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Testosterone Exacerbates Neuronal Damage Following Cardiac Arrest and Cardiopulmonary Resuscitation in Mouse

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Abstract

Male animals exhibit greater neuronal damage following focal cerebral ischemic injury in many experimental injury models, however the mechanism of this is unknown. This study used cardiac arrest and cardiopulmonary resuscitation (CA/CPR) in male mice exposed to physiological vs. pharmacological doses of testosterone and tested the hypothesis that testosterone increases damage following global cerebral ischemia. Analysis of histological damage 72 hrs after resuscitation revealed a complex dose-response curve for testosterone, such that low and high doses of testosterone exacerbated ischemic neuronal damage, while intermediate doses had no effect on neuronal survival. In agreement with these histological observations of neuronal damage, both low and high doses of testosterone increased sensorimotor deficit following CA/CPR compared to vehicle treated animals. Finally, the androgen receptor antagonist flutamide inhibited the increase in neuronal damage and sensorimotor impairment observed in testosterone treated mice. Our data showed that low and supra-physiological levels of testosterone increase neuronal damage following global cerebral ischemia and that blockade of androgen receptors limits this injury. Therefore, this study indicated that testosterone may have a role in determining sex-linked differences in cerebrovascular disease as well as having important health implications in clinical conditions of elevated testosterone.

Keywords

testosterone; ischemia; cardiac arrest

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Disclosure/Conflict of Interest

The authors have no conflict of interests.

INTRODUCTION

Each year over half a million people suffer from cardiac arrest (CA) and receive cardiopulmonary resuscitation (CPR) in the United States (Rosamond et al. 2008). Despite intense research over the past 4–5 decades, clinical outcome remains poor and neurological and neuropsychological deficits are prevalent (Roine et al. 1993; Lim et al. 2004; Saxon 2005). Unfortunately, no pharmacological interventions have proven successful in improving survival or outcome following CA/CPR. However, gender appears to have a large impact on incidence and outcome, with women having lower incidence of cardiac arrest and enjoying better outcomes compared to men (Kim et al. 2001; Wigginton et al. 2002; Vukmir 2003, Rosamond et al. 2008). Therefore, understanding the role of sex steroids in determining outcome following CA/CPR could lead to new insights into therapeutic interventions.

Experimental animal models of CA/CPR and stroke mimic the condition in humans, demonstrating sex-linked differences in histological and behavioral outcomes following cerebral ischemia (for reviews see Hurn and Brass 2003; Murphy et al. 2004; Herson et al. 2009). While a great deal of research has been undertaken to unravel the role of female sex steroids in cerebral ischemia, little is known about the effects of the male-specific androgens following ischemia. Indeed, the effect of testosterone on outcome following focal cerebral ischemia middle cerebral artery occlusion (MCAO) has recently begun to be investigated, and the studies performed to date are inconsistent. Multiple studies have observed a detrimental effect of androgens, by demonstrating that castration decreased damage (Hawk et al. 1998) and that testosterone replacement in castrated rodents exacerbated histological damage (Hawk et al. 1998; Yang et al. 2002; Cheng et al. 2007). In contrast, others have failed to observe an effect of castration on ischemic injury (Toung et al. 1998), with a recent study showing that a low dose of androgen provides histological protection (Uchida et al. 2010) and one report demonstrating improved functional recovery following experimental stroke (Pan et al. 2005). *In vitro*, the pattern is reversed, with multiple reports of androgen providing neuroprotection (Ahlbom et al. 1999; Hammond et al. 2001; Zhang et al. 2004) and a single report showing increased neuronal damage following exposure to androgens (Caruso et al. 2004).

Circulating levels of androgens vary significantly in normal males across time, cycling daily and possibly monthly (Simpkins et al. 1981). Men experience a progressive decline in testosterone levels with age, termed the andropause (Morely et al. 1997; Harman et al. 2001; Feldman et al. 2002; Kaufman and Vermeulen 2005; Mooradian and Korenman 2006). In addition, recent evidence indicated that testosterone levels decline rapidly with stress and illness. In order to tightly control plasma testosterone levels, this study determined the effect of testosterone replacement on neuronal damage following global cerebral ischemia in castrated male mice. Importantly, this study utilized our mouse model of CA/CPR that closely mimics the human clinical condition of complete loss of systemic circulation followed by CPR and return of spontaneous circulation (Burne-Taney et al. 2003; Kofler et al. 2004). Finally, to begin to determine the molecular mechanism underlying the effects of testosterone on neuronal outcome following cerebral ischemia, we tested the ability of an androgen receptor antagonist to lessen the effects of testosterone.

