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ORIGINAL ARTICLE

Synergistic inhibitory effect of nicotine plus oral contraceptive on mitochondrial complex-IV is mediated by estrogen receptor-β in female rats

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Abstract

Chronic nicotine and oral contraceptive (NOC) exposure caused significant loss of hippocampal membrane-bound estrogen receptor-beta (ER-β) in female rats compared with exposure to nicotine alone. Mitochondrial ER-β regulates estrogen-mediated mitochondrial structure and function; therefore, investigating the impact of NOC on mitochondrial ER-β and its function could help delineate the harmful synergism between nicotine and OC. In this study, we tested the hypothesis that NOC-induced loss of mitochondrial ER-β alters the oxidative phosphorylation system protein levels and mitochondrial respiratory function. This hypothesis was tested in hippocampal mitochondria isolated from female rats exposed to saline, nicotine, OC or NOC for 16 days. NOC decreased the mitochondrial ER-β protein levels and reduced oxygen consumption and complex IV (CIV) activity by 34% and 26% compared with saline- or nicotine-administered groups, respectively. We also observed significantly low protein levels of all mitochondrial-encoded CIV subunits after NOC as compared with the nicotine or saline groups. Similarly, the silencing of ER-β reduced the phosphorylation of cyclic-AMP response element binding protein, and also reduced levels of CIV mitochondrial-encoded subunits after estrogen stimulation. Overall, these results suggest that mitochondrial ER-β loss is responsible for mitochondrial malfunction after NOC.

Keywords: birth control pills, complex IV, cyclic-AMP response element-binding protein, mitochondrial complex activity, mitochondrial respiration, ROS.

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Despite global warnings and awareness of the detrimental effects of smoking-derived nicotine on health, 22.4% of American women of reproductive age of 18–44 years currently smoke cigarettes (CDC 2008; McClave *et al.* 2010). Women in this age group are also likely to use oral contraceptives (OC). Combination OC therapy (OC containing two hormones; e.g. estrogen and progestin) is the leading method of contraception in the United States (Mosher *et al.* 2004). Although OC confers benefits such as convenience, low failure rate, reduced risks of ovarian and endometrial cancer, and treatment of dysmenorrhea (Sherif 1999), the synergistic negative interaction between cigarette smoking and OC results in poor health outcomes, causing increased smoking-related mortality and an average loss of 14 years of life (Goldbaum *et al.* 1987; USDHH 2004). More importantly, women who smoke and use oral contraceptives are at increased riskof cardiovascular and cerebrovascular diseases compared with non-smoking women who use OC (Goldbaum *et al.* 1987; Hannaford 2000; Keeling 2003). However, the underlying mechanisms responsible for increased severity of diseases are poorly understood and require investigation.

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Abbreviations used: AS, antisense oligodeoxynucleotides; CA1, Cornu Ammonis layer 1; CIV, complex IV; CREB, cyclic-AMP response element-binding protein; COX, cytochrome *c* oxidase or CIV; E_2, 17β-estradiol; EE, ethinyl estradiol; ERs, estrogens receptors; ER-β, estrogens receptor-beta; mtDNA, mitochondrial DNA; N, nicotine; nDNA, nuclear DNA; ND5, NADH dehydrogenase; NOC, nicotine plus OC; OC, oral contraceptives; OXPHOS, oxidative phosphorylation system protein; p, phosphorylation; ROS, reactive oxygen species; TMPD, N,N,N′, N′-tetramethyl-p-phenylenediamine.

Apart from diverse physiological effects of chronic nicotine exposure common to both sexes owing to the activation of nicotinic acetylcholine receptors, in women, nicotine reduces circulating estrogen levels (Jensen *et al.* 1985; Windham *et al.* 1999). In laboratory studies on female rats, we confirmed the epidemiological findings suggesting that chronic nicotine exposure reduces circulating 17β-estradiol (E_2) levels, and also observed that chronic nicotine made female rats more susceptible to ischemic brain damage (Raval *et al.* 2009a, 2011). Brain ischemia occurs from loss or drastic reduction of blood supply to the brain, a common consequence of cardiac arrest or stroke. The hippocampus is the most vulnerable region of the brain following cerebral ischemia, and is thus the target of our current research. The severity of ischemic hippocampal damage is far greater in females simultaneously exposed to oral contraceptives than to nicotine alone (Raval *et al.* 2011). Based on these early findings, our next goal is to elucidate the mechanisms that are unique to, or different from, nicotine plus OC (NOC) compared with nicotine alone, which are also possibly responsible for the exacerbation of post-ischemic hippocampal damage in female rats (Raval *et al.* 2011).

Estrogen is neuroprotective against ischemia in both *in vivo* and *in vitro* injury models. The mechanism of neuroprotection governed by estrogen is likely to be multifactorial (Lee *et al.* 2004; Zhao *et al.* 2004). Estrogen activates rapid intracellular signaling pathways via transcription regulators such as cyclic-AMP response element-binding protein (CREB) and affects the genomic activity required for neuronal survival from ischemic stress in female rats (Lee *et al.* 2004; Raval *et al.* 2009b). These rapid and neuroprotective effects of estrogen in the hippocampus are mediated through its receptor subtypes alpha (ER-α) and beta (ER-β).

Estrogen receptor-mediated phosphorylation of CREB was abolished after chronic nicotine exposure in an *ex vivo* model of hippocampal slice cultures devoid of blood flow, which demonstrated that nicotine directly hinders estrogen-mediated cell-survival intracellular signaling (Raval *et al.* 2011). Furthermore, nicotine alone or in combination with OC decreased membrane-bound ER-β but not ER-α protein levels in the hippocampus of female rats (Raval *et al.* 2011). Interestingly, we observed significantly lower levels of membrane-bound ER-β protein in an NOC group as compared with a nicotine-alone group, which suggested a role for ER-β in the induction of harmful effects of NOC.

Estrogen receptors are also located in mitochondria and are known to play a direct role in estrogen-mediated preservation and regulation of mitochondrial function (Bettini and Maggi 1992; Klinge 2008; Mirebeau-Prunier *et al.* 2010). Therefore, understanding the role of mitochondrial ER-β in mitochondrial function after exposure to NOC could help delineate the harmful synergism between nicotine and OC. Here, we hypothesize that NOC exposure results in the loss of mitochondrial estrogen receptors, which then causes defects in mitochondrial respiration owing to alterations in the levels of some of the proteins in the oxidative phosphorylation system (OXPHOS).

Materials and methods

All protocols were approved by the Animal Care and Use Committee of the University of Miami. Animals were purchased from Charles River Laboratories (Wilmington, MA, USA).

