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The Impact of Aging and Estrogen Therapy on Synaptic Mitochondrial Bioenergetics in an Alzheimer's Mouse Model

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The Impact of Aging and Estrogen Therapy on Synaptic Mitochondrial Bioenergetics in an Alzheimer's Mouse Model

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Eric W. Hendricks

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The Impact of Aging and Estrogen Therapy on Synaptic Mitochondrial Bioenergetics in an Alzheimer's Mouse Model

Eric W. Hendricks, B.S.

Eastern Illinois University

A Thesis Presented to the Faculty of the Graduate School of Eastern Illinois University in Partial Fulfillment of the Requirements for the Degree of Master of Science (Research)

2015

Abstract

Mitochondrial dysfunction, such as reductions in ATP production via oxidative phosphorylation, is a recognized pathological symptom of neurodegenerative diseases including Alzheimer's disease (AD). One of the known risk factors for the development of AD is the inheritance of a specific genotype of a lipid transporting protein known as apolipoprotein E (apoE). How apoE contributes to the development and progression of AD is poorly understood. The purpose of this study was to determine if a relationship exists between mitochondrial dysfunction and genetic predisposition to AD via apoE. Mitochondrial bioenergetics and enzymatic activity were investigated in an AD mouse model (apoE KO) and in wild-type (WT) mice. Since AD develops with age, mitochondrial function was studied in both young (4-6 months old) and old (12-22 months old) mice. Additionally, estrogen therapy was utilized as a possible therapeutic to prevent mitochondrial dysfunction.

We isolated mitochondria located in nerve cell terminals (synaptosomes) using Percoll gradient centrifugation from WT (C57BL/6J) and apoE KO ($Apoe^{tm1Unc}$) mice. Mitochondria were isolated from the following three treatment groups: mice possessing ovaries injected with vehicle control solution (Sham), ovariectomized mice with vehicle control injection (OVX), and ovariectomized mice with 50 ng/g injection of 17β-estradiol (OVX + E2). Mitochondrial function was evaluated based on oxygen consumption of permeabilized and non-permeabilized synaptosomes and was measured using the OROBOROS Oxygraph-2k at 37°C. Succinate dehydrogenase (SDH) and citrate synthase (CS) activity was also assayed to help elucidate the bioenergetic profile of the isolated synaptosomes.

Our results revealed no significant differences in oxygen consumption from intact and permeabilized synaptosomes between Sham and OVX young WT and apoE KO mice. Additionally, no significant differences were detected in SDH and CS activity between young WT and apoE KO mice. However, estrogen treatment dramatically increased oxygen consumption and enzymatic activity in apoE KO for all respiration parameters, whereas, a minimal effect was observed in WT mice. No significant differences in oxygen consumption from permeabilized and intact synaptosomes were detected between young and old OVX and OVX + E2 treated mice, but old Sham mice had significantly higher oxygen consumption rates compared to young Sham mice for most all permeabilized parameters in both WT and apoE KO mice. Estrogen treatment had no effect on mitochondrial respiration in old mice, but estrogen treated old apoE KO mice had significantly higher SDH activity compared to its Sham.

Increases in respiration and enzymatic activity prompted investigation of mitochondrial quantity via Western blot. We used the voltage dependent-anion-channel (VDAC) as a mitochondrial quantity marker. The results revealed that estrogen treatment increased mitochondrial quantity in estrogen treated young apoE KO and old Sham mice. We speculate that increases in mitochondrial quantity in young apoE KO mice and old Sham mice were due to estrogen and reactive oxygen species (ROS) induced mitochondrial biogenesis, respectively. Our data suggests that predisposition of AD via apoE inheritance is not an underlying source of mitochondrial abnormalities; however, the effectiveness of estrogen as a neuro-therapeutic may be dependent upon an individual's apoE genotype, as well as, their age.

Dedication

To My Family and Friends for Their Steadfast Guidance and Support

Acknowledgements

I would like to give a special thanks to my co-advisor Dr. Britto P. Nathan for recognizing my potential as a scientist and for igniting my passion for research in science. I extend my heartfelt thanks to my co-advisor Dr. Michael A. Menze for his unwavering guidance throughout the duration of my program and for his immense commitment to help me succeed in all my academic endeavors. The training I received working in the laboratories of Drs. Nathan and Menze are invaluable. I would also like to thank Dr. Gary A. Bulla for his advisement and service on my thesis committee. I am also very appreciative for all the support I received from the College of Sciences and the Graduate School at Eastern Illinois University. Their contributions are a kindness that I will not soon forget. Finally, I am grateful for the life-long friendships that I was able to create through my experiences at EIU. Their company uplifted my strength of mind and helped me get through the long days.

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I. Introduction

A. The Role of the Mitochondrion in Alzheimer's Disease

According to the Centers for Disease Control and Prevention, nearly 16 million Americans will be diagnosed with Alzheimer's disease (AD) by the year 2050. By this time, patient care cost associated with AD is expected to reach $1.2 trillion, which will confer an impressive economic burden on society. AD is characterized by the presence of neurofibrillary tangles and extracellular amyloid-β (Aβ) deposition (Selkoe, 1999), but the earliest detectable neuropathological hallmark of AD is decreased cerebral metabolism (Blass, 2000; Devi et al., 2006). It has long been known that reductions in mitochondrial enzymatic activity, including cytochrome c oxidase and α-ketoglutarate dehydrogenase, as well as, pyruvate dehydrogenase, occur during the pathological progression of AD and can be identified in postmortem whole-brain tissue samples from AD patients (Parker et al., 1989; Gibson et al., 1998; Sorbi et al., 1983). Interestingly, deficits in these key metabolic enzymes have been observed prior to the onset of AD pathology in animal models (Yao et al., 2009). A large body of evidence suggests that alterations in enzymatic activity observed in AD are a result of oxidative damage due to mitochondrial dysfunction (Hirai et al., 2001; Nunomura et al., 2001), but the exact mechanism leading to mitochondrial dysfunction specific to AD is unknown (Devi et al., 2006).

B. The Role of ApoE in Alzheimer's Disease

One of the known risk factors for AD is the inheritance of a specific genotype of a lipid transporting protein known as apolipoprotein E (apoE). ApoE is a plasma protein

that is mainly expressed in the brain and liver (Elshourbagy et al., 1985). The gene for apoE is found on chromosome 19 and there have been three human isoforms identified: *apoε2*, *apoε3*, and *apoε4* (Strittmatter et al., 1993). Specifically, the inheritance of the *apoε4* allele markedly increases the incidence of AD (Strittmatter et al., 1994). Recent research suggests that the higher risk of developing AD, due to *apoε4* inheritance, is attributed to competitive binding of Aβ and apoE4-Aβ complexes to low-density lipoprotein receptor-related protein 1(LRP1) and very-low-density lipoprotein receptor (VLDL) (Verghese et al., 2012; Schmechel et al., 1993; Deane et al., 2004). LRP1 rapidly clears Aβ across the blood brain barrier (Bell et al., 2007; Shibata et al., 2000); however, apoE4 has been shown to bind with Aβ, which shifts the clearance from an LRP1-mediated action to a much slower clearing mechanism mediated by VLDL, which drastically increases brain retention of Aβ (Deane et al., 2008). Higher concentrations of cellular Aβ, due to decreased clearance, allows for the accumulation in organelles including the mitochondrion, which, along with increased ROS production, alters mitochondrial respiration (Hansson et al., 2004; Caspersen et al., 2005; Manczak et al., 2006).

As mentioned previously, reduced cerebral metabolism, as a result of a shift from glucose utilization to ketone bodies (Ishii et al., 1997), and altered mitochondrial respiration, due to reductions in mitochondrial enzymes (Yao et al., 2009; Hansson et al., 2004; Caspersen et al., 2005; Manczak et al., 2006), are the earliest detectable symptoms of AD. Furthermore, previous studies have linked dysfunctional mitochondria, like those observed prior to the onset of AD pathology, with increased ROS production and oxidative stress, which exacerbate the progression of AD pathology (Khan et al., 2000;

Swerdlow et al., 1997; Cardoso et al., 2004). Therefore a reasonable approach for slowing the progression of AD would be a therapeutic molecule that combats mitochondrial dysfunction and subsequent ROS production in an attempt to prevent the occurrence of Aβ and neurofibrillary tangles.

C. Estrogen as a Potential Therapeutic in Alzheimer's Disease

The female sex hormone, 17β-estradiol, is a lipophillic molecule that is able to cross the blood brain barrier and is synthesized from cholesterol *de novo* in the brain (Srivastava et al., 2011). In addition, previous studies have shown that mitochondria possess estrogen receptors (Yang et al., 2003; Chen et al., 2004), which have been shown to increase both the energetic efficiency of neurons and the antioxidant defense system for ROS elimination (Wang et al., 2001; Green and Simpkins, 2000). The properties of estrogen and its actions on the mitochondrion serves as a possible therapeutic compound for restoring the bioenergetic properties of dysfunctional mitochondria observed prior to the onset of AD pathology.

D. Thesis Objectives

Many studies have investigated the indirect effects of apoE on mitochondrial function, and its association with AD; however, a comprehensive investigation of the effects of apoE on mitochondrial bioenergetics is missing. The purpose of this study was to characterize the presence and absence of apoE and its effects on the bioenergetic properties of the nerve cell terminals, known as synaptosomes, using apoE knock out (KO) and wild type (WT) mice. Since AD is an age-related neurodegenerative disease we also investigated the impacts of aging on mitochondrial respiration, as well as, on the

activity of two key mitochondrial enzymes: citrate synthase and succinate dehydrogenase. We hypothesize treatment of estrogen will enhance mitochondrial performance with respect to age and genotype. Furthermore, we speculate that interventions aimed at reducing mitochondrial dysfunction in the early stages of AD development may slow or prevent progression of the disease.

II. Materials and Methods

A. Chemicals

Chemicals, reagents and consumables were supplied by Fisher Scientific, Pittsburgh, PA and Sigma-Aldrich, St. Louis, MO. Percoll was obtained from GE Healthcare, Buckinghamshire, UK. Female $Apoe^{tm1Unc}$ gene deficient/knockout (apoE KO) and C57BL/6J wild-type (WT) littermate mice were purchased from The Jackson Laboratories (Bar Harbor, Maine).

B. Surgeries

Young (3-6 months) and old (12+ months) female apoE KO ($Apoe^{tm1Unc}$) and WT (C57BL/6J) littermate mice were used in this study. Mice were anesthetized with 1 μL ketamine/xylazine per 1 g body weight. After deep anesthesia was observed, the mouse was shaved and swabbed with 100% ethanol just below the last rib. A midline incision was made to expose the peritoneal cavity. The ovaries were identified and an incision between the beginning of the oviduct and ovary was made. The wound was sutured and sealed with surgical glue. All mice underwent surgery; however, sham mice were left with intact ovaries. Mice were given two weeks to recuperate from ovariectomy and sham surgeries. Mice were divided into the 12 experimental groups: (1) young WT sham, (2) young WT ovariectomized control (ovx ctrl), (3) young WT estrogen (E2), (4) old WT sham, (5) old WT ovx ctrl, (6) old WT E2, (7) young apoE KO sham, (8) young apoE KO ovx ctrl, (9) young apoE E2, (10) old apoE KO sham, (11) old apoE KO ovx ctrl, and (12) old apoE KO E2. After recuperation, both sham and ovx ctrl mice were administered an intraperitoneal (i.p.) injection of a corn oil solution containing 1%

ethanol, whereas, the E2 mice were given an i.p. injection of 50 ng/g body weight of 17β-estradiol 24 h prior to sacrificing.

