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ORIGINAL ARTICLE

Oral Contraceptives and Nicotine Synergistically Exacerbate Cerebral Ischemic Injury in the Female Brain

Ami P. Raval · Raquel Borges-Garcia · Francisca Diaz · Thomas J. Sick · Helen Bramlett

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Abstract Oral contraceptives (OC) and smoking-derived nicotine (N) are known to synergistically increase the risk and severity of cerebral ischemia in women. Although it has been known for some time that long-term use of OC and nicotine will have an increased risk of peripheral thrombus formation, little is known about how the combination of OC and nicotine increases severity of brain ischemia. Recent laboratory studies simulating the conditions of nicotine exposure produced by cigarette smoking and OC regimen of women in female rats confirms that the severity of ischemic hippocampal damage is far greater in female rats simultaneously exposed to OC than to nicotine alone. These studies also demonstrated that the concurrent exposure of OC and nicotine reduces endogenous 17β-estradiol levels and inhibits estrogen signaling in the brain of female rats. The endogenous 17β-estradiol plays a key role in cerebrovascular protection in women during their pre-menopausal life and loss of circulating estrogen at reproductive senescence increases both the incidence and severity of cerebrovascular diseases. Therefore, OC and nicotine induced severe post-ischemic damage might be a consequence of lack of estrogen signaling in the brain. In the present review we highlight possible mechanisms by which OC and nicotine inhibits estrogen signaling that could be responsible for severe ischemic damage in females.

Keywords Hippocampus · Estrogen receptors · Synaptic plasticity · Mitochondria · Complex IV

A. P. Raval (✉) · R. Borges-Garcia · F. Diaz · T. J. Sick · H. Bramlett
Cerebral Vascular Disease Research Center, Department of Neurology, Leonard M. Miller School of Medicine, University of Miami, Two Story Lab (TSL), Room # 230A, 1420 NW 9th Avenue, Miami, FL 33101, USA
e-mail: ARaval@med.miami.edu

Introduction

Oral contraceptives (OC) are the leading method of contraception in United States [1]. An estimated 11.6 million American women use OC and one fourth of OC users also smoke cigarettes [2]. This population is continuously increasing and smoking-related mortality accounts for an average loss of 14 years of a woman's life [2–4]. Importantly, OC and smoking-derived nicotine (N) are known to synergistically increase the risk and severity of cerebral ischemia in women. Cerebrovascular disease is one of the leading causes of death in women in the United States [5]. However, how nicotine dependence in combination with OC increases the incidence and severity of cerebrovascular disease in women is not clearly understood.

Women are naturally protected from cerebrovascular diseases during pre-menopausal life and endogenous estrogen plays a key role in cerebrovascular protection [6–10]. The loss of circulating estrogen at reproductive senescence/menopause increases both the incidence and severity of cerebrovascular diseases [6, 11]. Apart from this natural loss of circulating estrogens at menopause, cigarette smoking-derived nicotine reduces the levels of endogenous estrogen and induces early onset of menopause in women of reproductive age [12–14]. In laboratory studies on female rats, we confirmed the previously mentioned epidemiological findings that chronic nicotine exposure reduced circulating 17β-estradiol (E_2) levels; furthermore, we found that chronic nicotine exposure makes female rats more susceptible to ischemic brain damage [15, 16]. Our study also demonstrated that the severity of ischemic brain damage is far greater in female rats simultaneously exposed to OC and nicotine (OC+N) than to nicotine alone (Fig. 1) [15–17]. In this study we simulated smoking behavior-induced nicotine levels in the human body by implanting an osmotic pump containing nicotine into female rats for 16 days. Habitual smokers

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regulate their smoking behavior to sustain a certain level of nicotine in their blood and the paradigm of continuous nicotine delivery with an osmotic pump mimicked this aspect [18]. In our study, we also confirmed nicotine release by measuring cotinine—a nicotine metabolite. Furthermore, we mimicked the use of OC in females by administering OC orally to the rat. Combination OC therapy (OC containing two hormones; e.g., estrogen and progestin) is the leading method of contraception in the United States [1]. Therefore, we used a combination OC pill containing 0.3 mg Norgestrel and 0.03 mg ethinyl estradiol, to investigate the effect of OC on the brain. The rats exposed to either nicotine alone or in combination with oral contraceptives were given an ischemic episode using a model of global cerebral ischemia, which results in a lack of blood flow in the forebrain similar to that occurring after cardiac arrest. Cardiac arrest is one of the conditions for which smoking-derived nicotine is a risk factor. Cardiac arrest-induced global cerebral ischemia causes selective neuronal death in the hippocampal CA1 region of the brain and remains the target of the current review [19].

