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ISOFLURANE PRECONDITIONING NEUROPROTECTION IN EXPERIMENTAL FOCAL STROKE IS ANDROGEN-DEPENDENT IN MALE MICE

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Abstract

Isoflurane preconditioning neuroprotection in experimental stroke is male-specific. The role of androgens in the ischemic sensitivity of isoflurane preconditioned male brain and whether androgen effects are androgen receptor dependent were assessed. Male C57BL/6 mice were implanted with flutamide (androgen receptor antagonist), or castrated and implanted with testosterone, dihydrotestosterone, flutamide, letrozole (aromatase inhibitor), or vehicle 7–13 days before preconditioning. P450 estrogen aromatase wild-type and knockout mice were also evaluated. All mice were preconditioned for 4 h with 0% (sham preconditioning) or 1% isoflurane (isoflurane preconditioning) and recovered for 24 h. Mice then underwent 2 h of middle cerebral artery occlusion and were evaluated 22 h later for infarct volume. For neurobehavioral outcomes, sham and isoflurane preconditioned castrated male±dihydrotestosterone groups underwent 1 h of middle cerebral artery occlusion followed by 9 days of reperfusion. Isoflurane preconditioning neuroprotection relative to infarct volume outcomes were testosterone and dihydrotestosterone dose-specific and androgen receptor-dependent. Relative to long-term neurobehavioral outcomes, front paw sensorimotor function improved in isoflurane preconditioned mice regardless of androgen status while androgen replacement independently improved sensorimotor function. In contrast, isoflurane preconditioning improved cognitive function in castrates lacking endogenous androgens, but this improvement was absent in androgen replaced mice. Our findings suggest that androgen availability during isoflurane preconditioning may influence infarct volume and neurobehavioral outcomes in male mice following experimental stroke.

Keywords

androgens; testosterone; dihydrotestosterone; androgen receptor; neuroprotection; preconditioning

Prior brain exposure to minor insults, chemicals, or pharmacological agents can “precondition” or increase the brain’s tolerance to future, more injurious events. Anesthetics in particular may play an important and unique role as brain preconditioning agents because of their clinical use and relevance during the perioperative period for cardiovascular procedures and neurosurgical interventions. Perioperative stroke is a serious complication that can occur during or following

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procedures like carotid endarterectomy or coronary artery bypass grafting (Selim, 2007). Clinically, there is much interest in pharmacologically preconditioning the human brain with anesthetics and its impact on perioperative stroke, as anesthetic agents are known to affect ischemic outcome in experimental stroke (Kitano et al., 2007a). However, it is currently unclear how anesthetic preconditioning may influence perioperative stroke outcomes in men versus women as experimental brain studies examining anesthetic preconditioning and its neuroprotective mechanisms have used primarily male animals, have not indicated sex, or have not stratified results relative to sex (Wang et al., 2008b).

Few studies have specifically addressed sex differences in the preconditioned brain's response to ischemia or other forms of brain injury (Wang et al., 2008b). Our prior work has shown that isoflurane preconditioning neuroprotection in experimental focal stroke is male-specific, with the neuroprotective benefits of isoflurane preconditioning being lost in female mice and present in ovariectomized mice lacking female sex steroids (Kitano et al., 2007b; Wang et al., 2008a). Even fewer studies have addressed the role of individual sex steroids in the brain's response to preconditioning, anesthetic or otherwise, before ischemia or other forms of brain injury. We have previously shown that estradiol attenuates isoflurane preconditioning neuroprotection in ovariectomized females (Wang et al., 2008a). However, no work has been done exploring the role of androgens in any type of experimental brain preconditioning or clinically in perioperative stroke.

Testosterone and dihydrotestosterone (DHT) are the primary androgens seen in rodents and men. Testosterone can be converted via 5 α -reductase to the more potent androgen and pure androgen receptor (AR) agonist, DHT or it can be aromatized to 17 β -estradiol via P450 estrogen aromatase. The few available data with androgens and human stroke have focused on testosterone levels in men after stroke that were either unaffected (Taggart et al., 1980) or decreased, presumably as a stress response (Jeppesen et al., 1996). Although androgens have been reported in some studies as having harmful effects on ischemic rodent brain injury (Cheng et al., 2007; Hawk et al., 1998; Li et al., 2009; Uchida et al., 2009; Yang et al., 2002, 2005), several studies would suggest that androgens may not be universally detrimental in experimental cerebral ischemia (Hammond et al., 2001; Li et al., 2009; Pan et al., 2005; Toung et al., 1998; Uchida et al., 2009). Androgens may even be required for ischemic preconditioning cardioprotection in experimental myocardial ischemia models (Liu et al., 2006; Song et al., 2003; Tsang et al., 2007) and may mediate their effects via the AR (Liu et al., 2006; Tsang et al., 2007). Little work has been done evaluating androgens and the AR in preconditioned ischemic male brain.