RESULTS

Injection of KCl resulted in immediate systolic cardiac arrest in all mice. Body weight, mean ischemia time and epinephrine dose were not different amongst experimental groups (Table 1). Neuronal histology and neurobehavioral analysis was performed after 3-day survival and importantly, the survival rate was not different among groups (Table 1). Separate animals

(n=5/group) were used to determine the effect of treatment on physiological parameters. No differences between treatment groups in blood gases, pH, blood glucose, lactate, sodium or potassium were observed in any sampling time (10 min. before, during or 30 min. after CA/CPR; gases and all data were well within physiological levels; data not shown). Mice were castrated and implanted at the same time with subcutaneous slow-release testosterone pellets of varying concentrations (0, 0.5, 1.5, 10.0 mg). Analysis of serum testosterone levels was performed 3 days after resuscitation, at the end of each experiment. Figure 1 demonstrates that implantation of testosterone pellets into castrated mice resulted in a dose-dependent increase in serum testosterone concentration.

Analysis of the caudoputamen (CP) at 3 days after CA/CPR revealed that low and high doses of testosterone exacerbate neuronal damage following global ischemia (Fig. 2). Low dose testosterone (0.5mg) increased neuronal damage in the caudal CP (Fig. 2A) from $24.5 \pm 6.7\%$ (n=15) to $60.2 \pm 8.7\%$ (n=17, $p < 0.05$) and from $30.7 \pm 8.7\%$ (n=15) to $50.7 \pm 9.1\%$ (n=17) in the rostral CP (Fig. 2B). Treatment with 1.5mg testosterone did not alter outcome in caudal or rostral CP, $25.7 \pm 7.4\%$ (n=14) and $31.2 \pm 8.7\%$ (n=14), respectively. Similarly, 5.0mg treatment did not alter outcome in caudal or rostral CP, $49.8 \pm 9.6\%$ (n=14) and $42.8 \pm 9.6\%$ (n=14). In contrast, the highest dose (10.0mg) increased damage to $71.5 \pm 4.7\%$ (n=10, $p < 0.05$) and $54.39.5\%$ (n=10) in the caudal and rostral CP, respectively.

Figure 3 illustrates a similar U-shaped dose-response relationship in the CA1 region of the hippocampus. Castrated male mice implanted with 0.5mg testosterone pellets had significantly greater neuronal damage in the CA1 region of the hippocampus compared to vehicle treated mice, $48.7 \pm 5.2\%$ (n=17) and $29.4 \pm 4.8\%$ (n=15, $p < 0.05$) damage, respectively. Moderate doses of testosterone (1.5 and 5.0mg) were not different from the vehicle group, $26.7 \pm 5.0\%$ (n=14) and $42.2 \pm 7.0\%$ (n=14) damage, respectively. In contrast, the highest dose of testosterone (10.0mg) significantly increased damage to $48.1 \pm 6.5\%$ (n=10, $p < 0.05$).

Testosterone interacts with its cognate androgen receptor (AR) to induce many of its physiological effects. Therefore, we tested the ability of the specific AR antagonist flutamide to prevent the increased neuronal damage observed in mice treated with low dose testosterone. The presence of 5.0mg flutamide prevented the increased damage observed with 0.5mg testosterone in all three brain regions analyzed (Fig. 4). The presence of flutamide decreased damage in the CA1 region from $44.7 \pm 5.7\%$ to $8.5 \pm 1.8\%$ (n=12, $p < 0.05$). Similarly, in the CP, flutamide decreased the damaging effect of 0.5mg testosterone, reducing damage from $60.2 \pm 8.7\%$ to $21.9 \pm 6.2\%$ (n=12, $p < 0.05$) and from $50.2 \pm 9.1\%$ to $26.8 \pm 12.3\%$ (n=12) in the caudal and rostral CP, respectively. Interestingly, mice treated with flutamide exhibited significantly less neuronal damage in the CA1 region than the vehicle treated group (Fig. 4). Flutamide did not alter neuronal survival in sham operated mice..

