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Review articles

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MEMBRANE ESTROGEN RECEPTORS – IS IT AN ALTERNATIVE WAY OF ESTROGEN ACTION?

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The functions of estrogens are relatively well known, however the molecular mechanism of their action is not clear. The classical pathway of estrogen action is dependent on ERα and ERβ which act as transcription factors. The effects of this pathway occur within hours or days. In addition, so-called, non-classical mechanism of steroid action dependent on membrane estrogen receptors (mER) was described. In this mechanism the effects of estrogen action are observed in a much shorter time. Here we review the structure and cellular localization of mER, molecular basis of non-classical mER action, physiological role of mER as well as implications of mER action for cancer biology. Finally, some concerns about the new estrogen receptor - GPER and candidates for estrogen receptors - ER-X and ERx, are briefly discussed. It seems that mER is a complex containing signal proteins (signalosome), as IGF receptor, EGF receptor, Ras protein, adaptor protein Shc, non-receptor kinase c-Src and PI-3K, what rationalizes production of second messengers. Some features of membrane receptors are almost identical if compared to nuclear receptors. Probably, membrane and nuclear estrogen receptors are not separate units, but rather the components of a complex mechanism in which they both co-operate with each other. We conclude that the image of the estrogen receptor as a simple transcription factor is a far-reaching simplification. A better understanding of the mechanisms of estrogen action will help us to design more effective drugs affecting signal pathways depending on both membrane and nuclear receptors.

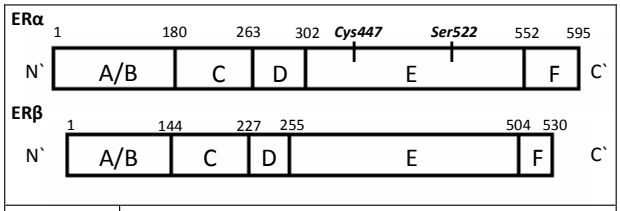
Key words: *membrane estrogen receptor, nuclear estrogen receptor, GPER, ER-X, ERx, signalosome, signal transduction, estrogens*

Abbreviations: Akt, AKT8 virus oncogene cellular homolog; CNS, central nervous system; c-Src, Rous sarcoma oncogene cellular homolog; E2, estradiol; EGFR, epithelial growth factor receptor; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; ERE, estrogen response element; ERK, extracellular signal regulated kinase; GPER, G protein-coupled estrogen receptor 1; Grb2, growth factor receptor-bound 2; Hsp, heat shock protein; IGFR, insulin-like growth factor receptor; LH, luteinizing hormone; MAPK, mitogen-activated protein kinases; MEK, MAPK/Erk kinase; mER, membrane estrogen receptor; NO, nitric oxide; PAT, palmitoyltransferase; PI-3K, phosphatidylinositol 3-kinase; 7-TM, 7-transmembrane-spanning receptor; WT, wild type.

INTRODUCTION

Estrogens are key hormones responsible for the regulation of mammal females' reproductive functions. Although the consequences of their actions for the whole organism are well known and described, the molecular mechanism of these changes is still not completely clear. Two different isoforms of estrogen receptor (ER) are known so far - ERα and ERβ, encoded by two different genes on chromosome 6 and 14, respectively. Both isoforms show significant structural homology and contain six functional domains (*Fig. 1*). Estrogen receptors belong to the nuclear transcription factors superfamily and represent the classical pathway of estrogen-dependent action. The action of estrogen receptors consists of binding lipophilic hormone molecule in cytoplasm, translocation the complex to the nucleus, dimerization, and interaction with appropriate response elements in gene promoters, what initiates transcription after coactivators binding. The effects of steroid hormones action dependent on ER occur within hours or even days (1, 2).

Nevertheless, numerous experimental studies indicate the possibility of existing another, so-called, non-classical mechanism of steroid action. By the early 20th century, it was noted that some effects of steroid hormone action, including estrogens, are observed in a much shorter time than those required for ER binding to DNA. These effects are not blocked by inhibitors of transcription (3). The rapid action of steroids was first described by Hans Selye in 1942 (4). He provided studies on hypnotic effects of progesterone, recently known as a consequence of the hormone interaction with GABA (γ-aminobutyric acid) receptor (3, 5). The first observation on estradiol (E2) action was described by Szego and Davis (6). They showed an increase in cAMP levels in the rat uterus, 15



A/B	AF-1 – hormone independent activation of transcription
C	DBD – DNA binding domain; dimerization
D	NLS – hinge domain; nuclear localization, dimerization
E	LBD – ligand binding domain; AF-2 – hormone dependent activation of transcription
F	poorly known, inhibition of dimerization (?)

Fig. 1. Schematic representation of the structure of ERα and ERβ receptor. The number of amino acids in domains and a function of domain A/B, C, D, E and F are presented.

seconds after the hormone administration. It was also found that estradiol influences prolactin secretion by prolactinoma cells line (7-9), and vasodilatation of vascular preparations (4).

For a long time, it has been difficult to explain the nature of the described phenomena. The growing amount of evidence for modulation of intracellular pathways by estrogens, *i.e*. flow of calcium ions (7, 10-13), activity of phospholipase C (14), adenylate (7, 15, 16) and guanylate cyclase (17, 18), MAPK (mitogen-activated protein kinases) pathway (11, 13, 19, 20, 21), PI-3K (phosphatidylinositol 3-kinase) (11, 22), and STAT (signal transducers and activators of transcription) proteins (23), as well as the identification of sites specific for binding ligands with high affinity in the cytoplasmic membrane of various cell types (3, 24), significantly pointed to this cellular compartment as a locus of action of those hormones.

STRUCTURE AND CELLULAR LOCALIZATION OF mER

According to current knowledge, membrane estrogen receptors (mER) are identical with classical nuclear receptors and it is highly probable that they do not belong to the group of heptahelical, transmembrane proteins similar to receptors for polypeptide hormones which do not cross the cell membrane. Studies on CHO cells (commonly used, ER-negative cell line, derived from Chinese hamster ovary) transfected with ERα and ERβ gene, demonstrated the presence of these receptors in both nucleus and cell membrane, indicating that they are derived from the same transcript (15). On the other hand, the knock-out of both genes makes the visualization of ER impossible in both compartments (25). The features of ER: weight, electrophoretic mobility and affinity to estradiol, are almost identical if we compare membrane and nuclear receptor (14, 15). Pedram *et al*. analyzing the mass spectrometry spectra of membrane proteins capable to bind estradiol (isolated by affinity chromatography) found that membrane and nuclear receptors are identical (11). In addition, experiments with the use of monoclonal antibodies, showed high similarity of membrane and nuclear receptor protein epitopes (26). However, the pool of mER is only 3–10% of the nuclear receptors (11, 15, 27).

It is still unclear how estrogen receptor joins the cytoplasmic membrane. Transmembrane localization of the receptor would require the existence of longer hydrophobic segment in the receptor protein sequence, but such a domain does not exist in the estrogen receptor (26, 28). Another possibility would be miristilation or palmitoylation of the receptor. Attachment of fatty acid residues is a posttranslational modification, which increases hydrophobicity, thereby facilitating the anchoring of protein in the cell membrane. Although early reports suggested that there are no such places (14, 29), later, an amino acid - cysteine 447 undergoing palmitoylation with resulted formation of thioester bond has been identified in the ligand binding domain E (27). This process is catalyzed by two newly identified palmitoylacyltransferases (PAT), DHHC-7 and DHHC-21, representing the enzyme family containing the catalytic DHHC (aspartate-histidine-histidine-cysteine) domain. So far, in human, there are known 23 enzymes which belong to that family. The processes catalyzed by DHHC-7 and DHHC-21 occur in the Golgi apparatus, where both enzymes are located. There is probably a vague cooperation between them, since an exclusion of each enzyme function separately disrupts ER palmitoylation at the same extent as exclusion both enzymes simultaneously. It is interesting that the same PAT are responsible for the modification of the progesterone and androgen receptor (30).

The above mentioned Cys 447, is a part of the highly-conservative motif F(XCysXXXX)LL (where F is phenylalanine, X is undefined amino acid and L is leucine or isoleucine). Similar sequences were found in heptahelical receptors coupled with G-proteins (GPCR), as well as in the androgen, progesterone and glucocorticoid receptors. Amino acids surrounding the cysteine facilitate receptor interaction with PAT. The entire 9-amino acid sequence is located deeply in the tertiary structure of the receptor, therefore an access to it and a possibility of modifying by PAT in the basic conformation of the receptor, is difficult (30, 31). Palmitoylation is accelerated by regulatory protein Hsp27 (heat shock protein 27) which joins ER monomer, changing its conformation to the more privileged for association with PAT. Oligomerization of the Hsp27 protein is essential for this action (32). Cooperation of mER and Hsp27 may also take place in such a way that the gene for Hsp27 is regulated in non-classical manner (33). It is still unclear what exactly is the factor limiting the small pool of receptors subjected to lipid modifications (34). Although they all reveal the required structure, the receptors present in the nucleus remain unchanged. At present, we know only, that the presence or absence of hormone has no effect on the percentage of palmitoylated proteins (30).

