The neurotoxic effects of estrogen on ischemic stroke in older female rats is associated with age-dependent loss of IGF-1

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Abstract

Hormone therapy to postmenopausal females increases the risk and severity of ischemic stroke. Our previous work using an animal model of menopause (reproductive senescence) shows that middle cerebral artery occlusion (MCAo) causes a larger cortical-striatal infarct in this older acyclic group as compared to younger females. Moreover, while estrogen treatment is neuroprotective in younger females, estrogen paradoxically increases infarct volume in acyclic females. We hypothesized that the neurotoxic effects of estrogen in older females occurs due to decreased availability of IGF-1, a neuroprotectant which decreases with advancing age and is down-regulated by estrogen treatment. Our data shows that plasma IGF-1 levels are significantly reduced in reproductive senescent females and further reduced by estrogen at all ages. Estrogen's neuroprotective effect on MCAo-induced cortical infarct volume in mature adult female is reversed by icv injections of IGF-1 receptor antagonist JB-1. Similarly, estrogens neurotoxic effects on cortical infarct volume in senescent females is attenuated by concurrent IGF-1 treatment, and reversed when IGF-1 is infused 4h after the onset of ischemia (delayed IGF-1 treatment). Delayed IGF-1/estrogen treatment also suppressed ischemia-induced ERK1 phosphorylation, reduced protein oxidation and stimulated an early increase in prostaglandin (PG) E2 at the infarct site. IGF-1 treatment was only protective in senescent females that received estrogen, indicating that the neuroprotective actions of this peptide require interaction with the steroid hormone receptor. These data support the hypothesis that stroke severity in older females is associated with decreased IGF-1 and further indicate that short term post-ischemic IGF-1 therapy may be beneficial for stroke.

Keywords

MCA occlusion; IGF-1R; estrogen receptor; MAP kinases; oxidative stress

Introduction

Stroke is the third major cause of death and the leading cause of long-term disability in the U.S. Although men are at greater risk for stroke, older women have a higher risk for stroke than men (Towfighi et al., 2007), more fatal strokes, greater post-stroke disability and mental impairment (Bushnell, 2008). Declining ovarian steroid hormones are thought to contribute to the increased stroke-associated disability and fatality in this group (Roquer et al., 2003; Niewada et al., 2005; Hochner-Celnikier et al., 2005). However, hormone therapy to postmenopausal women significantly increases the risk for stroke (Shumaker et al., 2004; Wassertheil-Smoller et al., 2003), while, paradoxically, low dose oral estrogen-based contraceptives does not significantly increase stroke risk in young women (Petitti et al.,...
1996; Siritho et al., 2003). These clinical studies suggest that age and the hormonal milieu interact to modify cerebrovascular disease, however most animal studies have not recapitulated this age-dependent estrogen effect. In ovariectomized rats, estrogen treatment has been shown to be neuroprotective in some cases, (Dubal et al., 1998; Rusa et al., 1999; Simpkins et al., 1997), and neurotoxic in others (Gordon et al., 2005; Carswell et al., 2004). Meta analyses (Macrae and Carswell, 2006; Strom et al., 2009) suggest that the overall effect of estrogen may depend on the type of ischemia (permanent vs transient), dose and timing of estrogen. Through our recent studies using the endothelin-1 model of MCA occlusion, we uncovered a novel age-dimorphic effect of estrogen that better mimics the clinical data. In this stroke model, estrogen reduced infarct volume in young ovariectomized females, but paradoxically increased infarct volume in acyclic (reproductive senescent) females (Selvamani and Sohrabji 2008). Thus, this system offers a unique opportunity to decipher the underlying mechanism of estrogen’s age-dimorphic effects in stroke.

We hypothesized that estrogen’s dimorphic effect on young and senescent females is related to the bioavailability of a neuroprotective intermediary product that is down-regulated both by advancing age and by estrogen. In view of its actions as a neuroprotectant in stroke models (Schabitz et al., 2001; Lin et al., 2009), insulin-like growth factor (IGF)-1 is an ideal candidate. Low normal levels of IGF-1 are associated with increased morbidity and mortality in ischemic heart disease and stroke (Schwab et al., 1997; Laughlin et al., 2004; Johnsen et al., 2005). Plasma IGF-1 levels decrease with advancing age (Walters et al., 2003; Denti et al., 2004) and are further down-regulated by estrogen treatment (Friend et al., 1996; Ho et al., 1996). Finally, IGF-1 and estrogen have been shown to act synergistically in experimental models of neurodegenerative diseases (Garcia-Segura et al., 2001).

The present studies therefore tested the hypothesis that circulating IGF-1 levels are reduced due to age in reproductive senescent females and further reduced by estrogen treatment. Additionally, these studies determined whether altering IGF-1 availability to reproductive senescent females would compensate or reverse the deleterious effect of estrogen. Our data indicates that estrogen and IGF-1 interaction is critical for neuroprotection in the ischemic cortex of reproductive senescent females.

**Methods**

**Animals**

All animals were purchased from Harlan Laboratories (IN), either as young adult virgin females (nulliparous, 3-4 months, 250gms, n=12) or as proven breeders with either four pregnancies (5-6 months, 230–320 g, n =24) or as retired breeder (9-11 months, 280–350 g, n = 72). This latter group typically arrive acyclic (usually constant estrus). Daily vaginal smears were performed for 3 weeks on all females to confirm estrus cycling, and retired breeders were assigned to the study as reproductive senescent females when characterized as constant diestrus (undetectable levels of estrogen) for at least 2 weeks. All females were then subject to the ovariectomy surgery and replaced with either a control or 17β-estradiol containing pellet. Therefore, reproductive senescent females are in an estrogen deficient stage for 4+ weeks, and an estrogen depleted state for 5 weeks (2+ weeks of constant diestrus, 3 weeks of ovariectomy). All animals were maintained in a constant 12-h dark:12-h light cycle. Food and water were available ad libitum.

