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# Deleterious effects of dihydrotestosterone on cerebral ischemic injury

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**Outcome from cerebral ischemia is sexually dimorphic in many experimental models. Male animals display greater sensitivity to ischemic injury than do their female counterparts; however, the underlying mechanism is unclear. The present study determined if the potent and nonaromatizable androgen, dihydrotestosterone (DHT), exacerbates ischemic damage in the male rat and alters postischemic gene expression after middle cerebral artery occlusion. At 22 h reperfusion, removal of androgens by castration provided protection from ischemic injury in both cortex and striatum (2,3,5-triphenyltetrazolium chloride (TTC) histology), whereas DHT replacement (50 mg subcutaneous implant) restored infarction volume to that of the intact male; testosterone (50 mg) had similar but less potent effects. We utilized microarray and real-time quantitative polymerase chain reaction (PCR) to identify genes differentially expressed at 6 h reperfusion in periinfarct cortex from castrated rats with or without DHT replacement. We identify, for the first time, a number of gene candidates that are induced by DHT with or without ischemia, many of which could account for cell death through enhanced inflammation, dysregulation of blood–brain barrier and the extracellular matrix, apoptosis, and ionic imbalance. Our data suggest that androgens are important mediators of ischemic damage in male brain and that transcriptional mechanisms should be considered as we seek to understand innate male sensitivity to cerebral ischemia.**
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**Keywords:** androgens; cerebral ischemia; dihydrotestosterone; sex steroids

## Introduction

Men are at increased risk for stroke as compared with women until the end of the reproductive years. Sex-linked differences in experimental stroke outcomes have been modeled in adult and reproductively senescent rodents. Available data from experimental stroke suggest that females enjoy a protected phenotype in cerebral ischemia and that estrogen mediates, in part, the neuroprotection enjoyed by females (Hall *et al*, 1991; Alkayed *et al*, 1998, 2000). However, sexual dimorphism in stroke outcomes is difficult to explain solely by the female phenotype. Males sustain larger ischemic damage for a comparable insult relative to age-matched females, suggesting that there is also a male 'ischemia-sensitive' phenotype. Furthermore, the role of androgens in male stroke is unclear, and current data are surprisingly few and contradictory. In male rats, castration and subsequent testosterone loss either decreases or has little effect on the ischemic histological damage (Hawk *et al*, 1998; Toung *et al*, 1998; Yang *et al*, 2002). Other reports indicate that testosterone replacement in castrates exacerbates injury (Hawk *et al*, 1998; Yang *et al*, 2002). In contrast, a single report suggests that androgens improve functional recovery after stroke (Pan *et al*, 2005). *In vitro*, androgens provide protection in primary neuronal culture after oxidative stress, $\beta$-amyloid toxicity, and serum deprivation (Ahlbom *et al*, 2001, 1999; Zhang *et al*, 2004; Hammond *et al*, 2001) or can amplify excitotoxicity (Caruso *et al*, 2004).

Although testosterone is the most abundant mammalian androgen, it acts as a 'prohormone' in brain that can be converted into dihydrotestosterone (DHT) or into 17$\beta$-estradiol (Lephart *et al*, 2001).

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Dihydrotestosterone is the more potent agonist of the single-known androgen receptor and frequently acts as an amplifier and executor of testosterone's actions. Thus, in the present study, we determined if DHT exerts deleterious effects on histological ischemic outcome after middle cerebral artery occlusion (MCAO) in rats. Because androgens are known to act by receptor-mediated, transcriptional mechanisms, we next examined DHT's effect on bcl-2 and bax mRNA levels. These genes have been shown to play essential roles in postischemic apoptotic signaling (Alkayed *et al*, 2001; Graham and Chen, 2001). Finally, to evaluate more comprehensively if DHT shifts brain transcriptional responses toward ischemic sensitivity, global gene expression was examined in castrated and DHT-replaced males using microarray technology.

## Materials and methods

### Experimental Animals

This study was conducted in accordance with National Institute of Health guidelines, and all protocols were approved by the Animal Care and Use Committee of Oregon Health and Science University. Male rats were castrated with or without subcutaneous hormone replacement under halothane anesthesia 1 week before MCAO, as previously described (Toung *et al*, 1998). Gonadally intact animals were sham operated under halothane anesthesia 1 week before MCAO. For infarction studies, four groups of sexually mature, age-matched adult (9- to 10-week-old) Wistar rats (Charles River Laboratories, Wilmington, MA, USA) were studied: intact ($n=9$), castrated ($n=14$), testosterone replaced, castrated males (50 mg/pellet, Innovative Research of America, Sarasota, FL, USA) ($n=14$) and 5$\alpha$-dihydrotestosterone (DHT, 50 mg/pellet, Innovative Research of America, Sarasota, FL, USA) replaced, castrated males ($n=8$). Postischemic *bcl-2* and *bax* transcription was examined in separate cohorts of intact, castrated, and DHT-replaced males ($n=6$). Microarray analysis and quantitative PCR (qPCR) confirmation was performed only in castrated ($n=3$) and DHT-replaced males ($n=4$).