Location of receptors in the membrane is not accidental. Mostly, they are placed in caveolaes, *i.e*. cell membrane invaginations involved in signal transduction and endocytosis (29). Lipid composition of caveolar membranes is different from the one of typical membranes. Caveolar membranes contain specific structural proteins - caveolins (35). It was found that the binding of caveolin-1 (35), is an essential step of ERα and ERβ joining to cell membrane, and this process is facilitated by prior ER palmitoylation (31, 35). Both proteins - ER and caveolin - are closely coupled, and serine 522 present in the domain E of ER is directly responsible for this interaction. An importance of this amino acid highlights the fact that the substitution of hydrophilic serine by hydrophobic alanine leads to a 62% decrease in ER number in the membrane (14). The role of the whole domain appears to be extremely important for generating cytoplasmic signals. Mutants that are devoid of domain E are not able to activate universal ERK kinase (extracellular signal regulated kinase), which is one of the last kinases of the MAPK pathway, whereas deletion of A/B and C domains does not impair this function (14, 20, 36). On the other hand, some authors attribute the role of "anchoring protein" of estrogen receptor in endothelial cell membranes to the striatin, which binds amino acids 183-253 of ERα, corresponding to domain C (37). Thus, it can not be excluded that this feature is cell-specific.

MECHANISM OF mER ACTION

Because of the ability of estradiol to diffuse through the lipid bilayer, studies on receptors face difficulties in distinguishing "membrane" and "nuclear" effects of the estradiol binding. This problem was resolved by combining molecules of estradiol with bovine serum albumin (BSA) (13, 38), horseradish peroxidase or biotin (39, 40). Due to the large size and charge characteristics, E2-protein conjugates can not pass through the hydrophobic membrane, but they can bind and activate membrane receptor. At a relatively early stage of studies, there occurred suggestions that the commercially available conjugates contain unbound E2 (41), so nowadays, simple but effective procedures for purifying them are performed before the experiment, to minimize errors (38, 42).

Generally, regardless of a receptor type, chronic stimulation of membrane receptors leads to their internalization, what is considered as a regulatory mechanism attenuating excessive stimulation. It was confirmed in electron and confocal microscopy that estrogen receptors are also subject to this process (43). Despite the lack of structural similarity, this process occurs in the model of β2-adrenergic receptor dependent on β-arrestin (40).

Dimerization, which occurs in the presence of hormone, is necessary for the proper functioning of mER. This is phenomenon analogous to that observed in the nucleus, where the creation of dimers precedes genes transactivation. Although, the presence of homodimers was extensively reported, it is well known that heterodimerization, thus forming ERα-ERβ complexes, is also possible (44). In endothelial cells, abolition of the ability to dimerize prevented the activation of ERK and PI-3K, as well as cAMP formation. In this case, a very early stage of signal transduction, crucial for the association and dissociation of G protein subunits, was impaired (25).

In amino acid sequences of ERα and ERβ protein, the motifs corresponding to the function of the cyclase, phospholipase or kinase, were not found, thus the synthesis of the second messenger must be carried out in cooperation with other molecules (4, 45). In fact, mER is not structurally a single protein, but rather a complex with many signal proteins (called signalosome), whose nature, and consequently activated pathway, depends on the cell type (4). ER plays a central role in this complex. It is in close proximity to such molecules as insulin-like growth factor 1 receptor (IGFR) (46), epidermal growth factor receptor (EGFR) (23, 45, 47), Ras protein (so called small G protein, which is one of the first activated in

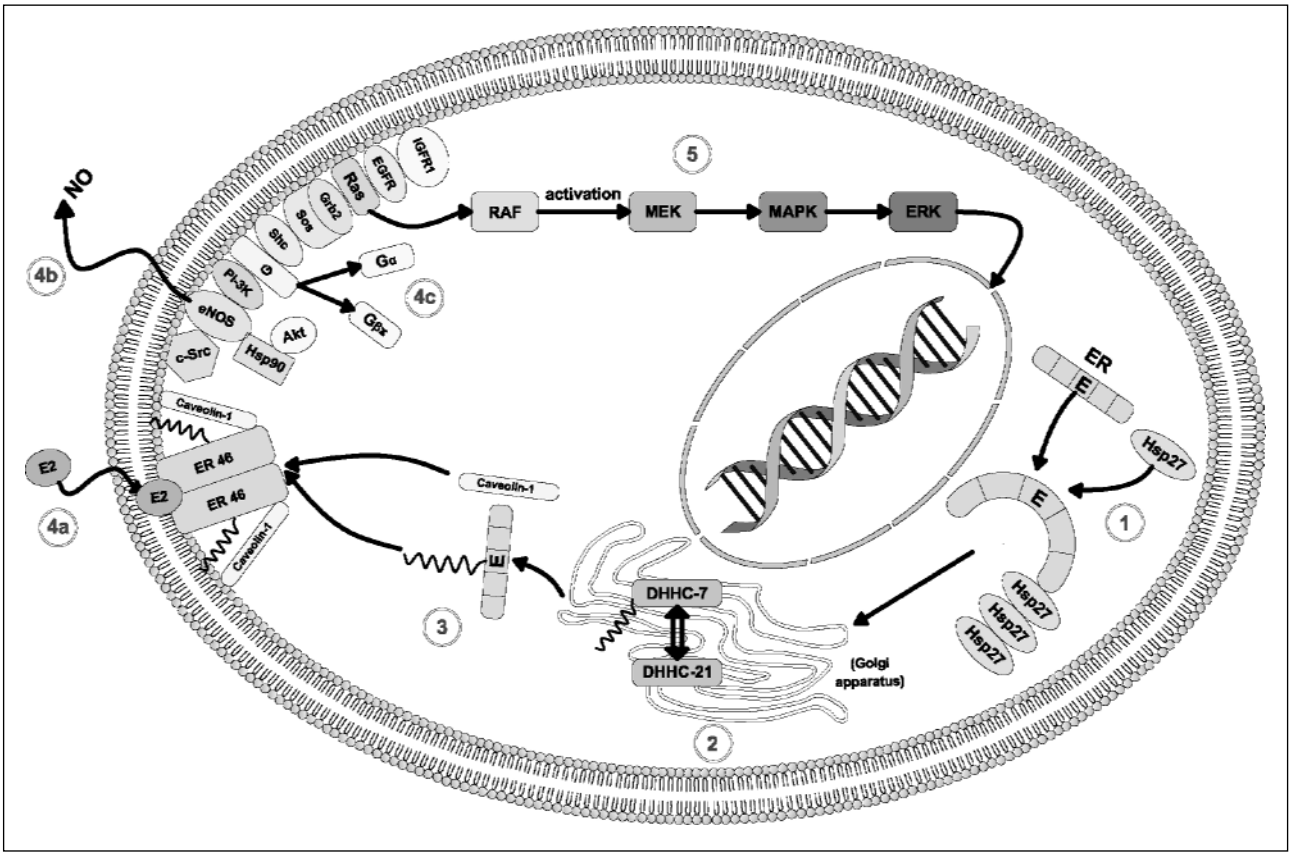


Fig. 2. The mechanism of membrane estrogen receptors action. The passage of estrogen receptor (ER) to the cell membrane requires association with heat shock protein Hsp27 oligomers in the cytoplasm (1). ER changes the conformation and its domain E is palmitoylated in the Golgi apparatus by palmitoiloacyltransferases DHHC-7 and DHHC-21 (2). Palmitoylated ER binds caveolin-1 in the cytoplasm (3) and then is anchored in the membrane as a dimer (in the endothelium composed of 46 kDa isoforms). Estradiol (E2) joins to the ER dimer (4a), which in cooperation with c-Src, PI-3K, Akt kinase and heat shock protein Hsp90 activates eNOS, thus increasing nitric oxide (NO) production (4b). Binding of ER with E2 may also result in dissociation of proteins G into Gα and Gβγ subunits (4c). EGFR and IGFR present in the signalosome participate in the activation of MAPK pathway. This includes binding of adaptor proteins: Shc, Grb2, Sos and activation of Ras GTP-ases, what ultimately leads to the cascade of RAF, MEK, MAPK and ERK kinases (5). ERK alters a function of transcription factors in the nucleus, what explains how estrogen action on the cell membrane may result in subsequent initiation of transcription.

