Biochimica et Biophysica Acta 1812 (2011) 1054–1060

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Review

Liganded and unliganded activation of estrogen receptor and hormone replacement therapies ☆

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ARTICLE INFO

Article history:
Received 11 January 2011
Received in revised form 29 April 2011
Accepted 1 May 2011
Available online 14 May 2011

Keywords:
Estrogen receptor
Hormone replacement therapy
Transcriptinal oscillation
Estrogen receptor pharmacology

ABSTRACT

Over the past two decades, our understanding of estrogen receptor physiology in mammals widened considerably as we acquired a deeper appreciation of the roles of estrogen receptor alpha and beta (ERα and ERβ) in reproduction as well as in bone and metabolic homeostasis, depression, vascular disorders, neurodegenerative diseases and cancer. In addition, our insights on ER transcriptional functions in cells increased considerably with the demonstration that ER activity is not strictly dependent on ligand availability. Indeed, unliganded ERs may be transcriptionally active and post-translational modifications play a major role in this context. The finding that several intracellular transduction molecules may regulate ER transcriptional programs indicates that ERs may act as a hub where several molecular pathways converge: this allows to maintain ER transcriptional activity in tune with all cell functions. Likely, the biological relevant role of ER was favored by evolution as a mean of integration between reproductive and metabolic functions. We here review the post-translational modifications modulating ER transcriptional activity in the presence or in the absence of estrogens and underline their potential role for ER tissue-specific activities. In our opinion, a better comprehension of the variety of molecular events that control ER activity in reproductive and non-reproductive organs is the foundation for the design of safer and more efficacious hormone-based therapies, particularly for menopause. This article is part of a Special Issue entitled: Translating Nuclear receptors from health to disease.

1. Introduction

In all metazoans, the ability of nuclear receptors (NR) to regulate large transcription gene programs provides a critical strategy for the control of complex physiological processes such as reproduction, development and homeostasis; this may explain why dysregulation of NR functions is associated with a large variety of diseases.

Among the NR gene family, the two mammalian estrogen receptors, estrogen receptor alpha (ERα, ESR1, NR3A) and estrogen receptor beta (ERβ, ESR2, NR3b) [1], are phylogenetically very ancient as are expressed in non-vertebrates as well as in vertebrates [2]. The complexity of ER mechanisms of activation and functions suggests that during the evolution these proteins were implicated in a variety of functions which stratified with time and are still functioning in vertebrates. Structurally similar to all nuclear receptors, ERs are composed of six functional domains (named A-F) [3] and are generally classified as ligand-dependent transcription factors because, after the association with their specific ligands, they bind specific genomic sequences (named Estrogen Responsive Elements, or EREs) and interact with co-regulators to modulate the transcription of target genes. Several lines of evidence showed that the unliganded ER may be transcriptionally activated by selected post-translational modifications (PTM). In addition to their capability to modulate the activity of selected promoters directly, the liganded or unliganded ERs regulate several intracellular pathways by molecular interference with other signaling molecules present in the nucleus (e.g. transcription factors like NF-Kb or AP-1) or in the cytoplasm (e.g. IP3K, G proteins and others) [4]. Because of their widespread expression and the variety of interactions with extracellular as well as intracellular signaling molecules it is conceivable that ERs may help to adjust single cell functions in relation with the overall body homeostasis. Indeed, ER ablation or dysregulation is associated with altered functions of several systems including the reproductive [5], cardiovascular [6,7], skeletal [8,9] immune [10] and nervous systems [4,11,12].

2. Mechanisms of ER transcriptional activation

2.1. Hormone-dependent

Transcriptional activation by ERs is a multistep process, occurring in a sequential order, that requires the interaction of the receptor with a wide variety of primary and secondary enzymatic activities to obtain

