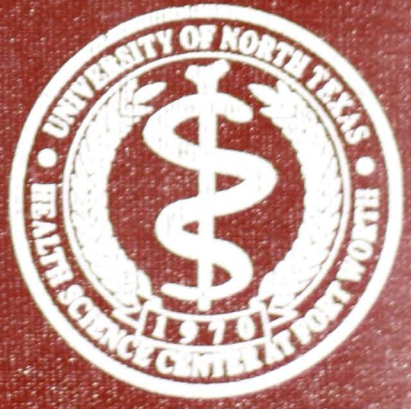

UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Gatson, Joshua Wayne, The Role of a Membrane Androgen Receptor in the Brain. Doctor of Philosophy (Biomedical Sciences), February 2007, pp187, 34 illustrations.

In the brain, depending on the insult type, androgens have been shown to protect from or exacerbate the levels of cell death. This discrepancy is partly due to the array of receptors that androgens may activate during injury. For example, activation of intracellular androgen receptors (AR) leads to the activation of pro-survival pathways and protects from various toxins such as beta-amyloid. In contrast, previous studies have demonstrated that testosterone causes an increase in lesion size following stroke. The damaging effects of androgens in the brain may be mediated by a membrane-associated AR (mAR), since activation of mAR in peripheral tissue results in a decrease in cell growth and an increase in apoptotic cell death during serum deprivation.

Here, I hypothesize that activation of a mAR in cortical astrocytes, suppressess the ERK and Akt signaling pathways and increases cell death in the presence of a metabolic and oxidative stressor. In this study, we found that glia express both isoforms of the AR (AR-B and AR-A) and that dihydrotestosterone (DHT) elicits ERK and Akt phosphorylation in rat glioma (C6) cells. The effect of DHT on the activation of these signaling pathways is AR dependent, since flutamide blocked this effect. In contrast to the intracellular receptor, we concluded that DHT-BSA (membrane impermeant form of DHT) binds to DHT displaceable sites on the plasma membrane. Also, treatment with DHT-BSA in the C6 cells resulted in a significant decrease in phospho-ERK and Akt

levels, suggesting the existence of two different pathways through which DHT can influence the activity of these signaling pathways.

With respect to cell survival, the C6 cells and primary cortical astrocytes were treated with the metabolic and oxidative insult, iodoacetic acid (IAA), in the presence or absence of DHT, DHT-BSA, or estradiol. Following treatment, DHT and estradiol protected the glia from IAA-induced toxicity, whereas DHT-BSA caused a significant increase in cell death in the presence of a sublethal concentration of IAA. These results indicate that activation of the intracellular pathway is protective and activation of a membrane pathway is damage-inducing during injury, further supporting our results from the ERK and Akt signaling studies. To further characterize this mAR in the brain, we decided to look for indices of apoptosis such as caspase activation and TUNEL staining. It was found that DHT-BSA treatment in the presence of IAA, resulted in an increase in caspase-3/7 activation and increased TUNEL staining. In addition, PKC-delta mediated DHT-BSA-induced cell death, since antagonism of PKC-delta with rottlerin afforded protection.

In conclusion, we have partially characterized a novel mAR in astrocytes during injury. Here, the damaging effects of androgens, at least in astrocytes, may in fact be mediated by a mAR, which may be a therapeutic target during stroke or reperfusion injury.

THE ROLE OF A MEMBRANE ANDROGEN RECEPTOR IN THE BRAIN

JOSHUA WAYNE GATSON, M.S.

APPROVED:

Major Professor

Committee Member

Committee Member

Committee Member

University Member

Chair, Department of Pharmacology and Neuroscience

Dean, Graduate School of Biomedical Sciences

THE ROLE OF A MEMBRANE ANDROGEN RECEPTOR IN THE BRAIN

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Joshua Wayne Gatson, M.S.

Fort Worth, Texas

February, 2007

ACKNOWLEDGMENTS

As I complete my Ph.D. studies, I would like to first thank my mentor, Dr. Meharvan "Sonny" Singh. Dr. Singh has provided me with the knowledge, skills, and confidence to pursue a career in academia as a scientific researcher. Throughout my academic years, Dr. Singh has been very supportive and has helped me overcome many obstacles. And, I am very appreciative of the opportunity that he has given me to conduct research in his laboratory and to obtain a doctoral degree in Pharmacology and Neuroscience.

As an extension of my appreciation, I would also like to thank my committee members: Dr. James Simpkins, Dr. Peter Koulen, Dr. Alakananda Basu, and Dr. Stephen Grant. These committee members have been very helpful in instructing how research is conducted by teaching me how to interpret various results, form conclusions, and design necessary experiments to test the hypothesis.

I also thank my family for their continued support over the years. Throughout my graduate school career there has been many times that I have needed help paying bills and staying focused on my goal to obtain my PhD. To my family, I love you for your continued support.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
Akt	Protein Kinase B
ANOVA	Analysis of variance
AR	Androgen receptor
ATP	Adenosine triphosphate
BAD	BCL- associated death promoter
BCL-2	B-Cell Leukemia/Lymphoma - 2
BSA	Bovine Serum Albumin
C6	Rat Glioma Cells
Ca^{2+}	Calcium
CMO	Carboxymethyloxime
CNS	Central Nervous System
DHT	Dihydrotestosterone
DHT-BSA	BSA-Conjugated DHT
DHT-CMO	5α-androstan-17β-ol-3-o-carboxymethyloxime
d.i.v.	Days In Vitro
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
E2	17-β Estradiol

EGF	Epidermal Growth Factor
ERK	Extracellular Signal-Regulated Kinases
FBS	Fetal Bovine Serum
FH	Forkhead
FITC	Fluorescein Isothiocyanate
Flut	Flutamide
GFAP	Glial fibrillary acidic protein
GnRH	Gonadotrophin Releasing Hormone
Hsp	Heat Shock Protein
HT-22	Rat Hippocampal Cells
HT	Hormone Therapy
IAA	Iodoacetic Acid
IACUC	Institutional Animal Care and Use Committee
ICE	Interleukin-1β Converting Enzyme
LDH	Lactate Dehydrogenase
LH	Luteinizing Hormone
LNCaP	Prostate Cancer Cell Line
MAPK	Mitogen-Activated Protein Kinase
mAR	Membrane Androgen Receptor
MCAO	Middle Cerebral Artery Occlusion
Mek	MAP Kinase Kinase
mER	Membrane Estrogen Receptor

P3	Postnatal Day 3
PAGE	Polyacrylamide Gel Electrophoresis
PC12	Rat Pheochromocytoma Cells
PI3K	Phosphoinositide-3 kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PTX	Pertussis Toxin
PVDF	Polyvinylidene Difluoride
Raf	MAP Kinase Kinase Kinase
Ras	Small Guanine Nucleotide Exchange Protein
RFU	Relative Fluorescence Units
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SHBG	Sex Hormone Binding Globulin
T47D	Ductal Carcinoma Human Breast Cancer Cells
T-BSA	Testosterone Conjugated to BSA
TBS-T	Tris-Buffered Saline Tween – 20
TRAIL	TNF-Related Apoptosis-Inducing Ligand
Tr-X	Triton X
TUNEL	Terminal dUTP Nick End Labeling
UO126	1,4 – diamino - 2,3 – dicyano - 1,4 – bis [2 - aminophenyalthio] butadiene

CHAPTER I

INTRODUCTION

Androgens and Androgen Receptors

Androgens are classically associated with the regulation of muscle growth, spermatogenesis, growth of bone, and the development of secondary sexual characteristics in males and females (1). Androgens, specifically testosterone, are produced in the testes by the Leydig cells. Production of testosterone in the testes is strictly regulated by luteinizing hormone stimulation (2). From the hypothalamus, gonadotrophin releasing hormone (GnRH) leads to the release of the LH from the gonadotrophs of the pituitary. Once released testosterone can act on various target tissues or feedback to negatively regulate the release of GnRH and LH from the hypothalamus and pituitary, respectively (1). In females, testosterone can be made by the adrenal glands as well as the ovaries (1).