MAPK pathway) (14), adaptor protein Shc (binds directly receptor protein kinases and activates MAPK pathway by recruiting Grb2/Sos) (23, 46), protooncogene non-receptor kinase c-Src (23, 48) and PI-3K (4, 49), what rationalizes production of second messengers.

The structure and function of the signalosome was particularly well recognized on the example of endothelial cells (*Fig. 2*). ER46 (46 kDa) - ER splicing variant lacking A/B domains, with a greater potential for stimulation of nitric oxide (NO) synthesis than full-length protein (66 kDa) plays a key role in the endothelium (36). To bind the cell membrane, ER must create a complex with c-Src (Rous sarcoma oncogene cellular homolog), which by SH2 domain joins phosphorylated tyrosine 537 of ER46 variant (48, 50). Although ERα can directly activate the PI-3K/Akt pathway by interacting with PI-3K kinase regulatory subunit (p85α) (49), some signalosomes include Gαq or Gαs proteins, bridging ER and effector kinases (15, 25, 32). In this case, binding hormone with its receptor results in dissociation of G protein into Gα and Gβγ subunits, which in turn leads to the kinase cascade activation. It is not clear how the interaction between ER and protein G occurs. Probably caveolin plays there an important role. It seems that the ER interactions depend on the type of caveolin. For example, in hippocampal neurons, the presence of caveoline-1 determines the interaction of ERα with the glutamate receptor - mGluR1, and caveoline-3 determines interaction of ERα and ERβ with mGluR2/3 receptor (39, 40, 51).

Most of the studies focused on isoform ERα. However, from the obtained results it can be concluded that the isoform ERβ acts in a similar manner, when it is co-expressed. It is very interesting that in case of nuclear receptor action, ERβ often has the opposite effect to ERα (52).

IN VIVO MODELS

Most of the studies on mER were performed *in vitro*. Recently, the murine models were described to explain the role of non-classical pathways of estrogen action *in vivo* (*Table 1*). In the so-called membrane only estrogen receptor (MOER) α model, mice were characterized by ligand binding domain E of ERα (the only one required for proper receptor function in non-classical manner) bound to the cell membrane, and simultaneous ERα gene knock-out (53). Although it was confirmed that after the exposure of selected cells of these mice to E2 *in vitro*, signal transduction through the mER took place, these mice were infertile. The reason of the later was significant atrophy of the uterus and vagina, and impaired development of the ovaries with a number of hemorrhagic cysts and the absence of corpora lutea. Hormonal status was disturbed. Luteinizing hormone (LH) secretion was not suppressed by estradiol whose concentration was high, while concentration of prolactin was low. Moreover, the presence of the domain E in the membrane did not ensure the proper development of the mammary gland. In these mice, 8-fold increase in visceral fat mass and 60% increase in body weight compared to the wild type (WT), despite comparable food consumption was observed. ERα knock-out (ERαKO) mouse females presented similar phenotypes. It should be noted that similar effect: increased body weight and decreased weight of uterus, was observed in ovariectomized rats because of estrogen depletion (54).

Thus, it seems that the action of nuclear ERα is necessary for the proper female phenotype development and for the ovarian cycle regulation. Signals initiated by membrane receptors do not compensate the absence of ERα in the nucleus. However, membrane and nuclear estrogen receptors should not be considered as separate units, but rather as components of a complex mechanism in which they both cooperate with each other. For example, it was shown in endothelial cells that 40–60 min. after the initial membrane signal, transcription attributed to nuclear receptor action occurs. Microarray analysis revealed that in these cells, transcription of at least 250 genes is dependent on PI3K/Akt, including genes of transcription factors (c-fos, c-myc, c-jun), signal transducers (Cot/Tpl-2 kinases, GTP-ase, TLR-2), enzymes (cyclooxygenase-2, proceruloplasmin), cytokines (IFN-β2, IGF-2, BMP-2a) and structural proteins (α-catenin, crystallin, vitronectin) (22). This is because of the fact that, on one hand, transcription factors and coactivators such as nuclear

Table 1. In vivo models and mouse strains used in the studies on estrogen receptors.

In vivo models		
Name		**Specification**
MOER	Membrane only estrogen receptor α mouse	Knock-out of estrogen receptor α gene but domain E of ERα is introduced with a vector and expressed in cell membrane
NERKI	Non-classical estrogen receptor α knock-in mutation mouse	The receptor is expressed; point mutation in one allel prevents binding of domain C of ERα with DNA; pure strain (homozygote) was not obtained because of infertility of $ER\alpha^{NERKI/WT}$
Mice strain		
Name		**Specification**
ERαKO	Estrogen receptor α knock-out mouse	Knock-out of estrogen receptor α gene
$ER\alpha^{KO/WT}$	Heterozygote ERαKO and wild type	Heterozygotes of ERα gene with one knock-out allele and second of wild type
$ER\alpha^{NERKI/WT}$	Heterozygote NERKI and wild type	Heterozygotes of ERα gene with one dysfunctional allele and second of wild type
$ER\alpha^{NERKI/KO}$	Heterozygote NERKI and ERαKO	Heterozygotes of ERα gene with one dysfunctional allele and second knock-out
DERKO	Double estrogen receptor knock-out mouse	Knock-out of estrogen receptor α and β genes

factor κB (NFκB), STAT and activator protein 1 (AP-1) are stabilized (22, 55, 56), but on the other hand, kinases phosphorylate nuclear ER, which act as transcription factors without binding a ligand (57). Several sites of phosphorylation were recognized as potential targets for Akt, MAPK, PKA, c-Src and glycogen synthase kinase 3β (GSK-3β) (57). Such a model is consistent with observations that in the cells which are subjected to treatment with E2-BSA followed by estradiol, there appears more intensive transcription of marker genes than during administration of estradiol alone (58). Taking these facts into consideration, the results of *in vivo* studies should be interpreted carefully until mice expressing only nuclear ER will be obtained. In addition, it should be noted that MOER organs not committed to reproduction have not been examined, yet (53).

The model known as non-classical ERα knock-in mutation mouse (NERKI), assumed the introduction of the mutation into one of the domain C zinc fingers, what prevents the binding of receptor with estrogen response element (ERE) in DNA (33). Interestingly, the heterozygous $ER\alpha^{NERKI/WT}$ females, but not heterozygous $ER\alpha^{KO/WT}$ and $ER\alpha^{KO/WT}$ in the model MOER, were infertile. This would indicate that in case of $ER\alpha^{NERKI/WT}$, one dysfunctional allele can impair a production of sufficient amount of protein to keep sexual performance. Theoretically, the introduced mutation could be manifested as a dominant and mutated protein would inactivate a product of proper gene. However, authors of the model experimentally ruled out this possibility, concluding that the observed phenotype is a consequence of imbalance between classical and non-classical pathways. In the ovaries of $ER\alpha^{NERKI/WT}$ mice, there were not corpora lutea, but numerous cysts and other features indicating of abnormal steroidogenesis, could be seen. In addition, development of the mammary gland was also inhibited. Surprisingly, the only change in tested hormone concentration was lower progesterone level, which can be explained by lack of ovulation and corpora lutea. In this context, a single functional allele seems sufficient to ensure the hormonal balance. Significant degree of the uterus atrophy in MOER (53) and $ER\alpha^{NERKI/KO}$ females (59) indicates that nuclear receptor plays a major role, however more than 2-fold increase in $ER\alpha^{NERKI/WT}$ uterus weight, size and hyperplasia of the mucosa shows that the interaction of non-classical and classical pathways is essential for proper development of the uterus (33).

Studies on the bone biology of mice with NERKI mutations provided interesting data. Heterozygotes $ER\alpha^{NERKI/KO}$ presented a paradoxical response to estrogens. While the ovariectomy in wild mice led to the loss of bone mass (reversible after E2 substitution), the ovariectomy of $ER\alpha^{NERKI/KO}$ mice resulted in an increase in cortical bone density. No similar changes were observed in ERαKO, so this phenomenon should be assigned to a specific action of ERE-independent pathways (59-61).

The main difference, comparing MOER and NERKI mice, is that in the latter model, mutation in the zinc finger blocks a direct binding of ERα to DNA sequences, however, the interaction between ERα and other transcription factors, and effective transcription dependent on these factors can not be excluded. Such an imbalance in ERE-dependent and ERE-independent gene transcription may explain the paradoxical increase in $ER\alpha^{NERKI/WT}$ uterus weight and unexpected response of $ER\alpha^{NERKI/KO}$ bone density. To clarify this issue it would be interesting to compare a response to estradiol by examining an expression of many genes by microarrays in different strains.