C. Synaptosome Isolation

Brain synaptosomes were isolated as described previously with minor modifications (Kristian, 2010). Briefly, synaptosomes were isolated 24 h post-treatment from all groups. The forebrain was removed, washed, and homogenized in 20 mL of isolation medium (IM) containing sucrose (225 mM), mannitol (75 mM), EGTA (1 mM), HEPES (5 mM), and 1 g/L BSA adjusted to pH 7.4 with Tris base. The homogenate was transferred evenly into two 10 mL polycarbonate tubes and centrifuged at 1300g for 3 min. The pellet was resuspended in 3 mL of IM and centrifuged at 1300g for an additional 3 min. The supernatants from the two centrifugations were combined and centrifuged at 21,000g for 10 min. A Percoll gradient in IM was prepared in a separate polycarbonate tube by first adding 3.7 mL of 24% Percoll and, using a transfer pipette, 1.5 mL of 40% Percoll was slowly added underneath the 24% Percoll layer. The supernatant from the previous centrifugation step was discarded and the pellet was resuspended in 3.5 mL of 15% Percoll, and the resuspended pellet was next layered on top of the 24/40% Percoll gradient using a transfer pipette, and then centrifuged at 30,700g for 8 min. The material at the 15% Percoll band, containing mostly myelin, was discarded, whereas the 24% Percoll band, containing mostly synaptic mitochondria, was collected. The fraction containing the synaptosomes was washed with 6 mL of IM and centrifuged at 16,700g for 10 min. This step was repeated and the supernatant was discarded both times. The resulting pellet was resuspended in 400 μL of IM and contained 6-7mg/mL synaptosomal protein, which was determined using a Modified

Lowry Protein Assay Kit according to the instructions of the manufacturer (Thermo Fisher Scientific, Waltham, MA).

D. Intact Synaptosome Respirometry

Oxygen consumption of synaptosomes was measured in 2 mL of incubation medium (120 mM NaCl, 15 mM glucose, 3.5 mM KCl, 2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM $Na2SO_4$, 0.4 mM KH_2PO_4) at 37°C using Clark-type polarographic electrodes. Mitochondrial bioenergetics of intact synaptosomes was evaluated in all treatment groups under the same set of conditions. Basal respiration was measured after the addition of synaptosomes to the incubation medium containing respiratory chamber. Under these conditions mitochondrial respiration was fueled by NADH and $FADH_2$ generated from the TCA cycle after decarboxylation of pyruvate, which was produced in the cytoplasm from the conversion of glucose in the media. Excess capacity for electron entry in the ubiquinone pool via NADH and $FADH_2$ was determined by uncoupling the synaptosomes in a step-wise manner with successive 1 µL injections of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (0.5 µM, FCCP). Proton leak was measured after inhibiting the F_0F_1 ATPase activity with the addition of oligomycin (2 µg/mL) in the absence of FCCP. Non-mitochondrial oxygen consumption was recorded in the presence of antimycin A (2.5 µM).

E. Permeabilized Synaptosome Respirometry

Oxygen consumption of permeabilized synaptosomes was measured in 2 mL of MIR05 Medium (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA) at 37°C using Clark-type

polarographic electrodes. Mitochondrial bioenergetics of permeabilized synaptosomes was evaluated in all treatment groups under the same set of conditions. Synaptosomes were permeated with digitonin (8.1 mM). Proton leak fueled by Complex I was measured after the addition of 5 µL malate (400 mM, M), 10 µL glutamate (2000 mM, G), and 5 µL pyruvate (2000 mM, P) in the absence of adenosine 5' diphosphate (ADP). Oxygen consumption under conditions of oxidative phosphorylation (OXPHOS) was measured after the addition of 5 µL ADP (500 mM) in the presence of MGP. Contributions of Complex II to OXPHOS capacity was measured with the addition of 20 µL succinate (1000 mM) in the presence of both MGP and ADP. Excess capacity for electron transfer produced by Complexes I & II was determined by uncoupling the synaptosomes in a step-wise manner with 1 µL injections of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (0.5 µM, FCCP). Proton leak fueled by Complexes I & II was measured by inhibiting the F_0F_1 ATPase activity with the addition of oligomycin (final concentration 2 µg/mL) in the absence of FCCP and in the presence of MGPDS. Non mitochondrial oxygen consumption was recorded in the presence of antimycin A (2.5 µM).

F. Succinate Dehydrogenase Assay

Succinate dehydrogenase activity in synaptosomal extracts was performed as described previously (Munujos et al., 1993). Briefly, 10 µL of isolated mitochondria was dissolved in 970 µL of the reaction mixture consisting of triethanolamine (100 mM, pH 8.3), EDTA (0.5 mM), NaCN (2 mM), iodonitrotetrazolium chloride (INT; 2 mM), and Kolliphor EL (12 g/L). The absorbance reading was set to zero and the assay was initiated through the addition of 20 µL of succinate (1 M). The change in absorbance

after the addition of succinate was recorded at room temperature every second for 6 minutes at 500 nm using an Evolution 300 UV-VIS spectrophotometer (Fisher Scientific, Pittsburgh, PA). Succinate dehydrogenase activity was calculated from the initial linear increase in absorbance at 500 nm and expressed as absorbance per minute per μg of protein.

G. Citrate Synthase Assay

A citrate synthase assay was performed as described elsewhere (Childress and Somero, 1979). Briefly, 10 μL of isolated mitochondria was dissolved in 980 μL of the reaction mixture consisting of imidazole (pH 8.0; 50 mM), acetyl-CoA (0.1 mM), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) (0.1 mM), and $MgCl_2$ (1.5 mM). The absorbance reading was set to zero and the reaction was initiated through the addition of 10 μL of oxaloacetate (50 mM). The change in absorbance after the addition of oxaloacetate was recorded at room temperature every second for one minute at 412 nm using an Evolution 300 UV-VIS spectrophotometer (Fisher Scientific, Pittsburgh, PA). Citrate synthase activity was calculated from the initial linear increase in absorbance at 412 nm and expressed as absorbance per minute per μg of protein.

H. Western Blot

Synaptosome samples were mixed at a 1:1 ratio with sample Laemmeli buffer (2% SDS, 25% glycerol, 5% mercaptoethanol, 0.01% bromphenol blue, and 62,5 mM Tris-HCl, pH 6.8), denatured at 95°C for 5 min and 20 μL of extract was loaded per lane. Synaptosomal samples were subjected to 10% SDS-PAGE using Bio-Rad Mini-PROTEAN precast gels (Bio-Rad Laboratories, Hercules, CA). The proteins in the gels

were electrophoretically transferred at 120 volts for 1 h in a transfer buffer containing 192 mM glycine, 20% methanol, 0.025% SDS, and 25 mM Tris onto a nitrocellulose membrane. The membrane was washed with 25 mL of Tris-buffered saline containing 0.05% Tween-20 (TBS-T), subsequently stained with Ponceus stain to confirm protein transfer, and the lanes were cut in a fashion to allow for 2 separate antibody incubations. The membrane was washed with TBS-T and blocked with 5% fat free dry milk in TBS-T for 1 h. After washing the membranes with TBS-T, rabbit anti-VDAC and rabbit anti-β-tubulin (Cell Signaling Technologies, Beverly, MA) were used as primary antibodies at a 1:5,000 dilution. The blots were incubated in a 5% BSA-TBS-T solution with the primary antibodies overnight at 4˚C. Membranes were washed three times for 5 min each with TBST, and incubated with an HRP-labeled goat anti-rabbit secondary antibody for 1 h at room temperature. Membranes were washed three times for 5 min each with TBST and proteins were visualized using a chemiluminescence detection kit (FujiFilm, Tokyo, Japan).

III. Results

A. ApoE KO versus WT Comparison

1. Respirometry of Intact Synaptosomes

Basal respiration of 3-6 month WT and apoE KO mice was measured in intact synaptosomes suspended in a medium containing glucose (Fig. 1A). Basal respiration rates reflect mitochondrial respiration fueled by NADH and $FADH_2$ provided by the glycolytic and mitochondrial conversion of glucose supplied by the medium and converted to pyruvate in the cytoplasm of the nerve cell terminal to fuel the mitochondrial Krebs cycle (Choi et al. 2009) via pyruvate decarboxylation. Ovariectomy did not change respiration in wild-type or apoE KO mice. However, estrogen treatment in apoE KO significantly increased oxygen consumption compared to the Sham ($p < 0.05$).

In order to assay the maximum respiratory capacity of non-permeabilized synaptosomes, the mitochondrial electron transport system (ETS) was uncoupled from the phosphorylation system with the addition of FCCP (Fig. 1B). The observed FCCP uncoupled respiration rates were 5-fold higher than basal respiration rates. Estrogen treatment did not affect uncoupled oxygen consumption rates in ovariectomized WT mice. Estrogen treatment in ovariectomized apoE KO mice significantly increased uncoupled respiration compared to Sham and estrogen treated WT ($p < 0.05$).

Leak respiration was measured in the absence of FCCP after the addition of oligomycin (2 μg/mL) to block the F_0F_1-ATP synthase. Among the three assessed respiration parameters, leak respiration accounted for the lowest respiration rates measured (Fig. 1C). Ovariectomy in apoE KO mice did not significantly alter respiration

compared to Sham. No differences in leak respiration were detected between sham and ovariectomized WT and apoE KO mice. Surprisingly, estrogen treated apoE KO mice had significantly higher respiration rates compared to estrogen treated WT mice.

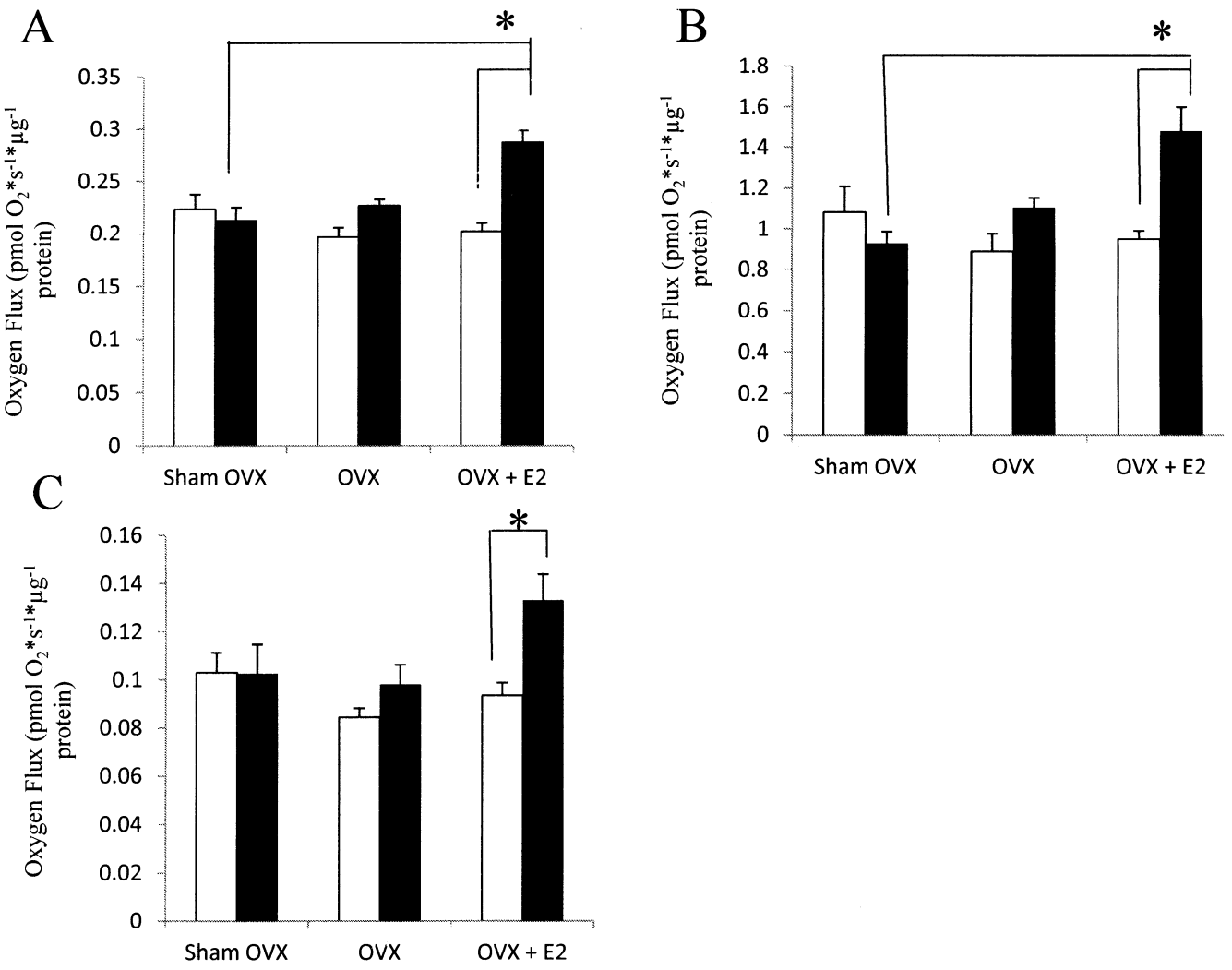


Figure 1. Oxygen flux in intact synaptosomes from WT and apoE KO mice. Synaptosomes were isolated from either WT (white bar) or apoE KO (black bar) mice in the diestrous cycle (Sham OVX), following ovariectomy (OVX), or following treatment with 50 ng E2/g body weight (OVX + E2). (A) Basal respiration, (B) Uncoupled respiration, and (C) Leak respiration are shown. Respiration rates were standardized based on protein content and are expressed as pmol of O_2 consumed per second per µg protein. Significant differences in oxygen flux are denoted with an asterisk (*). Data was analyzed using a two-way ANOVA ($p < 0.05$, n = 3-6). Data represent the mean ± S.E.M.

