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Mitochondrial signals to nucleus regulate estrogen-induced cell growth

Quentin Felty, Deodutta Roy*

Department of Environmental Health Sciences, University of Alabama at Birmingham, Birmingham, AL 35294-0022, USA

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Summary Classical genomic and non-genomic signaling pathways mediated by nuclear and cell membrane estrogen receptors are considered to contribute to estrogen-induced cell proliferation. Here we propose that mitochondrial signals to the nucleus regulate estrogen-induced progression of the cell cycle. The influence of estrogen on mitochondrial oxidative phosphorylation and mitochondrial gene transcription support the idea that mitochondria are significant targets of estrogen. Mitochondria are the major source of reactive oxygen species (ROS) in epithelial cells. Estrogen redox cycling within mitochondria also generates ROS. Antioxidants inhibit estrogen-induced cell growth. A-Raf, Akt, PKC, MEK, ERK, and transcription factors AP-1, NF-κB, and CREB are targets of both estrogen and ROS. We provide four lines of evidence in support of our hypothesis that estrogen-induced mitochondrial ROS stimulate redox sensor kinase A-Raf, Akt or PKC, which, in turn, activate transcription factors NF-κB, CREB, or AP-1 via the MEK/ERK pathway. Thus, estrogen-induced mitochondrial ROS leading to the activation of cell cycle genes containing AP-1, NF-κB, or CREB response elements are involved in the progression of the cell cycle of the estrogen-dependent cells. Our novel concept will contribute to the development of new targets in the prevention and control of estrogen-induced disease including cancer.

Introduction

Estrogens elicit different growth responses in various tissues that depend on the cell-type and dose of exposure. For example, the growth of breast, pituitary, endometrial, and testicular Leydig cells is stimulated by an estrogen concentration ranging from 0.1–1.0 pg/ml (0.1–100 pM) to 0.01–100 ng/ml (100 pM to 1 μM) while a high concentration ranging from 1 to 100 μg/ml (>1–100 μM) results in the inhibition of cell growth [1–3]. Upon hormone binding, the estrogen receptor (ER)α and ERβ initiate transcription by directly binding to estrogen response elements (EREs) of genes or indirectly through the phosphorylation of signaling proteins involved in cell cycle progression. However, data obtained from various laboratories show that the receptor binding activity of estrogen does not correlate with its influence on in vitro and in vivo growth of cells. For instance, 17 α-estradiol has a very weak affinity for the estrogen receptor compared to that of diethylstilbestrol (DES), 17 β-estradiol, and 17 α-ethinylestradiol; but it is able to stimulate the growth of Leydig cells and other cells

* Corresponding author. Tel.: +1-205-934-6081; fax: +1-205-975-6341.
E-mail address: royd@uab.edu (D. Roy).

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equivalent to that of DES and 17 β-estradiol [1]. Similarly, 4-hydroxyestradiol has a 50% receptor ligand binding affinity when compared to DES or 17 β-estradiol, but is able to produce stronger cell growth than either of these compounds in the uterus of mice [4]. Recently, we have identified that estrogen stimulated the growth of HEK 293 cells in an ER-independent manner given that this cell line does not contain ER (Singh KP, Venkat S, Roy D, unpublished). In contrast to the classical genomic pathways of estrogen action that occur over the course of several hours or days, recent studies have shown evidence of a rapid estrogen signaling pathway mediated by cell surface ERs and non-genomic estradiol-induced signal transduction pathways which contribute to cell proliferation [5]. These findings suggest that in addition to the receptor activity of estrogens, other factor(s) must be involved in the stimulation of cell growth by estrogen. Hence, these findings led to the generation of different perspectives on estrogen-induced signaling and cell proliferation. More recently, mitochondria have been implicated in the control of cell proliferation [6]. For instance, the mitochondrial peripheral benzodiazepine receptor (PBR) has been implicated in the regulation of human breast cancer cell proliferation [7]. Similarly, we have demonstrated that mitochondria can modulate the expression of nuclear cell cycle genes and human breast tumor growth [8,9]. The growth of estrogen-dependent and estrogen-independent cells is inhibited by controlling mitochondrial biogenesis [9]; and estrogen upregulates mitochondrial calcium in MCF-7 cells [8,10]. In this paper, we provide evidence in support of our postulate that signaling between the mitochondria and nucleus is involved in the control of estrogen-dependent cell growth. Estrogen-induced mitochondrial ROS stimulate redox sensor kinases. This in turn activates transcription factors NF-κB, CREB, or AP-1 which upregulate early genes involved in the progression of the cell cycle of estrogen-dependent cells.