PHYSIOLOGICAL ROLE OF mER

The attempts to determine the physiological role of signal transduction dependent on mER in relation to the location of these receptors in non reproductive tissues indicate that they may be responsible for the adaptation of the maternal organism for pregnancy. This reaction includes immune suppression as a response on fetal tissues, adaptation of the circulatory and respiratory system to altered hemodynamic conditions, strengthening of bone structure due to increasing body weight, and behavioral changes (62).

Protective effect of estradiol on the women cardiovascular system is well known, especially before menopause. In part, it may result from secretion of nitric oxide by endothelium. NO is responsible for vasorelaxation and anti-aggregation of platelets. NO is produced from arginine by nitric oxide synthase (eNOS) - an enzyme present in caveoles (4, 50, 63). The increase in eNOS activity is a result of mERα initiated activation of PI-3K kinase (4, 49), what indirectly leads to the phosphorylation of cytoplasmic kinase Akt. The direct target for Akt kinase is eNOS (4, 49, 50, 63). Akt kinase activity also supports the association of eNOS with its chaperone protein Hsp90 (63). In addition, estradiol stimulates in blood monocytes NO production depending on the release of Ca^{2+} from endoplasmic reticulum. NO, considered as a factor impeding cell adhesion to the vessel wall, impairs in this way the infiltration of monocytes into tissues, contributing to the overall immunosuppressive and antiatherogenic role of estrogens (12). Furthermore, it was observed in rats that under the influence of NO secreted by the endothelial cells, both cGMP production and secretion of gonadotropin-releasing hormone (GnRH) from neuronal endings in mediana eminence increases. Testosterone does not exhibit a similar effect (17). On the other hand, membrane receptors are responsible for suppression of LH secretion from gonadotrophes, and this function is rather ERα-, but not ERβ-dependent (42).

Estrogens inhibit cardiomyocyte hypertrophy induced by endothelin-1 (ET-1) and angiotensin II (ATII). Molecular basis of this phenomenon is very complex. On one hand, signaling *via* PI-3K (but not Akt) leads to increased expression of the natural inhibitor of calcineurin - MCIP-1 (modulatory calcineurin-interacting protein). The activity of calcineurin followed by the translocation of transcription factors NF-ATc3 (nuclear factor of activated T-cells) and c4 to the nucleus is responsible for cardiac muscle hypertrophy. On the other hand, estradiol stimulates the synthesis and release of cardiac natriuretic factors: atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), which serve as negative autocrine signals attenuating ERK kinases and protein kinase C (PKC) activity, also associated with cardiac hypertrophy, overexcited by endothelin-1 and angiotensin II (19). Moreover, E2 reduces the response of bronchial smooth muscle cells to histamine, measured by intracellular Ca^{2+} concentration, what is consistent with reports of its bronchodilatory action (64).

E2 is involved in two periovulatory events which mechanism is based on the rapid signal transduction. The first is rat dorsal lordosis, a stereotype female behavior meaning sexual receptivity. E2 leads to the dorsal lordosis by stimulation of neuropeptide Y secretion in the arcuate nucleus, which stimulates other neurons of the same nucleus leading to release of β-endorphin to preoptic area and then to desensitization of μ-opioid receptors on local neurons. Although the total time from estradiol exposure to μ-opioid receptor desensitization lasts 30 minutes (and can be induced by E2-biotin conjugate), the lordosis is not observed until 1–2 days. This is a reason of difficulties in interpretation, because using only the criterion of time, it could be argued that this phenomenon results from the excitation of nuclear receptors (39, 40, 65).

The second neurohormonal phenomenon is a positive feedback. Although, through most of the ovarian cycle E2 has a suppressive effect on LH, shortly before ovulation E2 induces high peak of LH. Recently, it is clear that the local progesterone synthesis in the hypothalamus (so called neuroprogesterone), is a

conditio sine qua non of LH surge and ovulation. In membranes of astrocytes which are responsible for neuroprogesterone synthesis, both mERα existence and its interaction with mGluR1 receptor, was shown. Estradiol, produced in the ovary, stimulates mERα in astrocytes what activates phospholipase C (PLC). As a result, inositol-3-phosphate is produced from the membrane phospholipids, calcium ions are released from the Golgi apparatus, and PLC-dependent neuroprogesterone synthesis takes place. Glutamic acid acts on mGluR1 receptor in a similar way. Neural and hormonal stimulation of astrocytes acts supra-additively on the neuroprogesterone synthesis, which stimulates neighboring neurons and induces the phenomena responsible for the GnRH and LH secretion (39, 40).

mERα stimulation is responsible for inhibition of osteoblast`s apoptosis and osteoblastogenesis at the same time. This is due to increased activity of the MEK kinase which results in the phosphorylation and premature degradation of the dimers of the Smad proteins. These proteins are responsible for a signal transduction in cells sensitive to bone morphogenetic proteins-2 (BMP-2), which in turn promotes the differentiation of osteoblasts (60).

ROLE OF mER IN CANCER CELL BIOLOGY AND METASTASIS - EXAMPLE OF BREAST CANCER

Because of important epidemiological and clinical significance, mER are mostly recognized and described in breast cancer cells. Estrogens promote the growth and survival of breast cancer cells. This is reflected in a modern histopathology and pharmacotherapy as a standard procedure of the determination of nuclear estrogen and progesterone receptor (PR) expressions together with HER-2/neu (belonging to the family of EGFR). However, a significant number of reports regarding mER refer to its role in the breast cancer biology. Tumor cell signalosome includes adapter proteins similar to those found in non transformed cells *i.e.*: c-Src (47), PI-3K (66), MNAR/PELP (modulator of nongenomic actions of the estrogen receptor), Shc (SH2-containing collagen-related protein) (45, 47), angiotensin II receptor - AT1 (67) and EGFR (47). In most studies on breast cancer, MCF-7 cell line which expresses ERα, ERβ and PR in the absence of HER-2/neu amplification, is used.

Disturbances in at least three processes are needed for a cancer to develop: deregulation of cell death, impaired DNA damage repair and uncontrolled cell division. mER participates in all of them.

First, estradiol inhibits breast cancer cells apoptosis induced by UV radiation or paclitaxel (taxane-mitotic spindle poison) (21). Apoptosis involves an increase in JNK (Jun N-terminal kinase) activity, which in turn inactivates antiapoptotic proteins Bcl-2 and Bcl-xl. This leads to the activation of caspases and ultimately, to cell death. An action of E2 leads to deactivation of JNK and consequently to blocking the entire signaling pathway. Ability to the inhibition of JNK kinase is one of a few examples of differences between mERα and mERβ because when isoform ERα inhibits JNK, the isoform ERβ does not exhibit such property (15). Furthermore, the signal from the mER stimulating PI-3K/Akt inactivates proapoptotic Bad protein, what prevents the death program (45).

Secondly, E2 interferes with DNA repair mechanisms. Natural cell response to damaging agents, such as ionizing radiation or hydroxyurea, is mobilization of repair mechanisms. ATR pathway (ATM and Rad3 related protein kinase) is initiated by the association of ATR with TopBP1 protein (DNA topoisomerase II beta-binding protein 1) which is attributed to numerous functions related to the integrity of the genome. It is followed by an activation of Chk1 kinase (Checkpoint kinase 1), which phosphorylates Cdc25C phosphatase and in cooperation with 14-3-3 protein leads to its degradation. This prevents the removal of inhibitory phosphate group from cyclin-dependent kinase 1 (CDK1) and passing by cell G2/M restriction point. Arresting cells in the G2 phase gives them time for repair damages before completing the cycle. E2 interference with the above mechanism is based on the Akt-mediated inhibition of TopBP1:ATR complex formation, and independently, on inhibition of Chk1 kinase. In addition, as a result of absence of ATR-dependent phosphorylation of p53, a cell can not be arrested in restriction point G1/S. Omitting checkpoints results in slowing down the reparation of damages, what inevitably leads to accumulation of mutations (66).

