

INVITED REVIEW

Non-genomic actions of sex steroid hormones

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Abstract

Steroid hormone receptors have been traditionally considered to act via the regulation of transcriptional processes, involving nuclear translocation and binding to specific response elements, and ultimately leading to regulation of gene expression. However, novel non-transcriptional mechanisms of signal transduction through steroid hormone receptors have been identified. These so-called 'non-genomic' effects do not depend on gene transcription or protein synthesis and involve steroid-induced modulation of cytoplasmic or cell membrane-bound regulatory proteins. Several relevant biological actions of steroids have been associated with this kind of signaling. Ubiquitous regulatory cascades such as mitogen-activated protein kinases, the phosphatidylinositol 3-OH kinase and tyrosine kinases are modulated through non-transcriptional mechanisms by steroid hormones. Furthermore, steroid hormone receptor modulation of cell membrane-associated molecules such as ion channels and G-protein-coupled receptors has been shown. Tissues traditionally considered as 'non-targets' for classical steroid actions are instead found to be vividly regulated by non-genomic mechanisms. To this aim, the cardiovascular and the central nervous system provide excellent examples, where steroid hormones induce rapid vasodilatation and neuronal survival via non-genomic mechanisms, leading to relevant pathophysiological consequences. The evidence collected in the past years indicates that target cells and organs are regulated by a complex interplay of genomic and non-genomic signaling mechanisms of steroid hormones, and the integrated action of these machineries has important functional roles in a variety of pathophysiological processes. The understanding of the molecular basis of the rapid effects of steroids is therefore important, and may in the future turn out to be of relevance for clinical purposes.

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Introduction

Steroid hormone receptors (SRs) are transcription factors regulating the expression of target genes (1). Binding of steroid hormones to the specific SR ligand-binding domain (LBD) induces a conformational modification of the receptor, followed by the separation of the receptor from cytoplasmic chaperone proteins such as heat shock protein 90 (Hsp90) and by the exposure of nuclear localization sequences. This allows nuclear translocation and homo/heterodimerization of the ligand-bound receptors, and their binding to steroid response elements (i.e. nucleotide sequences specifically recognized by SRs) on the promoter regions of the target genes, thus regulating gene expression by interacting with the transcription machinery (1) (Fig. 1).

However, the observation of effects elicited by steroid hormones which are too rapid to be mediated by activation of RNA and protein synthesis has prompted the search for alternative signaling mechanisms. Additional indications for new ways of steroid signaling have been provided by the discovery of receptor subfractions localized at the cell membrane level (2, 3), that

have been indicated as potentially responsible for the non-nuclear effects of steroid hormones.

Different terms have been used to distinguish these non-conventional signaling mechanisms, the more popular being 'non-genomic' or 'non-nuclear'. However, these mechanisms may be more properly indicated as 'non-transcriptional', so as to underline that DNA binding of the receptors and RNA synthesis are not required. The classification of these effects is still poor, due to the incomplete knowledge of the molecular basis, which explains the different phenomena that have been described as 'non-genomic' actions of SRs. The definition itself of these mechanisms is a 'negative' one, defining what these phenomena are not, instead of one describing their specific characteristics. As practical rules, non-transcriptional effects can be indicated as: (i) actions that are too rapid to be compatible with RNA and protein synthesis (i.e. that ensue within seconds to minutes from the challenge with the hormone); (ii) actions that can be reproduced in the presence of inhibitors of RNA or protein synthesis; (iii) actions that can be reproduced by using steroid hormones coupled

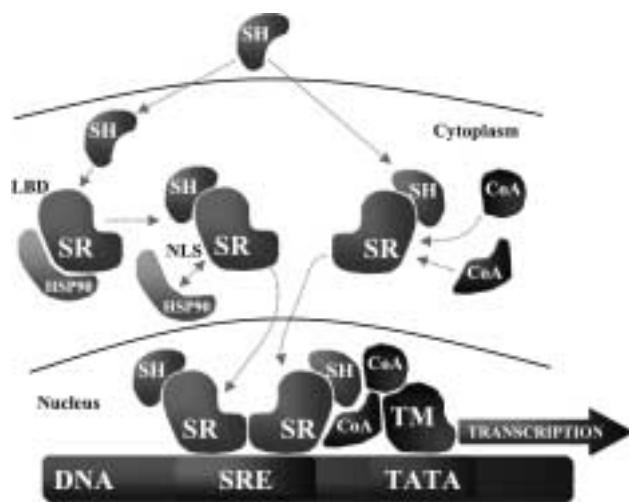


Figure 1 SRs' genomic actions. Binding of steroid hormones (SH) to the LBD of the SR induces a conformational modification of the receptor. This causes the separation of the receptor from cytoplasmic chaperone proteins such as Hsp90, the interaction with cell-specific co-activators (CoA), and the exposure of nuclear localization sequences (NLSs). This allows nuclear translocation and homo/heterodimerization of the ligand-bound receptors, and their binding to steroid response elements (SREs, i.e. nucleotide sequences specifically recognized by SRs) on the promoter regions of the target genes, thus regulating gene expression by interfering with the transcription machinery (TM).

to cell membrane-impermeable molecules; (iv) actions that steroid hormones induce in cells with highly compacted chromatin, in which RNA and protein synthesis are absent (such as spermatozoa); and (v) actions that are elicited by steroid hormones via binding to receptors containing mutations which make them incapable of activating transcriptional processes.

A full understanding of the nature of these mechanisms is still far from being accomplished, and up to now only descriptive information on different non-transcriptional effects of steroid hormones has been accumulated (4).

Cell membrane SRs

One of the most compelling arguments in favor of the presence of non-nuclear actions of steroid hormones is represented by the localization of a pool of SRs at the cell membrane level. Pioneering evidence for the existence of such a distinct subpopulation was provided in the late 1970s by the work of Pietras & Szego, who described the presence of cytoplasmic membrane binding sites for estradiol (E_2) in endometrial cells (2). Since then, several reports on these receptors have been contributed using imaging techniques, and evidence that these molecules mediate important cellular actions has accumulated (5). Indeed, plasma membrane estrogen receptors (ERs) have been advocated to be involved in the regulation of cell membrane ion channels (6–8), G-protein-coupled receptors (GPCRs) (9), tyrosine

kinases and mitogen-activated protein kinases (MAPKs) (10, 11). Moreover, they have also been shown to activate adenylate cyclase production (12), as well as to trigger phospholipase C (PLC) activation (13).

A lively debate has been ongoing for some time on the nature of these putative membrane ERs. After the failure of the identification of a structurally distinct ER isoform with membrane localization sequences, there is a growing consensus that the naturally occurring ER can *per se* localize at the cytoplasmic membrane, and that therefore these receptors would be in equilibrium with the pool of cellular ERs. In agreement, recent transfection studies suggest that both ER α and ER β can localize to the cell membrane (14). It is also possible that localization to the cell membrane may depend on the interaction with specific membrane structures, and indeed both ER isoforms have been shown to localize in caveolae (15, 16).

Cell membrane binding sites have been described also for other steroid hormones. Particularly, binding sites for testosterone have been identified on rat osteoblasts (17), in macrophages (18) and T lymphocytes (19), in prostate cells (20) as well as in vascular cells (21). Membrane progesterone receptors (PRs) have also been studied, and they have been found to be of particular relevance in sperm cells, where they are responsible for the rapid, non-genomic activation of Ca^{2+} and Cl^- channels, leading to the acrosomal reaction (22). Additional cell types where membrane progesterone-binding sites have been described are represented by osteoblasts (23), granulosa cells (24) and oocytes (25).

The specific function of these cell membrane SRs has not been fully ascertained, but there seems to be a solid link with the activation of rapid signaling. This is also suggested by the relative abundance of membrane receptors in organs and tissues where steroid non-genomic signaling has been more consistently observed, such as the cardiovascular (26) and central nervous (27) systems. However, although there is relevant evidence indicating the existence of cell membrane SRs, many studies suffer from technical limitations. Indeed, the best studies have been conducted using overexpression systems, which may generate artifacts by inducing non-specific localization of the receptors to non-physiological intracellular sites due to the excessive amounts of molecules produced in the cell.