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doi:10.1016/j.bbadis.2011.05.001

a productive interaction with the entire transcriptional machinery. ERs are generally maintained inactive by specific inhibitory proteins which must be removed to enable the ER-dependent transcriptional activity. Ligand-operated transcription by ERs is initiated by the binding of estrogenic compounds to the inactive ER-chaperon complex. The ligand binding occurs at the ER hormone binding domain (HBD) located in the C-terminus E region. The HBD consists of 12 α-helices arranged as a three-layered anti-parallel α-helical sandwich that forms the hydrophobic site to which the ligand binds. The accommodation of the ligand causes a reorientation of helix 12 toward the opening of the HBD allowing helices 3,5 and 12 to generate a novel activation function (AF) domain consisting of a hydrophobic grove on the LBD binding surface [13,14]. The ligand-dependent allosteric alteration mediates the dissociation of ER from its chaperones/nuclear matrix-associated binding proteins [15] unmasking the domains for receptor dimerization, nuclear localization, binding to the EREs (DBD, C region) and binding to other transcription proteins. Thus, by shedding the chaperons, ER enhances its ability to homo- or hetero-dimerize [16], changes the dynamics of partitioning between nuclear matrix and chromatin [17] and increases the intramolecular interactions thus strengthening the stability of ER interactions [18] with the ERE and the co-activators; this ultimately leads to the recruitment of the enzymatic complex necessary for transcription initiation.

Once bound to the DNA, ER transcriptional activity is dependent on two activating functions (AFs): AF-1 in the receptor's N-terminus A/B region and the AF-2 in the E region. The AF-1 operates in a ligand-independent mode, while AF-2 is ligand-dependent [19,20]. Although the two domains can function independently, the maximal ER transcriptional activity is achieved when there is synergy among the two AFs [21]. These sites are field of interaction for a large number of co-activator proteins. The presence of co-activators facilitates the interactions between the NR and the transcriptional machinery. The co-activators bind to the ligand-activated receptor through a highly conserved signature motif LXXLL termed the “nuclear receptor box” [22]. Extensive studies of the binding affinities of the members of the p160 family of co-activators and other chromatin modifiers (e.g. the histone acetyltransferase p300) indicate that p160 proteins (steroid receptor co-activators: SRC-1, SRC-2 and SRC-3) act as bridging proteins for the assembly of the complex regulatory framework required for the chromatin modifications and synthesis of the primary transcript [23,24]. The kinetics of the ERα binding to the pS2 promoter have been extensively studied in MCF-7 cells where the punctual interactions of the liganded receptor with all the components of the transcriptional apparatus as well as with co-regulators were described [17,25,26]. The ERα–ERE association facilitates the recruitment of very specific enzymatic activities necessary for chromatin remodeling (e.g. histones H3 K14 and H4 R3 acetylases or HATs, and methylases or HMTs) and, with a short lag period, the components of the preinitiation complex and Pol II. DNA transcription initiates with cycles of about 40–45 min in which the system becomes periodically refractory to the activated ERα with alternate activating and repressing epigenetic processes. In these cycles, ERα undergoes protein modifications leading to disassembling of the transcription complex and receptor degradation. This oscillations at the promoter is believed to be necessary to poise ER-dependent transcriptional activity to the mutable requirements of the cell metabolism [17].

A constellation of PTM (phosphorylation, acetylation, methylation, sumoylation) regulates ER activities prior and after its binding to the DNA modulating its ability to operate also in relation to the cell and whole organisms cues (Table 1). For instance, ERα phosphorylation may occur at 10 different serine/threonine/tyrosine residues and was shown to be necessary for receptor dimerization and recruitment of specific transcription factors such as p160 co-regulators and chromatin remodeling enzymes [27,28]. In addition, phosphorylation of Ser 305 was shown to facilitate the receptor acetylation required for the facilitation of DNA binding and ligand-induced transcriptional activity [29]. Interestingly, acetylation lys 266–268 increases ER activity, whereas the same PTM at Lys 299, 302 and 303 is associated with a decrease of ER-dependent transcription [30,31]. ERα palmitoylation anchors a pool of ERα to the plasma membrane where it may interact with other signaling proteins (such as Src or PI3K) [32]. Methylation in the hinge region of ERα (Lys 302 induced by the lysine methyl transferase Set7) increases the receptor stability [33], however when the methylation of ERα occurs at the Arg 260 residue, it facilitates the interactions with other signaling molecules such as Src or PI3K [34]. ERα ubiquitination may occur both on inactive receptor and on the receptor actively engaged in transcription. In the first case, it is responsible for the clearance of the misfolded protein; in the second, it causes inhibition of ERα degradation and decreases ERα [35,36] transcriptional activity. This latter observation indicates that the stabilization of the protein may be relevant for the regulation of the efficiency of ER-dependent transcription. Finally, ERα may be target of small ubiquitin-like modifiers (SUMO) enzymes that induce the covalent attachment of SUMO to Lys residues, this modification generally does not induce protein degradation, but facilitates interactions with other proteins. In the case of ERα, sumoylation occurs at two Lys in the hinge region (Lys 266 and Lys 268) following activation by the ligand and their mutation results in decreased receptor activity [37].