Finally, activation of mER induces MAPK pathway, together with its executive ERK kinase. In light of recent reports, MAPK induction does not occur directly, but rather through transactivation of EGFR (25). Therefore, pathways favorable for neoplastic transformation cross at a level of EGFR adapter proteins. The range of ERK activity is extensive. Numerous processes involved in proliferation, differentiation and cell death are controlled by ERK. Apart from modifying the Bad function (68), ERK is also responsible for the phosphorylation of Ets transcription factor (49). The latter increases the synthesis of cyclin D1 protein, which (in complex with activated CDK4) releases E2F from the retinoblastoma protein - E2 transcription factor (Rb-E2F) complex, and allows the transition from G1 to S phase of cell cycle. ERK similarly influences cyclin B1 and CDK1, what is a condition for transition from G2 to M phase (14, 69). Thus, not surprisingly, estradiol promotes DNA synthesis in MCF-7 cells, visualized by the degree of radiolabelled thymidine incorporation (11, 69). Application of MAPK kinase inhibitor (PD98059) leads to 60–85% reduction of replication, indicating a significant contribution of described pathways in hormone-dependent progression of breast cancer (15). In this context, Razandi *et al*. found an interesting role of breast cancer growth suppressor protein (BRCA1) (69). Apart from its participation in the DNA damage repair, it silences EGF, IGF-1 and estradiol activated ERK activity, by appropriate phosphatase activities. The most common mutations, which predispose to breast cancer development, deprive the BRCA1 of described tumor suppressor function. The results of clinical trials of simultaneous application of antiestrogens and trastuzumab, which is anti-HER2 (Human EGFR2) antibody, are in accordance with theoretical circumstances of EGFR and ER close relation. Similarly, therapeutic efficacy of gefitinib (EGFR inhibitor) and fulvestrant (antiestrogen) administered together, was observed in non small cell lung cancer therapy (47).

The role of membrane receptors in tumor cells survival is complicated. For example, in the breast cancer, estrogens stimulate an increase of cAMP level, but the consequences of this action vary depending on the mERα expression level. The general assumption may be adopted that an increase in cAMP concentration followed by increasing activity of protein kinase A (PKA) inhibits ERK kinase stimulants, such as Ras, Raf and MEK and, as a consequence, proliferation of MCF-7 cells. It seems to be contrary to the facts presented earlier. However, a detailed analysis of this effect may explain some of the observations. In the cytoplasm of cells of low mERα expression, low concentrations of cAMP were measured regardless of E2 concentration in the culture medium; this promoted cell divisions. In subpopulations with higher mERα expression, low concentrations of E2 (1 pM) still stimulated cell divisions, while higher levels (10 nM) inhibited them. Probably, E2 stimulates the opposing pathways, but only a significant stimulation is able to tilt the balance toward the inhibitory cAMP/PKA in relation to stimulating MAPK. Cells being for a long period deprived of E2, so called MCF-7 LTED (long term estrogen deprived), become highly sensitive to its

effects (16). It creates new therapeutic possibilities, since similar conditions occur during therapy with aromatase inhibitors (47).

Another issue concerning malignant tumor is invasion and metastasis. An ability of cancer cells to infiltrate surrounding tissues and homing places far from the primary tumor requires an expression of adhesive molecules, secretion of enzymes capable of degrading extracellular matrix and the ability to move. It has recently been demonstrated that estradiol increases the mobility and invasiveness of T47D and MCF7 breast cancer cell lines. In these cells, stimulation of mERα leads to the formation of membrane-bound complex, in which, first, G protein-containing Gα13 subunit (70, 71) dissociates, then, c-Src kinase is activated, and finally PI-3K kinase is activated *via* PELP1/MNAR adapter protein (72). On one hand, PI-3K may activate ILK1 kinase (integrin-linked protein kinase 1) closely neighbouring PELP1. On the other hand, the target for PI-3K and c-Src is FAK kinase (focal adhesion kinase) (72, 73). It should be noted that both FAK (also known as PTK2) and ILK1 have a recognized role in the regulation of cell adhesion and mobility. They both mediate activation of Cdc42 GTP-ase. However, at the same time, RhoA GTP-ase appears to be directly activated by protein Gα13. Both GTP-ases belong to the same family of so-called small Rho GTP-ases and play a role of molecular mediators or switches in the pathway leading to actin fibers remodeling. Afterward, neural Wiskott-Aldrich syndrome protein (NWASP) is directly activated by Cdc42 (73) and RhoA activates moesin indirectly by depending kinase ROCK-2 (71). Parallelly, JNK kinase and c-Src stimulate paxillin through the MAPK pathway (72). When taken together, in the presence of estradiol - FAK, NWASP, moesin and paxillin proteins translocate toward the cell membrane, where they participate in the formation of focal adhesion complex with integrins. It is a bridge between the cytoskeleton and extracellular matrix. In addition, Arp2/3 protein which initiates a polimerization of G-actin to F-actin, colocalizes closely to the cell membrane with the above mentioned complex (73). This cascade of events results in remodeling of cytoskeleton and creation of filipodia and lamellipodia in one of the cell poles (*Fig. 3*).

It should be clarified that for healthy breast tissue estradiol is not a mitogen *per se*. It rather acts on the ER-positive cells, and stimulates the synthesis of autocrine and paracrine growth factors (45). The following question would arise: do all the above described facts reflect the biology of the tumor *in vivo*? It seems so. By comparing the phosphorylation status of mERα in the healthy breast tissues, carcinoma *in situ* (DCIS) and invasive cancer, it was found that phosphorylation status corresponds to the potential of metastasis to surrounding tissues. Similarly, the intensity of the phenomena in the MAPK, PI-3K/Akt, Bad/Bcl-2 increases in the order as follows: healthy tissue, DCIS, and invasive cancer (68).

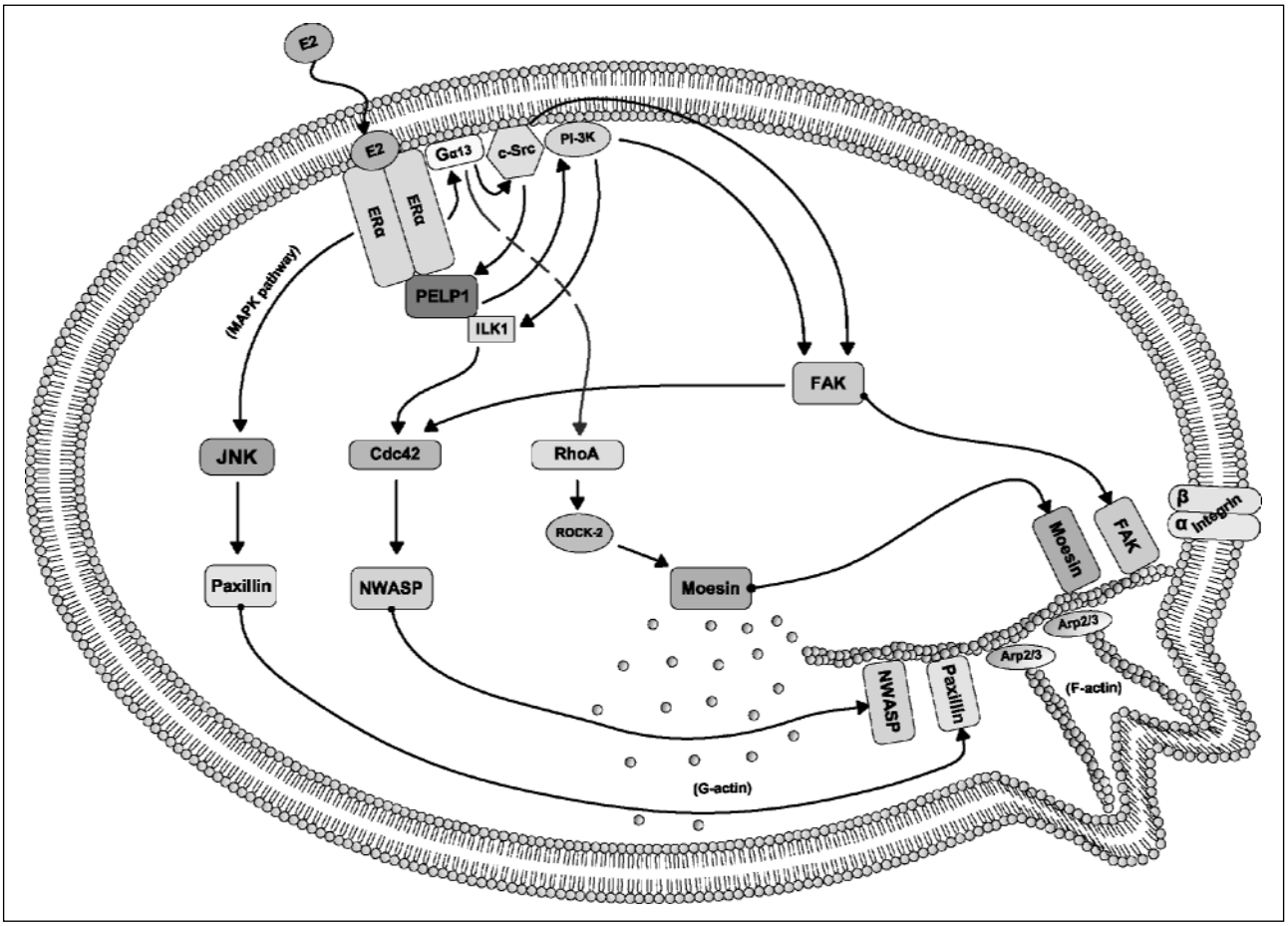


Fig. 3. The mechanism of breast cancer cell cytoskeleton remodeling that facilitates cancer cells migration and invasion. Among the proteins forming signalosome, which initiate tumor cell migration, are: ERα dimers, G protein with $G\alpha_{13}$ subunit, c-Src kinase, PI-3K, and PELP1 and ILK1 proteins. Signalosome transduces signal probably by MAPK pathway to JNK; but also to Cdc42 and RhoA GTP-ases. The consequence of their action is translocation of paxillin, NWASP, moesin and FAK proteins toward the cell membrane. These molecules create a focal adhesion complex with polymerized F-actin and integrins. Focal adhesion complex *via* integrins binds components of extracellular matrix and improve cell motility.