Smoking-derived nicotine has a number of well-known adverse systemic effects resulting in the increased risk of cerebrovascular diseases in both sexes. Apart from these systemic harmful effects, chronic nicotine exposure directly

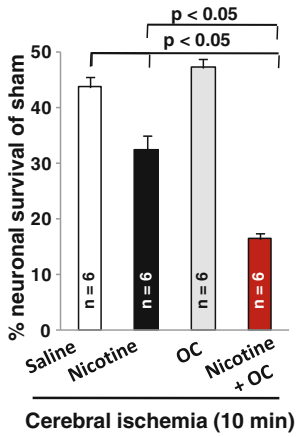

Fig. 1 Effect of nicotine alone or in combination with oral contraceptives on post-ischemic hippocampal neuronal survival in female rats. The graph shows number of normal neurons in the CA1 region of hippocampus. Data expressed as a percentage of sham (100 % normal neurons) in rat hippocampus 7 days after induction of cerebral ischemia. In this study we simulated nicotine dependence in rat by implanting an osmotic minipump, which produced a dose of 4.5 mg/kg/day. To mimic the OC (0.3 mg Norgestrel and 0.03 mg ethinyl estradiol) regimen in women, rats were given OC by oral gavage. The dose was prepared to mimic a woman's OC daily dose based on 1,800 cal/day. Taking into account that rats need an average of approximately 32 cal/100 g of BW per day; a typical 290±20 gm rat requires 96 cal/day. The rats were given OC treatment for three consecutive days and placebo on the fourth day based on the 4-day estrous cycle of rat, and to resemble OC administration in women. On the 16th day, after completion of approximately four estrous cycles, the rats in the OC/placebo groups were exposed to cerebral ischemia. Modified from Raval et al. [16]

hinders E_2-mediated intracellular signaling in hippocampal slice cultures (generated from female rat pups) which are devoid of blood flow [16]. At the cellular level, E_2 is known to protect neurons through genomic mechanisms. Estradiol is a lipophilic molecule and diffuses through the neuronal plasma membrane, where it binds with its receptor in cytosol and subsequently translocates to the nucleus where it regulates the transcription of genes [20]. Estrogen receptors are of two types: ligand-activated estrogen receptor-alpha (ER-α) and beta (ER-β). Both these receptors play a role in estrogen-mediated post-ischemic neuronal survival although the mechanisms of neuroprotection governed by these receptors might be different. A recent review also suggested that estrogen receptor-dependent mechanisms of neuroprotection could vary depending on the experimental injury model used, the level of estrogen administrated and the mode of administration of the steroid (see review [21]). Studies from various laboratories including ours suggested that estrogen activates rapid intracellular nongenomic pathways that indirectly affect genomic activity via other transcription regulators such as cyclic-AMP response element binding protein (CREB) [22–24]. Other studies show that E_2-mediated activation of its extranuclear receptors rapidly phosphorylates neuronal extracellular signaling-related kinase (ERK) and the phosphotidylinositol-3-kinase (PI3 K)-Akt pathway [25–28]. Estradiol-mediated ERK activation is known to phosphorylate/regulate numerous downstream targets which include transcription factors. It has been demonstrated that the activated ERK plays dual role in cerebral ischemia [29, 30]. Therefore, it is crucial to understand in which subcellular compartment ERK is activated following E_2 stimulation. On the other hand, differences in neuroprotective mechanisms might be dependent on the subcellular location of estrogen receptor activation. It is now known that these receptors are located in the nucleus, plasma membrane, and mitochondria [31–35]. Our recent studies demonstrated that the synergistic negative effects of OC and nicotine reduced protein levels of membrane-bound and mitochondrial ER-β (Fig. 2) [15–17, 36]. Therefore, ER-β signaling at these two locations will be discussed in subsequent sections in order to delineate deleterious effects of OC and nicotine on the female brain.