Within the plasma, approximately, ninety-five percent of testosterone is bound to serum proteins such as albumin and sex hormone binding globulin (SHBG) (1, 3). These serum proteins assist in the delivery of testosterone to its target tissue (4). Once testosterone has been delivered to its target tissue, it can then be converted to 5α-dihydrotestosterone (DHT) by 5-α-reductase. DHT is the major metabolite of testosterone and is more potent at the intracellular "classical" androgen receptor (AR) than the parent compound, testosterone (1).

Testosterone can be synthesized from cholesterol by various enzymes and can be converted to either estradiol or DHT. Within the central nervous system (CNS), testosterone is converted to 17β-estradiol and DHT by aromatase and 5α-reductase, respectively (1). Thus depending on the predominance of one enzyme over the other, the effects of testosterone in the brain can be very different. In addition to the brain, testosterone can also be aromatized to estrogen in Leydig cells, Sertoli cells, and adipose tissue. DHT is further metabolized to 3α-17β-androstanediol, and the metabolites are ultimately cleared from the plasma (1). Testosterone or DHT mediate their effects in various tissue types by binding to its nuclear receptor and initiating multiple effects within the cell (1, 5).

Steroid hormones acting through their nuclear "classical" receptors have been shown to be important for many cellular functions (6). The receptors that are activated by these steroid hormones belong to a super family of ligand inducible transcription factors. Upon binding of testosterone to the ligand binding region of the AR, the AR goes through a conformational change and chaperone proteins (including heat shock protein [HSP] 90) are released from the AR. Following release of the HSPs, the steroid-receptor complex translocates to the nucleus and modulates the transcription of various genes or regulates the activity of transcription factors (6). Two forms of the AR, AR-B (110 kDa) and AR-A (87 kDa), mediate the effects of androgens in the cell. The AR is transcribed from a single gene and the AR-A isoform is a N-terminally truncated form of the AR (7). Whether these two isoforms have different actions within the cell is not very well

understood, but, previously it was demonstrated that AR-A antagonizes the action of AR-B, which may be relevant to the activation/inhibition of signaling pathways, regulation of gene transcription, or cell death (8). In addition to the AR-B and AR-A isoforms of the AR, putative membrane ARs have been characterized in a variety of tissue types and may mediate the rapid signaling effects of testosterone or DHT in the cell. These receptor types have been shown to be instrumental in mediating the effects of testosterone and/or DHT through the genomic (classical) mechanism (6) and/or non-genomic mechanisms (9-12) to regulate growth, survival, or differentiation.

The MAPK/ERK Pathway

The Ras/Raf/MEK/MAP kinase (MAPK) pathway is a signal transduction pathway that is important for cell growth, differentiation, or survival (13, 14). Upon activation of a small guanine nucleotide exchange protein (Ras), subsequent activation of the MAP kinase kinase kinase (Raf) occurs. Following Raf activation, this serine/threonine kinase then activates the MAP kinase kinase (MEK), which is an upstream activator of a member of the MAP kinase family (ERK) (15). The pattern of ERK activation by steroid hormones such as testosterone or DHT may predict cell death or cell survival in the presence of various cytotoxins. A rapid (minutes) and sustained (hours) activation of ERK is associated with cell survival, differentiation or growth. In contrast, a delay in ERK activation, but protracted or prolonged pattern of activation, may lead to cell death (13, 14, 16). Steroid hormones such as estrogen, progesterone, and

testosterone or DHT have been shown to rapidly activate this signaling pathway, to influence cell growth, differentiation, or survival (17-21).

The PI-3 kinase/Akt Pathway

Protein kinase B (Akt) is a signaling protein that is instrumental in blocking programmed cell death (apoptosis) (22). In addition, this signaling protein is important for cell growth and the regulation of cellular metabolism (22). Activation of receptor tyrosine kinase receptors by various growth factors leads to the activation of phospoinositide-3 kinase (PI3K). Following activation of PI3K, this protein associates with the plasma membrane and phosphorylates phosphoinositol. Upon phosphorylation, two phosphates are generated, PIP_2 and PIP_3. PIP_2 and PIP_3 recruit Akt to the plasma membrane and this kinase is activated by proteins such as 3'-phosphoinositide-dependent kinase-1 (PDK1) (23-26). Akt is fully activated once phosphorylated at the Ser^{473} and Thr^{308} residues. Phosphorylated Akt can then act on downstream proteins that play important roles in apoptosis such as Bad and forkhead (27-30). Bad signaling results in an increase in cytochrome C release from the mitochondria, resulting in an increase in caspase-3 and 7 activation. Phosphorylation of Bad at Ser^{16} by Akt, decreases the association of this protein with Bcl-2 and this protein is sequestered by protein 14-3-3 (22, 31). In addition to Bad, activated Akt can phosphorylate caspase-9 and block its ability to activate other caspases that mediate the induction of apoptotic cell death (32). Akt has also been shown to regulate the transcription of apoptotic genes by

phosphorylating proteins such as forkhead (FH). FH regulates the transcription of genes such as the Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL), as well as proteins that interact with Bcl-2 to induce apoptosis (29, 33). In addition to growth factors such as insulin and epidermal growth factor (EGF), androgens can also rapidly activate the PI3K/Akt pathway (9, 10, 18).

Androgen Signaling Through the Nuclear Receptor

ERK/MAPK Pathway:

Androgens have been shown to modulate the activity of the ERK/MAPK pathway in many different tissue types (4, 20, 34-36). In a prostate cancer (LNCaP) and osteocytic cell line, androgen treatment resulted in the rapid (~5 min) increase in phospho-ERK levels. This increase in the levels of ERK phosphorylation by androgens was determined to be mediated through the association of the AR and c-Src. This was supported by the fact that inhibition of the AR or c-Src blocks the androgen-induced Raf-1 and ERK activation (4). Androgen-induced proliferation of prostate cancer cells has also been shown to be mediated by the ERK pathway (35). In addition to the ERK pathway, androgens have also been shown to modulate the activity of the PI3K/Akt pathway (9, 10, 18).

PI3K/Akt Pathway:

The PI3K/Akt pathway is another signaling pathway that can be activated by androgens in a receptor dependent manner (9, 10, 18). For example, the classical AR was

shown to mediate androgen-induced activation of the Akt pathway by interacting with various subunits of the upstream activators of Akt, PI-3 kinase and c-Src. In the HEK293 cells, the AR binds to PI3K at the inhibitory domain and increases the release of the catalytic domain, resulting in downstream activation of Akt in the presence of testosterone. The association of c-Src with the AR further activates PI3K (9, 10). This increase in the activation of Akt, leads to an increase in androgen-induced proliferation and cell survival through the classical receptor.

Membrane Androgen Receptors

Membrane steroid receptors have been described for estrogens, progestins, and androgens (11, 12, 37-43). To date, only the membrane receptor for progesterone has been cloned (41, 42, 44). This membrane progesterone receptor is a 7-transmembrane G-protein-coupled receptor. In addition, functional studies suggest that this membrane receptor is Gαi/o linked, since pertussis toxin blocked the ability of progesterone to decrease cAMP levels (41, 42). With respect to the membrane estrogen receptor, this receptor has yet to be cloned, but many functional studies support the existence of one or more membrane estrogen receptors (mER) through which estrogen mediates its effects on various signaling pathways (37-39). Various studies also suggest that the membrane ER may be a truncated form of ER-α, since ER-α can be detected at the level of the plasma membrane (37-39).