Functional consequence of testosterone treatment was assessed using a 23 point neuroscore scale that assesses general health and cognitive ability (Kofler et al., 2004). CA/CPR resulted in significant neurological deficit 1 day after surgery that was observed to improve significantly by day 3 after resuscitation (Fig. 5). Low (0.5mg) and high doses (10.0mg) of testosterone had no effect on initial impairment observed at day 1 after resuscitation, but prevented recovery after CA/CPR. Figure 5 illustrates that 0.5mg and 10.0mg testosterone worsened outcome 3 days after CA/CPR, increasing neurological deficit score from 7.5 ± 1.5 (n=14; vehicle) to 13.7 ± 1.6 (n=17), and 14.7 ± 1.7 (n=10), respectively. The 1.5 and 5.0mg dose did not alter functional outcome compared to untreated mice. Finally, the presence of 0.5mg testosterone prevented the increased impairment observed in the presence of 0.5mg testosterone without flutamide (Fig. 5).

DISCUSSION

The major finding of this study was that testosterone dose-dependently increased damage to the brain following global cerebral ischemia induced by CA/CPR. Testosterone exhibited a complex dose-dependence (U-shaped dose-response curve), such that histological and neurobehavioral deficits were exacerbated by low and high doses, with intermediate doses having no significant effect. The ability of flutamide to reverse the effects of testosterone indicated that the increased neuronal damage observed in the presence of testosterone was androgen receptor dependent. Therefore, we speculate that testosterone may play a role in sensitizing male brains to ischemic damage after cardiac arrest.

It has recently been observed that testosterone levels drop immediately following a variety of stressors, including traumatic brain injury and stroke (Elwan et al. 1990; Jeppesen et al. 1996; Dimopoulou et al. 2004; Dimopoulou et al. 2005). In addition, there is evidence that testosterone levels follow a daily rhythm, varying from 0.5–2.0ng/ml in rats (Simpkins et al. 1981). In order to avoid these possible confounding influences in our study, we chose to castrate male mice and implant them with slow-release pellets to achieve a reliable and continuous level of testosterone. We observed that a very low dose of testosterone (0.5mg pellet), resulting in low physiological levels of plasma testosterone (~1.0ng/ml), caused a significant increase in neuronal damage and sensorimotor impairment following CA/CPR (global ischemia). It is important to note that the increased neuronal damage was not attributed to changes in cardiac arrest parameters such as resuscitation time, epinephrine dose, or arterial blood pressure. This observation was consistent with several reports indicating that testosterone increased damage following experimental stroke (focal ischemia) (Ahlbom et al. 1999; Hammond et al. 2001; Zhang et al. 2004), although it is important to note that dose-dependence was not determined in these studies. Interestingly, this differs from a recent report in a mouse model of stroke which showed that low physiological levels of testosterone are protective and high dose testosterone exacerbates damage (Uchida et al. 2010). Surprisingly, testosterone replacement to near physiological levels had no significant effect on outcome following CA/CPR, as compared to castrated male mice. The lack of effect observed with intermediate doses of testosterone may in part be explained by a damaging effect of testosterone being countered by a protective effect of estrogen produced via aromatase conversion of testosterone. Taken together, these findings suggest that the decline in testosterone experienced as part of the normal aging process in rodents and in man may render them more sensitive to cerebral ischemia. Indeed, epidemiological data indicate that men experience more cardiac arrests as they age (Rosamond et al. 2008) and outcome is inversely related to testosterone levels (Jeppesen et al. 1996). Therefore, our results indicate that a decline in testosterone may increase risk of brain damage following cardiac arrest and may point to hormone replacement as a beneficial strategy for middle aged and elderly men.

The data with flutamide, a specific androgen receptor (AR) antagonist, suggest that the mechanism of protection and injury exacerbation involves AR signaling. ARs are widely distributed in brain, specifically in neurons (Simerly et al. 1990; DonCarlos et al. 2006), and in areas relevant to injury from CA/CPR in our model such as cortex and hippocampus (Simerly et al. 1990; DonCarlos et al. 2006; Sarkey et al. 2008; Pelletier 2000; Abdelgadir et al. 1999). We predicted that flutamide would have a greater impact on testosterone-mediated effects in the hippocampus as compared to the CP where AR is not as high. However, testosterone-exacerbated neuronal injury (at the low dose) was completely abolished by flutamide in all brain regions analyzed. These data indicate that the low expression level of AR detected in the CP is sufficient to exert maximal effects, even at very low levels of circulating testosterone. An unexpected finding was the protective effect in hippocampus with testosterone under conditions of AR blockade. This is an interesting observation that requires additional research in order to provide a mechanistic explanation but this may

involve the conversion of testosterone to estrogen via the aromatase mechanism (Bulun et al. 2002). In addition, these data indicate disparate cell death pathways and testosterone signaling in the hippocampus and CP.

