-dependent, but palmitoylation- independent, activation of p38 kinase, which, in turn, was responsible for Nar-mediated anti-proliferative effects in cancer cells (Totta et al., 2004; Galluzzo et al., 2008). These results imply that, besides its effects in the presence of ERβ (Totta et al., 2004), Nar works as a selective inhibitor of ERα-mediated proliferation (Totta et al., 2004; Galluzzo et al., 2008).

The data here reported on Nar ability to revert E2-induced cancer cell proliferation, strongly support our hypothesis that flavonoid such as naringenin and quercetin, at concentration achievable in the plasma, act as endocrine disruptors, able to exert antiproliferative effect mainly binding to, and in turn, modulating, ER activities also in presence of the endogenous hormone. The obtained data confirm that the Nar concentration required to half-saturate ERα is about 1000-fold higher than that reported for E2; however, in the presence of Nar, E2 affinity for ERα linearly decreased. This decreased affinity between E2:ERα did not impair the E2 ability, in the presence of Nar, to trigger gene transcription through the direct binding of ERα to ERE-containing reporter gene (i.e. pC3). This result implies that the coactivator recruitment on the ligand-bound ERα is not prevented by Nar as well as the arrangement of a macromolecular complex, which provides the platform on which the components of transcriptional machinery are assembled. However, ligand bound to ERα could mediate gene transcription even in a manner that does not require the ERα direct binding to DNA. This is referred to as ‘‘indirect genomic mechanism’’ which requires the ERα interaction with specific transcription factors such as Sp1 and AP-1. The ERα-Sp1 and ERα-AP-1 complexes interact with response elements (GC-rich and TRE, respectively) within target promoters (Safe and Kim, 2008). Intriguingly, in the presence of Nar, E2 lacks its ability to activate cyclin D1 promoter, suggesting that E2-induced ERα interaction to Sp1 and AP-1 is impaired. Our previous data indicate that E2 stimulation of ERα-containing HeLa cells induced an increase in AP-1 binding to DNA, whereas Nar or the other flavonoids utilized, quercetin, were unable to do this (Virgili et al., 2004).

Although evidence indicate the ability of flavonoids to bind both ER isoforms maintaining the ERs gene transcriptional ability (Kuiper et al.,

1997; Kuiper et al., 1998; Harris et al., 2005), current data indicate that Nar only allows the E2-induced direct transcriptional activity of ERα, highlighting a role for Nar as an antagonist of E2-induced indirect gene expression.

Furthermore the results of this study demonstrate that, even if Nar does not impair E2-induced ERE-dependent ERα transcriptional activity, Nar treatment reverts the proliferative effects of E2 impairing ERα-mediated rapid signals and inducing different proapoptotic signal transduction pathways. As previously said, the mechanisms by which E2 exerts proliferative effects is assumed to be exclusively mediated by ERα rapid membrane-starting actions (*i.e.* ERK1/2 and PI3K pathway activation) (Ascenzi et al., 2006; Castoria et al., 2001). In fact, these pathways enhance the expression of the antiapoptotic protein Bcl-2, promote G1-to-S phase transition through the enhancement of the cyclin D1 expression and block the activation of the p38/MAPK, responsible for the caspase-3-activated proapoptotic cascade. Thus, E2 inability to activate rapid signal transduction pathways, in the presence of Nar, was paralleled by the block of E2-induced proliferation and by the induction of the apoptotic cascade (i.e. caspase-3 activation and PARP cleavage).

As a whole, the assays with Nar against a background level of E2 allowed us, not only to assess the estrogenic versus antiestrogenic activity of this flavanone, but also to elucidate flavonoid disrupting action mechanism at concentration achievable in the plasma after a meal rich in flavonoids. These results increase the list of Nar and quercetin effects on human health adding up a possible therapeutic benefit of regular consumption of these flavonoids, which may counteract the E2 proliferative action. In addition, this study indicates that the studies, which only focus on the transactivation capacity of various naturally derived estrogenic ligands, could be misleading in that they are actually assaying just one of the diverse action mechanisms elicited by the ERs. Finally, although flavonoid chemoprotective effects is undisputable, we must take in account that E2 regulates a widespread of physiological processes, thus, since these compounds act interfering with ER activities, major study are necessary in order to investigate the effect of these compounds in other E2 tissue targets.

4. ANTIPROLIFERATIVE FLAVONOID EFFECTS IN MIXTURE WITH OTHER EDs

4.1 Introduction

Despite of flavonoids, a normal human diet leads to the exposure to a complex mixture of xenoestrogens resulting in systemic circulation of these compound in the body. As a consequence, in the last years, some evidence has become available to show the combined effects of endocrine disrupters (Kortenkamp, 2007). Nevertheless, the research on the effects of xenoestrogens mixtures, especially combinations of "dietary" and synthetic chemicals at relevant human levels, remain inconclusive. Evidence from study of the combined effects of endocrine disrupters, based on mixture of EDs belonging to the same category (e.g. mixture of anthropogenic pollutant with estrogenic properties) (Suzuki et al., 2001; Rajapakse et al., 2004; van Meeuwen , 2007), are often adverse and discrepant. It has been shown that combined MCF-7 cells exposure to ERα-interacting estrogenic compounds, belonging to the same class, (*i.e.* phytochemical mixtures or synthetic chemical mixtures) seems to act as (full) E2 agonists in ERα-mediated proliferative and transcriptional effects in MCF-7 cells. The estrogenic compound mixtures appear to interact with E2 both in an additive (van Meeuwen et al., 2007) and synergistic (Suzuki et al., 2001) way. However, also antagonistic effects for ERα-interacting estrogenic compounds, belonging to the same class, have been described (Rajapakse et al., 2004). A possible explanation for the discrepancy among the described effects could be the lack of knowledge of the multiplicity of mechanisms through which these compounds act.

Among the most widespread man-made food contaminant, bisphenol A (BPA, 2,2-bis (4-hydroxyphenyl) propane) deserves particular attention. BPA is a monomer of polycarbonate plastics and BPA-based resins used in many products including food cans, dental composites, baby bottles, mineral water bottles, toys (Brotons et al., 1995; Wetherill et al., 2007). The ester bond linking BPA molecules in resins easily undergoes hydrolysis, resulting in the release of free BPA into food and beverages whose represent the main exposure route of BPA in humans (Ikezuki et al., 2002; Yamamoto et al., 2001; Newbold et al., 2009). BPA is known to harbor estrogenic activity; this compound is a weak agonist of both ERα and ERβ (Kuiper et al., 1997) and is capable of stimulating moderate E2-independent proliferation in ERα-containing breast cancer cells and in ERα-transfected HeLa, a cervix carcinoma cell line at concentration achievable in the plasma (10^{-5}M) (Krishnan et al., 1993; Kurosawa et al., 2002; Bolli et al., 2008;

Ricupito et al., 2009). We have recently demonstrated that BPA effects in inducing cell proliferation is related to its ability to rapidly activate ERα-dependent ‘‘extranuclear’’ signals (Ricupito et al., 2009; Bolli et al., 2008).

On the contrary, scarce investigation directed at dissecting the importance of ERβ in the proliferative response to BPA have been established. ERβ, representing the predominant ER subtype in the human colon, plays a pivotal role in E2-induced in colorectal cancer protection (Galluzzo et al., 2007). Actually, ERβ decrease is associated with colonic tumorigenesis and loss of malignant colon cell de-differentiation (Campbell-Thompson et al., 2001; Bardin et al., 2004; Foley et al., 2000; Konstantinopoulos et al., 2003). The mechanisms underlying this protective effects start to be elucidated. In particular, upon E2 binding to ERβ increases receptor association to caveolin-1, a membrane scaffolding protein, and to p38, a member of the MAPK family, in ERβ-containing DLD-1 colon cancer cells (Galluzzo et al., 2007). The resulting signal transduction pathways are required for the downstream activation of a pro-apoptotic cascade involving caspase-3 activation (Caiazza et al., 2007; Galluzzo et al., 2007).

Contrarily to E2, BPA dramatically attenuated ERβ expression; this finding was specific to prostate tumor cells in which BPA induces cellular proliferation (Hess-Wilson et al., 2007). On the other hand BPA, like E2, enhances the receptor transcriptional activity in ERβ overexpressing HeLa cells (Wetherill et al., 2007). These conflicting data raise several concerns on the mimetic role played by BPA on E2-dependent cancers, rendering very difficult to predict if the interactions of BPA with ERβ can induce E2 agonistic or antagonistic proliferative responses.

The aim of this part of project is to evaluate if the above reported anticancer effects for Nar both in ERα and ERβ presence are maintained also in the presence of other EDs with a strong proliferative activity. In particular, the effect of Nar in two cancer cell lines, containing endogenous ERα (MCF-7, T47D) or endogenous ERβ (DLD-1) was evaluated in the presence of BPA.

4.2 Results

4.2.1 *BPA and Nar binding to ERα*

Analysis of data reported in figure 5.5 allowed the determination of the equilibrium dissociation constant (K_d) values for E2, Nar, and BPA binding to recombinant ERα: $(2.1 \pm 0.5) \times 10^{-10}$ M, $(1.4 \pm 0.8) \times 10^{-7}$ M, and $(1.2 \pm 0.3) \times 10^{-6}$ M, respectively. These K_d values are in good agreement with those previously reported (Carlson et al., 1997; Kuiper et al., 1997). Therefore, BPA and Nar bind to ERα although with less affinity (about 1000 and 10000 times less, respectively) that E2. Moreover, Nar shows an about 9-fold higher affinity for ERα than BPA

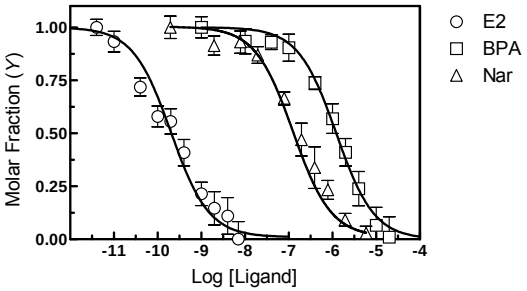


Figure 4.1: E2, BPA, and Nar binding to ERα. Dependence of the intrinsic ligand-bound ERα molar fraction (*Y*) on the free E2 (circles), BPA (squares), and Nar (triangles) concentration.

4.2.2 BPA and Nar mixture effect on breast cancer cells survival and proliferation

First of all, we confirmed ERα presence in MCF-7 cells. MCF-7 cells contain the ERα isoform, corresponding to the 66 kDa protein (Fig. 4.2a). As further confirmation, T47D and MDA-MB231 breast cancer cells have been used as positive and negative control of ERα presence, respectively. As expected, while T47D cells express ERα, MDA-MB231 cells don't express ERα (Fig. 4.2a).

It is well known that E2 proliferative effects in MCF-7 cells is ERα-dependent (Bolli et al., 2008; Ricupito et al., 2009). In order to evaluate BPA and Nar effects on MCF-7 survival and proliferation, cells have been treated with E2 (10^{-8} M) or BPA (10^{-5} M) or Nar (10^{-6}M) (Fig.4.2b),

according to the K_d values for E2, BPA and Nar binding to recombinant ERα (Fig. 4.1). After 24h of stimulation, BPA, like E2, increases cell number, whereas Nar stimulation causes a reduction of cell number (Fig. 4.2b). E2, BPA and Nar effect on MCF-7 cell number was completely prevented by cells pre-treatment with the pure antiestrogen ICI, confirming that the obtained effect are ERα-dependent. (Fig. 4.2b). These data confirm that BPA acts as an estrogen mimetic, while, in the presence of ERα, Nar exerts antiestrogenic effect in MCF-7 cells. In order to evaluate the effect of BPA and Nar mixture on MCF-7 cell survival and proliferation, MCF-7 cells have been treated for 24 h with increasing Nar concentration (10^{-9}M - 10^{-4}M) in presence of BPA (10^{-5}M). Interestingly, an increase of cell number has been observed at low Nar concentration (10^{-9}M and 10^{-8}M) in presence of 10^{-5}M BPA (Fig. 4.2c) with respect to control. On the contrary, a reduction of cell number has been observed at high Nar concentration (10^{-7}M-10^{-4}M) in presence of BPA (10^{-5}M) with respect to control (Fig. 4.2c). These data suggest that Nar, at concentration corresponding to the K_d values Nar binding to recombinant ERα, prevents BPA ability to induce cell proliferation leading cell to apoptosis. Successively, we analyzed the cleavage of the caspase-3 proform (32-kDa band) which results in the production of the active subunit of the protease (17-kDa band). Staurosporin (2μM) has been used as apoptosis positive control. No cleavage of caspase-3 was induced by E2 or BPA alone or BPA in presence of low nanomolar Nar concentration (10^{-9}M and 10^{-8}M) whereas BPA in the presence of nutritionally relevant Nar concentrations (10^{-7} M-10^{-4} M) induced the production of the active subunit (Fig. 4.3a). The increase in caspase-3 activity was confirmed by the cleavage of the known substrates of caspase-3, the DNA repair enzyme PARP (116kDa). Neither E2 or BPA alone induces any conversion of PARP in the inactive form (85 kDa band). On the contrary, cell stimulation with BPA in presence of 10^{-6}M Nar resulted in the conversion of PARP into the inactive 85-kDa fragment (Fig. 4.3b).