It is an intriguing fact that the rapid estrogen signaling has also implications for metabolism of cancer cells not associated with this hormone so obviously. Although, it paid less attention in the literature, the following should be mentioned: inhibition of human myeloid leukemia cells (HL-60) differentiation induced by retinoic acid (74), MAPK activation and MAPK-dependent transcription of c-fos in neuroblastoma cell lines (56) and Ca^{2+}-dependent release of prolactin from prolactinoma cell line GH3/B6 (7-9).

GPER RECEPTOR

In recent years there appeared a number of publications describing different proteins as estrogen receptors. First example would be Gpr30, which was discussed in the PubMed database by more than 300 times. Despite the fact, that there are many contradictory reports in the literature, Gpr30 was officially renamed as G protein-coupled estrogen receptor 1 (GPER).

GPER (7p22.3), a 7-transmembrane spanning protein unrelated to the steroid nuclear receptors, for a long time was considered as an orphan receptor. 7-TM structure suggested an existence of peptide ligand, therefore although GPER was known formerly, it was in 2005 when Revankar *et al*. (75) and Thomas *et al*. (76) presented evidences that GPER might be an estrogen receptor. The first group used the ER negative COS-7 cell line (75), stably transfected with GPER cDNA. It gave the ability to stimulate PI-3K activity and Ca^{2+} mobilization by physiological concentrations of estradiol. In addition, GPER gene silencing with antisense oligonucleotides, resulted in a weakening stimulation of PI-3K in SKBR3 breast cancer cells ($GPER^+$, $ER\alpha^-$, $ER\beta^-$).

Another attempt was to localize GPER intracellularly. Revankar *et al*. (75) prepared fluorochromes Alexa 546 and 633 covalently linked to estradiol. These fluorochromes stained only the cells with permeabilized membrane, what suggested, that the receptor is localized in intracellular structures. Based on the confocal images, the GPER positioning was estimated to endoplasmic reticulum and Golgi apparatus, but not to the cell membrane of both transfected cells and those not subjected to any manipulation breast cancer cells MCF-7, MDA-MB-231, SKBR3, choriocarcinoma JEG and uterine sarcoma HEC-50. According to Otto *et al*. (77), in both MDA-MB-231 and HEC-50 cells showing constitutive GPER expression, the receptor is located in the endoplasmic reticulum. Furthermore, they denied the fact, that the transfection resulted in cellular missorting or retention of newly synthesized protein in endoplasmic reticulum, what could explain the location of 7-TM receptor.

Such an unexpected location for the 7-TM receptor requires special attention taking into consideration the fact, that Thomas *et al*. independently indicated the cell membrane as a place of location of GPER (76). This observation was confirmed by Filardo *et al*. (78). The observed discrepancies were not a result of cell specificity, because in the all reported studies SKBR3 cells were used (75, 76, 78). In another study, in fibroblasts associated with breast cancer ($GPER^+$, $ER\alpha^-$, $ER\beta^-$) a number of antibodies were able to bound GPER in the nucleus. The functional implication of this observation arises from the fact that both GPER and EGFR can bind to promoter region of cyclin D1 gene (79). Suggestions that EGFR can directly activate transcription (reflecting the proliferative potential) appeared earlier, but in case of GPER it is completely new proof of its role as a transcription factor. Thus, the possible places in which GPER can be found are: cell membrane, endoplasmic reticulum, Golgi apparatus and nucleus.

Analysis of GPER mRNA and protein expression revealed, that this receptor is widely represented in the murine and rat tissues. Within central nervous system (CNS), a strong GPER expression occured in the isocortex, hippocampus, mitrial layer of the olfactory bulb, piriform cortex, ventromedial hypothalamic nucleus and in the pars compacta of substantia nigra. Poor expression was found in the amygdala, raphe nuclei and in the majority of the thalamus (80). In other organs: pituitary gland, heart, adrenal medulla, renal pelvis, lung, mammary gland, uterus, ovary and testis GPER expression was distinguishable. In the ovary the expression appeared mainly in granulosa cells; in the testis in Sertoli cells, Leydig cells, spermatocytes and spermatogonia (80, 81), suggesting an involvement of GPER in many cellular processes. There were no sex differences in the GPER expression both in the CNS and all other organs but on the other hand, there was no significant difference in the location of $ER\alpha$ and $ER\beta$ (80). Therefore, the conclusion that GPER acts as a sex hormone receptor can not be confirmed on this basis.

The cells characterized by GPER expression, exhibited increasing concentrations of cAMP after application of E2 and E2-BSA. Nevertheless, the concentrations used by some authors were rather high (20–100 nM), whereas the increase of cAMP did not exceed 20–30% over the control levels (76, 78). On the other hand, Pedram *et al*. stated over 200% increase of cAMP in MCF-7 cells which was most likely related to $mER\alpha$ action. They observed this effect using concentrations closer to physiological (10 nM) (11). Dissociation constant of GPER showed strong E2 binding (Kd=2.7 nM) (76), but affinity was about 10× lower than for $ER\alpha$ (Kd=0.283 nM) and 2× lower than for $ER\beta$ (Kd=1.23 nM) (15). Determination of maximal binding capacity in SKBR3 cells at a level of 114 pM (76), indicated that the density of receptors is extremely low.

GPER action in different cell and tissue types at physiological concentrations of estradiol (1–10 nM) was described. For example, in RAW 264.7 macrophage-like cells, 10 nM estradiol inhibited the expression of Toll-like receptor 4 (TLR4). GPER gene silencing by RNA interference prevented this effect, and GPER down-regulation prevented G1 (GPER agonist) activity, reinforcing the strength of evidence. G1 mimicked estradiol action, while selective agonists of $ER\alpha$ (PPT) and $ER\beta$ (DPN) did not exhibit these properties (82). G1 selectivity to GPER was verified within 27 receptors including adenosine A1, adrenergic, angiotensin AT1, chemokine, dopamine, estrogen, muscarinic, neurotensin NT1 and serotonin receptors (83). These observations indicate that the TLR4 down-regulation is dependent on GPER receptor. Furthermore, it was demonstrated that neuroprotection in hippocampal neurons is mediated by $mER\alpha$ and GPER, at hormone concentrations corresponding to physiological ones (84). In immortalized embryonic hippocampal cell lines mHippoE-14 and mHippoE-18, 10 nM estradiol led to the phosphorylation of the Akt kinase and STAT3 protein, thus promoting protection against glutamate-induced neurotoxicity. Using G1 and G15 (GPER antagonist) the authors demonstrated in embryonic female mHippoE-14 cells that the protection depends only on GPER activation but in embryonic male mHippoE-18 cells $mER\alpha$ is also required, thus pointing interesting sexual difference. An impact of GPER was also found in gametes. In pachytene spermatocytes 10 nM E2 led to phosphorylation of ERK. Classic estrogen receptors seemed to participate equally with GPER, since G1 mimicked the effect of estradiol and ERK phosphorylation was inhibited by fulvestrant. The consequence of G1 and E2 action was a decrease of cyclin A1 and B1 and an increase of Bax expression (85). Finally, Filice *et al*. (86) noted that in the isolated male rat hearts, even such low as 1 pM estradiol plays a role of both inotropic and chronotropic factor. Since the rat heart expresses $ER\alpha$, $ER\beta$ and GPER, an application of selective agonists was necessary to explain the molecular mechanism of inotropic and chronotropic effect. This revealed

that each agonist partially mimics the effect of E2. Pathways stimulated by E2 were PI-3K, PKA, ERK, eNOS and cGMP, of which GPER action certainly involves ERK and eNOS.