In the CNS and periphery the existence of a membrane androgen receptor (mAR) has been shown to mediate cellular growth, cell signaling, and survival (11, 12, 18). Previously, detection of this membrane receptor was performed using radio-ligand binding studies, confocal microscopy, and flow cytometry. These reports concluded the mAR exists at the level of the plasma membrane (11, 45-47). Binding studies revealed that testosterone had a K_d of 10 nM for this receptor in the ovary of the Atlantic Croaker (11). This binding was competed away by testosterone, dihydrotestosterone, progesterone, estrogen, and flutamide with varying affinities. Specific binding of testosterone was also detected in the kidney, liver, and brain of the Atlantic Croaker, indicating that this mAR may be biologically important in many different systems. In contrast, very little binding was detected in muscle and heart tissue (11). Using confocal microscopy and flow cytometry, Alexaki *et al.* showed that chromaffin cells (PC12) that differentiate into neuronal-like cells actually had a decrease in expression of the mAR (46). The binding of androgens to the mAR has also been shown in T cells, macrophages, smooth muscle cells, rat cardiac myocytes, prostate cancer cells, skeletal muscle, and C6 cells (18, 45, 47-50). From these studies it was determined that activation of the mAR by testosterone-BSA (plasma membrane impermeant form of testosterone used to target membrane sites) in tissue such as heart, muscle, and the immune cells, led to a rapid increase in calcium influx (< 5 sec). Additionally, it was determined that this influx of calcium is mediated by the Gαi/Go signaling pathway since pertussis toxin (PTX) blocked the increase in intracellular calcium (18, 45, 47-50). In skeletal muscle,

macrophages, and vascular smooth muscle, activation of a novel mAR with testosterone-BSA, led to a rapid increase in ERK phosphorylation (48, 51, 52), indicating that androgens can influence the activity of the ERK/MAPK through not only the nuclear receptor, but also through a membrane-associated receptor. Though numerous studies have supported the existence of a mAR in peripheral tissue, very little is known about the existence or role of mARs in the CNS.

To date, only one study has demonstrated that a mAR is present in the brain (18). In this study, the rat glioma (C6) cell line was used to show that the mAR exists in astroglia. Using flow cytometry, it was determined that DHT binds to membrane receptors and that this binding is displaceable. Interestingly, activation of the mAR in the C6 cells decreased the activity of the ERK/MAPK and PI3K/Akt pathways and blocked the ERK-inducing effects of other steroid hormones (18), suggesting that androgens acting through a mAR may block the beneficial effects of other steroids such as estrogen or progesterone during injury.

In addition to binding and cell signaling, the mAR has also been shown to be involved in cell survival in peripheral tissue. In LNCaP cells, testosterone-BSA treatment led to the inhibition of LNCaP cell growth and also led to the induction of apoptosis during serum deprivation. In addition, activation of the membrane AR also blocked the migration and invasion of these cancer cells. The action of testosterone-BSA on the LNCaP cells was independent of the classical AR, since flutamide and siRNA-mediated

knock down of the classical AR did not alter this effect (12). In another cancer cell line (T47D breast cancer cells), it was found that these cells express binding sites for testosterone-BSA on the membrane as indicated by radioligand binding studies and flow cytometry. In addition, testosterone-BSA enhanced apoptosis during serum deprivation and blocked the survival promoting effects of E_2-BSA (49).

Protective Effects of Androgens in the Brain

Previously, androgens in the CNS have been shown to protect from a wide array of insults such as β-amyloid, excitotoxicity, and serum deprivation. This protection afforded by androgens was also shown to be AR dependent (20, 53, 54). To determine if androgens may be useful in decreasing the pathology of Alzheimer's disease (AD), primary hippocampal cultures generated from Sprague Dawley male rats were treated with β-amyloid in the presence or absence of testosterone (55). It was concluded that testosterone significantly decreased β-amyloid toxicity. In addition, this protection by testosterone was not mediated by estrogen, since the estrogen receptor antagonist, droloxifene did not block the survival promoting effects of testosterone (55). It was also determined that the ability of testosterone to decrease the damaging effects of β-amyloid is mediated by activation of the ERK/MAPK pathway (20). In neuroblastoma cells and primary cortical neurons, it was also demonstrated that testosterone decreases the release of β-amyloid from the cell (56), as seen previously in estrogen treated cultures (57). In

addition to β-amyloid, androgens have also been instrumental in protecting from serum deprivation, as supported by the ability of androgens to protect primary cultures derived from human fetal brains from serum deprivation-induced cell death. Flutamide blocked the survival promoting effects of testosterone, indicating that the "classical" AR mediates the effects of testosterone in this model (54). Additionally in the CA2/3 region of the hippocampus, testosterone was shown to decrease kainic acid-induced lesion size (58). These studies demonstrate that testosterone protects the brain from various insults, and that this protection is likely mediated by the classical AR. Thus, the expression of the nuclear AR may be necessary for testosterone to protect from various insults.

Damaging Effects of Androgens in the Brain

In contrast to the protective effects of androgens, this hormone has also been shown to cause an increase in cell death following insult to the brain. This discrepancy as to whether androgens are protective or damage-inducing may be dependent on the tissue type or location in the brain, receptor expression, androgen levels, or type of insult. For example, increased levels of androgens in the brain have been shown to lead to a dramatic increase in cell death. In one study, human neuroblastoma cells treated with supraphysiological concentrations of testosterone led to a sustained increase in intracellular calcium, which in turn resulted in the activation of apoptosis, as seen by an increase in caspase activity, DNA damage, and annexin-V staining (59). In the presence

of excitotoxic insults, testosterone also increased cell death in a rat hippocampal cell line (HT-22) and primary oligodendrocyte cultures (60, 61). In addition, testosterone in the brain was shown to cause an increase in lesion size following stroke. These results suggest that an individual with high testosterone levels during brain injury may be more susceptible to the damaging effects of oxidative stress, excitotoxicity, or stroke. Also, since a mAR was implicated in the damaging effects of testosterone in peripheral tissue (12, 49, 62), the negative effects of androgens in the brain may also be mediated by a novel mAR.

Clinical Significance of the Current Research:

Stroke

Stroke in the brain is defined as a decrease in blood flow to various regions of the brain, resulting in ischemic injury. Ischemia in the brain results in a decrease in cellular ATP. This decrease in ATP, results in an inbalance of ion flow across the plasma membrane, increase in oxidative stress, an increase in excitotoxic events, and an increase in cell death. Additionally, dying cells (neurons and glia) in the brain, release more excitoxins, which can heighten the damage induced by stroke leading to an increase in brain inflammation. Also, there is an increase in brain lesions and scar tissue. As a result, the damaging effects of stroke can lead to a loss in the normal motor and sensory function

as well as speech, cognition, and memory (63, 64). There are two main types of stroke: Ischemic (thrombotic and embolic) and hemorrhagic stroke.

Ischemic stroke occurs when a blood clot occludes the cerebral arteries and leads to a decrease in blood flow throughout the brain. To relieve this blood clot, thrombolytics are used. The use of anti-thrombotics is another treatment for ischemic stroke. Hemorrhagic stroke occurs when a blood vessel ruptures, hemorrhages and leads to a decrease of blood flow throughout the brain. Treatment of hemorrhagic stroke usually requires surgery (65, 66).

In the United States, stroke is the third leading cause of death, with heart disease and cancer being the number one and two leading causes, respectively. Additionally, stroke in the united states is the leading cause of long-term disability, as well as dementia (67, 68). Previous studies have shown that men have a higher risk for stroke than women, which may correlate with higher testosterone levels in males vs. females. Previously, steroid hormones such as estrogen and progesterone protected from the damaging effects of stroke (18, 69) (70, 71). In contrast, testosterone has been shown to mediate an increase in cell death, following reperfusion injury (61, 72).

Role of Androgens During Ischemic Stroke

Following stroke, testosterone has been shown to play an important role in cell survival. In a recent study, stroke was induced in gonadectomized Sprague Dawley rats

by occluding the middle cerebral artery for one hour. Following a 24-hour reperfusion period, the brains were collected and analyzed for lesion volumes. To determine if testosterone excacerbates cell death following stroke, testosterone pellets were implanted 2 days prior to inducing stroke. At 1, 2, 4, and 6 hours prior to occluding the middle cerebral artery the testosterone pellets were removed. It was determined that removal of the testosterone pellet 6 hours prior to stroke and reperfusion injury resulted in a significant decrease in the lesion volume (61). Previously, it was also shown that during ischemia-induced stress (mild insult), testosterone levels are decreased and there is a decrease in lesion size (73-75). These results indicate that normal or high testosterone levels, following stroke, may result in a greater increase in brain cell death. Thus, treatment with testosterone in androgen replacement therapies may make an individual more susceptible to the damaging effects of androgens following brain injury.