Non-genomic signaling mechanisms of sex steroid hormones

Non-genomic actions of steroid hormones result from the recruitment of signaling pathways that are often associated with cell membrane receptors such as GPCRs, ion channels or enzyme-linked receptors (28). Through the recruitment of such pathways, steroids rapidly regulate multiple cellular functions, but can

also modulate longer-term processes such as gene expression, protein or DNA synthesis and cell proliferation (29).

GPCRs

Regulation of GPCRs by steroid hormones is one of the best characterized mechanisms of non-genomic signaling. ERs can couple to PLC beta by interacting with a G-protein in osteoblast cells (13). This leads to a rapid increase in intracellular Ca^{2+} concentration due to Ca^{2+} mobilization from the endoplasmic reticulum and to the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (13). Coupling with G-proteins seems to be restricted to certain isoforms, since in transfected COS-7 cells $\text{ER}\alpha$ interacts with $\text{G}\alpha_i$ but not with $\text{G}\alpha_q$ or $\text{G}\alpha_s$ (30). $\text{ER}\alpha$ can also interact with $\text{G}\beta\gamma$ subunits (31). No reports have been published up to now on interactions between the β isoform of ER and G-proteins.

Progesterone modulates the activity of GPCRs, such as the oxytocin receptor. However, while direct binding of the hormone to the oxytocin receptor has been shown (32, 33), resulting in interference with oxytocin binding, a direct involvement of the PR has not been reported.

Androgen receptors (ARs) have also been indicated to interact/modulate GPCRs, through indirect lines of evidence (34). Indeed, in a macrophage cell line expressing membrane AR, but lacking intracellular AR, rapid Ca^{2+} mobilization upon treatment with testosterone is prevented by the administration of pertussis toxin or by PLC inhibitors (35).

Modulation of membrane receptors linked to G-proteins may possibly explain a variety of cellular actions of sex steroids, such as regulation of the PLC/DAG/ IP_3 cascade (31, 36), intracellular Ca^{2+} mobilization and activation of protein kinase C (PKC) (13), and activation of the adenylate cyclase/protein kinase A pathway (37).

Cell membrane ion channels

One of the earliest observed rapid actions of sex steroids is the regulation of transmembrane ion fluxes. Several cell types are exquisitely sensitive to intracellular ion content variations, and rapid channel modulation by steroids results in relevant biological effects.

Sex steroids act as vasodilators, and part of this effect relies on the modulation of intracellular Ca^{2+} in endothelial and smooth muscle cells. In vascular smooth muscle cells (VSMCs), E_2 treatment inhibits the voltage-dependent L-type Ca^{2+} channels (7). E_2 also controls K^+ efflux in VSMCs, by opening Ca^{2+} - and voltage-activated K^+ (BKCa) channels via cGMP-dependent phosphorylation (38). It is unclear whether the ER has any direct role in the control of membrane ion channels, but E_2 can independently bind and activate

Maxi- K^+ channels (8). These structures consist of a pore-forming α subunit and of a regulatory β subunit which increases the channel sensitivity to Ca^{2+} . E_2 directly binds to the regulatory subunit therefore activating the Maxi- K^+ channel (8).

Progesterone administration is also known to regulate ion fluxes. Sperm cells are characterized by a highly compacted chromatin, and do not accomplish significant RNA or protein synthesis, thus representing an excellent model to study non-genomic actions of steroids. Progesterone induces rapid Ca^{2+} influx in spermatozoa by targeting a membrane Ca^{2+} channel (39) and therefore activating the acrosomal reaction. Control of Ca^{2+} influx at the cell membrane level may be linked to the local presence of PRs (40), although this is not yet clear. Similar effects can be found in osteoblasts, where a membrane-impermeable form of progesterone increases intracellular Ca^{2+} by opening L-type Ca^{2+} channels and activating Ca^{2+} mobilization from the endoplasmic reticulum (23). However, Ca^{2+} regulation by progesterone seems to be cell-specific, since in VSMCs progesterone exerts opposite effects, reversibly inhibiting KCl-induced elevation of cytosolic-free Ca^{2+} by blunting L-type Ca^{2+} channel inward current, therefore decreasing the peak inward current (41). This Ca^{2+} -antagonist action may explain part of progesterone's vasodilatory actions observed *in vivo* (42).

It is noteworthy that in VSMCs, E_2 , progesterone and testosterone have similar effects, reducing contractility and cell Ca^{2+} influx in de-endothelialized coronary artery strips isolated from castrated male pigs (43). However, testosterone effects are more pronounced than those of estrogen, possibly due to the parallel activation of large-conductance, BKCa channels (44, 45).

Protein kinase signaling pathways

Between the signaling machineries that are regulated by sex steroids are the MAPK cascades, several tyrosine kinases and lipid kinases. These pathways are ubiquitous transducers of the signals coming from different growth factors, and their activation is linked to a variety of important cellular events including gene expression, cell proliferation and survival.

MAPKs and tyrosine kinases The activation of the MAPK pathways by sex steroids has been characterized in several tissues. Although large overlaps exist, MAPKs are organized in three step-modules, where an upstream kinase phosphorylates and activates the downstream MAPK (46). Three main cascades are described, the extracellular signal-regulated kinase (ERK) 1/2, the p38 and the stress-activated protein kinase (SAPK) or c-Jun NH₂-terminal kinase (JNK) cascades (46, 47).

The relationship between MAPKs and SRs is complex, as several levels of mutual regulation exist (48).

On the one hand, MAPKs can lead to ligand-independent activation of SRs. Indeed, ERs, PRs, ARs, retinoic acid receptors, retinoid X receptors and vitamin D receptors all exhibit ligand-independent activation under appropriate conditions, although the specific role of MAPKs is not known for all of these receptors. In the case of the ER there is good evidence that receptor phosphorylation plays a role in ligand-independent activation, and MAPKs are responsible for such phosphorylation (49) (Fig. 2).

On the contrary, exposure to estrogens leads to rapid activation of the ERK 1/2 module in various cell types. In nerve cells, membrane-impermeable E_2 rapidly triggers ERK 1/2 activation, leading to c-Fos immediate-early gene transcription (11). A similar rapid activation can be found in osteoblasts (50) as well as in white adipocytes (51).

Activation of MAPK may be finely tuned by the coordinated interaction with other signaling cascades. For instance in adipocytes, pertussis toxin, PP2 and wortmannin reduce the magnitude of MAPK activation by E_2 (51) suggesting the parallel involvement of the Gi-protein/Src family kinase/phosphatidylinositol 3-OH kinase (PI3K) pathways for the functional activation of MAPKs (Fig. 2). In breast cancer cells, E_2 -induced ERK activation is mediated by a heregulin/human epidermal growth factor receptor-2/PKC- δ /Ras pathway, and leads to E_2 -dependent growth-promoting effects (52). Moreover, estrogen deprivation causes breast cancer cells to develop hypersensitivity to the mitogenic effect of E_2 , and there is evidence that hypersensitivity to E_2 depends on enhanced MAPK activity (53), therefore

suggesting a strong interrelationship between the ER and the MAPK pathways.

In breast cancer cells, progesterone plays a relevant role in the regulation of ERK 1/2. Indeed, the B isoform of the PR stimulates the activated ER to recruit the Src/p21^{ras}/ERK pathway (54), revealing a functional cross-talk between SRs, which could be relevant for their growth-promoting effects on cancer cells. But PR can also *per se* interact with p42 MAPK, which in turn is able to phosphorylate PR *in vitro* (25).