In vivo experiments

Female Sprague–Dawley rats weighing 290 ± 20 g were used for this study. The stages of estrous cycle were monitored as described (Marcondes *et al.* 2002) and only rats showing at least three consecutive normal period (4 days) estrous cycles were used for our experiments. We demonstrated that the higher circulating plasma estrogen level at proestrus stage protects Cornu Ammonis layer 1 (CA1) neurons against cerebral ischemia, and thus rats were killed on proestrus stage of estrous cycle in the following experiments (Raval *et al.* 2009a). The treatment groups were:

Group 1: saline: rats were implanted with an osmotic pump containing 0.9% saline for 16 days as described previously (Raval *et al.* 2011).

Group 2: nicotine: rats were implanted with an osmotic pump containing nicotine bitartrate dissolved in 0.9% saline (dose 4.5 mg/kg/day) for 16 days as described before (Raval *et al.* 2011). Previously, we used this paradigm of nicotine delivery and monitored plasma cotinine-nicotine metabolite levels on last day of nicotine treatment to confirm nicotine delivery (Raval *et al.* 2011).

Group 3: oral contraceptive (OC): rats were treated with an oral contraceptive (OC; 0.3 mg norgestrel and 0.03 mg ethinyl estradiol) for 16 days (approximately four estrous cycles) as described previously (Raval *et al.* 2011). The dose was prepared to mimic a woman's OC daily dose based on 1800 calories per day. The rats were given OC treatment (oral gavage) for three consecutive days and a placebo on the fourth day based on the 4-day estrous cycle of the rat to resemble OC administration in women (Eleftheriades *et al.* 2005). For the control experiment, rats were treated with placebo (oral gavage) for 16 days.

Group 4: nicotine + OC (NOC): rats were treated with nicotine and OC (described for group 3 and 4; Fig. 1a) for 16 days.

Isolation of mitochondria from hippocampus

After respective treatment rats were anesthetized using 5% isoflurane, decapitated, and the hippocampi were immediately immersed in cold (4°C) isolation medium [250 mM sucrose, 10 mM HEPES (pH 7.4), 1 mg/mL BSA-IV, 0.5 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 mM ethylene glycol bis(-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) dipotassium salt). The tissue was minced and suspended in isolation media containing nagarse (2.5 mg/g tissue), and incubated in this solution for 2 min. At the end of incubation, tissue was homogenized in a hand-operated glass Teflon homogenizer by seven up-and-down strokes. The homogenate was diluted to yield 10% (W/V) homogenate, and centrifuged at 2000 *g* for 3 min in a Sorvall RC5 centrifuge at 4°C. The supernatant was decanted and centrifuged at 12 000 *g* for 8 min at 4°C. The resulting supernatant

was discarded and the pellet was re-suspended in the isolation medium. The suspension was centrifuged at 12 000 *g* for 10 min. The resulting pellet was re-suspended in 0.25 M sucrose and centrifuged at 12 000 *g* for 10 min. The pellet was suspended in 0.25 M sucrose and was used as the source of crude mitochondria (non-synaptic and synaptic). The suspended mitochondria sample was divided into two parts, one part was used for polarographic studies, effect of estrogen receptor agonist on mitochondrial complex IV (CIV) activity and reactive oxygen species (ROS) measurements, while other part was stored at −80°C and utilized for mitochondrial complex activities measurements and western blotting.

Polarographic studies in isolated mitochondria

Mitochondrial [permiabilized in digitonin (0.007% (w/v)] oxygen consumption was measured in a Clark type oxygen electrode as described before (Davey *et al.* 1997; Dave *et al.* 2001). The rate of respiration was measured in the presence of 5 mM pyruvate and 2.5 mM malate to determine the respiration rates of states 3 and 4. State 3 respiration rate was initiated by the addition of 1 μL of 0.1 M ADP and state 4 respiration rate was ensued after the depletion of added ADP. Results were expressed as nmol oxygen/min/mg protein. For these experiments, 20–35 μg of mitochondria were used in the assay. The oxidation of 5 mM pyruvate plus 2.5 mM malate was measured in presence of excess ADP (500 μM final concentration). The rate of oxygen consumption was recorded up to 2–3 min. The reaction was inhibited by addition of 10 μL rotenone (5 μM), a complex I inhibitor. In the same assay, 8 mM succinate plus 4 mM glycerol-3-phosphate was added. The rate of oxygen consumption was recorded up to 2–3 min. The reaction was inhibited by addition of 10 μL antimycin (10 μM), a complex III inhibitor. Subsequently, 500 μM ascorbate and 200 μM N,N,N′,N′–tetramethyl-p-phenylenediamine (TMPD) were added. The rate of oxygen consumption was recorded up to 2–3 min. The reaction was inhibited by addition of 3 μL potassium cyanide (200 mM), a CIV inhibitor.

Mitochondrial complex activities

A total of 20–40, 5–10, 1–5, and 7–15 μg of mitochondrial proteins were used to determine the activities of complexes I, II, III, and IV, respectively via spectrophotometer (Dave *et al.* 2001, 2009). Activities were measured at 37°C in a final volume of 1.0 mL.

Mitochondria from hippocampus of naïve female rats were exposed to ER-α [25 nM; 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole] or ER-β [40 nM; 2,3-bis(4-hydroxyphenyl) propionitrile] agonists for 15 min followed by CIV activity measurements as described above. These agonists were dissolved in dimethylsulfoxide and same was use for control experiment.

Measurement of the rate of H_2O_2 production

The rate of H_2O_2 production was measured using Amplex Red hydrogen peroxide assay kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. In brief, H_2O_2 release from mitochondria was measured in 100 μL of reaction mixture containing 50 μm Amplex Red reagent, 0.01 U of horseradish peroxidase, 145 mm NaCl, 5.7 mm sodium phosphate, 4.86 mm KCl, 0.54 mm $CaCl_2$, 1.22 mm $MgSO_4$, 5.5 mm glucose, pH 7.35, and 50–100 μg of mitochondrial protein at 37°C. The fluorescence (excitation: 545 nm and emission: 590 nm) was recorded for 30 min. The linear rate of reaction was considered to be the rate of H_2O_2 production.

