Published in final edited form as:

Neuroscience. 2008 June 23; 154(2): 720–731.

Neuroprotective Actions of Ovarian Hormones Without Insult in the Raphe Region of Rhesus Macaques

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Abstract

Using a nonhuman primate model of surgical menopause, our laboratory has shown that ovarian hormone treatment (HT) improves serotonin neural function in the dorsal raphe nucleus (DRN). We further hypothesize that HT may increase serotonin neuronal resilience. Recent data from microarray analysis indicated that HT regulates gene expression in pathways that lead to apoptosis. In this study, we questioned whether HT alters protein expression in caspase-dependent and independent pathways. Ovariectomized monkeys received Silastic implants containing placebo (empty), estrogen (E) or E+ progesterone (P). A small block of the midbrain containing the DRN was dissected and subjected to subcellular fractionation, yielding cytosolic, nuclear and mitochondrial fractions (n=4/group). The pro-apoptotic protein, JNK1 and its phosphorylation were decreased by E+P treatment in the cytosolic fraction. Downstream of JNK are proteins in the caspase-dependent and independent pathways. First, in the caspase-dependent pathway, cytoplasmic and mitochondrial fractions were immunoblotted for Bcl-2 family members, cytochrome c, Apaf1 and XIAP. However, the expression of these proteins did not differ among treatments. Pro-caspase 3 was decreased by E+P, but there was no evidence of active caspase in any group. Then, we examined the involvement of a protein in the caspase-independent pathway, called apoptosis-inducing factor (AIF). AIF mRNA (n=3/group) and AIF mitochondrial protein tended to decrease with hormone treatment. However, AIF protein in the nuclear fractions in E+P treated monkeys was significantly reduced. This indicates that HT is reducing the translocation of AIF from mitochondria to nucleus, thus inhibiting AIF-mediated apoptosis. AIF was immunocytochemically localized to large serotonin-like neurons of the dorsal raphe. This data suggests that in the absence of global trauma or ischemia, HT may act through the caspase-independent pathway to promote neuroprotection in the serotonin system.

Introduction

A large body of literature indicates that the ovarian hormones, estrogen (E) and progesterone (P) are neuroprotective (Simpkins et al., 2005, Suzuki et al., 2006, Singh et al., 2008). Animal models have shown that administration of E, prior to or coincident with, trauma or global ischemia decreases tissue damage (McCullough et al., 2001, Hoffman et al., 2006). Recent reports have found activation of caspases in the damaged tissue suggesting that the caspase-dependent pathway is involved in apoptosis resulting from ischemia (Springer, 2002, Faubel and Edelstein, 2005). Moreover, E treatment decreases caspase activation in the damaged area

of the brain (Jover-Mengual et al., 2007). In the clinical arena, some evidence suggests that E may delay the onset of overt neurodegeneration in Alzheimer's (Henderson, 2006) or Parkinson's disease (Benedetti et al., 2001, Ragonese et al., 2006) although the precise mechanism(s) of action remain obscure.

Neurodegeneration is usually thought of in the context of severe deficits in motor or cognitive function. However recently, it has been suggested that even the psychopathologies may involve functional degeneration of critical central neural systems and many are thought to have a serotonergic neurodegenerative etiology (Tejani-Butt et al., 1995, Benninghoff et al., 2002). The dorsal and medial raphe nuclei contain the serotonergic neurons that project to all areas of the forebrain and serotonin neurons modulate a wide range of autonomic functions, cognitive domains, affect or mood, anxiety, and stress-related disorders. Thus, any loss or degeneration of serotonin neurons could have profound ramifications.

This laboratory has devoted significant effort to understanding the actions of ovarian steroids in the serotonin neural system. Our studies indicate that the ovarian hormones, estrogen (E) and progesterone (P), regulate gene and protein expression in serotonin neurons to increase serotonin production, serotonin turnover, neural firing and decrease degradation (Bethea et al., 2002, Lu and Bethea, 2002, Lu et al., 2003, Sanchez et al., 2005). Recently, using Affymetrix array analysis on RNA extracted from the dorsal raphe region or from laser captured serotonin neurons of rhesus macaques, we found that several genes involved in neurotoxicity or programmed cell death were regulated by one month of hormone treatment (HT) (Reddy and Bethea, 2005, Bethea and Reddy, 2008).

It is important to note that our model of HT in primates after surgical menopause does not involve a gross insult to the brain. So, although the neuroprotective effect of HT has been extensively studied in models of injury to the brain, the neuroprotective actions of HT in a non-injured environment are less understood. However, there is a continuum of lesser insults to the central nervous system over the course of life such as stress, illness and psychological trauma. Thus, we question the potential of ovarian steroids to increase the resilience of serotonin neurons to normal life stress.

Other work from this laboratory has shown that monkeys who secrete lower levels of E and P during the menstrual cycle have fewer serotonin neurons than monkeys with higher levels of E and P. Moreover, the monkeys with fewer serotonin neurons are stress sensitive and they will rapidly cease ovulation upon exposure to a combination of diet, exercise and housing change (Bethea et al., 2005). Thus, it is reasonable to question whether women who lose ovarian steroid support during menopause, may also gradually lose serotonin neurons for the next several decades of life.