Although less is known about the regulation of the other MAPK modules, the p38 cascade is activated by estrogen in endothelial cells (55). In this cell type, E_2 rapidly activates p38 β , leading to MAPKAP-2 kinase activation and to phosphorylation of Hsp27. Through this pathway, E_2 preserves stress fiber formation, and actin and membrane integrity. Moreover, E_2 -induced p38 activation prevents hypoxia-induced apoptosis, and induces the migration of endothelial cells and the formation of primitive capillary tubes (55).

Opposite to the other MAPK modules, estrogens downregulate the activity of JNK (56). In breast cancer cells, E_2 inhibits Taxol- or UV-stimulated JNK activity, therefore abrogating Bcl-2 and Bcl-x1 phosphorylation and caspase activation (57). In parallel, the steroid also activates ERK protein kinase activity (57). These molecular events may play a role in E_2 -dependent prevention of chemotherapy or radiation-induced apoptosis in breast cancer cells.

Testosterone is also able to regulate the MAPK family of protein kinases. Recent work indicates that, via a cell membrane AR, testosterone induces a rapid rise in the intracellular free Ca^{2+} concentration in macrophages (18). Testosterone does not in itself activate the ERK 1/2, p38, or JNK/SAPK cascades; however, testosterone attenuates the lipopolysaccharide-dependent activation of p38 (but not that of ERK 1/2 and JNK/SAPK) (18).

A prominent feature of sex steroids is also represented by activation of tyrosine kinases. Estrogen treatment of various cell types induces protein tyrosine phosphorylation. This has been shown in different cell types, including osteoclasts, where E_2 stimulates a profound increase in phosphotyrosine immunostaining of proteins within 1 min of treatment (58). Src kinase is one of the phosphorylated intermediates and it appears to translocate to the plasma membrane and to have enhanced kinase activity during estrogen treatment (58). Tyrosine kinase activation by estrogen seems also to be involved in the recruitment of other non-genomic pathways, such as MAPK. For instance, E_2 signaling to ERK 1/2 can occur via a G-protein-dependent, pertussis toxin-sensitive pathway that requires Src-related tyrosine kinase activity and tyrosine phosphorylation of the Shc adapter protein (59). Indeed, E_2 treatment is associated with transactivation of the epidermal growth factor receptor (EGF-R), and E_2 signaling to ERK 1/2 can be blocked by inhibiting EGF-R tyrosine kinase activity (59). Moreover, recent data

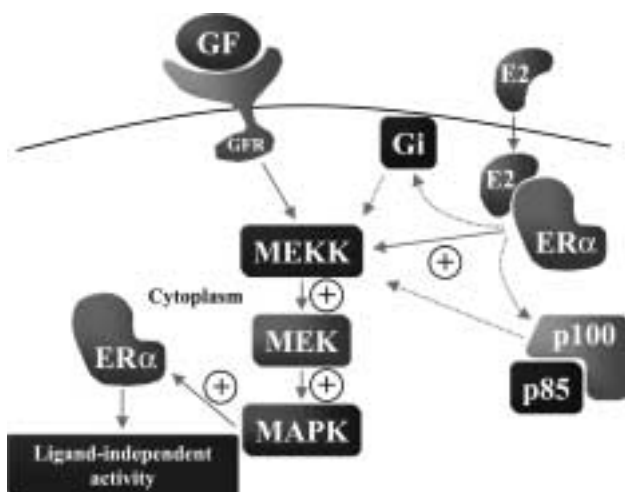


Figure 2 ERs and MAPKs. Binding of growth factors (GF) to the specific membrane receptors induces activation of the MAPK cascade (MEKK → MEK → MAPK). Active MAPK phosphorylates and activates ER independently of the presence of E_2 . On the other side, E_2 -bound ER triggers the activation of the three-step ERK 1/2 MAPK module. ER activation of MAPK is finely modulated by Gi protein (Gi)-dependent and PI3K (p85/p100)-dependent intracellular pathways.

also indicate that the transactivation of EGF-R may represent another cell membrane-based effect of E₂, being mediated by the GPCR, GPR30 (60), although other data support a direct interaction between ER and EGF-R (61). The interaction of tyrosine kinases and MAPK pathways may be of particular importance in breast cancer cells, where estrogen may drive growth factor-dependent cellular responses through these mechanisms, but there is evidence that it may be relevant also in neurons (62).

Rapid activation of tyrosine kinases is accomplished also by progesterone. In sperm cells, cell membrane PR interaction with protein tyrosine kinases accounts for the ion channel regulation that is required for the acrosomal reaction (40). Although the mechanism of interaction of PR with tyrosine kinases is unclear, recent evidence indicates the existence of a specific polyproline motif in the NH₂-terminal domain of PR that mediates direct progestin-dependent interaction of PR with SH₃ domains of various cytoplasmic signaling molecules, including c-Src tyrosine kinases. PR interaction with SH₃-containing tyrosine kinases may also be the mechanism for progesterone-dependent MAPK activation (63).

PI3K In addition to protein kinase signaling pathways, non-transcriptional actions of sex steroid hormones can be mediated by the recruitment of lipid kinases. Indeed, upon binding with E₂, ER α physically and functionally couples with the regulatory subunit of the lipid kinase PI3K, thus triggering an activation of the catalytic subunit and increasing intracellular production of phosphoinositides (64) (Fig. 3). PI3K phosphorylates the D-3 position of the phosphatidylinositol ring, catalyzing the synthesis of lipid mediators that act as second messengers transferring the signaling cascade to intracellular protein kinases. One of the principal targets of this cascade is the serine-threonine protein kinase Akt/protein kinase B. Activation of Akt mediates many of the downstream cellular effects of PI3K triggered by E₂, including rapid activation of the endothelial isoform of the nitric oxide (NO) synthase (eNOS) (64). The relevance of this interaction has been confirmed *in vivo*, where estrogen induces a dramatic reduction in leukocyte adhesion to endothelium in vessels after ischemia/reperfusion, which is completely reversed by treatment with PI3K as well as eNOS inhibitors (64). Activation of PI3K by estrogens is important also in breast cancer cells, where E₂ rapidly triggers association of ER α with Src and p85 (65). This ternary complex probably favors hormone activation of Src- and PI3K-dependent pathways, which converge on cell cycle progression (65).

Interaction with PI3K extends to other SRs, such as the AR and the glucocorticoid receptor (GR) (64). Particularly, GR interacts with PI3K upon binding with different synthetic glucocorticoids, resulting in NO-dependent

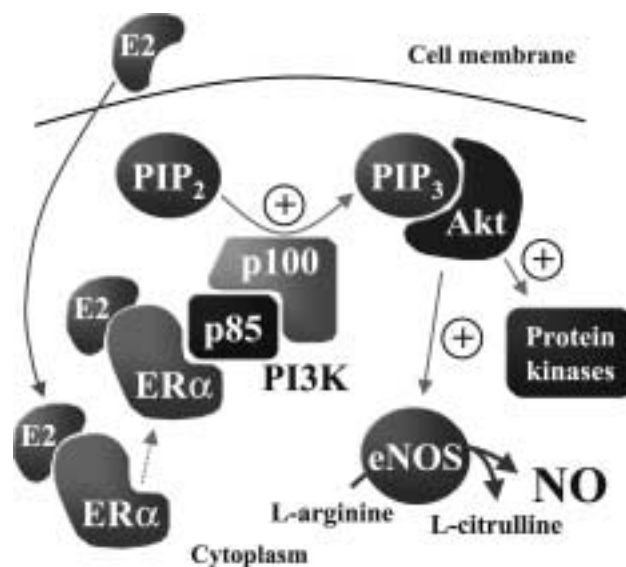


Figure 3 ER and PI3K. Ligand (E₂)-bound ER interacts with the regulatory subunit (p85) of PI3K (p85/p110). The catalytic subunit (p110) is thus activated, leading to phosphorylation of phosphoinositides (PIP₂, PIP₃) on the D-3 position of the inositol ring. PIP₃ is recognized by specific docking sites on protein kinases, which trigger the activation of protein kinase Akt. Akt regulates, via phosphorylation, several intracellular enzymes (protein kinases), including eNOS, leading to increased NO synthesis.

anti-inflammatory effects on blood vessels, and in potent anti-ischemic effects in the mouse heart (66).