2. Respirometry of Permeabilized Synaptosomes

Respiration rates observed under permeabilized conditions in the presence of saturating substrate concentrations were approximately 10-fold higher than respiration rates of intact synaptosomes in presence of glucose alone (Figs. 1 and 2). Leak respiration driven by NADH in the absence of ADP was assayed after the addition of MGP (malate, glutamate, and pyruvate) and no significant changes in the rate of oxygen consumption was observed in ovariectomized WT mice as compared to Sham (Fig. 2A). Despite the fact that estrogen treatment did not change leak respiration in WT mice, a significant increase in respiration rates was observed in apoE KO mice compared to estrogen treated WT mice ($p < 0.05$). Oxidative phosphorylation (OXPHOS) was initiated by the addition of 0.5 M ADP. Neither ovariectomy nor estrogen treatment led to a change of OXPHOS rates compared to the sham control in WT mice (Fig. 2B). Ovariectomy and subsequent estrogen treatment in apoE KO mice resulted in a significant increase in OXPHOS respiration as compared to Sham ($p < 0.05$). Furthermore, estrogen treated apoE KO mice had significantly higher OXPHOS rates compared to estrogen treated WT mice ($p < 0.05$).

Succinate was added to assess the simultaneous entry of electrons provided by complex II and complex I into the ubiquinone pool under OXPHOS conditions. Ovariectomy and estrogen treatment had no effect on respiration rates fueled by NADH and $FADH_2$ (complex I + II) supplying substrates in WT mice (Fig. 2C). In contrast to WT mice, ovariectomy in apoE KO mice resulted in significantly higher respiration rates measured under these conditions as compared to the Sham ($p < 0.05$). Estrogen treatment in apoE KO mice significantly increased respiration rates engaging complex I + II as

compared to the Sham, and estrogen treated WT mice ($p < 0.05$). Maximum respiratory capacities of the electron transport system (ETS) in permeabilized synaptosomes were examined after the addition of FCCP (Fig. 2D). Similar to the results observed under OXPHOS conditions, estrogen treatment in apoE KO mice significantly increased uncoupled respiration as compared to the Sham ($p < 0.05$). In contrast, Sham and ovariectomized apoE KO and WT mice had similar uncoupled respiration rates. Leak respiration rates in the presence of ADP were examined by inhibiting the F_0F_1-ATPase with the addition of oligomycin. Both ovariectomy and estrogen treatment had no effect on respiration in WT mice. Likewise, ovariectomy in apoE KO had no effect on leak respiration rates, but estrogen treatment significantly increased leak respiration in apoE KO mice as compared to Sham. No significant differences in leak respiration were detected between apoE KO and WT.

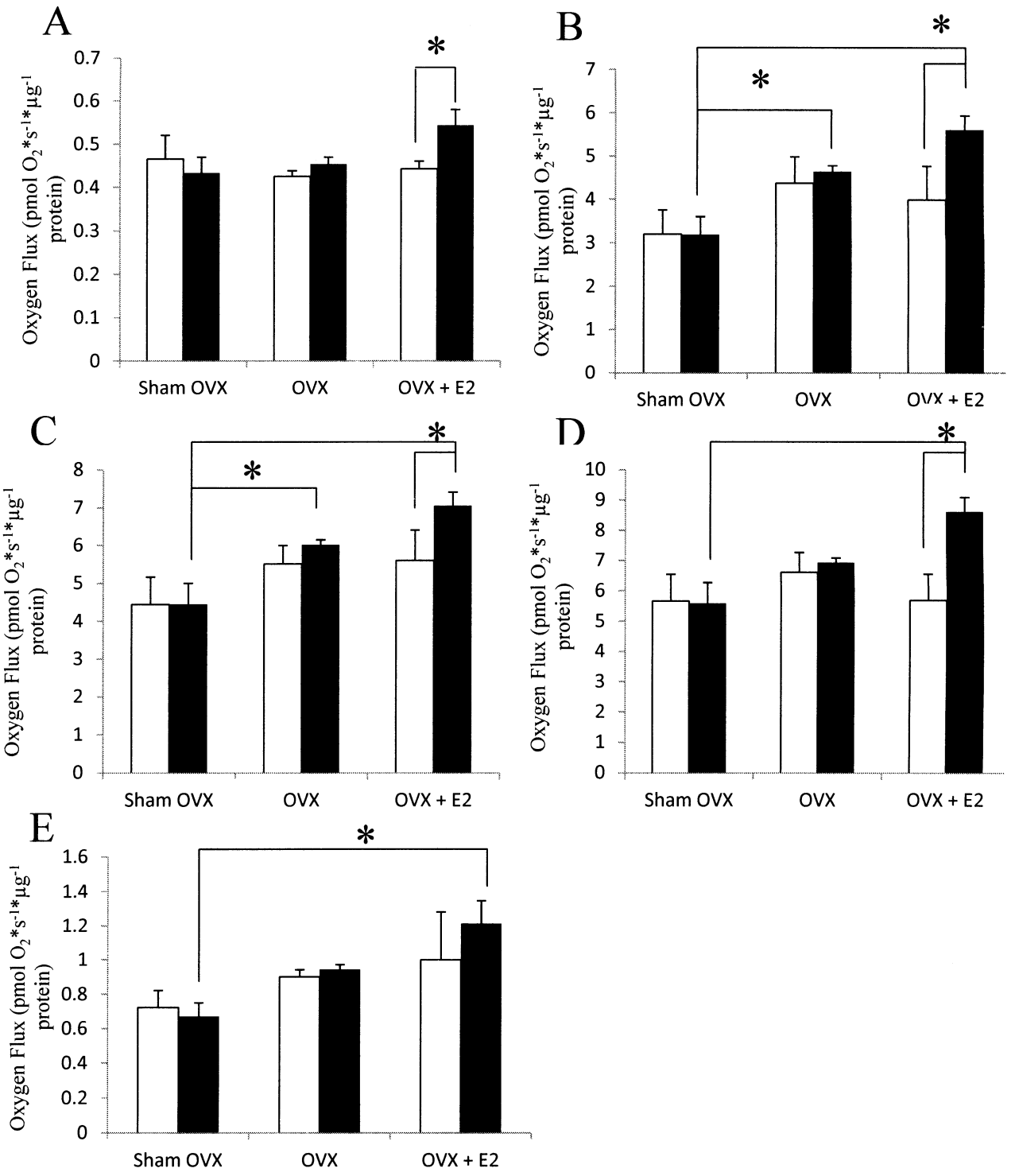


Figure 2. Oxygen flux in permeabilized synaptosomes from WT and apoE KO mice. Synaptosomes were isolated from either WT (white bar) or apoE KO (black bar) mice in diestrous cycle (Sham OVX), following ovariectomy (OVX), or following treatment with

50 ng E2/g body weight (OVX + E2). (A) Leak respiration rates driven by MGP in the absence of ADP, (B) oxidative phosphorylation rates after the addition of ADP in presence of MGP, (C) response to addition of succinate under complex I fueled OXPHOS conditions, (D) uncoupled respiration in presence of the above mentioned substrate combination, and (E) leak respiration in the absence of FCCP after the addition of oligomycin to block the F_0F_1-ATP synthase. Respiration rates were standardized based on protein content and are expressed as pmol of O_2 consumed per second per μg protein. Significant differences in oxygen flux are denoted with an asterisk (*). Data was analyzed using a two-way ANOVA ($p < 0.05$, $n = 3$-6). Data are presented as the mean ± S.E.M.

3. Respirometry of Permeabilized Synaptosomes in Presence of Succinate and Rotenone

Next we examined $FADH_2$ fueled respiration alone in the presence of succinate and rotenone. Rotenone is a potent complex I inhibitor, which allowed for the assessment of complex II-derived electrons into the ubiquinone pool. Leak respiration was investigated after the addition of succinate and rotenone but in absence of ADP. No significant differences were detected between both phenotypes under sham conditions or after ovariectomy (Fig. 3a). Furthermore, estrogen treatment had no effect on leak respiration in WT mice but in apoE KO mice led to a significant increase in respiration rates compared to the Sham ($p < 0.05$). $FADH_2$ fueled rates of OXPHOS was assessed after the addition of ADP in the presence of rotenone and succinate (Fig. 3B). Ovariectomy or estrogen administration had no effect on respiration in WT mice, but in apoE KO both treatments led to significantly increased OXPHOS respiration rates compared to Sham ($p < 0.05$). These results were also reflected in the FCCP uncoupled respiration rates. Only in apoE KO ovariectomy and estrogen treatment led to significant increases in respiration but both treatments were without effect in wild type mice. However, maximum respiration rates did not exceed coupled OXPHOS rates for both WT and apoE KO mice (Fig. 3C).