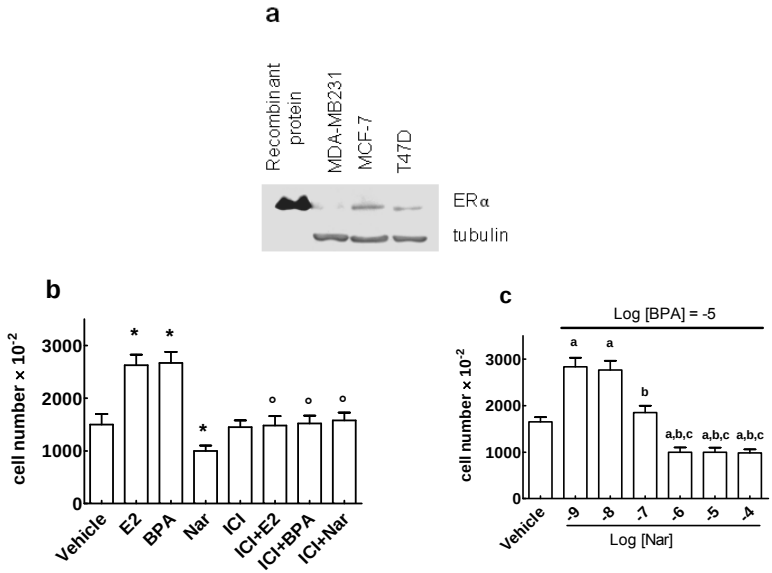


Figure 4.2: BPA and Nar effects on breast cancer cell line survival and proliferation. (**a**) Western blot analysis of ERα in different breast cancer cell lines. (**b**) MCF-7 cells were counted after 24 h treatment with vehicle or E2 (10^{-8} M) or BPA (10^{-5} M) or Nar (10^{-6} M).When indicated cells were pre-treated with estrogen receptor inhibitor (ICI, 10^{-6} M). Data are the mean of four different experiments ±SD. * P< 0.001 was calculated with Student *t*-test with respect to samples treated with vehicle (*) or to samples treated with E2 or BPA or Nar (o). (**c**) MCF-7 cells were counted after 24 h treatment with vehicle or BPA (10^{-5} M) in presence of increasing concentration of Nar (10^{-9}M -10^{-4} M). Data are the mean of four different experiments ±SD. * P< 0.001 was calculated with Student *t*-test with respect to samples treated with vehicle (a) or to samples treated with BPA (10^{-5} M) in presence of 10^{-9} M or 10^{-8} M Nar (b) or in presence of 10^{-7}M Nar (c). Data are the mean of four different experiments ±SD. * P< 0.001 was calculated with ANOVA followed by post-hoc Bonferroni test with respect to samples treated with vehicle(a), Nar (10^{-8}M) (b) and Nar (10^{-7}M) (c) .

4.2.3 BPA and Nar mixture effect on ERα-activated extranuclear signals

We have previously demonstrated that, as well as E2, BPA-mediated induction of ERα-transfected cell proliferation is due to AKT phosphorylation (Bolli et al., 2008). As expected, Nar failed in activating AKT but induced the ER-dependent p38 kinase activation which in turn is responsible for Nar-mediated antiproliferative effects in cancer cells via a proapoptotic cascade (caspase-3 and PARP cleavage) (Totta et al., 2004; Galluzzo et al., 2008). Indeed, the activation of p38/MAPK pathway has been associated with the regulation of apoptosis and differentiation processes (Ambrosino et al., 2001; Harper et al., 2001; Talapatra and Thompson 2001; Shimada et al., 2003; Porras et al., 2004). According to cell proliferation experiments (Fig. 4.2 a, b) and to proapoptotic cascade activation (caspase-3 and PARP cleavage) (Fig. 4.3 a, b), after 60 min of stimulation, Nar (10^{-6}M) prevents BPA-induced activation of AKT (Fig.4.4a) and in turn the increase of Bcl-2 levels (24hours) (Fig. 4.4.c). Furthermore, at 10^{-6}M Nar is able to rapidly (60 min) induce, also in presence of BPA, p38 /MAPK phpsphorylation (Fig. 4.4b). Some experiments were performed with 2×10^{-6}M Staurosporin to evaluate the cell capability to undergo to apoptosis. Staurosporin treatment for 4h induces caspae-3 activation and PARP cleavage I MCF-7 cell line (data not shown).

According to the obtained data in MCF7 cell line, a reduction of T47D cell number has been observed when cells are treated with Nar (10^{-6}M) alone or in presence of a background of E2 (10^{-8}M) or of BPA (10^{-5}M) (Fig.4.5a). Furthermore, T47D cell pretreatment with p38/MAPK inhibitor SB 203580 completely prevents Nar ability to reduce cell number also in presence of a background E2 (10^{-8}M) or BPA (10^{-5}M) (Fig. 4.5a) rescuing the proliferative effect of both E2 and BPA (Fig. 4.5a). Finally, the ERα-dependence of E2, BPA and Nar effect on cell survival and proliferation, has been also confirmed in MDA-231B cells (Fig.4.5b). As shown in figure 4.2a, this cell line doesn't express ERα. According to ERα absence, no change in cell number has been observed upon cell stimulation with any of the considered compounds (Fig.4.5b).

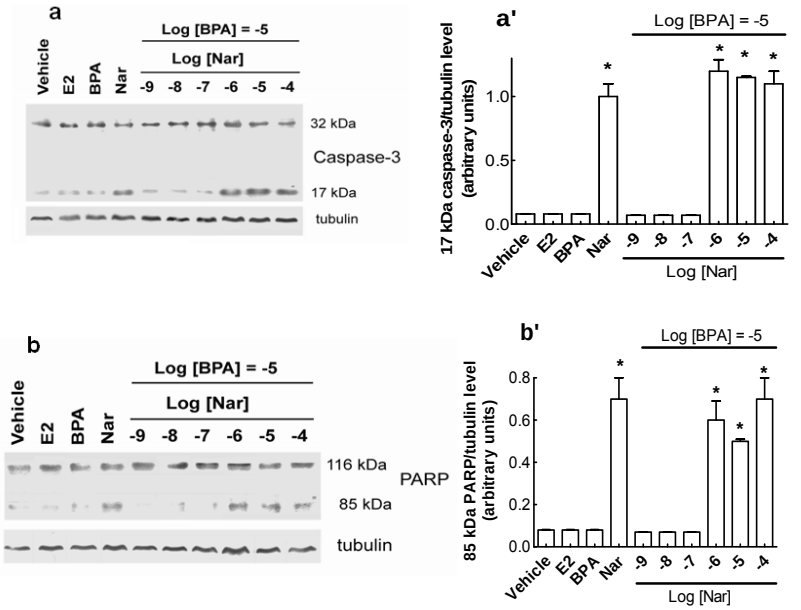


Figure 4.3: BPA and Nar mixture effect on proapoptotic cascade activation. Western blot analysis of Caspase- 3 (a) and PARP (b) after 24h treatment with E2 (10^{-8} M) or BPA (10^{-5} M) or Nar (10^{-6} M) or BPA (10^{-5} M) in presence of increasing concentration of Nar (10^{-9} -10^{-4} M) or after 4h of stimulation with 2μM Staurosporin in MCF-7 cells. Tubulin expression was used for protein level normalization (a, b). Related densitometric analysis are shown in Panels a' and b', respectively. Data are the mean values of four different experiments ± SD. *P < 0.001, calculated with Student's *t*-test, with respect to samples treated with vehicle (*).

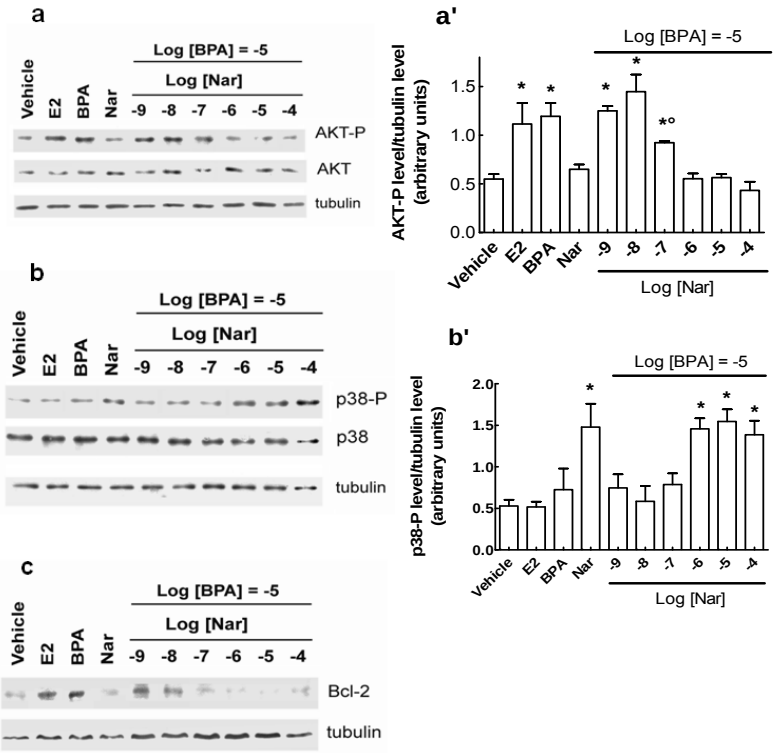


Figure 4.4: BPA and Nar mixture effect on signaling pathway activation. Western blot analysis of AKT (60 min), p38 phosphorylation (60 min) and Bcl-2 proteic level (24h) in MCF-7 cells stimulated with E2 (10^{-8} M) or BPA (10^{-5} M) or Nar (10^{-6} M) or BPA (10^{-5} M) in presence of increasing concentration of Nar (10^{-9} - 10^{-4} M) (a, b,c). Tubulin expression was used for protein level normalization (a, b, c). Related densitometric analysis are shown in Panels a' and b', respectively. Data are mean values of four different experiments ± SD. *P < 0.001, calculated with Student's *t*-test, with respect to samples treated with vehicle (*).

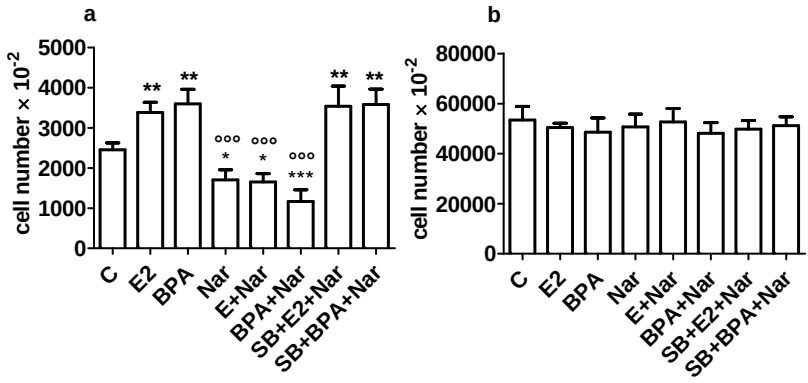


Fig.4.5: BPA and Nar mixture effect on T47D and MDA-231B breast cancer cells. T47D (a) and MDA-231B cells (b) were counted after 24 h treatment with E2 (10^{-8} M) or BPA (10^{-5} M) or Nar (10^{-6} M) or Nar(10^{-6} M) plus E2 (10^{-8} M) or BPA (10^{-5} M). When indicated cells have been preatreated with p38 inhibitor, SB (5×10^{-6} M). Data are the mean of four different experiments ±SD. * $P< 0.001$ was calculated with Student's *t*-test with respect to samples treated with vehicle (*) or to samples pretreated with SB (o).

4.2.4 BPA binding to ERβ

It is well established that BPA mimics E2-induced proliferation in several cancer cells by binding to ERα (Bolli et al., 2008; Ricupito et al., 2009). However, scarse and conflicting data are available concerning the effect of BPA on ERβ-mediated functions. Thus, before assessing BPA and Nar mixture effect on ERβ-mediated cellular functionswe evaluated BPA effect, alone or in combination with E2 in ERβ-expressing colon cancer cells (DLD-1 cells).

Our first objective was to confirm BPA ability to bind to human recombinant ERβ. As shown in Figure 4.6, BPA binds to human recombinant ERβ. The value of the dissociation equilibrium constant (*i.e.*, K_d) for BPA binding to ERβ ($4.8 \pm 0.6)\times10^{-7}$ M) is approximately three-fold higher than that for E2 association (= ($3.5 \pm 0.5)\times10^{-10}$ M). Notably, the K_d value for BPA binding to ERβ is lower by about three-orders of magnitude than that for BPA association to ERα (Kuiper et al., 1997; Matthews et al., 2001; Bolli et al., 2008). Since the full receptor occupancy is achieved for ligand concentrations higher than $10 \times K_d$, the concentration of E2 and BPA

used in the present study was 10^{-8} M and 10^{-5} M, respectively.

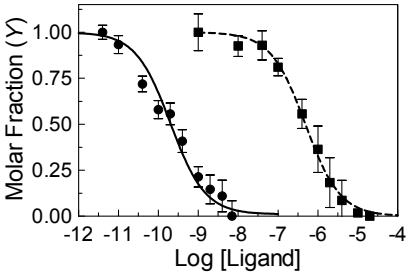


Figure 4.6: E2 and BPA binding to ERβ. Dependence of the molar fraction of the ligand-bound ERβ (*Y*) on the free E2 (circles) and BPA (squares) concentration.

4.2.5 Effects of E2 and BPA on DLD-1 cell survival and propoapototic cascade activation.

As above reported, upon E2 stimulation, DLD-1 cell number decreases, due to the activation of a proapoptotic cascade (Galluzzo et al., 2007). As expected, 30 h of 10^{-8} M E2 stimulation reduces DLD-1 cell vitality of about 50% with respect to un-stimulated cells, whereas 10^{-5} M BPA stimulation does not affect the cell growth (Fig. 4.7a). Notably, the E2 effect on cell vitality is completely prevented when DLD-1 cells are stimulated with BPA in the presence of E2 background (10^{-8} M). This effect is also present at 10^{-6} M BPA concentrations. On the other hand, higher concentrations (10^{-3} M) result cytotoxic, further decreasing the DLD-1 cell number (Fig. 4.7a). This result, indicating an antagonism of BPA toward E2 in colon cancer cells, has been confirmed by evaluating the effects of BPA on caspase-3 activation. Indeed, E2 activates caspase-3 increasing the level of both active caspase (*i.e.*, 17 kDa band) and the cleavage of its substrate PARP. BPA alone does not affect caspase-3 activation and PARP cleavage, but prevents E2 effects on pro-apoptotic proteins when DLD-1 are co-stimulated with BPA and E2 (Figs 4.7b and 4.7c).

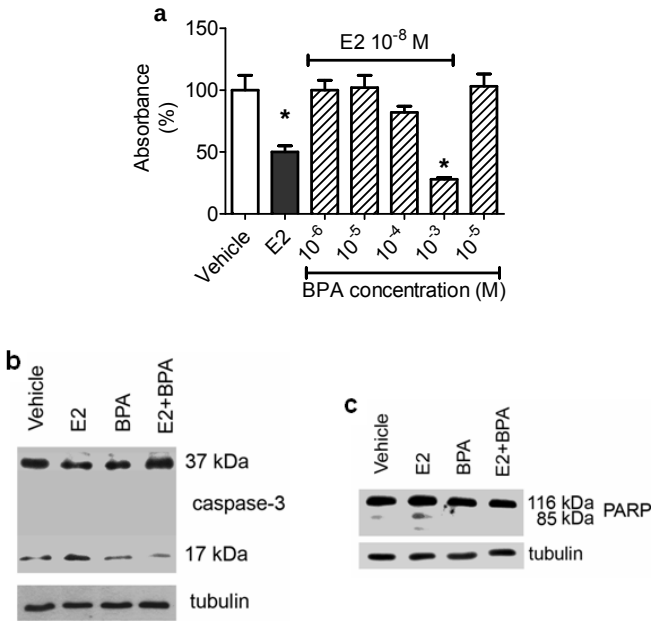


Figure 4.7: Effects of E2 and BPA on DLD-1 cell survival and activation of pro-apoptotic cascade. (a) XTT assay of DLD-1 cells treated 30 h with 17β estradiol (E2, 10^{-8}M) in the absence or presence of different concentrations of BPA (from 10^{-6}M to 10^{-3}M). Absorbance was determined at 490 nm. Data are the mean of six different experiments ±SD. (*) $P < 0.001$ with respect to vehicle. Western blot analyses of caspase-3 activation (b) and PARP cleavage (c) performed on DLD-1cells treated with E2 (10^{-8}M), BPA (10^{-5}M) or E2(10^{-8}M) plus BPA (10^{-5}M). β-tubulin expression was used for protein level normalization. Western blots are representative of three different experiments.