Estrogen and mitochondria

Besides the well-known mitochondrial functions of apoptosis, respiration, and oxidative phosphorylation; mitochondria also regulate inorganic ion homeostasis and the synthesis of heme, lipids, amino acids, and nucleotides. Mitochondria are also an integral component of steroidogenesis. Key enzymes of estrogen biosynthesis (3 β-hydroxysteroid dehydrogenase and aromatase) have been demonstrated in the mitochondria of ovarian tumor epithelial cells [11]. Although estrogen synthesis occurs in the mitochondria, exogenously added estrogen is also transported to this organelle. For instance, in vivo exposure of ovariectomized rats to tritiated estrogen showed with increasing time, the translocation of this hormone from the plasmalemma mainly to the mitochondria (75%) rather than the nuclei in liver, adrenal gland, and spleen tissues [12]. The function of estrogen at the mitochondria is not clear, however, recent studies have identified ERα and ERβ within the mitochondria implicating its role in the regulation of mitochondrial genome transcription [13,14]. Partial EREs reported in genes cytochrome oxidase (CO I and CO II) may account for the observed estrogen-induced increase of these mitochondrial transcripts in rat GH4C1 pituitary cells and rat hepatocytes [15–17]. Similarly, long-term estrogen treatment increases mitochondrial transcripts CO II, CO III, and ATP synthase subunit 6 (ATPase 6) in rodents [18]. Other mitochondrial genome regions that also contain EREs include 12S rRNA, 16S rRNA, tRNA-gln, cytochrome oxidase b, unidentified reading frame (URF) 4, URF5, and the D-loop region [15]. In the human mitochondrial genome, we have identified partial or ERE 1/2 sites in the D-loop region, CO II, tRNA-met, 12S rRNA, 7S rRNA, URF1, and URF5 (unpublished Felty Q and Roy D).

Besides transcription estrogen has also been demonstrated to effect mitochondria at the protein level. For instance, estrogen and estrogen receptor agonists have been demonstrated in several studies to inhibit mitochondrial respiratory complex I, II, III, IV, and mitochondrial ATP synthase (F_0F_1-ATPase) [19–22]. Since most of these studies used cytotoxic doses of estrogen (μM), any physiological responses by mitochondria were probably overlooked. Estrogen is known to act as either an antioxidant or pro-oxidant depending on the concentration [23]. Estrogens redox cycle within mitochondria, which also generate ROS such as hydrogen peroxide, superoxide, and hydroxyl radicals [24–27]. Whether physiological concentrations of estrogen can stimulate mitochondrial ROS is not clear because most studies have been performed with cytotoxic doses. In addition, most experiments were conducted on isolated mitochondria, which neglected the significance of interorganelle cross-talk between the mitochondria and nucleus. Although several studies have reported estrogen specific inhibition of mitochondrial respiratory proteins, it is not clear whether estrogen can modify mitochondrial proteins at the post-translational level. There is a single report that estradiol increased the phosphorylation of a 76 kDa protein in the

mitochondrial fraction of the rat corpus luteum [28]. The presence of protein kinases within the mitochondria along with this last report of estradiol increased phosphorylation, indicates that estrogen may regulate mitochondrial physiology at the post-translational level. In summary, the influence of estrogen on mitochondrial gene transcription and mitochondrial respiratory complexes support the idea that mitochondria are a major target of estrogen within the cell.

Estrogen-induced growth of cells in relation to mitochondria

Four lines of evidence suggest that estrogen-induced mitochondrial ROS is involved in the proliferation of estrogen-dependent cells. A characteristic of rapidly dividing cancer cells is their capacity to produce significant amounts of intracellular reactive oxygen species (ROS), which has been implicated in the promotion of accelerated cell cycle activity in neoplastic cells [30]. Mitochondria have long been suspected to play a role in the development and progression of cancers. In a recent study of renal cell carcinoma, a decreased content of oxidative phosphorylation (OXPHOS) complexes II, III, IV, and V correlated with tumor aggressiveness [31]. In line with their data, Simonnet et al. [31] postulated that a decreased OXPHOS capacity may favor tumor growth and invasiveness. It is of particular interest that estrogen can modulate OXPHOS at the transcriptional level and perhaps the post-translational level since increased free radical formation has been shown to occur in the target organ of cancer in Syrian hamsters treated with estradiol [3].

