Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model

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Yang, Shao-Hua, Evelyn Perez, Jason Cutright, Ran Liu, Zhen He, Arthur L. Day, and James W. Simpkins. Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model. J Appl Physiol 92: 195–201, 2002.—Increasing evidence has demonstrated striking sex differences in the outcome of neurological injury. Whereas estrogens contribute to these differences by attenuating neurotoxicity and ischemia-reperfusion injury, the effects of testosterone are unclear. The present study was undertaken to determine the effects of testosterone on neuronal injury in both a cell-culture model and a rodent ischemia-reperfusion model. Glutamate-induced HT-22 cell-death model was used to evaluate the effects of testosterone on cell survival. Testosterone was shown to significantly increase the toxicity of glutamate at a 10 μM concentration, whereas 17β-estradiol significantly attenuated the toxicity at the same concentration. In a rodent stroke model, ischemia-reperfusion injury was induced by temporal middle cerebral artery occlusion (MCAO) for 1 h and reperfusion for 24 h. To avoid the stress-related testosterone reduction, male rats were castrated and testosterone was replaced by testosterone pellet implantation. Testosterone pellets were removed at 1, 2, 4, or 6 h before MCAO to determine the duration of acute testosterone depletion effects on infarct volume. Ischemic lesion volume was significantly decreased from 239.6 ± 25.9 mm³ in control to 122.5 ± 28.6 mm³ when testosterone was administered 2, 4, or 6 h before MCAO. Reduction of lesion volume was associated with amelioration of the hyperemia during reperfusion. Our in vitro and in vivo studies suggest that testosterone possesses neuroprotective properties (3, 12, 33, 42), which have led to a growing appreciation of the positive impact of estrogens on the central nervous system. In contrast, effects of androgens on the central nervous system are much less studied.

Testosterone has been shown to be a survival factor for axotomized motoneurons and promotes motor axon regeneration (21, 22). Recently, several in vitro studies suggested that testosterone possessed neuroprotective effects on cerebellar granule neuron (1, 2). In view of the proposed neuroprotective effects both of estrogens and androgens, effects of sex difference on the outcome of stroke (3, 27, 44) could not be explained by sex hormones. We have previously reported that chronic testosterone replacement increased while chronic castration and chronic 17β-estradiol treatment decreased ischemic damage related to middle cerebral artery occlusion (MCAO) in male rats (19) and that the decrease of ischemic lesion volume with chronic 17β-estradiol treatment was associated with a marked reduction of testosterone level in intact males (19). In the present study, effects of acute testosterone depletion on ischemic stroke were evaluated. Our objective was twofold. First, direct effects of testosterone on neuronal survival were evaluated in a HT-22 cell-culture model using glutamate insult. Second, effects of acute testosterone depletion on ischemic lesion volume from MCAO were assessed in male rats. Our strategy was to compare the ischemic lesion volume from MCAO between testosterone depletion animals and animals with physiological level testosterone. Sustained physiological testosterone levels were obtained by castration and steroid pellet replacement technique, which our

GONADAL STEROID HORMONES such as androgens and estrogens may affect various target tissues throughout the body, including central nervous system. Clinical evidence has demonstrated striking sex differences in the incidence and outcome of stroke (27), which precipitated the studies of the potential impact of gonadal steroid hormones in disturbances of the central nervous system. A major focus in basic and clinical research in the last decade has been related to the activities of estrogens. Although the impact of postmenopausal estrogen-replacement therapy on stroke prevention and stroke severity remains inconsistent (7, 26), data from experimental studies in laboratory animals suggest that estrogens may have neuroprotective properties (3, 12, 33, 42), which have led to a growing appreciation of the positive impact of estrogens on the central nervous system. In contrast, effects of androgens on the central nervous system are much less studied.

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laboratory has previously reported (19). Acute testoster-
one depletion was achieved by pellets withdrawn 2
days after castration and testosterone pellet implanta-
tion, whereas sham withdrawal was used to maintain
physiological testosterone levels in control. By using
this strategy, the effects of timed depletion of testos-
terone before ischemic insult on the lesion volume and
regional cerebral blood flow (CBF) from temporary
ischemia before ischemic insult on the lesion volume and
this strategy, the effects of timed depletion of testos-
terone Silastic pellets containing crystalline steroid were
implanted subcutaneously immediately thereafter. Blood
samples (0.5 ml each time) were taken via jugular vein at 24
(n = 4) and 48 h (n = 4) after the implantation of steroid
pellets under methoxyflurane inhalant anesthesia. Then the
pellets were removed and blood samples were taken via
jugular vein at 1, 2, 4, 8, 12, and 24 h (n = 4 each time point)
after steroid pellet removal. Serum was separated from blood
cells by centrifugation and stored frozen (−20°C). Serum
testosterone concentrations were determined by using dupli-
cate serum aliquots in a radioimmunoassay (Diagnostic Sys-
tems Laboratories, Los Angeles, CA). Animals used for tes-
tosterone assessment were not used for ischemia outcome
studies.