2.2. Hormone-independent

PTM of ERs are relevant for the transcriptional activation of unliganded ER as demonstrated by several groups following the initial observation by O'Malley's group who first reported that, in the absence of the cognate ligand, the progesterone receptor could be transcriptionally activated by phosphorylation [66]. ERα was shown to be activated by epidermal growth factor (EFG) [67]; IGF-1 [43,68] and neurotransmitters like dopamine, via cAMP/PKA activation [69,70]. However, in spite of the solid evidence demonstrating the possibility of ER transcriptional activation in the absence of ligands, the characterization of the mechanisms involved in this process has proven quite difficult. Initial studies in transfected cells pointed to the relevance of selected kinases (MAPK, PKA and p21 ras/ERK) [42,60,71–73] for ER activation; furthermore, the use of mutants of ERα showed that in each cell system specific ER phosphorylation sites were necessary for the unliganded ER activity (e.g. Ser 118 in COS-1 cells and Tyr 537 in neuroblastoma cells) [45]. This observation indicated that unliganded ER activation might fulfill a variety of cell-specific functions. Yet, in some cases, the direct phosphorylation of ERα was reported to be insufficient by itself for the transcriptional activation of the receptor even if allowed the recruitment of co-regulators and splicing factors [46]. More recently, in MCF-7 cells it was shown that for PKA-induced activation of the unliganded ERα the direct phosphorylation of the receptor is dispensable, but the phosphorylation of the coactivator-associated arginine methyltransferase 1 (CARM1) at a single serine is sufficient for its direct binding to the HBD of the receptor and its activation [74].

Several studies have underlined the importance of unliganded ERα activation in mammalian physiology: it was shown that growth hormone/neurotransmitter-dependent activation of ER is required in reproductive and non-reproductive organs (such as IGF-1 growth of the uterine epithelium or neonatal behavior modulated by dopamine in specific brain areas) [75] and that aberrant mechanisms of unliganded ERα activation may underpin the development of ER-positive cancer cells towards resistance to endocrine therapy [76]. The use of ERE-Luc reporter mice further underscored the concept of independence of ER activity from circulating estrogens, rather showing that activation of ERs by other signaling pathways such as growth factors and their intracellular signal transducers may play the dominant role in non-reproductive organs like bone and brain [77]. It

Table 1
ER post-translational modifications.

PTM	Domain	Kinase	Function	Ref.
ERα phosphorylation				
Ser 102, 104, 106	N-terminus (AF-1)	glycogen synthase kinase-3 (GSK3)	transcription activation	[38]
Ser 102, 104, 106		Cyclin A CDK2	AF-2 independent transcription activation	[39]
Ser 104 and 106		TPA-dependent	trancription activation	[40]
Ser 118		p42/p44 MAPK; CDK7	E2 and Tamoxifen-dependent ER activation	[41]
Ser 118		CDK7	ligand-dependent interaction with TFIIH/CDK7	[42]
Ser 118			apo-ER transcriptional activation	[43]
Ser 118		MAPK	recruitment of p68 helicase	[44]
Ser 104, 106, 118		MAPK	ligand-dependent dimerization	[45]
Ser 104, 106, 118		MAPK	ER mRNA splicing	[46]
Ser 104, 106, 118		growth factors	ligand-dependent and independent binding to p160 coactivators	[47]
Ser 154		unknown	unknown	[28]
Ser 167		casein kinase II	activation of unliganded receptor	[48]
Ser 167		p90 ribosomal S6 kinase 2 (Rsk2)	activation of unliganded ER	[49]
Ser 167		Akt, MAPK	ligand-independent rapid signalling	[50]
Ser 236	DBD	PKA	ER dimerization and DNA binding	[51]
Ser 236		PKA	ligand-mediated receptor degradation	[52]
Ser 305	LBD (AF-2)		negative regulation of ER acetylation	[29]
Ser 305		p21 kinase	increased transactivation	[53]
Ser 559		CK2	Transcription inhibition	[54]
Thr 311	LBD	p38 prot kinase and MEKK	regulation nuclear export and inhibits p160 interactions	[55]
Tyr 52, 219		c-Abl	Transcription activation	[56]
Tyr 537	LBD (AF-2)	src	cell proliferation	[57–59]
Tyr 537	LBD (AF-2)	MAPK	hormone-independent ERA activation	[60]
ERß phosphorylation				
Ser 94, Ser 106	N-terminus (AF-1)	Erk	ubiquitination and degradation of unliganded ERb	[61]
Ser 106,124		AKT	activation unliganded receptor and SRC-1 interaction	[62]
Ser 106,124		PKA	ERb activation	[63]
Ser255	hinge region	AKT	inhibits Erb activity by inhibiting CBP interaction	[62]
ERα acetylation				
Lysine 266; 268		p300	stimulates DNA-binding and ligand-dependent activity	[30]
Lysine 299, 302, and 303		p300	diminishes response to agonists	[31]
ERα methylation				
Arg 260				[34]
Lysine 302		SET7methyltransferase	ER stabilization	[64]
ERα ubiquitylation				
Lysines 302-534			Ligand-independent ubiquitination	[65]
Residues 535–595 (lysine 581)			Ligand-dependent ubiquitination and receptor degradation	[35]
ERα sumoylation				
Lysines 266; 268	hinge region	PIAS1 and PIAS3	ligand-dependent activity	[37]