Mitochondrial to nuclear DNA ratio

Total DNA was obtained from hippocampal tissue by proteinase K digestion, phenol–chloroform–isoamyl alcohol extractions and ethanol precipitation. Mitochondrial DNA (mtDNA) content from each sample was calculated in triplicates by real-time quantitative PCR in a Bio-Rad CFX96 detection system using the SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and primers shown below. Standard curves were generated for each set of primers to ensure linearity of the amplification reaction and 2.5 ng of total DNA of all samples were used to determine the threshold cycle (ΔC_t) for each gene. We calculated the ΔC_t of Cox 3 and ND5 for the estimation of mtDNA and the ΔCt of actin and GAPDH for nuclear DNA (nDNA). The ratio of mtDNA/nDNA was calculated for each treatment. The following sets of primers were used: Actin: ggcgcttttgactcaggatt and gggatgtttgctccaaccaa; GAPDH: gaaatcccctggagctctgt and ctggcaccagatgaaatgtg; NADH dehydrogenase (ND5) ND5: caataccccacccccttatc and gaggctcatcccgatcatag; Cox3: acataccaaggccaccaac and cagaaaaatccggcaaagaa.

Silencing of hippocampal ER-α or β after respective antisense oligodeoxynucleotides infusion

To knockdown ER-α/β in the hippocampus, we administered ER-α or β-antisense oligodeoxynucleotides (AS) or scrambled missense (10 nmol of AS mixed with 5 μL of vivo-jetPEI; Polyplus Transfection, New York, NY, USA) by bilateral cerebroventricular infusion every 24 h for 4 days, as described previously by (Zhang *et al.* 2009). Rats were killed 30 min after the last infusion. The antisense sequences used in this study were 5′-CATGGTCATGGTCAG-3′ for ER-α; 5′-GAATGTCATAGCTGA-3′ for ER-β (Edinger and Frye 2007). The same dose of a scrambled missense oligo (MS; 5′-ATCGTGGATCGTGAC-3′) was used as control. To stimulate estrogen-signaling, rats were injected with a single bolus of E_2 (5 μg; oil-vehicle; Sigma, St. Louis, MO, USA) or estrogenic component of OC ethinyl estradiol (EE; 5 μg; oil-vehicle; concentration based on Picazo *et al.* 2011) on the second day (48 h prior to killing the rats) of AS treatment. We selected the 48-h duration based on our previous study demonstrating that a single bolus of E_2 phosphorylates CREB protein in the hippocampus of female rats after 48 h (Raval *et al.* 2009b).

Immunohistochemistry

To confirm the delivery of the AS to the hippocampus after cerebroventricular infusion, we used Alexa 488-labeled missense (Alexa 488-MS). Rats were perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline 3 h after Alexa 488-MS injection. Coronal brain sections that included the hippocampus were cut at 30 μm thickness on a cryostat. Staining was performed on free-floating sections in phosphate-buffered saline + 0.8% Triton-X 100. Following pre-blocking with 10% goat serum, sections were incubated for 24 h at 4°C with neuronal marker NeuN (mouse monoclonal, 1 : 400; Chemicon, Temecula, CA, USA). Following overnight washing, the sections were then incubated with fluorescent secondary antibodies (rhodamine-labeled anti-mouse) for 24 h at 4°C. The sections were, mounted onto slides and viewed on a Carl Zeiss LSM-510 confocal microscope (Raval *et al.* 2005).

ER-β knockout mouse hippocampus

Brain tissue samples of knockout/wild-type mice were obtained from Dr Kenneth Korach's laboratory at the National Institute of Environmental Health Science at Research Triangle Park, North Carolina. We performed western blot analysis to investigate pCREB protein levels in hippocampus.

Isolation of nuclear fraction

Hippocampi of rats were collected after the various experimental treatments (groups 1–4). The hippocampal samples were homogenized in buffer (4 mM ATP, 100 mM KCl, 10 mM imidazole, 2 mM EGTA, 1 mM $MgCl_2$, 20% glycerol, 0.05% Triton X-100, 17 μg/mL phenylmethylsulfonyl fluoride, 20 μg/mL soybean trypsin inhibitor, 25 μg/mL leupeptin, 25 μg/mL aprotinin, and 1 mM Na_3 VO_4). The homogenate was then centrifuged at 4° C at 480 *g* for 10 min. The resulting (i) pellet and (ii) supernatant were treated separately to acquire the nuclear and cytosolic fractions respectively. (i) The pellet was washed twice, resuspended in nuclear lysis buffer (pH 8.0; 20 mM HEPES, 400 mM NaCl, 0.5 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, proteinase inhibitor (5 μL proteinase inhibitor cocktail/1 mL lysis buffer), and 1 mM Na_3VO_4) and centrifuged for 15 min. The resulting supernatant was the nuclear fraction. (ii) The resulting supernatant was re-centrifuged at 32000 *g* for 20 min to isolate the cytosolic fraction; the pellet was discarded. The cytosolic or nuclear fractions were analyzed for protein content using the Bio-Rad protein assay kit.

Western blot analysis

The proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Equal amounts of protein from each group were run on the same gel and analyzed at the same time. Proteins were transferred to an Immobilon-P (Millipore Corporation, Bedford, MA, USA) membrane and incubated with primary antibodies against Tim23 (mitochondrial marker; monoclonal; 1 : 1000; BD Biosciences, San Jose, CA, USA), lamin (nuclear marker), rabbit polyclonal anti-ER-α/ER-β (1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-CREB (pSer133; rabbit polyclonal, 1 : 1000; Cell Signaling Technology, Beverly, MA, USA), CREB (rabbit polyclonal; 1 : 1000; Cell Signaling Technology), subunits of complex I subunit NDUFB8 (~20 kD), complex II subunit-CII-30 (~30 kD), complex III subunit core 2 (~47 kD), CIV subunit I (~39 kD), ATP synthase subunit alpha (~53 kD) (total OXPHOS rodent WB Antibody Cocktail from Mitosciences, Eugene, OR, USA), and cytochrome *c* oxidase (COX) 1, 2, 3 and 4. Immunoreactivity was detected using enhanced chemiluminescence (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). Western images were digitized at 8-bit precision by means of a charge-coupled device-based camera (Xillix Technologies Corp., Richmond, BC, Canada) equipped with a 55 mm Micro-Nikkor lens (Nikon, Melville, NY, USA). The camera was interfaced to an advanced image-analysis system (MCID Model M2, Imaging Research, Inc.). The digitized immunoblots were subjected to densitometric analysis using MCID software.

Statistical analysis

The data are presented as mean value ± SEM. Results from the densitometric analysis or enzyme activities were analyzed by a two-tailed Student's 't' test and a $p < 0.05$ was considered statistically significant.