Using a mouse model of isoflurane preconditioning and experimental stroke, we evaluated the role of testosterone and DHT in male-specific isoflurane preconditioning neuroprotection. We also determined if these androgens act via an AR-dependent mechanism in isoflurane preconditioned ischemic male brain.

EXPERIMENTAL PROCEDURES

Experiments were carried out in accordance with the National Institutes of Health guidelines for research animal care and approved by the Oregon Health and Science University Animal Care and Use Committee.

Experimental groups

Experiments were carried out on male C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA), 8–14 weeks of age and weighing 20–25 g. The following experimental groups underwent sham or isoflurane preconditioning and were analyzed for infarct volumes at 22 h reperfusion following 2 h of middle cerebral artery occlusion (MCAO)

(Fig. 1): gonadally intact males ± 5 mg flutamide ($n=39$); castrated males ± 5 mg flutamide ($n=37$); castrated males+testosterone (1.5 or 5 mg) ± 5 mg flutamide ($n=58$); castrated males +DHT (0.5 or 1.5 mg) ± 5 mg flutamide ($n=57$); castrated males+1.5 mg testosterone+vehicle ($n=22$); and castrated males+1.5 mg testosterone +1 mg/kg/day letrozole ($n=23$). Infarct volumes at 22 h reperfusion after 2 h MCAO were also determined in sham and isoflurane preconditioned P450 estrogen aromatase wild-type ($n=24$) and knockout ($n=24$) male mice (8–14 weeks of age, 20–25 g). Based on infarct outcomes, the lowest neuroprotective DHT dose (0.5 mg) was used to assess neurobehavioral outcomes. Sham and isoflurane preconditioning castrated males ± 0.5 mg DHT ($n=75$) subjected to 1 h of MCAO were therefore evaluated for long-term neurobehavioral outcomes during the 9 day period following MCAO. All mice were maintained on a 12/12-h light/dark cycles and permitted *ad libitum* access to food and water.

Hormone and drug treatments

Under isoflurane (Hospira, Lake Forest, IL, USA) anesthesia (induction 4%; maintenance 1.5%), male mice were castrated 7–8 days before preconditioning to allow endogenous serum sex steroid hormone levels to decrease and exogenous serum sex steroid hormone levels in implanted mice to come to steady state levels. At the time of castration, select groups of castrated mice were implanted s.c. with 21-day release pellets (Innovative Research of America, Sarasota, FL, USA) containing testosterone (1.5 or 5 mg), DHT (0.5 or 1.5 mg), and/or the AR antagonist, flutamide (5 mg). Selected groups of gonadally intact male mice were also treated with flutamide (5 mg). We have previously confirmed the antagonist properties of flutamide at equimolar or higher doses as concurrently administered testosterone doses in ischemic male brain (Uchida et al., 2009).

In experimentally naive gonadally intact C57BL/6 males, we have previously reported a range of 0.1–21.7 ng/mL for total testosterone, 0.0–55.1 pg/mL for free testosterone and 0.0–3.0 ng/mL for DHT (Uchida et al., 2009). Serum androgen levels following experimental stroke in gonadally intact males, untreated castrates, and castrates treated with the testosterone (1.5, 5 mg) or DHT (0.5, 1.5 mg) implant doses used in this study have been previously characterized by our group (Uchida et al., 2009). In testosterone treated castrates, we have shown that total and free testosterone levels increased relative to testosterone dose and were within the broad physiological range for naive gonadally intact male mice described above (Uchida et al., 2009). We have also shown that DHT treatment in castrates had no effect on total or free testosterone levels as compared to untreated castrates but increased serum DHT levels in a dose-dependent manner, with values within the physiological range described above for naive gonadally intact males (Uchida et al., 2009).

Letrozole (1 mg/kg/day; gift from Novartis Pharma AG, Basel, Switzerland), an aromatase inhibitor that is able to cross the blood-brain barrier (Hume and Wynne-Edwards, 2006), and its associated vehicle (propylene glycol; SIGMA, St. Louis, MO, USA) were dispensed via s.c. implantation of osmotic pumps (Model 1002, Alzet Osmotic Pumps, Durect Corporation, Cupertino, CA, USA) concurrently with castration and implantation of 1.5 mg testosterone pellets in male C57BL/6 mice 13 days before preconditioning. The letrozole dose selected and the timing of its administration prior to preconditioning for the proposed studies is based on biologically effective doses used in rodent studies involving CNS effects (Hume and Wynne-Edwards, 2006; Park et al., 2009).

P450 estrogen aromatase knockout (ArKO) mice

Generation of P450 estrogen aromatase knockout (ArKO) mice is as previously described (Fisher et al., 1998). This strain originated on a C57BL/6J; 129SvEv background and has been backcrossed to C57BL/6J for at least 10 generations. In ArKO mice, exon IX of the *Cyp 19* gene is disrupted by insertion of a neo cassette using homologous recombination. Homozygous

ArKO mice produce an abnormal transcript that generates a nonfunctional protein. Consequently, estrogen aromatase activity is lacking in all cell types and estrogen synthesis is blocked. Breeding colony was maintained utilizing heterozygous breeding trios (two females, one male). Genotyping was done by polymerase chain reaction (PCR) amplification of tail DNA as previously described (Liu et al., 2008).