**Estrous cycle determination**

Vaginal smears were obtained daily between 10:00 and 11:00 a.m. Smears, obtained with a cotton swab, were placed on a slide and later examined under a microscope (Olympus, Leeds Instruments, TX; 20×objective) and staged according to commonly accepted criteria. Smears
were obtained over a 14–20 day period and rats that persisted in any one stage for 7 days were considered acyclic. The estrous cycle has four stages: proestrus (round nucleated epithelial cells), estrus (enucleated cornified cells), metestrus (proportional numbers of leukocytes and cornified cells) and diestrus (few cells, predominantly leukocytes, with the presence of thick mucus). Reproductive senescent animals were included in the study if they were in constant diestrus for a period of 2 weeks. Mature adult females had a mean estrus cycle length of 8 days.

**Surgery**

All animals were ovariectomized and replaced with a control pellet or 17β-estradiol containing pellet, using our previously established procedures (Jezierski and Sohrabji, 2001). Ovaries were removed and 17β-estradiol pellets (1.0 mg) 60-d time-release or control pellets (Innovative Research, Sarasota, FL) were placed subcutaneously before closing the incision. These pellets are designed to maintain a plasma hormone level of 60-80 pg/ml (Jezierski and Sohrabji, 2001; Nordell et al., 2003). Previous studies have shown that a stable level of plasma estradiol is maintained at 3, 4 and 6 weeks, although a recent study reports a high supraphysiologic burst of hormone levels soon after pellet implantation (Singh et al., 2008). Control pellets do not contain hormone. At sacrifice, the uterus was removed, cleaned of fat and weighed to determine the effectiveness of estrogen replacement.

**Stereotaxic surgery**—Three weeks after ovariectomy, the animals were subjected to stereotaxic surgery to occlude the left middle cerebral artery (MCAo) as reported in Selvamani and Sohrabji (2008). MCA occlusion was induced by microinjecting 3 μl of Endothelin-1 (American Peptide Company INC, CA; 0.5μl in 1 μl PBS). Complete details of this procedure are described in Selvamani and Sohrabji (2008). This experimental stroke model results in a prolonged ischemia and gradual reperfusion, which lasts approximately 7h in the striatum and 16h in the cortex (Biernaski et al., 2001). Optical imaging studies using a small fluorescent probe (Cy5.5, 1kD mw) indicated that ET-1 induced vasoconstriction is clearly visible 5h post ET-1, while reperfusion is apparent at 10h in the midline (striatum) and is fully re-established when imaged at 21h post ET-1 (Supplementary Fig I).

**ICV infusions**—1) Concurrent IGF-1 or JB-1 administration: Immediately after the ET-1 injection, a 28-gauge guide cannula (Alzet Brain Infusion kit II; DURECT Co., Cupertino, CA) was implanted in the lateral ventricle: ICV coordinates: -1.0 mm anterior posterior, +1.4 mm medial lateral, -3.5 mm relative to the dural surface (Paxinos et al., 1985) and cemented in place with loctite 454 (Durect Co., Cupertino, CA). For icv infusions, Alzet osmotic minipumps (model 1007D; 0.5 μl/hr) with a reservoir volume of 96 ± 2 μl was filled with vehicle (artificial cerebral spinal fluid; aCSF), icv IGF-1 (100 μg/ml; total range 9.4 –9.8 μg, Bachem, San Carlos, CA) or JB-1 (20 μg/ml; total range 1.88 –1.96 μg, Bachem, CA) and primed before implantation as recommended by the manufacturer. Osmotic minipumps were implanted subcutaneously over the latissimus dorsi muscles and connected via catheters to the infusion cannula in the lateral ventricle. Doses used for icv administration of IGF-1 were similar to those reported in Quesada and Miceyvich (2004). 2) Delayed IGF-1 administration – IGF-1 was administered four hours after the ET-1 injection.

During surgery the rats were maintained at 37 °C throughout and oxygen saturation and respiratory rate were constantly monitored using the Mouse Oximeter (STARR life sciences corp. PA). Acceptable parameters for oxygen saturation and respiratory rate were above 85% and 50 brpm, respectively (see Supplementary Table I for mean ± SD for each treatment group). At termination, the brain was rapidly removed and processed for TTC staining and biochemical analysis. The uterus was also removed, cleaned and weighed.
Infarct volume

Brain slices (2 mm thick) between -2.00 mm and +4.00 mm from Bregma were incubated in a 2% TTC solution at 37 °C for 20 min and later photographed using a Nikon E950 digital camera attached to a dissecting microscope. Infarct volume was determined from digitized images using the Quantity One software package (Bio-Rad CA). Typically 3 such slices were used for analysis. The area of the cortical and striatal infarct was measured separately in all slices as well as the contralateral hemispheres. Details of the volume determination are described in Selvamani and Sohrabji (2008). The volume of the infarct was normalized to the volume of the contralateral (non-occluded) hemisphere. To ensure reliable and consistent detection of the infarct zone, images were digitally converted to black and white and magnified, and all traces were performed by the same author (AS).