Androgen Replacement Therapy

With age, there is a gradual decrease in the plasma levels of testosterone in both males in females. In men, testosterone levels start to decrease around age 40. By the age of 85, there is an approximately 50% reduction compared to the age 40 group (76). Decreases in testosterone in men, results in a decrease in the number of Leydig cells, muscle mass, physical function, bone density, and libido. Decreased testosterone, also leads to an increase in body fat. Also, a change in cognition and mood has been

associated with a decrease in testosterone (76-78). In these studies, treatment with testosterone, resulted in a decrease in the amount of body fat, increase muscle mass and strength, increase in libido, and improved cognition and memory (78-84). In contrast to the beneficial effects of testosterone, there are several side effects that can arise from androgen replacement therapy (78). Very little is known about the damaging effects of androgen therapy, but several clinical trials have shown that there is a link between androgen therapy and diseases such as cardiovascular disease and prostate cancer (78, 85).

In women, by the age of 50, there is a significant decrease in plasma testosterone levels compared to the average 20 year old female. However, it should be noted that the decline in testosterone is not precipitous (i.e., is not strongly associated with menopause), since the decline begins by the second decade of life (86). Women that experience this decrease in testosterone have decreased libido, as well as a decrease in motivation to perform various tasks such as exercise. These women also have a decrease in personal confidence (86, 87). Androgen therapy in women improved libido in pre and postmenopausal women. In addition, treatment with testosterone dramatically improved self confidence and motivation (86, 88-91). Side effects from androgen therapy in women are very mild. The main side effects that were observed were weight gain, hirsutism, and acne (86).

To limit the severity of side effects seen with androgen therapy in both males and females, a better understanding should be gained from the biology of testosterone. Also, the type(s) of androgen receptors that are expressed should be analyzed, since the expression of one receptor type over another may explain why androgens are protective or damage inducing. Ultimately, these factors may influence the vulnerability of individuals to diseases such as Alzheimer's disease and stroke.

Goals of the Current Research

Currently, with respect to normal cell function and survival, androgens have been shown to be biologically important in the periphery and in the brain. In all instances, either the classical/nuclear or mAR has been shown to be required for androgens to influence the activity of various signaling pathways. Interestingly, androgens have been shown to be protective as well as damage-inducing in the brain, but the mechanism underlying this duality is unclear. My hypothesis to explain whether testosterone is protective or damaging-inducing is based on the type of receptor that is activated by testosterone during injury. For example, during acute brain injury, activation of the AR may protect from oxidative stress, excitotoxicity, and ischemia, whereas testosterone acting through the mAR, most likely worsens the condition, as seen in peripheral tissue. Thus, my hypothesis is that during acute brain injury, increased activation of the mAR competitively decreases the survival-promoting effects of androgens through the

intracellular AR, resulting in suppression of protective signaling and exacerbation of stroke-induced toxicity.

In this dissertation, the binding affinity of DHT for the mAR was determined in primary glia. To further characterize the mAR in the brain during injury, the effects of DHT-BSA during metabolic inhibition in primary astroglia was also determined. Additionally, the effect of DHT-BSA on intracellular signaling pathways such as the ERK, PI3K/Akt, protein kinase C (PKC), and protein kinase A (PKA) was analyzed to determine which pathways mediate the effects of DHT-BSA during metabolic inhibition. In addition, the effects of DHT-BSA on estradiol and DHT-induced survival was also tested. To determine the type of cell death that is initiated by DHT-BSA, markers of apoptosis were also observed. Understanding the mechanism of action and identifying the pathways through which androgens mediate negative effects during brain injury is the main focus of this study.

REFERENCES

1. **Mooradian AD, Morley JE, Korenman SG** 1987 Biological actions of androgens. Endocr Rev 8:1-28
2. **Berthois Y, Pourreau-Schneider N, Gandilhon P, Mittre H, Tubiana N, Martin PM** 1986 Estradiol membrane binding sites on human breast cancer cell lines. Use of a fluorescent estradiol conjugate to demonstrate plasma membrane binding systems. J Steroid Biochem 25:963-72
3. **Mendel CM** 1989 Modeling thyroxine transport to liver: rejection of the "enhanced dissociation" hypothesis as applied to thyroxine. Am J Physiol 257:E764-71
4. **Heinlein CA, Chang C** 2002 The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. Mol Endocrinol 16:2181-7
5. **Gobinet J, Poujol N, Sultan C** 2002 Molecular action of androgens. Mol Cell Endocrinol 198:15-24
6. **Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, et al.** 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-9
7. **Wilson CM, McPhaul MJ** 1996 A and B forms of the androgen receptor are expressed in a variety of human tissues. Mol Cell Endocrinol 120:51-7

8. **Liegibel UM, Sommer U, Boercsoek I, Hilscher U, Bierhaus A, Schweikert HU, Nawroth P, Kasperk C** 2003 Androgen receptor isoforms AR-A and AR-B display functional differences in cultured human bone cells and genital skin fibroblasts. Steroids 68:1179-87

9. **Baron S, Manin M, Beaudoin C, Leotoing L, Communal Y, Veyssiere G, Morel L** 2004 Androgen receptor mediates non-genomic activation of phosphatidylinositol 3-OH kinase in androgen-sensitive epithelial cells. J Biol Chem 279:14579-86

10. **Sun M, Yang L, Feldman RI, Sun XM, Bhalla KN, Jove R, Nicosia SV, Cheng JQ** 2003 Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85alpha, androgen receptor, and Src. J Biol Chem 278:42992-3000

11. **Braun AM, Thomas P** 2004 Biochemical characterization of a membrane androgen receptor in the ovary of the atlantic croaker (Micropogonias undulatus). Biol Reprod 71:146-55

12. **Hatzoglou A, Kampa M, Kogia C, Charalampopoulos I, Theodoropoulos PA, Anezinis P, Dambaki C, Papakonstanti EA, Stathopoulos EN, Stournaras C, Gravanis A, Castanas E** 2005 Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo. J Clin Endocrinol Metab 90:893-903

13. **Marshall C** 1995 Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179-85

14. **Stanciu M, DeFranco DB** 2002 Prolonged nuclear retention of activated extracellular signal-regulated protein kinase promotes cell death generated by oxidative toxicity or proteasome inhibition in a neuronal cell line. J Biol Chem 277:4010-7.

15. **Marshall CJ** 1994 MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr Opin Genet Dev 4:82-9

16. **Stanciu M, Wang Y, Kentor R, Burke N, Watkins S, Kress G, Reynolds I, Klann E, Angiolieri MR, Johnson JW, DeFranco DB** 2000 Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. J Biol Chem 275:12200-6

17. **Singh M** 2001 Ovarian hormones elicit phosphorylation of Akt and extracellular-signal regulated kinase in explants of the cerebral cortex. Endocrine 14:407-15.

18. **Gatson JW, Kaur P, Singh M** 2006 Dihydrotestosterone differentially modulates the mitogen-activated protein kinase and the phosphoinositide 3-kinase/Akt pathways through the nuclear and novel membrane androgen receptor in C6 cells. Endocrinology 147:2028-34

19. **Singh M, Setalo G, Jr., Guan X, Warren M, Toran-Allerand CD** 1999 Estrogen-induced activation of mitogen-activated protein kinase in cerebral

cortical explants: convergence of estrogen and neurotrophin signaling pathways. J Neurosci 19:1179-88

20. **Nguyen TV, Yao M, Pike CJ** 2005 Androgens activate mitogen-activated protein kinase signaling: Role in neuroprotection. J Neurochem 94:1639-51

21. **Peterziel H, Mink S, Schonert A, Becker M, Klocker H, Cato AC** 1999 Rapid signalling by androgen receptor in prostate cancer cells. Oncogene 18:6322-9

22. **Hanada M, Feng J, Hemmings BA** 2004 Structure, regulation and function of PKB/AKT--a major therapeutic target. Biochim Biophys Acta 1697:3-16

23. **van der Heide LP, Ramakers GM, Smidt MP** 2006 Insulin signaling in the central nervous system: learning to survive. Prog Neurobiol 79:205-21

24. **Vanhaesebroeck B, Alessi DR** 2000 The PI3K-PDK1 connection: more than just a road to PKB. Biochem J 346 Pt 3:561-76

25. **Vanhaesebroeck B, Waterfield MD** 1999 Signaling by distinct classes of phosphoinositide 3-kinases. Exp Cell Res 253:239-54

26. **Storz P, Toker A** 2002 3'-phosphoinositide-dependent kinase-1 (PDK-1) in PI 3-kinase signaling. Front Biosci 7:d886-902