Isoflurane preconditioning model

Isoflurane preconditioning model is as described previously (Kitano et al., 2007b; Wang et al., 2008a). Mice were placed for 4 h in an air-tight, temperature-controlled chamber flushed with 1.0% isoflurane (isoflurane preconditioning) or 0% isoflurane (sham preconditioning) in oxygen-enriched air. We have previously shown that physiological systemic variables (glucose, pH, partial arterial pressure carbondioxide [P_aCO_2]) evaluated after 1 and 4 h of isoflurane preconditioning showed no major perturbations imposed by isoflurane preconditioning other than mild hypotension and partial arterial pressure oxygen (P_aO_2) values consistent with animals exposed to oxygen-enriched air (Kitano et al., 2007b). Twenty-four hours after preconditioning, mice underwent transient focal cerebral ischemia.

Transient focal cerebral ischemia

Preconditioned male mice were subjected to 1 or 2 h of reversible MCAO via the intraluminal filament technique as previously described (Kitano et al., 2007b; Wang et al., 2008a). Mice were anesthetized with isoflurane (induction 4%; maintenance 1.5%) during surgery until unilateral MCAO was achieved and then allowed to awaken during ischemia. Towards the end of ischemia, mice were briefly reanesthetized with isoflurane and reperfusion was initiated by intraluminal filament withdrawal. Rectal temperatures in anesthetized mice were monitored and maintained at 36.4 ± 0.7 °C throughout MCAO surgery and during induction of reperfusion. Anesthetized mice were warmed as needed using an infrared heating lamp (115 VAC, 50/60 Hz, Cole-Parmer Instrument Company, Vernon Hills, IL, USA). Cortical blood flow was monitored throughout MCAO surgery, initial induction of MCAO, end-ischemia, and initiation of reperfusion by laser Doppler flowmetry (LDF; model MBF3D, Moor Instruments Ltd., Oxford, England). The effectiveness of vascular occlusion was confirmed by sustained reduction in LDF signal while restoration of blood flow following release of vascular occlusion was assessed by increases in LDF signal towards baseline levels. Animals were excluded if intra-ischemic LDF was greater than 25% pre-ischemic baseline. All animals undergoing cerebral ischemia regardless of preconditioning status had comparable exposure times (26.5 ± 0.4 min, $n=316$) as well as dose relative to isoflurane delivered during MCAO and reperfusion.

For infarct volume outcomes, animals were euthanized after 22 h of reperfusion, brains removed, and blood obtained for hormone analysis. Infarct volume was estimated using 2,3,5-triphe-nyltetrazolium chloride (SIGMA) staining of 2-mm thick coronal sections (five slices total) (Bederson et al., 1986). Both sides of each stained coronal slice were photographed via a digital camera (see Fig. 2A for examples of coronal images) and then evaluated by digital image analysis (SigmaScan Pro, Jandel, San Rafael, CA, USA). Infarcted area was integrated across sections, and volume expressed as % of contralateral structure (cortex, caudate putamen). Animals used for neurobehavioral evaluations were euthanized after 9 days of reperfusion.

Femoral arterial catheters were placed in separate groups of sham and isoflurane preconditioned castrated males ($n=9$) and castrates treated with 0.5 mg DHT, in the presence or absence of 5 mg flutamide ($n=20$) subjected to 2 h of MCAO in order to determine mean arterial blood pressure, blood gases (pH, P_aO_2 , P_aCO_2), and blood glucose values at 1 h of ischemia and at 10 min of reperfusion. Animals were anesthetized with 1.5% isoflurane

throughout ischemia and reperfusion. Mice were euthanized after the final blood sample was taken 10 min into the reperfusion period.

Hormone assay

Serum hormone levels (total and free testosterone, DHT, 17β -estradiol) were measured in duplicate by radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA, USA). The lower limits of detection for total and free testosterone, DHT, and estradiol were 0.01 ng/mL, 0.5 pg/mL, 0.01 ng/mL, and 10 pg/mL, respectively. When calculating mean hormone levels per treatment group, a value of zero was assigned to hormone values less than the lower limits of detection.

Neurobehavioral evaluations

We selected neurobehavioral tests that have consistently provided reliable outcome evaluations across experimental focal cerebral ischemia studies in mice in our laboratories (Craft et al., 2006; Li et al., 2004; Uchida et al., 2009). All mice undergoing behavioral testing were single-housed on a 12/12-h light/dark cycle. All neurobehavioral assessments were performed during the second half of the light cycle between 1200 and 1800 h. The observer who performed all neurobehavioral assessments was blinded to treatment group. All equipment was cleaned with 10% ethanol between trials.