### Middle Cerebral Artery Occlusion

Animals were anesthetized and operated as previously described (Toung *et al*, 1998) under halothane anesthesia (1% to 2% via mask in $O_2$-enriched air). A femoral artery catheter was placed to monitor mean arterial blood pressure continuously and measure arterial blood gases. Rectal and temporalis muscle temperature were continuously monitored (Mon-a-therm, Mallinckrodt Medical Inc., St Louis, MO, USA) and maintained with heating lamps. Middle cerebral artery occlusion (2 h) was achieved with a modified intraluminal filament technique as previously described (Alkayed *et al*, 1998). Adequacy of the vessel occlusion and reperfusion was assessed by laser-Doppler flow signal (LDF, Moor Instruments LTD, Oxford, England), obtained with a probe placed 6 mm lateral and 2 mm posterior to bregma. Occlusion was accomplished by introducing a 4.0 nylon monofilament surgical suture with a silicon-coated tip through right common carotid artery and internal carotid artery until an abrupt and significant reduction was observed in LDF. Laser-Doppler flow was continuously monitored during the artery occlusion and 30 min of reperfusion to confirm a uniform insult among animals. For tissue damage analysis, rats were decapitated at 22 h reperfusion, and plasma testosterone and DHT levels were determined by radioimmunoassay. The brain was removed and sectioned into seven 2-mm-thick coronal sections. The slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St Louis, MO, USA) for 10 min on each side at 37°C, fixed in 10% formalin overnight, and then photographed. Images were analyzed with Image Analysis Software (Sigma Scan Pro, Jandel, San Rafael, CA, USA). Infarction size was expressed as a percentage of the contralateral structure as a correction for edema.

### Microdissection and RNA Preparation

For qPCR measurement of bcl-2 and bax, brains were harvested at 6 or 22 h reperfusion and sectioned into seven 2-mm-thick coronal sections. Two slices that encompass the middle cerebral artery (MCA) territory (between +2 and −2 mm relative to Bregma) were frozen on dry ice, and micropunched from cortical regions (periinfarct zone, ischemic core, and transition zone between periinfarct zone and ischemic core) and two striatal regions (periinfarct zone and ischemic core), using 1 mm internal diameter Micron Punch (Zivic Laboratories, Pittsburgh, PA, USA) (Figure 1), as previously described (Alkayed *et al*, 2001, 2002). Identical locations were harvested from contralateral tissue. Total RNA was isolated using the

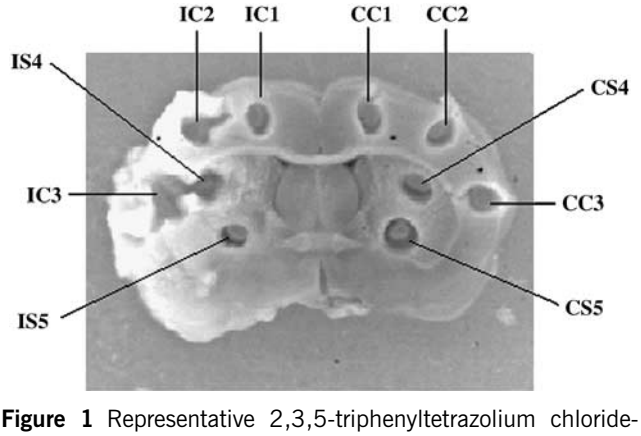


**Figure 1** Representative 2,3,5-triphenyltetrazolium chloride-stained brain sections (slice 4) of rat at 22 h reperfusion after 2 h MCAO. Infacted tissue is white and live tissue is darkly stained by 2,3,5-triphenyltetrazolium chloride. The micropunch regions, which are represented by holes, were analyzed for bax and bcl-2 mRNA levels by qPCR techniques and microarray analysis. IC1, periinfarct zone in cortex; IC2, transition zone in cortex; IC3, ischemic core in cortex. IS4, ischemic core in striatum; IS5, periinfarct zone in striatum.

RNAqueous-Micro kit (Ambion, Austin, TX, USA) as per the manufacturer's instructions. Briefly, each micropunch was homogenized in 100 $\mu$L lysis buffer. Total RNA was isolated, eluted from column with 20 $\mu$L RNase-free elution buffer, and further treated with Turbo DNase (Ambion, Austin, TX, USA,) and then stored at $-80^{\circ}$C.

### Microarray Analysis

For each animal, tissue was collected from cortical ipsilateral periinfarct zones and the corresponding sites of the contralateral sides of the slice 3 and 4 at 6 h reperfusion with micropunch techniques as mentioned above (Figure 1). To obtain adequate RNA samples, tissue from each punch was pooled from both slices for each animal. Quantity and quality of total RNA were determined using Agilent Bioanalyzer 2100, and microarray study was performed at the OHSU Gene Microarray Shared Resource using Affymetrix Expression Analysis (RAT genome 230 2.0 chip). Briefly, 1 $\mu$g RNA was extracted from each pooled sample and used for synthesis of double-strand complementary DNA (cDNA). Biotin-labeled complementary RNA (cRNA) was transcribed *in vitro* from cDNA according to the Affymetrix One-cycle Target Labeling Protocol. Then, the biotin-labeled cRNA was fragmented and hybridized to an expression chip, containing 31,000 oligonucleotide probe sets that represent 28,000 genes and expression sequence tags (ESTs). The fluorescence image of hybridized microarray was scanned with GeneChip scanner 3000. Data analysis was conducted using Affymetrix GeneChip Operation System (Version 1.2), and values were converted into $\log_2$-transformed format. For comparison between different chips, global scaling was used, scaling all probe sets to a predefined target intensity. Two types of data analyses were conducted, as previously described (Xu *et al*, 2006). The first analysis consisted of a pairwise comparison between ipsilateral hemispheres of DHT-treated and untreated castrated animals (DI–CI) to isolate genes that exhibited two- or higher fold change in at least 6 out of 12 pairwise comparisons. Fold changes were calculated from the $\log_2$-transformed signal ratios for each transcript in the sample groups. In addition, genes differentially expressed between ipsilateral and contralateral hemispheres (I–C) were compared between DHT-treated (DI–DC) and untreated castrated groups (CI–CC).