Demonstration of the signal transduction dependent on GPER at a wide range of E2 concentration covering physiological estrogen levels, clearly confirmed important GPER function. However, the question arised, if E2 is the only specific ligand for GPER or maybe, a type of ligand is cell-dependent? In this context, an interesting observation was that aldosterone acts *via* GPER in the endothelial cells and vascular smooth muscle cells, what includes ERK and myosin light chain phosphorylation, thus promoting the contraction of the latter. A salient issue is the fact that the peak of ERK phosphorylation (about 120% of the control level) was obtained at a concentration of aldosterone of 10 pM, whereas estradiol applied at a dose of 10 nM increased ERK phosphorylation only by 50% (87). In this work, eplerenone known as a blocker of mineralocorticoid receptor, appeared to be a partial GPER antagonist. This report confirmed that it should be verified which of the steroids is a physiological ligand for GPER. The examination of other cell types targeted by aldosterone should be helpful. It can not be ruled out, that the type of ligand for GPER is dependent on microenvironment, its availability, and physiological or pathological conditions. The selection of pharmaceuticals used in such studies must also be very careful, because it is known, for example, that tamoxifen and fulvestrant are both agonists of GPER (75, 76).

Another question is, whether GPER mediates the action of estrogen alone or in cooperation with ER when both receptors are co-expressed. It was found, that it is not a case in the liver, where ERα is easy detectable but GPER is absent (88). On the other hand, in SKBR3 breast cancer cells, GPER is present, but ERα and ERβ are not expressed (15, 75). In some studies on the breast and ovarian cancer, coexpression of ERα and GPER was not confirmed, although the cooperation between GPER and HER2/neu was observed (89). Thus, it can not be excluded that GPER is not required for all ER actions, GPER functions within tumor cells are changed, or GPER at the moment cooperates with another (still unexplored) estrogen receptor.

Nevertheless, it appeared that in some cells the cooperation between GPER and ER is possible or even obvious. The context in which estrogen receptors are found is of particular importance and determines an effect of their stimulation. For example, in the BG-1 ovarian cancer cells the expression of c-fos, pS2, cyclin A and E genes (of which promoter sequence does not contain estrogen response elements) is both ERα- and GPER-dependent. Down-regulation of ERα and GPER inhibits proliferation and c-fos transcription, indicating the use of the same signaling cascade (90). It is also belived that in the heart functional cross talk between receptors takes place, since independent stimulation of GPER, ERα and ERβ by specific agonists resulted in similiar effects as estradiol (86), however, pathways activated by ERα or ERβ were not specified.

In the double positive ($ER\alpha^+$, $GPER^+$) cells, for example: BG-1, Ishikawa and 2008 cells, both receptors promoted cell division, while the silencing of each individual inhibited it (90). Similary, in the GPER-expressing SKBR3 cells, the receptor excitation promoted mitosis (90, 91). Unfortunately, this can not be considered as a rule since GPER silencing in MCF-7 cells also intensified their divisions. In this case, activation of the receptor resulted in arresting phase G1 by accumulation of p53 and p21 proteins (91). Perhaps an impact of estrogen receptors on proliferation depends on the signalosome composition and thus, proteins that modulate the function of receptors might be responsible for cellular specificity.

Because receptor interactions are complex and highly dependent on cellular context, the cooperation between estrogen receptors and some other receptors is suggested. It is commonly believed, that EGFR takes an important place within the signal transmitting proteins functionally associated with GPER and mERα (85, 90, 92). Transactivation of EGFR initiates a cascade including EGFR/c-Src/PI-3K/MAPK and finally Elk-1 transcription factor, directly coupled with the c-fos transcription (92). It is still uncertain, what is a factor directly stimulating EGFR and which of estrogen receptors participates in this pathway. The study on a mechanism of ovarian and breast cancer development as a consequence of estrogenic activity of herbicide Atrazine, showed that it neither directly binds to ERα and ERβ nor activates them. Atrazine is a xenoestrogen, as some pesticides (93) and chemical compounds (94), and it can enhance transcription of estrogen target genes. Silencing of ERα and GPER genes inhibited transcription of c-fos stimulated by Atrazine. However, ERα- and ERβ-negative cells responded to Atrazine through the GPER. This may indicate that Atrazine acts *via* GPER, and this action is followed by ERα activity, pointing predominant role of GPER as compared to ERα (95).

In the cancer cells the expression of GPER is often higher, comparing to the cells from which the tumor originates, suggesting the participation of the receptor in neoplastic transformation (75, 81, 89). However, diversifying the levels of GPER expression in ovarian cancer, its relationship with grading, histological type, or an overall survival was not found (89). It would suggest that GPER participates in the early stages of cancer development. The question is if GPER has a biological significance for the advanced development of these tumors? It is known, that the breast cancer hormone therapy with most commonly used selective ER modulator tamoxifen predisposes to the development of endometrial cancer. Tamoxifen is GPER agonist. An active metabolite of tamoxifen - hydroxytamoxifen, estradiol and GPER agonist - G1, stimulated cells of Ishikawa endometrial cancer and KLE cells proliferation by the MAPK pathway, what was dependent on the presence of GPER. The invasiveness of the tumor was rising, in part due to increased production of MMP-2 and MMP-9 (96). On the other hand, the resistance to tamoxifen increased in MCF-7 cells with the duration of a treatment (97). It was found in MCF-7 cells, continuously cultured by 6 months in medium containing 10 nM tamoxifen that after this time, the drug had no longer an inhibitory effect on the proliferation of MCF-7 (thus, called TAM-R). In TAM-R cells, the kinetics of MAPK and Akt phosphorylation was accelerated after exposure to estradiol or G1, and what is more, an application of tamoxifen enhanced cell growth *via* MAPK itself. This was not due to increased expression of GPER, MAPK or Akt, although the expression of EGFR was slightly elevated. In addition, growth induction and MAPK phosphorylation was blocked by inhibition of c-Src kinase and EGFR receptor, and down regulation of GPER with antisense vectors. It is also interesting, that the resistance to tamoxifen can be obtained by a long-term incubation in medium containing G1. So it seems that, in specific circumstances, such as long-term SERM therapy, breast cancer cells adapt to the adverse conditions *via* cross talk between GPER and EGF receptor (97).

Apart from works enthusiastic to the idea that GPER mediates estrogen response, several studies clearly question that. In the studies of Otto *et al*., in both MDA-MB-231 and HEC-50 cells showing constitutive GPER expression, as well as in COS-7 and HEK293 cells stably transfected with GPER-GFP (green fluorescent protein), neither 10 nM E2, nor 100 nM G1 stimulated an increase in cAMP concentration or calcium mobilization (77). Pedram *et al*. failed to demonstrate phosphorylation of ERK and Akt, and an increase of cAMP level in SKBR3 cells after application of 10 nM E2. Similarly, in lysates of MCF-7 cells, they failed the searching (by chromatography with solid phase sephadex-E2) for estradiol binding proteins other than ER. In this study, estradiol failed to trigger rapid phosphorylation of ERK and Akt, as well as cAMP

increase in endothelial cells from DERKO mice (15). Recently, also the doubts about the selectivity of G1 have been growing. Although there are no doubts in G1 selectivity to GPER as compared to ERα and ERβ, it is not quite clear in relation to other 7-TM receptors. Kuo *et al*. did not find GPER in the cell membranes of astrocytes influenced by G1 although they observed an increase in intracellular Ca^{2+} concentration and the progesterone synthesis (83, 98). The authors concluded that this was due to a direct interaction of G1 with mGluR1a.

According to some reports of *in vivo* studies, G1 caused 3–4 times increase in the endometrial proliferative index thus mimicking the effect of estradiol in 25%, while the administration of the GPER antagonist G15 inhibited estradiol-induced cell proliferation by 50%. Similarly, the use of G1 imitated the antidepressant effect of estrogens (99). According to others, G1 does not have any effect on the uterus and mammary gland. Otto *et al*. found, that estradiol allows uterine epithelial cells to achieve mitotic phase S and thus it affects the expression of cyclin E, while G1 does not have such properties. In the mammary gland estradiol promoted growth and increased the expression of indicative genes such as Wnt-4, IGF-1 and Frizzled-2, while GPER agonist not (77).