27. **Krasilnikov MA** 2000 Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation. Biochemistry (Mosc) 65:59-67

28. **Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P** 1997 Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol 7:261-9

29. **Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME** 1999 Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96:857-68

30. **Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ** 1996 Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L) [see comments]. Cell 87:619-28

31. **Downward J** 1999 How BAD phosphorylation is good for survival. Nat Cell Biol 1:E33-5

32. **Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC** 1998 Regulation of cell death protease caspase-9 by phosphorylation. Science 282:1318-21

33. **Burgering BM, Medema RH** 2003 Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. J Leukoc Biol 73:689-701

34. **Kayampilly PP, Menon KM** 2004 Inhibition of extracellular signal-regulated protein kinase-2 phosphorylation by dihydrotestosterone reduces follicle-stimulating hormone-mediated cyclin D2 messenger ribonucleic acid expression in rat granulosa cells. Endocrinology 145:1786-93

35. **Bell WC, Myers RB, Hosein TO, Oelschlager DK, Grizzle WE** 2003 The response of extracellular signal-regulated kinase (ERK) to androgen-induced proliferation in the androgen-sensitive prostate cancer cell line, LNCaP. Biotech Histochem 78:11-6

36. **Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Barone MV, Ametrano D, Zannini MS, Abbondanza C, Auricchio F** 2000 Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. Embo J 19:5406-17

37. **Toran-Allerand CD, Guan X, MacLusky NJ, Horvath TL, Diano S, Singh M, Connolly ES, Jr., Nethrapalli IS, Tinnikov AA** 2002 ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. J Neurosci 22:8391-401

38. **Taguchi Y, Koslowski M, Bodenner DL** 2004 Binding of estrogen receptor with estrogen conjugated to bovine serum albumin (BSA). Nucl Recept 2:5

39. **Thomas P, Pang Y, Filardo EJ, Dong J** 2005 Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 146:624-32

40. **Stevis PE, Deecher DC, Suhadolnik L, Mallis LM, Frail DE** 1999 Differential effects of estradiol and estradiol-BSA conjugates. Endocrinology 140:5455-8

41. **Zhu Y, Bond J, Thomas P** 2003 Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci U S A 100:2237-42

42. **Zhu Y, Rice CD, Pang Y, Pace M, Thomas P** 2003 Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci U S A 100:2231-6

43. **Peluso JJ, Fernandez G, Pappalardo A, White BA** 2001 Characterization of a putative membrane receptor for progesterone in rat granulosa cells. Biol Reprod 65:94-101

44. **Sakamoto H, Ukena K, Takemori H, Okamoto M, Kawata M, Tsutsui K** 2004 Expression and localization of 25-Dx, a membrane-associated putative progesterone-binding protein, in the developing Purkinje cell. Neuroscience 126:325-34

45. **Benten WP, Lieberherr M, Giese G, Wrehlke C, Stamm O, Sekeris CE, Mossmann H, Wunderlich F** 1999 Functional testosterone receptors in plasma membranes of T cells. Faseb J 13:123-33

46. **Alexaki VI, Dermitzaki E, Charalampopoulos I, Kampa M, Nifli AP, Gravanis A, Margioris AN, Castanas E** 2006 Neuronal differentiation of PC12 cells abolishes the expression of membrane androgen receptors. Exp Cell Res 312:2745-56

47. **Benten WP, Lieberherr M, Stamm O, Wrehlke C, Guo Z, Wunderlich F** 1999 Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Mol Biol Cell 10:3113-23

48. **Estrada M, Espinosa A, Muller M, Jaimovich E** 2003 Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. Endocrinology 144:3586-97

49. **Kampa M, Nifli AP, Charalampopoulos I, Alexaki VI, Theodoropoulos PA, Stathopoulos EN, Gravanis A, Castanas E** 2005 Opposing effects of estradiol- and testosterone-membrane binding sites on T47D breast cancer cell apoptosis. Exp Cell Res 307:41-51

50. **Vicencio JM, Ibarra C, Estrada M, Chiong M, Soto D, Parra V, Diaz-Araya G, Jaimovich E, Lavandero S** 2006 Testosterone induces an intracellular calcium increase by a nongenomic mechanism in cultured rat cardiac myocytes. Endocrinology 147:1386-95

51. **Benten WP, Guo Z, Krucken J, Wunderlich F** 2004 Rapid effects of androgens in macrophages. Steroids 69:585-90

52. **Somjen D, Kohen F, Gayer B, Kulik T, Knoll E, Stern N** 2004 Role of putative membrane receptors in the effect of androgens on human vascular cell growth. J Endocrinol 180:97-106

53. **Ramsden M, Nyborg AC, Murphy MP, Chang L, Stanczyk FZ, Golde TE, Pike CJ** 2003 Androgens modulate beta-amyloid levels in male rat brain. J Neurochem 87:1052-5

54. **Hammond J, Le Q, Goodyer C, Gelfand M, Trifiro M, LeBlanc A** 2001 Testosterone-mediated neuroprotection through the androgen receptor in human primary neurons. J Neurochem 77:1319-26

55. **Pike CJ** 2001 Testosterone attenuates beta-amyloid toxicity in cultured hippocampal neurons. Brain Res 919:160-5

56. **Gouras GK, Xu H, Gross RS, Greenfield JP, Hai B, Wang R, Greengard P** 2000 Testosterone reduces neuronal secretion of Alzheimer's beta-amyloid peptides. Proc Natl Acad Sci U S A 97:1202-5

57. **Xu H, Gouras GK, Greenfield JP, Vincent B, Naslund J, Mazzarelli L, Fried G, Jovanovic JN, Seeger M, Relkin NR, Liao F, Checler F, Buxbaum JD, Chait BT, Thinakaran G, Sisodia SS, Wang R, Greengard P, Gandy S** 1998 Estrogen reduces neuronal generation of Alzheimer beta-amyloid peptides. Nat Med 4:447-51

58. **Ramsden M, Shin TM, Pike CJ** 2003 Androgens modulate neuronal vulnerability to kainate lesion. Neuroscience 122:573-8

59. **Estrada M, Varshney A, Ehrlich BE** 2006 Elevated testosterone induces apoptosis in neuronal cells. J Biol Chem 281:25492-501

60. **Caruso A, Di Giorgi Gerevini V, Castiglione M, Marinelli F, Tomassini V, Pozzilli C, Caricasole A, Bruno V, Caciagli F, Moretti A, Nicoletti F, Melchiorri D** 2004 Testosterone amplifies excitotoxic damage of cultured oligodendrocytes. J Neurochem 88:1179-85

61. **Yang SH, Perez E, Cutright J, Liu R, He Z, Day AL, Simpkins JW** 2002 Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model. J Appl Physiol 92:195-201

62. **Verzola D, Gandolfo MT, Salvatore F, Villaggio B, Gianiorio F, Traverso P, Deferrari G, Garibotto G** 2004 Testosterone promotes apoptotic damage in human renal tubular cells. Kidney Int 65:1252-61

63. **White AC, Jr.** 2000 Neurocysticercosis: updates on epidemiology, pathogenesis, diagnosis, and management. Annu Rev Med 51:187-206

64. **Martin LJ, Brambrink A, Koehler RC, Traystman RJ** 1997 Primary sensory and forebrain motor systems in the newborn brain are preferentially damaged by hypoxia-ischemia. J Comp Neurol 377:262-85

65. **Del Zoppo GJ, Zeumer H, Harker LA** 1986 Thrombolytic therapy in stroke: possibilities and hazards. Stroke 17:595-607

66. **Zivin JA** 1999 Thrombolytic stroke therapy: past, present, and future. Neurology 53:14-9

67. **Foulkes MA, Wolf PA, Price TR, Mohr JP, Hier DB** 1988 The Stroke Data Bank: design, methods, and baseline characteristics. Stroke 19:547-54

68. **Hankey GJ, Jamrozik K, Broadhurst RJ, Forbes S, Anderson CS** 2002 Long-term disability after first-ever stroke and related prognostic factors in the Perth Community Stroke Study, 1989-1990. Stroke 33:1034-40

69. **Wassertheil-Smoller S, Hendrix SL, Limacher M, Heiss G, Kooperberg C, Baird A, Kotchen T, Curb JD, Black H, Rossouw JE, Aragaki A, Safford M, Stein E, Laowattana S, Mysiw WJ** 2003 Effect of estrogen plus progestin on stroke in postmenopausal women: the Women's Health Initiative: a randomized trial. Jama 289:2673-84

70. **Chen Q, Moulder K, Tenkova T, Hardy K, Olney JW, Romano C** 1999 Excitotoxic cell death dependent on inhibitory receptor activation. Exp Neurol 160:215-25.