### Quantitative Polymerase Chain Reaction

Complementary DNA was reverse transcribed from total RNA (500 ng) using a high-capacity cDNA synthesis kit (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative PCR reactions were performed on ABI Prism 7000 DNA Detection System (total 50 $\mu$L volume). Primers and Taqman probes for rat bcl-2 and bax were designed from known sequences for rat bcl-2 mRNA (GenBank accession no. NM_016993) and bax (GenBank no. AF235993) using Primer Express (Version 2.0, Applied Biosystem, Foster City, CA, USA). For bcl-2, the sequences were 5′-TGG GAT GCC TTT GTG GAA CTA T-3′ (forward primer), 5′-AGA GAC AGC CAG GAG AAA TCA AAC-3′ (reverse primer), and 5′-FAM-TGG CCC CAG CAT GCG ACC TC-TAMRA (probe). For bax, the sequences were 5′-GGG TGG TTG CCC TTT TCT ACT-3′ (forward primer), 5′-CCC GGA GGA AGT CCA GTG TC-3′ (reverse primer), and 5′-FAM-ACT GGT GCT CAA GGC CCT GTG CA-TAMRA (probe). 18S RNA was also assessed to serve as an internal control for each sample (Eurogentec North America Inc., San Diego, CA, USA). Final data were normalized to 18S RNA and expressed as the ratios to contralateral values.

Ten candidate genes with a differential expression pattern between DI and CI were selected for confirmation by qPCR. Real-time qPCR confirmation was performed on total RNA isolated from newly prepared cortical tissue ($n=3$ for each group). The primers and probes were obtained from Applied Biosystem for qPCR confirmation of prostaglandin-endoperoxide synthase 2 (COX-2, cat. no. Rn00568225_m1), interleukin-6 (IL-6, cat. no. Rn00561420_m1), tissue inhibitor of metalloproteinase 1 (Timp 1, cat. no. Rn00587558_m1), prepronociceptin (Pnoc, cat. no. Rn00564560_m1), tissue factor pathway inhibitor 2 (Tifpi2, cat. no. Rn00597628_m1), intercellular adhesion molecule-1 (ICAM-1, cat. no. Rn00564227_m1), interleukin 1β (IL-1$\beta$, cat no. Rn00580432_m1), SNF1-like kinase (Snf1lk, cat. no. Rn01429325_m1), and wingless-related mouse mammary tumor virus integration site 4 (Wnt4, cat. no. Rn00584577_m1). Primers and probe for qPCR confirmation of trombospondin-1 were designed from the known sequence (AF309630) using Primer Express (Version 2.0, Applied Biosystem, Foster City, CA, USA): 5′-GGA AGA GCA TCA CGC TGT TTG-3′ (forward primer), 5′-GGG CTC TCC ATC TTG TCA CA-3′ (reverse primer), and 5′-FAM-CCA AGA AGA CAG GGC CCA GCT CTA CA-TAMRA (probe).

### Statistical Analysis

Values were expressed as mean $\pm$ s.e.m. and subjected to one-way or two-way analysis of variance (physiological variables) with *post hoc* Newman–Keuls test. Statistical significance was set at $P<0.05$. All statistical analyses were performed using SigmaStat Statistical Software, Version 2.0 (SPSS Inc., Chicago, IL, USA).

## Results

### Effects of Castration, Dihydrotestosterone, and Testosterone on Infarct Volume

There were no differences in physiologic data among the treatment groups (Table 1). Plasma total testosterone and DHT were $4.8\pm2.4$ and $0.5\pm0.2$ ng/mL, respectively, in intact males. Castration reduced both steroids to below the detection threshold. Testosterone and DHT replacement resulted in plasma concentrations of $37.6\pm6.9$ and $2.6\pm1.3$ ng/mL, respectively. Intraischemic LDF was not different among the groups: $30\%\pm2\%$ (intact), $30\%\pm2\%$ (castrates), $35\%\pm2\%$ (DHT-replaced), and