The AR is a member of the steroid hormone receptor superfamily and acts as a transcription factor by binding to androgen response elements in the promoters of androgen “responsive” genes (for review see Quigley 1998; Roy et al. 1999; Heinlein and Chang 2002). The effector genes important in mediating testosterone effects on outcome following cerebral ischemia have not been well-studied. However, recent data from our group implicates several families of candidate genes, including pro-inflammatory cytokines, chemokines, adhesion molecules, as well as pro-apoptotic genes such as Bax (Cheng et al. 2007). Others have described rapid non-genomic AR signaling modalities in the brain. For example, AR activation has been observed to rapidly activate mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and CREB which are known to be protective in neurons (Pike et al. 2007). Therefore, a variety of pathways may contribute to the complex response of the male brain to androgen-androgen receptor signaling after global cerebral ischemia.

In summary, the present data show that testosterone can exacerbate neuronal damage and increase functional impairment following global cerebral ischemia induced by CA/CPR. To our knowledge, this study is the first to demonstrate that testosterone has a detrimental effect in brain following CA/CPR, indicating that gender differences in outcome following cerebrovascular disease may result in part from the damaging effect of testosterone in the male brain.

EXPERIMENTAL PROCEDURE

All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with National Institutes of Health guidelines for the care and use of animals in research. All experiments were performed in a blinded, randomized fashion using adult male C57Bl6 mice approximately 12 weeks old (20–25g). A total of 154 mice were enrolled in the study, with 35 being lost to mortality, leaving a total of n=119 animals included in the current study. All mice were castrated under isoflurane anesthesia 7 days prior to cardiac arrest as described previously (Toung et al. 1998). Animals were randomized to treatment groups with subcutaneous pellets (Innovative Research of America, Sarasota, FL) of varying concentrations of testosterone (0, 0.5, 1.5, 5.0, 10.0 mg) at the time of castration. Additionally, 5.0mg flutamide pellets were implanted alone or with 0.5mg testosterone pellets. Serum testosterone was analyzed by radioimmunoassay 3 days after resuscitation from cardiac arrest and yielded a clear dose response.

Cardiac Arrest Model

Male mice were subjected to CA/CPR as previously described (Kofler et al. 2004). Briefly, anesthesia was induced with 3% isoflurane and maintained with 1–1.5% isoflurane in O₂ enriched air via face mask. Temperature probes were inserted into the left temporalis muscle and rectum to monitor head and body temperature simultaneously. For drug administration, a PE-10 catheter was inserted into the right internal jugular vein and flushed with heparinized 0.9% saline. A second PE-10 catheter was introduced into the right femoral artery and connected to a pressure transducer to continuously monitor mean arterial blood pressure (MABP) (Gould Instruments, Valley View, OH). Animals were then endotracheally intubated, connected to a mouse ventilator (Minivent, Hugo Sachs Elektronik, March-Hugstetten, Germany) and set to a respiratory rate of 160 min⁻¹. Cardiac arrest was induced by injection of 70µl cold (4°C) 0.5M KCl via the jugular catheter and confirmed by the immediate drop of MAPB. The endotracheal tube was disconnected and anesthesia stopped.

During cardiac arrest, body temperature was cooled to 28°C and head temperature increased to 38.8°C. CPR was begun 10 min. after induction of cardiac arrest by injection of 0.5–1.0ml pre-warmed epinephrine solution (16.0µg epinephrine/ml 0.9 saline), chest compressions at a rate of 300 min⁻¹, and ventilation with 100% oxygen at a respiratory rate of 190 min⁻¹ and a 25% increased tidal volume. Cardiac massage was stopped as soon as spontaneous circulation was restored, defined as a sustained systolic blood pressure of 60mmHg (Idris et al. 1996). CPR was abandoned if spontaneous circulation was not restored within 2.5 min. Simultaneously with initiation of CPR, head temperature was cooled to 38°C and the body re-warmed using a heating lamp or pad. Both catheters and temperature probes were then removed and skin wounds closed.

Neuroscore

A neurological score was performed on each mouse daily after CA/CPR according to a graded scoring system ranging from 0–3,4 for each category, with 0 = no deficit and 3,4 = most impaired. Consciousness, interaction, eye appearance, breathing, food/water intake and overall activity were assessed. Maximum score was 23, while a score of 0 was assigned for no impairment.