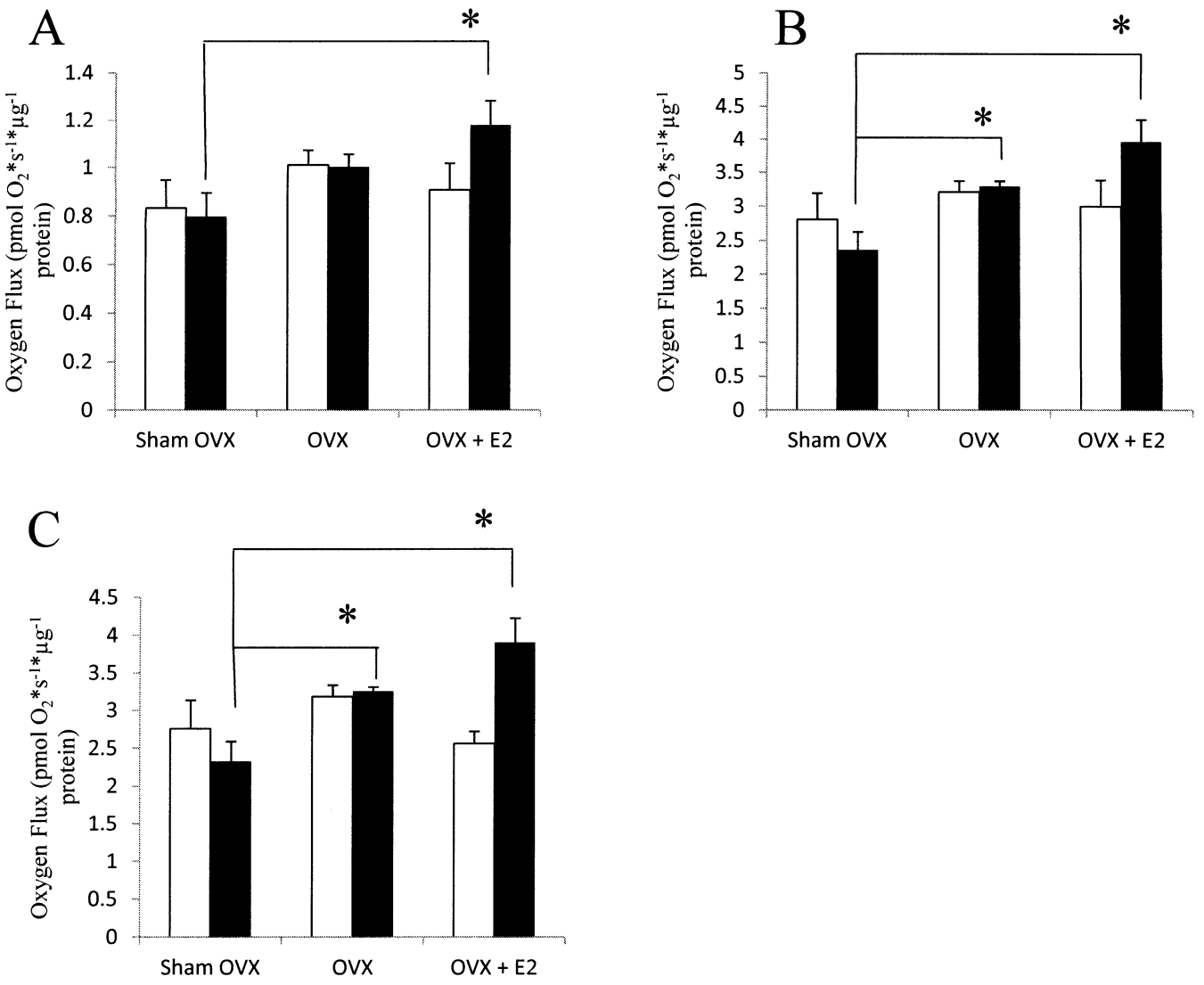


Figure 3. Complex II fueled respiration in permeabilized synaptosomes from WT and apoE KO mice. Synaptosomes were isolated from either WT (white bar) or apoE KO (black bar) mice in the diestrous cycle (Sham), after ovariectomy (OVX), or OVX followed by treatment with 50 ng E2/g (Ovx + E2). (A) Leak respiration was assessed in the presence of rotenone and succinate. (B) Respiration under conditions of OXPHOS was initiated by addition of 3 mM ADP. (C) In order to uncouple oxygen utilization from ADP phosphorylation increments of 0.5 µM FCCP were added until maximum oxygen flux was observed. Respiration rates were standardized based on protein content and are

expressed as pmol of O_2 consumed per second per μg protein. Significant differences in oxygen flux are denoted with an asterisk (*). Data was analyzed using a two-way ANOVA ($p < 0.05$, $n = 3$-6). Data are presented as the mean ± S.E.M.

4. Activity of Succinate Dehydrogenase and Citrate Synthase

In order to evaluate whether increases in respiration rates observed in ovariectomized and estrogen treated WT or apoE KO mice were due to an increase in mitochondrial content per synaptosome, or an increase in the specific activity of respiratory complexes per mitochondrion, we investigated the activity of both succinate dehydrogenase (SDH; Fig. 4A) and citrate synthase (CS; Fig. 4B) in our preparation. Ovariectomized WT mice had a significant increase in SDH activity as compared to the Sham and estrogen treatment significantly reduced SDH activity in WT mice compared to the ovariectomy control ($p < 0.05$). Likewise, ovariectomy in apoE KO mice significantly increased SDH activity compared to the Sham, but estrogen treatment had no effect on SDH compared to the ovariectomy control. Furthermore, no significant differences in SDH activity were observed between apoE KO and WT mice under any condition.

Citrate synthase (CS) activity is commonly used to judge mitochondrial content of cells (Remels et al., 2010). Neither ovariectomy nor estrogen treatment had any effect on CS activity in WT mice. Likewise, ovariectomy in apoE KO mice had no effect on CS activity but, interestingly, estrogen treatment nearly doubled CS activity in apoE KO mice compared to the Sham. Therefore, CS activity in apoE KO mice was highly sensitive to estrogen treatment whereas estrogen treatment in WT mice had no effect

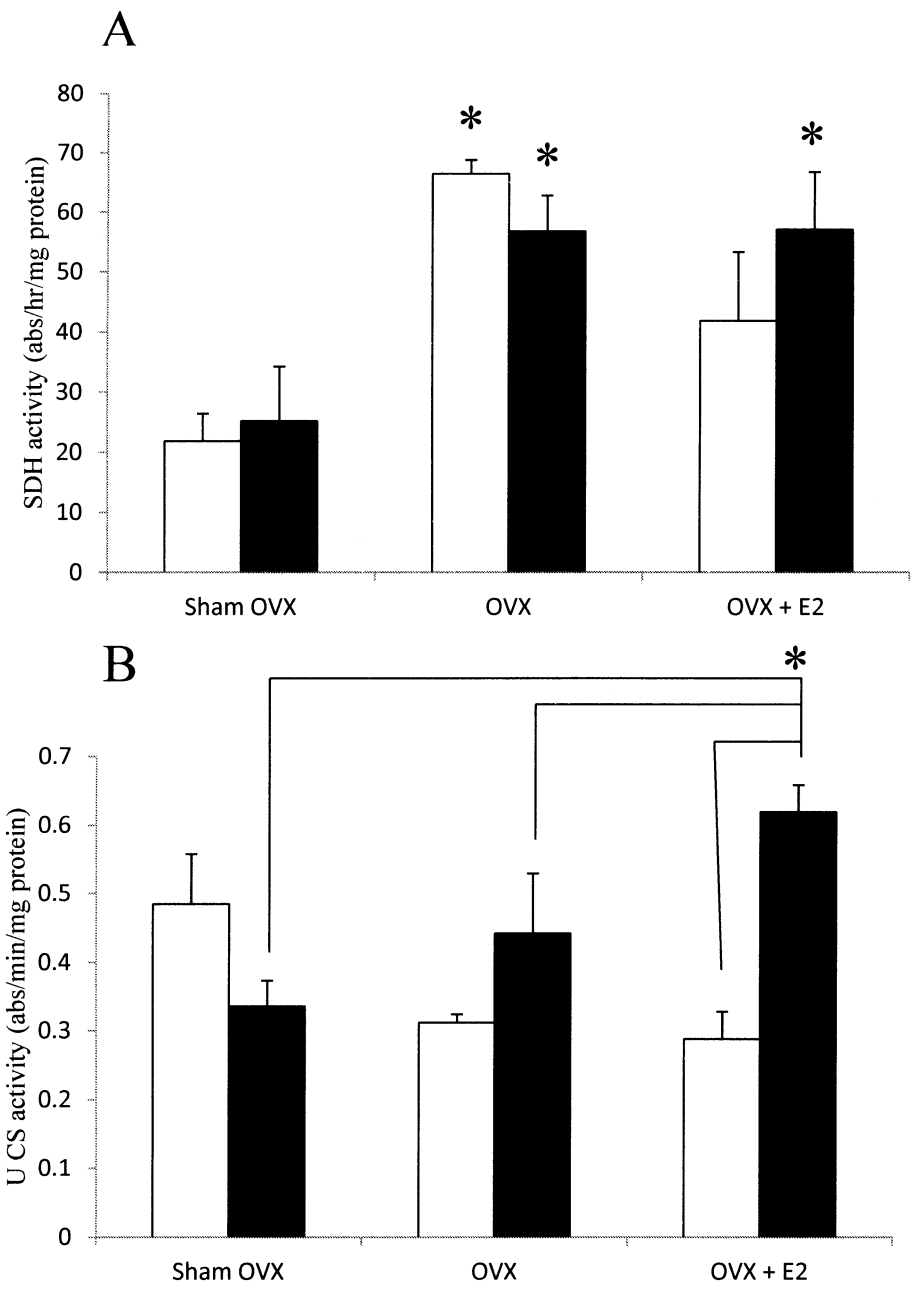


Figure 4. Succinate dehydrogenase and citrate synthase activity in synaptosomes from WT and apoE KO mice. (A) Succinate dehydrogenase (SDH) activity and (B) citrate synthase (CS) activity were measured in synaptosome samples isolated from either WT (white bar) or apoE KO (black bar) mice in the diestrous cycle (Sham), after ovariectomy (OVX), or ovariectomy followed by treated with 50 ng E2/g (OVX + E2). SDH activity was standardized to total protein content and expressed as absorbance per

hour per milligram of total protein. CS activity was also standardized based on total protein and was expressed as absorbance per min per milligram of total protein. Significant differences in SDH and CS activity are denoted with asterisks (*). Data was analyzed using a two-way ANOVA ($p < 0.05$, $n = 3$-6). Data are presented as the mean ± S.E.M.

5. Western Blot Analysis of the Voltage Dependant Anion Channel

Immunoblotting of the voltage-dependent anion channel (VDAC) was used as an additional mitochondrial marker to further validate if the observed increases in SDH and CS activities were due to an increase in total number of mitochondria (Fig. 5A). Relative to VDAC expression levels observed in sham, ovariectomy in WT mice resulted in a 26 % increase in the level of the protein. Estrogen treatment in WT mice slightly decreased VDAC expression compared to the ovariectomy, but was still 21% higher than in Sham (Fig. 5B). Similarly, ovariectomy significantly increased VDAC expression levels in apoE KO mice by 17% compared to the Sham, but in contrast to WT mice, estrogen treatment in apoE KO mice led to a further increase in VDAC expression reaching 29% above Sham (Fig. 5B).

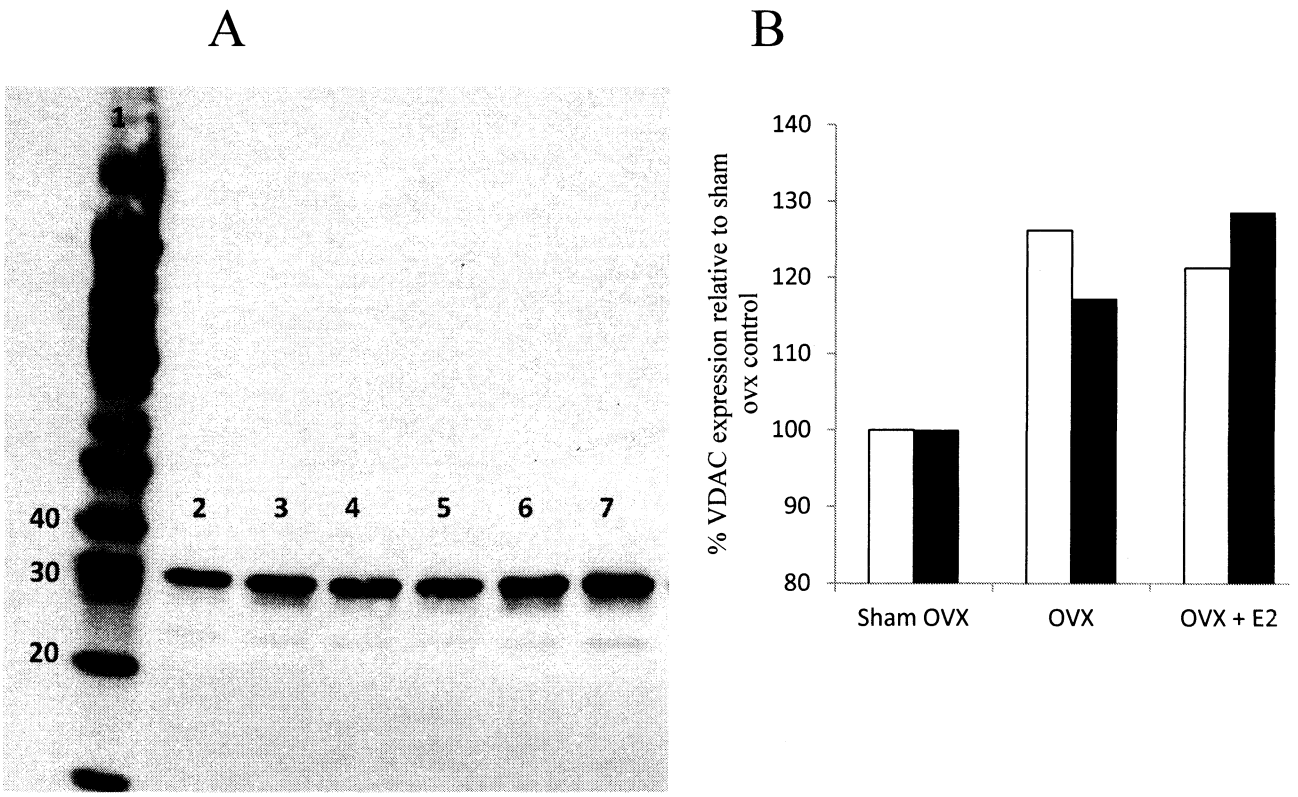


Figure 5. Immunoblotting of VDAC in WT and apoE KO mice. Synaptosome samples (20 μg total protein per lane) were subjected to SDS-PAGE and immunoblotted for VDAC. (A) Lane 1, molecular weight standards; lane 2, WT sham OVX; lane 3, WT OVX; lane 4, WT OVX + E2; lane 5, apoE KO sham OVX; lane 6, apoE KO OVX; lane 7, apoE KO OVX + E2. (B). Optical density of VDAC bands were quantified using Image J software. WT mice (white bar); apoE KO (black bar). Values were converted to percent increase relative to the Sham.