Results

NOC reduced the level of hippocampal mitochondrial ER-β protein

Because our previous results demonstrated that nicotine reduced the level of membrane-bound ER-β (Raval *et al.* 2011), in this study we investigated the availability of ER-β in hippocampal mitochondria and nuclear fractions after NOC exposure (Fig. 1a). Our results demonstrated that NOC significantly reduced hippocampal mitochondrial ER-β protein levels as compared with the nicotine and saline groups (Fig. 1b). In contrast, mitochondrial ER-α or nuclear ER-α/β protein levels did not change following either nicotine alone or NOC. ER-β protein levels after nicotine, OC and NOC showed a 75% ($n = 8$), 91% ($n = 8$; NS) and 38% ($n = 8$; $p < 0.001$) reduction, respectively, as compared with saline (100%; $n = 8$) (Fig. 1c).

NOC impaired mitochondrial respiration

Because NOC reduces mitochondrial ER-β protein levels, we investigated how the loss of mitochondrial ER-β affected mitochondrial functions. We measured mitochondrial oxygen consumption and determined the respiratory control index (average respiratory control index was 2.16 ± 0.2) in mitochondria isolated from rat hippocampi from saline, nicotine, OC, and NOC-exposed female rats (Fig. 2a). Subsequently, we investigated the function of mitochondrial respiratory chain complexes by measuring the rate of oxidation of different substrates in isolated hippocampal mitochondria (Fig. 2b). The mean rate of mitochondrial respiration (state 3 in presence of ADP) in the presence of different substrates is presented in Fig. 2c. When pyruvate and malate were added as substrates providing electrons to complex I (CI), oxygen consumption in the nicotine and NOC groups was lowered by 27% (104 ± 17; $n = 5$) and 43% (83 ± 6; $n = 5$; $p < 0.001$), respectively, when compared with the saline group (144 ± 22; $n = 10$). No significant differences were found between the saline- and OC-treated groups. The rate of respiration was comparable among all groups when succinate and glycerol-3-phosphate were used as complex II (CII) and III (CIII) substrates, respectively. Oxygen consumption in the nicotine, OC and NOC groups was reduced by 8% (794 ± 105; $n = 5$), 17% (715 ± 65; $n = 5$) and 34% (571 ± 71; $n = 4$; $p < 0.01$) as compared with saline-treated group (859 ± 103; $n = 10$) in the presence of the CIV substrates ascorbate and TMPD, respectively.

NOC impaired hippocampal mitochondrial CIV activity

Because we observed an impaired rate of respiration in the presence of pyruvate plus malate and ascorbate plus TMPD substrates, we decided to measure the enzymatic activity of the individual respiratory complexes spectrophotometrically (Fig. 3a–d). Our results demonstrated that neither nicotine

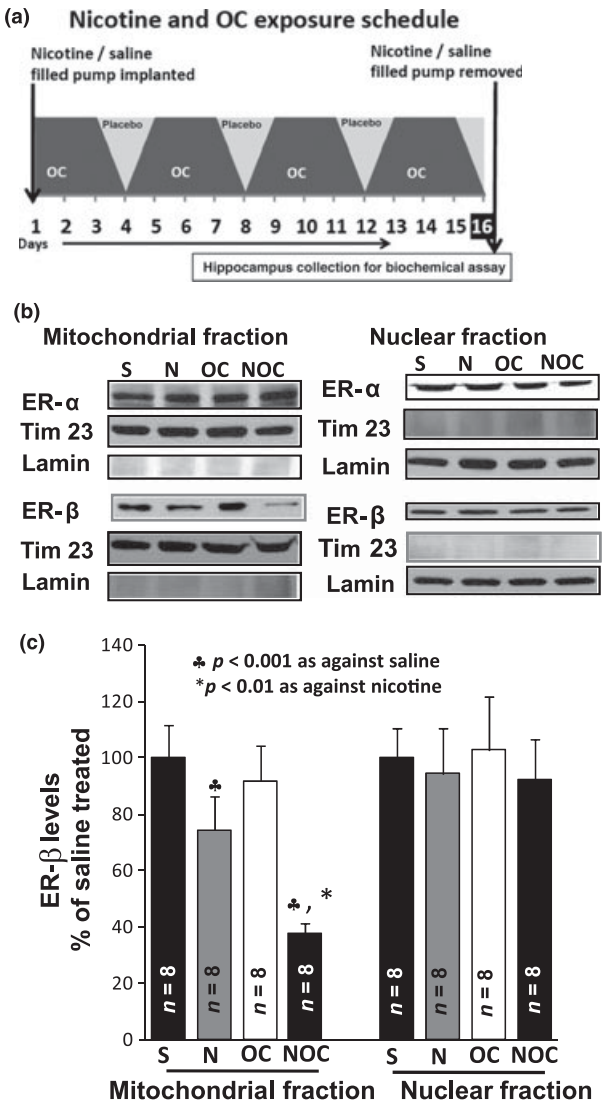

Fig. 1 Loss of hippocampal mitochondrial ER-β but not ER-α after NOC. (a) Rats were implanted with an osmotic mini-pump, which produced a dose of 4.5 mg/kg/day. To mimic the OC regimen of women, rats were given OC by oral gavage. The rats were given OC treatment for three consecutive days and placebo on the fourth day based on the 4-day estrous cycle of rat, and to resemble OC administration in women. On the 16th day, the rats in the OC/placebo groups were sacrificed for tissue collection. (b) Representative immunoblots showing the protein levels of ER-α and ER-β in the mitochondrial fraction for different experimental conditions. Tim 23 (mitochondrial marker) and lamin (nuclear marker) were used as loading controls. Note the absence of immunoreactivity for lamin in the mitochondrial fraction panel, confirming purity of the mitochondrial fraction. (c) Densitometric analysis of scanned western blots revealed that nicotine/NOC exposure decreased the levels of mitochondrial ER-β protein in hippocampus. In contrast, mitochondrial ER-α or nuclear ER-α and ER-β protein levels were not changed following nicotine or NOC treatment.

nor NOC treatment to rats altered CI, CII or CIII activities as compared with the saline group. Interestingly, we observed significant decreases in the activity of CIV in NOC-exposed groups as compared with the rest of the experimental groups (Fig. 3d).

ER-β enhances mitochondrial CIV function

Because NOC reduced the levels of ER-β, we investigated whether ER-β had a direct effect on CIV activity. Mitochondria from hippocampus of female rats were exposed to ER-α [25 nM 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole] or ER-β [40 nM 2,3-bis(4-hydroxyphenyl) propionitrile] agonists for 15 min and CIV activity was measured. We observed an increase in CIV activity by 36% (1572 ± 65; $n = 4$; $p < 0.05$) in the presence of ER-β agonist compared with the presence of ER-α agonist (1151 ± 116; $n = 4$) suggesting that ER-β activation directly regulates CIV activity (Fig. 3e). This result also confirms the presence of ER-β in isolated hippocampal mitochondria.