Vibrissae-elicited forelimb placement test

Stroke injury was assessed by the forelimb placing test which was performed both before and after the MCAo as described in Woodlee et al. (2005) and Selvamani and Sohrabji (2008). Animals were subjected to same-side placing trials and cross-midline placing trials elicited by the ipsi- and contra-lesional vibrissae. Detailed description of same-side and cross-midline placing tests, pre- and post-test sessions and scoring during each test session are reported in Selvamani and Sohrabji (2008). Vibrissae-evoked forelimb placement revealed a significant loss of right paw placement in all animals post-stroke, indicative of left-sided cortical-striatal infarction (Supplementary Figs II-IV).

Cytokine and prostanoid expression

Expression levels of a panel of inflammatory cytokine/chemokine were quantified using a rat cytokine/chemokine panel (Milliplex™ MAP kit, Millipore corp., Billerica, MA). The Bio-Plex Suspension System was calibrated using CAL2 (High PMT, Bio-Plex Calibration Kit) and standard/sample preparation was performed according to manufacturer's directions. Filter plate was pre-wet with assay buffer and vacuum filtered before adding standard, control or samples to the appropriate wells. Premixed beads were then added to each well and incubated overnight at 4°C with shaking. Following 2 washes, 25 μl of detection antibody was added to each well and incubated for 2 hrs at room temperature and then treated with streptavidin-phycoerythrin (25 μl) to each well for 30 min at room temperature. The plate was then washed twice with wash buffer and the beads were resuspended in 150 μl BioReady assay buffer and read on the BioPlex Suspension System. Cytokines levels were normalized to total protein values.

PGE$_2$ levels were measured using a commercial enzyme immunoassay kit (Cayman, Ann Arbor, MI) as per manufacturer's instruction. Samples, standards, controls, prostaglandin E$_2$ AChE tracer and prostaglandin E$_2$ monoclonal antibody were added to appropriate wells in a 96 well plate as stated in the kit protocol and incubated at 4°C overnight. After the wash Ellman's reagent was added to each well and incubated for 2 hrs at room temperature and then treated with streptavidin-phycoerythrin (25 μl) to each well for 30 min at room temperature. The plate was then washed twice with wash buffer and the beads were resuspended in 150 μl BioReady assay buffer and read on the BioPlex Suspension System. Cytokines levels were normalized to total protein values.

Protein extraction and Western blot analysis—Cell proteins from cortical tissue (core and penumbra) and striatal tissue from the left and right hemispheres were harvested in lysis buffer [50 mm Tris (pH 7.4), 150 mm NaCl, 10% glycerol, 1 mM EGTA, 1 mM Na-orthovanadate (pH 10), 5 μM ZnCl$_2$, 100 mM NaF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride in dimethylsulfoxide, 1% Triton X-100] and centrifuged at 20,000 rpm for 30 min. Supernatant was collected and stored at -20°C until further analysis. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Samples were size fractionated on 10% PAGE and transferred to Hybond-C membranes.

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Blots were blocked with 1× Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk. Subsequently, blots were probed for either ERα or ERK. Blots were incubated with primary (1:200, ERα (MC20); Santa Cruz, CA; p-ERK, 1:200, Santa Cruz) and secondary antibodies (1:2000, goat anti-rabbit; Santa Cruz, CA; Goat anti-mouse 1:2000, BD Pharmagen) subjected to three washes in 1× Tween Tris-buffered saline buffer between the two incubations. The immunosignal was detected by x-ray film (Bio-Rad) using chemiluminescence reagents (Renaissance; NEN Life Science Products, Boston, MA). Blots were stripped and reprobed for either tubulin or pan-ERK (1:200; Santa Cruz, CA). Immunosignal was quantified using computer-assisted densitometric analysis (Quantity One; Bio-Rad). ERα immunosignal was normalized to tubulin, while phospho-ERK immunosignal was normalized to pan-ERK.

**Immunoprecipitation**—Cortical protein lysates (300 μg) from left and right hemispheres were incubated with ERα antibody (1:100) overnight on a nutator at 4°C. Samples were then immunoprecipitated with sheep-anti-rabbit IgG Dynabeads® M280 (Invitrogen Dynal AS, Oslo, Norway) for 3 hrs. Following washes the immunoprecipitate was size fractionated on 10% PAGE and transferred to nylon membranes (Millipore, Bedford, MA). Blots were blocked with 1× Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk. Subsequently, blots were incubated with IGF-1R antibodies (1:200, IGF-1R (C-20); Santa Cruz, CA) and secondary antibodies (1:2000, goat anti-rabbit; Santa Cruz, CA), subjected to three washes in 1× Tween Tris-buffered saline buffer between the two incubations. In parallel experiments, cortical lysates were immunoprecipitated with IGF-1R antibody and later resolved on 10% PAGE and probed with the ERα antibody. In each case, immunosignal was detected on x-ray film (Bio-Rad) using chemiluminescence antibody (Renaissance; NEN Life Science Products, Boston, MA).

**Detection of oxidized proteins**

The Oxyblot™ protein oxidation detection kit (Millipore, MA) was used to detect and quantify oxidized proteins in cortical lysates. A total of 10 μl aliquot of protein was denatured using 6% SDS and derivatized with 1× DNPH at room temperature for 15 min. Following which the samples were treated to a neutralization solution and reduced with 2-mercaptoethanol. Proteins were separated by 10% SDS-PAGE and transferred to a Hybond-C membrane (Amersham Pharmacia Biotech, NJ). Blocking and dilution buffers for primary and secondary antibodies were prepared per manufacturer's instructions. The DPN-derived protein expression was normalized to α-tubulin (1:2000, Sigma, St. Louis, MO).