B. Age Comparison: Old Mice versus Young Mice

1. Intact Synaptosomes

A comparison of basal respiration in young (3-6 month) and old (12-24 month) WT and apoE KO mice was measured in intact synaptosomes suspended in a medium containing glucose as the sole carbon source (Fig. 6A & 6B). Basal respiration rates reflect mitochondrial respiration fueled by NADH and $FADH_2$ provided by the glycolytic and mitochondrial conversion of glucose supplied by the medium and converted to pyruvate in the cytoplasm of the nerve cell terminal to fuel the mitochondrial Krebs cycle (Choi et al. 2009) via pyruvate decarboxylation.

i. Respirometry of Intact Synaptosomes from Wild-Type Mice

No differences in basal respiration were detected between young and old WT mice (Fig. 6A). Furthermore, ovariectomy and estrogen treatment had no effect on basal respiration in old mice. For a detailed comparison of treatment effects within young WT mice please refer to section III.A.1. In order to assay the maximum respiratory capacity of non-permeabilized synaptosomes, the mitochondrial electron transport system (ETS) was uncoupled from the phosphorylation system with the stepwise addition of FCCP (Fig. 6C). The observed uncoupled respiration rates were approximately 5-fold higher than basal respiration rates. Old WT Sham mice had significantly higher uncoupled respiration rates compared to the young Sham group ($p < 0.05$; Fig. 6C). No differences in the uncoupled respiration rates were detected after ovariectomy and subsequent estrogen treatment in old mice. Leak respiration was measured in the absence of FCCP after the addition of oligomycin (2 μg/mL) to block the F_0F_1-ATP synthase (Fig. 6E).

Among the three non-permeabilized respiration parameters, leak respiration accounted for the lowest respiration rates and no differences were detected between young and old mice (Fig. 6E). Furthermore, ovariectomy or ovariectomy followed by estrogen treatment had no effect on leak respiration in old mice.

ii. Respirometry of Intact Synaptosomes from ApoE KO Mice

Similar to the results observed in wild-type mice no differences in basal respiration were detected between young and old apoE KO mice (Fig. 6B). Furthermore, ovariectomy and estrogen treatment had no effect on basal respiration in old mice. For a detailed comparison of treatment effects within young apoE KO mice please refer to section III.A.1. Maximum respiratory capacity was again measured after the stepwise addition of FCCP), and respiration rates increased similar to the increase observed in WT mice (Fig. 6D,C). Unlike Old WT Sham mice no significant increase in uncoupled respiration rates between young and old Sham apoE KO was observed ($p > 0.05$; Fig. 6D). A comparison among treatment groups revealed no differences in the uncoupled respiration rates after ovariectomy and subsequent estrogen treatment in old mice. As in WT mice leak respiration accounted for the lowest respiration rates and no differences were detected between young and old mice or among treatment groups (Fig. 6F).

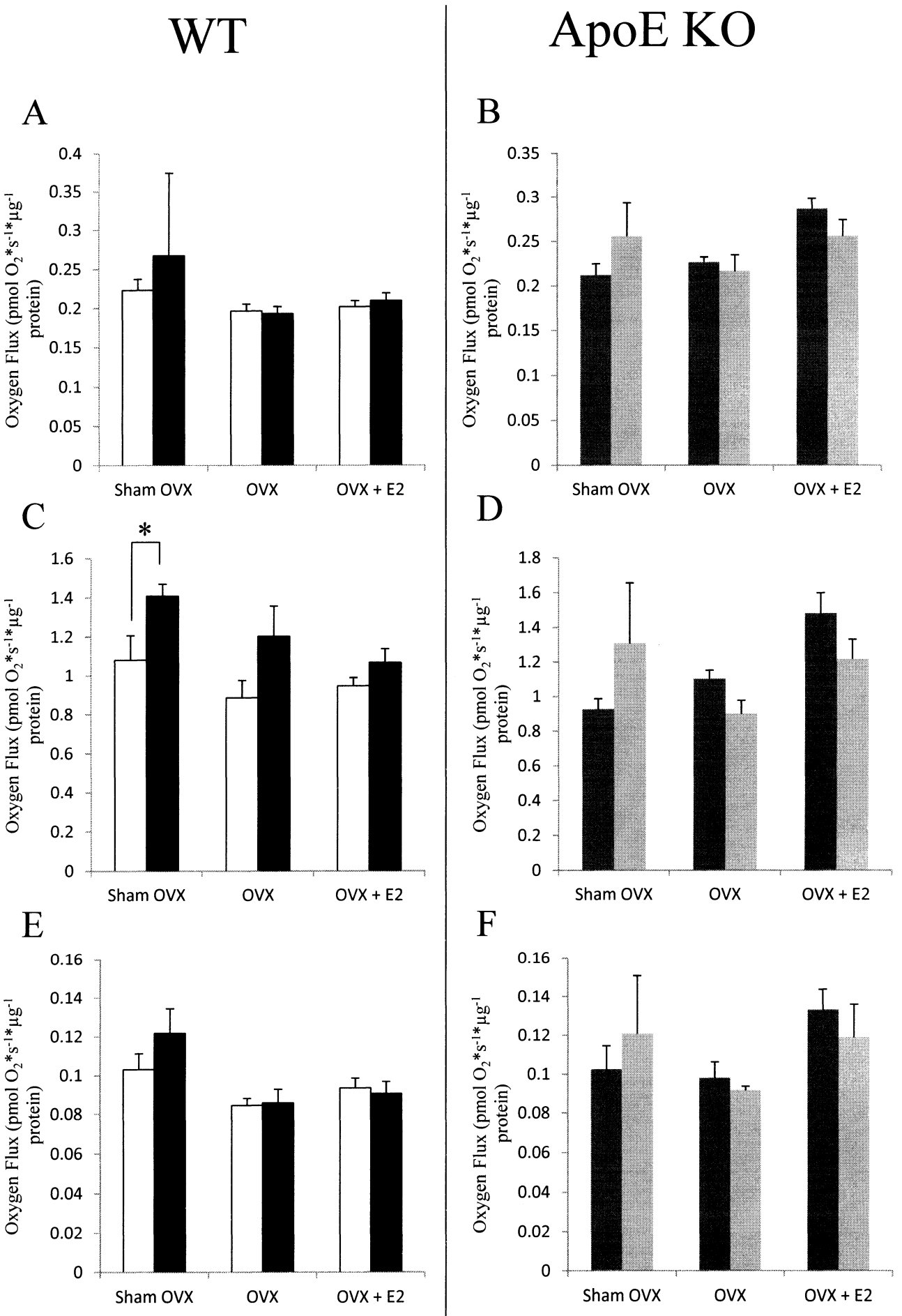

WT
ApoE KO
A
B
C
D
E
F
Oxygen Flux (pmol O_2*s^{-1}*μg^{-1} protein)
Sham OVX
OVX
OVX + E2
*

Figure 6. Oxygen flux in intact synaptosomes from old and young WT and apoE KO mice. Synaptosomes were isolated from either young WT (white bar), old WT (black bar), young apoE KO (dark gray bar), or old apoE KO (light gray bar) mice in the diestrous cycle (Sham OVX), after ovariectomy (OVX), or after ovariectomy following by treatment with 50 ng E2/g (OVX + E2). (A & B) Basal respiration, (C & D) Uncoupled respiration, and (E & F) depicts leak respiration. Respiration rates were standardized based on protein content and are expressed as pmol of O_2 consumed per second per μg protein. Significant differences in oxygen flux are denoted with an asterisk (*). Data was analyzed using a two-way ANOVA ($p < 0.05$, $n = 3\text{-}6$). Data are presented as the mean ± S.E.M. [#]young wild-type and ApoE KO are added for reasons of comparison and are from figures 1A, B, C.

2. Respirometry of Permeabilized Synaptosomes

The titration protocol used for permeabilized synaptosomes from old mice was the same as used for young mice. Briefly, leak respiration driven by NADH in the absence of ADP was assayed after the addition of MGP and OXPHOS was stimulated by addition of ADP. Succinate was added to assess any impact of electron entry provided by complex II and complex I into the ubiquinone pool and maximal respiration was recorded after uncoupling with FCCP. Again leak respiration was measured in presence of oligomycin. Across the parameters investigated, rates observed under permeabilized conditions were approximately 5 to 10-fold higher than respiration rates of intact synaptosomes in the presence of glucose alone.

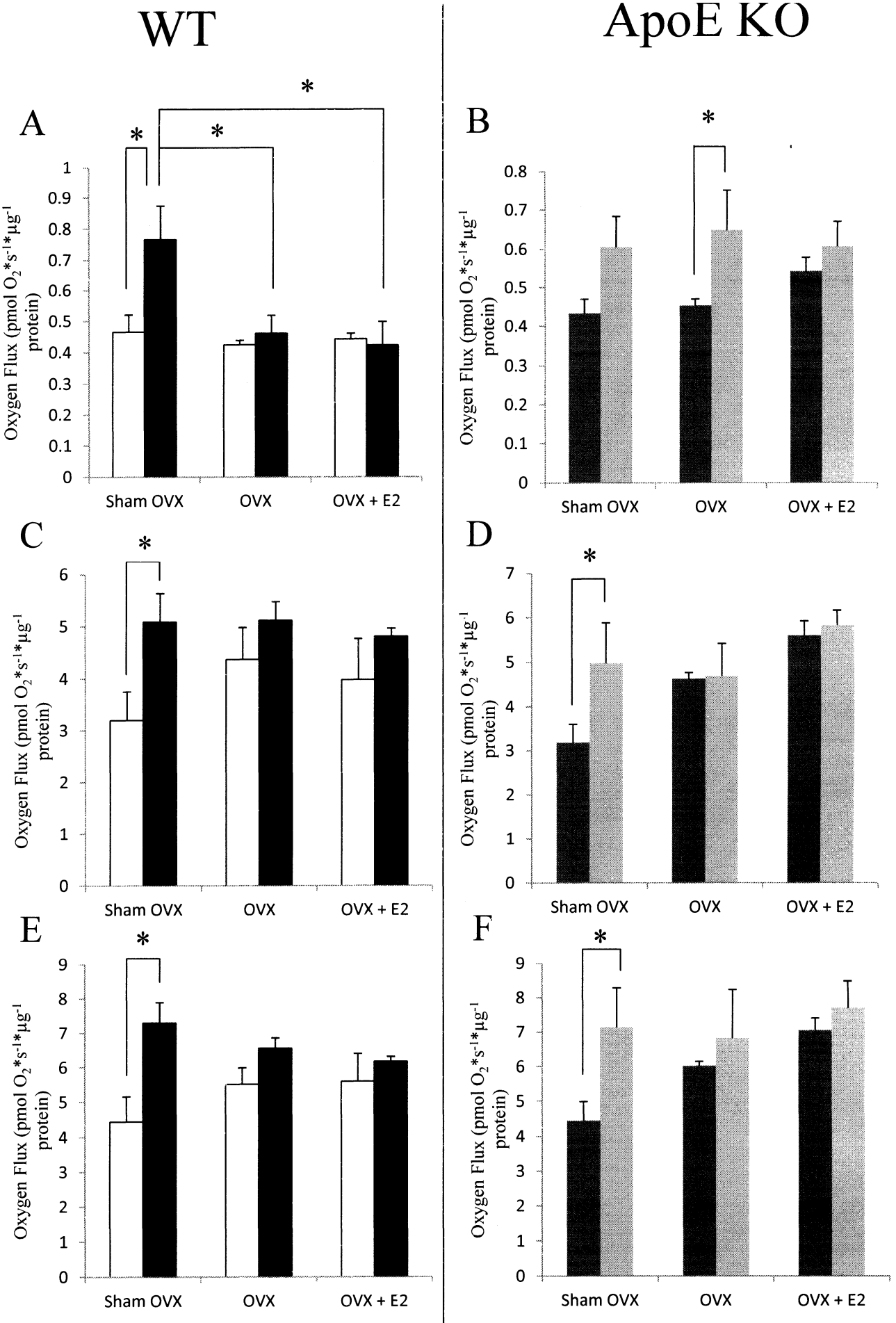
i. Respirometry of Permeabilized Synaptosomes from Wild-Type Mice