Knockdown of ER-β inhibits estrogen-mediated phosphorylation of CREB in hippocampus of female rat

As it has been reported that stimulation of ER-β increases phosphorylation of CREB in neurons (Boulware *et al.* 2007; Luoma *et al.* 2008), we decided to test if knocking down ER-β gene would result in the abrogation of CREB phosphorylation. To knock down ER-α or β we used the AS approach. First, we confirmed delivery of the AS to the hippocampus after cerebroventricular infusion using Alexa 488-labeled missense (Alexa 488-MS). Rats were killed 3 h after Alexa 488-MS injection and the brains were processed for immunohistochemistry of the neuronal marker NeuN. Confocal microscopic images of hippocampal CA1 region showed Alexa488-MS signal (green) co-localizing with the neuronal marker NeuN (red), suggesting the presence of MS in hippocampal CA1 cells (Fig. 4a). Second, we confirmed the efficacy of the ER-α/β-AS in knocking down ER-α and β in the hippocampus by western blot analysis (Fig. 4b). Quantification of western blots showed that the ER-α-AS and ER-β-AS caused a significant reduction in ER-α and ER-β protein levels, respectively (Fig. 4c). Finally, to test our hypothesis, we isolated mitochondrial and nuclear fractions of hippocampus following ER-α/β-AS treatment and performed western blotting for pCREB. The western blot images presented in Fig. 4d show that ER-β-AS, but not ER-α-AS, attenuated pCREB in both the nuclear and mitochondrial fractions of hippocampus following E_2/EE treatment. These results suggested that the E_2/EE-induced phosphorylation of CREB is mediated via ER-β and silencing of ER-β caused low protein levels of pCREB.

Because pCREB has been implicated in modulating mitochondrial gene expression (Lee *et al.* 2005; Ryu *et al.*

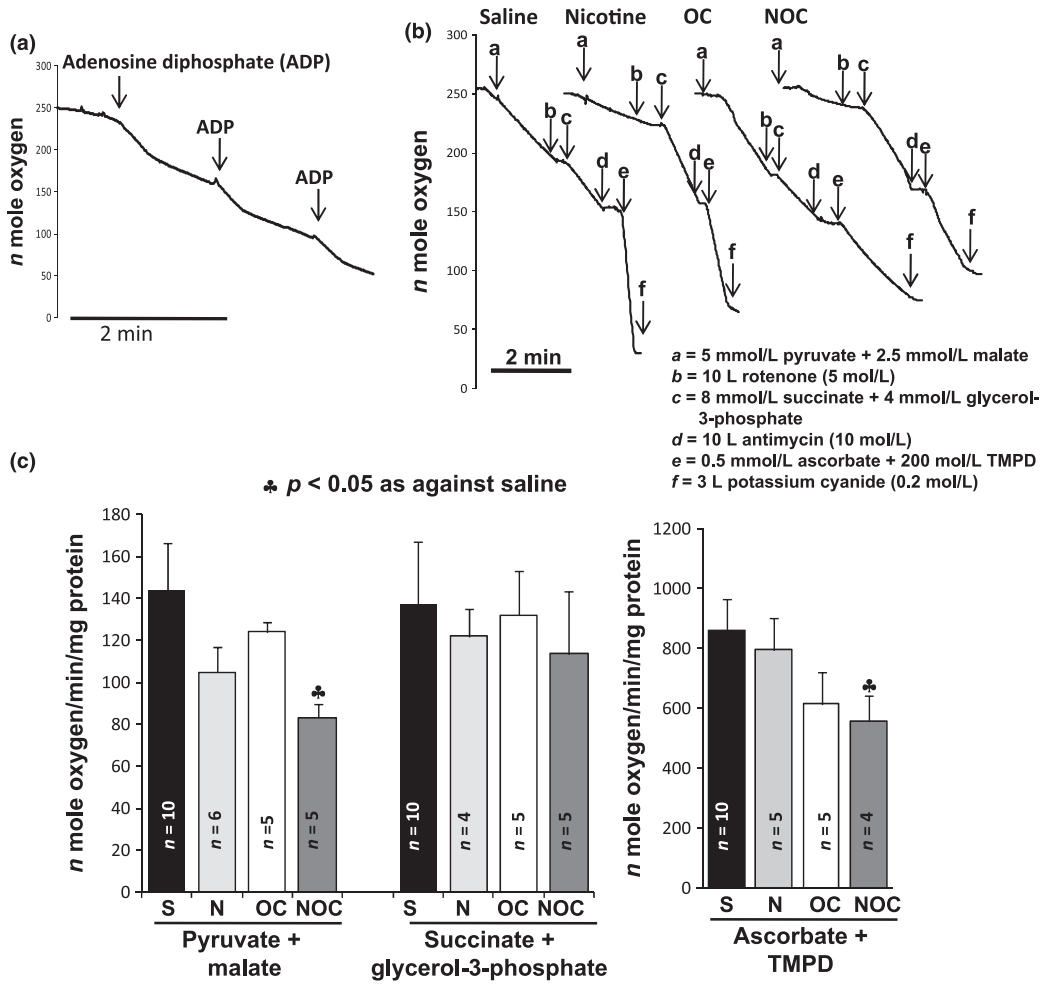

Fig. 2 NOC reduced hippocampal mitochondrial respiration. (a) Typical polarographic traces indicating respiratory control index and state 3 and 4 measurements. Arrows indicate addition of adenosine diphosphate (ADP). (b) Typical polarographic traces for the four animal groups showing oxygen consumption in the presence of different substrates and cofactors. (c) Represents data from pyruvate-malate and succinate-glycerol-3-phosphate; and ascorbate plus TMPD.

2005; De Rasmo *et al.* 2009), we tested if the reduced mitochondrial pCREB following ER-β-AS plus E_2/EE treatment affected the levels of mitochondrial-encoded subunits of CIV. Figure 4e shows that inhibition of ER-β signaling with AS resulted in dramatic reduction of CIV subunit levels and this correlated with the reduced levels of pCREB. To corroborate that a decreased level of pCREB is directly related to knockdown of ER-β, we investigated the levels of pCREB in ER-β knockout mice. Figure 4f shows dramatically reduced pCREB protein levels in knockout hippocampal tissue as compared with wild-type.

NOC reduced mitochondrially encoded CIV subunit protein levels

The above results clearly demonstrated that knock down of the ER-β resulted in reduced mitochondrial-encoded CIV subunits. Next, we tested whether NOC-mediated loss of ER-β was correlated with reduction in other OXPHOS proteins. We analyzed the steady-state protein levels of CI subunit NDUFB8 (~20 kD); CII subunit-CII-30 (~30 kD); CIII subunit core 2 (~47 kD); CIV subunit 1 (~39 kD); and ATP synthase subunit alpha (~53 kD) in isolated mitochondria. Quantification of steady-state levels demonstrated no significant difference in protein levels in all subunits tested, with the exception of the CIV subunit Cox1 (Fig. 5a). As the steady-state level of Cox1 changed, we determined the levels of other CIV subunits after NOC. Figure 5b demonstrated significant decreases only in mitochondrially encoded subunits (Cox 1, 2 and 3) and not in nuclear-encoded (Cox4) protein levels after NOC.