**Experiment protocol.** Two days after castration and testos-
terone pellet implantation, ischemic stroke was induced in
animals after testosterone pellet removal or sham removal.
Pellets were removed in the testosterone depletion groups at 1
(n = 7), 2 (n = 5), 4 (n = 5), or 6 h (n = 5) before ischemia
under methoxyflurane inhalant anesthesia. Sham pellet re-
moval was performed in the physiological testosterone level
group as control in the same condition as pellet removal at 1
(n = 5), 4 (n = 5), and 6 h (n = 5) before MCAO. Ischemic
stroke was induced by MCAO described as before (18, 31).
Briefly, animals were anesthetized by intraperitoneal injec-
tion of ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal
temperature was monitored and maintained between 36.5
and 37.5°C during the procedure. With the aid of an operat-
ing microscope, the left common carotid artery (CCA), exter-
nal carotid artery (ECA), and internal carotid artery (ICA)
were exposed through a midline cervical skin incision. CCA
and ECA were permanently cauterized. A 3–0 monofilament
suture was introduced into the ICA via ECA lumen and
advanced until resistance was encountered. The distance
between the CCA bifurcation and the resistive point was
~1.9 cm. The middle cerebral artery was occluded for 1 h,
and then the suture was withdrawn for reperfusion. ICA was
coagulated, and the skin incision was closed.

Animals in each group were decapitated 24 h after reper-
fusion. Then the brain was harvested and placed in a metallic
brain matrix for tissue slicing (Harvard Apparatus, Holli-
ston, MA). Seven slices were made at 3, 5, 7, 9, 11, 13, and 15
mm posterior to the olfactory bulb. Each slice was incubated
for 30 min in a 2% solution of 2,3,5-triphenyltetrazolium
chloride (TTC) in physiological saline at 37°C and then fixed
in 10% formalin. Stained slices were photographed by a
digital camera (Sony MVC-FD5, Tokyo, Japan) and subse-
quenty measured for the surface area of the slices and the
ischemic lesion (Image-Pro Plus 4.1, Media Cybernetics, Sil-
ver Spring, MD).

**Regional CBF measurement and physiological parameters
monitor.** In a separate study, MCAO was induced 6 h after
pellet (n = 6) or sham removal (n = 6). Left femoral artery
was canialized and connected to a blood pressure monitor.
Arterial blood samples (150 μl each time) were taken before,
30 min during, and 30 min after MCAO, respectively. Phys-
iological parameters were measured by an ISTAT portable
clinical analyzer (East Windsor, NJ).

Hydrogen clearance blood flowmeter (Digital UH meters,
Unique Medical, Tokyo, Japan) was used for regional CBF
measurement. Two Teflon-coated platinum probes were ste-
reotaxically inserted into the core area of ischemia (0.5 mm
posterior bregma, 4 mm lateral, and 5 mm depth). Regional
CBF was monitored bilaterally during occlusion and within
30 min after reperfusion in testosterone pellet removal
and sham removal groups.

**Statistical analysis.** All data are presented as means ± SE.
Cell death, CBF, ischemic volumes, and physiological param-
eters in each group were compared by one-way ANOVA
followed by Tukey tests. A probability of <0.05 was consid-
ered significant.

**RESULTS**

**Effect of testosterone and 17β-estradiol on glutamate
toxicity.** Ten micromolar testosterone significantly in-
creased glutamate toxicity to 87.5 ± 3.7% of cells
killed, compared with 71.9 ± 6.9% at 0 μM testosterone.
Opposite to the deleterious effect of testosterone,
10 μM 17β-estradiol ameliorated glutamate toxicity to 40.3 ± 3.1% of cells killed, compared with 78.3 ± 3.3% at 0 μM 17β-estradiol (Fig. 1).

Testosterone concentrations in testosterone replacement and withdrawal animals. Subcutaneous implantation of testosterone pellets increased serum testosterone concentrations to 2.58 ± 0.47 and 1.83 ± 0.13 ng/ml at 1 and 2 days after implantation, respectively, both of which are within the reported physiological range of testosterone in male rats (Fig. 2). Serum testosterone concentrations decreased to 0.24 ± 0.01 ng/ml at 1 h after removal of the pellets. Thereafter, testosterone concentrations decreased to <0.08 ng/ml, the limits of sensitivity of the assay (Fig. 2).