Old WT Sham mice had significantly higher MGP induced leak respiration rates compared to the young Sham ($p < 0.05$; Fig. 7A). Ovariectomy and subsequent estrogen treatment resulted in a significant reduction in leak respiration in old mice compared to its Sham ($p < 0.05$). OXPHOS rates for old WT Sham was significantly higher than those observed in the young WT Sham mice and neither ovariectomy nor estrogen treatment had any effect on rates in old mice compared to its Sham ($p < 0.05$; Fig. 7C). Respiration rates fueled by NADH and $FADH_2$ supplying substrates in old WT Sham mice was significantly higher than those rates observed in young WT mice. However, ovariectomy and estrogen treatment had no effect in old WT mice compared to its Sham on respiration rates (Fig. 7E). No age effects were observed on uncoupled respiration rates and all treatment groups displayed similar rates of oxygen consumption (Fig. 7G).

Proton leak in presence of oligomycin was significantly higher in old Sham mice compared to young Sham mice ($p<0.05$) and both ovariectomy and estrogen treatment resulted in a significant reduction in leak respiration in old WT mice ($p < 0.05$; Fig. 7I).

ii. Respirometry of Permeabilized Synaptosomes from ApoE KO Mice

In contrast to WT mice old apoE KO Sham were not different from young ApoE KO Sham in MGP induced leak respiration rates. However, ovariectomized old apoE KO mice had significantly higher leak respiration compared to young ovariectomized mice and estrogen treatment did not rescue respiration rates in old mice (Fig. 7B). After induction of OXPHOS, respiration rates in old Sham mice were significantly higher than those observed in young Sham mice, but ovariectomy and estrogen treatment had no effect in old mice (Fig. 7D). As observed for wild-type mice under concurrent electron entry into the ubiquinone pool by NADH and $FADH_2$ supplying substrates old ApoE KO Sham rates were also significantly higher than those rates observed in the young age group. Again ovariectomy and estrogen treatment had no effect on respiration rates ($p < 0.05$; Fig. 7F, E). Uncoupled respiration rates were similar between young and old mice and did not differ among treatment groups (Fig. 7H). Similar to the results observed in WT leak respiration rates were higher in old ApoE KO Sham than in young ApoE KO Sham but neither ovariectomy and estrogen impacted respiration rate in old ApoE KO (Fig. 7G).


WT
ApoE KO
A
B
C
D
E
F
Oxygen Flux (pmol O_2*s^{-1}*μg^{-1} protein)
Sham OVX
OVX
OVX + E2
*

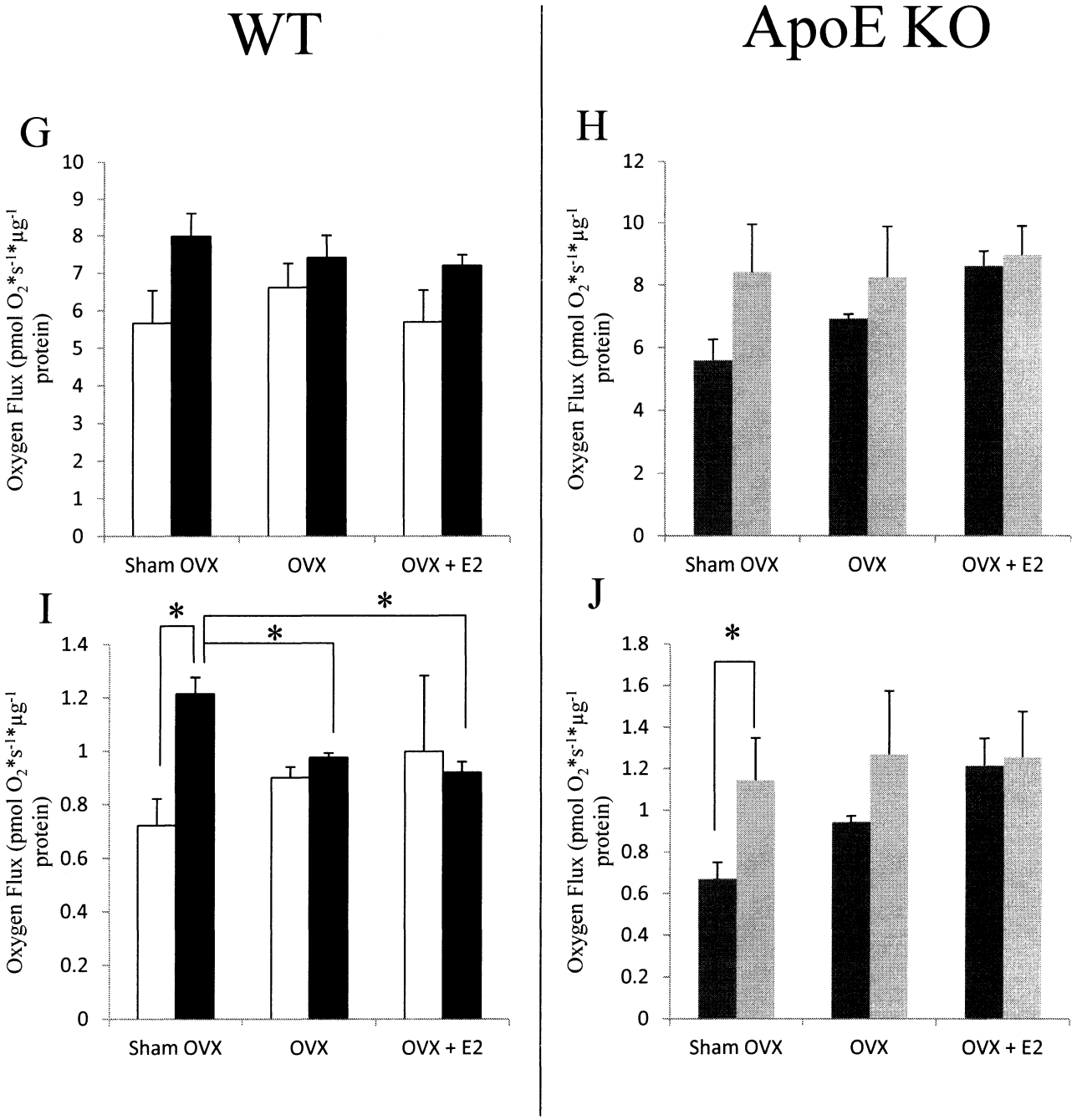


Figure 7. Oxygen flux in permeabilized synaptosomes from old and young WT and apoE KO mice. Synaptosomes were isolated from either young WT (white bar), old WT (black bar), young apoE KO (dark gray bar), or old apoE KO (light gray bar) mice in the diestrous cycle (Sham OVX), after ovariectomy (OVX), or ovariectomy followed by treatment with 50 ng E2/g (OVX + E2). (A & B) Leak respiration rates driven by MGP in the absence of ADP, (C & D) complex I and II OXPHOS rates after the addition of ADP,

(E & F) OXPHOS rates in the presence of succinate and MGP, (G & H) uncoupled respiration after the addition of FCCP, and (I & J) leak respiration after the addition of oligomycin. Respiration rates were standardized based on protein content and are expressed as pmol of O_2 consumed per second per μg protein. Significant differences in oxygen flux are denoted with an asterisk (*). Data was analyzed using a two-way ANOVA ($p < 0.05$, $n = 3$-6). Data are presented as the mean ± S.E.M. [#]young wild-type and ApoE KO are added for reasons of comparison and are from figures 2A, B, C, D, E.

3. Respirometry of Permeabilized Synaptosomes in Presence of Succinate and Rotenone

Complex II respiration in permeabilized synaptosomes was assessed in a separate titration scheme. Briefly, Complex II fueled leak respiration and OXPHOS was investigated in the presence of rotenone, a potent complex I inhibitor (Fig. 8A & 8B).

i. Respirometry of Permeabilized Synaptosomes from Wild-Type and ApoE KO Mice

Old WT Sham mice had significantly higher complex II leak respiration rates in comparison to rates observed in young WT sham mice ($p< 0.05$). Furthermore, ovariectomy and estrogen treatment resulted in a significant decrease in complex II leak respiration in old WT mice ($p < 0.05$; Fig. 8A). Similar to WT mice, old apoE KO Sham mice had significantly higher complex II leak respiration rates compared to the rates observed for young apoE KO mice. In contrast to old WT mice, ovariectomy and estrogen treatment in old apoE KO mice had no effect on complex II leak respiration. After addition of ADP to induce OXPHOS, old WT Sham mice had significantly higher complex II OXPHOS rates compared to young WT sham mice and rates were significantly reduced after ovariectomy and estrogen treatment in old WT mice ($p < 0.05$; Fig. 8C). However, no age effect in OXPHOS rates was observed in apoE KO mice and among treatment groups (Fig. 8D). Maximum respiration rates did not exceed coupled OXPHOS rates and were strikingly similar to rates generated in presence of succinate and ADP for both WT and apoE KO mice (Fig 8E,F).