4.2.6 BPA effect on ERβ genomic and extra-nuclear activities

Since, the obtained results indicate an antagonism of BPA toward E2 in colon cancer cells, we evaluated also the effects of BPA on ERβ genomic and extra-nuclear activities. Figure 4.8 shows the effect of BPA on the ERE-containing pC3 reporter plasmid activity in the presence and absence of 10^{-8} M E2. BPA does not exert any effect, whereas the E2 treatment induces a two-fold increase of the pC3 promoter activity (Fig. 4.8a). When added with E2, BPA prevents the E2-induced ERβ transcriptional activity (Fig. 4.8a). BPA treatment also impairs the E2-induced extra-nuclear activities of ERβ. In fact, BPA and E2 co-treatment inhibits the E2-induced increase of p38 phosphorylation (Fig. 4.8b). Previously, we reported that both genomic and extra-nuclear ERβ activities are required for E2-induced increased expression of ERβ, representing an important step for the protection exerted by E2 against colon cancer cell growth (Caiazza et al., 2007). This prompted us to evaluate the impact of BPA on the E2-induced increase of ERβ level. Figure 4.8c shows that BPA specifically impairs the E2-induced increase of ERβ level further confirming the antagonistic behavior of this compound on the E2:ERβ complex formation.

4.2.7 BPA effect on ERβ molecular interactions with caveolin-1 and p38

In ERβ-containing DLD-1 colon cancer cells, upon E2 binding to ERβ, increases receptor association to caveolin-1and to p38 (Galluzzo et al., 2007) resulting in a signal transduction pathways required for the downstream activation of a pro-apoptotic cascade involving caspase-3 activation (Fig. 2; Caiazza et al., 2007; Galluzzo et al., 2007). Thus, we assessed the effect of BPA on ERβ molecular interactions with caveolin-1 and p38 (Galluzzo et al., 2007). In the resting state (*i.e.*, vehicle treated), ERβ is associated with caveolin-1, whereas the association of phoshorylated p38 to ERβ is barely detectable (Fig. 4.9). Intriguingly, both in the absence and in the presence of E2, BPA stimulation prevents the ERβ:p38 association without affecting the ERβ:caveolin-1 complex formation (Fig. 5.4).

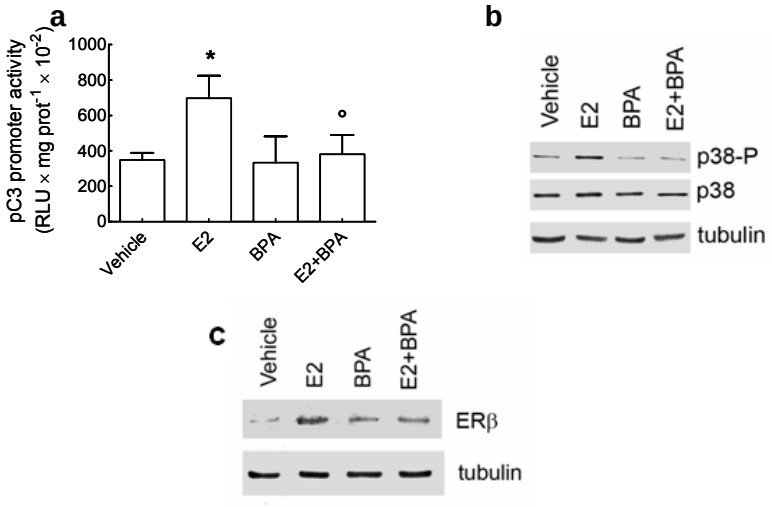


Figure 4.8: BPA effect on ERβ–mediated pC3 promoter activity, p38 activation and ERβ level. Luciferase assay detection in DLD-1 cells transfected with pC3-luciferase (pC3) reported plasmid and stimulated 24h with vehicle, E2 (10^{-8} M) or BPA (10^{-5} M), or E2 (10^{-8} M) plus BPA (10^{-5} M) (a). Data are the mean of six different experiments ±SD. $P < 0.001$ with respect to samples treated with vehicle (*) or E2 (°). Representative Western blot analysis of p38 phosphorylation (b) and ERβ level (c) in DLD-1 cells treated 24h with vehicle, E2 (10^{-8} M) or BPA (10^{-5} M), or E2 (10^{-8} M) plus BPA (10^{-5} M). β-tubulin expression was used for protein level normalization.

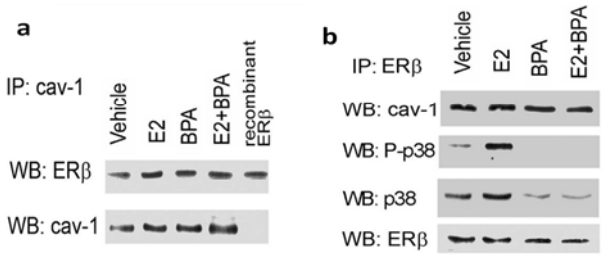


Figure 4.9: BPA effect on ERβ molecular interactions with caveolin-1 and p38. After 24h oh stimulation with vehicle, E2 (10^{-8} M) or BPA (10^{-5} M), or E2 (10^{-8} M) plus BPA (10^{-5} M), DLD-1 cells were lysated and subjected to caveolin-1 immunoprecipitation (a) or ERβ immunoprecipitation (b) followed by Western blot with anti-caveolin-1, anti-ERβ, anti Phosphorylated-p38, or anti-p38 antibodies. Western blot analysis is representative of 3 different experiments.

4.2.8 Effect of BPA and Nar mixture on DLD-1 cancer cells growth.

The above reported results indicate that BPA acts as an E2 antagonist in ERβ-containing colon cancer cell line. This result prompt us to evaluate if Nar maintain its antiproliferative effect in the presence of BPA. DLD-1 cells have been treated with E2 (10^{-8} M) or BPA (10^{-5} M) or Nar (10^{-6}M) (Fig.4.10b), according to the K_d values for E2, BPA and Nar binding to recombinant ERβ (Fig. 4.6, Kuiper et al., 1997). As expected (Totta et al., 2004, Galluzzo et al., 2007) after 24h of stimulation, Nar, like E2, decreases cell number, whereas BPA has no effect on cell proliferation (Fig. 4.10a). Nar and E2 mixture shows an effect comparable to E2 and Nar alone, suggesting that these compounds share the same pathway. Accordingly, ER inhibitor ICI (10^{-6}M) completely prevent Nar (10^{-6}M) and Nar (10^{-6}M) and E2 (10^{-8}M) mixture-induced reduction of cell number ((Fig.4.10a). On the other hand BPA completely prevents E2-ability to decrease cell number. However, DLD-1 cell stimulation with Nar and BPA mixture results in a decrease of cell number, suggesting that Nar is able to decrease cell number also in the presence of BPA. As shown in figure 4.10 b, Nar (10^{-7}M-10^{-4}M) decreases cell number also in the presence of BPA, at concentration corresponding to the K_d values Nar binding to recombinant ERβ.

Since E2- and Nar- induced decrease of cell number is due to p38 activation (Caiazza et al., 2007), and in turn, to the activation of a proapototic cascade (Totta et al., 2004, Galluzzo et al., 2007) we evaluate

the correlation between cell number decrease and the activation of both p38 and of a pro-apoptotic cascade. Staurosporin (2μM) has been used as apoptosis positive control. As shown in figure 4.11a both E2 (10^{-8}M) and Nar (10^{-6}M) activates p38 MAPK, while BPA (10^{-5}M) doesn't lead to p38 phosphorylation. However, Nar (10^{-6}M), in presence of BPA (10^{-5}M) is still able to induce p38 phosphorylation (Fig. 4.11a). Accordinglyboth E2 and Nar induced the cleavage of the caspase-3 proform (32-kDa band), which results in the production of caspase active subunit (17-kDa band) (Fig. 4.11b). No cleavage of caspase-3 was induced by BPA alone (Fig. 4.11b). However, cells co-stimulation with both Nar and BPA results in the production of the active subunit of caspase-3. (Fig.4.11b) The increase in caspase-3 activity was confirmed by the cleavage of the known substrates of caspase-3, the DNA repair enzyme PARP (116kDa). Whereas any PARP inactive 85-kDa fragment has been detected in BPA-stimulated cells, E2, Nar and Nar plus BPA stimulation result in the conversion of PARP into the inactive 85-kDa fragment (Fig. 4.11b).

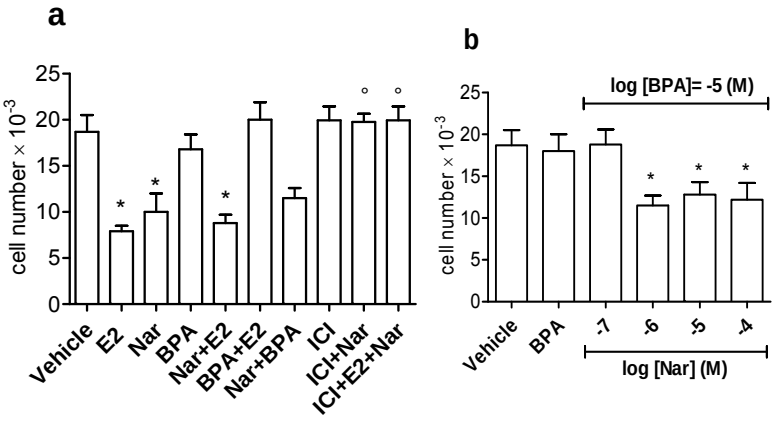


Figure 4.10: BPA and Nar mixture effects on DLD-1 cell growth. DLD-1 cells were counted after 24 h treatment with vehicle or E2 (10^{-8} M) (a) or Nar (10^{-6} M) (a) or BPA (10^{-5} M) (a, b) or Nar plus E2 (a) or BPA plus E2(a) or BPA (10^{-5} M) plus Nar (10^{-6} M) (a) or BPA (10^{-5} M) in presence of increasing concentration of Nar (10^{-7}M -10^{-4} M) (b). Data are the mean of four different experiments ±SD. * P< 0.001 was calculated with Student *t*-test with respect to samples treated with vehicle (*) or to samples treated with E2 or BPA or Nar (o).

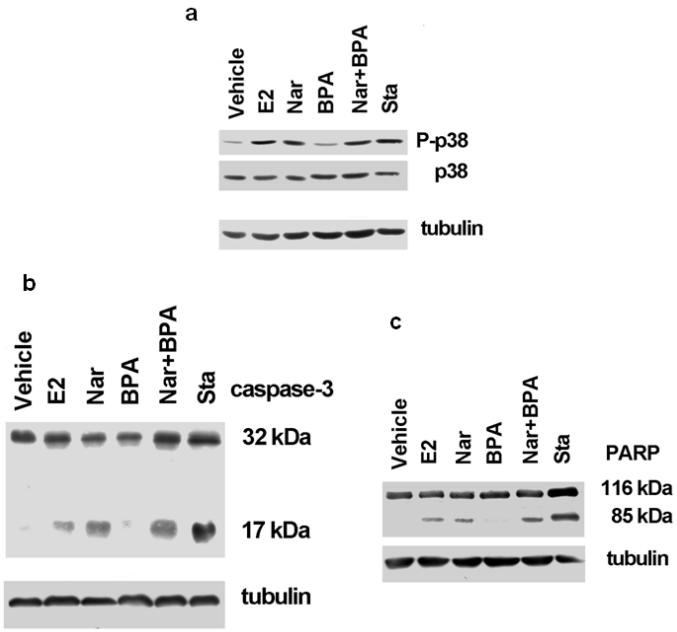


Figure 4.11: BPA and Nar mixture effect on p38 activation and proapoptotic cascade activation. Western blot analysis of p38 phosphorylation (60 min) (a) caspase 3 (24h) (b) and PARP (24h) (c) in DLD-1 cells stimulated with E2 (10^{-8} M) or BPA (10^{-5} M) or Nar (10^{-6} M) or BPA (10^{-5} M) plus Nar (10^{-6}M). Tubulin expression was used for protein level normalization (a, b, c). Western blot analysis is representative of 3 different experiments.

4.2.9 Effects of BPA and Nar mixture on ERβ levels

As previously said, E2-induced increased expression of ERβ represent an important step for the protection exerted by E2 against colon cancer cell growth (Caiazza et al., 2007). Since Nar acts as an estrogen mimetic preserving its ability to leads colon cancer cells to apoptosis also in presence of BPA, we assessed Nar and Nar and BPA mixture effect on ERβ level. As shown in figure 4.12, Nar (10^{-6}M), like E2 (10^{-8}M), increases ERβ levels. Furthermore, Nar and E2 mixture shows an effect comparable to E2 and Nar alone. Notably, unlike E2, Nar preserves its ability to increase ERβ levels also in presence of BPA (Fig.4.12), confirming its E2-mimetic protective role against colon cancer growth also in mixture with other EDs.

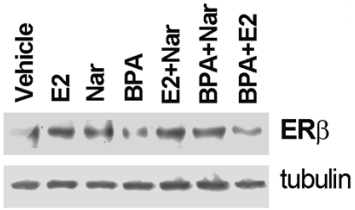


Figure 4.12: Nar and BPA mixture effect on ERβ level. Representative Western blot analysis of ERβ level in DLD-1 cells treated 24h with vehicle, E2 (10^{-8} M) or Nar (10^{-6}M) BPA or (10^{-5} M) or E2 (10^{-8} M) plus Nar or E2 (10^{-8} M) plus BPA (10^{-5} M) or Nar (10^{-6}M) plus BPA (10^{-5} M). β-tubulin expression was used for protein level normalization.