is perhaps not surprising that ERs are regulated in this manner, as they are a member of a large family of transcription factors that evolved from ancestors that were unable to bind ligands [78] and relied on other common regulatory signals like phosphorylation. Even today, other nuclear receptors, in particular the estrogen receptor related proteins, may lack natural endogenous ligands, suggesting that the regulation of NR activities by alternative mechanisms is likely to be functionally important.

The functional significance of unliganded ER activation still requires further investigation to be fully understood. The systematic, comparative, analysis of the cistrome related to ER activity in different cell systems after activation by PTM or interaction with estrogen will clarify the role of these alternative ways to activate ER which plays a role in the selection of targets [79].

3. ERα and ERβ: why two receptor for a single hormone?

Intracellular ER exists in two forms ERα and ERβ which are transcribed from different genes, but have a strong structural similarities, particularly in the DBD (97% amino acid identity) and HBD (56% amino acid identity); lower homology is found in the A/B amino-terminus (about 20% identity) [3]. The two ERs have different cellular and tissue distribution and activities: studies with animals in which each of the two receptors were ablated and with selective ERα and ERβ ligands show that after 17β-estradiol (E2) activation the two ER isoforms have a differential effect both at systemic and cellular level: ERα is essential for reproductive development and functions while ERβ activity is more relevant in non-reproductive organs in spite of the fact that its presence and activity in the granulosa cells which contributes to provide full fertility to mice [5]. The mechanisms responsible for these differential physiological effects are still matter of study. It is well known, that despite similar *in vitro* E2 and DNA binding properties, ERβ has a substantially lower transcriptional activity than ERα [80,81]: the underlying mechanism for such differences is not fully characterized, undoubtedly, the absence of a strong AF-1 domain in the ERβ A/B domain may explain the lower transcriptional capacity of this isoform. It was also proposed that ERβ contains a repression domain within its amino-terminus lowering the efficiency of this receptor protein [82].

In several cell systems the two isoforms are co-expressed, often at concentrations which may be significantly modified by physiopathological events [7,83]. Several studies have addressed the issue of specificity of action of ERα and ERβ and questioned their ability to recognize specific ERE binding sites in the homo- and hetero-dimeric form [16,84]. A recent study on the genome-wide dynamics of ER chromatin binding has finally provided a very helpful view on the intracellular interactions of the two ER by demonstrating that each ER subtype when present alone bind most of the EREs available, but when both are expressed there is a mutual competition restricting significantly the number of the sites shared and a shifting of each ER to new sites. Interestingly, when co-expressed, ERα has a predominance on ERβ because it occupies most of the common sites causing a major shift of ERβ to novel EREs. A potential explanation for these findings is associated to the different affinities for the EREs of the ERα

homodimers (higher affinity), heterodimers (medium affinity) and ERβ homodimers (lower affinity), which increases the residence time of ERα homodimers on its DNA target, however several studies showed that each of the two isoforms associates preferentially with different co-regulators, it is therefore conceivable that in each cell system each isoform adopts a specific set of co-modulators to best fulfill their cell specific functions [85,86].