Effect of testosterone on ischemic lesion volume. Ischemic lesion volume was significantly decreased when testosterone pellets were removed at 6 h before MCAO. Lesion volume was 217.8 ± 24.69, 192.6 ± 13.90, 151.3 ± 45.54, and 122.5 ± 28.62 mm³ at 1, 2, 4, and 6 h after pellet removal, respectively, compared with 239.6 ± 25.89 mm³ in control animals in which physiological testosterone concentrations were maintained (Fig. 3). As no differences in ischemic lesion volume were found between sham pellet removal animals at 1, 4, or 6 h before MCAO, all sham pellet removal animals were pooled together as controls.

Effect of testosterone on blood pressure, gases, pH, ions, and regional CBF. Physiological parameters are shown in Table 1. There were no significant differences between testosterone and testosterone-depletion groups for any parameters measured.

Regional CBF decreased to 8.7 ± 2.1 and 7.5 ± 1.9 ml·min⁻¹·100 g tissue⁻¹ during MCAO in the testosterone and testosterone-depletion groups, respectively. Hyperemia was observed during reperfusion in the testosterone group, which showed a CBF of 82.2 ± 12.2 ml·min⁻¹·100 g tissue⁻¹ compared with the nonspheric side in the testosterone and testosterone-depletion groups (P < 0.05), which had CBF of 32.0 ± 1.7 and 46.0 ± 3.6 ml·min⁻¹·100 g tissue⁻¹, respectively. In the testosterone-depletion group, no significant hyperemia was observed (Fig. 4).

Fig. 2. Testosterone concentrations in testosterone-replacement and -depletion animals. A: rats (n = 4) were castrated, and two testosterone pellets were implanted subcutaneously. Testosterone concentrations were maintained at 2.58 ± 0.47 and 1.83 ± 0.13 ng/ml at 1 and 2 days after implantation, respectively. B: testosterone concentration decreased to 0.24 ± 0.01 ng/ml at 1 h after removal of the pellets. Thereafter, testosterone concentration decreased to <0.08 ng/ml. Values are means ± SE. *P < 0.05 vs. pellets in.
did not affect the ischemia-reperfusion injury by using a similar model (36). Two reasons could contribute to the different result between these studies: 1) difference in the duration of MCAO, which was 1 h in the former study compared with 2 h in the latter study; and 2) wide range of testosterone concentrations in noncastrated animals in the latter study, which ranged from 0.05 to 1.62 ng/ml. The wide range of testosterone concentrations in the intact male animals could have resulted from the different kinds of stress and daily rhythms of testosterone. Testosterone had a daily rhythm in young male rats, with daily troughs as low as ~0.5 ng/ml and peaks as high as 2.0 ng/ml (32). In the present study, castration and testosterone-replacement techniques were used to evaluate the effects of acute testosterone depletion on ischemia-reperfusion injury. This technique produces a sustained physiologically relevant testosterone level and avoids the influence of stress and daily rhythms in testosterone levels. Testosterone levels decline rapidly in response to both physical and psychological stress (14), and testosterone levels are reduced in stroke patients (10, 16). Testosterone levels have been shown to be inversely associated with stroke severity and 6-mo mortality, whereas estradiol levels were not reduced in stroke patients (20). We have also shown that testosterone levels decrease significantly after MCAO (Fig. 5). Physiological consequences of this response are still unclear. It has been shown that adrenomedullary activation may be influenced by the stress-induced decline in testosterone (15). Testosterone receptor blockade using flutamide appeared to ameliorate the depressed adrenal function in males after trauma and severe hemorrhagic shock (5). Stress-induced testosterone reduction could positively influence stroke outcome through adrenomedullary activation. In the present study, acute depletion of testosterone significantly decreased the ischemic lesion volume, which suggests that stress-related testosterone reduction could be a protective response.

Interestingly, acute depletion of testosterone before ischemic insult caused a time-dependent improvement in MCAO outcome. One of the reasons for the time-dependent effects of testosterone depletion could have resulted from the delayed degradation of testosterone in the brain. Our previous study suggested that plasma testosterone was a primary determinant of the size of ischemic lesions following MCAO in male rats (19). The half-life of serum testosterone is very short, and serum testosterone decreased to an undetectable level within 2 h after pellet removal. Testosterone is highly hydrophobic and is cleared much more slowly from lipid-rich tissue, such as brain tissue, than from blood. So central nervous system effects of testosterone can persist after androgen depletion (13). Decreased effects of testosterone depletion also suggest that the effects could be mediated through a transcriptional mechanism, which could take 4 to 6 h to terminate.