One very important pro-apoptosis gene, JNK1 (c-jun n-terminal kinase or MAPK8 or SAPKs), was markedly decreased by HT in a previous microarray analysis and this was confirmed with qRT-PCR (Reddy and Bethea, 2005). JNK is a member of the mitogen activated protein kinase (MAPK) superfamily, originally identified as stress-activated protein kinases (SAPKs) (Kyriakis and Avruch, 1990). JNK has been shown to mediate cell death by c-Jun transcriptional events, release of cytochrome c and AIF from the mitochondria and phosphorylation of various Bcl-2 family members, leading to caspase-dependent or – independent cell death (Aoki et al., 2002, Ameyar et al., 2003, Bogoyevitch and Kobe, 2006).

HT altered several other genes in the dorsal raphe region involved in programmed cell death, such as Bcl-2 family members, inhibitors of apoptosis (IAPs), and SOD1. Moreover, in laser captured serotonin neurons, apoptotic peptidase activating factor 1 (Apaf1), apoptosis inducing factor (AIF) and Diablo (also called SMAC, second mitochondrial activator of caspases) were markedly reduced (Bethea and Reddy, 2008). These findings led us to hypothesize that HT is

neuroprotective by altering various proteins involved in either caspase-dependent and/or – independent apoptotic pathways. However, it was necessary to determine whether changes in gene expression on the microarray were translated to the protein level. Therefore, we examined the expression and activity levels of JNK1, Bcl-2 family members (Bcl-2, Mcl-1, Bak and Bax), Apaf1, XIAP, caspase-3, cytochrome c and AIF in subcellular fractions of the dorsal raphe region to determine the mechanism by which hormone therapy may promote neuroprotection in the absence of global injury.

Materials and Methods

The Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee approved this study.

Animals and treatments

Twenty-one adult female rhesus monkeys (*Macaca mulatta*) were spayed (ovariectomized only) by the surgical personnel of ONPRC between 3 and 6 months before assignment to this project according to accepted veterinary surgical protocol. All animals were born in China, were aged between 7-14 years by dental exam, weighed between 4 and 8 kg, and were in good health.

Animals were either treated with placebo (**ovx-control group; n=7**), or treated with estrogen (E) for 28 days (**E group; n=7**), or treated with E for 28 days and then supplemented with P for the final 14 of the 28 days (**E+P group; n=7**). The placebo treatment of the spay-control monkeys consisted of implantation with empty Silastic capsules (s.c.). The E-treated monkeys were implanted (s.c.) with a 4.5-cm E-filled Silastic capsules (i.d. 0.132 in.; o.d. 0.183 in.; Dow Corning, Midland, MI). The capsule was filled with crystalline estradiol (1,3,5(10)-estratrien-3,17-b-diol; Steraloids, Wilton, NH). The E+P- treated group received E-filled capsules, and 14 days later, received one 6-cm capsule filled with crystalline P (4-pregnen-3,20 dione; Steraloids). All capsules were placed in the periscapular area under ketamine anesthesia (ketamine HCl, 10mg/kg, s.c.; Fort Dodge Laboratories, Fort Dodge, IA). Two days before necropsy or at necropsy, blood samples were drawn for measurement of serum E and P concentrations by radioimmunoassay.

The monkeys were euthanized at the end of the treatment periods according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Each animal was sedated with ketamine, given an overdose of pentobarbital (25 mg/kg, i.v.), and exsanguinated by severance of the descending aorta.

Tissue Harvest for Quantitative RT-PCR (n=3/treatment group)

The left ventricle of the heart was cannulated and the head of each animal was perfused with 3 liters of 1X cold RNA-later buffer (Ambion Inc., Austin, TX). The brain was removed from the cranium and blocked. The dissected midbrain block displayed the rounded central canal on its anterior surface and the wing-shaped canal on its caudal surface. This section was microdissected and a small square piece of tissue was harvested which extended from the middle of the central gray to the decussation of the cerebellar peduncles. We call this small piece the dorsal raphe nucleus (DRN) block. The piece was the width of the central gray and contained the major portion of the dorsal raphe nucleus (approximately 5mm wide, 6 mm high and 3mm thick). The DRN block was immediately frozen in liquid nitrogen. For RNA extraction, the frozen block was dropped directly into TriReagent (Sigma, St. Louis, MO).

Tissue Harvest for Subcellular Fractionation and Western Blot (n=4/treatment group)

The brain was removed from the cranium and dissected yielding a block of tissue from the pontine midbrain. This block was either (1) immediately microdissected as described above and processed for subcellular fractions, or (2) frozen for up to one week and then thawed, microdissected and the raphe region was processed for subcellular fractions. Preliminary tests compared the yields of the different fractions from DRN blocks that were processed immediately (never frozen) to DRN blocks that were frozen and then processed.