Neurological deficit score—A neurological deficit score was recorded in each mouse 1 and 3 days after MCAO (Day 1 and Day 3) as previously described (Li et al., 2004; Uchida et al., 2009). The graded scoring systems ranged from 0 to 2 to 0 to 5 depending on the behavior assessed, with 0 indicating no deficit and 2–5 indicating the most impaired. The behaviors assessed included consciousness (0–3), interaction (0–2), eye appearance (0–2), breathing (0–2), food/water intake (0–2), ability to grab wire top (0–2), motor function (0–5), and activity (0–2). The final neurological deficit score was a summation of these graded scoring systems and ranged from 0 (least impaired) to 20 (most impaired).

Cylinder test—We performed the cylinder test (Schallert et al., 2000; Uchida et al., 2009) to analyze forelimb use bias. Each mouse was placed in a transparent cylinder measuring 9 cm in diameter and 15 cm in height. The cylinder was wide enough for the mouse to move freely and small enough to encourage rearing behavior. Four separate video cameras were placed around the cylinder at 90° intervals in order to record rearing behavior from all angles. We recorded a maximum of two paw touches for one rearing event. If the mouse touched the cylinder wall with the same paw two times, we designated these motions as “independent.” If the mouse touched the cylinder wall the first time with one paw and a second time with the other paw, we designated these motions as “independent” and “both,” respectively. A total of 20 forelimb touches was recorded during the 10-min test and was found to be sufficient to determine bias without habituating the mouse to the apparatus. Mice with fewer than 20 forelimb touches in this time period were excluded from neurobehavioral analysis. The final score was calculated as the percentage of total touches that used the impaired forelimb. Baseline paw preference was assessed one day before MCAO just prior to preconditioning. Subsequent assessments were made at 3 and 7 days after MCAO.

Locomotor activity in the open field—To evaluate spontaneous locomotor activity, we used the open field protocol (Craft et al., 2006). Mice were placed individually into a 41 cm (W) × 41 cm (D) × 38 cm (H) plastic enclosure equipped with video cameras mounted above to record movement in four arenas simultaneously. The video was analyzed off-line using Noldus software (Ethovision 2.3, Noldus, Leesburg, VA, USA). Total distance traveled and mean velocity were assessed at baseline, one day prior to MCAO and 5 days following MCAO.

Novel object recognition test—Mice were placed individually into a 41 cm (W) × 41 cm (D) × 38 cm (H) plastic enclosure and allowed to habituate to the arena before novel object recognition testing. During the sample session 6 days after MCAO, two identical objects were placed in opposite corners of the arena, approximately 1 in. from the wall. Time spent exploring each object during the sample session was hand scored with stopwatches and each mouse was removed from the arena after accumulating 38 s of exploration time on either of the sample objects (maximum of 10 min total allotted), in agreement with previous studies (Hammond et al., 2004). After a 24 h delay, mice were again placed in the arena for a test session (day 7 after MCAO), with one object replaced with a novel object (location of novel object randomized). A 5 min test session was performed and the time exploring each object was recorded. Novel object preference ratio is the time spent exploring the new object divided by the total exploration time during the test session. All objects used in this study were characterized previously in our laboratory to ensure that mice prefer each object equally.

Statistics

Values are expressed as mean ± standard error of the mean (SEM). Differences in infarct volumes in each isoflurane preconditioning group as compared to its corresponding sham preconditioning group were determined with a Student's *t*-test in order to examine the effect of isoflurane preconditioning relative to sham preconditioning under different experimental conditions (castration, hormone or drug administration, presence or absence of aromatase). Differences in serum hormone levels between male P450 estrogen aromatase wild-type (ArWT) and ArKO mice and between testosterone replaced castrated males treated with letrozole or vehicle were determined by a Student's *t*-test. Mean arterial blood pressure, blood gases (pH, P_aO₂, P_aCO₂), and blood glucose were subjected to two-way analysis of variance (ANOVA) with post hoc Newman–Keuls test. Behavioral outcomes for neurological deficit scores and the paw preference test were analyzed by one-way ANOVA repeated measurement with post hoc Newman–Keuls test. Behavioral outcomes for the novel object recognition test were analyzed by one-way ANOVA with post hoc Newman–Keuls test. Statistical significance was *P* < 0.05. Statistical analyses were performed using SigmaStat Statistical Software, Version 3.1 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Mortality and exclusions in infarct volume outcome studies

Overall mortality was 13 mice out of a total of 284 mice, with mortality ranging from 0 to 2 mice within the experimental groups. Overall number of mice excluded due to intra-ischemic LDF greater than 25% pre-ischemic baseline was 13 mice out of a total of 284 mice, with exclusions ranging from 0 to 3 mice within the experimental groups. At initiation of reperfusion, all experimental groups reperfused to greater than 50% pre-ischemic baseline (data not shown).