**Table 1** Physiologic data before, during, and after occlusion

| *Group (n)* | *pH* | *MAP (mm Hg)* | *PaCO2 (mm Hg)* | *PaO2 (mm Hg)* | *Glucose (mg/dL)* | *Rectal temperature (°C)* | *Temporalis muscle temperature (°C)* |
|---|---|---|---|---|---|---|---|
| *Baseine* | | | | | | | |
| Intact (9) | 7.40±0.01 | 91±2 | 50±1 | 140±7 | 122±4 | 36.7±0.2 | 36.2±0.1 |
| Cas (14) | 7.42±0.01 | 97±2 | 49±1 | 150±5 | 124±3 | 36.7±0.2 | 36.2±0.1 |
| DHT (8) | 7.41±0.01 | 92±1 | 49±2 | 143±5 | 123±7 | 37.1±0.2 | 36.2±0.1 |
| Tes (14) | 7.40±0.01 | 93±3 | 50±2 | 152±5 | 124±5 | 37.0±0.2 | 36.1±0.1 |
| *60 mins MCAO* | | | | | | | |
| Intact (9) | 7.40±0.01 | 99±2 | 49±1 | 128±5 | 131±5 | 37.0±0.4 | 36.4±0.1 |
| Cas (14) | 7.40±0.01 | 100±4 | 50±1 | 135±6 | 136±3 | 36.8±0.2 | 36.3±0.1 |
| DHT (8) | 7.41±0.01 | 100±2 | 48±2 | 139±6 | 126±7 | 37.4±0.2 | 36.6±0.1 |
| Tes (14) | 7.42±0.01 | 100±2 | 49±1 | 133±5 | 121±6 | 37.5±0.2 | 36.4±0.1 |
| *15 mins reperfusion* | | | | | | | |
| Intact (9) | 7.40±0.02 | 89±4 | 51±1 | 130±5 | 132±7 | 36.8±0.3 | 36.8±0.1 |
| Cas (14) | 7.41±0.01 | 84±3 | 51±1 | 140±5 | 136±4 | 37.3±0.2 | 36.5±0.1 |
| DHT (8) | 7.44±0.01 | 86±3 | 48±1 | 150±4 | 124±5 | 37.5±0.1 | 36.9±0.2 |
| Tes (14) | 7.42±0.01 | 90±3 | 48±1 | 139±4 | 121±6 | 37.5±0.2 | 36.6±0.1 |

Cas, castrated male; DHT, DHT (50 mg/pellet)-treated castrated male; intact, intact male; MAP, mean arterial pressure; Tes, testosterone (50 mg/pellet)-treated castrated male.
Data are expressed as mean±s.e.m.

31%±2% (testosterone-replaced). As shown in Figure 2, castrated males sustained smaller infarcts than intact males in cerebral cortex (15.2%±5.73% versus 33.6%±4.2%) and striatum (42.5%±6.3% versus 75.3%±5.0%). Dihydrotestosterone replacement reversed the beneficial effect of castration and increased cortical and striatal infarcts to 42.3%±4.4% and 88.1%±4.4%, respectively, which were equivalent to that of intact males. Testosterone replacement yielded larger striatal infarcts (82.2%±3.7%) as compared with castrated males; however, cortical infarction damage was unchanged (24.8%±4.1%).

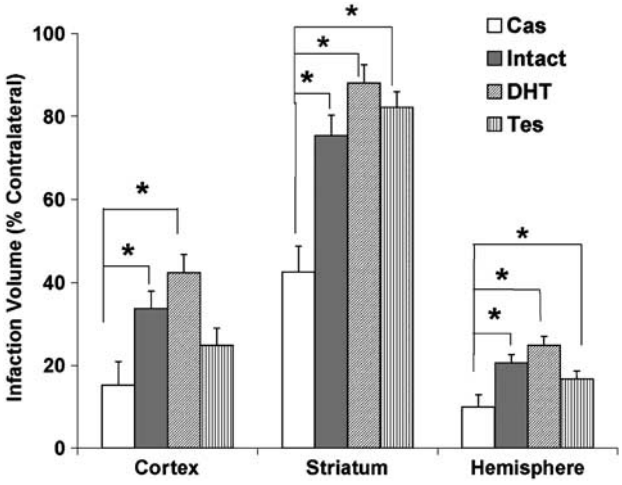


**Figure 2** Infarct volume in castrated males, intact males, DHT-supplemented, and testosterone-supplemented castrated males. A significant cortical infarct size decrease was seen in castrated males compared with intact and DHT-supplemented castrated males. A significant decrease in striatal infarcts was also seen in castrated males when compared with intact, DHT-supplemented, and testosterone-supplemented castrated males. * $P < 0.05$.

### Effects of Dihydrotestosterone Replacement on Postischemic Bcl-2 and Bax mRNA

At 22 h reperfusion after MCAO, remarkable RNA degradation was detected in ischemic core but not in periinfarct zones as assessed by Agilent Bioanalyzer or 18S RNA qPCR (data not shown). Accordingly, we assessed bcl2 and bax by brain region (Figure 1) at both 6 h and 22 h reperfusion. Intact and DHT-treated animals, but not castrated animals, displayed elevated bax mRNA as compared with contralateral samples at 22 h reperfusion (Figure 3). There was no differential transcription of bcl-2 at any region or time point.

### Dihydrotestosterone Replacement and Microarray

A large number of genes showed a differential expression pattern at 6 h reperfusion. In DHT-treated animals, 422 genes/ESTs were increased by MCAO and 358 were decreased. In castrates, 271 genes/ESTs were increased and 350 were depressed by ischemia. In total, 184 candidates overlapped in these comparisons. When ischemic cortex was compared between DHT and castration treatment groups (DI–CI), 421 genes were increased by DHT treatment and 239 genes were decreased.