Subcellular fractionation

Subcellular fractionation was performed following the method of Booth and Clark (1978). Briefly, the DRN block was homogenized in 1 ml of isolation medium [0.32M Sucrose, 1mM K-EDTA (pH 7.4), 20mM Tris-HCl (pH 7.1)] in a Dounce glass homogenizer. The homogenate was spun at $1,500 \times g$. The pellet (nuclear and membrane fraction) was resuspended in isolation medium. The supernatant was spun at $15,000 \times g$ using Beckman swing bucket SW-60Ti. The supernatant from this high speed spin was kept as the cytosolic fraction. The crude mitochondria pellet was resuspended in isolation medium and mixed with 12% Ficoll solution [12% (w/w) Ficoll, 0.32 M Sucrose, 50 μ M K-EDTA (pH 7.4)] in a centrifuge tube (Polyallomer Centrifuge Tubes, Beckman). A layer of 7% Ficoll solution [7% (w/w) Ficoll, 0.32 M Sucrose, 50 μ M K-EDTA (pH 7.4)] was added on top of the mitochondria suspension and then isolation medium was added on top of the 7% Ficoll solution. The samples were then spun at $100,000 \times g$ and mitochondria were pelleted at the bottom.

Western blot analysis

The lysates were denatured at 95°C for 5 min in sample buffer [2% SDS, 10% glycerol, 60 mM Tris (pH 6.8), 5% β -mercaptoethanol, 0.01% bromophenol blue]. Samples were resolved by 10-15% SDS-PAGE, and transferred onto nitrocellulose membrane sheets. The blots were first incubated in blocking buffer [5% (wt/vol) nonfat dry milk in Tris-buffered saline (TBS) + Tween 20 (TBS-T)] for 1 h. The blots were then incubated with primary antibody over night at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The antibody-antigen complex was visualized by SuperSignal West Pico Chemiluminescent (Pierce Biotechnology, Rockford, IL). Equal amounts of protein from the different fractions were loaded for comparison between treatment groups. Protein concentrations were determined with BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Loading consistency was confirmed by reprobing the blots with either actin, Cox1 or Histone H1 or by staining the blot with Coomassie blue.

Immunocytochemistry

Perfusion fixed sections (25 μ) of the midbrain from an Ovx-placebo treated monkey containing the dorsal raphe were prepared as previously described (Sanchez et al., 2005) and stored at -80°C in cryoprotectant (30% ethylene glycol, 20% glycerol in 0.05 M PBS). Sections were removed from storage, washed in KPBS buffer 4 times for 15 min each, immersed in methanol plus 10% hydrogen peroxide for 10 min, washed in KPBS buffer 4 times for 15 min each and then incubated with the following blocking solutions: Vector normal goat serum (NGS) for 30 min, Vector Avidin for 20 min, and Vector Biotin for 20 min. Sections were then incubated for 48 hours in antibody to AIF (1/400 in KPBS). Sections were then rinsed in KPBS buffer 4 times for 15 min each, incubated in Vector biotinylated goat anti-rabbit serum for 60 min, washed in KPBS buffer 4 times for 15 min each, incubated with Vector ABC reagent for 60 min, washed in KPBS buffer 4 times for 15 min each, washed in Tris buffer pH 7.6 for 5 min, incubated with DAB (100 mg/200 ml Tris HCl buffer, pH 7.6 plus 80 μ l of 30% hydrogen peroxide) for 20 min, washed in Tris buffer for 5 min, washed in KPBS buffer for 5 min and then mounted on Super Frost Plus slides (Fisher Scientific). After adherence to the slide, the

sections were dehydrated through a graded series of ethanols, xylene and HistoClear. Sections were mounted under glass with DPX.

Antibodies

Rabbit polyclonal antibodies to JNK1, phospho-JNK1, Bcl-2 family members, AIF, cytochrome c, caspase 3, Lamin A/C (1:500) were purchased from Cell Signaling Technology (Danvers, MA). The mouse monoclonal antibody to XIAP was from MBL (Nagoya, Japan) and the rabbit polyclonal antibody to Apaf1 was from QED Biosciences (San Diego, CA). Antibodies were diluted at 1/1000 for all immunoblot assays unless otherwise noted.

Quantitative (q) RT-PCR

RNA was extracted from the raphe region of the 9 rhesus midbrains (n=3/treatment group; perfused with RNA later) using TriReagent and further cleaned with a Qiagen RNAeasy column (Valencia, CA). The quality of the RNA from the Qiagen column was examined on an Agilent Bioanalyzer and found acceptable and of equal quality. This RNA was subjected to quantitative (q)RT-PCR for AIF.

Complementary DNA (cDNA) synthesis was performed using Oligo-dT 15 primer (Invitrogen, Carlsbad, CA) and Superscript III reverse transcriptase (200 U/μg of RNA, Invitrogen) at 50° C for 1 hr. A pool of RNAs from different rhesus tissues was used to generate a standard curve. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene, as previous data indicated that this gene was not regulated by E or P (Reddy and Bethea, 2005).