Infarct volumes in preconditioned males, castrates, and androgen-replaced castrates

Isoflurane preconditioning significantly decreased infarct volumes in intact male mice as compared to sham preconditioned males (Fig. 2). In contrast, reduction of endogenous androgen levels via castration prevented the decrease in infarct volumes in isoflurane versus sham preconditioned mice (Fig. 2). Isoflurane preconditioning neuroprotection was restored in castrates treated with low dose (1.5 mg) testosterone (Fig. 3A) or (0.5 mg) DHT (Fig. 3B). In castrates treated with high dose (5 mg) testosterone (Fig. 3A), isoflurane preconditioning neuroprotection was only reinstated in the caudate putamen. Isoflurane preconditioning neuroprotection was not re-established in castrates treated with high dose (1.5 mg) DHT (Fig. 3B).

Infarct volumes in flutamide-treated groups and in mice with aromatase deletion or inhibition

The protective effects of isoflurane preconditioning on infarct volumes in intact male mice were blocked when males were treated with flutamide, an AR antagonist (Fig. 4A). However, when endogenous androgen levels were reduced by castration, flutamide had no effect on infarct volumes regardless of preconditioning status (Fig. 4A). The protective effects of isoflurane preconditioning in castrates treated with low-dose testosterone (1.5 mg) or DHT (0.5 mg) were attenuated by concurrent exposure to flutamide (Fig. 4B). The protective effects of isoflurane preconditioning on infarct volumes were not altered in gonadally intact males by genetic deletion of aromatase versus wild-type (Fig. 5A) or by pharmacologic inhibition of aromatase with letrozole versus vehicle in castrates treated with low dose (1.5 mg) testosterone (Fig. 5B).

Baseline and intra-ischemic arterial blood pressure and blood gases

Overall mortality was three mice out of a total of 29 mice, with mortality ranging from 0 to 1 mouse within the experimental groups. No mice were excluded due to intra-ischemic LDF greater than 25% pre-ischemic baseline. At initiation of reperfusion, all experimental groups reperused to greater than 50% pre-ischemic baseline (data not shown). Mean arterial blood pressure, blood gases (pH, P_{aO_2} , P_{aCO_2}), and blood glucose were comparable between castrates, castrates treated with 0.5 mg DHT, and castrates treated with 0.5 mg DHT and 5 mg flutamide sham and isoflurane preconditioning groups at each time point evaluated (Suppl. Table 1). There were no differences between ischemic and reperfusion values within each experimental group (Suppl. Table 1).

Hormone levels

Regardless of preconditioning group, there were no differences between male ArWT and ArKO mice in free (ArWT, 0.5 ± 1.0 pg/mL, $n=18$; ArKO, 0.6 ± 0.1 pg/mL, $n=20$) and total (ArWT, 0.5 ± 0.1 ng/mL, $n=21$; ArKO, 0.5 ± 0.1 ng/mL, $n=21$) testosterone and DHT (ArWT, 0.2 ± 0.0 mg/mL, $n=21$; ArKO, 0.3 ± 0.1 mg/mL, $n=20$) levels. However, estradiol levels were ($P<0.05$) lower in male ArKO mice (16 ± 2 pg/mL, $n=10$) as compared to male ArWT mice (28 ± 2 pg/mL, $n=15$). Estradiol levels were also ($P<0.05$) lower in testosterone-replaced (1.5 mg) castrates treated with letrozole (3 ± 2 pg/mL, $n=17$) versus vehicle (14 ± 3 pg/mL, $n=15$).

Neurobehavioral testing

Neurobehavioral testing was performed over a 9 day period following 1 h of MCAO in sham and isoflurane preconditioned castrated C57BL/6 mice and sham and isoflurane preconditioned castrated C57BL/6 males treated with 0.5 mg DHT. Mortality in each experimental group was as follows: sham preconditioned castrates, 12% (2/17 in total); isoflurane preconditioned castrates, 29% (6/21 in total); sham preconditioned castrates treated with 0.5 mg DHT, 21% (4/19 in total); and isoflurane preconditioned castrates treated with 0.5 mg DHT, 17% (3/18 in total). Only two mice, one from each sham and isoflurane preconditioned groups of castrates treated with 0.5 mg DHT, were excluded as neurobehavioral testing outliers (unable to perform tasks).

Neurological deficit score was assessed at 1 and 3 days after MCAO as an indicator of general health. There were no differences in neurological deficit scores between the experimental groups on each day tested (Fig. 6A). All experimental groups showed similar levels of impairment 1 and 3 days after MCAO, with declining neurological deficit scores over time. Motor dysfunction was assessed on the affected, contralateral side (left paw/forelimb) using the cylinder test. No asymmetries were observed in evaluations done one day before MCAO, just prior to preconditioning (Fig. 6B). At 3 and 7 days following MCAO, isoflurane preconditioning minimized contralateral forelimb impairment regardless of whether castrates

were untreated or treated with DHT (Fig. 4B). DHT also reduced forelimb asymmetry at 3 and 7 days following MCAO regardless of preconditioning status (Fig. 6B).