Thus the differential expression of the two receptor isoforms may constitute a mechanism for the modification of the overall transcriptional ability of ERs in response to estrogens: this view is in line with the initial speculations of ERβ role as a modulator of ERα proliferative action in specific tissues (e.g., mammary cells) [87]. More recently, the finding that unliganded ERβ may interact SRC-1 inducing ligand-independent transcription and has the tendency to reside on the DNA bound to co-repressors [88] led to further speculations on the differential responsibilities of the two receptor isoform within each target cell. ERα would be the primary target for fluctuating levels of circulating estrogens, while ERβ could be a permissive regulator of estrogen-responsive genes via ERE-independent nuclear/cytoplasmic signaling pathways. However this further hypothesis does not take into account the fact that also ERα may be transcriptionally activated in the absence of the ligand.

4. Achieving a cell-specificity of action

One of the most striking features of ERs is their wide distribution in the mammalian organisms and the variety of functions fulfilled in each cell system. So far, at least eight distinct mechanisms contributing to ER diversification of functions may be envisaged: 1) the epigenetic events that select the EREs transcriptionally available in each cell system; 2) the cell-specific expression of the co-regulators and enzymes necessary for ER activation/inactivation; 3) the stoichiometry of ERα and ERβ; 4) the presence of truncated versions of ERs due to differential transcriptional initiation at ER promoter or to alternative splicing; 5) the levels of circulating estrogens; 6) the composition of circulating estrogens each having a differential ability to induce a allosteric conformation of the LBD; 7) the levels of paracrine or endocrine hormones other than estrogens able to regulate ER transcriptional ability at the promoter of each target gene; 8) the complexity of the promoter itself that may significantly alter the receptor actions. The number of combinations in which these events may occur provides a clear picture of the potential multiple outcomes of ER activation and underlines the difficulties we are encountering in the definition of efficacious replacement therapies with either synthetic or natural estrogens.

Yet a question arises: if these receptors have so many disparate functions why the ablation of one or both ERs is not lethal? In view of the evolutionary age of these proteins we speculate that in less evolved organisms ERs were common transcription factors activated by the signaling of membrane receptors (growth factors, neurotransmitter or cytokines receptors). During evolution, membrane receptors might have acquired more specialized pathways for their intracellular signaling, but ERs were maintained as salvage pathway. At the same time, novel synthetic pathways evolved to generate ligands able to bind ERs and to convert them into ligand-operated transcription factors able to modulate novel target genes. How ERs gained the control over reproduction, the most important of the biological functions, has not been object of intense study so far. However, if we believe that in multicellular organisms these proteins were originally involved in the transduction of most signals regulating cell homeostasis, we might speculate that the selection of ERs as regulator of reproduction was favored by their ability to sense and respond to a variety of homeostatic and metabolic signals. Because of that, ERs were able to ensure that pregnancy occurred only under environmental and metabolic favorable conditions. If this was the case, the understanding of the evolutionary cues that favored the selection of ER as the key element in reproduction might constitute a significant advancement in our insights on ER physiology and, consequently, in our ability to conceive hormone replacement therapies (HRT) much more efficacious than those currently available [52].

5. Could exogenous administration of estrogens ever substitute for the ovarian functions?

In the field of estrogen action, the assessment of the necessity and usefulness of HRT remains as one of the most unanswered questions. Clinical and epidemiological studies demonstrated that the natural or surgical cessation of ovarian functions is associated with increased risk of incidence of cardiovascular, skeletal, immune and neurological pathologies [6–12]. However, to date, any attempt to reinstate the beneficial effects of the cyclic hormone production by HRT has not provided the desired results [89]. The major obstacle encountered with the administration of natural estrogens is associated with excessive cell proliferation in reproductive organs. Indeed, HRT with estrogenic compounds causes uterine hyperplasia and an increased risk of mammary, uterine and ovarian cancer [90]. The finding that synthetic estrogens such as tamoxifen were able to bind ERs and cause unique allosteric conformations enabling the receptor to interact efficiently with the transcription machinery only in selected tissues led to propose the use of these Selective Estrogen Receptor Modulators (SERMs) for a safe hormone replacement therapy [91]. This initiated a race for the identification of the wonder molecule able to activate ER only in non-reproductive tissues thus sparing the negative effects of HRT in the reproductive organs. Interestingly, none of the synthetic molecules so far identified proved to be the so desired magic bullet, generally because none of the molecules synthesized proved to be antagonist uniquely in the reproductive organs.