Quantitative polymerase chain reaction (qRT-PCR) was conducted with Platinum SYBR Green qPCR Super Mix UDG (Invitrogen Life Technologies, Carlsbad, CA). This mix contains a modified Platinum DNA Taq Polymerase, uracil DNA glycosylase (UDG) and Sybr green I fluorescent dye, which is specific for double stranded DNA. There is a linear increase in fluorescence detected as the concentration of amplified double stranded product cDNA increases during the reaction. The fluorescence was detected with the ABI 7900 thermal cycler during forty cycles. The reaction (final volume 20 μl) contained dilutions from 1 – 100,000 pg of pool cDNA (standard curve) or 250 ng of sample cDNA, 100 nM of forward and reverse primers and 1X Invitrogen PCR mix. The amount of cDNA from the samples was determined by measurement of the input ssDNA with a Nanodrop Spectrophotometer. A standard curve was generated for AIF by performing a linear regression analysis on the log of the input cDNA versus the cycle time at which saturation was reached. The standard curve was used to calculate the relative pg of AIF in the RNA extracted from the raphe blocks. Then, the ratio of each transcript to GAPDH was calculated.

Primer selection

For AIF, the probe set ID from the Affymetrix Rhesus Gene Chip was launched to query at Affymetrix NetAffix program on the web. This program enabled retrieval of the annotation list and provides direct access to Genbank sequence at NCBI. Then, using NetAffix, the oligonucleotide location in the AIF gene was identified. Based upon the oligoset distribution, a target area of the AIF gene was selected. The target sequence was then loaded into Primer Express software, which chooses the primers for optimum qRT-PCR. The primers were obtained from Invitrogen (Carlsbad, CA). The AIF primers utilized for qRT-PCR on the RNA extracted from the DRN block are as follows: TCAGCATCCCCTCTAGCTCCTT (305bp-326bp) and CCGCAAATCACCCGT (509bp-491bp).

Hormone Assays

Assays for estradiol and progesterone were performed utilizing a Roche Diagnostics 2010 Elecsys assay instrument. Prior to these analyses, measurements of estradiol and progesterone on this platform were compared to traditional RIA's as previously reported (Bethea et al., 2005). The E+P treatment regimen has been shown to cause differentiation of the uterine endometrium in a manner similar to the normal 28-day menstrual cycle (Brenner and Slayden, 1994).

Statistical analysis

The animals were treated and euthanized in sets containing one Ovx, one E-treated and one E+P treated animal. For the western blot data (n=4 sets), each set of animals was run on the same gel and the optical density (OD) of the protein band in each treated animal was expressed as a fraction of the band from the matched Ovx animal in the respective set. The normalized ODs for each treatment group were compared using ANOVA with Student Newman Keuls post-hoc pairwise comparison ($p < 0.05$ was considered significantly different). For the qRT-PCR data (n=3 sets) the ratio of AIF/GAPDH was calculated for each animal and the means of the Ovx, E and E+P treated groups were compared using ANOVA with Student Newman Keuls post-hoc pairwise comparison. All statistical analyses were conducted with the Prism Statistic Program (GraphPad, San Diego, CA).

Results

Characterization of subcellular fractions of the raphe region

We obtained the DRN block from test animals to determine the efficiency of the subcellular fractionation on fresh tissue compared to frozen tissue. Nuclear, cytoplasmic and mitochondrial fractions were obtained and western blots were probed with COX I (Complex IV subunit I or cytochrome c oxidase), Lamin A/C and actin. The yield of mitochondria was equivalent whether the tissue was processed fresh or frozen. Figure 1 illustrates the results of the successful subcellular fractionation on tissue that was frozen for up to one week. The top panel shows that the nuclear membrane components, Lamin A/C, are only present in the nuclear fraction. Similarly, the mitochondrial protein, COX I, was only detected in the mitochondrial fraction (bottom panel). The ability to freeze the midbrain block for subsequent fractionation was an important technical advantage because of the time required for necropsy and fractionation on the same day.

JNK1 expression and activity is down regulated in hormone-treated animals

We first examined the pro-apoptosis gene, JNK1, which was down regulated at the gene level in the DRN block of hormone-treated animals (Reddy and Bethea, 2005). Consistent with our JNK1 gene expression data, JNK1 protein in E+P treated monkeys was down regulated compared to the control Ovx monkeys (Figure 2B, left panel). JNK1 is activated by phosphorylation by MAPK kinases MKK4/7. Thus, we immunoblotted to determine the phosphorylation status of JNK1 (P-JNK1) by which the activity of JNK1 can be inferred. P-JNK1 was reduced in E+P-treated animals compared to the Ovx animals (Figure 2B, right panel). Because the reduction of P-JNK1 could be a consequence of the reduction in JNK1 protein, we calculated the ratio of P-JNK1 to JNK1. There was no difference between treatment groups in the ratio of P-JNK1/JNK1 suggesting that the decrease in JNK1 protein was producing the decrease in detectable P-JNK1. Representative blots of JNK1 and P-JNK1 are shown in Figure 2A, top and middle panels. The actin blot is shown in Figure 2A, bottom panel, as a loading control. Actin signals were not different across the gels so the JNK1 data were not normalized to actin. Since JNK1 is a pro-apoptotic kinase that can activate both caspase-

dependent and independent pathways, we examined both downstream pathways in our different treatment groups.