Effects of NOC on mitochondrial DNA

As we observed specific decreases in the levels of mitochondrial-encoded subunits after NOC, we examined whether this was related to alteration in the levels of mitochondrial DNA. Quantification of mtDNA/nDNA ratio using two different sets

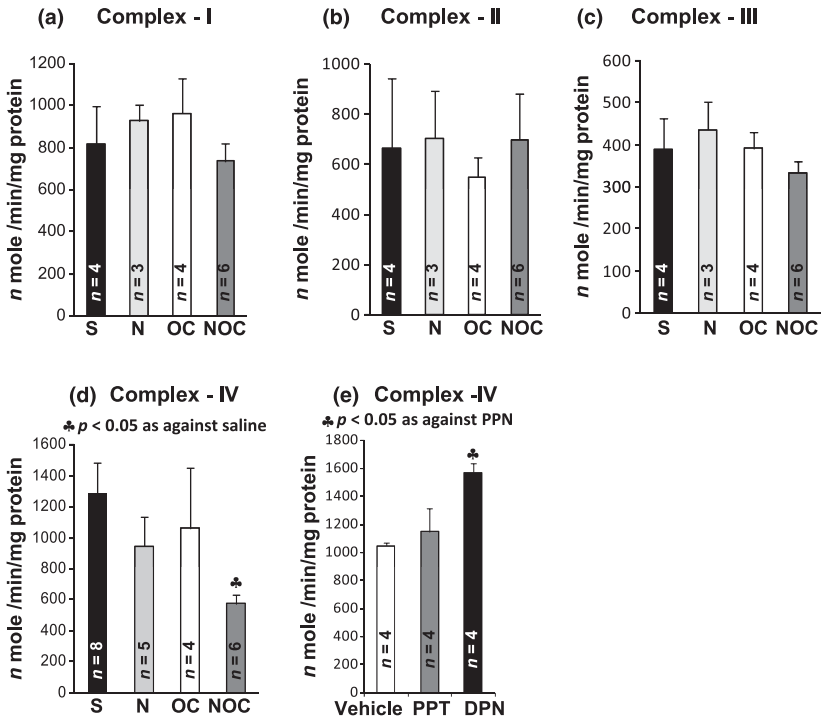

Fig. 3 NOC impaired hippocampal mitochondrial CIV activity. (a–d) Represents data of complex I–IV activity measured by spectrophotometer in isolated mitochondria from different experimental groups. (e) Represents data of complex IV activity after estrogen receptor specific ER-α (25 nM) or ER-β (40 nM) agonists.

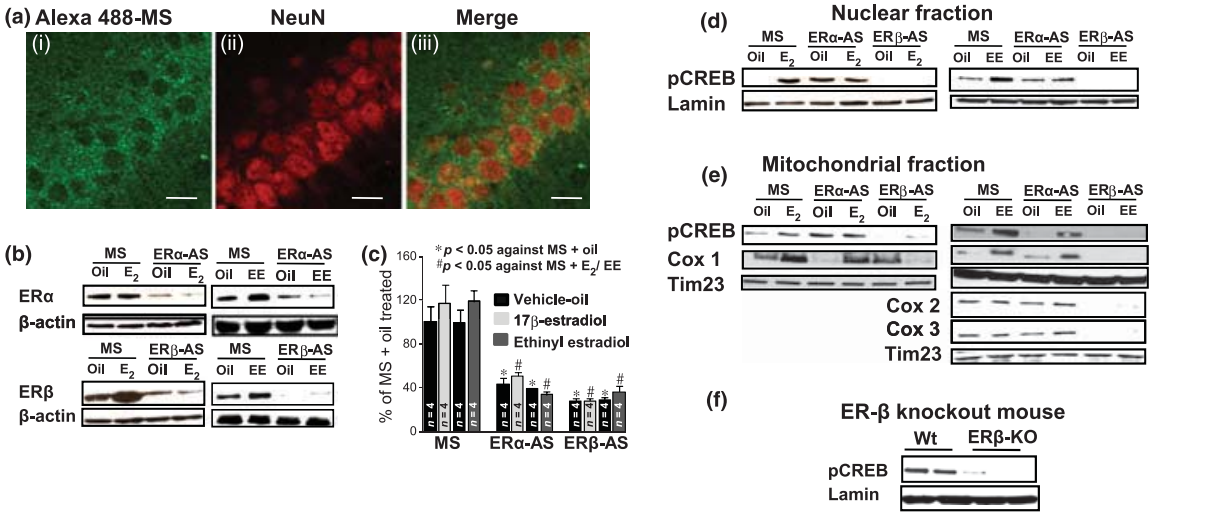

Fig. 4 Knockdown of ER-β in hippocampus of female rat. (a) Confocal microscopic images of hippocampal CA1 region depicting the presence of Alexa 488-MS (green) signal co-localization with the neuronal marker NeuN (red) (40× magnification, bar is 20 μM). Note: distribution of fluorescent MS in the cytoplasm of hippocampal CA1 cells. (b, c) Western blot analysis demonstrates a significant reduction in ER-α and ER-β protein levels after treatment with ER-α/β AS compared with MS control. (d) Immunoblots showing pCREB content in the nuclear (a) and mitochondrial (e) fractions of rat hippocampus following MS/ER-α/β-AS. Note: ER-β-AS, but not ER-α-AS, attenuated the pCREB in nuclear and mitochondrial fractions of hippocampus following E_2/EE treatment. Furthermore, the reduced mitochondrial pCREB following ER-β-AS plus E_2 or EE treatment correlates with lower complex-IV subunit 1 or subunit 1, 2 and 3 mitochondrial-encoded protein levels, respectively. Lamin and Tim23 were the loading controls for nuclear and mitochondrial fractions, respectively. (f) The representative western blot image showing dramatically reduced pCREB protein levels in knockout hippocampal tissue as compared to wild-type.

of genes Cox3 and ND5 versus actin and GAPDH revealed no differences in mtDNA content among the different experimental groups (Fig. 5c and d). These results suggested that the changes in CIV subunits upon NOC treatment might be owing to changes in gene expression or post-translational instability of CIV subunits and not to changes in mtDNA.