The Membrane-Bound ER-β Signaling in the Brain: a Target of OC and Nicotine

The membrane-bound estrogen receptors exist within caveolar rafts tethered to the inner face of the plasma membrane. In the caveolar rafts, estrogen receptors are bound to the specific membrane proteins such as caveolin-1/2/3, isoforms of G-protein (i.e., $G_{s\alpha}$ and $G_{q\alpha}$) and L-type calcium channels [37, 38]. The caveolae are signaling regulators that

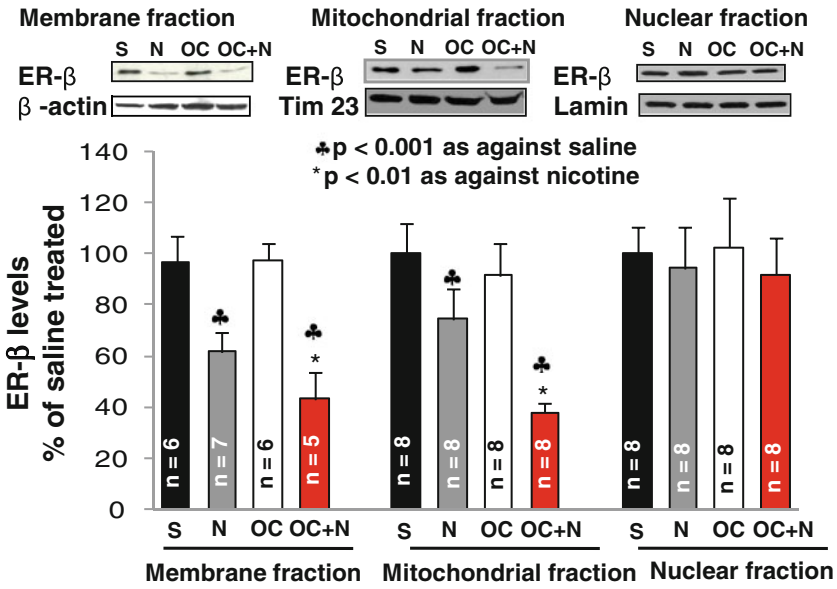

Fig. 2 Effect of nicotine alone or in combination with oral contraceptives on ER-β protein levels in sub-cellular fractions of hippocampus. *Top* representative immunoblots showing the protein levels of ER-β in the membrane, mitochondrial, and nuclear fractions for the hippocampus different experimental conditions viz. saline (*S*), nicotine (*N*), oral contraceptives (*OC*), and oral contraceptives in combination with nicotine (*OC+N*). Beta-actin (cytoskeletal), Tim 23 (mitochondrial marker), and lamin (nuclear marker) were used as loading controls. *Bottom* graphs present densitometric analysis of scanned Western blots. Note the OC and nicotine exposure decreased the protein levels of membrane-bound and mitochondrial ER-β protein in hippocampus of female rats. Figure modified from Raval et al. [11, 36]

serve to orchestrate the interaction of receptors and signaling molecules, modulating trans-membrane signaling in a rapid and specific manner. A necessary step in estrogen receptors localization to caveolae is palmitoylation of the receptor [34, 38, 39].

Palmitoylation is a post-translational modification that regulates membrane–protein interactions [40–43]. Protein S-palmitoylation refers to the addition of palmitate (16-carbon fatty acid) to a cysteine residue [40–42]. The unique reversibility of protein palmitoylation allows proteins to shuttle between intracellular membrane compartments. Various physiological stimuli regulate this palmitate cycling and contribute to cellular homeostasis [42]. Recently, palmitoyl acyl transferases have been identified as enzymes responsible for protein palmitoylation [40]. It has been demonstrated that the palmitoylation of human estrogen receptor at cysteine 447 is essential for receptor interaction with caveolin-1 and its subsequent localization to the plasma membrane [33]. Mutation of cysteine 447 to an alanine has been found to result in loss of membrane estrogen receptors in Chinese hamster ovarian cells [34, 44]. In addition, the physical interaction between estrogen receptors and caveolin-1 is abolished, and membrane estrogen effects are eliminated [34, 44]. This suggests that palmitoylation is a crucial modification for membrane translocation of estrogen receptor(s) and disturbances in the processes of palmitoylation reflect on estrogen signaling. In an unpublished study, we observed that chronic nicotine impairs the process of palmitoylation of ER-β in an in vitro model of hippocampal slice cultures. This result indicates possible transportation defects which are reflected in low levels of membrane bound ER-β after nicotine in the hippocampus of female rats [16]. We observed significant lower levels of membrane-bound ER-β protein in OC and nicotine group as compared to the nicotine-alone group. This finding suggested that the combination of OC+N aggravates nicotine toxicity in the hippocampus [16].