Bcl-2 family members are not altered by HT-treatment

The Bcl-2 family members consist of pro- and anti-apoptotic members, which play a crucial role in the regulation of apoptosis by regulating mitochondrial membrane permeability. To determine whether HT is neuroprotective as a result of an alteration in the expression ratio of the pro- or anti-apoptotic proteins, we examined the expression of the Bcl-2 family proteins. There were no significant changes in the anti-apoptotic proteins Bcl-2 and Mcl-1 or in the pro-apoptotic protein, Bak, in the mitochondrial fraction of hormone-treated animals (Figure 3A and 3C, top panels). In addition, we examined pro-apoptotic Bax expression both in the cytosolic and mitochondrial fractions (Figure 3A, 3B and 3C, bottom panels). There was no effect of hormone treatment on Bax expression in the cytosol or mitochondria. Representative western blots for each of the examined proteins are shown in Figure 3, parts A and B and the quantitation of each protein is shown in Figure 3, part C. Cox1 signal is shown as a loading control for the mitochondrial proteins, and actin signal is shown as a loading control for the cytoplasmic protein. There was no difference in Cox1 or actin signals across the gels, so the Cox1 and actin signals were not used to normalize the data. In summary, HT did not exert a significant effect on the Bcl-2 family members.

The effect of hormone treatment on cytochrome c and Apaf1

The release of different pro-apoptotic proteins present in the mitochondria, such as cytochrome c or AIF, is observed in both caspase-dependent or -independent apoptotic cell death. Thus, we examined the expression of cytochrome c in mitochondria and cytosolic fractions. There was no difference in total cytochrome c (data not shown) and cytochrome c was present in the cytoplasm in all treatment groups. However, the amount of cytochrome c present in the cytoplasm was independent of treatment (Figure 4A and Figure 4B). The blot was stained with Coomassie blue and demonstrated consistent loading with equal band density of various visible proteins. Cytochrome c activates the adaptor molecule apoptotic peptidase activating factor 1 (Apaf1), generating the complex known as the apoptosome. Each apoptosome recruits procaspase-9, which leads to cell death. Therefore, we determined whether Apaf1 was influenced by HT. As shown in Figure 4C and 4D, there was no significant effect of hormone treatment on Apaf1 expression. The Apaf1 blot was reprobbed for actin as a loading control and there was no difference in actin signals across the gels. Therefore, the Apaf1 data was not normalized by actin.

The effect of hormone treatment on caspases and IAP

In our recent study with Affymetrix Rhesus Macaque Gene Chips, there was no effect of E or E+P on caspase 3 in the DRN block (Bethea and Reddy, 2008). This seemed inconsistent with a neuroprotective action of ovarian hormones. Thus, we examined the expression level of pro- and cleaved (active) caspase 3 in the DRN block. We saw little to no detectable cleaved caspase 3 in any treatment group (Figure 5a). Unlike the gene expression detected on the microarray, the total protein level of pro-caspase 3 was decreased in E+P-treated animals compared to Ovx animals (Figure 5B, representative blot in Figure 5A). Another class of proteins called inhibitors of apoptosis (IAPs) suppresses apoptosis by interacting with and inhibiting the enzymatic activity of caspases (Nishihara et al., 2003, Saito et al., 2004). HT increased gene expression of the most potent and well-known member, X-chromosome-linked IAP (XIAP), in the DRN block (Bethea and Reddy, 2008). However, we did not observe any effect of hormone treatment on XIAP by western blot analysis (representative blot in Figure 5B). Each blot was reprobbed for actin to demonstrate consistent loading of samples, but the data was not normalized by actin.

HT acts through the caspase-independent pathway

In our recent study with Affymetrix Rhesus Macaque Gene Chips, AIF gene expression was reduced in laser captured serotonin neurons from E and E+P- treated animals (Betheda and Reddy, 2008). Examination of AIF in mRNA extracted from the DRN block with qRT-PCR reflected the microarray results with the laser captured serotonin neurons, but the decrease did not reach statistical significance due to the variance, which is potentially due to the presence of other cell types. (Figure 6A). Nonetheless, it was important to examine AIF in the nuclear and mitochondrial fractions from the raphe blocks. There was a declining trend in AIF in the mitochondria and significantly less AIF had translocated to the nucleus in animals treated with E+P (Figure 6B). The lower expression of AIF in the mitochondria and nuclei suggest that total AIF may be reduced by hormone treatment, reflecting the mRNA expression. Thus, although cytochrome c release was not altered, HT significantly decreased AIF release from the mitochondria. The mitochondrial blot was stained with Coomassie blue and demonstrated consistent loading with equal density of multiple visible protein bands. The nuclear blot was reprobbed for Histone H1 and showed that the decrease in nuclear AIF was not due to a decrease in protein loaded. However, the data was not normalized by Histone H1.

AIF localized to DRN neurons

AIF was detected in large neurons of the DRN and median raphe, but not outside of these areas. Figure 7 illustrates the immunostaining for AIF in the DRN. The median raphe was largely excluded in the dissection of the DRN block.