4.3 Discussion

Our previous reports show BPA ability to stimulate moderate E2-independent proliferation in ERα-containing breast cancer cells and in ERα-transfected HeLa, a cervix carcinoma cell line through the activation of ERα-dependent membrane starting signals (Bolli et al., 2008; Ricupito et al., 2009).

On the contrary an antagonistic behavior of Nar with respect to E2 in ERα-transfected HeLa cells have been not reported before.

In light with these results and the evidence of the antagonistic behaviour of Naringenin with respect to BPA in ERα-dependent cancer cell growth, we assessed the effect of BPA and Nar mixture in ERα-expressing breast cancer cells. Our data confirm that the binding affinity constant value of both BPA and Nar for ERα are quite similar, and comparable to BPA and Nar concentration achievable in the plasma. However, although Nar and BPA binding affinity costant for ERα are quite similar, the 9 fold higher affinity for Nar binding to ERα with respect to BPA is sufficient to Nar to block BPA-cellular effects. Thus, at concentration achievable in the plasma Nar preserve its ability to induce apoptosis in presence of BPA in ERα-expressing cancer cells. Accordingly to the data obtained in E2 and Nar costimulated ERα-expressing cells (Bulzomi et al., 2010), the activation of a proapoptotic cascade in ERα-expressing cells depends on Nar-induced p38 phosphorylation and on downregulation of the antiapoptotic protein Bcl-2. The Nar-dependent p38 phosphorylation is necessary for caspase-3 and PARP cleavage, whereas the Nar-mediated block of BPA-induced Bcl-2 increase is dependent on Nar ability to prevent BPA-dependent PI3K/AKT pathway. On the other hand, contrarily to BPA stimulated E2-independent proliferation in ERα-expressing cells, scarce and conflicting investigation have been aimed at dissecting the importance of ERβ in the proliferative response to BPA (Wetherill et al., 2007; Hess-Wilson et al., 2007). In transiently transfected HeLa cells where ERβ, co-activators, and reporter genes were all over-expressed (Kurosawa et al., 2002; Wetherill et al., 2007) a general agonistic behavior of the ERβ:BPA complex on E2-induced ERβ transcriptional activity have been reported without any information on the BPA effect on cell proliferation. In contrast, in prostate cancer cells, where the loss of ERβ signaling results in increased cell proliferation, BPA reduced ERβ levels (Hess-Wilson et al., 2007) as an E2 antagonist.

The present data demonstrate that in an endogenous ERβ expressing colon cancer cells BPA behaves as a subtype-specific E2 antagonist by

blocking the E2 ability to reduce cancer cell proliferation. Notably, only higher BPA concentrations (≥ 1.0×10-3 M) result cytotoxic in DLD-1 cancer cells, as previously reported in HeLa cells (Bolli et al., 2008).

E2 ability to confer protection against colorectal cancer depends on E2:ERβ complex ability to associate with caveolin-1 and with p38/MAPK, leading to the activation of a proapoptotic cascade (Galluzzo et al., 2007, Caiazza et al., 2007). Accordingly, colorectal cancer, the second most common malignancy in industrialized countries, is more frequent in men than women (DeCosse et al., 1993; Slattery et al., 2004). Furthermore, a lower incidence of colon cancer in young women than that in men, and an increased risk to develop colon cancer in postmenopausal women have been reported (Marino and Galluzzo, 2008).

The ER-dependent molecular mechanisms underlying BPA interference with E2 protective effects involve both genomic and extra-nuclear ERβ activities. In fact, BPA prevents the E2-induced transcriptional activity of ERβ toward an ERE containing reporter gene in colon cancer cells. Furthermore, BPA prevents the E2 ability to increase ERβ levels, which requires both the genomic and the extra-nuclear ERβ activities (Caiazza et al., 2007), further sustain the observed antagonist character of the BPA:ERβ complex.

Accordingly, ERβ decreased level is associated with colonic tumorigenesis and loss of malignant colon cell differentiation (Foley et al., 2000; Campbell-Thompson et al., 2001; Konstantinopoulos et al., 2003; Bardin et al., 2004). Moreover, BPA was able to completely silence also the E2-induced ERβ extra-nuclear activities further supporting its role as an ERβ-specific E2 antagonist in colon cancer cells. In particular, BPA does not affect the ERβ interaction with the plasma-membrane protein caveolin-1, but it impairs ERβ association with the signaling protein p38, thus decoupling ERβ from the downstream signals important for the E2-induced pro-apoptotic cascade. Therefore, the BPA-induced modulation of the ERβ:p38 interaction indicates that different ligands, by inducing distinct conformational changes in ERβ, could play an active role also in ER extra-nuclear functions.

As a whole, we describe an ERβ-mediated mechanism of BPA action, which was never reported before. This mechanism, along with the well know BPA ability to induce and/or promote cancer cell proliferation in an ERα-dependent manner (Bolli et al., 2008), allow us to depict BPA as a double sided endocrine disruptor, which promotes tumor incidence in breast and other target organs that predominantly express ERα but inhibits the E2 protective effects in the ERβ-expressing colon.

These two divergent aspects could act synergistically, thus increasing

the E2-disrupting potential of this widespread environmental polluter.

In light with these results we also assessed the effect of BPA and Nar mixture in ERβ-expressing cells. Notably, as well in ERα-expressing cells, Nar preserves its ability to induce apoptosis in presence of BPA also in ERβ-expressing cells. Contrarily to E2 and BPA costimulation in ERβ-expressing cell (Bolli et al., 2010), Nar and BPA costimulation in ERβ-expressing cell cotreatment results in a Nar-dependent acvtivation of p38 MAPK, and in turn the activation of a proapoptotic cascade (i.e. caspase-3 and PARP cleavage). Furthermore, unlike E2, Nar sustains its ability to increase ERβ levels also in the presence of BPA.

Since E2 failed in increasing ERβ levels in presence of BPA, Nar ability to induce ERβ level increase in the presence of BPA confirms its E2-mimetic protective role against colon cancer growth also in mixture with other EDs and represents an important step for the protection exerted by this natural compound against colon cancer cell growth.

Unlike the papers reporting contrasting and confusing data about the effect of endocrine disruptor mixtures (Suzuki et al., 2001; Rajapakse et al., 2004; van Meeuwen et al., 2007), our data highlight the necessity to understand ED underlying mechanisms of action in order to full understand the mixture effects. In fact, as we assumed, the final outcome is strictly dependent on the interaction among the EDs and the specific ER isoform present, which, in turn, will generate different signal transduction pathways.

As a whole, we can affirm that also Nar could be point out as a double sided protective agent, which is able to reduce tumor incidence both in breast and other target organs that predominantly express ERα and colon and other organs ERβ-expressing at concentration achievable in the plasma. Thus, our data highlight a chemopreventive role of this natural molecule, occurring in fruit and vegetable widely present in Mediterranean diet, against cancer promoting effects of compounds such as BPA.

5. NARINGENIN EFFECT ON E2- INDUCED SKELETAL MUSCLE PROTECTION.

5.1 Introduction

As reported, E2 effects go beyond the regulation of cell proliferation/apoptosis. Besides the well established E2 effects on the reproductive system, bone, cardiovascular and nervous system, E2 exerts its effects also in skeletal muscle. Epidemiological data show gender-related differences in skeletal muscle and in women treated with hormone replacement therapy (HRT) the menopause-related decline in muscle performance is reduced (Phillips et al., 1993, Heikkinen et al., 1997; Sirola and Rikkonenn, 2005). Variation in skeletal muscle strength during the human menstrual cycle has been reportedand muscle mass and strength diminish during the postmenopausal years, leading to sarcopenia (Dionne et al., 2000; Lemoine et al., 2003; Wiik et al., 2003). In addition, female muscles are more fatigue-resistant and recover faster than male muscles and estrogens increase skeletal muscle force production (Glenmark et al., 2004). *In vivo* experiment demonstrate that female rat muscles show fewer histopathological changes after repeated eccentric contractions than male muscles; male and ovariectomized female rats exhibit higher indexes of exercise-induced muscle membrane damage, which disappear in ovariectomized female rats after estradiol treatment (Fulco et al., 1999; Glenmark et al., 2004; McCormick et al., 2004). All together, these data indicate that estrogens can regulate skeletal muscle mass.

We have recently demonstrated that skeletal muscle express both ER isoforms and that E2 leads to skeletal muscle differentiation (Galluzzo et al., 2009). Particularly, E2 is able to promote differentiation in rat myoblast cell (L6) increasing the expression of well known differentiation markers of myogenesis (*i.e.,* GLUT-4 membrane translocation, the transcription factor myogenin and the contractile protein myosin heavy chain, MHC, expression) (Galluzzo et al., 2009). GLUT-4 translocation to the plasma membrane, the major glucose transporter expressed in skeletal muscle, precedes other muscle specific protein increase (*i.e.,* myogenin and MHC) which are necessary for the appearance of the morphological muscle phenotype (Luμs et al., 2006). The E2-induced increase of GLUT-4 translocation to plasma membrane, and of myogenin and MHC protein levels requires ERα-depedent rapid extra-nuclear signals (*i.e.* p38/MAPK, PI3K/AKT activation). ERα-depedent AKT phosphorylation is necessary for the rapid (15 min) translocation of GLUT-4 to membranes in L6 cells. This event is the first one necessary for the E2 promotion of differentiation

process in L6 cells. E2-induced AKT phosphorylation is also required for the subsequent (6-24 h) expression of myogenin, confirming the role played by this kinase in muscle differentiation (Sipila et al., 2001). Thus, ERα activities (i.e. both rapid and long term mechanisms) are not only important for E2-induced cell proliferation, but are also important for E2-induced skeletal muscle differentiation. These results rises the hypothesis that Nar stimulation may interfere with E2-induced differentiation by impairing rapid ERα-dependent signals. Besides estrogens, also male steroid hormones (i.e. dyhidrotestosterone and testosterone) possess the same ability of E2 to induce myoblast differentiation rendering this experimental model unique to evaluate if a different susceptibility to ED occurs in male and female sex steroid hormones.

However, both ERα and ERβ are expressed in human skeletal muscle in both sexes (Lemoine et al., 2003; Wiik et al., 2003), but ERβ role in muscle remain unclear. Particularly the involvement of ERβ specific activities in the regulation of important cellular function in a cellular context, such as skeletal muscle, where both ERs are expressed has not been defined.

Recently, it has been demonstrated that E2 protects Retinal Pigmented Epithelium (ARPE-19) cells from oxidative stress through an ERβ-dependent mechanism. Particularly, E2 mediated cytoprotection was through preservation of mitochondrial function, reduction of ROS production and induction of cellular anti-oxidant genes (Giddabasappa et al., 2010). In muscle cells, reactive oxygen species (ROS) are continually generated, and it is believed that these molecules have a well-established role as physiological modulators of skeletal muscle functions, including development, metabolism and contractile functions. Moreover, studies in the past two decades suggest that, during strenuous muscle activity, in some pathological conditions, or in aging, the generation of ROS in the skeletal muscle cells may be elevated to a level that overwhelms the antioxidant defense systems (Ji, 1995; Ji, 2001) and can contribute to the development of muscle fatigue, inflammation, and degeneration (Meydani et al., 1992) leading to many muscle diseases. Intriguingly, in a study conducted to determine the existence of a gender-related difference in recover after muscle injury, oxidative stress, and apoptosis induced by eccentric exercise, it has been demonstrated that at baseline and independently from exercise, females had higher E2 and superoxide dismutase (SOD) in conjunction when compared with men. After eccentric exercise, men reported greater soreness levels at 24, 48, and 72 h. Furthermore, Bax protein level increased in both genders, whereas Bcl-2 increased only in women. The bax/bcl-2 ratio in women significantly decreased after 6 h and returned to baseline levels after 24 h. Men exhibited greater cell death at all time points whereas

myofibrillar protein content and total DNA content decreased in both genders at 24 h after exercise (Kerksick et al., 2008). Thus, it is very important to define if E2 is included among the factors that could determine skeletal muscle cell protection from oxidative damage (Caporossi et al., 2003) and if ERβ is the mediator of the E2-dependent skeletal muscle protection from ROS injury.

To verify the hypotheses that Nar stimulation may interfere with E2-induced differentiation by impairing rapid ERα-dependent signals, to evaluate if a different susceptibility to EDs occurs in male and female sex steroid hormones, and to verify E2 role in skeletal muscle protection from ROS-injury, evaluating ER isoform involvement, L6 rat myoblast and C2C12 mouse myoblast committed to differentiation by lowering serum concentration in culture media (2%) were used as experimental model.

In these model we assessed Nar effect on skeletal muscle differentiation marker in absence or in presence of E2, Nar effect on ROS-induced skeletal muscle injury, and Nar ability to interfere with male steroid hormones.

5.2 Results

5.2.1 ER expression in differentiation-induced L6 cells

We have previously demonstrated that in growing medium (10% serum) cultured L6 cells express both ER isoform with a different balance between ERα and ERβ *ratio* (Fig. 5.1 line 0). Although an increase of both ER level is evident 24h after serum reduction in the medium (2% serum), ERα increase is major with respect to ERβ increased expression (Fig. 5.1a). However, any significant variation in ERα/ERβ *ratio* during differention has been observed.(Fig. 5.1b)

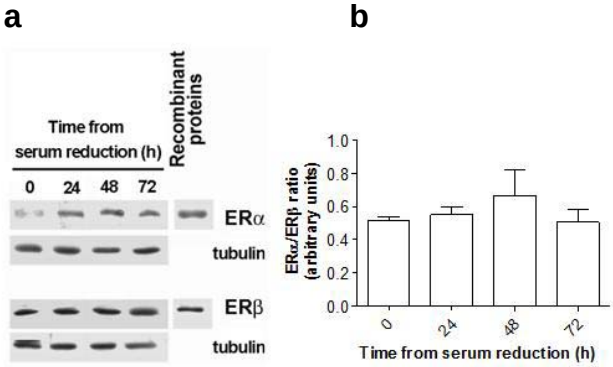


Figure 4.1: ER isoform level during L6 differentation. (a) Western blot analysis of recombinant ER proteins and ERα and ERβ protein level in L6 cells induced to differentiation by reducing serum concentration in the medium (2% serum). Typical blot of 3 independent experiments. (b) ERα/ERβ *ratio* during L6 differentiation.