As mentioned at the beginning of this section, the membrane-bound ER-β are located within caveolae. In hippocampal neurons estrogen receptors co-localized with metabotropic glutamate receptors (mGluR) in the caveolae and the cross-talk between estrogen receptors and mGluR confers estrogen-mediated phosphorylation of CREB (see review [37]). The phosphorylated CREB is key for post-ischemic neuronal survival and pCREB plays a major role in both short-term and long-term synaptic plasticity in hippocampus and other brain structures [45]. It has been shown that loss of synaptic function leads to neuronal cell death in the hippocampus after cerebral ischemia [46]. Therefore, defects of ER-β CREB signaling after OC and nicotine might hinder hippocampal synaptic plasticity which could be a contributing factor for severe post-ischemic injury in OC and nicotine-exposed female rats.

Estrogen receptor-beta is predominantly expressed in the hippocampus and synaptic ER-β is suggested to be a more responsive target to E_2. Loss of synaptic function leads to

neuronal cell death in the hippocampus after cerebral ischemia. Our previous study demonstrated that hippocampal slices harvested 24 h after global cerebral ischemia exhibited no long-term potentiation (LTP; a cellular correlate of learning and memory) response, even when no histopathological abnormalities were observed, suggesting that synaptic dysfunction precedes neuronal cell death in the hippocampus after global cerebral ischemia [46]. In female mice, activation of ER-β potentiates LTP in CA1 neurons and improves hippocampus-dependent cognition [47]. Furthermore, the use of selective ER-β agonists increases the levels of key synaptic proteins such as the post-synaptic density, synaptophysin and the AMPA-receptor (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) in the hippocampus of mice [47]. These changes in synaptic proteins mediated by ER-β activation are related to morphological changes such as increased dendritic branching and increased density of mushroom-type spines in hippocampal neurons [47]. Overall, these studies showed that ER-β is a key mediator of hippocampus-dependent synaptic functions.

We found a reduction of short-term synaptic plasticity at the Schaffer Collateral-CA1 synapse of rats chronically exposed to nicotine indicated by the impairment of paired pulse facilitation and post-tetanic potentiation (Fig. 3) [48]. In contrast to our results demonstrating that nicotine attenuated short-term plasticity, prior studies demonstrated enhancement of LTP following nicotine application. However, those studies mainly investigated acute effects of nicotine on LTP or were conducted on male experimental animals [49, 50]. Biological responses to nicotine are gender-specific [51]. Therefore, discrepancies observed between our results and prior literature might be due to differences in the duration of nicotine exposure or sex. Furthermore, our results that chronic nicotine exposure reduced membrane-bound ER-β protein and impaired short-term plasticity, suggest a possible role of ER-β in mediating short-term plasticity.

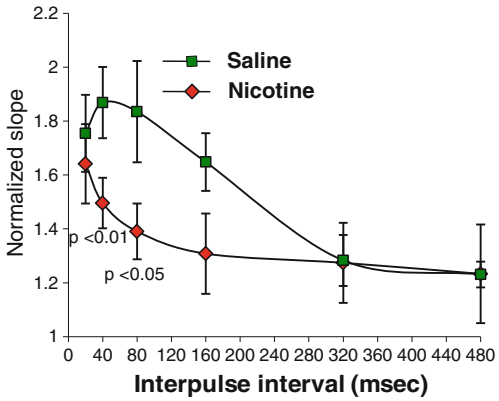

Fig. 3 Nicotine attenuates short-term synaptic plasticity in female rats. Relative slope of paired-pulse stimulation given at intervals of 20, 80, 160, 320, and 480 ms recordings from saline and nicotine exposed rat hippocampal slices are presented. The results showed significant depression at 40 ms (p=0.01), and 80 ms (p=0.05) of paired-pulse stimulations in nicotine-exposed rats as compared to the saline group. Modified from Raval et al. [48]