Moreover, it was shown in four strains of currently available GPER-knock out mice that obtained phenotypes overlap only partially (*Table 2*). For example, GPER deficiency did not affect the reproductive system (88, 100-102). The weight and development of the uterus and mammary gland, as the fertility of mice of both sexes were unchanged. In the ovaries normal follicles and corpora lutea were found. The expression of target genes: lactoferrin, Wnt-4 and cyclin E in the uterus, and indolamine-pyrrole 2,3 dioxygenase in the mammary gland, was also unchanged (88). These results may indicate that GPER does not play a major role in estrogen signaling. On the other hand, in the mentioned above models, a number of morphological, metabolic and immunological abnormalities were found. Unfortunately, most of these observations are contrary when individual models are compared with one another, thus it can not be excluded that the differences result from genomic changes not connected immediately with GPER gene expression. For example, the majority of authors observed that GPER deletion does not affect animal weight and body fat (88, 102) even if high-fat diet was applied (100) whereas some others observed weight decrease (101) or increase (103, 104). Moreover, male bones in knock-out mice were longer and bone mineral density (BMD) was higher than in males of the wild strain. Changed IGF-1 and glucose serum level, impaired tolerance to glucose due to incorrect first phase of insulin secretion, increased blood pressure and reconstruction of the mesenteric resistance vessels were noted in Martensson`s (101), but not in other models. Finally, Wang *et al*. (105) and Isensee *et al*. (100) reported disturbances in the immune system. Physiologically, the effect of E2 on thymus includes two-steps: ERα inhibits very early stage of lymphocyte $CD44^{+},CD25^{-},CD4^{-},CD8^{-}$ development, and GPER participates in the subsequent apoptosis of lymphocytes $TCR\beta^{-/low},CD4^{+},CD8^{+}$. According to the first group, GPER knock-out prevents estradiol-dependent thymus atrophy.

Table 2. Comparison of effects observed in four models of GPER-knock-out mice (87, 97-102, 104). M – Males, F – Females, ↑ – increased, ↓ – decreased, n.a. – not analyzed, BMD – bone mineral density.

<table>
<tr><th>Phenotypic features</th><th>Otto`s model</th><th>Martensson`s model</th><th>Wang`s model</th><th>Isensee`s model</th></tr>
<tr><td rowspan="2">Weight</td><td rowspan="4">No effects
in M and F</td><td>No effects in M</td><td rowspan="2">↑ in M and F</td><td rowspan="3">No effects in M and F</td></tr>
<tr><td>↓ in F</td></tr>
<tr><td>Body fat</td><td>No effects in M and F</td></tr>
<tr><td rowspan="2">Serum chemistry</td><td>No effects in M</td><td>No effects on serum IGF-1 concentration in M</td></tr>
<tr><td>↑ glucose,
impaired glucose tolerance,
↓ serum IGF-1; in F</td><td rowspan="2">n.a. in F</td><td rowspan="2">No effects in F</td></tr>
<tr><td>Reproductive system</td><td colspan="2">No effects in F</td></tr>
<tr><td rowspan="2">Cardiovascular system</td><td>Impaired left ventricular function in M</td><td>No effects in M</td><td rowspan="2">Impaired G1-dependent relaxation of carotid arteries
in M and F</td><td rowspan="3">No effects in M and F</td></tr>
<tr><td>No effects in F</td><td>↑ mean arterial blood pressure,
↑ thickness of the wall of mesenteric arteries; in F</td></tr>
<tr><td rowspan="2">Skeleton</td><td rowspan="3">n.a.</td><td>No effects in M</td><td>↑ bone length,
↑ growth plate proliferation,
↑ BMD,
↑ trabecular bone volume; in M</td></tr>
<tr><td>↓ bone length,
unchanged BMD,
impaired estrogen influence on growth plate; in F</td><td>n.a. in F</td></tr>
<tr><td rowspan="2">Immune system</td><td colspan="2">n.a. in M</td><td rowspan="2">↓ number of $CD4^{+}$, $CD8^{+}$ and CD62L cells in M and F</td></tr>
<tr><td>No effects in F</td><td>Partial estrogen-dependent atrophy of the thymus in F</td></tr>
</table>

According to the second group the percentage of $CD4^+$ and $CD8^+$ cells is reduced, what contradicts such a mechanism. However, this point of view was not confirmed by other authors (102). They did not find any effect of GPER gene knock-out on thymus involution or imbalance in lymphocyte subpopulations.

In the opinion of Langer *et al*. (106), Otto`s model (88) seems to be the most well designed. This is due to applied method of knock-out, a proper analysis of the vector integration and removal of neomycin resistance cassette which could interfere with the function of genes other than GPER in the genome. Therefore, it seems reasonable to continue studies on this mouse strain. Although the GPER knock-out did not affect reproductive system of Otto`s mice, the exploration of the rest of the organs is warranted. Finding of abnormal left ventricular cardiac function in males, manifested by its enlargement and systolic-diastolic dysfunction is encouraging (107).

To sum up, many studies have demonstrated that GPER mediates estrogen signaling both at non physiological and physiological concentrations of estrogens, but in the light of earlier studies and new findings about GPER, we can conclude that further studies are needed to better understand the role of GPER in the different tissues.

ER-X AND ERx RECEPTORS

It seems that the mER and GPER do not exhaust all of the possibilities of estrogen action at the level of cell membrane. In 2002, Toran-Allerand *et al*. discovered the existence of a new estrogen receptor in the brain neocortex and the uterus, temporary named ER-X (108). 62-63 kDa membrane protein could be detected with antibodies against domain E and C of ERα receptor. This might suggest that ER-X is a splicing variant, if it did not exhibit unique pharmacological properties and the presence in ERαKO mice. ER-X does not stimulate ERK kinase in the presence of selective ERα and ERβ agonists. In addition, it is more strongly stimulated by 17α- than 17β-estradiol, which, obviously, makes it different from the previously described receptors (109). Thus, ER-X is probably 17α-estradiol receptor. However, it is difficult to assess what role it actually plays, because this issue has not received sufficient attention. Moreover, the structure of the receptor is not really known.

As it has already been mentioned above, an action of estrogens on cell membrane can result in subsequent initiation of transcription. It has been found very recently that application of ERα, ERβ and GPER antagonists, and simultaneous administration of E2-BSA construct to selected cell lines of breast cancer, reveals receptor-specific pattern of transcribed gene (109). However, what is really interesting, in ERα-positive cell lines MCF-7 and TD47, transcription of 10–15% genes occurs under the influence of E2-BSA in spite of blocking all three receptors simultaneously. Among the regulated genes, there were: transcription factor SMAD6, proteins of the ID group (inhibitor of DNA-binding protein), RARA (retinoic acid receptor alpha) and EGFR. In other words, there were genes involved in the regulation of transcription, apoptosis and growth factor signaling. With regard to the foregoing facts, the presence of a new membrane estradiol binding factor, misleadingly named ERx, was suggested. It should be clarified that, based on the current knowledge, there is no equality between ER-X (108) and ERx (109). This happens because the structure of both proteins and pharmacological properties of ERx are not known so far. Furthermore, it can not be unambiguously indicated that the ERx is a single protein, because transcriptional effect may correspond to excitation of several unknown receptors. Characteristics of ERx are currently in progress, so it will soon be known, whether in fact we are dealing with a new estrogen receptor or not.

CONCLUSIONS

In recent years, the knowledge about mechanisms of estrogen action has shown significant progress, extending previously described paradigm. It has been demonstrated that ERα and ERβ may be localized in the cell membrane. The probable mechanism of their translocation in the cell has been described. Moreover, a lot of facts about composition of the signalosome and signaling pathways have been discovered. The non-classical mechanism seems to be a good explanation for some physiological estrogen actions in the endocrine, cardiovascular and nervous system but it also extends the knowledge about the influence of estrogens on cancer development. Finally, a new estrogen receptor - GPER, with surprising structure and localization has been discovered, and other candidates to this function already wait for a more detailed examination. Among others, a new splicing variant of ERα known as ERα36 focus considerable attention. It is localized mainly in the cell membrane, both in ER-positive and ER-negative cells. It has interesting pharmacological properties and its transcription is regulated in a different manner as compared to the full-length receptor. ERα36 directly binds GPER agonist G1 and it is activated by G1. Antiestrogens act as their agonists. There are also strong evidences of its relationship to GPER, what may be crucial for the proper interpretation of its actions (110, 111).

To conclude, the image of the estrogen receptor as a simple transcription factor is a far-reaching simplification. The GPER and ER-X/ERx position in the estrogens` world will be undoubtedly intensively studied as a consequence of unresolved questions about their real role, also outlined in this paper. Further studies on new estrogen receptors help us better understand the mechanism of estrogen action. An examination of this mechanism is a base for the safe clinical introduction of drugs such as estrogen receptor modulators. Such drugs are increasingly used and a better understanding of their effects will help us to design more effective therapies reducing side effects at the same time.

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