5.2.2 Naringenin effect on L6 cell differentiation

E2 is able to promote differentiation of active proliferating rat myoblast cell (L6) increasing the expression of well known differentiation markers of myogenesis (*i.e.,* GLUT-4 membrane translocation, the transcription factor myogenin and the contractile protein myosin heavy chain, MHC, expression) (Galluzzo et al., 2009). As well as for growing medium cultured L6 cell (Galluzzo et al., 2009), E2 increases differentiation marker levels also in differentiating L6 myoblast with respect to the control. E2 effect in differentiating myoblast is dose- and time-dependent. GLUT-4 translocation to cell membrane is evident after only 15 min of E2 stimulation (Fig. 5.2a), while myogenin (Fig. 5.2b) and MHC level (Fig. 5.2c) increase is evident after 6 and 24 h respectively. The maximum of E2 effect has been detected from 10^{-8}M to 10^{-6}M E2 for GLUT-4 translocation (Fig. 5.2 a'), from 10^{-10}M to 10^{-8}M E2 for myogenin level increase (Fig.5.2b') and from 10^{-9}M to 10^{-8}M E2 for MHC level increase (Fig.5.2c'). On the contrary, Nar stimulation has no effect on the differentiation muscle marker at any of the tested time (Fig. 5.2a, b, c) and concentrations (Fig. 5.2a', b', c'). However, Nar stimulation (10^{-7}M-10^{-5}M) of L6 cells in presence of E2 (10^{-8}M) completely prevents E2-induced differentiation marker increase (Fig. 5.3a, b, c), suggesting that Nar antagonizes E2-induced of myoblasts.

5.2.3 Nar specifically impairs E2- induced differentiation in L6 cells.

As previously reported, E2-induced differentiation in proliferating L6 cells requires rapid signal activation (AKT and p38 MAPK). While AKT activation is necessary for the rapid GLUT-4 plasma membrane translocation, p38 activation is necessary for both myogenin and MHC increase (Galluzzo et al., 2009). However, among several muscle trophic factors, Insulin-like growth factor I (IGF-I), like E2, is able to induce skeletal muscle differentiation (Sheffield-Moore and Urban, 2004; Lluìs et al., 2006) activating the same rapid signals (AKT and p38) (Galluzzo et al., 2009). In order to verify Nar effect on AKT phosphorylation, L6 cells were co-stimulated with Nar and E2 and Nar and IGF-I. As shown in figure 6.4a both E2 and IGF-I increase GLUT-4 plasma membrane translocation (30 min), whereas no change in GLUT-4 plasma membrane translocation has been observed upon Nar stimulation. Cell pretreatment with AKT inhibitor completely prevent both E2- and IGF-I-induced GLUT-4 translocation to plasma membrane (Fig. 5.4a) confirming the pivotal role of this pathway in this event. Interestingly, unlike Nar, both E2 and IGF-I induce AKT phosphorylation (Fig.5.4 b), but upon cell co-stimulation with both Nar (10^{-}

^{6}M) and E2 (10^{-8}M) or Nar (10^{-6}M) and IGF-I, AKT phosphorylation is blocked only in E2 and Nar costimulated cells (Fig.5.4 b), suggesting that Nar ability to hamper E2-induced differentiation depends on the block of ERα-mediated AKT phosphorylation.

As previously said, p38 pathway is involved in both myogenin and MHC increase in proliferating L6 cells (Galluzzo et al., 2008), thus we evaluated Nar and E2 ability to activate p38, and in turn to increase myogenin and MHC level. As shown in figure 5.4a, Nar and E2 alone as well as Nar and E2 costimulation lead to p38 phosphorylation. Cell pretreatment with the p38 inhinitor (SB) prevent E2- and IGF-induced myogenin and MHC increase (Fig. 5.4b, c), confirming p38 involvement in E2- and IGF-induced myogenin and MHC increase. However the lack of myogenin and MHC increase in Nar-stimulated cell indicate that p38 alone is not sufficient to lead L6 cell to differentiation (Fig.5.4b, c). Accordingly, Nar, impairs only E2-induced increase of Myo (Fig. 5.5a) and MHC (Fig. 5.5b) in Nar and E2 cotreated L6 cells, withouth affecting IGF-induced differentiation in Nar and IGF costimulated cells (Fig. 5.5a, b).

Together these data indicate Nar ability to specifically hamper E2-induced L6 cell differentiation by blocking ERα action impeding AKT phosphorylation.

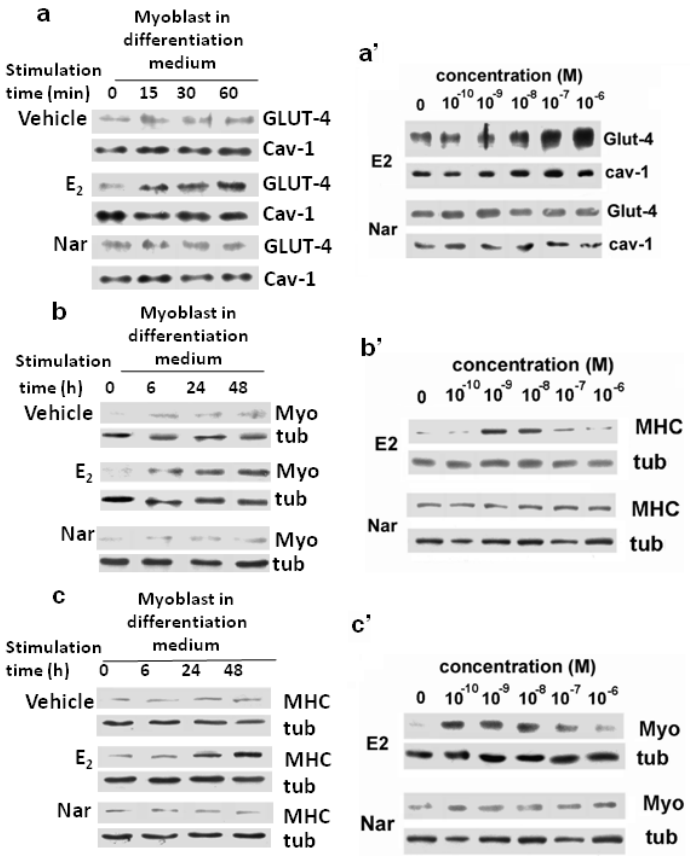


Figure 5.2: E2 and Nar effect on skeletal muscle differentiation markers in L6 cells. Time-course analysis of E2 (10^{-8}M) and Nar (10^{-6}M) treatment on GLUT-4 plasma membrane translocation (panel a) and on myogenin (Myo, panel b) and MHC (panel c) levels, in L6 cells grown in differentation medium containing 2% serum. Panels a', b', and c', show E2 dose-dependent (10^{-10}M-10^{-6} M) effects and Nar dose-dependent (10^{-10}M-10^{-6} M) effects on plasma membrane GLUT-4 translocation (30 min of stimulation) and on myogenin (Myo) and MHC levels (24 hrs of stimulation), respectively. The amount of protein was normalized by comparison with cav-1 (panels a, a') or tubulin (panels b, b', c, c') levels. The data are typical Western blots of 3 independent experiments.

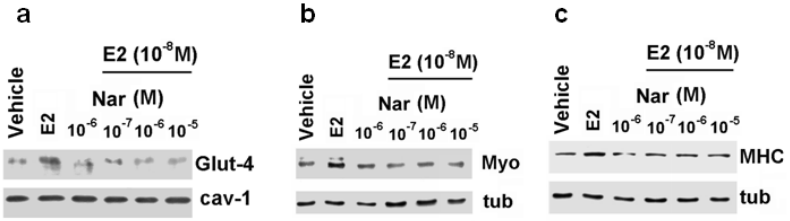


Figure 5.3: E2 and Nar coadministration effect on skeletal muscle differentiation markers in L6 cells. Western blot analysis of GLUT-4 plasma membrane translocation (panel a) and myogenin (Myo, panel b) and MHC (panel c) levels in L6 cell maintained in differentation medium (2% serum) for 30 min (panel a) or 24 h (panel b, c) in the presence of vehicle or E2 (10^{-8}M) or Nar (10^{-6}M) or different concentration of Nar (10^{-7}M to 10^{-5}M) in presence of a background of E2 (10^{-8}M). The data are typical Western blots of 3 independent experiments.

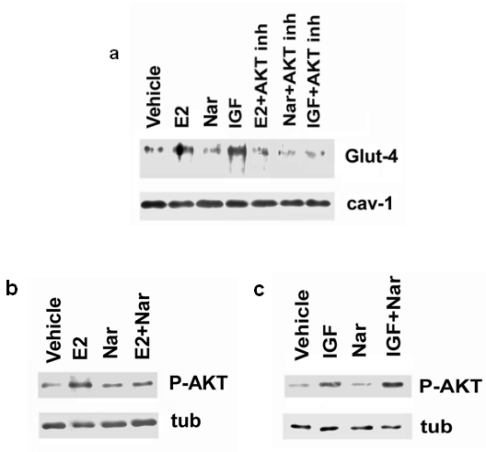


Figure 5.4: Nar effect on GLUT-4 plasma membrane translocation and AKT activation. Western blot analysis of GLUT-4 plasma membrane translocation (a) and AKT phosphorylation (b, c) upon differentiation induced L6 cells stimulation for 30 min (a) or 1h (b, c) with vehicle, E2 (10^{-8}M), Nar (10^{-6}M), IGF. When indicated cells have been pretreated (30 min) with AKT inhibitor. The data are typical Western blots of 3 independent experiments.

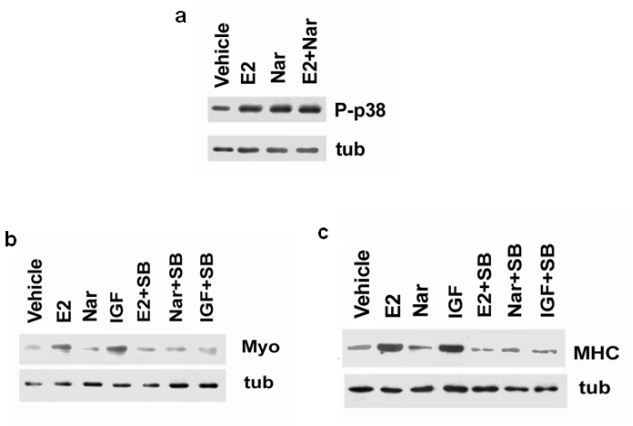


Figure 5.5: p38 involvment in E2, Nar, and IGF effect on myogenin and MHC level. Western blot analysis of p 38 phosphorylation (a), myogenin (Myo) (b) and MHC (c) upon differentiation induced L6 cells stimulation for 1h (a) or 48h (b, c) with vehicle or E2 (10^{-8}M) or Nar (10^{-6}M) or IGF. When indicated cells have been pretreated (20 min) with p38 inhibitor (SB). The data are typical Western blots of 3 independent experiments.

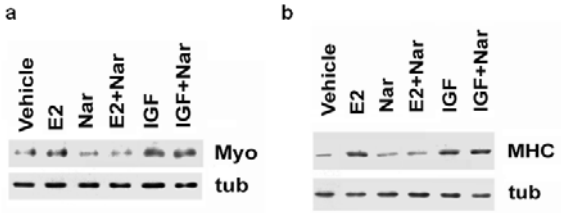


Figure 5.6: Nar effect on E2-and IGF- induced increase of myogenin and MHC level. Western blot analysis of myogenin (Myo) (a) and MHC (b) upon differentiation induced L6 cells stimulation for or 48h with vehicle or E2 (10^{-8}M) or Nar (10^{-6}M) or IGF. The data are typical Western blots of 3 independent experiments.

5.2.4 Narigenin effect on L6 cell growth

Since Nar-dependent p38 activation leads to cancerous cell apopotic death both in ERβ-expressing cells (Totta et al., 2005) and in ERα-expressing cells (Galluzzo et al., 2008), also in the presence of E2 (Bulzomi et al., 2010) we assessed the impact of Nar on differentiation medium cultured L6 myoblast growth in comparison with E2. As shown in figure 5.7 Nar stimulation (10^{-6}M 24h), as well as E2 stimulation (10 nM, 24 h) did not affect L6 cell cycle (Fig. 5.7a) nor caspase-3 activation (Fig. 5.7b), demonstrating that Nar did not influence differentiating myoblast proliferation or apoptosis.

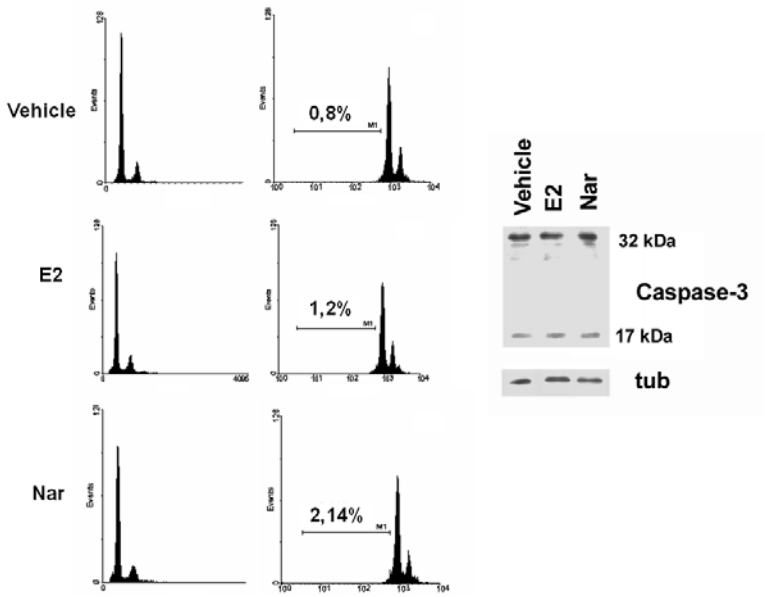


Figure 5.7: Nar effect on L6 cell growth. Flow cytometric analysis of L6 cells grown in differentiation medium with 2% serum in presence of vehicle or E2 (10^{-8}M) or Nar (10^{-6}M). The plots indicate cell cycle distribution present in G1, S, and G2/M phases, respectively (panel a). Western blot analyses of caspase-3 activation performed on un-stimulated (vehicle) and 24 h E2-treated (10^{-8}M) or Nar-treated (10^{-6}M) L6 cells growing in differentiation medium (b). The amount of proteins was normalized by comparison with tubulin level.

5.2.5 Nar effect in C2C12 cells

In order to verify Nar ability to affect E2-induced differentiation in L6 cells is not linked to this particular cellular context, and to evaluated if Nar was able to affect also the differentiation effect of the male sex steroid hormones testosterone (T) and dihydrotestosterone (DHT), some experiments have been performed in C2C12 mouse myoblasts. Figure 5.8a confirmed that this cell line express both ER isoform. Furthermore, C2C12 cells express Androgen Receptor (AR) as shown by the comparison with the prostate cancer cell DU145 (Fig.6.8b).