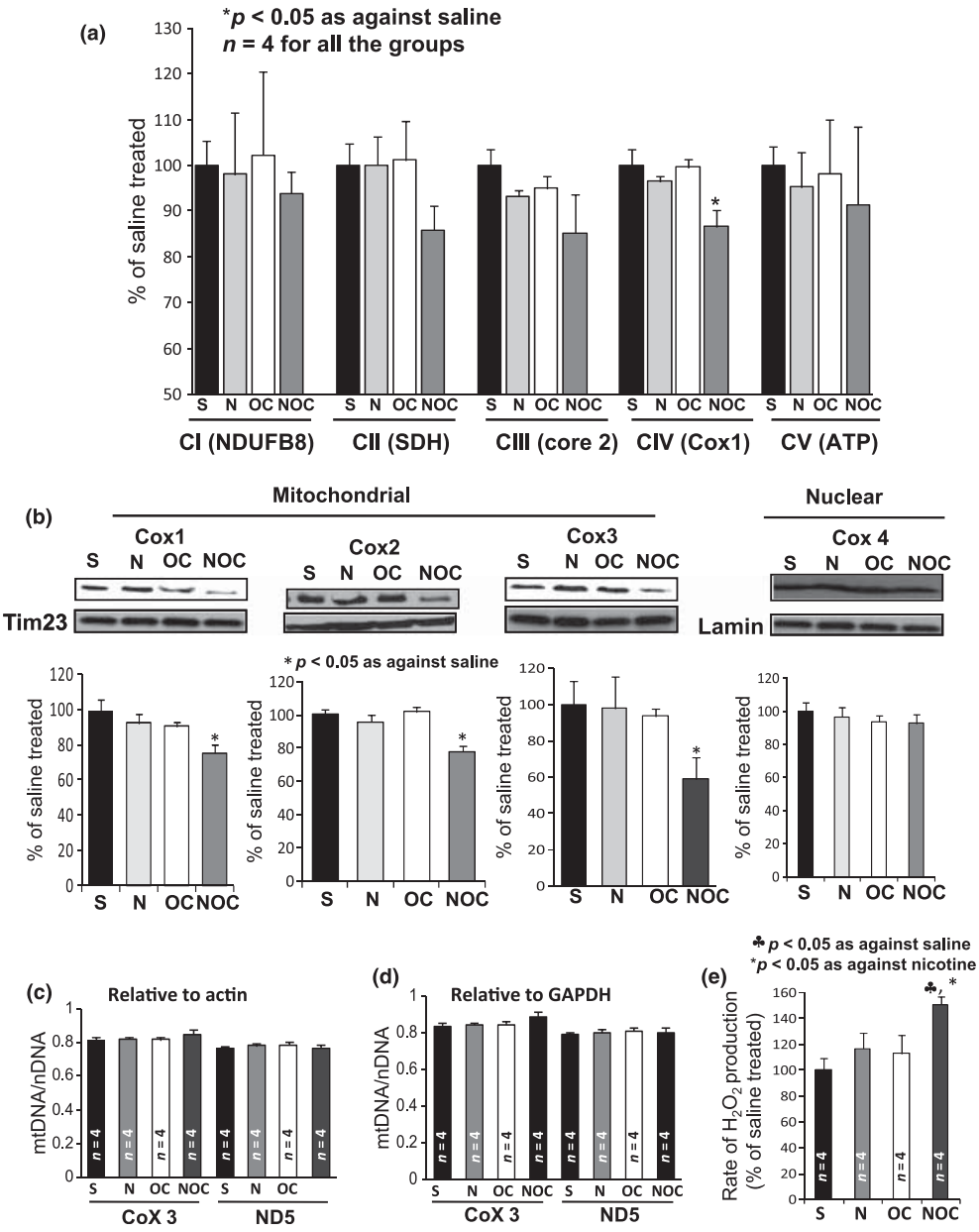

Fig. 5 NOC reduced hippocampal mitochondrial Cox 1, 2 and 3 protein levels. (a) Quantification of immunoblots showing the steady-state protein levels of subunits of complex I–V in isolated mitochondria. Note the significant decrease in complex IV protein levels in NOC-exposed rats. (b) Representative immunoblots showing the protein levels of complex IV subunits 1, 2, 3 and 4. Quantification of western blots demonstrated significant decrease in Cox-1 and 3 protein levels in nicotine alone or NOC groups. (c, d) Quantification of mtDNA/nDNA ratio using two different two different sets of genes (Cox3 and ND5 vs. actin and GAPDH) revealed no difference in mtDNA content among different experimental groups. (e) The rate of hydrogen peroxide production in the hippocampal mitochondria was measured using Amplex Red in different experimental groups and the rate of H_2O_2 production is presented as percentage of saline.

NOC increased hippocampal mitochondrial hydrogen peroxide production

Because the impairment of mitochondrial function has been associated with increased production of reactive oxygen species, and we observed CIV defects, we decided to investigate the effect of NOC on ROS release. We measured the rate of H_2O_2 production in hippocampal mitochondria. Results demonstrated 50% and 34% increase ($n = 4$; $p < 0.05$) in the rate of H_2O_2 production in hippocampal mitochondria obtained from NOC rats as compared with the

saline and nicotine groups, respectively (Fig. 5e). This increased ROS production might contribute to the observed harmful effects of NOC.

Discussion

In previous research, we found that long-term nicotine exposure alone or in combination with oral contraceptives decreased membrane-bound ER-β availability and inhibited 17β-estradiol-mediated intracellular signaling in the hippocampi of female rats (Raval *et al.* 2009a, 2011). In the present study, we also observed reduced mitochondrial ER-β protein levels in the hippocampi of female rats after nicotine or NOC exposure. Importantly, the hippocampal ER-β loss from both of these sub-cellular sites was more pronounced in NOC-exposed female rats compared with the group exposed to nicotine alone, which suggests that the compounded toxic effects of NOC were more harmful than nicotine by itself (Raval *et al.* 2011). Furthermore, these results indicate that the harmful effects of NOC in the hippocampus are induced via a different mechanism from the one inducing the effects of nicotine alone. Here, we present the first evidence that NOC impaired ER-β-mediated mitochondrial respiration owing to altered OXPHOS protein levels. These effects were absent in the hippocampal mitochondria harvested from nicotine-exposed female rats.