We grouped the DI–CI candidates into functional categories based on their ontology (Table 2). The functional group related to inflammation and signaling had the largest number of regulated genes. In total, 17 inflammatory genes uniformly showed increased expression pattern in DHT-treated brain

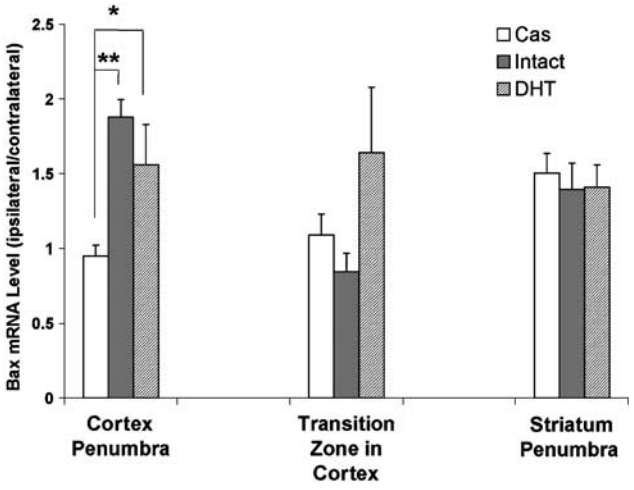


**Figure 3** Bax mRNA levels at 22 h reperfusion determined by qPCR assay. Castrated males had significantly decreased levels of bax mRNA in the cortex periinfarct zone compared with intact males and DHT-supplemented castrated males at 22 h reperfusion. The bax mRNA levels in the striatum periinfarct zone did not differ among the groups. * $P < 0.05$ and ** $P < 0.01$ ($n = 6$).

versus castrated brain at 6 h reperfusion. Twelve out of 17 genes are proinflammatory. *IL-1rap* and *IL-2rg* are required for proinflammatory cytokine IL-1 and IL-2 signaling transduction. CD14 is the biomarker for proinflammatory lymphocytes and crucial for proinflammatory cytokine generation. It is unclear whether CD9 and CD44 are pro or antiinflammatory.

Dihydrotestosterone also regulated a variety of genes related to extracellular matrix, channels, signaling cascades, and metabolism. Most of the genes related to signaling and metabolisms have not been implicated in the cerebral ischemic etiology. The results of this analysis imply that several mechanisms, such as enhanced inflammation, apoptosis, blood–brain barrier damage, protein kinase signaling, and ionic dysregulation, could be involved in DHT's deleterious effect on cerebral ischemia. We chose a small sample of these candidates for qPCR confirmation (Figure 4), selected for their potential importance in ischemia. Seven of 10 samples were confirmed to be present and altered by DHT.

## Discussion

This study presents two major findings. First, DHT, the principal nonaromatizable metabolite of testosterone, increases ischemic injury after experimental stroke. The steroid was more effective than testosterone in restoring infarct size in the cortex of castrated animals. Second, DHT replacement enhances transcription of a variety of genes early in reperfusion, particularly inflammatory genes, but also those functionally associated with apoptosis, maintenance of the extracellular matrix, kinase signaling, and metabolism. We speculate that DHT plays a vital role in male susceptibility to focal cerebral ischemia, in part by altering postischemic gene expression.

It is now well known that the magnitude of brain damage after experimental stroke is gender-specific (Alkayed *et al*, 1998; Hall *et al*, 1991; Hurn *et al*, 1995). Enhanced damage in the male versus female brain cannot be fully explained by estrogen's protective properties, and available data suggest that testosterone contributes to sexually dimorphic outcomes from cerebral ischemia (Hawk *et al*, 1998; Yang *et al*, 2002). The present findings extend this hypothesis, by showing that when equal doses of testosterone or DHT are replaced in castrates, DHT is the more potent steroid in exacerbating injury, at least in the cortex. Further, androgen aromatization products are not required. Although it is known that estrogen provides neuroprotection in part through vascular mechanisms (McCullough *et al*, 2001), effects of testosterone on periischemic cerebral blood flow are not known. The large differences in infarct volume in the present study are not explained by differences in intraischemic LDF as values were similar among treatment groups. However, LDF measures changes in cortical perfusion relative to baseline signal; so we cannot exclude the possibility that absolute cerebral blood flow was different in androgen-replaced versus castrated animals during ischemia or reperfusion. Further study is needed to elucidate potential vascular mechanisms by which androgens enhance ischemic damage.

In the present study, we utilized a continuous hormone replacement strategy, recognizing that this does not simulate the physiological, diurnal rhythms of androgen production. Young male animals usually exhibit low-basal total testosterone titers in plasma (e.g., 1 ng/mL), interspersed by transient 20-fold elevations (Aikey *et al*, 2002). Previously reported DHT levels in intact male rodents are similar to those of the DHT-replaced rats of the present study (e.g., 2.1 ng/mL; Angele *et al*, 2003). Accordingly, the androgen implants in this study achieved physiologically relevant plasma steroid levels.

Androgens exhibit an array of actions that could potentially account for their ability to exacerbate ischemic injury. Typically, androgen acts in most cell types by binding to its cognate receptor, which translocates into the nucleus and acts as a transcription factor. However, recent evidence also suggested that androgens exhibit rapid, nontranscriptional effects on signal transduction pathways (Doncarlos *et al*, 2006). Our intent in the present study was to describe and confirm a broad group of androgen-responsive genes that are also activated by cerebral ischemia, primarily for the purpose of future hypothesis generation. Although we focused on DHT's transcriptional effects in this study, we do not exclude the steroid's potential mechanisms of