**Statistics**

Infarct volume was analyzed by 2-way ANOVA (independent variables: +/- hormone, +/- IGF-1 or +/-JB-1) with planned comparisons. The effect of stroke injury on the forelimb placement test was analyzed by paired t-test (pre-MCAo and post-MCAo). A 3-way analysis of variance was used for ERK activation, ERα expression, cytokines and protein oxidation, with “hemisphere” as the repeated measure, and +/- hormone, +/- IGF-1 as independent variables. Statistical analysis was performed using the software package SPSS® 13.0.

**Results**

**Estrogen treatment**

Hormone replacement was confirmed by uterine weights which is a cumulative indicator of estrogen stimulation. Uterine weights were similar in ovariectomized young adult (3-4 months old) (0.15 g ± 0.024), mature adult (5-7 months old) (0.17 g ± 0.016) and reproductive senescent (9-11 months old) (0.18 g ± 0.03) females. Three weeks of estrogen treatment increased uterine weights in all groups (young adult: 0.49 g ± 0.09, mature adult: 0.51 g ± 0.086, reproductive
senescent: 0.52 g ± 0.098). There were no differences in uterine weights in ovariectomized young, mature and senescent females. Similarly, estrogen treatment had comparable effects at all ages.

**Local and circulating levels of IGF-1**—Plasma samples were obtained from ovariectomized young adult (3-4 months), mature adults (5-7 months) and reproductive senescent females (9-11 months) that were ovariectomized and replaced with an estrogen or placebo pellet. As shown in Fig 1A, IGF-1 was detected in plasma from all reproductive ages, however there was a statistically significant decline in the level of this peptide with reproductive age (F(2,21): 24.83, p<0.05). Furthermore, IGF-1 levels were also significantly suppressed in estrogen-treated animals (F(1,21): 5.26, p<0.05), at each age group. Specifically, estrogen reduced IGF-1 levels by 22% in young adults, 14% in mature adults and 30% in reproductive senescent females. Consequently, reproductive senescent females treated with estrogen had the lowest circulating levels of IGF-1. Post-hoc analysis of the treatment groups indicates that IGF-1 levels in the estrogen-treated senescent females are significantly lower than those in estrogen-treated young adults and estrogen-treated mature adults (p<0.05, in both cases). Local IGF-1 levels were measured from the micropunches of cortical tissue obtained from the cortex of mature and senescent females. Similar to plasma levels, cortical IGF-1 levels were ~40% lower in reproductive senescent females as compared to mature adult females (Fig 1B). Thus local and circulating levels of IGF-1 were suppressed in older acyclic females.

**Effect of inhibition of IGF-1 signaling on ischemia infarct volume in mature adult females**—Adult multiparous females with normal estrus cycles (see Methods) were ovariectomized and replaced with estradiol or a placebo pellet. Three weeks later animals were subject to MCA ischemic stroke induced by endothelin-1 and immediately infused with the IGF-1 receptor antagonist JB-1 directed to the lateral ventricles (ICV). Seven days later all animals were terminated and their brains removed and assessed for cell death by TTC staining (Fig 2A). As shown in Fig 2B, cortical infarct volume was modified by a complex interaction of estrogen and the IGF-1 inhibitor, JB-1 (F(1,14): 5.794, p<0.05, interaction effect). Similar to previous data, estrogen treatment to ovariectomized mature adult females reduced (39%) the volume of the cortical infarct. However, JB-1 treatment completely attenuated the neuroprotective effects of estrogen in this group, so that infarct volumes in this group were similar to non-estrogen treated controls. Notably, JB-1 did not affect cortical infarct volume in animals that were not treated with estrogen (Fig 2). Furthermore, JB-1 had no effect on the striatal infarct (Fig 2; overall ANOVA F(3,14): 0.751; p>0.05).

**Effect of IGF-1 replacement on ischemic infarct volume in reproductive senescent females**—Reproductive senescent females (multiparous, acyclic females that were in constant diestrus) were ovariectomized and replaced with estrogen or control pellets and subject to ET-1 induced MCAo. IGF-1 was infused ICV either concurrent with the MCAo or delayed 4h after MCAo.

**Concurrent IGF-1 treatment**—Concurrent IGF-1 treatment had a significant effect on cortical infarct size (F(1,14): 6.028533, p<0.05). As reported previously, cortical infarct volume was significantly worse in estrogen-treated animals as compared to untreated ovariectomized animals (Fig 3). Planned statistical comparisons indicate that IGF-1 infusion to estrogen-treated females reduced cortical infarct volume as compared to the group treated with estrogen alone (p<0.05) but was equivalent to the baseline group (no estrogen, no IGF-1) (p>0.05). IGF-1 had no further effect on infarct volume in groups deprived of estrogen treatment. Neither IGF-1 nor estrogen treatment affected the striatal infarct (Fig 3B; overall ANOVA F(3,14): 1.72; p>0.05).
Delayed IGF-1 treatment—The next study tested the efficacy of delayed IGF-1 treatment on infarct volume. Reproductive senescent animals were prepared as before and infused with IGF-1, 4h after the MCAo was initiated. Delayed IGF-1 treatment had a remarkable neuroprotective effect on cortical infarct volume (F(3,18): 10.125; p<0.05) and a significant interaction effect of estrogen and IGF-1 (F(3,18): 13.09; p<0.05). Thus while IGF-1 had an overall effect on lowering infarct volume this effect was mainly driven by the interaction with estrogen and IGF-1. While estrogen replacement alone to senescent females increased infarct volume as compared to untreated controls, IGF-1 treatment to this group not only reversed estrogen’s toxic effects but also reduced cortical infarct volume as compared to baseline (untreated controls). As before, IGF-1 did not affect the cortical infarct volume in the untreated ovariectomized group of animals. IGF-1 and estrogen also did not affect striatal infarct volume (Fig 4; overall ANOVA F(3,18): 0.696; p>0.05).