More recently, to overcome the proliferative effects associated with HRT, attempts were made to exploit the differential physiology of the two ERs and to develop selective agonists for ERβ. The ERβ selective agonists so far developed belong to three classes: the first is represented by ERB-041 (WAY-202041) which binds to ERβ much better than ERα; the second derived from plants (MF101, nyasol and liquiritigenin) bind similarly to both ERs, but activate transcription only with ERβ; the third diarylpropionitrile which selectivity is due to a combination of greater binding to ERβ and high transcriptional activity. So far, the data available on the biological/pharmacological activities of these compounds are too limited to draw any definitive conclusion on their suitability for HRT.

The selective ERα and ERβ ligands are a very important tool to decipher the roles of ERα and ERβ in different organs and to elucidate how ligands, acting through either of the two ERs, can prevent or treat various age- or sex-specific diseases. Appropriate clinical studies are necessary to validate these compounds as agents for the prevention and treatment of diseases [92].

Our group recently proposed a novel strategy aimed at the study of HRT which is based on the selection of molecules able to mimic at the systemic level the oscillatory state of ER activation observed in healthy females in the reproductive age [93,94]. The availability of the ERE-Luc reporter mouse enabled to follow temporally, by means of *in vivo* imaging, ERs transcriptional activity at systemic level. This model enabled to demonstrate that in each organ ER activity oscillates in time with a periodicity of about 4 days that is independent from the ovarian production of estrogens. In fact, in the various organs, ER activity on the promoters of the reporter as well as of endogenous genes is asynchronous [69,94,95]; furthermore stress (e.g. calorie restriction), or changes in the reproductive state (e.g. ovariectomy, lactation, pregnancy) affect significantly the extent of ER oscillatory activity in terms of frequency and amplitude [94]. Altogether, these observations suggest that factors other than circulating estrogen may activate ERs and indicate that ER transcriptional activity adapts to the overall state of animal health.

If the hypothesis that evolution selected ER as reproduction controller because of its capacity to interact and react to most homeostatic cues were true, we would predict that ovarian hormones do not regulate ER activity, particularly in non-reproductive organs. In reality, we would expect changes of ER activity in relation to endogenous and exogenous signals affecting body homeostasis. More research is needed to clarify how, in each organ, the changes in the frequency of ER activity influence ER-dependent gene expression programs and which are the hormonal cues networking ER activity at systemic level. It is likely that an efficacious HRT should reproduce the ultracircadian ER oscillations typical of a healthy cycling organism.

To verify the extent to which the oscillatory activity found in healthy young fertile mice could be mimicked by the administration of a synthetic estrogen, we performed a series of long term (21 days) treatments of ovariectomized female mice with most of the known ER ligands. The goal was therefore to assess whether any of these treatments could reproduce a state of systemic ER activity comparable to cycling mice. We demonstrated that each ligand was characterized by a specific *spatio*-temporal profile of ER transcriptional oscillations. Further analysis of the data obtained by means of agglomerative hierarchical clustering proved a strict correlation between the structure of the compound tested and their effect on ER oscillation. In addition, the clustering analysis allowed the identification of families of compound more likely to reproduce the effect of endogenous hormones, thus paving the way to a novel methodology to identify novel chemical entities suitable for HRT.

6. Conclusions

The results of the last years of study have demonstrated an unsought complexity of ER action at both cellular and systemic level, possibly providing an explanation for the difficulties found in the design of appropriate HRT. These difficulties appear to be shared with a number of other members of this important family of receptors which revealed to be very complex targets for drugs, an example being the PPAR ligands which revealed an unexpected amount of side effects. The complexity of action of NR may require a revisitation of the methodologies generally applied in drug discovery programs to take into account the multiplicity of effects that ligands binding to NR may induce at systemic level. From this standpoint the application of *in vivo* imaging might provide novel opportunities of analysis particularly for their ability to measure NR action in space and time in living organisms [94].

Acknowledgments

Work in A. Maggi's lab is supported by EU (STREP EWA LSHM-CT-2005-518245 and CRESCENDO LSHM-CT-2005-018652); NIH (RO1AG027713); Pharmaceutical Company Pfizer.

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