Hormone Concentrations

Estradiol concentrations (pg/ml) in the serum of the OvX, E-, P- and E+P-treated groups (n=7) equaled 23.4±4.5, 136.0±17.2, 24.5±5.9 and 136.0±11.2, respectively (ANOVA, $p < 0.0001$). Progesterone concentrations (ng/ml) in the serum of the OvX, E-, P- and E+P-treated groups (n=7) equaled 0.14±0.03, 0.56±0.17, 6.94±0.92 and 5.95±0.65, respectively (ANOVA, $p < 0.0001$). The sensitivity of the estradiol assay was 5 pg/ml and the sensitivity of the progesterone assay was 0.02 ng/ml. The interassay coefficient of variation based upon multiple determinations of a pool of rhesus macaque serum did not exceed 10% for either assay.

Discussion

In animal models of global trauma, estrogen appears to decrease apoptosis by actions through the caspase dependent pathway (Rau et al., 2003). This pathway is activated by severe injury such as ischemia or neurotoxic agents (Roof and Hall, 2000). In another model that does not involve insult, estrogen decreases apoptosis in the sexually dimorphic nucleus of the preoptic hypothalamus in postnatal rats by increasing Bcl-2 and decreasing Bax (Tsukahara et al., 2008) although downstream effectors have not been elucidated. In this study, we show that HT may increase DRN neuronal resilience in the absence of injury through actions on JNK1 and downstream by actions on AIF, a pivotal protein in the caspase –independent pathway.

This study was initiated to determine whether changes in gene expression in pathways leading to apoptosis as detected by microarray analysis were manifested at the protein level. In the microarray study, gene expression changes were examined in a small block of tissue containing the DRN, such as was used for this protein study, and in laser captured serotonin neurons. We proposed that gene changes observed in the DRN block would be translated to protein changes that would be detectable by western blot. However, the changes in gene expression observed in the DRN block, although significant, were not robust fold changes. Thus, lack of translation to a detectable change in protein was also feasible. We expected that gene changes in the laser-captured neurons might be masked at the protein level. Nonetheless, we used the latter data set to provide hints of important regulatory events.

The JNK pathway is activated in response to environmental stress and largely, the activation leads to apoptosis. However, expression and activity of JNK under basal conditions and by non-noxious environmental stimuli also indicate a physiological role of JNKs in the nervous system (Xu et al., 1997). JNKs appear to be involved in both neuronal regeneration and neuroplasticity, thus the activation of JNK pathways elicits very different types of cellular responses depending on the stimuli ranging from cell proliferation to cell death (Herdegen et al., 1997). In this study, we showed that JNK1 and phospho-JNK1 were significantly decreased by E+P treatment. Similarly, E has been shown to attenuate hepatocellular injury following ischemia-reperfusion injury by significantly downregulating JNK activity (Vilatoba et al., 2005).

JNK1 can activate either the caspase-dependent pathway or the caspase-independent pathway although this is somewhat of a misnomer since caspase 3 can be recruited in the so-called independent pathway. In either case, mitochondria pores are critical. Depending on the stress, mitochondrial permeability can lead to the release of cytochrome c, which in turn activates the caspase dependent pathway. Alternatively, mitochondrial permeability can result in the release of Smac/Diablo or AIF. Diablo can inhibit IAPs, but AIF translocates to the nucleus and precipitates chromatin condensation. This process is called the caspase independent pathway although AIF can recruit caspases. Co-occurrence of both mechanisms is common in apoptosis, but the caspase independent pathway can lead to apoptosis even when the caspases are blocked (Krantic et al., 2007).

Mitochondrial Bcl-2 family members play an important role in mitochondrial permeability. Anti-apoptotic members maintain pore integrity and prevent the release of cytochrome c, which leads to caspase activation. Pro-apoptotic members bind anti-apoptotic members and thereby open pores through which cytochrome c is released. Several studies have suggested that E effects the expression of the Bcl family members (Garcia-Segura et al., 1998, Dubal et al., 1999, Zhang et al., 2003, Koski et al., 2004, Yao et al., 2007). These studies involve gross insults, such as glutamate toxicity, TNF α or β -Amyloid peptide-induced apoptosis, and ischemic brain injury, which elevate pro-apoptotic Bcl-2 family members. E treatment decreased pro-apoptotic Bcl-2 family members and increased anti-apoptotic Bcl-2 family members and ameliorated the damage. However, in our non-insult model, there was no difference in anti-apoptotic Bcl-2 protein with treatment, which is consistent with the lack of difference observed at the level of gene expression (Bethea and Reddy, 2008). Anti-apoptotic Mcl-1 gene expression was previously found to increase with HT and it exhibits a similar, though nonsignificant, trend at the protein level. However, pro-apoptotic Bak and Bax were unchanged with HT at both gene and protein levels. Thus, there was little or no effect of HT on these members of the Bcl family.