**Table 2** Genes regulated by dihyrotestosterone at 6 h reperfusion after 2 h MCAO

| *Category* | *Name* | *Symbol* | *GenBank* | *Fold change* |
|---|---|---|---|---|
| Inflammation related | Prostaglandin-endoperoxide synthase 2 (COX-2) | Ptgs2 | U03389 | I (1.8) |
| | CD14 antigen | Cd14 | NM_021744 | I (2.6) |
| | CD 9 antigen | Cd9 | AI227627 | I (1.8) |
| | CD 44 antigen | Cd44 | BI302830 | I (1.9) |
| | Intercellular adhesion molecular 1 | Icam1 | NM_012967 | I (2.1) |
| | Chemokine (C-X-C motif) ligand 2 | Cxcl2 | NM_053647 | I (6.6) |
| | Chemokine (C-X-C motif) ligand 1 | Cxcl1 | NM_030845 | I (4.1) |
| | Chemokine (C-C motif) ligand 2 | Ccl2 | NM_031530 | I (5.9) |
| | Chemokine (C-C motif) ligand 7 | RGD:1359152 | BF419899 | I (11.6) |
| | Chemokine (C-C motif) ligand 3 (MIP-1$\alpha$) | Ccl3 | U22414 | I (4.4) |
| | Small inducible cytokine A4 (MIP-1$\beta$) | Ccl4 | U06434 | I (3.6) |
| | Interleukin 1$\alpha$ | IL1a | NM_017019 | I (3.8) |
| | Interleukin 1$\beta$ | IL1b | NM_031512 | I (4.4) |
| | Interleukin 6 | IL6 | NM_012589 | I (8.6) |
| | G4 protein (tumor necrosis factor | TNF | AA819227 | I (2.5) |
| | Interleukin 1-receptor accessory protein | IL1rap | BF391914 | I (2.3) |
| | Interleukin 2 receptor,$\gamma$ | IL2rg | AI178808 | I (2.8) |
| Extracellular matrix related | Thrombospondin 1 | Thbs1 | AI406660 | I (4.0) |
| | Tissue inhibitor of metalloproteinase 1 | Timp1 | NM_053819 | I (4.7) |
| | Matrix metallopeptidase 12 | Mmp12 | NM_053963 | I (3.9) |
| | Aggrecan 1 | Agc 1 | BM384639 | I (2.0) |
| | Chondrotin sulfate proteoglycan | Cspg2 | AF072892 | I (2.8) |
| | Claudin 5 | Cldn5 | BI281680 | D (2.7) |
| Apoptosis related | Sspingosine kinase 1 | Sphk1 | AB049572 | I (4.3) |
| | B-cell leukemia/lymphoma 2 related protein A1 | Bcl2a1 | NM_133416 | I (2.1) |
| | Inhibitor of apoptosis protein 1 | Birc3 | NM_023987 | I (2.3) |
| Signaling | Dual specificity phosphatase 5 | Dusp5 | NM_133578 | I (3.5) |
| | Dual specificity phosphatase 6 | Dusp6 | AI602811 | I (1.8) |
| | Regulator of G-protein signaling 2 | Rgs2 | AF321837 | I (2.3) |
| | Gastric-inhibitory polypeptide receptor | Gipr | NM_012714 | I (2.6) |
| | Proprotein convertase subtilisin/kexin type 1 | Pcsk1 | NM_017091 | I (3.7) |
| | SNF-like kinase | Snf1lk | NM_021693 | I (2.6) |
| | Phosphodiesterase 4D | Pde4d | NM_017032 | I (2.0) |
| | Rad and gem related GTP-binding protein | Rem2 | NM_022685 | I (2.3) |
| | GTP cyclohydrolase 1 | Gch | NM_024356 | I (2.0) |
| | Suppressor of cytokine signaling 2 | Socs2 | BM384088 | I (1.9) |
| | Suppressor of cytokine signaling 3 | Scos3 | NM_053565 | I (1.8) |
| | cAMP responsive element modulator | Crem | NM_017334 | I (3.9) |
| | Adenylyl cyclase | Adcy8 | NM_017142 | I (2.1) |
| | Adenylate cyclase activating-polypeptide | Adcyap1 | NM_016989 | I (2.9) |
| | MAS 1 oncogene | Mas1 | NM_012757 | I (7.0) |
| | Neuropilin 1 | Nrp1 | AF016296 | I (1.7) |
| | Protein phosphatase 1, regulatory (inhibitor) subunit 12A | Ppp1r12a | BF398081 | I (2.3) |
| | Phosphoglycerate kinase 1 | Pgk1 | NM_05329 | I (1.6) |
| | Phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 1 | Pik3r1 | D64048 | D (1.7) |
| Growth factor, hormone | Connective tissue growth factor | Ctgf | NM_022266 | I (2.6) |
| | Interferon-related developmental regulator 1 | Ifrd1 (PC4) | NM_019242 | I (1.8) |
| | Brain-derived neurotrophic factor | BDNF | NM_012513 | I (5.7) |
| | Inhibin$\beta$ A | Inhba | NM_017128 | I (12.1) |
| | Corticotrophin releasing hormone-binding protein | Crhbp | NM_139183 | I (2.3) |
| Immediate early gene | Fos-like antigen (Fra-1) | Fosl1 | NM_012953 | I (5.3) |
| | Homer homolog 1 (Drosophila) | Homer1 | AF030088 | I (3.2) |
| | S100 calcium binding protein A8 (calgranulin A) | S100a8 | NM_053822 | I (4.2) |
| | S100 calcium binding protein A9 (calgranulin B) | S100a9 | NM_053587 | I (4.6) |
| | Early growth response 4 | Egr4 | NM_019137 | I (1.6) |
| Channels | Potassium channel, subfamily V, member 3 | KcngV3 | BF391696 | I (1.8) |
| | Pannexin 1 | Panx1 | AI136392 | I (1.9) |
| | Cholinergic receptor, nicotinic $\alpha$ polypeptide 5 | Chrna5 | NM_017078 | I (2.1) |
| Cell cycle | Cyclin L1 | Ccnl1 | NM_053662 | I (1.8) |
| | Deleted in bladder cancer chromosome region candidate 1 | Dbccr1 | NM_080482 | I (2.2) |