Membrane-bound estrogen receptors are also implicated in mediating estrogen's effects on hippocampus-dependent enhancement of cognitive performance [52, 53]. The membrane-mediated effect of estrogen is evident from a study using an E_2:bovine serum albumin (BSA) conjugate [54]. The BSA-conjugated E_2 fails to penetrate cell membranes and could not induce activation of an E_2-response [54, 55]. Application of BSA-conjugated E_2 to the hippocampus produced behavioral performance-enhancing effects as that of free E_2, suggesting that BSA-conjugated E_2 mediates effects of estrogen via membrane-bound estrogen receptors in the hippocampus [52, 54]. Additionally, studies have demonstrated a key role for ER-β in hippocampus-dependent memory and cognition [56]. ER-β knockout mice treated with estradiol show impairments in acquisition of a spatial reference memory, implicating a role for ER-β in hippocampus-dependent cognition [57]. However, the preceding two studies did not identify the sub-cellular location of ER-β that is responsible for mediating its effects on cognition. Taken together, the above literature and our findings that a combination of OC and nicotine reduces ER-β-mediated pCREB signaling in hippocampus, suggest that OC and nicotine may have deleterious effects on cognition. However, the clinical significance of this effect will require further investigation. Additionally, it will be essential to investigate the extent to which the effect of nicotine and OC on synaptic plasticity and cognition persists after cessation of nicotine alone or nicotine plus OC. This investigation could guide future translational research addressing women smokers trying to give up smoking. The investigation of this nature could explain why women addicted to nicotine might respond differently to hormone replacement therapy (HRT). Further knowledge regarding how nicotine interacts with female endocrinology is essential for understanding the effects of nicotine addiction on HRT and for understanding why the Women's Estrogen for Stroke Trial and the Women's Health Initiative failed to show any benefit of HRT [58–62].

Synergistic Inhibitory Effect of Oral Contraceptives Plus Nicotine Inhibits ER-β-Regulated Mitochondrial Functions

Mitochondrial estrogen receptors play a direct role in estrogen-mediated regulation of mitochondrial respiratory function [63–65]. The mitochondrial respiratory chain converts substrates generated from glycolysis, Krebs cycle, and β-oxidation into energy in the form of ATP, a process known as oxidative phosphorylation (OXPHOS) [66–68]. The OXPHOS system is composed of five multi-enzyme complexes (complexes I through V) and two electron carriers, a

quinone (coenzyme Q) and a small heme containing protein (cytochrome c) that are located in the inner mitochondrial membrane. These respiratory complexes are formed by subunits encoded by both the mitochondrial and the nuclear genome with the exception of complex II which is entirely encoded by nuclear DNA. In this energy-generating pathway, the reducing equivalents NADH and FADH2 formed during the TCA cycle and β-oxidation enter into the electron transport chain (complexes I through IV of the OXPHOS system) at the level of complex I (NADH:ubiquinone oxidoreductase) or at the level of complex II (succinate dehydrogenase), respectively. Electrons are subsequently transferred to the non-protein electron carrier coenzyme Q and then to complex III (ubiquinol:cytochrome c oxidoreductase or bc1 complex). The second electron carrier, cytochrome c, acts as a bridge for the transfer of electrons between complexes III and IV (cytochrome c oxidase). Once the electrons have reached complex IV they are transferred to molecular oxygen to form water [66]. During the transfer of electrons through the electron transport chain (ETC, complexes I–IV), protons are translocated simultaneously from the matrix to the mitochondrial intermembrane space by complexes I, III, and IV. This proton translocation creates an electrochemical gradient that is utilized by complex V (ATPase synthase) to generate ATP with the concomitant translocation of protons back into the matrix.

Complex IV enzyme activity is significantly increased upon ER-β agonist treatment in isolated mitochondria whereas ER-α agonist treatment failed to do so [36]. This study once more confirmed the presence of ER-β in mitochondria that was demonstrated by other labs earlier and identified that ER-β regulates the complex IV function [32, 36]. The mammalian complex IV is a 200-kDa complex composed of 13 different subunits, three (COX1, COX2, and COX3 subunits) of which form the catalytic core and are encoded by mitochondrial DNA (mtDNA), whereas the rest of the subunits are encoded by the nuclear genome (COX4, COX5a, COX5b, COX6a, COX6b, COX6c, COX7a, COX7b, COX7c, and COX8 subunits; Fig. 4) [66, 69].

Mitochondrial DNA (mtDNA) is an intronless circular double-stranded DNA containing 37 genes. Thirteen out of 37 mitochondrial genes are encoded for proteins that serve in the electron transport system, with the remainder encoding for tRNA and rRNA required for expression. MtDNA transcription is initiated at two promoters (PL and PH) located in the D-loop regulatory region through the binding of mitochondrial RNA (mtRNA) polymerase and the mitochondrial transcription factors Tfam (mtDNA maintenance factor; mtTFA) and TFB (mitochondrial transcription factors B2, TFB1M and TFB2M) making polycistronic transcripts that are processed later [70, 71]. The processing of these

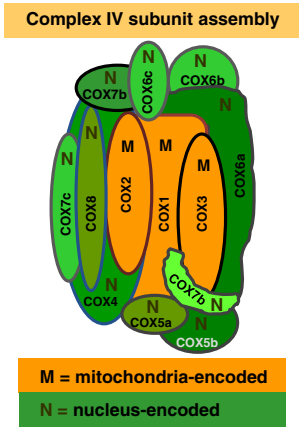

Fig. 4 Dual genetic origin of complex IV (CIV) subunits. This figure depicts the origin of mitochondrial CIV subunits and their assembly. Note the central three largest forms of the catalytic core and are encoded by the mitochondria. Modified from Diaz [72]

polycistronic transcription units remains to be fully characterized [73].