Cytochrome c is a peripheral protein of the mitochondrial inner membrane and functions as an electron shuttle between complex III and complex IV of the respiratory chain and its activity is necessary for life. Upon apoptotic stimuli, cytochrome c is released from mitochondria into the cytoplasm where it oligomerizes with Apaf-1 forming a complex known as apoptosome. Each apoptosome then recruits caspase 9, which leads to activation by self-proteolysis. Recent data suggests that cytochrome c release does not act in an all-or nothing manner, but rather acts in a biphasic fashion (Marsden et al., 2002, Scorrano et al., 2002, Garrido et al., 2006). Although cytochrome c plays a pivotal role in glutamate-induced neuron death, which is ameliorated by pretreatment with estrogens (Zhang and Bhavnani, 2005), we observed no change in cytochrome c with hormone treatment in the DRN region.

Active caspase 9 cleaves the effector caspases (caspases 3, 7, and 10), and effector caspases then cleave and activate many substrates that commit the cell to death (Wang, 2001). Caspase 3 is considered the central and final apoptotic effector caspase responsible for much of

biological apoptosis. There are several studies showing that E prevents the up-regulation of caspase 3 induced by glutamate, H₂O₂, or β -Amyloid apoptosis (Zhang et al., 2003, Soustiel et al., 2005, Lu et al., 2007, Park et al., 2007). We did not observe any regulation of the caspases at the gene level in the DRN block (Bethea and Reddy, 2008). Because this could be inconsistent with neuroprotection it was important to examine this at the protein level. Unlike the microarray suggestion, pro-caspase 3 protein was decreased with HT in the DRN block; however, there was no active caspase-3 expression in any of the treatment groups. The discrepancy between gene and protein expression in the DRN block suggests that mechanisms involving procaspase degradation may be in effect. However, future study of procaspase in serotonin neurons by immunohistochemistry is needed. In addition, caspase cleavage can be regulated independently of the apoptosome.

The IAP proteins are potent inhibitors of caspase cleavage and the most significant IAP is XIAP. Gene expression of XIAP was increased by HT in the DRN block (Bethea and Reddy, 2008), which suggested that this could be mediating the neuroprotective effect of ovarian steroids. However, there was no effect of treatment on XIAP at the protein level. Altogether, these results did not provide compelling evidence for an action of HT on the caspase dependent pathway. In addition, the data reinforce the importance of determining whether changes in gene expression are truly manifested at the protein level.

Although caspase activation is considered a hallmark of apoptotic cell death, other pathways have been described that do not require caspase activation. In addition to cytochrome c release, mitochondria can also release factors involved in caspase-independent cell death such as AIF. AIF is believed to play a central role in the regulation of caspase-independent cell death (Krantic et al., 2007). Upon apoptotic stimuli, AIF is released from the mitochondria and translocates to the nucleus where it triggers chromatin condensation and large DNA fragmentation. Under nonstressed conditions, AIF is essential for mitochondrial health and function. In healthy cells, AIF is retained in the mitochondria where it is believed to have an oxidoreductase function (Miramar et al., 2001). However, in response to apoptotic stimuli, AIF becomes an active executioner of the cell. AIF nuclear translocation is thought to be a commitment point to neuronal cell death, which occurs prior to caspase activation and thus may account for the limited effects of caspase inhibitors on AIF. We previously showed that AIF gene expression was significantly decreased by HT in laser captured serotonin neurons (Bethea and Reddy, 2008) and there was a similar trend in the DRN block (this study). At the protein level, AIF exhibited a downward trend in the mitochondria and significantly less AIF was translocated to the nucleus with hormone treatment. The lower expression of AIF in the mitochondria and nuclei suggest that total AIF may be reduced by hormone treatment in a manner similar to the mRNA. Thus, under normal ovarian conditions, E and P act to maintain mitochondrial integrity, which retains AIF in the mitochondrial membrane, and HT may decrease total AIF expression.

In general, when an effect of HT was observed, the effect of E+P appeared more robust than the effect of E alone. This suggests that P plays an important role, which needs further clarification. Currently, we are comparing microarray gene expression between the E and E+P treated groups to discover potentially unique actions of P.

Although the serotonin neural population is prevalent in the DRN block that was used for this protein analysis, it remained a possibility that there could be changes in the caspase cascade within serotonin neurons that are masked by the presence of other cells and glia. It was also possible that the changes in JNK1 and AIF occurred in most or all of the cells in the DRN block and not only in serotonin neurons. Indeed, JNK1 was immunostained in neurons both within the DRN and elsewhere (data not shown). However, AIF was immunostained only in large, serotonin-like neurons of the DRN and MRN. Our DRN block excluded the MRN, suggesting

that the observed changes in AIF on the western blots were contributed by the large neurons of the DRN. Moreover, we previously found that HT significantly decreased AIF mRNA in laser captured serotonin neurons (Reddy and Bethea, 2008). Double immunocytochemistry with fluorescent secondary antibodies is anticipated to phenotypically define the neurons. However, as technology advances, the ability to perform a proteomic analysis of laser captured serotonin neurons may be the next step. Nonetheless, the data highlight an important difference in the mechanism of action of ovarian hormones on neuroprotection in the presence or absence of gross injury. Moreover, these data suggest that ovarian steroids may protect serotonin neurons from signals that arise from normal life stresses such as cytokines from illness, glutamate excitotoxicity or psychological stress and trauma.