Our data show that there was a similar CBF reduction in both the testosterone and testosterone-depletion groups during MCAO. Hyperemia was shown clearly in the ischemic side within 30 min after reperfusion compared with the contralateral side in the testosterone group but not in the testosterone-depletion group. This suggests that the deleterious effects of testosterone could be CBF related. Reactive hyperemia and delayed hyporemia have been found during reper-

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**Table 1. Physiological parameters in rats subjected to transient MCAO**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before MCAO</th>
<th>During MCAO</th>
<th>After MCAO</th>
<th>Before MCAO</th>
<th>During MCAO</th>
<th>After MCAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP, mmHg</td>
<td>87.0 ± 2.5</td>
<td>77.8 ± 2.5</td>
<td>87.0 ± 5.4</td>
<td>84.3 ± 1.9</td>
<td>82.0 ± 5.6</td>
<td>80.4 ± 2.7</td>
</tr>
<tr>
<td>Po2, Torr</td>
<td>48.8 ± 2.9</td>
<td>46.9 ± 1.3</td>
<td>45.3 ± 1.3</td>
<td>56.1 ± 2.3</td>
<td>47.6 ± 4.8</td>
<td>42.1 ± 3.9</td>
</tr>
<tr>
<td>PO2, Torr</td>
<td>78.2 ± 7.2</td>
<td>73.0 ± 3.1</td>
<td>86.0 ± 4.3</td>
<td>67.2 ± 4.1</td>
<td>71.0 ± 7.2</td>
<td>80.6 ± 8.3</td>
</tr>
<tr>
<td>HCO3−, mmol/l</td>
<td>26.6 ± 0.4</td>
<td>25.8 ± 0.2</td>
<td>25.0 ± 0.3</td>
<td>27.0 ± 0.7</td>
<td>26.2 ± 1.1</td>
<td>25.2 ± 0.9</td>
</tr>
<tr>
<td>SO2, %</td>
<td>93.2 ± 2.3</td>
<td>93.2 ± 0.9</td>
<td>95.7 ± 0.8</td>
<td>88.8 ± 2.9</td>
<td>90.8 ± 3.7</td>
<td>94.2 ± 1.8</td>
</tr>
<tr>
<td>pH</td>
<td>7.35 ± 0.02</td>
<td>7.35 ± 0.01</td>
<td>7.35 ± 0.01</td>
<td>7.29 ± 0.02</td>
<td>7.36 ± 0.03</td>
<td>7.39 ± 0.03</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>14.4 ± 0.2</td>
<td>14.0 ± 0.5</td>
<td>14.6 ± 0.5</td>
<td>14.5 ± 0.2</td>
<td>14.0 ± 0.0</td>
<td>14.4 ± 0.2</td>
</tr>
<tr>
<td>Na+, mmol/l</td>
<td>141.4 ± 0.9</td>
<td>140.0 ± 0.9</td>
<td>140.2 ± 1.6</td>
<td>141.2 ± 1.4</td>
<td>138.0 ± 1.1</td>
<td>139.8 ± 1.5</td>
</tr>
<tr>
<td>K+, mmol/l</td>
<td>4.5 ± 0.1</td>
<td>5.3 ± 0.2</td>
<td>5.0 ± 0.3</td>
<td>4.8 ± 0.1</td>
<td>5.8 ± 0.2</td>
<td>5.1 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6 for each group). MCAO, middle cerebral artery occlusion; MABP, mean arterial blood pressure. There were no significant differences between treatment groups at any sampling time.
fusion, and both are thought to be harmful to ischemic tissue (34, 37). Ischemic edema and blood-brain barrier disruption have been found to be exacerbated after acute reperfusion, which is related to the sudden surge reperfusion with hyperemia (23, 41). Gradual blood flow restoration could significantly reduce the exacerbation of ischemic edema and blood-brain barrier opening (17). As such, the damaging effects of testosterone could have partially resulted from reactive hyperemia during reperfusion.