Growth factor-induced ROS and cell proliferation

The rapid stimulation of intracellular ROS by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and nerve growth factor (NGF) suggests that this underlying mechanism of cell growth may be shared with other growth factors including estrogen [32]. Exogenous addition of low concentrations of H_2O_2 and/or $O_2^{\cdot-}$ has been demonstrated to stimulate cell growth in a variety of cell types including muscle cells, fibroblasts, amnion cells, prostate cancer cells, and aortic endothelial cells [30]. Tumor necrosis factor alpha (TNF-α) induces gene expression via mitochondrial respiratory chain dependent activation of NF-κB, AP-1, JNK, and MAPKK [33]. Although the molecular signaling mechanism that initiates ROS production by mitochondria is not clear, other cell processes besides apoptosis may be coupled to this signaling event. For instance, a recent report of integrin mediated mitochondrial ROS demonstrated that another signaling mechanism besides TNF-α can activate the transcription factor NF-κB [33]. Similarly, the proliferative response of endothelial cells to hypoxia was demonstrated to be initiated upstream by mitochondrial ROS generation that activates the MEK/ERK pathway [34]. Although other endogenous ROS sources besides mitochondria such as NAD(P)H oxidase exist, mitochondria will be the focus of this paper for the following reasons: (i) mitochondria are the principal source of intracellular ROS in epithelial cells. (ii) the growth of adenocarcinomas occur in tissue of epithelial cell origin.

Mitochondrial ROS and cell proliferation

An increasing body of evidence suggests that a moderate level of mitochondrial ROS is a stimulus for cell proliferation. The ROS molecules hydrogen peroxide and nitric oxide have been demonstrated to stimulate mitochondrial biogenesis, a process that depends on the flow of molecules into and out of the organelle [35,36]. Since mitochondrial proteins are encoded in two separate genomes (mitochondria and nuclear genome), biogenesis is a coordinated effort in which mitochondria transmit signals to the nucleus and vice versa. The question of how mitochondria transmit these signals in the process of cell proliferation has risen from reports of its involvement in cell growth. For example, in the developing rat brain mitochondrial nitric oxide synthase (mtNOS) dependent H_2O_2 production was maximal in newborns and decreased in the adult stage [37]. Cerebral granular cells isolated from newborn rats with high mtNOS activity were reported to exhibit maximal proliferation rates which depended on NO and H_2O_2 levels. In addition, manganese superoxide dismutase (MnSOD) displayed an increased pattern of activity similar to mtNOS [37]. NO has been proposed to inhibit respiratory complex IV in favor of superoxide production [38] and therefore MnSOD may work in unison with mtNOS dismutating superoxide generated by NO-dependent inhibition to H_2O_2. Since H_2O_2 is a highly diffusible signaling molecule and both MnSOD and NOS are modulated by estrogen [39,40], it is biologically plausible that estrogen-induced redox signaling at the level of mitochondria contributes to cell proliferation in a manner similar to the cerebral granular cells in newborn rats. For

example, an increase in mitochondrial superoxide generation has been demonstrated in cultured rat hepatocytes and HepG2 cells when treated with ethinyl estradiol, estradiol, and estrogen catechol metabolites at a dose range of 0.25—5 μM [41]. Stilbene estrogen-induced proliferation of Syrian hamster renal proximal tubular cells can be inhibited in vitro when treated with liposomes containing superoxide dismutase or catalase [42]. In human fibroblast cells, exposure to interleukin-1 beta (IL-1β) or TNF-α causes these cells to release significant levels of superoxide. Co-treatment with an inhibitor of IL-1β and TNF-α synthesis, pentoxifylline, inhibited stilbene estrogen-induced increase in myeloperoxidase activities, 8-hydroxydeoxyguanosine (8-OHdG) formation, mutations in the testicular genome, and prevented estrogen-induced testicular preneoplastic lesions [3]. Recently, we have shown that estrogen-induced stimulation of macrophage cells and MCF-7 cells in part occurs through reactive oxygen species [43,44]. We have also observed that estrogen-induced growth of MCF-7 cells is inhibited by reactive oxygen scavengers such as *N*-acetylcysteine, ebselen, and catalase (Singh M, Felty Q, Roy D, unpublished). In summary, these findings support the role of ROS as an autocrine growth signal in estrogen-induced cell proliferation.