In view of the neuroprotective effects of combined estrogen/delayed IGF-1 treatment to senescent females at 7d post-stroke, the next series of experiments determined the early biochemical effects of combined estrogen/delayed IGF-1 treatment on ischemic tissue (24h post MCAo). In this study, local cortical and striatal levels of IGF-1 were first measured to determine whether IGF-1 infusion increased local levels of the peptide. Cortical levels of IGF-1 were significantly elevated by IGF-1 infusion (1.6 fold, p<0.05) (Fig 5A), however ICV infusion of IGF-1 did not increase striatal IGF-1 levels. Whether this is due to the location of the cannula (which favored cortical rather than striatal distribution of the peptide) or due to poor sequestration of the peptide in the striatum, these data are consistent with the absence of IGF-1-dependent changes in striatal infarct volume seen in the above studies.

Overall, IGF-1 levels were approximately 50% lower in the striatum as compared to the cortex. As shown in Fig 5B, cortical IGF-1 levels were increased by 60% in the ischemic hemisphere (F(1,6): 31.46, p<0.05, repeated measure) as compared to the non-ischemic side. Estrogen treatment significantly decreased IGF-1 levels in the ischemic and non-ischemic cortex (F(1,6): 10.09, p<0.05). Striatal IGF-1 levels were also higher in the ischemic hemisphere (27%) although this was not statistically significant (F(1,6): 4.58, p=0.076, repeated measure), and estrogen did not alter IGF-1 levels in this region.

ERK activation in the ischemic site—ERK signaling is associated with oxidative stress and cell death (Murray et al., 1998, Poddar and Paul, 2009) and suppression of ischemia-induced ERK activation is associated with reduced infarct volumes (Wakade et al., 2008). Cortical tissue from reproductive senescent females subject to MCAo was harvested at 24h and size-fractionated by SDS/PAGE and probed for phospho-ERK and pan (total)-ERK. As expected, ERK-1 activation was enhanced 2-4 fold in the ischemic cortex as compared to the non-ischemic side. Estrogen treatment significantly decreased IGF-1 levels in the ischemic and non-ischemic cortex (F(1,6): 10.09, p<0.05). Striatal IGF-1 levels were also higher in the ischemic hemisphere (27%) although this was not statistically significant (F(1,6): 4.58, p=0.076, repeated measure), and estrogen did not alter IGF-1 levels in this region.

Cytokine expression in the ischemic site—Ischemia is associated with neuroinflammation resulting from activation of local microglia and astrocytes as well as infiltration of leukocytes due to breakdown of the blood brain barrier. In the next analysis, cortical lysates from the ischemic and non-ischemic hemisphere from the “24h delayed IGF-1” study were also assayed by multiplex ELISA for a panel of cytokines. A large number of cytokines are elevated in the ischemic cortex as compared to the contralateral hemisphere (Supplementary data Table II; main effect of “hemisphere”, indicated by upward or downward pointing arrow). Virtually none of these were regulated by estrogen and/or IGF-1 at 24h post ischemia. Furthermore, only one inflammatory mediator was regulated by an interaction of
IGF-1/estrogen, PGE2. PGE2, which is synthesized enzymatically from arachidonic acid, is reportedly elevated following ischemia and neural injury. As shown in Fig 7, at 24h post ischemia, PGE2 was modestly elevated in the ischemic hemisphere (F(1,14): 6.047, main effect of hemisphere, repeated measure, p<0.05). Furthermore, animals that received estrogen+IGF-1 had the highest levels of PGE2 at this early time point (F(1,14): 4.86, interaction effect of estrogen and IGF-1, p<0.05). At 7d post ischemia, PGE2 was elevated in the ischemic hemisphere in all groups to a similar extent (F(1,14): 165.64; main effect of hemisphere, p<0.05), indicating that estrogen and IGF-1 promotes an early elevation of this prostanoid.

Protein oxidation—Oxidation of carbonyl groups is an indicator of oxidative stress on proteins and is correlated with cell death (Sultana et al., 2004; Niebroj-Dobosz et al., 2004). Carbonyl groups on proteins that react with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazone (DNP) were examined by immunoblotting. 2,4-DNP-modified proteins were seen in all groups (Fig 8) but the intensity of the immunosignal (normalized to tubulin) was significantly reduced in the groups exposed to IGF-1 (F(1,11): 15.49, p<0.05), indicative of lower protein oxidation in these groups.

Immunoprecipitation of estrogen and IGF receptors—Since the previous studies indicate that estrogen and IGF-1 interact to regulate crucial measures such as infarct volume, ERK-1 phosphorylation and PGE2 expression in the ischemic cortex, the next studies determined estrogen receptor expression in the ischemic cortex and its interaction with IGF-1R, the tyrosine kinase receptor that transduces the actions of IGF-1, ERα, a 68kD protein that has been implicated in the neuroprotective/neuroinflammatory actions of estrogen (Dubal et al., 2001; Vegeto et al., 2003), was examined by Western blot analysis. As shown in Fig 9A, both ischemic and non-ischemic cortices expressed ERα. However, ERα expression was significantly reduced in the ischemic cortex, as compared to the contralateral hemisphere, in groups that received aCSF infusion. In groups infused with IGF-1, ERα levels were no different from the contralateral hemisphere (interaction effect, hemisphere × IGF-1 treatment, F(1,10): 7.02, p<0.05), indicating that the peptide serves to maintain estrogen responsiveness in infarcted tissue.