Non-genomic actions of sex steroid hormones in steroid 'non-target' tissues

Non-genomic signaling by steroid hormones seems to be particularly important in tissues considered non-traditional targets of steroid hormones. These sites are those which, due to the relatively lower concentration of SRs, have been neglected in the past as possible steroid-regulated sites (67). On the contrary, while growing evidence indicates that there may be no cells where steroid hormones are unable to exert regulatory actions, non-genomic mechanisms are found to play a prominent role in the modulation of these tissues by steroids.

Cardiovascular system

The cardiovascular system offers a paramount example of this phenomenon; in the past few years, the cardiovascular system has been shown to be an important target for estrogens (68). Non-transcriptional signaling represents a particularly important mechanism of action for ERs at the vascular wall level (26, 69). Female sex steroid hormones exert diverse effects on the vascular system, inducing rapid vasodilatation, blocking the vessel wall's response to injury, and decreasing the development of atherosclerosis (26, 69). All of these

effects are the result of a multi-faceted series of actions on the various components of the vascular wall, from endothelial cells to VSMCs, to macrophages and stromal vascular cells, and are the result of a complex interplay of SR-mediated transcriptional as well as non-transcriptional actions.

A prototypical 'non-genomic' action of SRs is represented by vasodilatation induced by estrogens that occurs in a matter of seconds to minutes (70). This acute effect is the result of a regulation of ion fluxes as well as of vasoactive molecules release on endothelial and VSMCs (Fig. 4).

Regulation of NO synthesis is a major target of estrogens at the endothelial level. NO is a central controller of vascular function, playing a role as a potent vasodilator as well as an anti-inflammatory molecule (71). Estrogen-induced endothelium-dependent vasodilatation *in vivo* is reliant on NO production (72) (Fig. 4), and estrogen has been shown to regulate NO release by several means (Fig. 3). The principal mechanism is probably represented by the rapid activation of eNOS. ER α is involved in the genesis of this phenomenon, which is independent of gene transcription and has been proposed to be in part due to the activation of MAPK- or tyrosine kinase-dependent pathways (73). Unpublished data from our laboratory confirm this hypothesis, indicating that E₂ treatment of human endothelial cells results in a ultra-rapid activation of the Raf-1/MEK 1-2/ERK 1/2/MAP kinase module (T Simoncini and AR Genazzani, unpublished observations). Additionally, ER α rapidly activates eNOS through the PI3K/Akt pathway (64). Another possible mechanism of regulation of eNOS by estrogen may involve the chaperone protein Hsp90, which basally interacts with the ER and dissociates from it upon binding with the ligand. In this regard, Hsp90 has

been demonstrated to interact with eNOS and to dynamically regulate its enzymatic activity (74). Additional mechanisms for non-genomic estrogen-induced vasodilatation are found in the potent and rapid regulation of Ca²⁺ mobilization (7) and in the control of the cell membrane K⁺ channels (38) in VSMCs, that produces vessel relaxation and increased blood flow (Fig. 4). The vasodilatory effects of estrogens in the blood vessels have recently been found to be shared by progesterone (42) as well as by testosterone (44, 75) (Fig. 4).

Central nervous system

A variety of actions of sex steroid hormones in the central nervous system have been linked to non-genomic mechanisms (27). Sex steroids exert potent anesthetic and anxiolytic actions by acting on brain cells (76). These effects largely depend on direct non-genomic modulation of the γ -aminobutyric acid type A (GABA-A) receptor, which is a highly specific, structure-selective steroid effect (77). In agreement, GABA-A receptor signaling is recruited by the progesterone metabolite 5 α -pregnane-3 α -ol-20-one in pituitary cells (78), but the 3 β isomer is completely inactive (78). Estrogens and other steroids also affect neuronal excitability and non-genomic mechanisms play a major role in these phenomena. For instance, it has been known for a long time that E₂ rapidly decreases the spontaneous firing of neurons in the medial preoptic area (79) and rapidly increases the firing rate of pituitary cells (80). Recently, non-genomic regulation of gonadotropin-releasing hormone (GnRH)-secreting neurons by androgens has been found (81). In these cells, treatment with 5 α -dihydrotestosterone (DHT) inhibits forskolin-stimulated accumulation of cAMP through a pertussis toxin-sensitive G-protein,

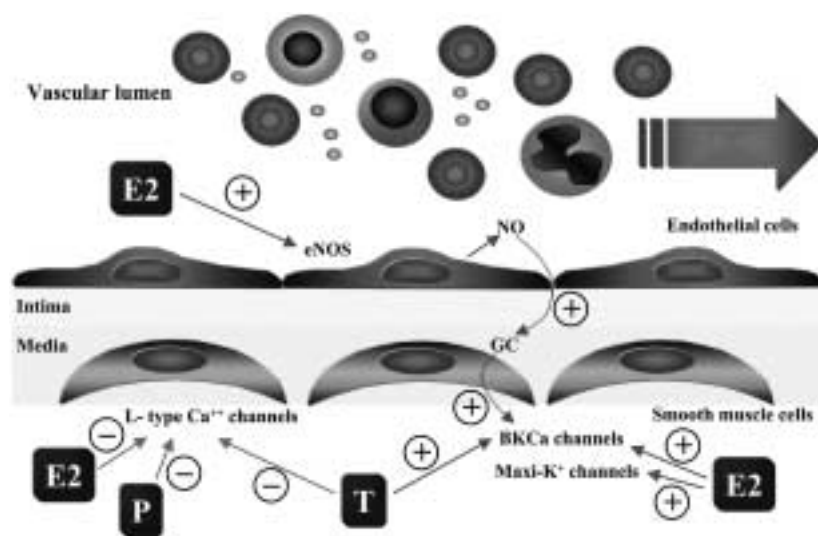


Figure 4 Non-genomic actions of steroid hormones on the vascular wall. Steroid hormones induce rapid vasodilatation by acting via non-genomic mechanisms on several targets in vascular cells. E₂ induces rapid NO synthesis in endothelial cells via activation of eNOS. Endothelial-derived NO triggers activation of smooth muscle cell guanylate cyclase (GC), therefore inducing BKCa channel activation. Additionally, E₂ directly induces the activation of BKCa and Maxi-K⁺ channels, and the inhibition of voltage-dependent L-type Ca²⁺ channels in smooth muscle cells. Inhibition of L-type Ca²⁺ channels in smooth muscle cells is also induced by progesterone (P) and testosterone (T). Testosterone is also able to activate BKCa channels in the same cells.

and the effect of DHT on cAMP accumulation is blocked by hydroxyflutamide, a specific inhibitor of ARs. In addition, evidence for a cell membrane 110 kDa protein which is recognized by anti-AR antibodies has also been found in GnRH neurons (81), supporting a role of membrane ARs in the regulation of cellular cAMP.

As in other organs, sex steroids modulate protein kinase cascades in the brain. In neuroblastoma cells E_2 activates MAPKs (11). This action is independent of the classic nuclear mechanisms of action, but is not blocked by ICI 182,780 or by tamoxifen (11), indicating that a non-classical ER may be involved.

Ion fluxes play a critical role in the control of neuron function. E_2 activates a GPCR in rat neurons, thus rapidly suppressing ion currents mediated by L-type Ca^{2+} channels (82). Association with GPCRs and rapid intracellular signaling has been demonstrated in neuronal cell lines also for progesterone (33). Moreover, estrogen has been shown to attenuate K^+ conductance and to induce depolarization of hypothalamic neurons via cAMP (83).