71. **Simpkins JW, Yang SH, Liu R, Perez E, Cai ZY, Covey DF, Green PS** 2004 Estrogen-like compounds for ischemic neuroprotection. Stroke 35:2648-51

72. **Toung TJ, Traystman RJ, Hurn PD** 1998 Estrogen-mediated neuroprotection after experimental stroke in male rats. Stroke 29:1666-70

73. **Jeppesen LL, Jorgensen HS, Nakayama H, Raaschou HO, Olsen TS, Winther K** 1996 Decreased serum testosterone in men with acute ischemic stroke. Arterioscler Thromb Vasc Biol 16:749-54

74. **Yang SH, Liu R, Wen Y, Perez E, Cutright J, Brun-Zinkernagel AM, Singh M, Day AL, Simpkins JW** 2005 Neuroendocrine mechanism for tolerance to cerebral ischemia-reperfusion injury in male rats. J Neurobiol 62:341-51

75. **Elwan O, Abdallah M, Issa I, Taher Y, el-Tamawy M** 1990 Hormonal changes in cerebral infarction in the young and elderly. J Neurol Sci 98:235-43

76. **Allan CA, McLachlan RI** 2004 Age-related changes in testosterone and the role of replacement therapy in older men. Clin Endocrinol (Oxf) 60:653-70

77. **Neaves WB, Johnson L, Porter JC, Parker CR, Jr., Petty CS** 1984 Leydig cell numbers, daily sperm production, and serum gonadotropin levels in aging men. J Clin Endocrinol Metab 59:756-63

78. **Liu PY, Swerdloff RS, Veldhuis JD** 2004 Clinical review 171: The rationale, efficacy and safety of androgen therapy in older men: future research and current practice recommendations. J Clin Endocrinol Metab 89:4789-96

79. **Marin P, Holmang S, Gustafsson C, Jonsson L, Kvist H, Elander A, Eldh J, Sjostrom L, Holm G, Bjorntorp P** 1993 Androgen treatment of abdominally obese men. Obes Res 1:245-51

80. **Janowsky JS, Oviatt SK, Orwoll ES** 1994 Testosterone influences spatial cognition in older men. Behav Neurosci 108:325-32

81. **Janowsky JS, Chavez B, Orwoll E** 2000 Sex steroids modify working memory. J Cogn Neurosci 12:407-14

82. **Schroeder ET, Singh A, Bhasin S, Storer TW, Azen C, Davidson T, Martinez C, Sinha-Hikim I, Jaque SV, Terk M, Sattler FR** 2003 Effects of an oral androgen on muscle and metabolism in older, community-dwelling men. Am J Physiol Endocrinol Metab 284:E120-8

83. **Wittert GA, Chapman IM, Haren MT, Mackintosh S, Coates P, Morley JE** 2003 Oral testosterone supplementation increases muscle and decreases fat mass in healthy elderly males with low-normal gonadal status. J Gerontol A Biol Sci Med Sci 58:618-25

84. **Hajjar RR, Kaiser FE, Morley JE** 1997 Outcomes of long-term testosterone replacement in older hypogonadal males: a retrospective analysis. J Clin Endocrinol Metab 82:3793-6

85. **Barqawi AB, Crawford ED** 2005 Testosterone replacement therapy and the risk of prostate cancer: a perspective view. Int J Impot Res 17:462-3

86. **Bolour S, Braunstein G** 2005 Testosterone therapy in women: a review. Int J Impot Res 17:399-408

87. **Braunstein GD** 2002 Androgen insufficiency in women: summary of critical issues. Fertil Steril 77 Suppl 4:S94-9

88. **Goldstat R, Briganti E, Tran J, Wolfe R, Davis SR** 2003 Transdermal testosterone therapy improves well-being, mood, and sexual function in premenopausal women. Menopause 10:390-8

89. **Shifren JL, Braunstein GD, Simon JA, Casson PR, Buster JE, Redmond GP, Burki RE, Ginsburg ES, Rosen RC, Leiblum SR, Caramelli KE, Mazer NA** 2000 Transdermal testosterone treatment in women with impaired sexual function after oophorectomy. N Engl J Med 343:682-8

90. **Sherwin BB, Gelfand MM** 1987 The role of androgen in the maintenance of sexual functioning in oophorectomized women. Psychosom Med 49:397-409

91. **Sherwin BB, Gelfand MM, Brender W** 1985 Androgen enhances sexual motivation in females: a prospective, crossover study of sex steroid administration in the surgical menopause. Psychosom Med 47:339-51

DIHYDROTESTOSTERONE DIFFERENTIALLY MODULATES THE MAPK AND THE PI-3 KINASE/AKT PATHWAYS THROUGH THE NUCLEAR AND NOVEL MEMBRANE ANDROGEN RECEPTOR IN C6 CELLS

JOSHUA W. GATSON, PARAMJIT KAUR, MEHARVAN SINGH*

Department of Pharmacology and Neuroscience and the Institute for Aging & Alzheimer's Disease Research, University of North Texas Health Science, Fort Worth, Texas

Running Title: Androgen signaling in glia via the membrane AR

Endocrinology 2006, 147(4):2028-34.

Corresponding author:

Meharvan Singh, Ph.D.
Department of Pharmacology and Neuroscience
University of North Texas Health Science Center at Fort Worth
3400 Camp Bowie Blvd.
Fort Worth, Texas 76107-2699
Phone: (817) 735-5429
Fax: (817) 735-0408
E-mail: msingh@hsc.unt.edu

CHAPTER II

DIHYDROTESTOSTERONE DIFFERENTIALLY MODULATES THE MAPK AND THE PI-3 KINASE/AKT PATHWAYS THROUGH THE NUCLEAR AND NOVEL MEMBRANE ANDROGEN RECEPTOR IN C6 CELLS

ABSTRACT

Androgens such as dihydrotestosterone (DHT) are known to exert their effects through the activation of intracellular receptors that regulate the transcription of target genes. Alternatively, "non-genomic" mechanisms, including the activation of such signaling pathways as the mitogen-activated protein kinase (MAPK) pathways have been described. It is unclear, however, whether this latter mechanism of action is mediated by the classical androgen receptor (AR), or some alternative mechanism. Here, using a glial cell model (C6 cells) that we found to express the AR, we identified that DHT increased the phosphorylation of both ERK and Akt, key effectors of the neuroprotection-associated MAPK and phosphoinositide-3 kinase (PI-3K) signaling pathways, respectively, and ERK phosphorylation was blocked by the AR antagonist, flutamide. In contrast, the membrane-impermeable, BSA-conjugated androgen (DHT-BSA), caused a dose-dependent suppression of ERK and Akt phosphorylation, suggesting the existence of a novel membrane-associated AR that mediates this opposite effect on neuroprotective signaling. This is further supported by the observation of DHT-displaceable binding sites on the cell surface of live C6 cells. Collectively, these data support the existence of a

novel membrane-associated AR in glial cells, and argue for the existence of two, potentially competing, pathways in a given cell or tissue. This mutual antagonism was supported by the ability of DHT-BSA to attenuate DHT-induced ERK phosphorylation. Thus, depending on the predominance of one receptor mechanism over another, the outcome of androgen treatment may be very different, and as such, could help explain existing discrepancies as to whether androgens are protective or damage-inducing.

INTRODUCTION

Androgens are classically associated with the regulation of muscle growth, spermatogenesis, growth of bone, and the development of secondary sexual characteristics in males and females (1, 2). Within the brain, testosterone can be aromatized to estradiol or alternatively, reduced at the 5α position to dihydrotestosterone (DHT) (1). DHT, however, is non-aromatizable and can elicit its effects via either genomic (classical) (1, 2) or non-genomic mechanisms (1, 3, 4). With regards to the latter, androgens have been shown to rapidly activate the phosphoinositide-3 (PI-3) kinase/Akt and/or mitogen-activated protein kinase (MAPK) pathways in a variety of peripheral tissues (3-5). However, whether these "non-genomic" effects are mediated by the classical androgen receptor or by some alternative mechanism remains controversial and unclear.