Immunoprecipitation of ERα/IGF-1R complexes from cortical tissue obtained from ischemic and non-ischemic hemispheres were assessed using both ERα and IGF-1R antibodies as a probe (Fig 9B). ERα precipitated lysates probed for IGF-1R indicated a 78kD band consistent with the IGF-1Rb segment. Similarly, cortical lysates immunoprecipitated with IGF-1R probed for ERα revealed an expected 68kD band, indicating that ERα dimerizes with IGF-1R. Immunoprecipitation of IGF-1R and ERα was seen in all treatment groups, and Fig 9B shows expression of IGF-1R complexed to ERα in the reproductive senescent group that received both estrogen and IGF-1 infusion.

%Discussion

The present data demonstrates, for the first time, that the decline in estrogen’s effectiveness in the aging brain is associated with declining levels of IGF-1. Similar to humans (Walters et al., 2003; Denti et al., 2004), circulating IGF-1 levels decrease with age and is further suppressed in estrogen-replaced rats. In older acyclic females where constitutive IGF-1 levels are low, compared to all other groups, exogenous replacement of IGF-1 attenuates the neurotoxic effects of the steroid hormone. In fact, delaying IGF-1 treatment for a few hours after the onset of ischemia completely reverses estrogen’s neurotoxic effects, resulting in reduced protein oxidation and suppression of pERK-1 at 24h with reduced infarct volume at 7d post-stroke.

While both concurrent and delayed IGF-1 attenuate estrogen’s toxic effects on infarct in older females, delayed IGF-1 is paradoxically more effective. Although not tested in the current
studies, we hypothesize that concurrent IGF-1 delivery attenuates the development of compensatory post-stroke cellular events. Following ischemia, several cell death and cell protective responses are generated in brain tissue, and the eventual size and severity of the infarct reflects the balance between these opposing cellular processes. For example, inflammation is a critical post-ischemic event, and IGF-1 is reported to promote T-cell maturation and delay apoptotic cell death of neutrophils (Himpe et al., 2008; Law et al., 2008), which is consistent with its growth promoting actions, but may create a less favorable environment for neurons post-ischemia. Thus, early IGF-1 treatment may hinder the development of a full neuroprotective effect.

While the MCAo results in a cortical-striatal infarction, all our treatment effects (estrogen or IGF-1) are restricted to the cortical infarction. Striatal infarct volume is resistant to both estrogen and IGF-1 treatment, during the time frame of this study (7d). This lack of improvement in the striatal infarct is consistent with the lack of improvement noted in the vibrissae-evoked forelimb placement task, since most recovery-of-function tasks involve a motor response. A related possibility is that the time frame of these studies is too short to detect functional recovery from stroke. For example, the “cross-midline” version of this task reportedly recovers in 28d while the “same side” version of this test remains impaired 128d later (Woodless et al., 2005). However, since behavioral improvement is almost always associated with recovery and reduced neuronal loss, the mechanisms underlying cortical neuroprotection described here provide a crucial insight into the aging brain’s response to stroke.

The reproductive senescent female rat mimics salient aspects of the postmenopausal human female, namely decreased reproductive capacity and diminished availability of ovarian hormones (estropause). In this model, estrogen replacement is proinflammatory (Johnson and Sohrabji, 2005) and neurotoxic in stroke and excitotoxic inflammation models (Selvamani and Sohrabji, 2008; Nordell et al., 2003). Currently, the effect of estrogen treatment on ischemic injury in older females is controversial, with some studies indicating a beneficial effect (Alkayed et al., 2000; Dubal and Wise, 2001; Toung et al., 2004) and others a neurotoxic effect (Selvamani and Sohrabji, 2008). These differences may be due to the animal species (rat vs mice), the determination of estrus acyclicity as well as the nature of the ischemic insult itself. The ubiquitously-used suture model, for example, where a nylon suture is used to occlude the MCA, results in a rapid ischemia and a rapid reperfusion, while the vasoconstrictive (endothelin-1) stroke model causes a gradual ischemia and a gradual reperfusion. Hence the rate of infarct development and its pathophysiology may markedly vary between the two models and this difference may crucially predict whether estrogen exerts protective or non-protective actions.