The mechanism of testosterone’s effect on CBF is unclear. Testosterone has been shown to be vasoactive in the peripheral artery system. Treatment with testosterone causes a vasorelaxant response in rabbit coronary arteries (43). Other studies (29, 39) also indicated that testosterone infusion into coronary arteries in men with coronary artery disease induced vasodilation and that intravenous administration of testosterone reduced exercise-induced ischemic response in men with coronary artery disease. Testosterone’s effect on vascular tone could be because of aromatization of testosterone into estradiol, as aromatase has been identified in the arterial wall (11). However, 17β-estradiol inhibits Ca$^{2+}$ entry, whereas testosterone causes coronary relaxation by inhibiting other mechanisms in addition to Ca$^{2+}$ entry (8). Furthermore, testosterone has been shown to exacerbate, whereas estrogen decreases, the vulnerability of lateral striatal artery to chemical hypoxia (25). The direct mechanism of testosterone action on arteries should also be taken into account.

Consistent with our in vivo study, testosterone was shown to exacerbate glutamate toxicity in an in vitro model. Toxic insults by glutamate in neuronal cell culture mimic a key component of ischemic brain injury. Microdialysis studies have shown that there is a severalfold increase in extracellular glutamate during global ischemia, beginning within 1–2 min (6, 24). There is a similar rise during focal ischemia, beginning within 2 min of MCAO (38). Furthermore, glutamate can cause both apoptosis and necrosis (28). In HT-22 cells, glutamate competes with cystine for uptake, leading to a reduction in glutathione, accumulation of reactive oxygen species, and ultimately cell death (35). The present study shows that testosterone treatment exacerbates glutamate toxicity to HT-22 cells, whereas 17β-estradiol treatment decreases the cells’ susceptibility to glutamate toxicity, which provides us in vitro evidence to support our in vivo study. Although the deleterious effects of testosterone are only present at the micromolar level in vitro, which is thousands of times higher than peak physiological levels in reproductive males, physiological levels of testosterone exert damaging effects on ischemia-reperfusion injury in vivo.

It has been shown that in vivo treatment of postnatal rats with testosterone rendered cerebellar granule neurons less vulnerable to oxidative stress-induced apoptosis in vitro, which was associated with increases in catalase activity as well as in the activity of superoxide dismutase (1). However, the decreased susceptibility to oxidative stress induced by the postnatal treatment with testosterone was more likely due to an accelerated maturation with a consequent development dependent increase in the antioxidant defense (30). Effects of testosterone could be different in mature animals, as was shown with cerebral ischemia in our study. Testosterone treatment in vitro has also been shown to be neuroprotective for cerebellar granule neurons (2). As 17β-estradiol is also neuroprotective in cerebellar granule neurons (9), the neuroprotective effects of testosterone could be due to the conversion of testosterone into 17β-estradiol by aromatization in vivo. However, 17β-estradiol inhibits Ca$^{2+}$ entry, whereas testosterone causes coronary relaxation by inhibiting other mechanisms in addition to Ca$^{2+}$ entry (8). Furthermore, testosterone has been shown to exacerbate, whereas estrogen decreases, the vulnerability of lateral striatal artery to chemical hypoxia (25). The direct mechanism of testosterone action on arteries should also be taken into account.

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![Fig. 4. Effect of testosterone on regional cerebral blood flow (CBF). T-ipsilateral, ischemic side in testosterone group; T-contralateral, contralateral side in testosterone group; T-depletion ipsilateral, ipsilateral side in testosterone-depletion group; T-depletion contralateral, contralateral side in testosterone-depletion group; MCAO, middle cerebral artery occlusion. Values are means ± SE. *P < 0.05 vs. T-contralateral and T-depletion contralateral. **P < 0.01 vs. T-depletion contralateral during MCAO.](image1)

![Fig. 5. Effect of ischemia-reperfusion injury on serum testosterone level in normal male rats (n = 7). Blood samples were taken before MCAO and 24 h after reperfusion. All blood samples were taken in the morning. Testosterone concentration was reduced to 0.73 ± 0.19 ng/ml at 24 h after reperfusion, compared with 2.46 ± 0.31 ng/ml before MCAO (P < 0.01). Values are means ± SE. ***P < 0.01 vs. testosterone concentration before MCAO.](image2)
matase. Furthermore, testosterone has been reported to attenuate neuronal death in mice in response to excitotoxins, which were blocked by aromatase (4).

In summary, the present data show that testosterone can increase neuronal toxicity and exacerbate ischemia-reperfusion injury. These results suggest that sex differences in the outcome after stroke may have resulted from both the protective effects of estrogens and the damaging effects of testosterone. Furthermore, acute depletion of testosterone provides neuroprotective effects on ischemia-reperfusion injury, which could be partially related to the amelioration of hyperemia during reperfusion.

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