Two isoforms of the classical androgen receptor (AR) have been described, (AR-B and its N-terminally truncated form, AR-A), and are expressed in many different cell

types (6-8). While the precise role of AR-A remains unclear, what has been described is that AR-A can antagonize the action of AR-B, a modulatory mechanism that may be relevant to the activation/inhibition of signaling pathways, regulation of gene transcription, as well as the regulation of cell survival (9). Alternatively, the existence of a plasma membrane receptor for androgens has also been proposed (18), like that described (or postulated) for estrogen and progesterone (11-17). This membrane AR has been described primarily in non-central nervous system tissue, including vascular tissue, macrophages, the ovary and in T cells (10, 19-24). Of interest is that this membrane-associated AR is linked to the activation of signaling pathways that may be important in regulating cell death, survival, or growth (18).

In order to clarify the role of the classical AR in the "non-genomic" effects of androgens on glia, we evaluated the effects of DHT and the membrane impermeable, BSA-conjugated androgen (DHT-BSA), on the phosphorylation of ERK and Akt, two key effectors within the MAPK and PI-3K signaling pathways, respectively. We found that while DHT induced the phosphorylation of ERK, DHT-BSA resulted in a dose-dependent suppression of both ERK and Akt phosphorylation and even blocked the effects of DHT. The suggestion that this effect of DHT-BSA was mediated by a novel membrane-associated AR was further supported by the identification of DHT-displaceable binding sites on the cell surface of live C6 cells. Collectively, these data support the existence of a novel plasma membrane-associated androgen receptor and

suggest the existence of two, potentially competing pathways, within a specific cell or tissue type.

MATERIALS AND METHODS

Cell culture. Rat glioma cells (C6; American Type Culture Collection, Manassas, VA) were propagated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% charcoal-stripped fetal bovine serum (FBS; Hyclone, Logan, UT), and maintained at 37°C in a humidified environment containing 5% CO_2.

Following treatment of the cells with the appropriate duration and dose of hormone, the cells were harvested, homogenized, and centrifuged and the supernatant was subsequently collected and analyzed for total protein concentration using the Biorad DC protein assay kit. Pre-prepared cell lysates from the prostate cancer cell line, LNCaP, (Santa Cruz Biotechnology, Santa Cruz, CA) were obtained and used as positive controls for the detection of the androgen receptor.

Treatment of cultures. C6 cells were treated with either vehicle control (DMSO, 0.1%), dihydrotestosterone (DHT) (Steraloids Inc., Newport, RI), or 5α-androstan-17β-ol-3-one-3-o-carboxymethyloxime:BSA (DHT-BSA) (Steraloids Inc.) at the concentrations indicated for 30 minutes, to assess the effects of these hormones on ERK and Akt phosphorylation. Controls for the BSA-conjugated DHT (DHT-BSA) treatment included treatment with equimolar concentrations of 5α-androstan-17β-ol-3-one-3-o-

carboxymethyloxime (DHT:CMO) (Steraloids Inc.) or bovine serum albumin (BSA) alone (Fisher Scientific, Fair Lawn, NJ) for a similar duration (30 min). Inhibition of the classical androgen receptor was achieved using the androgen receptor antagonist, Flutamide (3 μM; Sigma-Aldrich, St. Louis, MO), which was applied 30 minutes prior to treatment with the hormone. Inhibition of MEK, the signaling protein upstream of ERK was achieved using UO126 (10 μM; Cell Signaling, Beverly, MA), also pre-incubated for a period of 30 minutes prior to hormone administration.

Western blot analysis. Following treatment with hormone and/or inhibitor, C6 cells were harvested into lysis buffer containing protease and phosphatase inhibitors as described previously (25). Following homogenization, samples were centrifuged at 99,000 x g for 15 min at 4°C, and the resulting supernatants were evaluated for total protein concentrations using the BioRad DC (Biorad Labs, Hercules, CA) protein assay kit [based on the method of Lowry; (26)]. Sample lysates were loaded onto a sodium dodecyl sulfate (SDS), 10% polyacrylamide gel (PAGE), subjected to electrophoresis, and subsequently transferred onto a polyvinylidene difluoride membrane (PVDF; 0.22 μm pore size, BioRad). The membrane was blocked for 6 hrs with a 3% BSA in 0.2% Tween-containing TBS (TBS-T) solution prior to application of the primary antibody. The following primary antibodies were used: for the detection of the androgen receptor, anti-AR (C-19) (1:200; Santa Cruz Biotechnology); for the detection of the phosphorylated form of Akt: rabbit anti-phospho-Akt (Ser473; 1:1000; Cell Signaling, Beverly, MA), for the detection of total Akt, anti Akt (1:1000; Cell Signaling); for the

detection of the phosphorylated form of ERK1/2, rabbit anti phospho-p44/42 Map Kinase (Thr202/Tyr204, 1:1000; Cell Signaling); for the detection of total ERK1 and ERK2, goat anti-ERK1 (C-16, 1:500)/goat anti-ERK2 (C-14, 1:500; Santa Cruz Biotechnology). Antibody binding to the membrane was detected using a secondary antibody (either goat anti-rabbit or rabbit anti-goat) conjugated to horseradish peroxidase (1:20,000; Pierce, Rockford, IL.) and visualized using enzyme-linked chemiluminescence (ECL, Amersham, Arlington Heights, IL) with the aid of the UVP imaging system. Phospho-Akt and phosphoERK blots were re-probed with anti-Akt or anti ERK1/2 antibodies to ensure equal loading across lanes.

Flow cytometry. C6 cells (10^6 cells) were pipetted into a 1.5 ml micro-centrifuge tube, centrifuged at 250 x *g* for 5 minutes and washed twice with 1ml of PBS. After the last wash, the cells were re-pelleted, and suspended in 100 µl of PBS and treated with DHT-BSA-FITC (50 µM; Sigma Aldrich) in the presence or absence of dihydrotestosterone (DHT; 1 mM), for 30 minutes at 4°C. In parallel, cells were re-suspended in PBS treated with BSA-FITC (50 µM; Sigma Aldrich), serving as the control for the detection of "non-specific binding". Following this incubation period, the cells were washed twice in PBS and resuspended in 500 µl of PBS. The labeled C6 cells were injected into a EPICS XL-MCL flow cytometer (Beckman Coulter) and analyzed with System 2 software (Beckman Coulter). Graphical representation of the data was generated using the FloJo software (Tree Star, Inc., San Carlos, CA).

Statistical Analysis. Densitometric analysis of the Western blots was conducted using the LabWorks Image Acquisition and Analysis Software (UVP Inc., Upland, CA). Densitometric data from at least three independent experiments was subjected to an analysis of variance (ANOVA), followed by a Tukey's *post hoc* analysis for the assessment of group differences, and presented as a bar graph depicting the average ± S.E.M., using the GraphPad Software (San Diego, CA).

RESULTS

C6 cells express the androgen receptor (AR)

Genomic and non-genomic pathways activated by androgens may involve one or both receptor isoforms of the androgen receptor (AR-B or AR-A). Using Western blot analysis, we evaluated whether the C6 glial cells express either of these receptor isoforms. Figure 1 confirms the presence of immunoreactive AR, and identifies two distinct bands corresponding to molecular weights of 110 and 87 kDa, respectively. These two bands are of the same size described in the literature for the full length, AR-B and the truncated, AR-A. Interestingly, a doublet was observed in lysates from C6 cells. Given that in protein bands may appear as doublets (such as for ERK1 and ERK2), reflecting the phosphorylated and unphosphorylated forms of the protein, we suggest that this doublet may also reflect two dominant post-translational states of the AR. Nevertheless, the bands identified in Figure 1 co-migrated with the AR-B and AR-A

bands seen in the positive control, a cell lysate derived from the prostate cancer cell line, LNCaP cells.