Both estrogen and progesterone have neuroprotective effects *in vitro* (84), but the molecular basis may be different. E_2 has originally been reported to promote cell viability under the effects of serum deprivation. These actions of E_2 are present at physiological concentrations, and enhance rat hypothalamic neuronal survival in a serum-free medium by an ER-dependent mechanism (85). One possible way through which estrogens protect neural cells may be the reduction of oxidative stress (86) that may be mediated, at least in part, by the ability to alter free radical production and/or free radical action through chemical scavenging. In addition, estrogen is neuroprotective against N-methyl-D-aspartate (NMDA)- and kainate-mediated neurotoxicity through the recruitment of tyrosine kinase/MAPK pathways (62). As in breast cancer cells, estrogen-mediated enhancement of long-term potentiation in the hippocampus is mediated by activation of a Src tyrosine kinase pathway (62). Thus, estrogen, by activating a Src tyrosine kinase and the ERK/MAPK signaling pathway, enhances NMDA receptor function and long-term potentiation and retains neuroprotective properties. Additional *in vivo* mechanisms of neuroprotection may be represented by the reduction of neuronal death during ischemia (87, 88). These actions of estrogens are largely exerted via non-transcriptional mechanisms, and are thought to be the basis for the reduction of neuronal loss that would explain the suggested prevention of dementia of Alzheimer's type in women taking estrogens (89, 90).

Sex steroid hormones non-genomic signaling: open questions and speculations

Non-transcriptional SR signaling is to many extents still a conundrum. Indeed, there is still a large number of rapid effects of steroid hormones which have to be fully characterized. Additionally, we ignore whether there are physiological or pathophysiological conditions where non-genomic signaling by SRs may be specifically important *in vivo*, or, on the contrary, whether conditions exist where modifications of steroid hormones concentrations can be found which are sufficiently rapid and consistent to trigger non-genomic signaling *in vivo*.

These questions are relevant and we can suppose that in some ways tissues could be sensitive to either general or local hormonal variations which would lead to non-genomic effects. If this is found to be true, we can also hypothesize that conditions in which the dynamic changes of hormonal concentrations are lost or reduced may suffer from the loss of such regulatory mechanisms, therefore possibly identifying situations where altered non-genomic steroid signaling may specifically contribute to determine pathological conditions. This is a formal possibility which we still have to explore; indeed, there have never been clinical studies considering the effects of steroid hormones where the non-transcriptional actions had been experimentally excluded, or, on the other hand, no clinical study has ever been designed to demonstrate that the systemic effects observed do not depend on non-genomic signaling mechanisms, either.

A promising avenue of investigation which may be of help in dissecting the role of the classic from the non-genomic signaling of steroids has recently been offered by the development of animals carrying point mutations which impair the binding of the receptors to DNA, therefore knocking out the transcriptional function of these molecules. This technique has been used to introduce mutations in the first zinc finger of the ER (E207A/G208A and E207G/G208S) DNA-binding domain. These mutations abrogate ER binding to estrogen response elements but allow partial or full activity through non-classical mechanisms such as the modulation of the AP-1 promoter (91). Recently, a knock-in mouse has been generated introducing this mutated form of ER (92), and the comparison of the phenotype of these animals with that of ER α knock-out (KO) mice is beginning to unveil precious information on the possible role of ER-mediated non-genomic actions. The heterozygous female mice are infertile due to a deficit in ovulatory function, and have a severe phenotype (92), which is somewhat unexpected, since a single copy of the ER α gene is sufficient in heterozygous ER α KO mice to maintain a nearly normal function of ER α . The reason for this is

unknown, but a possible explanation may be that a correct balance between non-genomic and genomic pathways is important for a functional cell regulation. Notably, the effect of this possible unbalance is different in the various organs, since knock-in mice show cystic hyperplasia in the endometrium but hypoplasia in the breast (92). This observation stimulates intriguing hypotheses on the possible differential relevance of non-genomic actions of steroid hormones in different cell types.

Even if non-genomic signaling mechanisms are interpreted as pharmacological phenomena, the chance of activating or repressing selectively SRs' actions that are exerted through non-transcriptional mechanisms could open new avenues of pharmacological possibilities, with the chance of designing drugs which could differentially recruit transcriptional or non-transcriptional effects in selected tissues. Since non-genomic signaling has been consistently found to be of importance for relevant pathophysiological processes, molecules able to recruit selective rapid actions of steroids may turn out to be of use for diverse clinical applications.

Non-genomic SR signaling: a tentative interpretation

The understanding of the non-genomic signaling pathways for SRs represents a highly promising and exciting horizon. Based on the evidence gathered up to now on genomic and non-genomic signaling mechanisms by SRs, it is possible to submit a tentative interpretation of their relative role.

Genomic signaling mechanisms may represent the ways through which steroid hormones program the target cells, organs and systems for complex functions, providing the single cells and the cell networks with the tools to accomplish these tasks. These mechanisms require a relatively long time to get into action and could determine the medium- and long-term fate of the cell. The generation of cell specificity is mediated by a complex system of interacting proteins, which are differentially expressed in the distinct cells (Fig. 6).

Non-genomic signaling mechanisms may instead represent the system through which SRs rapidly activate the cellular functionalities needed to adapt to dynamic changes in the surrounding milieu. These mechanisms would rapidly enable the cell to use tools which are already present and which become functionally activated or repressed. The time of onset of these effects can be extremely rapid, but the modifications of the cell program may be long-lasting anyway (Fig. 6). A possible example of such a role can be found in the recently identified rapid regulation of signal transducer and activator of transcription (STAT) 3 and 5 in vascular cells (93). STATs are a family of transcription factors regulating target genes

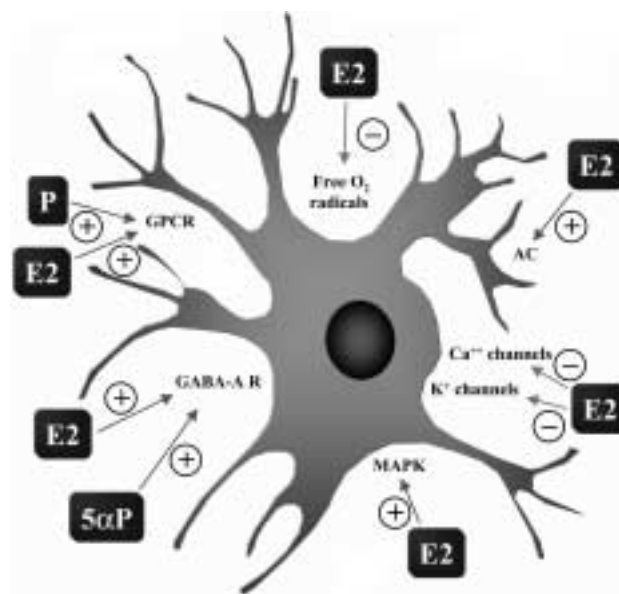


Figure 5 Non-genomic actions of steroid hormones on neurons. E₂ and progesterone (P) rapidly activate GPCRs in neural cells. E₂ and selected progesterone metabolites, such as 5 α -pregnane-3 α -ol-20-one (5 α P) have been shown to enhance the activity of the GABA-A receptor (GABA-A R). Additional effects of estrogens on neurons are the activation of MAPKs and adenylate cyclase (AC), as well as the inhibition of cell membrane K⁺ and Ca²⁺ channels and the scavenging of free oxygen radicals.

through binding to consensus sequences in the promoters. Recent data indicate that estrogens can trigger the genomic actions of STAT3 and STAT5 by inducing rapid cytoplasmic phosphorylation of these factors through MAPK-, Src kinase- and PI3K-dependent pathways (93). This newly identified signaling cascade may therefore be one of the first pieces of evidence for a tight interplay between non-genomic and genomic signaling mechanisms of steroid hormones.