Estrogen receptors bind to the estrogen responsive element (ERE) located in the D-loop of the mtDNA, suggesting that estrogen receptors are involved in modulation of mitochondrial gene expression [74, 75]. However, the exact mechanisms of regulation of mtDNA transcription induced by this hormone remain obscure. One of the reasons is that if mitochondrial transcription is regulated by estrogen binding to the D-loop in the mtDNA, one would expect that all genes encoded in the mtDNA would be equally increased upon estrogen binding but that does not seem to be the case. Only certain transcripts have been reported to increase in expression upon estrogen exposure. For example, estrogen increased and estrogen receptor antagonists (ICI 182,780) decreased mRNA levels of the cytochrome c oxidase subunits 1 and 2 in a human breast epithelial cell line [76]. In HepG2 estrogen increased the levels of cytochrome c oxidase subunit 3, ATP6 (subunit of Complex V) and ND1 (subunit of complex I) mRNAs [77]. Another study on ovariectomized female rats demonstrated that estrogen replacement significantly increased cytochrome c oxidase subunit 3 mRNA levels in the hippocampus [65]. Importantly, cytochrome c oxidase subunits 1, 2, and 3 are mtDNA-encoded genes and the preceding two studies confirmed a role of the estrogen receptor in expression of mtDNA-encoded subunits of the cytochrome c oxidase gene.

The question that remains to be investigated is whether the increase in specific mitochondrial transcripts upon estrogen stimulation is related to a subsequent processing of the polycistronic translational unit and the stability of the different mRNAs, or if other mechanisms yet to be analyzed are involved. Interestingly, there are partial estrogen responsive element sequences that might account for the differential

transcription of mitochondrial genes. These ERE sequences are distributed in different genes on the murine mtDNA: D-loop, tRNA-leu, 12S rRNA, and cytochrome oxidase subunits 1 and 3. Likewise, partial ERE in humans has been described in the 12S and 18S rRNAs, tRNA-leu, and D-loop [78].

Additional studies demonstrated that mitochondrial pCREB promotes the expression of mitochondrial genes [79–82]. ER-β regulates estrogen-mediated phosphorylation of CREB and silencing ER-β reduced mitochondrial pCREB following ethinyl estradiol (estrogenic component of OC) treatment in hippocampus [36]. Furthermore, silencing of ER-β lowered protein levels of mitochondria-encoded complex IV subunits 1, 2, and 3 (Cox 1, 2, and 3), indicating the role of ER-β in pCREB-mediated OXPHOS protein expression [36]. Importantly, owing to the dual genomic origin of complex IV subunits, the assembly process of this complex is very complicated and highly regulated [69]. To assemble complex IV, subunits translated from both genomes must come together in a coordinated and regulated manner [66]. Defects in complex IV subunits assembly and/or stability cause mitochondrial dysfunction [68, 83–85]. Mitochondrial dysfunction remains a central cause of ischemic neuronal death [86–88]. The previously observed defects in mitochondrial complex IV subunits protein and function after OC plus nicotine could aggravate mitochondrial dysfunction after brain ischemia, possibly explaining the exacerbation of post-ischemic damage after use of OC plus nicotine [16]. Figure 5 depicts the putative mechanism of OC plus nicotine induced action on mitochondrial functions and neuronal survival. Mitochondrial functional abnormalities observed after OC plus nicotine were not observed in the nicotine alone group, suggesting that these harmful effects of OC plus nicotine might be due to a unique synergy of nicotine with OC. However, this may be a timing or dosage issue, meaning that longer or higher nicotine exposure could produce similar effects; therefore, future studies are needed to dissect the effects of time and dosage of nicotine exposure in female rats.