Estrogen and mitochondrial oxidative stress

Recently, it has been reported that oxidative stress affects mitochondrial proteins of chronically estrogenized Syrian hamster kidney [45]. More specifically, a decrease in thiol/sulfhydryl groups is reported to occur in the mitochondrial fraction at a preneoplastic stage of carcinogenesis. The significance of this finding is that these post-translational modifications of mitochondrial proteins may occur through estrogen redox cycling and/or mitochondrial ROS. In terms of cell signaling, redox reactions involving cysteine thiol groups transduce signals by breaking or forming protein dithiol/disulfide bridges [46]. Since estrogen can induce mitochondrial ROS, we infer that the oxidation of thiols in response to estrogen converts the oxidative stress to a change in protein function involved in cell growth. Oxidative stress modifies mitochondrial matrix protein thiols [47]. Similarly, thiols on protein subunits 51- and 75-kDa of complex I have been recently reported to form mixed disulfides with glutathione (glutathionylation) in response to mitochondrial oxidative stress. This post-translational modification was reversible and correlated with an increase in mitochondrial superoxide production [48]. When MCF-7 human breast tumor cells are co-treated with the mitochondrial inhibitor rotenone (10 nM) and 17-β-estradiol (10 nM) ornithine decarboxylase activity is inhibited by 86% [49]. The inhibition of NADH dehydrogenase activity was suggested to block ROS modulated signal transduction pathways [49]. The dose of rotenone (10 nM) used in this study to produce complex I inhibition is 100-fold lower than micromolar concentrations reported to disrupt microtubule assembly and arrest mammalian cells [50]. Since ornithine decarboxylase activity is a marker for cell growth, it appears that a signal transduction pathway for estrogen-induced cell growth may originate in the mitochondria assuming that rotenone inhibition is specific to complex I. Interestingly, the antitumor arotinoid, mofarotene (Ro 40-8757), down-regulates gene expression of mitochondrial encoded NADH dehydrogenase subunit 1 (MTND1) in breast cancer cell lines MDA-MB-231, ZR-75-I, and MCF-7 [51]. Since MTND1 has been reported to form part of the rotenone-binding site in complex I [52], the absence of MTND1 may remove an important site of estrogen action in mofarotene treated cells and may account for the anti-proliferative effects of this compound. Whether these protein interactions and/or modifications can occur as a result of estrogen exposure remains to be investigated. From these investigations, we infer that estrogen mediated cell growth via mitochondrial generated ROS signaling molecules may exist and merits future exploration to address this novel pathway.

Estrogens and thioredoxin redox balance

Further evidence in support of the role of mitochondria in estrogen-induced cell proliferation comes from the study of the mitochondrial thioredoxin system. In general, the two antioxidant oxidoreductase enzymes thioredoxin (Trx) and thioredoxin reductase (TrxR) that compose the system modulate signal transduction properties of ROS by the reduction of intracellular disulfides. Trx acts as a protein disulfide reductant for ribonucleotide reductase and several transcription factors including p53, NF-κB, and AP-1 [52]. Once oxidized the active disulfide site is reduced by TrxR re-generating the reductant form of Trx. Enzyme isoforms Trx-2 and TrxR2 are reported to exist in the mitochondria [52]. Recently, a dominant negative form of TrxR2 (TrxR2DN) was expressed in HeLa cells to investigate its biological role [53]. The analysis of TrxR2DN induction by flow cytometry revealed an increase in the G_1 to S phase

transition and an increase in cell growth as measured by the MTT assay. In addition, cell cycle genes involved in the G_1 and S phases of the cell cycle were modulated by TrxR2DN expression. The dominant negative inhibition of TrxR2 was suggested to cause an increase in H_2O_2 accumulation, which activated cell proliferation [54]. Although it is not clear whether estrogen can modulate the H_2O_2 levels of mitochondria, estrogen (<10 nM) is reported to increase the expression of Trx protein in human brain SH-SY5Y cells and may play a role in cell viability [40]. In vitro estrogen (10—100 nM) treatment of primary human endometrial stromal cells show an increase in Trx protein and mRNA which implicate Trx involvement in cell growth and differentiation of estrogen responsive tissue [54]. Further evidence that supports the role of ROS in estrogen responsive tissue comes from an in vivo study that demonstrated sex hormone modulation of redox status in human endometrial cells occurs during the menstrual cycle [55]. Alterations in cellular redox status by increased expression of TrxR2 have been suggested to play a role in the growth of hepatocellular carcinomas [56]. Whether estrogen can specifically increase the expression or activity of Trx2 and/or TrxR2 is not known, but these findings suggest that estrogen may modulate signal transduction of mitochondrial derived ROS via the thioredoxin system. From these studies it appears that mitochondria may be involved in the growth of estrogen-dependent cells.