Conclusions

In summary, non-transcriptional sex steroid hormone signaling represents a vast array of actions triggered by SRs through distinct molecular mechanisms in different tissues. There is growing evidence to suggest that part of these actions can be ascribed to SRs localized to the plasma membrane, but this does not seem to be an absolute requirement. Non-transcriptional signaling mechanisms are particularly important in non-traditional steroid target tissues, such as the cardiovascular and the central nervous systems, where they have been found to mediate important pathophysiological processes. Understanding in-depth the molecular mechanisms through which these actions are exerted represents an important frontier in order to engineer newer and more selective pharmacological tools for endocrine therapies.

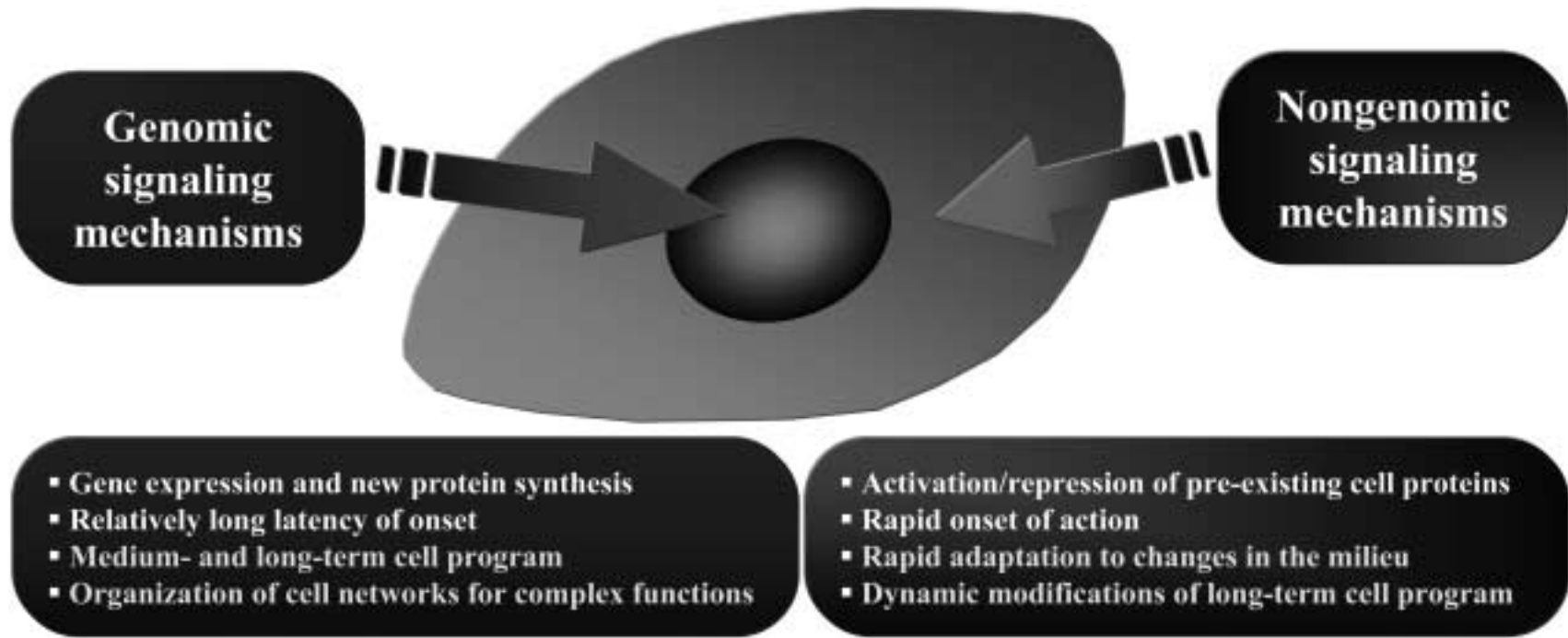


Figure 6 Genomic and non-genomic actions of steroid hormones: a tentative interpretation. Genomic actions of steroid hormones may serve to program cells, organs and systems for complex steroid hormone-regulated functions, providing the single cells and the cell networks with the tools to accomplish these tasks via gene expression and new protein synthesis. They are usually characterized by a relatively longer latency of action and could be meant to determine the medium- and long-term program of the cells. Non-genomic actions of steroids may instead serve to signal to the cells changes in the surrounding milieu, and to rapidly activate or repress the cellular functionalities needed to adapt to these changes, which are already present in the cells. The time of onset of these effects is usually extremely rapid, but the effects of these mechanisms may also serve to dynamically modulate the long-term cell program.