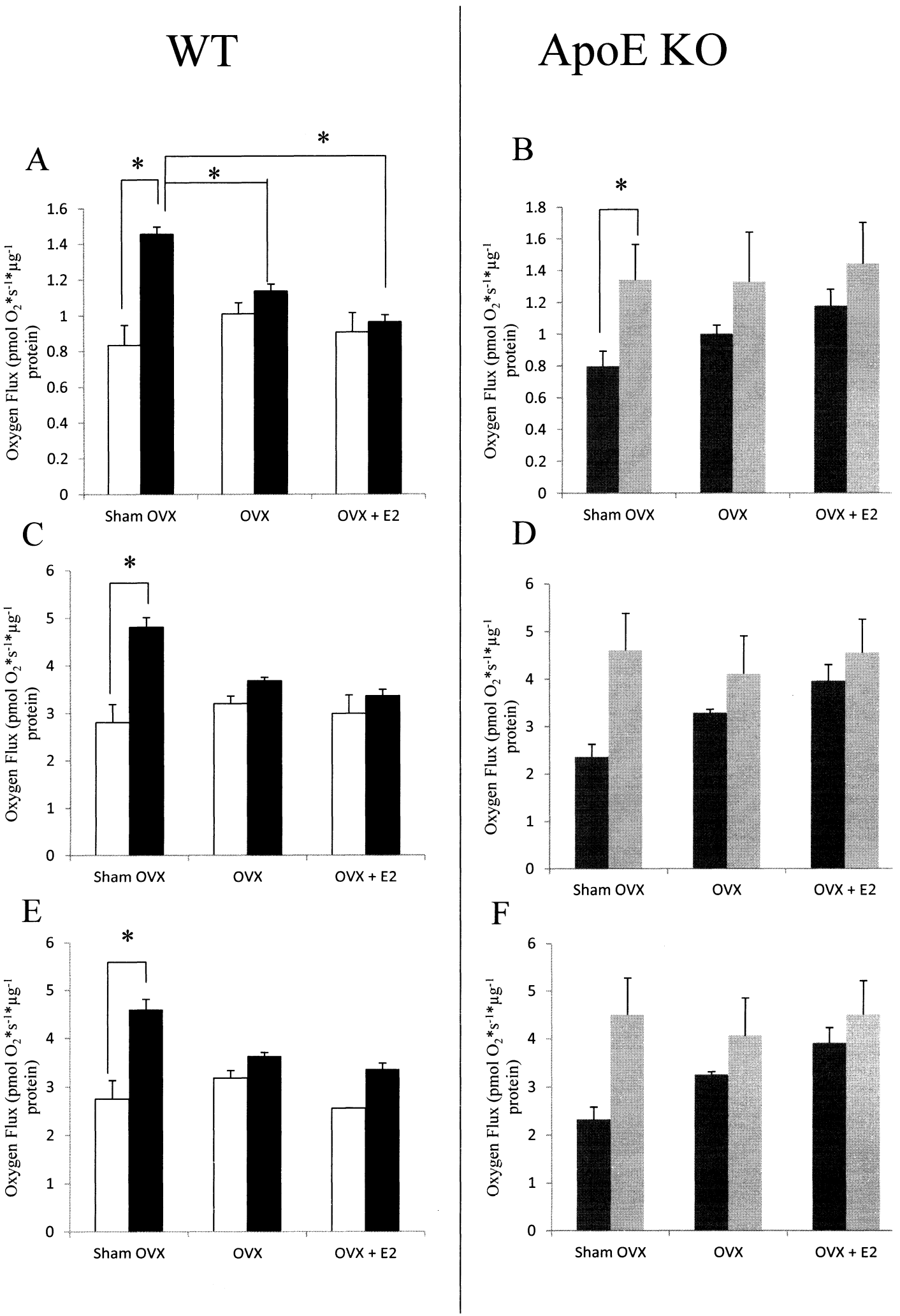

WT
ApoE KO
A
B
C
D
E
F
Oxygen Flux (pmol O_2*s^{-1}*μg^{-1} protein)
Sham OVX
OVX
OVX + E2
*

Figure 8. Complex II analysis in permeabilized synaptosomes from old and young WT and apoE KO mice. Synaptosomes were isolated from either young WT (white bar), old WT (black bar), young apoE KO (dark gray bar), or old apoE KO (light gray bar) mice in the diestrous cycle (Sham OVX), after ovariectomy (OVX), or ovariectomy followed by treatment with 50 ng E2/g (OVX + E2). (A & B) Complex II driven leak respiration was assessed in the presence of rotenone and succinate. (C & D) OXPHOS respiration initiated after the addition of 3 mM ADP. (E & F) Oxygen utilization was uncoupled from ADP phosphorylation with the addition of 0.5 μM increments of FCCP until maximum oxygen flux was observed. Respiration rates were standardized based on protein content and are expressed as pmol of O_2 consumed per second per μg protein. Significant differences in oxygen flux are denoted with an asterisk (*). Data was analyzed using a two-way ANOVA ($p < 0.05$, $n = 3$-6). Data are presented as the mean ± S.E.M. [#]young wild-type and ApoE KO are added for reasons of comparison and are from figures 3A, B, C.

4. Enzymatic Activity of Succinate Dehydrogenase and Citrate Synthase

Respiration data was corroborated by investigating the activity of succinate dehydrogenase (SDH; Fig. 9A & 9B) and citrate synthase (CS; Fig. 9C & 9D). Old WT Sham mice had significantly higher SDH activity compared to young WT Sham mice (Fig. 9A; $p < 0.05$). SDH activity after ovariectomy and estrogen treated did not change in old WT mice. In contrast to WT mice, no age effects were observed on SDH activity in apoE KO mice. However, old ovariectomized and estrogen treated apoE KO mice had significantly higher SDH activity compared to its Sham. CS activity in old WT mice was the same as observed for young WT mice and no differences were observed among treatment groups in old mice (Fig 9C). In contrast to WT mice, old apoE KO Sham mice had significantly higher CS activity compared to the young Sham mice. However, as in WT ovariectomy and estrogen treatment in old apoE KO mice had no effect on CS activity (Fig. 9B; $p < 0.05$).

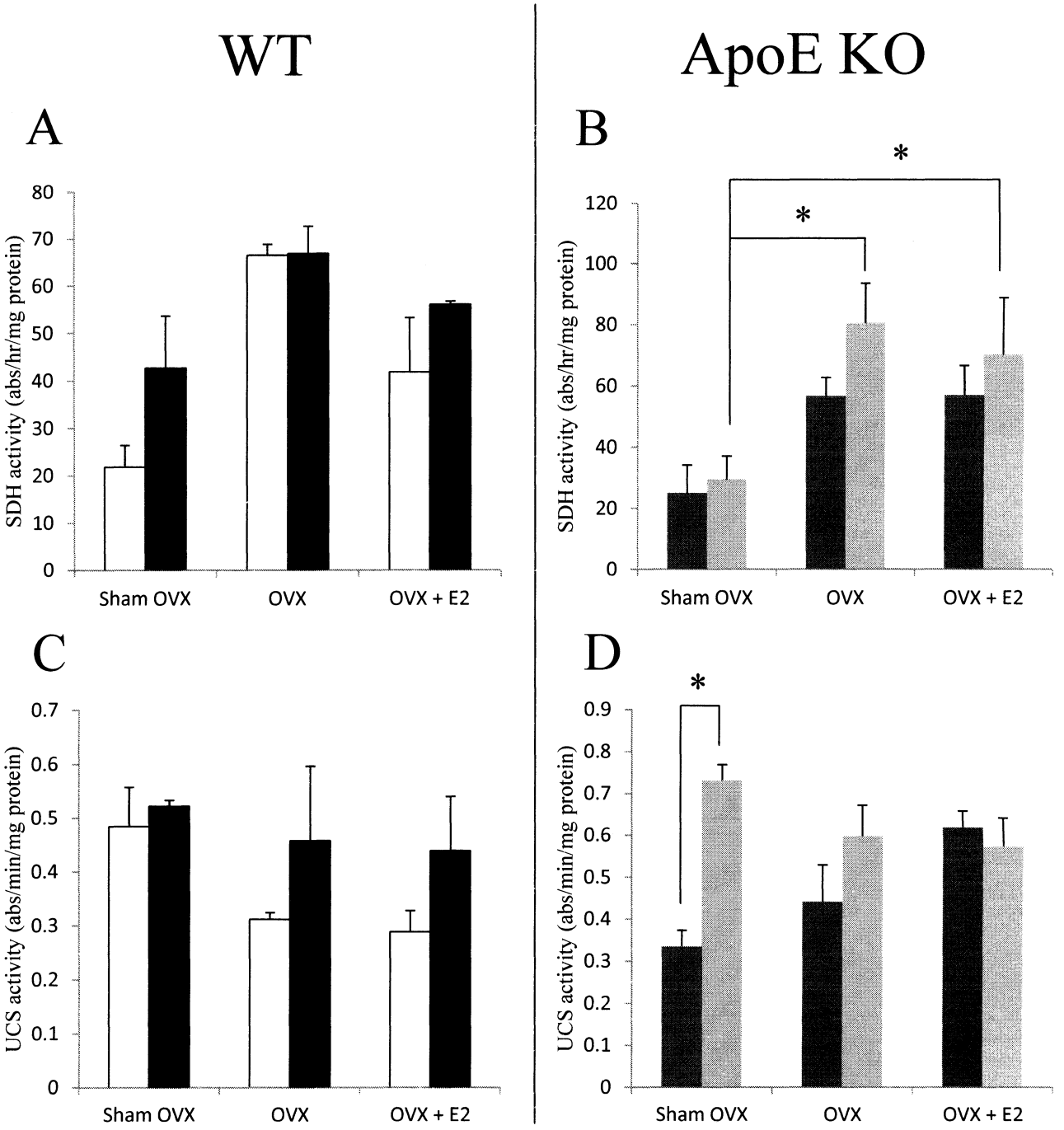


Figure 9. Succinate dehydrogenase and citrate synthase activity in synaptosomes from young and old WT and apoE KO mice. (A & B) Succinate dehydrogenase (SDH) activity and (C & D) citrate synthase (CS) activity were analyzed in synaptosome samples isolated from either young WT (white bar), old WT (black bar), young apoE KO (dark gray bar), or old apoE KO (light gray bar) mice in the diestrous cycle (Sham OVX), after ovariectomy (OVX), or ovariectomy followed by treatment with 50 ng E2/g (OVX

+ E2). SDH activity was standardized to total protein content and expressed as absorbance per h per milligram of total protein. CS activity was also standardized based on total protein and was expressed as absorbance per min per milligram of total protein. Significant differences in SDH and CS activity are denoted with asterisks (*). Data was analyzed using a two-way ANOVA ($p < 0.05$, $n = 3\text{-}6$). Data are presented as the mean ± S.E.M. #young wild-type and ApoE KO are added for reasons of comparison and are from figures 4A & 4B.

5. Western Blot Analysis of the Voltage Dependant Anion Channel

Expression levels of the voltage dependent anion channel (VDAC) were used as a mitochondrial marker to determine if increases in SDH and CS activities are contributed to an increase in total number of mitochondria (Fig. 10). Old WT Sham mice had 29% higher expression levels of VDAC compared to the young Sham. Ovariectomy and estrogen treatment caused insignificant increases in VDAC expression in old WT mice by 7% and 1%, respectively, compared to ovariectomized and estrogen treated young WT mice. In contrast to WT mice, young apoE KO mice had higher VDAC expression levels compared to old apoE KO mice for sham and estrogen treated mice. Young apoE KO sham VDAC expression was 19% higher than old sham mice, whereas, young estrogen treated apoE KO mice had 6% higher VDAC expression compared to old mice. Interestingly, old ovariectomized apoE KO mice had a 6% higher expression of VDAC compared to young ovariectomized mice. A detailed comparison of treatment groups within old mice revealed that relative to the Sham expression, ovariectomy in old WT mice resulted in a minor 4% increase in VDAC expression. Estrogen treatment decreased VDAC expression by 8% in old WT mice. Surprisingly, ovariectomy increased VDAC expression by over 36% in old apoE KO mice compared to levels observed in Sham. In striking contrast to WT mice, estrogen treatment in apoE KO mice led to a further increase in VDAC levels by 38% compared to the Sham.