Relationship between the mitochondrial signals and progression of estrogen-induced cell cycle

The exact mitochondrial signals that control the process of cell proliferation are not clear. However, based on the studies described above it appears that mitochondria may control estrogen-induced proliferation in multiple ways. In this section, we provide evidence in support of mitochondrial signals that may be involved in cell cycle progression of estrogen-dependent cells. Mitochondrial ROS fulfill the prerequisites of a second messenger since they are short-lived (rapidly generated and degraded), produced in response to a stimulus, highly diffusible (H_2O_2), and ubiquitously present in most cell types. Several oxidant sensitive proteins involved in cell signaling have been identified in the mitochondria. For example, src protein tyrosine kinase, which is stimulated by ROS, was recently identified in the intermembrane space of rat brain mitochondria [57]. The close proximity of src to mitochondrial ROS raises the interesting question of whether this organelle participates in pathways regulated by src family kinases such as cell growth via redox signaling [32]. In addition, other oxidant sensitive kinases are localized at the mitochondria, which include a-raf, Akt, and PKC.

Estrogen-mediated changes in redox sensor proteins and activation of transcription factors

A large class of DNA-binding proteins that include the glucocorticoid receptor (GR), estrogen receptor (ER), and thyroid hormone receptor contain zinc finger domains and are localized in the mitochondria [58]. In addition to hormone receptors, there are several protein kinases such as a-raf and PKC that have zinc finger domains. Zinc finger structures within a protein consist of at least two zinc-coordinated thiolates. Oxidation of a zinc finger converts cysteine thiol groups to disulfide releasing zinc from the protein. A change in protein conformation results from the mobilization of zinc that may affect protein function. Although protein kinases are known to participate in phosphorylation signal cascades, they may also participate in redox signaling networks due to these zinc finger domains. For instance, ROS can activate protein kinase C (PKC) that triggers the release of zinc ions [59]. Another protein kinase, c-Raf, known to participate in the MAPK signal cascade was also demonstrated to be redox activated at the zinc finger domain [60]. Similarly, ROS has been reported to modulate ERα and ERβ protein expression in various cell lines. In human breast cancer cells MCF-7 and T-47D, exposure to 2.5 μM of H_2O_2 increased the protein level of ERβ while having a minimal effect on ERα [61]. Endogenous oxidative stress induced by PMA (100 ng/ml) in the macrophage cell line J774A.1 also increased the expression of ERβ [61]. Since zinc-finger domains within these proteins were reported to act as oxidant sensors, a mode of cross-communication appears to exist between redox and phosphorylation networks. The mitochondrial localization of protein kinases a-raf, PKCδ, and PKCϵ is evidence that this subcellular compartment harbors redox sensors [29,62]. Although the role of the protein kinase a-raf in the mitochondria is not clear, a-raf mRNA is highly expressed in normal murine tissues such as the epididymis, ovary, kidney, and urinary bladder [63]. In Hela cells, epidermal growth factor rapidly (2 min) and transiently activated a-raf, which in turn phosphorylated the MAP kinase activator MEK1

[64]. In addition to a-raf and Akt, other redox sensitive proteins associated with mitochondria, PKCδ and PKCϵ, have been demonstrated to activate the raf/MEK/ERK pathway or directly activate MAPKs, respectively [62,65]. Interestingly, 17 β-estradiol can stimulate the phosphorylation of a-raf and cell cycle progression in ER positive MCF-7 cells [66]. Rapid effects of estrogen have been demonstrated to mediate the DNA binding activity and phosphorylation of transcription factors. For instance, a 10 nM estradiol treatment of rat adipocytes doubled AP-1 DNA binding in 15 min [67]. In addition, CREB protein was quickly phosphorylated by estradiol (10 nM) treatment within 15 min. Another transcription factor, NF-κB, is regulated by its interactions with the inhibitory co-factor Iκ-B that sequesters NF-κB to the cytoplasm. Akt is known to phosphorylate an upstream kinase, IKKα, which stimulates the degradation of Iκ-B [68]. Therefore, estrogen-induced mitochondrial ROS may stimulate Akt leading to the degradation of Iκ-B and activation of the transcription factor NF-κB [68]. Whether estrogen treatment can activate Akt via mitochondrial derived ROS is not clear, however, phosphorylation and translocation of Akt to the mitochondria was demonstrated when cells are treated with estrogen [69]. Akt can activate raf kinase [68]. Given that estradiol can stimulate mitochondrial ROS generation; ER, a-raf, Akt, and PKC are targets of oxidative stimuli localized at the mitochondria; and the transcription factors AP-1, NF-κB, and CREB are stimulated by oxidants [32,70,71]; it is possible that estrogen specific effects at the level of mitochondria can activate these transcription factors. Based on these studies we postulate that estrogen exposure to cells increases mitochondria ROS, which stimulates redox sensor proteins a-raf, Akt, and PKC that sense a change in redox state. Hence, estrogen-induced mitochondrial ROS stimulates a-raf, Akt, or PKC, which in turn activates transcription factors NF-κB, CREB, or AP-1 via the MEK/ERK pathway (scheme shown in Fig. 1) resulting in the transcription of cell cycle genes containing DNA responsive elements for NF-κB, CREB, or AP-1 and ultimately estrogen-induced cell proliferation.