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References

- 1 Truss M & Beato M. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocrine Reviews* 1993 **14** 459–479.
- 2 Pietras RJ & Szego CM. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature* 1977 **265** 69–72.
- 3 Pietras RJ & Szego CM. Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membranes. *Biochemical Journal* 1980 **191** 743–760.
- 4 Moggs JG & Orphanides G. Estrogen receptors: orchestrators of pleiotropic cellular responses. *EMBO Report* 2001 **2** 775–781.
- 5 Levin ER. Cellular Functions of the plasma membrane estrogen receptor. *Trends in Endocrinology and Metabolism* 1999 **10** 374–377.
- 6 Tesarik J & Mendoza C. Nongenomic effects of 17 beta-estradiol on maturing human oocytes: relationship to oocyte developmental potential. *Journal of Clinical Endocrinology and Metabolism* 1995 **80** 1438–1443.
- 7 Nakajima T, Kitazawa T, Hamada E, Hazama H, Omata M & Kurachi Y. 17beta-Estradiol inhibits the voltage-dependent L-type Ca^{2+} currents in aortic smooth muscle cells. *European Journal of Pharmacology* 1995 **294** 625–635.
- 8 Valverde MA, Rojas P, Amigo J, Cosmelli D, Orio P, Bahamonde MI *et al.* Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit. *Science* 1999 **285** 1929–1931.
- 9 Kelly MJ & Wagner EJ. Estrogen modulation of G-protein-coupled receptors. *Trends in Endocrinology and Metabolism* 1999 **10** 369–374.
- 10 Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E *et al.* Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO Journal* 1996 **15** 1292–1300.
- 11 Watters JJ, Campbell JS, Cunningham MJ, Krebs EG & Dorsa DM. Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. *Endocrinology* 1997 **138** 4030–4033.
- 12 Aronica SM, Kraus WL & Katzenellenbogen BS. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *PNAS* 1994 **91** 8517–8521.
- 13 Le Mellay V, Grosse B & Lieberherr M. Phospholipase C beta and membrane action of calcitriol and estradiol. *Journal of Biological Chemistry* 1997 **272** 11902–11907.
- 14 Razandi M, Pedram A, Greene GL & Levin ER. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Molecular Endocrinology* 1999 **13** 307–319.
- 15 Pyo Kim H, Young Lee J, Kim Jeong J, Won Bae S, Kyu Lee H & Jo I. Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae. *Biochemical and Biophysical Research Communications* 1999 **263** 257–262.
- 16 Chambliss KL, Yuhanna IS, Anderson RGW, Mendelsohn ME & Shaul PW. ER beta has nongenomic action in caveolae. *Molecular Endocrinology* 2002 **16** 938–946.
- 17 Lieberherr M & Grosse B. Androgens increase intracellular calcium concentration and inositol 1,4,5-trisphosphate and diacylglycerol formation via a pertussis toxin-sensitive G-protein. *Journal of Biological Chemistry* 1994 **269** 7217–7223.
- 18 Guo Z, Bente WP, Krucken J & Wunderlich F. Nongenomic testosterone calcium signaling. Genotropic actions in androgen receptor-free macrophages. *Journal of Biological Chemistry* 2002 **277** 29600–29607.
- 19 Bente WP, Lieberherr M, Giese G, Wrehlke C, Stamm O, Sekeris CE *et al.* Functional testosterone receptors in plasma membranes of T cells. *FASEB Journal* 1999 **13** 123–133.
- 20 Kampa M, Papakonstanti EA, Hatzoglou A, Stathopoulos EN, Stournaras C & Castanas E. The human prostate cancer cell line LNCaP bears functional membrane testosterone receptors that increase PSA secretion and modify actin cytoskeleton. *FASEB Journal* 2002 **16** 1429–1431.
- 21 Figueroa-Valverde L, Luna H, Castillo-Henkel C, Munoz-Garcia O, Morato-Cartagena T & Ceballos-Reyes G. Synthesis and evaluation of the cardiovascular effects of two, membrane impermeant, macromolecular complexes of dextran-testosterone. *Steroids* 2002 **67** 611–619.
- 22 Tesarik J, Moos J & Mendoza C. Stimulation of protein tyrosine phosphorylation by a progesterone receptor on the cell surface of human sperm. *Endocrinology* 1993 **133** 328–335.
- 23 Grosse B, Kachkache M, Le Mellay V & Lieberherr M. Membrane signalling and progesterone in female and male osteoblasts. I. Involvement of intracellular Ca^{2+} , inositol trisphosphate, and diacylglycerol, but not cAMP. *Journal of Cellular Biochemistry* 2000 **79** 334–345.
- 24 Machelon V, Nome F, Grosse B & Lieberherr M. Progesterone triggers rapid transmembrane calcium influx and/or calcium mobilization from endoplasmic reticulum, via a pertussis-insensitive G-protein in granulosa cells in relation to luteinization process. *Journal of Cellular Biochemistry* 1996 **61** 619–628.
- 25 Bagowski CP, Myers JW & Ferrell JE Jr. The classical progesterone receptor associates with p42 MAPK and is involved in phosphatidylinositol 3-kinase signaling in *Xenopus* oocytes. *Journal of Biological Chemistry* 2001 **276** 37708–37714.
- 26 Mendelsohn ME & Karas RH. The protective effects of estrogen on the cardiovascular system. *New England Journal of Medicine* 1999 **340** 1801–1811.
- 27 McEwen BS & Alves SE. Estrogen actions in the central nervous system. *Endocrine Reviews* 1999 **20** 279–307.
- 28 Watson CS. Signaling themes shared between peptide and steroid hormones at the plasma membrane. *Science's STKE* 1999 **1999** PE1.
- 29 Castoria G, Barone MV, Di Domenico M, Bilancio A, Ametrano D, Migliaccio A *et al.* Non-transcriptional action of oestradiol and progestin triggers DNA synthesis. *EMBO Journal* 1999 **18** 2500–2510.
- 30 Wyckoff MH, Chambliss KL, Mineo C, Yuhanna IS, Mendelsohn ME, Mumby SM *et al.* Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through Galpha(i). *Journal of Biological Chemistry* 2001 **276** 27071–27076.
- 31 Le Mellay V, Lasmoles F & Lieberherr M. Ga(q/11) and Gbg proteins and membrane signaling of calcitriol and estradiol. *Journal of Cellular Biochemistry* 1999 **75** 138–146.
- 32 Grazzini E, Guillon G, Mouillac B & Zingg HH. Inhibition of oxytocin receptor function by direct binding of progesterone. *Nature* 1998 **392** 509–512.
- 33 Burger K, Fahrenholz F & Gimpl G. Non-genomic effects of progesterone on the signaling function of G protein-coupled receptors. *FEBS Letters* 1999 **464** 25–29.
- 34 Heinlein CA & Chang C. The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. *Molecular Endocrinology* 2002 **16** 2181–2187.
- 35 Bente WP, Lieberherr M, Stamm O, Wrehlke C, Guo Z & Wunderlich F. Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. *Molecular Biology of the Cell* 1999 **10** 3113–3123.
- 36 Estrada M, Liberona JL, Miranda M & Jaimovich E. Aldosterone- and testosterone-mediated intracellular calcium response in

- skeletal muscle cell cultures. *American Journal of Physiology* 2000 **279** E132–E139.
- 37 Kelly MJ, Lagrange AH, Wagner EJ & Ronnekleiv OK. Rapid effects of estrogen to modulate G protein-coupled receptors via activation of protein kinase A and protein kinase C pathways. *Steroids* 1999 **64** 64–75.
 - 38 White RE, Darkow DJ & Lang JL. Estrogen relaxes coronary arteries by opening BKCa channels through a cGMP-dependent mechanism. *Circulation Research* 1995 **77** 936–942.
 - 39 Mendoza C, Soler A & Tesarik J. Nongenomic steroid action: independent targeting of a plasma membrane calcium channel and a tyrosine kinase. *Biochemical and Biophysical Research Communications* 1995 **210** 518–523.
 - 40 Revelli A, Massobrio M & Tesarik J. Nongenomic actions of steroid hormones in reproductive tissues. *Endocrine Reviews* 1998 **19** 3–17.
 - 41 Barbagallo M, Dominguez LJ, Licata G, Shan J, Bing L, Karpinski E *et al.* Vascular effects of progesterone: role of cellular calcium regulation. *Hypertension* 2001 **37** 142–147.
 - 42 Minshall RD, Pavcnik D, Browne DL & Hermmsmeyer K. Nongenomic vasodilator action of progesterone on primate coronary arteries. *Journal of Applied Physiology* 2002 **92** 701–708.
 - 43 Crews JK & Khalil RA. Antagonistic effects of 17 beta-estradiol, progesterone, and testosterone on Ca²⁺ entry mechanisms of coronary vasoconstriction. *Arteriosclerosis, Thrombosis and Vascular Biology* 1999 **19** 1034–1040.
 - 44 Deenadayalu VP, White RE, Stallone JN, Gao X & Garcia AJ. Testosterone relaxes coronary arteries by opening the large-conductance, calcium-activated potassium channel. *American Journal of Physiology* 2001 **281** H1720–H1727.
 - 45 Ding AQ & Stallone JN. Testosterone-induced relaxation of rat aorta is androgen structure specific and involves K⁺ channel activation. *Journal of Applied Physiology* 2001 **91** 2742–2750.
 - 46 Chang L & Karin M. Mammalian MAP kinase signalling cascades. *Nature* 2001 **410** 37–40.
 - 47 Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K *et al.* Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocrine Reviews* 2001 **22** 153–183.
 - 48 Weigel NL & Zhang Y. Ligand-independent activation of steroid hormone receptors. *Journal of Molecular Medicine* 1998 **76** 469–479.
 - 49 Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H *et al.* Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 1995 **270** 1491–1494.
 - 50 Endoh H, Sasaki H, Maruyama K, Takeyama K, Waga I, Shimizu T *et al.* Rapid activation of MAP kinase by estrogen in the bone cell line. *Biochemical and Biophysical Research Communications* 1997 **235** 99–102.
 - 51 Garcia Dos Santos E, Dieudonne MN, Pecquery R, Le Moal V, Giudicelli Y & Lacasa D. Rapid nongenomic E2 effects on p42/p44 MAPK, activator protein-1, and cAMP response element binding protein in rat white adipocytes. *Endocrinology* 2002 **143** 930–940.
 - 52 Keshamouni VG, Mattingly RR & Reddy KB. Mechanism of 17-beta-estradiol-induced Erk1/2 activation in breast cancer cells. a role for HER2 and PKC-delta. *Journal of Biological Chemistry* 2002 **277** 22558–22565.
 - 53 Yue W, Wang J-P, Conaway M, Masamura S, Li Y & Santen RJ. Activation of the MAPK pathway enhances sensitivity of MCF-7 breast cancer cells to the mitogenic effect of estradiol. *Endocrinology* 2002 **143** 3221–3229.
 - 54 Migliaccio A, Piccolo D, Castoria G, Di Domenico M, Bilancio A, Lombardi M *et al.* Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. *EMBO Journal* 1998 **17** 2008–2018.
 - 55 Razandi M, Pedram A & Levin ER. Estrogen signals to the preservation of endothelial cell form and function. *Journal of Biological Chemistry* 2000 **275** 38540–38546.
 - 56 Srivastava S, Weitzmann MN, Cenci S, Ross FP, Adler S & Pacifici R. Estrogen decreases TNF gene expression by blocking JNK activity and the resulting production of c-Jun and JunD. *Journal of Clinical Investigation* 1999 **104** 503–513.
 - 57 Razandi M, Pedram A & Levin ER. Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. *Molecular Endocrinology* 2000 **14** 1434–1447.
 - 58 Brubaker KD & Gay CV. Estrogen stimulates protein tyrosine phosphorylation and Src kinase activity in avian osteoclasts. *Journal of Cellular Biochemistry* 1999 **76** 206–216.
 - 59 Filardo EJ, Quinn JA, Bland KI & Frackelton AR Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Molecular Endocrinology* 2000 **14** 1649–1660.
 - 60 Filardo EJ. Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. *Journal of Steroid Biochemistry and Molecular Biology* 2002 **80** 231–238.
 - 61 Marquez DC, Lee J, Lin T & Pietras RJ. Epidermal growth factor receptor and tyrosine phosphorylation of estrogen receptor. *Endocrine* 2001 **16** 73–81.
 - 62 Bi R, Broutman G, Foy MR, Thompson RF & Baudry M. The tyrosine kinase and mitogen-activated protein kinase pathways mediate multiple effects of estrogen in hippocampus. *PNAS* 2000 **97** 3602–3607.
 - 63 Boonyaratankornkit V, Scott MP, Ribon V, Sherman L, Anderson SM, Maller JL *et al.* Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. *Molecular Cell* 2001 **8** 269–280.
 - 64 Simoncini T, Hafezi-Moghadam A, Brazil D, Ley K, Chin WW & Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 2000 **407** 538–541.
 - 65 Castoria G, Migliaccio A, Bilancio A, Di Domenico M, de Falco A, Lombardi M *et al.* PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. *EMBO Journal* 2001 **20** 6050–6059.
 - 66 Hafezi-Moghadam A, Simoncini T, Yang E, Limbourg FP, Plumier JC, Rebsamen MC *et al.* Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nature Medicine* 2002 **8** 473–479.
 - 67 Ciocca DR & Roig LM. Estrogen receptors in human nontarget tissues: biological and clinical implications. *Endocrine Reviews* 1995 **16** 35–62.
 - 68 Manolagas SC & Kousteni S. Perspective: nonreproductive sites of action of reproductive hormones. *Endocrinology* 2001 **142** 2200–2204.
 - 69 Simoncini T & Genazzani AR. Direct vascular effects of estrogens and selective estrogen receptor modulators. *Current Opinion in Obstetrics and Gynecology* 2000 **12** 181–187.
 - 70 Gilligan DM, Quyyumi AA & Cannon RO III. Effects of physiological levels of estrogen on coronary vasomotor function in postmenopausal women. *Circulation* 1994 **89** 2545–2551.
 - 71 Liao JK. Endothelial nitric oxide and vascular inflammation. In *Endothelium, Nitric Oxide and Atherosclerosis*, pp 119–132. Eds JA Panza & ROI Cannon. Armonk, NY: Futura Publishing Co. Inc., 1999.
 - 72 Guetta V, Quyyumi AA, Prasad A, Panza JA, Waclawiw M & Cannon RO III. The role of nitric oxide in coronary vascular effects of estrogen in postmenopausal women. *Circulation* 1997 **96** 2795–2801.
 - 73 Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME & Shaul PW. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *Journal of Clinical Investigation* 1999 **103** 401–406.