The open field test was performed 1 day before (Pre) and 5 days after MCAO as a control for recovery of motor function, in order to avoid this possible confounding factor during the novel object recognition test. No significant change in total distance moved or velocity of movement was observed during the 30-min open field test, regardless of treatment (data not shown). To determine the effect of MCAO on cognitive function using hippocampal-dependent tasks, the novel object recognition test was performed 6–7 days after MCAO. Only mice with an intact memory will recognize and preferentially explore a new or novel object, thus showing an increased novel object preference ratio. In castrated mice, isoflurane preconditioning prevented while DHT had no effect on the loss of novel object recognition and preference after MCAO observed in sham preconditioned castrates (Fig. 6C). However, the protective effect of isoflurane preconditioning on novel object recognition and preference was blocked in castrates treated with DHT (Fig. 6C).

DISCUSSION

This study demonstrates four important findings. First, the protective effects of isoflurane preconditioning on infarct volume in ischemic male brain are androgen-dependent. Second, isoflurane preconditioning neuroprotection relative to infarct volume outcomes is testosterone and DHT dose-specific. Third, the effects of testosterone and DHT on infarct outcomes in isoflurane versus sham preconditioned ischemic male brain are likely mediated through the AR. Last, isoflurane preconditioning shows a complex pattern of protection against long-term neurobehavioral deficits. Isoflurane preconditioning improved long-term sensorimotor outcome regardless of androgen status. Similarly, androgen replacement improved sensorimotor outcome regardless of preconditioning status. In contrast, isoflurane preconditioned mice in the absence of endogenous androgens showed improved cognitive outcome that was prevented by androgen replacement.

We have previously reported that neuroprotection due to isoflurane preconditioning is male-specific in ischemic brain (Kitano et al., 2007b). The current study demonstrates that castration ameliorated the protective response to isoflurane preconditioning in ischemic male brain, suggesting that androgens may be important to the male brain's response to isoflurane preconditioning. Because androgen levels are not constant during life and can begin to decline during the middle years of life (andropause), it seems likely that androgen loss during aging would have a similar effect on the aging male brain's response to isoflurane preconditioning as castration did in the young male brain. Therefore, future studies will need to address the role of androgens in isoflurane preconditioning neuroprotection in aging ischemic male brain as age can increase perioperative stroke risk following procedures like carotid endarterectomy (Bond et al., 2005; Schaller, 2007).

A potential difficulty in interpreting results from experiments using intact male mice is that endogenous androgen levels can be quite variable depending on time of day and environmental stresses as evidenced by the wide range of androgen values reported for mice (Overpeck et al., 1978; Quimby, 1999; vom Saal et al., 1994). Low endogenous androgen levels may therefore explain why in this study we failed to observe the protective effect of castration described in other rodent ischemic stroke models (Cheng et al., 2007; Hawk et al., 1998; Yang et al., 2002) on infarct volume outcomes in sham preconditioned intact versus castrated male mice. Furthermore, our findings indicate that the neuroprotective response to isoflurane preconditioning is androgen dose-dependent, with lower doses restoring and higher doses failing to fully re-establish isoflurane preconditioning neuroprotection in ischemic brain. Thus,

androgens may have a complex dose-response relationship in preconditioned (anesthetic or otherwise) ischemic brain.

The findings from our experiments with DHT (a non-aromatizable direct AR agonist), flutamide (anti-androgen with high AR specificity and ability to cross the blood-brain barrier) (Neri et al., 1972; Singh et al., 2000), and P450 estrogen aromatase deletion or inhibition with letrozole suggest that the effects of androgens on infarct outcomes in isoflurane preconditioned brains are mediated via the AR. The results from these experiments also suggest that testosterone is predominantly binding either directly or indirectly through its conversion via 5 α -reductase to DHT to the AR in preconditioned ischemic brain rather than being chiefly aromatized to 17 β -estradiol by P450 estrogen aromatase. Since the AR was implicated by our results in mediating the effects of androgens in isoflurane preconditioned ischemic brain, we evaluated physiological parameters during ischemia and early reperfusion in preconditioned castrates and castrates treated with 0.5 mg DHT in the presence or absence of 5 mg flutamide. Our findings relative to infarct volume are not explained by differences in physiological parameters among treatment groups involving AR manipulations.