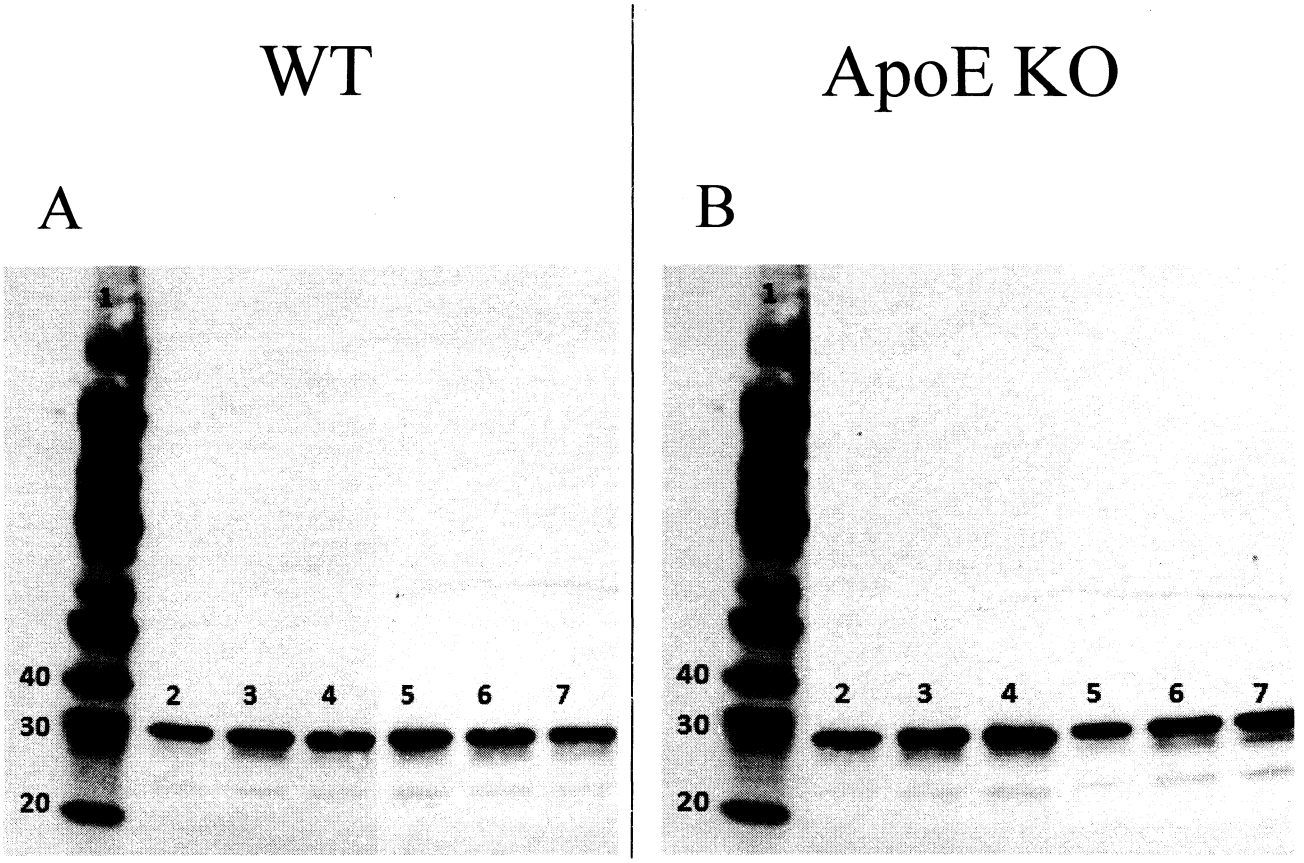


Figure 10. Immunoblotting of VDAC in young and old WT and apoE KO mice. Synaptosome samples (20 μg total protein per lane) were subjected to SDS-PAGE and immunoblotted for VDAC. (A) Old and young WT mice; Lane 1 = molecular weight standards, lane 2 = WT sham OVX, lane 3 = WT OVX, lane 4 = WT OVX + E2, lane 5 = old WT sham OVX, lane 6 = old WT OVX, lane 7 = old WT OVX + E2. (B). Old and young apoE KO mice; Lane 1 = molecular weight standards, lane 2 = apoE KO sham OVX, lane 3 = apoE KO OVX, lane 4 = apoE KO OVX + E2; lane 5 = old apoE KO OVX, lane 6 = old apoE KO OVX; lane 7 = old apoE KO OVX + E2. Optical density of VDAC bands were quantified using Image J software.

IV. Discussion

It is well known that the inheritance of the *apoe4* allele increases the likelihood of sporadic and late-onset familial AD (Corder et al. 1993; Bullido et al. 1998; Mahley et al., 2006). Expression of the *apoe4* allele has also been shown to drastically lower apoE concentrations in the brain (Poirier et al., 1995), as well as, decrease brain mitochondrial enzymatic activities (Risner et al., 2006). In addition, decreases in glucose utilization and mitochondrial dysfunctions have been observed before the pathological onset of AD in patients predisposed by *apoe4* inheritance (Reiman et al., 1996; Lin and Beal, 2006). In light of these key findings, we chose to use apoE KO mice as our experimental AD model in order to better our understandings of the effects of apoE on mitochondrial respiration and on enzymatic function in synaptosomes.

A. Synaptosomal Respiration: The Effect of ApoE

High-resolution respirometry of intact synaptosomes from apoE KO Sham and ovariectomized mice revealed no significant differences when compared with WT Sham and ovariectomized mice (Figure 1). However, estrogen treated apoE KO mice had significantly higher basal, uncoupled, and leak respiration in intact synaptosomes compared to age matched WT mice; a trend that extended into oxygen consumption rates of permeabilized synaptosomes (Figures 1& 2). Our results indicate that apoE KO and WT mice have approximately the same capacity for glucose transportation and utilization under sham and ovariectomized conditions, since no differences were detected in mitochondrial oxygen consumption. Previous studies have demonstrated that 17β-estradiol up-regulates glucose transporter proteins in the neurons of non-human primates

(Shi et al., 1997; Cheng et al., 2001). Therefore, increases in intact respiration were expected after the administration of estrogen; however, only apoE KO mice revealed increased respiration in intact synaptosomes, whereas, no effect was observed in WT mice (Figure 1). In addition, respiration rates in permeabilized synaptosomes were also significantly higher in estrogen treated apoE KO mice compared to its sham control, as well as, in age matched estrogen treated WT mice, whereas estrogen treatment had no effect in WT mice (Figures 2 & 7). In order to further understand this trend, enzymatic analysis of SDH and CS was conducted and revealed significantly higher enzymatic activities in estrogen treated apoE KO mice, whereas no effect was observed in estrogen treated WT mice (Figure 4). In light of these key findings, we concluded that increases in respiration were due to increases in mitochondria quantity via mitochondrial biogenesis, which was further validated via a Western Blot using VDAC as a mitochondrial housekeeping marker (Figure 5). In addition, we speculate that the protein apoE plays a regulatory role in estrogen induced mitochondrial biogenesis, thus explaining the differences observed between genotypes.

B. Mitochondrial Biogenesis

Mitochondrial biogenesis is a highly integrated process that requires immense coordination between nuclear and mitochondrial gene products including NRF-1, NRF-2, Tfam, PGC-1α, and mortalin (Kelly and Scarpulla, 2004; Londono et al., 2012). In particular, PGC-1α has been considered the master regulator for mitochondrial biogenesis in mammals, and is responsible for the activation of the required transcription factors needed for the initiation of mitochondrial biogenesis (Ventura-Clapier et al., 2008; Wu et al., 1999). Previous studies have shown that 17β-estradiol, as well as, oxidative stress

increase mRNA and protein levels of PGC-1α (Hsieh et al., 2005; Lee and Wei, 2005). It is also well known that aging increases mitochondrial driven oxidative stress (Berlett and Stadtman, 1997; Kokoszka, 2001; Lin and Beal, 2006), thereby increasing PGC-1α expression which explains the increases in synaptosome respiration observed in old mice when compared to their younger counterpart (Figure 6, 7 & 8). Currently there is no evidence suggesting that PGC-1α is differentially regulated in WT and apoE KO mice via estrogen treatment and/or under oxidative stress. However, mitochondrial biogenesis relies heavily on the import of proteins from the cytosol via mitochondrial-specific translocase proteins like mortalin (Koehler, 2004). Mortalin is a mitochondrial heat shock protein that is associated with the voltage dependent anion channel (VDAC) and plays a vital role in mitochondrial biogenesis, cell proliferation, and oxidative stress (Londono et al., 2012; Kaul et al., 1993; Wadhwa et al., 1993a). Interestingly, mortalin has a substrate binding domain that has been shown to bind with apoE *in vivo*, however, the function of this interaction is unknown (Londono et al., 2012). We propose that the mortalin-apoE interaction has an inhibitory effect on mitochondrial biogenesis by preventing the translocation of PGC-1α induced cytosolic proteins into the mitochondrion. Therefore, mice lacking apoE have increased mitochondrial biogenesis through the combined efforts of mortalin and PGC-1α in comparison to WT mice.

C. Antioxidant Defense Mechanisms

Another interesting result observed in our study was that ovariectomy surgery induced higher respiratory rates, as well as enzymatic activity, in both WT and apoE KO mice (Figures 2, 3, 4, 7 and 9). It is well known that mitochondria are the main source of ROS during metabolism (Chen et al., 2003). Mitochondria have enzymatic defense

systems in place to combat ROS production, such as, superoxide dismutase and glutathione peroxidase. Previous studies have shown that removal of ovaries drastically lowers expression levels of superoxide dismutase in mitochondrial fractions, thus increasing the amount of ROS exposure over time on the cellular level (Ha, 2004; Borras et al., 2003). Furthermore, increases in ROS production have been linked to increases in mitochondrial quantity (Barrientos et al., 1997a; Lee et al., 2002), which has been speculated to play a compensatory role for defective mitochondria (Lee and Wei, 2000). Our results, specifically increases in respiration observed in ovariectomized mice under permeabilized conditions, support the phenomenon of ROS induced mitochondrial biogenesis. In addition, Western blot analysis revealed increased expression levels of VDAC and increased enzymatic activity of complex II and the Krebs cycle in ovariectomized mice compared to the Shams, which provides substantial evidence that mitochondrial quantity was increased (Figures 5 and 10).

D. Estrogen

Estrogen's mechanism of action mediates targeted protein synthesis, including superoxide dismutase (Stirone et al., 2005b), which reduces ROS exposure. Ovariectomized WT mice injected with estrogen revealed oxygen consumption levels that resembled its Sham (Figures 1, 2, and 3). This was an expected result since estrogen most likely increased expression levels of antioxidant enzymes, such as superoxide dismutase, but an immunoblot would be needed to confirm this hypothesis. However, in most cases a single injection of estrogen was insufficient to bring oxygen fluxes all the way back to sham levels indicating a need for multiple estrogen injection to induce significant affects on mitochondrial activity. In contrast to WT mice, estrogen treated

apoE KO mice had significantly higher respiration rates and enzymatic activity compared to age matched WT mice (Figures 1, 2, 3, and 4). Previous studies have shown that synaptosomes isolated from apoE KO mice are structurally different from WT mice (Keller et al., 2000; Lauderback et al., 2001). Specifically, apoE KO synaptosomes possess increased membrane bilayer fluidity as a result of increases in oxidation of cholesterol (Maor et al., 2000). It is possible that increases in bilayer fluidity may increase synaptosomal uptake of estrogen, thereby increasing the activity of mitochondrial targeted estrogen-receptors, thus providing another explanation for estrogen induced increases in respiration and enzymatic activity observed in apoE KO mice compared to WT mice. In addition, increases in membrane fluidity are associated with increases in macromolecule interactions with receptors (Lenaz, 1987). As a result, estrogen induced signal transduction in apoE KO mice may be more active compared to WT mice.

Estrogen treatment had little to no effect in old mice (Figures 6, 7, and 8). Previous studies have shown that increases in rodent age are associated with decreases in estrogen receptors located in synapses (Adams et al., 2002). In light of this finding, it is not unreasonable to assume that the response to estrogen in aged mice will be blunted for all respiration parameters and enzymatic activities. However, the standard errors reported for old mice were relatively high compared with young mice, so the effects of estrogen may have been concealed. This may indicate that age plays a larger role in synaptosomal respiration and in order to reduce error in future studies the age range may need to be narrowed.

V. Conclusions

The conclusions of this thesis can be summarized in five key points:

1. Sham and ovariectomized young WT and apoE KO mice had similar respiration rates for intact and permeabilized synaptosomes, as well as, similar enzymatic activity.

2. Estrogen treatment dramatically increased respiration in intact and permeabilized synaptosomes, and enzymatic activity in young apoE KO mice, whereas, no effect was observed in young WT mice. Additionally, estrogen treatment had no effect in old mice from both genotypes.

3. Overall, age had little effect on respiration rates observed in intact synaptosomes, but significant increases in old WT and apoE KO Sham mice, compared to their younger counterparts, were detected for all permeabilized parameters except for uncoupled respiration.

4. Interestingly, ovariectomy and estrogen treatment increased VDAC expression in young and old apoE KO mice. However, VDAC expression in young WT mice were remarkably similar among treatment groups, whereas, slight decreases were observed in old ovariectomized and estrogen treated WT mice, in comparison to the Sham control.

5. We speculate that the observed increases in respiration and enzymatic activity are due to increases in mitochondrial quantity, which was supported via our VDAC expression data. We propose that mitochondrial biogenesis was stimulated through a concerted effort of translocating PGC-1α induced proteins via estrogen by mortalin. We are also suggesting that apoE plays a regulatory role on mortalin's translocating ability thus

explaining the differences observed between apoE KO and WT mice. In addition, it is most likely that increases in respiration from old mice was due to ROS induced mitochondrial biogenesis and the abolished effects of estrogen in old mice was most likely due to decreases in estrogen receptor expression.

VI. Works Cited

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