- 74 Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A *et al.* Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* 1998 **392** 821–824.
- 75 Chou TM, Sudhir K, Hutchison SJ, Ko E, Amidon TM, Collins P *et al.* Testosterone induces dilation of canine coronary conductance and resistance arteries *in vivo*. *Circulation* 1996 **94** 2614–2619.
- 76 Gee KW. Steroid modulation of the GABA/benzodiazepine receptor-linked chloride ionophore. *Molecular Neurobiology* 1988 **2** 291–317.
- 77 McEwen BS. Non-genomic and genomic effects of steroids on neural activity. *Trends in Pharmacological Sciences* 1991 **12** 141–147.
- 78 Vincens M, Shu C, Moguilewsky M & Philibert D. A progesterone metabolite enhances the activity of the GABA_A receptor complex at the pituitary level. *European Journal of Pharmacology* 1989 **168** 15–21.
- 79 Kelly MJ, Moss RL & Dudley CA. The effects of microelectrophoretically applied estrogen, cortisol and acetylcholine on medial pre-optic-septal unit activity throughout the estrous cycle of the female rat. *Experimental Brain Research* 1977 **30** 53–64.
- 80 Dufy B, Vincent JD, Fleury H, Du Pasquier P, Gourdj D & Tixier-Vidal A. Membrane effects of thyrotropin-releasing hormone and estrogen shown by intracellular recording from pituitary cells. *Science* 1979 **204** 509–511.
- 81 Shakil T, Hoque ANE, Husain M & Belsham DD. Differential regulation of gonadotropin-releasing hormone secretion and gene expression by androgen: membrane versus nuclear receptor activation. *Molecular Endocrinology* 2002 **16** 2592–2602.
- 82 Mermelstein PG, Becker JB & Surmeier DJ. Estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor. *Journal of Neuroscience* 1996 **16** 595–604.
- 83 Minami T, Oomura Y, Nabekura J & Fukuda A. 17 beta-estradiol depolarization of hypothalamic neurons is mediated by cyclic AMP. *Brain Research* 1990 **519** 301–307.
- 84 Roof RL & Hall ED. Gender differences in acute CNS trauma and stroke: neuroprotective effects of estrogen and progesterone. *Journal of Neurotrauma* 2000 **17** 367–388.
- 85 Chowen JA, Torres-Aleman I & Garcia-Segura LM. Trophic effects of estradiol on fetal rat hypothalamic neurons. *Neuroendocrinology* 1992 **56** 895–901.
- 86 Behl C, Skutella T, Lezoualc'h F, Post A, Widmann M, Newton CJ *et al.* Neuroprotection against oxidative stress by estrogens: structure–activity relationship. *Molecular Pharmacology* 1997 **51** 535–541.
- 87 Simpkins JW, Rajakumar G, Zhang YQ, Simpkins CE, Greenwald D, Yu CJ *et al.* Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat. *Journal of Neurosurgery* 1997 **87** 724–730.
- 88 Hawk T, Zhang YQ, Rajakumar G, Day AL & Simpkins JW. Testosterone increases and estradiol decreases middle cerebral artery occlusion lesion size in male rats. *Brain Research* 1998 **796** 296–298.
- 89 Simpkins JW, Green PS, Gridley KE, Singh M de Fiebre NC & Rajakumar G. Role of estrogen replacement therapy in memory enhancement and the prevention of neuronal loss associated with Alzheimer's disease. *American Journal of Medicine* 1997 **103** 19S–25S.
- 90 Resnick SM, Metter EJ & Zonderman AB. Estrogen replacement therapy and longitudinal decline in visual memory. A possible protective effect? *Neurology* 1997 **49** 1491–1497.
- 91 Jakacka M, Ito M, Weiss J, Chien PY, Gehm BD & Jameson JL. Estrogen receptor binding to DNA is not required for its activity through the nonclassical AP1 pathway. *Journal of Biological Chemistry* 2001 **276** 13615–13621.
- 92 Jakacka M, Ito M, Martinson F, Ishikawa T, Lee EJ & Jameson JL. An estrogen receptor (ER) (alpha) deoxyribonucleic acid-binding domain knock-in mutation provides evidence for nonclassical ER pathway signaling *in vivo*. *Molecular Endocrinology* 2002 **16** 2188–2201.
- 93 Bjornstrom L & Sjoberg M. Signal transducers and activators of transcription as downstream targets of nongenomic estrogen receptor actions. *Molecular Endocrinology* 2002 **16** 2202–2214.

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