Histological Analysis

Three days after CA/CPR, mice were deeply anesthetized with 3% isoflurane and transcardially perfused and fixed with 10% formalin as previously described (Kofler et al. 2004). Brains were removed, embedded in paraffin and 6µm coronal sections were serially cut. The CA1 region of the hippocampus was analyzed, three levels (100µm apart), beginning from –1.5mm bregma. Rostral and caudal CP were analyzed at three levels (100µm apart, beginning from 1.0 and –0.5mm bregma respectively). Sections were stained with hematoxylin and eosin (H&E) for analysis of damaged neurons, determined by the presence of pink eosinophilic cytoplasm and dark pyknotic nucleus. In order to specifically analyze neuronal damage in the CP, sections containing rostral or caudal CP were stained with the neuronal-specific marker NeuN (anti-NeuN antibody) in addition to H&E. The percentage of damaged neurons was calculated for each brain region (average of 3 levels per region). The investigator was blinded to treatment before analyzing neuronal damage.

Statistical Analysis

All data are expressed as mean ± SEM. Treatment groups were compared by one-way analysis of variance (ANOVA) and post-hoc Newman-Keuls test. Differences were considered statistically significant with $p < 0.05$.

Research Highlights

- Testosterone increases neuronal cell death following cardiac arrest
- Testosterone reduces neurobehavioral outcome following cardiac arrest
- Flutamide attenuates neuronal cell death and neurobehavioral outcome following cardiac arrest

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Figure 1. Dose-dependent increase in serum testosterone

Quantification of serum testosterone levels obtained from mice implanted with various testosterone pellets. Pellets implanted into castrated mice 7 days prior to CA/CPR and serum collected for analysis 3 days after resuscitation. Inset is expanded view of vehicle and 0.5mg dose. Number of animals is indicated in each bar. Data presented as mean $\pm$ SEM. * $p < 0.05$ compared to vehicle.

Figure 2. Dose-dependent effect of testosterone on histological outcome in caudoputamen
Quantification of ischemic neurons in caudal (A) and rostral (B) caudoputamen 3 days after CA/CPR. Castrated mice were implanted with various testosterone pellets 7 days prior to CA/CPR. Number of animals is indicated in each bar. Data presented as mean $\pm$ SEM. * $p < 0.05$ compared to vehicle.

Figure 3. Dose-dependent effect of testosterone on histological outcome in hippocampus
Quantification of ischemic neurons in CA1 region of hippocampus 3 days after CA/CPR. Castrated mice were implanted with various testosterone pellets 7 days prior to CA/CPR. Number of animals is indicated in each bar. Data presented as mean $\pm$ SEM. * $p < 0.05$ compared to vehicle.

Figure 4. Flutamide prevents deleterious effect of low dose testosterone

Quantification of ischemic neurons in mice treated with vehicle or 0.5mg testosterone with and without 5.0mg flutamide pellet (Flu). Castrated mice were implanted with various testosterone pellets 7 days prior to CA/CPR. Number of animals is indicated in each bar. Data presented as mean $\pm$ SEM. * $p < 0.05$ compared to vehicle.

Figure 5. Testosterone increases neurological deficit

Quantification of neuroscore days 1–3 after recovery from CA/CPR. Neuroscore is a 23 point scale with 0 corresponding to no impairment. Castrated mice were implanted with various testosterone pellets 7 days prior to CA/CPR. Data presented as mean $\pm$ SEM.

* $p < 0.05$ compared to vehicle.

Table 1

Bodyweight, cardiac arrest parameters and survival rates

	Vehicle	0.5mg	0.5mg+Flu	1.5mg	5.0mg	10.0mg
Body Weight (gms)	23.0±0.5	23.9±0.6	22.2±0.2	24.9±0.4	23.7±0.4	23.9±0.4
Baseline MABP (mmHg)	88.2±4.3	88.8±2.4	88.8±2.8	89.2±3.2	81.6±3.1	80.2±3.2
Total Ischemia Time (min.)	11.14±0.09	11.31±0.10	11.05±0.09	11.02±0.09	11.13±0.09	11.27±0.16
Epinephrine (µg)	10.01±0.26	11.4±0.29	11.0±0.29	9.9±0.35	10.1±0.28	10.1±0.21
Survival	15/22 (68%)	17/25 (68%)	11/16 (69%)	15/20 (75%)	14/17 (82%)	10/14 (71%)

No differences found among groups. MAPB = mean arterial blood pressure; Flu = flutamide. Total ischemia time represents total time from induction of cardiac arrest to successful resuscitation (spontaneous circulation). Data are presented as mean ± SEM.