**Table 2** *Continued*

| *Category* | *Name* | *Symbol* | *GenBank* | *Fold change* |
|---|---|---|---|---|
| Transcription | Nuclear factor, interleukin-3 regulated | Nfil3 | NM_053727 | I (2.7) |
| | MAD homolog 7 (Drosophila) | Madh7 | NM_030858 | I (2.0) |
| | Nuclear receptor subfamily 4, group A, member 2 | Nr4a2 | U72345 | I (1.9) |
| | Nuclear receptor subfamily 4, group A, member 3 | Nr4a3 | NM_031628 | I (2.1) |
| | Activating transcription factor 3 | Atf3 | NM_012912 | I (2.1) |
| | Musculoskeletal embryonic nuclear protein 1 | Mustn1 | AW251450 | I (3.2) |
| | Core promoter element-binding protein | Copeb | BF565718 | I (2.3) |
| | Discs, large homolog 1 (Drosophila) | Dlgh1 | NM_012788 | I (1.9) |
| Transporter | Solute carrier family 31 (copper transporters), member 1 | RGD: 620059 | NM_133600 | I (2.1) |
| | Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6 | Slc17a6 | NM_053427 | I (2.3) |
| | Synaptotagmin IV | Syt4 | L38247 | I (2.0) |
| Metabolism | Farensyl diphosphate synthase | Fdps | NM_031840 | I (1.8) |
| | Insulin-induced gene 1 | Insig1 | NM_022392 | I (2.0) |
| | UDP-glucose dehydrogenase | Ugdh | NM_031325 | I (1.7) |
| | Sterol-C4-methyl oxidase-like | Sc4 mol | NM_080886 | I (1.7) |
| | Isopentenyl-diphosphate delta isomerase | Idi1 | NM_053539 | I (2.1) |
| | Branched chain aminotransferase 1, cytosolic | Bcat1 | NM_017253 | I (2.0) |
| | Deiodinase, iodothyronine, type II | Dio2 | AW526991 | I (2.1) |
| | Glutathione-*S*-transferase, mu type 3 | Gstm3 | NM_031154 | I (1.6) |
| | Neuropeptide Y receptor Y1 | Npy1r | BI395810 | D (1.7) |
| Others | Hemoglobin $\beta$chain complex | Hbb | NM_033234 | I (2.2) |
| | Oxidized low-density lipoprotein (lectin-like) receptor 1 | Oldlr1 | NM_133306 | I (3.5) |
| | Serine (or cysteine) proteinase inhibitor, clade E, member 1 | Serpine 1 | NM_012620 | I (3.9) |
| | *N*-arginine dibasic convertase 1 | Nrd1 | BE115812 | I (1.8) |
| | Activin A receptor, type 1 | Acvr1 | BM389711 | I (1.9) |
| | Fc receptor IgG, low affinity III | Fcgr3 | NM_053843 | I (2.0) |
| | Secretory leukocyte peptidase inhibitor | Slpi | NM_053372 | I (2.0) |
| | Secretogranin 2 | Scg 2 | NM_022669 | I (1.8) |
| | Exportin 1, CRM1 homolog (yeast) | Xpol | AW533924 | I (1.9) |
| | Stress-induced phosphoprotein | Stip1 | BI283691 | I (1.9) |
| | Myosin heavy polypeptide 4 | Myh4 | BI277586 | D (2.0) |
| | Peroxisomal membrane protein 4 | Pxmp4 | AI232414 | D (2.3) |
| | $\beta$galactoside $\alpha$2,6 sialyltransferase 2 | St6gal2 | AW524749 | D (1.7) |
| | Brain and kidney protein | Bk | U30831 | D (1.8) |

D, decrease; I, increase.

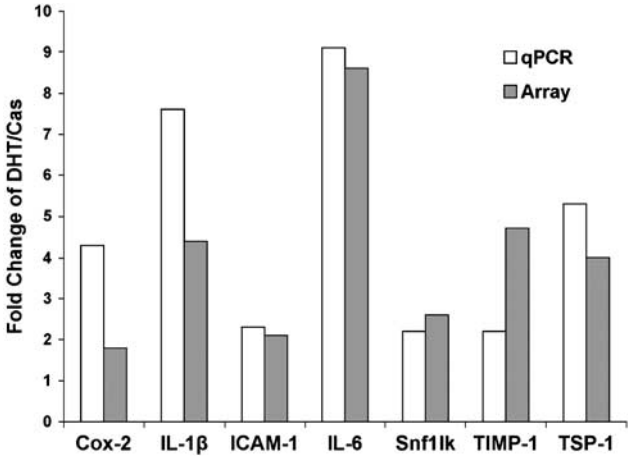


**Figure 4** Confirmation of the differential RNA expression for seven selected genes at 6 h reperfusion using real-time qRT-PCR techniques. The results are expressed as fold change of specific gene in DHT-treated animals to that in castrated animals and compared with array results ($n = 3$ for confirmation of each gene).

deleterious action through nonreceptor-mediated effects or receptor-mediated nongenomic actions.