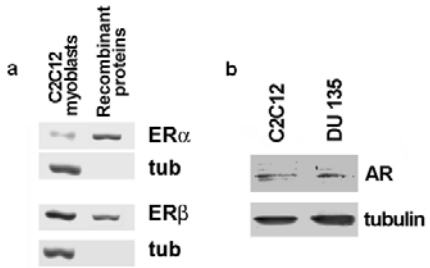


Figure 5.8: ER and AR expression in C2C12 cells. Western blot analysis of ERα and ERβ protein level in C2C12 cells and recombinant ER proteins (a) and AR protein level in C2C12 cells and DU145 cells (b).

As well as in differentiating L6 myoblast, E2 (10^{-8}M) increases myogenin (Fig. 5.9a) and MHC (Fig. 5.9b) levels in differentiation medium (2% fetal serum) coltured C2C12-myoblasts, whereas Nar (10^{-6}M) has no effect on myogenin and MHC level increase (Fig. 5.9a, b). An increase of both myogenin (Fig. 5.9a) and MHC (Fig. 5.9b) levels was observed also upon DHT (10^{-9}M) and T (10^{-9}M) stimulation, confirming the male sex steroid hormones ability to promote myoblast differentiation (Singh et al., 2003; Wannenes et al., 2008). Notably, Nar ability to block differentiation was evident only in E2-stimulated myoblast (Fig. 5.9a, b) whereas no block of myogenin and MHC increase levels was observed in Nar (10^{-6}M) and DHT (10^{-9}M) nor Nar (10^{-6}M) and T (10^{-9}M) co-stimulated differentiating C2C12 cells (Fig. 5.9a, b).

Since T effect, the common precursor of both E2 and DHT, was not blocked by Nar (Fig. 5.9a, b), it seems that in this cell lines T- induced

differentiation depends on AR presence. As confirmation of this hypothesis, C2C12 myoblast pretreatment with androgen receptor antagonist (Nilutamide, 10^{-6}M) completely prevents DHT and T-induced MHC increase (Fig. 5.9c), while cells pretreatment with estrogen receptor antagonist (ICI, 10^{-6}M), prevents only E2-induced MHC increase (Fig. 5.9c).
Collectively, these data confirm Nar as a specific E2 antagonist in ERα-mediated skeletal muscle differentiation.

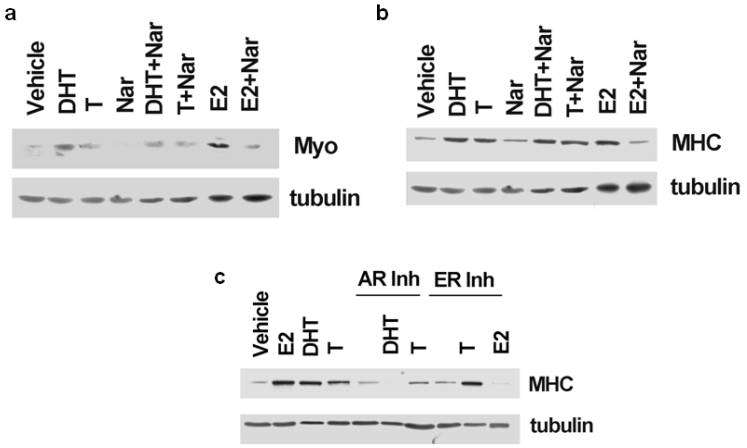


Figure 5.9: Nar effect on 17β-estradiol- and androgen- induced differentiation in C2C12 cells. Western blot analysis of myogenin (Myo, a) and MHC (b, c) levels in L6 cell maintained in differentiation medium (2%FHS) for 48 h (a, b, c) in the presence of either vehicle or E2 (10^{-8}M) or DHT (10^{-9}M) or T (10^{-9}M) or Nar (10^{-6}M). When indicated, the cells were pre-treated with the AR inhibitor (Nilutamide, 10^{-6}M) or ER inhibitor, ICI (10^{-6}M). The amount of proteins was normalized by comparison with tubulin (a, b, c) level. Data are representative Western blots of 5 independent experiments.

As above reported, MHCs are terminally differentiated muscle cells markers. In normal adult skeletal muscle fibres, four MHC isoforms may be expressed: one slow, the MHC-I, and three fast, MyHC-IIa, MyHC-IIb and MyHC-IIx/d (Schiaffino and Reggiani,1996). While *in vivo* data (Morano et

al., 1990) and *in vitro* data (Singh et al., 2003; Wannenes et al., 2008) show that DHT and T induce MHC fast isoform increase, few and conflicting data are available on E2-induced MHC isoform increase. In order to evaluate Nar effect on E2 and androgen-induced MHC isoform, differentiation medium growing C2C12 myoblasts (Fig. 5.10 a, b) and L6 cells (data not shown) have been treated for 48 h with DHT (10^{-9}M) , T (10^{-9}M), E2 (10^{-8}M) and Nar (10^{-6}M) . As shown in figure 5.10a, DHT and T increase only MHC fast levels, confirming the literature data, on the contrary, in our cellular systems E2 induce only MHC slow level increase (Fig. 5.10 b). Nar alone has not effect on any MCH isoform level (Fig. 5.10 a, b), however Nar costimulation with E2 completely prevents E2-induced increase of MHC slow level (Fig. 5.10b). On the contrary, Nar doesn't affect DHT and T dependent increase of MHC fast level (Fig. 5.10a).

All together these results clearly indicate that Nar specifically affect female sex steroid induced muscle differentiation, highlighting an endocrine disrupting action of Nar on E2-induced myoblasts differentiation.

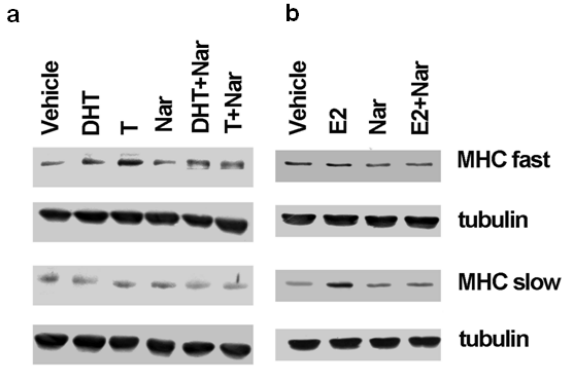


Figure 5.10: Nar effect on estrogen - and androgen- dependent MHC fast and slow increase level in C2C12 cells. Western blot analysis of MHC fast and slow levels in C2C12 cells maintained in differentiation medium (2%FHS) for 48 h in the presence of either vehicle or or DHT (10^{-9}M) (a) or T (10^{-9}M) (a) E2 (10^{-8}M) (b) or Nar (10^{-6}M) (a, b). The amount of proteins was normalized by comparison with tubulin (a, b) level. Data are representative Western blots of 5 independent experiments.

5.2.6 Nar effect on ROS production in L6 myoblasts

Several studies demonstrated a protective role of flavonoids against oxidative stress, however, as previously demonstrated (chapter 3), flavonoids are not present in the circulation at high enough concentrations to contribute significantly to total antioxidant capacity. In muscle cells, reactive oxygen species (ROS) are continually generated but when ROS levels overwhelms the antioxidant defense systems (Ji, 1995; Ji, 2001) they become cytotoxic. H_2O_2 cytotoxicity in rat myoblasts seemed correlated to the induction of apoptosis (Caporossi et al., 2003). The apoptosis of myoblasts is a physiological process during myogenesis and regeneration (Miller and Stockdale, 1986), but inappropriate myoblasts apoptosis may contribute to the pathological degeneration seen in various muscular dystrophies and spinal muscular atrophies (Adams et al., 2001).

Recent studies support the evidence for estrogens as strong antioxidant and important factor in maintaining membrane stability and protection from damaged muscle in female animals (Tiidus, 2005), thus we evaluated Nar effect, in comparison with E2, on H_2O_2-induced ROS production and the contribution of each ER isoform in mediating this putative effect.

In order to determine the pro-oxidant/antioxidant effect of E2, L6 cells were exposed to H_2O_2 (2×10^{-4}M) for 15 minutes, and then ROS generation (Fig. 5.11a) was measured evaluating the changing in DCF (2',7'-Dichlorofluorescein) fluorescence. The stimulation with E2 (10^{-8}M) for 24h before H_2O_2 addition caused a marked decrease in ROS generation preventing the H_2O_2 effect (Fig. 5.11a). The contribution of each ER isoforms in the E2 antioxidant effect has been evaluated by the use of ERα and ERβ selective agonists, PPT and DPN, respectively, and ERβ selective antagonists THC. The results show that DPN completely mimicked the E2 effect, whereas ERβ selective antagonist, THC, and the ERα selective agonist, PPT, were unable to prevent the ROS production (Fig. 5.11a). These data suggest that, even if ERβ is not primary for E2-induced L6 myoblast differentiation, ERβ is the only ER isoform involved in the E2 protective effect against H_2O_2-induced ROS production dangerous for cells. Interestingly, also Nar stimulation (10^{-6}M) for 24h before H_2O_2 addition caused a marked decrease in ROS generation (Fig. 5.11b, c). Furthermore, like E2, Nar effect is completely abolished by the use of ERβ selective antagonist, THC (Fig. 5.11c), demonstrating that Nar antioxidant activities are dependent on ERβ activities.

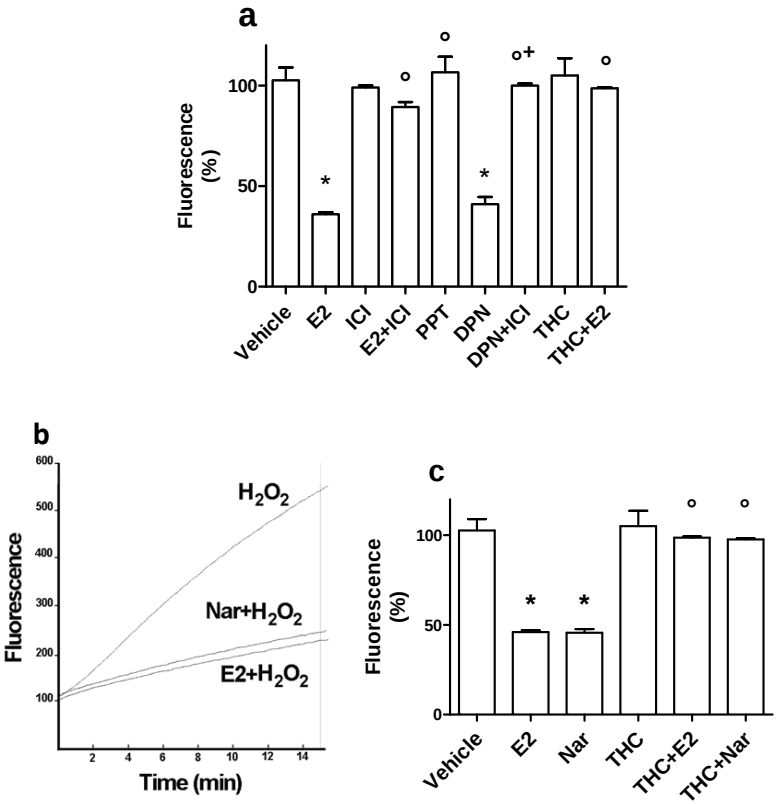


Figure 5.11: Nar and E2 effect on H_2O_2-induced ROS production in L6 myoblasts. L6 cells were pre-treated, as indicated, with vehicle, E2 (10^{-8}M) (a, c) Nar (10^{-6}M) (c) or PPT (10^{-8}M) (a) or DPN (10^{-8}M) (a), THC (10^{-6}M) (a, c) before exposition to H_2O_2 (2×10^{-4}M) for 15 minutes. Data, expressed as % of variation between H_2O_2-stimulated fluorescence versus basal fluorescence for each stimulation, are the mean ± S.D. of 3 independent experiments carried out in duplicate. $P<0.001$ was calculated with ANOVA followed by Turkey-Kramer post test comparing the samples to the vehicle- (*) or to E2- or to Nar- (°) treated samples. Panel b represent original outputs (arbitrary units) of the registrations captured by the spectrofluorimeter during 15 minutes substance administration.

5.3 Discussion

The present part of the project was aimed to determine the impact of the flavonoid Nar on E2 protective effect in non cancerous cells when both receptor isoforms are present. As well as in myoblasts growing in 10% serum (Galluzzo et al., 2009), after E2 treatment, we detected a dose- and time-dependent increase of well known skeletal muscle differentiation markers (*i.e.*, GLUT-4 membrane translocation, the transcription factor myogenin and the contractile protein MHC expression). Notably, the hormone dose response displays a bell-shaped curve, as is already known for other hormones that typically interact with plasma membrane receptors (*i.e.*, insulin, atrial natriuretic factor). The lack of effect at higher concentrations could be considered the expression of a receptor down-regulation phenomenon, by which the cells protect themselves against high hormone levels. Although to minor extent, E2 activates similar pathways as IGF-I, a hypertrophic factor for muscle cells (Jacquemin et al., 2004; Harridge, 2007). In fact, both hormones rapidly increase the AKT-dependent translocation of GLUT-4 glucose transporter. GLUT-4 translocation to the plasma membrane precedes other muscle specific protein increase (*i.e.*, myogenin and MHC) which are necessary for the appearance of the morphological muscle phenotype (Lluìs et al., 2006). This E2-induced effect, confirmed in C2C12 cells, further indicates that the hormone enhances the differentiation process in differentiation-induced myoblasts. On the contrary, Nar is not able to enhance myoblast differentiation at any of the concentration and time considered. Notably, Nar completely hampers E2 ability to induce myoblast differentiation. Since Nar acts as an E2 partial antagonist in presence of ERα and as E2 agonist in presence of ERβ in cancerous cell lines, we investigated Nar underlying mechanism of action in this cellular context expressing both ER isoforms.

As previously reported, E2-induced L6 differentiation is dependent on ER-initiated rapid signals (Galluzzo et al., 2009). Particularly, E2 rapidly induced the activation of AKT and p38/MAPK in proliferating myoblasts cells (Galluzzo et al., 2009). AKT has been linked to muscle development, regeneration, and hypertrophy (Lawlor et al., 2000; Rommel et al, 2001; Lai et al., 2004; Sandri et al., 2004) whereas p38/MAPK signaling pathway is crucial for the transcriptional control of skeletal muscle differentiation and for the fusion of myoblasts into myotubes (Lluìs et al., 2006). Although several studies suggest that the two pathways are parallel (Keren et al., 2006), the present data demonstrate that the only p38 MAPK activation, induced by E2 or Nar binding to ERβ, is not sufficient to enhance myoblast differentiation, highlighting the pivotal role of ERα in promoting E2-mediated cell myoblast differentiation. In fact, Nar, specifically blocking

ERα-mediated AKT activation, hampers E2-induced differentiation.