ROS mediated reorganization of cytoskeleton

Mitochondrial ROS may also affect cell growth by its actions on actin filaments. Actin is one of the most abundant proteins in eukaryotic cells involved in various processes such as cell cycle control, cell signaling, and cell structure [72]. Mechanical signals associated with cytoskeletal tension and restructuring are a requirement for anchorage-dependent cells to pass through the late G_1 restriction point of the cell cycle [73]. Whether mitochondrial ROS can affect the elasticity of the actin network is not clear, however, hydrogen peroxide was demonstrated to induce actin filament reorganization that increased cell motility

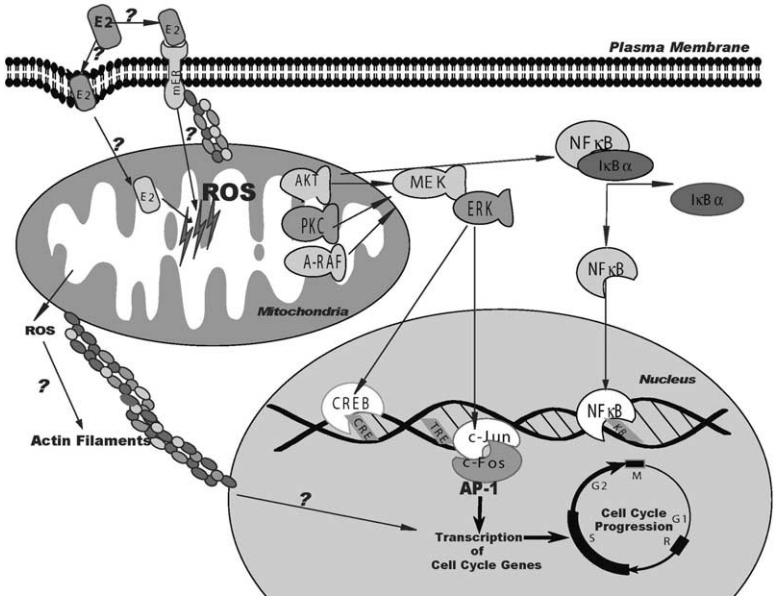

Figure 1 A scheme showing estrogen-induced mitochondrial ROS-mediated stimulation of redox sensor kinases A-Raf, Akt or PKC leading to activation of transcription factors NF-κB, CREB, or AP-1 via the MEK/ERK pathway, which, in turn, regulate cell cycle genes containing AP-1, NF-κB, or CREB response elements.

and in vitro angiogenesis [72]. Therefore, we postulate that estrogen-induced mitochondrial ROS may directly regulate progression of cell cycle through mechanical signals associated with cytoskeletal tension.

Conclusion

Although CREB, AP-1, and NF-κB are responsible for the transcription of a large number of genes in the nucleus, the localization of these transcription factors in the mitochondria raises an interesting question of whether estrogen can modulate mitochondria gene expression independent of the nuclear estrogen receptor [74—76]. Hence, the simultaneous actions of estrogen on both the nuclear genome and mitochondrial genome may be a complex series of events involved in the process of cell growth. We conclude that in addition to the mitochondria being an integral component of steroidogenesis, the influence of estrogen on various components of mitochondrial oxidative phosphorylation and mitochondrial gene transcription supports the idea that mitochondria are a major target of estrogen. Estrogen-induced mitochondrial ROS stimulates redox sensor kinases A-Raf, Akt or PKC leading to activation of transcription factors NF-κB, CREB, or AP-1 via the MEK/ERK pathway. This, in turn, regulates early cell cycle genes containing AP-1, NF-κB, or CREB response elements. This concept will contribute to the development of new targets in the prevention and control of estrogen-induced diseases.

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