Functional analysis of known genes with differential expression patterns in DI and CI suggests that both destructive and protective pathways are triggered in the periinfarct zone at 6 h reperfusion. For example, upregulation of genes such as brain-derived neurotrophic factor and adenylate cyclase-activating polypeptide 1 may represent a defensive response to the more severe ischemic damage conferred by DHT. Without doubt, DHT is a pleiotrophic steroid and alters multiple transcripts in ischemic tissue. The microarray data implicate enhanced expression of inflammation genes as one contributor to DHT-enhanced ischemic injury. Seventeen inflammatory genes showed uniformly increased expression in DHT-treated versus castrated brain at 6 h reperfusion, including proinflammatory cytokines (e.g., IL-1a, IL-1b, IL-6, and TNF), chemokines (e.g., MIP1a, MIP1b, CXCL1, CXCL2, and CCL2), and adhesion molecules such

as intercellular adhesion molecule. Many of these gene products are known to contribute to ischemic cell death (Zheng and Yenari, 2004). For example, pharmacological inhibition or genetic deletion of IL-1 and TNF is neuroprotective in focal ischemia models (Reiton *et al*, 1996; Loddick and Rothwell, 1996; Ohtaki *et al*, 2003), and high peak plasma IL-6 levels after clinical stroke correlate with poor outcomes (Smith *et al*, 2004). Inhibition of adhesion molecules and chemokines reduces experimental stroke damage, in part by depressing recruitment of peripheral inflammatory cells into injured brain (Takami *et al*, 2001; Zhang *et al*, 1994; Zhang *et al*, 1995). For example, the chemokine Ccl2 not only contributes to postischemic inflammation but also confers damage to the blood–brain barrier (Dimitrijevic *et al*, 2006). In this subcategory, DHT induction of Cox-2 is of interest, as ischemic neuronal damage is enhanced through inflammation and $Na^+$–$Ca^{2+}$ homeostasis via activation of E-prostanoid receptor 1 receptor by prostaglandin $E_2$ (Kawano *et al*, 2006).

Dihydrotestosterone also regulated postischemic transcription of six extracellular matrix-related genes. For example, Claudin 5 transcripts were suppressed by DHT. Claudin 5 is a component of the normal blood–brain barrier and its downregulation has been implicated in barrier damage after ischemia (Yang *et al*, 2006). Dihydrotestosterone also increased expression of MMP12, a member of superfamily of matrix metalloproteinases that are well-known players in ischemic brain damage by disrupting cell–matrix signaling (Lo *et al*, 2003). Because our initial qPCR protocols detected induction of proapototic bax at 22 h in the periinfarct areas by DHT treatment, we closely scanned the microarray data for genes related to apoptosis. Emphasizing the complexity of postischemic transcriptional processes, we observed that three antiapoptosis genes are upregulated by DHT at 6 h reperfusion. Among the three channel-forming genes regulated by DHT, pannexin 1 (Pan X1) may be relevant. Opening of Pan X1 channels after oxygen–glucose deprivation may contribute to the unregulated ion flux associated with ischemia (Thompson *et al*, 2006). Analysis of signaling genes reveals that two dual-specificity protein phosphatases, DUSP5 and DUSP6, which exhibit similar activities toward both tyrosine and serine/threonine-phosphorylated substrates, and guanosine 5′-triphosphate (GTP) cyclohydrolase (Gch) were induced after MCAO in the presence of DHT. After MCAO, Gch inhibition results in protection via reduction of inducible nitric oxide synthase activity (Kidd *et al*, 2005). Accordingly, enhancement of Gch may represent a mechanism, in addition to those mentioned above, of DHT-mediated ischemic injury.

Many genes that were altered in the present study are reminiscent of findings from other studies of periischemic gene transcription utilizing microarray technologies. For example, in our study, of 86 genes that were upregulated in the postischemic periinfarct zone by DHT (Table 2), 32 were also shown to be increased in rat cortex by KCl-induced spreading depression (Urbach *et al*, 2006). The remarkable uniformity in gene induction pattern between the two studies suggested that DHT could alter periinfarct depolarizations as one mechanism by which DHT enhances ischemic sensitivity. Comparison with selected studies of gene transcription after rat MCAO suggested a more limited overlap of gene candidates with the present study (Lu *et al*, 2003, 2004; Kastner-Schmidt *et al*, 2002).

One limitation of microarray techniques is the high potential for false-positive results. In our study, we confirmed differential expression of 7 of 10 genes by qPCR, although further study is requisite to confirm that functional proteins result from transcriptional upregulation. Our inability to confirm all genes by qPCR may be due to (1) microarray probes and those employed in the qPCR assays may target different parts of mRNA sequence, so the splicing variants can occur and (2) microarray and qPCR confirmation were performed on RNA prepared from different animals, so stroke size heterogeneity may influence perfect agreement between the techniques. Another limitation of the present study is that only changes in mRNA transcripts were evaluated, and protein translation remains to be confirmed for all the many responsive genes.

In conclusion, androgens are important mediators of stroke outcome in the male, and DHT, the nonaromatizable androgen, is more potent than testosterone in mediating the male's sensitivity to cerebral ischemia. By using microarray techniques, we identify, for the first time, a number of gene candidates that may be induced by DHT and which could account for cell death and tissue destruction through enhanced inflammation, dysregulation of the blood–brain barrier, and other mechanisms. Our data provide new insight into the role of androgens in brain injury.

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