Mitochondrial estrogen receptors play a direct role in estrogen-mediated preservation and regulation of mitochondrial structure and function (Bettini and Maggi 1992; Klinge 2008; Mirebeau-Prunier *et al.* 2010). In the present study, we demonstrated that NOC exposure, apart from reducing mitochondrial ER-β protein levels, caused mitochondrial dysfunction. Specifically, NOC significantly increased ROS generation and reduced oxygen consumption at respiratory chain CIV in mitochondria isolated from the hippocampus. The fact that we did not observe an overall defect in oxygen consumption when using succinate and glycerol-3-phosphate substrates, indicates that they become a limiting step in the reaction. Therefore, we measured the enzymatic activity of each respiratory complex and found that only CIV activity was affected. Alterations of enzymatic activity could have reflected decreased levels of its subunits or problems with complex assembly/stability (Moraes *et al.* 2002; Diaz and Moraes 2008).

It is known that ER-β mediates its effect through CREB phosphorylation, and in turn pCREB can bind directly to the D-loop of mtDNA (the control region of mtDNA), and regulates gene expression of OXPHOS subunits (Lee *et al.* 2005; Ryu *et al.* 2005; De Rasmo *et al.* 2010). Silencing of ER-β reduced nuclear and mitochondrial pCREB following E_2/EE treatment, suggesting that the ER-β is essential for CREB phosphorylation at both sub-cellular locations. Furthermore, the reduction in mitochondrial pCREB following ER-β-AS plus E_2/EE treatment correlates with lower Cox 1, 2 and 3 mitochondrial-encoded protein levels, indicating the role of ER-β in pCREB-mediated mitochondrial OXPHOS protein expression. In parallel, NOC significantly decreased Cox 1, 2 and 3 protein levels in isolated mitochondria. The mitochondrial respiratory chain enzymes are multimeric proteins; specifically, complexes I and IV are formed by a large number of subunits of dual genetic origin (from the nuclear and mitochondrial genomes) (Moraes *et al.* 2002; Diaz and Moraes 2008). Complex IV is a homodimer made up of thirteen subunits, three of which form the catalytic core and are encoded by the mtDNA, whereas the rest of the subunits are encoded by the nuclear genome. Owing to its dual origin, the assembly process of this complex is also very complicated and highly regulated. Thus, observed lower levels of oxygen consumption at respiratory chain CIV after NOC might arise from defects in complex assembly. Furthermore, CIV subunit 1, 2 and 3 proteins originate from mtDNA, thus suggesting possible defects in mitochondrial DNA arising from chronic NOC exposure. As we did not observe any alterations in the levels of mtDNA after NOC treatment, the decrease of CIV subunit level is likely caused by alterations in their expression levels or to post-translational protein instability. The possibility of protein instability also arises from the fact that we observed increased CIV enzyme activity in isolated hippocampal mitochondria after ER-β agonist treatment. Phosphorylation of CIV subunit is implicated in mitochondrial energy fluxes (Acin-Perez *et al.* 2011). Thus, the observed effect of ER-β agonist on CIV enzyme activity was within a short time interval and the possibility of mechanisms other than ER-β-mediated pCREB could not be denied and remains a caveat of the present study. Overall, the present results support our hypothesis that NOC mediated reductions in mitochondrial ER-β-mediated CREB phosphorylation and thus altered the levels of

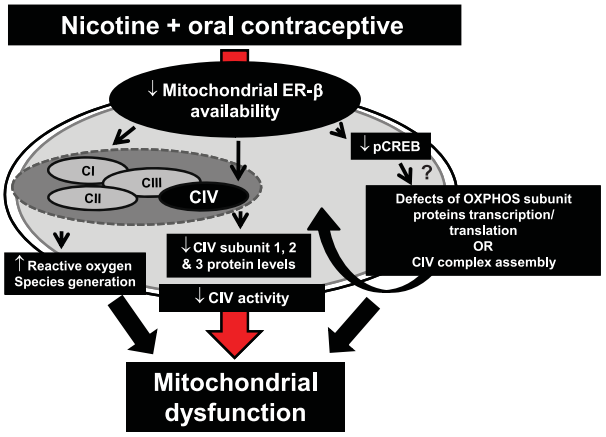

Fig. 6 Schematic diagram. This figure depicts the main hypothesis that nicotine with oral contraceptives induces ER-β loss from hippocampus and causes mitochondrial dysfunction at complex IV (CIV) in female rats.

oxidative phosphorylation system proteins in the hippocampus of female rats.

Mitochondrial dysfunction is the main cause of ischemic neuronal death (Chan 2004; Kristian 2004; Niizuma *et al.* 2009) and the observed defects in mitochondrial OXPHOS proteins and respiration after NOC can aggravate mitochondrial dysfunction, thereby exacerbating the post-ischemic damage after NOC, as previously reported by our lab (Raval *et al.* 2011). Future studies investigating CIV assembly/stability could help characterize the exact target(s) of NOC.

In contrast to ER-β, hippocampal ER-α protein levels were not altered in either nicotine- or NOC-exposed female rats. In a separate experiment using the ER-α-AS approach, silencing of ER-α did not decrease pCREB protein levels nor alter the levels of CIV subunits (Fig. 4d and e), which suggested that estrogen-mediated CREB phosphorylation occurs via ER-β. However, the fact that pCREB protein levels were more or less similar between vehicle and estrogen treatment after ER-α-AS suggested the possibility of other compensatory mechanisms. The current study did not investigate other mechanisms of ER-α activation including phosphorylation at serine 167, 118 or 104/105 sites and this constitutes another caveat (Murphy *et al.* 2011). Additionally, there are contradictory findings from different labs portraying roles for both estrogen receptors in neuroprotection from ischemic injury (Dubal *et al.* 2006; Lebesgue *et al.* 2009; Noppens *et al.* 2009; Zhang *et al.* 2009). Thus, one cannot overrule a possible role for ER-α in the harmful effects of NOC in the hippocampus, and this aspect requires future investigation.

In conclusion, this study demonstrated that NOC exposure impaired ER-β-mediated mitochondrial respiration at the CIV level, owing to lower protein levels of its catalytic subunits in the hippocampus of female rats (Fig. 6). Additionally, NOC increased mitochondrial production of ROS that could also facilitate mitochondrial dysfunction. Such dysfunction could be manifested after ischemic stress, therefore exacerbating hippocampal neuronal death in female rats as observed previously by our laboratory (Raval *et al.* 2011). Mitochondrial functional abnormalities observed after NOC were not found in the nicotine alone group, suggesting that these harmful effects of NOC might be due to a unique synergy of nicotine with oral contraceptives. However, this may be a timing or dosage issue, and longer or higher nicotine exposure could produce similar effects; therefore, future studies are needed to dissect the effects of time and dosage. Finally, based on these results we suggest that mitochondrial ER-β loss is the central reason that hippocampal damage is increased after cerebral ischemia in NOC rats.

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Mitochondrial complex activity measurement using spectrophotometer.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

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