Notably, Nar is not able to prevent IGF induced AKT activation, and in turn has not effect on IGF-dependent myoblast differentiation. These data strongly indicate that Nar affects E2-mediated myoblast differentiation specifically hampering ERα ability to activate AKT without any direct effect on the kinase activity (Totta et al., 2004; Galluzzo et al., 2008). Thus, since Nar specifically impairs ERα-activities also in this cell system, confirming its role as an endocrine disruptors on E2:ERα regulated functions.

Despite of E2, also male sex steroid hormones have been reported to play a role in maintaining muscle mass and strength in humans. Testosterone supplementation of healthy, hypogonadal men results in muscle hypertrophy (Sheffield-Moore and Urban, 2004). Our data confirmed androgens ability to promote myoblast differentiation, however, while testosterone encourages myoblast differentiation towards the formation of fast fiber type (Type II), E2, promotes the formation of slow fiber type (Type I). Our results are in good agreement with the epidemiological data showing that female muscles are more fatigue-resistant and recover faster than male muscles (Glenmark et al., 2004) whereas, testosterone's effects on muscle are related to its dose (Bhasin et al., 1996; Bhasin et al., 2001) and the improvement of maximal voluntary strength increases after T replacement therapy (Bhasin et al., 1997). Nar only affects E2 increase of MHCI level in C2C12 myoblast, expressing both ERs and AR, without affecting androgen ability to increase MHCII level, raising the existence of a hormone-dependent susceptibility to flavonoids. Because of Nar ability to affect only E2-induced differentiation, premenopausal women consuming food rich in Nar, could incur the loss of the E2-enhanced muscle fatigue resistant. However, despite of its endocrine disrupting activity on ERα-mediated differentiation, Nar does not impair muscle homeostasis increasing apoptosis of muscle cells. On the other hand, our data demonstrate that Nar exerts a protective role on muscle, preventing reactive oxygen species (ROS)-induced damage. In muscle cells, ROS are continually generated. It is believed that these molecules have a well-established role as physiological modulators of skeletal muscle functions, including development, metabolism and contractile functions. Moreover, studies in the past two decades suggest that, during strenuous muscle activity, in some pathological conditions, or in aging, the generation of ROS in the skeletal muscle cells may be elevated to a level that overwhelms the antioxidant defense systems (Ji, 1995; Ji, 2001) and can contribute to the development of muscle fatigue, inflammation, and degeneration (Meydani et al., 1992) leading to many muscle diseases. When rat myoblasts were

exposed to moderate or high intensity H_2O_2-induced oxidative stress (5×10^{-5}-3×10^{-5}M for 1–6 h), cell death induction became evident and the activation of the apoptotic pathway could be evaluated very early (2 h) after treatment (Caporossi et al., 2003). H_2O_2 cytotoxicity in rat myoblasts seemed correlated to the induction of apoptosis, since cell loss evaluated by MTS assay was related to the percentage of Hoechst positive nuclei and to the degree of caspase-3 activation (Caporossi et al., 2003). The apoptosis of myoblasts is a physiological process during myogenesis and regeneration (Miller and Stockdale, 1986), but inappropriate myoblasts apoptosis may contribute to the pathological degeneration seen in various muscular dystrophies and spinal muscular atrophies (Adams et al., 2001). Our data, confirm that E2 exerts a protective effect on H_2O_2-induced ROS production via ERβ.

Particularly, our data demonstrate for the first time, that the ERβ activities are necessary and sufficient for preventing the H_2O_2-induced ROS production in skeletal muscle cells; while the selective ERα agonist (PPT) and ERβ selective antagonist (THC) didn't impair H_2O_2-induced ROS production, ERβ selective agonist (DPN) prevents the H_2O_2-induced ROS production, completely mimicking the E2 effect on ROS production. According to this data E2:ERβ complex-mediated cytoprotection from oxidative stress has been reported also in ARPE cells (Giddabasappa et al., 2010). Notably, Nar completely mimics E2 protective effect against ROS production. As a consequence the data here reported strongly support our hypothesis that Nar-mediated skeletal muscle protection from ROS-induced oxidative stress, at concentration achievable in the plasma, depends on its ERβ agonist activity rather than a free radical quenching compound activity.

Thus, our data indicate that, the normal intake of this flavonoid with the diet, although it can affect the E2-ehnanched muscle fatigue resistant impairing E2:ERα function, can supply E2 protective role, both in men and women, particularly post-menopausal women, counteracting the increase in free radicals production and in turn protecting muscle from ROS-induced damage via ERβ (Meydani et al., 1992).

As a whole, beside the knowledge of the mechanisms underlying the (anti)estrogenicity of dietary compounds such as Naringenin, overall these data bring to light the new important role played by ERα and ERβ in skeletal muscle. ERα and ERβ mediate different E2 effects in skeletal muscle, thus, rises the complexity of E2 actions, whose are also dependent on the relative expression of ER isoform and on the balance between the signals originated by each isoform (Galluzzo et al., 2009).

6.CONCLUSION

EDs have recently drawn immense attention to the scientific community as they are now recognized as potentially hazardous factors for human health. Over the years these concerns grew with the advancement of biochemical, biomedical, and biotechnological industries and with the increasing possibility of bioterrorism and chemical-warfare. The "man-made" EDs are found abundantly in the environment on residential buildings, cars, furniture, plastics, products such as baby feeding bottles, lining, tin-food containers, and even in children's toys, whereas the "natural" EDs, flavonoids, occur in humans exclusively trough dietary intake, representing, therefore, the most abundant "minor components" in the diet (Manach et al., 2004). Flavonoids have been studied for more than 50 yr, and now it is definite that they exert a wide range of biochemical and pharmacological effects. The most investigated effects of flavonoids refer to their cancer preventive activities, whose have been predominantly associated, among other (Laughton et al., 1991; Harmon and Patel, 2004; Moon et al., 2006), with their antioxidant properties (Amic et al., 2007).

Much of the *in vitro* studies have been conducted using pharmacological doses of flavonoids ($\geq 5\times10^{-5}$M) with little regard to the bioavailability and metabolism of the compounds studied. Even after an extensive flavonoid intake, the maximal flavonoid plasma concentrations achieved are not more than low micromolar range, in part because of rapid metabolism by human tissues and colonic bacteria (Halliwell et al., 2005). Furthermore, many of the metabolism products, such as methylated and glucuronidated forms, have a decreased antioxidant activity because of the blocking of radical-scavenging phenolic hydroxyl groups (Halliwell et al., 2005). Although the correlation between the decreased risk of developing several diseases and the antioxidant properties of flavonoids at concentration achievable in the plasma after a meal rich in flavonoid are still confusing and equivocal, the consumption of flavonoid-rich food has been usually considered beneficial for the human health and according to this hypothesis, a huge number of preparations are commercially available on the market in the form of plant extracts or mixtures, containing varying amounts of isolated phytochemicals as dietary supplements and as health food products. The commercial success of these supplements is evident, even though the cellular mechanisms involved in the flavonoid anticancer activities are still largely unknown. In addition, in the most of the studies, the experimental design did not include the effect of the endogenous hormones, thus the physiological relevance of these findings is not clear.

Last but not least, distinct action mechanisms, possibly interacting one another, for nutritional and man-made molecules on cell signaling and response can be evoked. However, until now, the relative importance of these pathways and their putative cross-talk remained to be established.

The overall aim of this thesis was to investigate the correlation between the effects of some natural compounds, alone or in presence of the endogenous hormones or in mixture with other man-made compounds, and the underlying mechanisms of action, evaluating their possible interaction.

First of all, our data demonstrated that the well known growth inhibition and cell death effects of the flavonol quercetin, one of the most frequently studied and ubiquitous bioactive flavonoid, are not related to its high-concentration requiring (5×10^{-5}M) antioxidant activity but to quercetin ability to activate, in a very small amount (i.e., 10^{-6}M), a pro-apoptotic cascade mainly modulating both ER activities. Particularly quercetin, is able to bind both ERs, and in turn to act as an E2-mimetic, leading cancer cell to apoptotic death, in tissue expressing ERβ, such as colon (Galluzzo et al., 2007), or to antagonize E2 proliferative effect in tissue where ERα isoform is expressed, such as breast (Bulzomi et al., 2010). Quercetin underlying action mechanism requires ER activity modulation: in the ERβ presence quercetin activates the same E2 pathways (both genomic and extranuclear signal), whereas in presence of ERα, quercetin only allows ERα direct transcriptional activity, impairing ERα-mediated rapid signals important for ERα-induced cell proliferation.

Thus, at nutritionally relevant concentration, quercetin antiproliferative activities depends on ER activity modulation, rather than its antioxidant activity. As evidence, the antioxidant properties of flavonoids must be considered a simplified approach to the function of molecules of nutritional interest due to the fact that their antioxidant capacities are a chemical property which is not necessarily associated to an equivalent biological function and required flavonoid concentrations by far higher than those recovered in plasma after a meal rich in vegetables (Galluzzo et al., 2009a).

Our data also demonstrate that flavonoids, such as the flavanone Naringenin, preserve their ability to induce apoptosis in ERα-expressing cancer cells also in the presence of E2.

Nar is a flavanone highly present both in glycoside and in, even if in a little amount, aglycone form in tomato skin and citrus fruits. This flavanone is one of the best absorbed in the human gastrointestinal tract, and the peak of plasma aglycone naringenin ranges from 0.7 to 14.8×10^{-6} M (Erlund et al., 2001; Bugianesi et al., 2002; Manach et al., 2004). According to the reported plasma concentration the data obtained in this thesis demonstrate

that 10^{-6} M Nar, although didn't affect E2-induced direct transcriptional activity of ERα, reverts the proliferative effect of E2 impairing ERα-mediated rapid signals and inducing different proapoptotic signal transduction pathways in ERα-expressing cancer cells.

As a whole, the assays with nutritionally relevant concentration of Nar, against a background of physiological level of E2 allowed us to elucidate Naringenin disrupting action mechanism giving a physiological meaning to the antiproliferative activity of this compound.

These results increase the list of Nar and quercetin effects on human health adding up a possible therapeutic benefit of regular consumption of these flavonoids, which may counteract the E2 proliferative action.

The importance of the chemoprotective properties of flavonoids is strongly supported also by the data obtained on flavonoid and man-made ED mixtures, whose indicate that the small amount of naringenin recovered in human plasma (Manach et al., 2004) is sufficient to counteract BPA cancer promoting effect. BPA is a monomer of polycarbonate plastics which molecules easily undergoes hydrolysis, resulting in the release of free BPA into food and beverages whose represent the main exposure route of BPA in humans (Ikezuki et al., 2002; Yamamoto et al., 2001; Newbold et al., 2009). Our data indicate that BPA, is, like Nar, a double sided action mechanism compound, which promotes tumor incidence in breast and other target organs that predominantly express ERα but inhibits the E2 protective effects in the ERβ-expressing colon cell. These two divergent aspects could act synergistically by increasing the E2-disrupting potential of this widespread environmental polluter. In light with these results women may be considered a highly susceptible population with an increased risk of colon cancers after BPA exposures. Our data, demonstrating Nar ability to revert BPA estrogenic activity in ERα expressing cancer cells and to preserve its antiproliferative activity in ERβ-expressing cancer cells, strongly support the theory of the cell fate as the resulting balance of the ED-activated pathway, highlighting the importance of investigating the chemopreventive effect of flavonoids.

Since E2 effects go beyond cell proliferation, and ERα and ERβ are coexpressed in several tissue, we assessed the impact of the Nar on E2 protective effect in non cancerous cells when both receptor isoforms are present. The data obtained on E2 and Nar mixture in skeletal muscle myoblasts, expressing both ERs, allow us to affirm that ERα and ERβ mediate different E2 effect in skeletal muscle. ERα-activated rapid signals are essential for E2-induced skeletal muscle differentiation, while ERβ-activated pathways are the only involved in the E2-protective effect from

ROS-induced oxidative stress. Nar ability to specifically affect only E2-induced differentiation raise the existence of a gender-related susceptibilities to flavonoids in the different physiological stages of life.
It has been theorized that the insurgence of different pathologies may be due to the exposition to EDs during a critical window of pre-natal development (Selevan et al., 2000). Several studies confirmed that exposure during prenatal period alter gender-specific characteristics, developmental programming, and later pubertal development without the need for a second exposure (Fenton, 2006). Since flavonoids are EDs, it could be possible that these compounds may exert prominent effects during vulnerable developmental stages as *in uterus* or during puberty where EDs may pose a risk of developing disease later in life. Unfortunately, at the present, the contribution of each ER isoform in all the tissues has not been elucidated. However, if confirmed, these data could indicate that *in utero* exposition to flavonoids could be more critical for males, whose development is mainly dependent from testosterone produced by testis in the prenatal period, whereas females represent the sex more susceptible during the pubertal and premenopausal stages, since estrogens represent the main actors in the regulation of all female physiology in these physiological stages.
As a consequence, even if the importance of investigating on the nutraceutical effect of this natural compounds is undisputable, age and sex must be take in account before the administration of flavonoids as dietary supplements and healthy products.

As a whole these data enlarge our knowledge of the mechanisms underlying the (anti)estrogenicity of dietary compounds pointing to rapid mechanisms as the most susceptible target of endocrine disruptors. In fact nor Nar or Quercetin modify direct ERE-containing promoter transcription, but decoupling ERα from rapid signals, drive cells to different destiny, highlighting the importance of investigating flavonoid effect in different stages of life that could be characterized by a different level and role of this receptor isoform. Furthermore, these data, beside the knowledge of the mechanisms underlying the (anti)estrogenicity of dietary compounds such as Naringenin and their putative interaction, bring to light a new important issue in E2 action mechanisms. The view of E2 effects as balance of the relative expression of ER isoform and as the balance between the signals originated by each isoform (Galluzzo et al., 2009), must be integrated with ERα and ERβ ability to mediate E2 different effect, in a tissue coexpressing both ERs, thus increasing the complexity of E2 action. As a consequence, also ED action is more complex than originally considered, since different ligands induce ERs to assume different conformations responsible for specific signaling pathway activation.