While the fundamental event in menopause is the “built-in obsolesence” (Mason 1976) of the ovary, other endocrine glands are also affected by age and declining stimulation from ovarian hormones. The growth hormone (GH)/Insulin-like growth factor (IGF-1) axis also declines with age (“somatopause”), causing reductions in lean body mass, bone mass and immune function. Aging and estrogen reduces the levels of circulating IGF-1 in young and middle aged females (Bottner and Wuttke, 2006), and while this phenomenon increases longevity (Brown-Borg et al., 2007; Bartke et al., 2003), the age-related decline in IGF-1 may be deleterious for the injured brain. Exogenous IGF-1 reduces ischemic injury in many species (Gluckman et al., 1992; Lee et al., 1993; Johnston et al., 1996; Guan et al., 2001), stimulates stroke-induced neurogenesis (Yan et al., 2006) and promotes neuronal survival, neuronal myelination and angiogenesis (Smith, 2005; Wang et al., 2000). Similarly, estrogen is also known to decrease cell death following ischemia in rats (Simpkins et al., 1997; Dubal et al., 1998; Rusa et al., 1999) mice (Sawada et al., 2000) and gerbils (Jover et al., 2002). Thus, in young adult animals, estrogen and IGF-1 are independently neuroprotective.
Garcia-Segura and colleagues have shown that IGF-1 and estrogen also act cooperatively to prevent neuronal loss in neurodegenerative disease models (Garcia-Segura et al., 2001; Mendez et al., 2005; Torres-Aleman, 2005). Estrogen and IGF-1 receptors are co-localized in neuronal and glial populations (Cardona-Gomez et al., 2000; Garcia-Segura, 2000; Quesada et al., 2007), promote the survival and differentiation of the same groups of neurons and cooperatively modulate pro-survival signaling pathways (Cardona-Gomez et al., 2002). Furthermore, IGF-1-dependent neuronal differentiation and survival require estrogen receptors and estradiol neuroprotection requires ongoing IGF-1 signaling (Garcia-Segura et al., 2006). Intriguingly, inhibiting the IGF-1 receptor with JB-1 attenuates the neuroprotective effects of both estrogen and IGF-1 (Quesada et al., 2004). Our data uncover a unique interactive role for IGF-1 and estrogen in the aging brain, where IGF-1 supplementation is only neuroprotective in animals that are replaced with estrogen. Furthermore, IGF-1 alone promotes specific protective actions, such as reducing protein oxidation, although, in the present model, this does not result in a full neuroprotective effect. IGF-1 stabilizes the ischemia-induced loss of ERα expression, indicating that IGF-1 may provide a substrate to enhance ERα/IGF-1R interactions. If estrogen and IGF-1 synergy is critical for neuroprotection, then the combination of advanced age coupled with estrogen treatment places the postmenopausal female and the reproductive senescent rat at risk for more severe cell loss following stroke.

IGF-1's neuroprotective actions are principally transduced by the receptor tyrosine kinase IGF-1R, while ERα is the estrogen receptor that mediates neuroprotection and neuroinflammation (Dubal et al., 2001; Vegeto et al., 2003). In vivo, estradiol treatment transiently increases the association between brain IGF-1R and ER-α (Mendez et al., 2003), while brain injury, as indicated in this study, prolongs the formation of ER-α/IGF-1R dimers. ERα /IGF-1R dimerization most likely occurs at the plasma membrane which strategically positions these receptors to exploit several signaling molecules. Both estrogen and IGF-1 receptors activate the MAP kinases, a signaling platform responsive to a broad range of stimuli including cell proliferation, apoptosis, cell differentiation and gene regulation. Oxidative stress, caused by ischemia, induces ERK phosphorylation (Namura et al., 2001) while suppression of ERK reduces cell death in vivo and in vitro (Murray et al., 1998; Williams et al., 1998; Noshita et al., 2002; Wang et al., 2003). Tamoxifen, which binds ERα, reduces ischemic infarction by a mechanism involving the suppression of ERK activation (Wakade et al., 2008), although estradiol has been shown to reduce cell death by activating ERK/MAPK (Jover-Mengual et al., 2007). In the present study, IGF-1 infusion to estrogen treated older rats reduces both ERK-1 activation and infarct size.

Although the specific neuroprotective mechanism initiated by estrogen/IGF-1 remains to be elucidated, one possibility is the suppression of stroke-induced neuroinflammation. Of the large number of inflammatory proteins elevated by ischemia, only PGE2 was specifically elevated by the combination of estrogen and IGF-1. PGE2 belongs to a family of lipid-signaling molecules implicated in neuronal injury and inflammation, although recent evidence indicates that PGE2 promotes neuronal survival when bound to specific receptor subtypes. Specifically, the EP2 receptor promotes cell survival in permanent ischemia (Liu et al., 2005) and ischemia/reperfusion (McCullough et al., 2004) models. Neuronal EP2 expression is rapidly reduced in the ischemic core but is retained in peri-infarct neurons and induced in endothelial cells, where it remains elevated even 24h later (Li et al., 2008). If this early increase in EP2 receptors peri-infarct neurons and endothelial cells is a general feature of ischemic injury, then the rapid elevation of PGE2 in the IGF-1/estrogen-treated animals seen in this study may be critical for promoting cell survival.

The present studies emphasize the convergence of multiple endocrine changes in the aging animal and their impact on the pathophysiology of stroke and ischemic injury. A recent study by Liu et al. (2009) clearly demonstrates that age is a crucial factor in the severity of stroke,
and the present study points to a substrate (IGF-1) that may underlie this age-related susceptibility to increased tissue damage. The present study also offers the exciting possibility of a therapeutic intervention to reduce the extent of tissue damage following stroke in postmenopausal females, a population both likely to use estrogen therapy and at greater risk for stroke. The fact that IGF-1 treatment can be neuroprotective even when delivered after the onset of ischemia indicates a broad window of therapeutic opportunity for this compound.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.
Circulating and local levels of IGF-1. (A) IGF-1 levels in plasma of young adults, mature adults and reproductive senescent females were determined by ELISA. Both age (a: main effect of age, p<0.05) and estrogen treatment (b: main effect of hormone, p<0.05) significantly reduced IGF-1 levels. Planned comparisons indicate that IGF-1 levels in the reproductive senescent females are significantly lower than those in the young adult and mature adults. (B) IGF-1 levels in somatosensory cortex: Mature adult females had significantly higher levels of IGF-1 as compared to reproductive senescent females. (*: p<0.05). YA: Young adults (3-4 months old); MA: Mature adult females (5-6 months); RS: reproductive senescent females (9-11 months).
Effects of estrogen and concurrent JB-1 treatment on infarct size in mature adult females: MCAo induced by ET-1 injections resulted in cortical and striatal lesions as indicated by TTC-stained sections (A). The volume of the cortical infarct was significantly reduced in the estrogen-treated mature adult females (E) as compared to the control-replaced females (O), while JB-1 treatment reversed this effect. Neither estrogen nor JB-1 affected striatal infarct volume. Histogram depicts mean ± S.E.M. of cortical (B) and striatal (C) infarct volume as a ratio of the contra-lateral cortex and striatum respectively. n = 4-6 in each group, *$p < 0.05$. 