This study did not examine which mechanisms may be involved downstream of ARs such as transcriptional regulation of target genes through AR binding. Several possible target genes have already been identified that are induced by DHT in ischemic cortex and that could alter cell death outcomes through their involvement with inflammation, cell signaling, and blood-brain barrier regulation (Cheng et al., 2007). AR-independent mechanisms might also explain the observed androgen effects on ischemic outcomes in isoflurane preconditioned male brain. For example, we have shown that isoflurane preconditioning alone in males enhances Akt activation, a non-genomic neuronal survival-signaling pathway (Brunet et al., 2001; Dudek et al., 1997), but does not alter Akt activation in females at 24 h following preconditioning (Kitano et al., 2007b). Current literature suggests that testosterone enhances Akt signaling in heart (Bai et al., 2005). Therefore, it is possible that androgens may be beneficial in isoflurane preconditioned ischemic brain through effects on Akt activation. Regional differences in the mechanisms downstream or independent of ARs through which androgens exert their dose-specific effects in preconditioned ischemic brain may also explain why high dose testosterone restored isoflurane preconditioning in ischemic caudate putamen but not in cortex. Future studies from our laboratories will be done to determine which AR-dependent and independent mechanisms underlying the effects of androgens on ischemic outcomes in isoflurane preconditioned male brain are involved.

The lack of success in translating promising experimental rodent data into clinical trials has led to guidelines recommending evaluation of ischemia-induced functional deficits in addition to histological outcomes (Fisher et al., 2009). Therefore, we used several behavioral tasks to assess functional outcome during the first week following MCAO to extend our initial finding that isoflurane preconditioning protects the male brain in an androgen-dependent manner. For these longer term experiments, we used a significantly less severe insult (60 versus 120 min ischemia) in order to improve survival and recovery of gross sensorimotor deficits as cognitive function tests would be complicated by alterations in gross motor activity. Our data using a neurological deficit score to evaluate general sensorimotor performance and overall health did not show differences between treatment groups. We evaluated contralateral forelimb impairment using the cylinder test because we have previously demonstrated that this task detects impairment for several weeks after MCAO in mice (Li et al., 2004; Uchida et al., 2009). We observed that isoflurane preconditioning resulted in a decreased sensorimotor deficit regardless of androgen status, being equally effective in castrates and DHT replaced mice. Similarly, low dose DHT replacement in sham preconditioned mice improved long term sensorimotor outcome as measured by paw preference. Therefore, isoflurane preconditioning or low dose DHT appears to improve sensorimotor deficits at early and later time points

following cerebral ischemia. We conclude from these data that subtle sensorimotor testing provides insight into “neuroprotection” that is not apparent in histological analysis of infarct volume following MCAO.

It is becoming increasingly apparent that studying cognitive and sensorimotor deficits following cerebral ischemia provides additional insight into outcomes and efficacy of experimental treatments. We used the novel object recognition test to assess long-term cognitive outcome. In the present study, isoflurane preconditioning prevented the decline in memory retention observed following MCAO in castrated male mice. Our findings that isoflurane preconditioning improves post-ischemic cognitive function are in agreement with isoflurane preconditioning studies in rat using the same cognitive test (McAuliffe et al., 2009). However, two studies have observed no effect of isoflurane preconditioning on post-ischemic cognitive outcomes when other tests like the Morris water maze, Y maze, and social recognition (McAuliffe et al., 2007; Zhao et al., 2007) were used to evaluate cognitive function. Interestingly, we observed that low dose DHT blocked the protective effect of isoflurane preconditioning on novel object recognition and preference. This finding seems at odds with the observation that isoflurane preconditioned DHT-replaced castrates showed reduced infarct volumes. However, infarct volumes may not necessarily predict long-term functional outcomes as infarct volume determinations only discriminate between live and dead neurons but does not provide information on the functional status of the remaining neurons. Distribution of regional cell death may also have a greater impact on neurobehavioral functional outcomes than infarct size (DeVries et al., 2001), indicating the need to perform more detailed histopathology in the future to better correlate histological and functional outcomes in isoflurane preconditioned brain following ischemia. In addition, this finding is somewhat surprising in conjunction with the improved sensorimotor function observed in these experiments, indicating complex interactions between androgen signaling and isoflurane preconditioning during the 9 day period following transient focal cerebral ischemia.

CONCLUSION

In summary, we have demonstrated that male-specific ischemic outcomes in isoflurane preconditioned brain are androgen dose- and AR-dependent. Few studies have examined androgens as mediators in the ischemic response to anesthetic or other types of brain preconditioning. More research is needed to further delineate the role of androgens and ARs in the brain’s response to anesthetic preconditioning relative to histological versus neurobehavioral outcomes as well as determine which pathways are involved downstream of ARs or independently of ARs. Clinically, our findings suggest that isoflurane anesthesia and androgen availability during “at-risk” cardiovascular surgical procedures may influence perioperative stroke outcomes in men.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ANOVA	analysis of variance
AR	androgen receptor
ArKO	aromatase knockout
ArWT	aromatase wild-type
DHT	dihydrotestosterone
LDF	laser Doppler flowmetry
MCAO	middle cerebral artery occlusion
P _a CO ₂	partial arterial pressure carbon dioxide
PCR	polymerase chain reaction
P _a O ₂	partial arterial pressure oxygen
SEM	standard error of the mean

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APPENDIX

Supplementary data

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Fig. 1.