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APPENDIX

MATERIALS AND METHODS

Reagents

17β-estradiol (E2), Testosterone (T), dyhidrotestosterone (DHT), naringenin (Nar), quercetin (Q), Bisphenol A (BPA) insulin-like growth factor I (IGF-I), gentamicin, penicillin, and other antibiotics, GenElute plasmid maxiprep kit, 'Dulbecco Modified Eagle Medium' (DMEM, without phenol red), RPMI-1640 medium (without phenol red), charcoal stripped fetal calf serum, charcoal stripped fetal horse serum, Staurosporin (2μM) and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The XTT Assay Kit was purchased from Roche (Basel, Switzerland).The ER inhibitor ICI 182,780, the ERα selective agonist PPT (4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol), the ERβ selective agonist DPN (2,3-bis(4-Hydroxyphenyl)-propionitrile), the ERβ selective antagonist, THC (R,R)-5,11-Diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol, were obtained from Tocris (Ballwin, MO, USA). The AR inhibitor, Nilutamide was purchased from Sigma-Aldrich (St. Louis, MO, USA). The p38/MAPK inhibitor SB 203,580 (SB), the AKT inhibitor, were obtained from Calbiochem (San Diego, CA, USA). Lipofectamine Reagent was obtained from GIBCO-BRL Lifetechnology (Gaithersburg, MD, USA). Bradford Protein Assay was obtained from BIO-RAD Laboratories (Hercules, CA, USA). The monoclonal anti-phospho-ERK, anti-AKT, anti-ERα MC20 (C-terminus), and anti-ERα D12 (N-terminus), anti AR C19 (C-terminus), anti-caspase-3, anti-poly(ADP-ribose)polymerase (PARP), and anti-β-actin as well as the polyclonal anti-ERK, anti-caveolin-1, anti-Bcl-2, anti-ERα MC20 (C-terminus), and anti-ERβ L20 (C-terminus), and anti-ERβ H150 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The polyclonal anti-phospho-AKT, anti-phospho-p38, and anti-p38 antibodies were obtained by New England Biolabs (Beverly, MA, USA). The monoclonal anti-ERβ 14C8 (N-terminus) antibody was purchased from Genetex (San Antonio, TX, USA). The anti-glucose transporter type 4 (Glut-4), anti-myogenin, and anti-myosin heavy chain (MHC) were purchased from Abcam (Cambridge, UK). The anti- myosin heavy chain I (MHC slow) and the anti-myosin heavy chain II (MHC fast) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-β-tubulin was purchased from MP Biomedicals (Solon, OH, USA). CDP-Star, chemiluminescence reagent for Western blot was obtained from NEN (Boston, MA, USA). All the other products were from Sigma-Aldrich (St.

Louis, MO, USA). Analytical or reagent grade products were used without further purification.

Ligand competition analysis

Working stocks of competitors (E2, Nar and BPA) were prepared through serial dilutions in binding buffer. Dilutions of the recombinant ERα or ERβ (Panvera) were incubated for 2h at 25°C in binding buffer containing the radioligand ([^{3}H]E2, final concentration 2.0×10^{-9} M or 5.0×10^{-9} M) and the competitor (ranging between 4.0×10^{-10} M and 7.0×10^{-7} M for E2, between 1.0×10^{-7} and 4.0×10^{-4} for Nar and between 1.0×10^{-8} M and 2.0×10^{-5} M for BPA, respectively). Free and bound radioligand was separated by vacuum filtration through a 12-samples Millipore filter manifold, holding glass microfibre filters. Briefly, each filter was washed with 15 ml of pre-wash buffer prior to the addition of the binding reaction samples. Each filter was washed with 10 ml of post-wash buffer and the amount of [^{3}H]E2 retained was counted in 7 ml of scintillation fluid with a 2100TR Tri-Carb liquid scintillation analyzer. Values of the intrinsic molar fraction of E2- and Nar- bound ERα (*Y*) and E2- and BPA- bound ERβ (*Y*) were obtained at pH 7.4 and 25°C from *Y*app values according to following equation 1:$Y = Y_{app} / (1 + ([B] / H))$ where [B] is the fixed [3H]-E2 concentration (= 2 nM or 5 nM) and *H* is the equilibrium dissociation constant for [3H]-E2 binding to ERα (= 0.2 ± 0.05 nM) or to ERβ (=3.5 ± 0.05 nM. Values of the intrinsic equilibrium dissociation constant for E2 and BPA binding to ERα (*K*d) were obtained at pH 7.4 and 25 °C according to by a nonlinear four-parameter logistic model.

Cell culture

The ERs-devoid human cervix epithelioid carcinoma cell line (HeLa; Marino et al. 2002) and breast adenocarinoma cell line (MDA-MB231), the ERα containing breast adenocarcinoma cell lines (MCF-7 and T47D) were routinely grown in air containing 5% CO_2 in modified, phenol red-free, DMEM media, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml), and penicillin (100 U/ml). Cells were passaged every 2 days and media changed every 2 days.

The ERα containing hepatocellular carcinoma cell line (HepG2; Marino et al., 2001) and the ERβ containing human colon adenocarcinoma cell line (DLD-1; Marino et al. 2006c) were routinely grown in air containing 5% CO2 in modified, phenol red-free, RPMI media, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM),

gentamicin (0.1 mg/ml), and penicillin (100 U/ml). Cells were passaged every 2 days and media changed every 2 days.

The ERα and ERβ containing rat myoblasts cell line (L6, ATCC, Manassas, VA) were routinely grown in air containing 5% CO2 in modified, phenol red-free, DMEM containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml), and penicillin (100 U/ml). Cells were passaged every 2 days and media changed every 2 days. In some experiments L6 cells were treated with differentiation medium containing 2% (v/v) charcoal-stripped fetal calf serum.

The ERα, ERβ and AR containing mouse myoblasts cell line (C2C12, Prof. Daniela Caporossi, IUSM, Rome Italy) were routinely grown in air containing 5% CO2 in modified, phenol red-free, DMEM containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml), and penicillin (100 U/ml). Cells were passaged every 2 days and media changed every 2 days. In some experiments C2C12 cells were treated with differentiation medium containing 2% (v/v) charcoal-stripped fetal horse serum.

Cell stimulation, cell viability and cell cycle

Cells were simultaneously treated either with vehicle (ethanol/PBS 1:10, v/v) or different concentrations of E2 or DHT or T or Nar or Q or BPA (DMSO/PBS 1:10, v/v) or PPT (final concentration, 10 nM) or DPN (final concentration, 10 nM) or IGF-I (final concentration, 100 ng/ml). In some experiments cells were treated with E2 (10.0 nM) or BPA (final concentration, 10 μM) and different concentrations of Nar or Q (0.01 to 100 μM). When indicated, the anti-estrogen ICI 182,870 (final concentration 1 μM), or the AKT inhibitor (final concentration 1 μM), or the p38 inhibitor, SB 203580, (final concentration 5 μM) the ERβ inhibitor, THC (final concentration 1 μM), and the AR inhibitor, Nilutamide (final concentration 1 μM), were added 30 min before compound administration**.** For cell viability, cells, grown to ~60-70% confluence in 6 wells plates, were stimulated as indicated and then harvested with trypsin, centrifuged, and stained with the Trypan blue solution and counted in a hemocytometer (improved Neubauer chamber) in quadruplicate. In some experiments, cells were plated in 96-well culture plates at a density of 4,000 cells per well and stimulated as reported. After 24 h, cell growth was assessed by using the XTT reaction solution (sodium 30-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]- bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate; mixed in the 50:1 (v/v) ratio) according to the manufacturer's instructions (Roche). For the cell cycle analysis, 10^6 cells stimulated for 24 h as indicated were fixed with 1 ml ice-

cold 70% ethanol and subsequently stained with 2 mg/ml DAPI/PBS solution. The fluorescence of DNA was measured with a DAKO Galaxy flow cytometer (DAKO Cytomation, Glostrup, Denmark) equipped with HBO mercury lamp and the percentage of cells present in sub-G1, G1, S, and G2/M phases was calculated using a FloMax©Software (DAKO, Glostrup, Denmark).

Plasmids

The reporter plasmid containing the promoter of complement component 3 gene, retaining a natural estrogen responsive element (ERE), linked to the gene of luciferase (pC3), the reporter plasmid containing the promoter of cyclin D1 pXP2-D1K2966-luciferase (pD1), the expression vector pCR3.1-β-galactosidase, wild type human ERα pSG5-HE0, and human ERβ (pCNX2-ERβ) have been described elsewhere (Herbert et al. 1994, Marino et al. 2002, Acconcia et al. 2004, 2005a). A luciferase dose-response curve showed that the maximum effect was obtained when 1.0 μg of plasmids was transfected together with 1.0 μg of pCR3.1-β-galactosidase to normalize for transfection efficiency (approximate 55–65%). Plasmids were purified for transfection using the GenElute plasmid maxiprep kit according to the manufacturer's instructions.

Transfection and luciferase assay

HeLa and HepG2 cells were grown to ~ 70% confluence and then transfected using lipofectamine reagent according to the manufacturer's instructions. Six hours after transfection, the medium was changed and 24 h after the cells were stimulated with 10.0 nM E2 or Nar 1 μM or Q 1 μM or BPA 10 μM for 6 h. The cell lysis procedure as well as the subsequent measurement of luciferase gene expression was performed using the luciferase kit according to the manufacturer's instructions with an EC & G Berthold luminometer (Bad Wildbad, Germany).

True-blot immunoprecipitation

After E2 (10nM) or BPA (10μM) stimulation, DLD-1 cells were washed in ice-cold PBS, harvested by with trypsin (1%, v/v), and lysed in 50 ml lysis buffer, and lysed in 50 μl lysis buffer [10.0 mM Tris, pH 7.5, 1.0 mM EDTA, 0.5 mM EGTA, 10.0 mM NaCl, 1% (v/v) Triton X-100, and 1% (w/v) sodium cholate] containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1.0 μg/ml leupeptin, and 5.0 μg/ml aprotinin).

The cell lysates were clarified by centrifugation and immunoprecipitated with TrueBlot™ (eBioscience, San Diego, CA, USA) which preferentially detects the native disulfide form of mouse or rabbit IgG, reducing interference by the ~55 kDa heavy and ~23 kDa light chains of the immunoprecipitating antibody. Briefly, after stimulation equal amounts of soluble cell extracts were incubated with either 2.0 µg of anti-caveolin-1 or anti-14C8 ERβ antibody. The lysates and antibodies were incubated at 4°C for 1 h, then 20 µl of Anti Mouse or Anti Rabbit IgG Beads (eBioscience, San Diego, CA, USA) were added and samples incubated for 1 h on a rocking platform at 4°C. Samples were centrifuged at 10,000×g for 10 min, the supernatant was removed completely and beads (pelleted) were washed 3 times with 100 µl of lysis buffer. SDS-Reducing sample buffer (20 µl, containing 50 mM DTT) were added and samples were boiled at 100°C for 5 min. Proteins were resolved using 7 or 10% SDS-PAGE at 100 V for 1 h and then electrophoretically transferred to nitrocellulose for 45 min at 100 V at 4°C. The nitrocellulose was treated with 5% (w/v) non-fat dry milk (BIO-RAD Laboratories, Hercules, CA, USA) in 150 mM NaCl, 50.0 mM Tris HCl (pH 8.0), 0.1% (w/v). Tween-20, and then probed at 4°C overnight with either 2.0 µg of anti-caveolin-1 or or anti-p38 or anti-phospho p38 or anti-ERβ antibodies. The antibody reaction was visualized with the chemiluminescence reagent for Western blot (Amersham Biosciences, Little Chalfont, UK).

Lysate preparation, electrophoresis and immunoblotting

After treatments, cells were lysed and solubilized [in 0.125 M Tris, pH 6.8, containing 10% (w/v) SDS, 1.0 mM phenylmethylsulfonyl fluoride, and 5.0 lg/ml leupeptin] and finally boiled for 2 min. In some experiments cells were homogenized by using Teflon pestle homogenizer until about 90% of the cells were broken.; homogenates were centrifuged at 1,000x g for 10 min to pellet the nuclear fraction. Membrane rich fractions were obtained by centrifuging the supernatants at 100,000x g for 30 min. Proteins were then solubilized as described above. Total proteins were quantified using the Bradford Protein Assay. Solubilized proteins (20 µg) were electrophoretically resolved by SDS–PAGE (7.5-10%) at 100 V, 1 h, 24 °C and then transferred to nitrocellulose for 45 min at 100 V and 4°C. The nitrocellulose membrane was treated with 3% (w/v) BSA in 138.0 mM NaCl, 25.0 mM Tris, pH 8.0, at 24 °C for 1 h and then probed overnight at 4 °C with specific antibodies (anti-ERα or anti ERβ, or anti-AR, or anti-GLUT-4, or anti-Myogenin or anti-MHC, or anti-MHC slow or anti-MHC fast, or antiphospho-ERK or anti-phospho-AKT or antiphospho-p38 or anti-

caspase-3 or anti-PARP or anti-Bcl-2 antibodies). The nitrocellulose membrane was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical Company, Rockford, IL, USA) for 10 min at room temperature and then probed with anti-α-tubulin or anti-caveolin-1 or β-actin antibodies to normalize total lysate or membrane fractions respectively. Moreover, the nitrocellulose incubated with anti-phospho-ERK, anti-phospho-AKT, anti-phospho-p38 was stripped and probed with anti-ERK, anti-AKT, and anti-p38, respectively. To evidence ERα and ERβ levels electrophoreses were performed in the presence of 5 ng of recombinant ERα and ERβ; moreover, a standard curve of recombinant proteins showed that the band intensity was proportional to the protein amount. Antibody reaction was visualized with chemiluminescence Western blotting detection reagent (Amersham Biosciences, Little Chalfont, UK). Densitometric analyses were performed by ImageJ software for Windows.

Measurements of reactive oxygen species (ROS)

ERα-transfected Hela cells or L6 cells were grown to ~70% confluence and treated as indicated, then were harvested, and re-suspended in PBS with 10 μmol/l dichlorodihydrofluorescein diacetate (DCF; Molecular Probes, Eugene, OR, USA) for 30 min at 37°C in the dark. After additional 30 min, to allow the equilibrium, the fluorescence was measured under continuous gentle magnetic stirring at 37° C in a Perkin-Elmer LS-50B spectrofluorimeter. Excitation wavelengths were set at 498 nm and Emission at 530 nm, respectively. The fluorescence was registered as arbitrary unit for 15 min. In some experiments 600 μmol/l H_2O_2 (final concentration) was added after quercetin stimulation and fluorescence was registered after 15 min.

Statistical analysis.

A statistical analysis was performed by utilising Student's t test with the INSTAT software system for Windows. Some data were analyzed by one-way ANOVA and post hoc Bonferroni's test or Turkey-Kramer post test (INSTAT software system for Windows). In all cases probability (P) values below 0.05 were considered significant.