Fig 2.
Fig 3.
Effects of estrogen and concurrent IGF-1 treatment on infarct size in reproductive senescent females: MCAo induced by ET-1 injections resulted in cortical and striatal infarcts, as indicated by the TTC staining (A). The volume of the cortical infarct was significantly greater in the estrogen-treated reproductive senescent females (E) as compared to the control-replaced females (O). ICV infusion of IGF-1 concurrent with the onset of ischemia attenuated infarct size (α: main effect of IGF-1, p<0.05). Planned comparisons indicate that cortical infarct size is reduced in the E+IGF-1 group compared to the E+Veh, but is equivalent to the O+Veh group. Neither estrogen nor IGF-1 had any effect on striatal infarct volume. Histogram depicts mean ±S.E.M. of cortical (B) and striatal (C) infarct volume as a ratio of the contra-lateral cortex and striatum respectively. n = 4-6 in each group.
Fig 4.
Effect of estrogen and delayed IGF-1 treatment on infarct size in reproductive senescent females: (A) TTC stained sections from the brain of animals subject to ischemic stroke by induced by ET-1 injections. Cortical infarct volume was significantly greater in estrogen-treated reproductive senescent females (E) as compared to the control-replaced females (O). ICV infusion of IGF-1 4h after ischemia, (delayed IGF-1) reduced cortical infarct volume (a: main effect of IGF-1, p<0.05). There was a significant interaction effect of E+IGF-1 (c: interaction effect, p<0.05). Planned comparisons indicate that the cortical infarct volume was significantly reduced in the E+IGF-1 group as compared to all other groups including E+Veh and the O+Veh. No effect of estrogen or delayed IGF-1 was seen on striatal infarct volume. (b) Histogram depicts mean ± S.E.M. of cortical (B) and striatal (C) infarct volume as a ratio of the contra-lateral cortex and striatum respectively. n = 4-7 in each group.
Fig 5.
IGF-1 expression in the cortex and striatum of reproductive senescent females: (A) IGF-1 was infused 4h after the onset of ischemia and was measured 24h later. ICV infusions of IGF-1 significantly increased peptide accumulation in the cortex, but not in the striatum. (B) Endogenous levels of IGF-1 levels were increased in the ischemic cortex as compared to the non-ischemic (right) hemisphere ($a$: main effect of hemisphere, repeated measure, $p<0.05$), while estrogen-treated animals had lower levels of IGF-1 ($b$: main effect of estrogen, $p<0.05$). No significant differences were observed in the striatum. Key *: $p<0.05$
Fig 6.
Western blot analysis of phospho- and pan-ERK expression in cortical lysates from the ischemic and non-ischemic hemispheres of reproductive senescent females. While pERK-1 expression (normalized to ERK-1) was elevated in the ischemic hemisphere in most treatment groups, estrogen treatment combined with delayed IGF-1 infusion suppressed ischemia-induced ERK-1 activation ($\alpha$; interaction effect of hemisphere, estrogen, IGF-1). $n = 3-4$ per group.
Fig 7. PGE2 expression in the ischemic and non-ischemic cortex. PGE2 levels were measured by ELISA and normalized to total protein. At 24h post MCAo, PGE2 levels were modestly elevated in the ischemic cortex (a: main effect of hemisphere repeated measure, p<0.05). The levels of PGE2 were highest in the group that received estrogen and IGF-1 (c: interaction effect of E+IGF-1, p<0.05). At 7d post MCAo, PGE2 levels were elevated in the ischemic cortex of all groups (p<0.05, main effect of hemisphere, repeated measure) to a similar extent. n = 4-6 per group.
Fig 8.
Western blot analysis of 2,4-DNP-modified proteins in ischemic cortical lysates from senescent animals treated with estrogen or control pellet and later treated icv with either IGF-1 or vehicle 4h post ischemia. Proteins were derivatized by incubating tissue lysates with 2,4-dinitrophenylhydrazine. Immunosignal for 2,4-DNP proteins normalized to a loading control (tubulin) was significantly decreased in groups that received IGF-1 infusion (a: main effect of IGF-1, p<0.05). The bracket indicates the region in each lane that was quantitated by densitometry. n = 3 per group.
Fig 9.
ERα expression and immunoprecipitation with IGF-1R in the cortex of reproductive senescent females: (A) Western blot assays showing ERα expression in both the ischemic and non-ischemic cortex. In the non-ischemic cortex, ERα expression, normalized to tubulin, was similar in all groups. In the ischemic hemisphere, ERα expression was significantly higher in groups that received IGF-1 (c: interaction effect of hemisphere and IGF-1, p<0.05). (B) ERα/IGF-1R immunoprecipitation; Cortical lysates immunoprecipitated with ERα antibody revealed IGF-1R b segment (78kD) immunosignal (i) and parallel experiments of cortical lysates immunoprecipitated with IGF-1R revealed ERα (68kD) immunosignal (ii). ERα/IGF1R complexes were seen in all treatments, shown here are representative samples from the reproductive senescent animals treated with an estrogen pellet and ICV infusion of IGF-1.