The mechanisms of cell death after brain ischemia are complex. Generation of reactive oxygen species and release of cytochrome c, and their consequent effects on mitochondrial dysfunction, are considered key factors for the induction of cerebral ischemic injury. Previous studies from our laboratory demonstrated that the post-ischemic release of cytochrome c into the cytosol during early reperfusion activates the caspase cascade, which in turn activates protein kinase C isozyme delta (δPKC) by proteolysis [89]. Activation of δPKC in turns mediates ischemic injury by putatively acting on caspase 3 and causing secondary mitochondrial dysfunction [89]. The binding of activated δPKC to mitochondria inhibits anti-apoptotic B-cell lymphoma-2 (Bcl-2) protein activation. The Bcl-2 stabilizes the mitochondrial membrane potential and prevents the release of mitochondrial cytochrome c, thus blocking activation of the caspase cascade and the onset of cell death [90, 91]. Estrogen's putative effects mediating neuroprotection include attenuation of post-ischemic caspase-3-activation [92] and cytochrome c release from the mitochondria [93] in the hippocampus and is correlated with neuroprotection [94]. Estradiol has been shown to increase transcription of pro-survival bcl-2 in vivo after ischemic stroke [95]. Based on this literature, the loss of anti-apoptotic signaling might be another factor responsible for the observed post-ischemic hippocampal injury after nicotine and OC treatment; however, this aspect needs further investigation.

Cerebral Vasculature Estrogen Receptors: a Possible Candidate Responsible for Harmful Effects of OC and Nicotine on Female Brain

The cerebral vasculature is specialized to preserve brain function, maintaining cerebral blood flow constant (autoregulation) during systemic pressure changes, protecting the brain against the influx of toxic agents through the blood–brain barrier, and transporting required materials and metabolites across [96]. Estrogen regulates cerebral blood flow through multiple mechanisms such as (1) increase in endothelium-dependent nitric oxide (NO) production (2), decrease in vascular tone (3), and suppression of thrombosis and inflammation disruptive to the regulation of cerebrovascular function [96–101].

The ability of estrogen to enhance NO production by endothelial NO synthase (eNOS) is the most extensively studied effect of ovarian hormones on the cerebrovasculature [96]. Stirone et al. noted that in vivo estrogen treatment resulted in a 100 % increase in eNOS mRNA copy number and increased eNOS protein levels by 47 % in the cerebral blood vessels of mice [102]. The eNOS gene expression is regulated by estrogen receptors [103]. Cerebral vessels express estrogen receptors (ER-α and -β) in both the smooth muscle and endothelial cell layers of cerebral blood vessels [97]. Isolated cerebral vessels express more eNOS protein after in vitro incubation with physiological concentrations of 17β-estradiol; and this effect is blocked by the estrogen receptor antagonist (ICI 182,780) [104]. Estrogen receptor-α protein expression is upregulated in cerebral blood vessel endothelial cells after estrogen treatment to ovariectomized mice [102]. Estrogen-mediated vascular protection after ischemia is achieved via ER-α, which increased vascular expression of angiopoietin-1 and stimulated angiogenesis in the brain [105, 106].

Cerebrovascular dysfunction and pathology include endothelial dysfunction, thrombosis, inflammation, and atherosclerosis which contribute to the pathogenesis of stroke and brain ischemia. Newer-generation OC formulations in use to-date persistently increase the risk of thrombus formation; however,