Acknowledgements

We are deeply grateful to the dedicated staff of the Division of Animal Resources including the staff of the Departments of Surgery and Pathology for their expertise and helpfulness in all aspects of monkey management.

Supported by NIH grants: MH62677 to CLB, U54 contraceptive Center Grant HD 18185, and RR00163 for the operation of ONPRC

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Figure 1. Subcellular fractionation of the DRN block. Nuclear, cytoplasmic and mitochondria fractionation was obtained by Ficoll/sucrose gradient. Immunoblotting with antibodies against Lamin A/C (top panel), Actin (middle panel) COX I (bottom panel) confirms the integrity of nuclear, cytosolic and mitochondrial fractions, respectively.

Figure 2.

HT decreases JNK1 expression and activity. (A) Cytoplasmic fraction from representative Ovx control, E and E+P-treated animals were immunoblotted for JNK1 and phospho-JNK1. JNK1 and phospho-JNK1 expression were reduced in E+P-treated animals. Actin was used as a loading control. (B) Histograms illustrating the optical density of JNK1 and phospho-JNK1 as the percentage of Ovx control (n=4/group). There was a significant difference between the groups for both JNK1 and phospho-JNK1 (ANOVA $p < 0.05$). JNK1 was significantly decreased in E treated animals and in E+P-treated animals, phospho-JNK1 was significantly reduced (posthoc comparison $p < 0.05$).

Figure 3.

HT does not alter the expression of Bcl-2 family members. (A) Mitochondrial fractions from representative Ovx control, E and E+P treated animals were immunoblotted for Bcl-2, Mcl-1, Bak and Bax. COX I was used as loading control. (B) Cytoplasmic fractions from representative animals of each treatment group were immunoblotted for Bax. Actin was used as loading control. (C) Histograms illustrating the optical density of Bcl-2 family members (Bcl-2, Mcl-1, Bak, and Bax) as the percentage of Ovx control (n=4/group). There was no significant difference between the groups for any of the Bcl-2 family members in either the mitochondrial or cytoplasmic fractions (ANOVA $p > 0.1$).

Figure 4.

HT did not affect apoptosome components. (A) Immunoblots showing cytochrome c in mitochondria and cytoplasm from representative Ovx control, E and E+P-treated animals (B) Histograms illustrating the optical density of cytochrome c as the percentage of Ovx control in cytoplasm and mitochondria (n=4/group). There was no significant difference between the groups for either cytoplasmic or mitochondrial cytochrome c expression (ANOVA $p > 0.1$). (C) Immunoblots showing Apaf1 expression in representative Ovx control, E and E+P treated animals. (D) Histogram illustrating the optical density of Apaf1 as the percentage of Ovx control (n=4/group). There was no significant difference between the groups for Apaf1 (ANOVA $p > 0.1$).

Figure 5.

Effect of HT on effector caspase-3 and caspase inhibitor. (A) Immunoblot examining procaspase 3 and cleaved caspase 3 in representative OvX control, E and E+P treated animals. (B) Histograms illustrating the optical density of pro-caspase 3 as the percentage of OvX control (n=4/group). There was a significant difference between the groups for pro-caspase 3 (ANOVA $p < 0.05$). Pro-caspase 3 was decreased in the E+P-treated animals (posthoc comparison $p < 0.05$). (C) XIAP expression examined by immunoblot in representative OvX, E and E+P treated animals. Actin was used as loading control. (D) Histograms illustrating the optical density of XIAP as the percentage of OvX control (n=4/group). There was no difference in XIAP with hormone treatment.

Figure 6.

Effect of HT on caspase-independent protein AIF expression and nuclear translocation from the mitochondria. (A) Histogram illustrating the relative expression of AIF in the DRN block as determined with qRT-PCR ($n=3/\text{group}$). Although the difference was not statistically significant, there was a downward trend in AIF gene expression in E and E+P-treated animals. (B) Immunoblot examining AIF expression in the mitochondria (mAIF) and in the nucleus (nAIF) of representative Ov control, E and E+P treated animals. There appears to be more AIF in the mitochondrial fraction and less AIF in the nuclear fraction with E+P treatment. (C) Histograms illustrating the optical density of mitochondrial AIF and nuclear AIF as the percentage of Ov control ($n=4/\text{group}$). There was a significant difference between the groups

for nuclear AIF (ANOVA $p < 0.05$). Nuclear translocation of AIF from mitochondria was significantly decreased in E+P-treated animals (posthoc comparison $p < 0.05$).

Figure 7.

Photomicrographs of AIF immunostaining in the midbrain region containing the dorsal and median raphe nuclei. AIF immunostaining was detected only in the large serotonin-like neurons of the raphe nuclei and not in other areas. (A) Low power photomicrograph of the AIF positive neurons in the dorsal raphe nucleus. (B) High power photomicrograph of the AIF positive neurons in the median raphe region, which was not part of the DRN block used for protein analysis. (C) High power photomicrograph of the AIF positive neurons in the dorsal raphe nucleus.