Schematic overview of the time line and experimental design for castration, hormone or drug administrations, preconditioning and ischemia. Abbreviations: ArKO, P450 estrogen aromatase knockout; ArWT, P450 estrogen aromatase wild-type; DHT, dihydrotestosterone; IsoPC, isoflurane preconditioning; Sham PC, sham preconditioning.

Fig. 2.

Cortical and caudate putamen infarction volumes (% contralateral structure) determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining in male C57BL/6 mice preconditioned for 4 h with 0% (Sham PC) or 1% isoflurane (IsoPC). Preconditioning occurred 24 h before 2 h of middle cerebral artery occlusion followed by 22 h of reperfusion. (A) Representative images of TTC stained brains from Sham PC and IsoPC male and castrated (CAST) mice. (B) Effects of IsoPC in male and CAST mice. All values are mean $\pm$ SEM. * $P < 0.05$.

Fig. 3.

Cortical and caudate putamen infarction volumes (% contralateral structure) determined by 2,3,5-triphenyltetrazolium chloride staining in male C57BL/6 mice preconditioned for 4 h with 0% (Sham PC) or 1% isoflurane (IsoPC). Preconditioning occurred 24 h before 2 h of middle cerebral artery occlusion followed by 22 h of reperfusion. S.c. testosterone (T) and dihydrotestosterone (DHT) implants were placed in selected groups of male mice concurrently undergoing castration 1 week prior to preconditioning. (A) Effects of different T doses (1.5, 5 mg) in castrated (CAST) mice on the response to isoflurane preconditioning in ischemic mouse brain. (B) Effects of different DHT doses (0.5, 1.5 mg) in castrates on the response to isoflurane preconditioning in ischemic mouse brain. All values are mean $\pm$ SEM. * $P < 0.05$.

Fig. 4.

Cortical and caudate putamen infarction volumes (% contralateral structure) determined by 2,3,5-triphenyltetrazolium chloride staining in male C57BL/6 mice preconditioned for 4 h with 0% (Sham PC) or 1% isoflurane (IsoPC). Preconditioning occurred 24 h before 2 h of middle cerebral artery occlusion followed by 22 h of reperfusion. S.c. testosterone (T), dihydrotestosterone (DHT), and flutamide (F) implants were placed in selected groups of male mice concurrently undergoing castration 1 week prior to preconditioning. (A) Effects of isoflurane preconditioning in male and castrated (CAST) mice treated with *F* (5 mg), an androgen receptor antagonist. (B) Effects of isoflurane preconditioning in T (1.5 mg) or DHT (0.5 mg) replaced castrates treated with *F* (5 mg). All values are mean $\pm$ SEM. * $P < 0.05$.

Fig. 5.

Cortical and caudate putamen infarction volumes (% contralateral structure) determined by 2,3,5-triphenyltetrazolium chloride staining in male mice preconditioned for 4 h with 0% (Sham PC) or 1% isoflurane (IsoPC). Preconditioning occurred 24 h before 2 h of middle cerebral artery occlusion followed by 22 h reperfusion. S.c. testosterone (T) implants (1.5 mg) and osmotic pumps containing either vehicle (polyethylene glycol) or letrozole (1 mg/kg/day) were placed in selected groups of male mice concurrently undergoing castration 13 days prior to preconditioning. (A) Effect of isoflurane preconditioning on infarct volume in P450 estrogen aromatase knockout (ArKO) and wild-type (ArWT) gonadally intact male mice. (B) Effect of isoflurane preconditioning on infarct volume in castrated (CAST) testosterone-replaced C57BL/6 mice treated with either the aromatase inhibitor, letrozole (L), or its corresponding vehicle (V). All values are mean $\pm$ SEM. * $P < 0.05$.

Fig. 6. Neurobehavioral assessments in castrated (CAST) male C57BL/6 mice preconditioned for 4 h with 0% (Sham PC) or 1% isoflurane (IsoPC). Preconditioning occurred 24 h before 1 h of MCAO followed by 9 days of reperfusion. A 0.5 mg s.c. dihydrotestosterone (DHT) implant was placed in selected groups of male mice concurrently undergoing castration (CAST) 1 week prior to preconditioning. (A) Neuroscore was assessed one (Day 1) and 3 days (Day 3) after MCAO. No significant differences were observed across treatment groups. (B) Contralateral paw use was tested one day before MCAO (Pre), 3 days (Day 3) and 7 days (Day 7) after MCAO. * $P < 0.05$ compared to same group Pre. (C) Novel object recognition was assessed 7 days post MCAO in each group. The dashed line represents a preference ratio of 0.5, indicating lack of retention of novel object. All values are mean $\pm$ SEM. * $P < 0.05$ compared to all other groups.