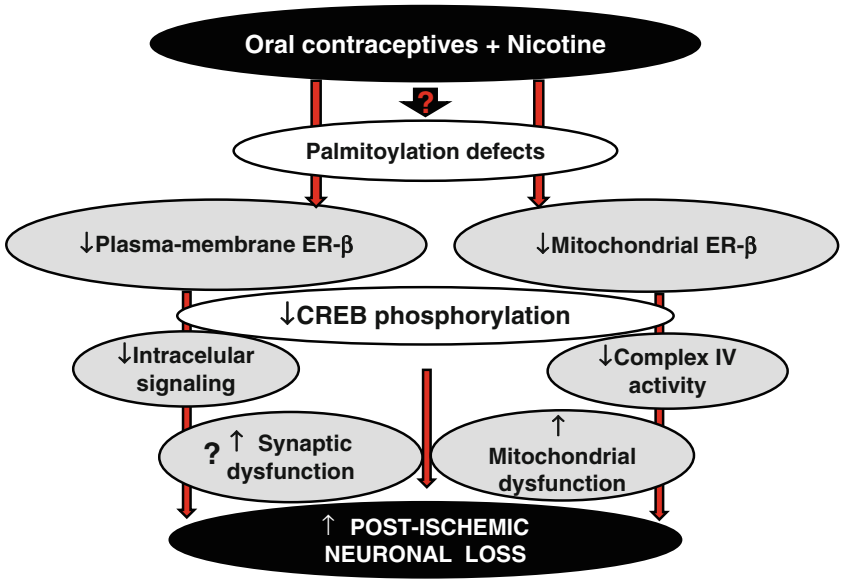

Fig. 5 Putative mechanism of action of nicotine and oral contraceptives on hippocampus of female. This figure depicts the main theme of this review that oral contraceptives along with nicotine induces ER-β loss from hippocampus and subsequently alters intracellular estrogen signaling, synaptic, and mitochondrial functions which could be responsible for severe ischemic injury in female rats

the mechanism is not understood [107]. The formation of a clot requires interaction of the blood with a biochemical or mechanical lesion in the vascular wall. Platelets in the blood produce thrombin and play a critical role in thrombus formation. Platelets express estrogen receptors [108, 109]. ER-β is found in membrane and cytosolic fractions of platelets [110]. Knockout of ER-β resulted in increased expression of P-selectin in platelets causing their activation. The decline or deprivation of ovarian hormones is associated with decreased ER-β expression and increased platelet activation/adhesion [111]. Estrogen treatment of ovariectomized pigs decreased platelet aggregation and secretion compared with untreated ovariectomized pigs [112]. Decline in ovarian hormones is also associated with increased expression of adhesion molecules, phosphatidylserine, and CD40, which allow the platelet to interact with leukocytes and endothelial cells of the vascular wall. This is implicated in the progression of arterial disease [113–117]. Risk for arterial diseases is compounded by other clinical risk factors such as nicotine addiction.

Given the presence of estrogen receptors in cerebral arteries, information about the role of estrogen receptors in the cerebral vasculature after nicotine addiction in women is limited and needs to be investigated [96, 118]. Additionally, it is well known that the chronic nicotine usage increases thrombus formation and alters cerebrovascular endothelial cell function [119–127]. It is important to emphasize the fact that nicotine and OC are independent risk factors for endothelial dysfunction and thrombus formation; however, the synergistic deleterious effect of nicotine and OC on endothelial and platelet function in women is not known. Furthermore, how long the risk of thrombus formation persists after cessation of smoking in OC users requires investigation. Exploring the possible impact of chronic nicotine exposure alone or in combination with OC on vascular or endothelial estrogen receptors might help understand the underlying mechanism causing increased severity of cerebrovascular diseases in women smokers using OC.

Conclusion

Despite global warnings and awareness of the detrimental effects of smoking on health, smoking-derived nicotine addiction makes it more difficult for women smokers to relinquish the habit than for men smokers [128, 129]. The rise in the number of women smokers continues to be a major public health concern in the United States. The United States is currently dealing with veterans of the Iraq and Afghanistan wars and women constitute a growing segment of the military veteran population and women veterans of the Iraq and Afghanistan wars are smoking at alarming rates. Smoking-derived nicotine in female rats renders them more susceptible to ischemic brain damage, a consequence that could occur from brain injury in combat. Smoking is the one preventable risk factor, and relinquishing this habit could reduce the risk for cerebrovascular diseases. In recent years, women smokers trying to give up smoking have switched to the new electronic nicotine delivery systems (e-Cigarettes) that deliver dosages of nicotine that mimics mild, moderate, and heavy smoking-derived nicotine. The e-Cigarettes are marketed as a "healthy tool for smoking cessation" and advertised with an aim to attract women. Although the e-Cigarettes are devoid of other compounds in cigarettes, their safety is questionable and the Food and Drug Administration (FDA) warns the public about the potential negative effects of nicotine on health. The effects of nicotine are more harmful for

women who use OC and studies are needed to (1) indentify specific effects on nicotine vs. nicotine in combination with OC on the female brain and (2) define for how long harmful effects of nicotine alone or in combination with OC on female brain persist after respective withdrawals. Currently multimillion national "smoke cessation" programs (e.g., Tobacco-free Florida, smokefree.gov) are creating a numerically important subset of the women who are "oral contraceptive users and ex-smokers". The studies directed to better understand the consequences of nicotine withdrawal specific to OC-exposed women could guide future translational research.

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Conflict of Interest None.

Compliance with Ethics Requirements Vertebrate animal use: In this review we have cited studies conducted at our lab. All institutional and national guidelines for the care and use of laboratory animals were followed at the time we conducted those studies.

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