The nuclear AR mediates DHT-induced phosphorylation of ERK

Given that androgens have been shown to elicit activation of cell signaling pathways in a variety of tissues, we evaluated whether DHT elicits the phosphorylation of ERK, a key effector of the MAPK pathway, in glia. Treatment of C6 cells with 10 nM DHT resulted in a robust (2.5-fold) increase in the phosphorylation of ERK. This effect was inhibited by the classical androgen receptor antagonist, Flutamide (Fig. 2). The effect of DHT on ERK phosphorylation was also blocked by the MEK1/2 inhibitor, UO126 (data not shown), suggesting that the effect of DHT on ERK required the activation of the upstream signaling kinase, MEK. Interestingly, higher concentrations of DHT (0.1 and 1 μM) did not result in an increase in ERK phosphorylation (Fig. 2). DHT also induced an increase in Akt phosphorylation, but required a slightly higher concentration than that which was required to elicit ERK phosphorylation. Another distinction was that the antagonist to the classical androgen receptor, flutamide, failed to inhibit the effect of DHT on Akt phosphorylation (Fig. 3).

Activation of a membrane AR decreased the phosphorylation of ERK

In an attempt to assess if the effect of DHT was mediated by the classical intracellular/intranuclear androgen receptor or alternatively, if a membrane androgen

receptor may be involved, we determined whether the membrane-impermeable androgen, DHT-BSA, would also elicit ERK phosphorylation. In contrast to the effect of DHT, the membrane-impermeable androgen not only failed to elicit an increase in the phosphorylation of ERK, but resulted in a substantial suppression of ERK phosphorylation, particularly at the higher concentrations of 100 nM and 1 μM (Fig. 4). This suppression of ERK phosphorylation by DHT-BSA was also insensitive to Flutamide (Fig. 5). To ensure that the inhibition of ERK was not due to the chemical modification of the parent DHT molecule, we evaluated the effect of DHT:CMO(5α-androstan-17β-ol-3-one-3-o-carboxymethyloxime). As expected, the carboxymethyloxime (CMO) group alone did not alter the ability of the androgen to elicit ERK phosphorylation (Fig. 6), and recapitulated the data seen with DHT alone (Fig. 2). Further, the effect of DHT:CMO on ERK phosphorylation was also blocked by the androgen receptor antagonist, flutamide (Fig. 6). As an added control, we determined whether the inhibitory effect of DHT-BSA could have been attributed to the bulky globulin (BSA) that was attached. Administration of BSA by itself failed to alter the basal phosphorylation state of ERK, suggesting that the inhibition was not due to BSA (Fig. 7).

Activation of the membrane androgen receptor results in a dose-dependent decrease in the phosphorylation of Akt

In order to assess if the activation of the putative membrane androgen receptor influenced another growth and/or survival promoting signal transduction pathway in a similar manner to what was observed with the MAPK pathway, we evaluated the effect of DHT-BSA on the downstream effector of the PI-3 kinase pathway, Akt. DHT-BSA, at concentrations of 0.01, 0.1 and 1 μM resulted in a dose-dependent suppression of Akt phosphorylation (Fig. 8).

DHT-BSA blocks DHT-induced ERK phosphorylation in a dose-dependent manner

In view of the inhibitory effects of DHT-BSA on ERK and Akt phosphorylation, and the stimulatory consequence of activating the intracellular androgen receptor (using DHT), we evaluated if activating the putative membrane androgen receptor would inhibit the classical AR-mediated induction of ERK phosphorylation. As such, we co-applied DHT and DHT-BSA to the C6 cells for 30 min and found that DHT-BSA blocked the effect of DHT (10 nM) on ERK phosphorylation at all concentrations tested (0.01, 0.1, 1 μM), and in a dose dependent fashion (Fig. 9). These findings support the idea that DHT may modulate the MAPK through at least two, competing pathways.

C6 glial cells express binding sites for DHT-BSA on the cell surface.

In order to determine if DHT-BSA binds to a specific site on the cell surface (indicative of a membrane-associated androgen receptor), we evaluated the binding of the fluorescently-labeled DHT-BSA (DHT-BSA-FITC) in C6 cells. Non-"fixed" and non-permeabilized C6 cells were treated with DHT-BSA-FITC (50 μM) in the presence or absence of 20-fold molar excess of DHT (1mM). Incubation of the cells with BSA-FITC alone for 30 min at 4°C provided a measure of non-specific binding. The fluorescence-intensity histograms (Fig. 10) obtained through flow cytometric analysis revealed the presence of specific, DHT-displaceable binding sites on the cell surface of the C6 cells.

DISCUSSION

In this study, we show that androgens regulate the activity of two signal transduction pathways, the MAPK and the PI-3K/Akt signaling pathways, in C6 glial cells. The existence of immunoreactive androgen receptors (AR-B and AR-A) in the C6 cells (Fig. 1), coupled with the fact that DHT-induced ERK phosphorylation was inhibited by the pharmacological antagonist of the classical androgen receptor (Fig. 2) supported the involvement of this "classical" receptor in regulating cell signaling. However, the opposite effect of DHT-BSA on ERK (Fig. 4) and Akt (Fig. 8) phosphorylation suggested that androgens may also regulate cell signaling via a distinct, and potentially, competing receptor mechanism. Supporting this further was the finding that the inhibitory effect of DHT-BSA on ERK phosphorylation was not blocked by the

classical androgen receptor antagonist, flutamide (Fig. 5), unlike the effects of DHT (Fig. 2). In addition, analysis of the binding of fluorescently-labeled DHT-BSA to live C6 cells revealed specific, DHT-displaceable binding sites on the cell surface (Fig. 10).

In order to exclude the possibility that chemical modification of the parent compound, DHT, was responsible for the observed effects of DHT-BSA, several important controls were performed. First, we ensured that the carboxymethyloxime (CMO) "linker" that enables the attachment of BSA to DHT, did not alter the ability of DHT to regulate cell signaling. Figure 6 shows that DHT:CMO elicited the same dose-dependent regulation of ERK phosphorylation as did the parent compound (Fig. 2). It is also unlikely that the effect of DHT-BSA was due to the presence of "free" DHT present in the DHT-BSA preparation, since the effect of DHT-BSA was opposite to that observed with DHT. Nevertheless, we took steps to ensure that no free DHT was present in the DHT-BSA solution. This was achieved by pre-filtering the DHT-BSA using a 30 kDa nominal cut-off column. The retentate was then eluted and used in the experiments. All data shown were derived from experiments in which the filtered DHT-BSA was used. Further, no differences were observed between the effects of filtered DHT-BSA and unfiltered DHT-BSA (data not shown). Finally, we ensured that BSA by itself was not responsible for the effect of DHT-BSA. By testing increasing concentrations of BSA, we found no differences in ERK phosphorylation relative to controls (Fig. 7).

Interestingly, while low concentrations of DHT elicited ERK and Akt phosphorylation, higher concentrations failed to do so (Fig. 2 and 3). We suggest that at

higher concentrations, DHT binds to the membrane androgen receptor, resulting in activation of an antagonistic mechanism reducing and/or preventing the induction of ERK phosphorylation via the classical receptor. This assertion is consistent with the dose response data depicted in Figures 2 and 4. That is, the inhibitory effect of DHT-BSA on either ERK or Akt phosphorylation becomes evident at the 0.1μM concentrations, and only at this same concentration (or higher) does DHT not activate ERK phosphorylation. Further, Figure 9 also demonstrates that DHT-BSA – induced activation of the putative membrane androgen receptor inhibits the ERK-inducing effects of DHT. Altogether, these data support the existence of two, competing mechanisms through which DHT regulates cell signaling. In addition, there also appeared to be a slight difference in potency by which DHT elicited ERK and Akt phosphorylation. The precise mechanism underlying this difference is still unclear, but possible explanations may include the existence of different androgen receptor subtypes or isoforms that mediate the effects of androgens on ERK and Akt separately. Ongoing research is aimed at addressing this difference further.

Membrane receptors have also been proposed for estrogen and progesterone, but only the membrane progesterone receptor has been successfully cloned. Zhu and colleagues (17) characterized a novel membrane-associated progesterone receptor that appears not to exhibit the stereotypical modular structure seen with other members of the steroid hormone receptor superfamily, but instead, contains a seven transmembrane spanning domain. As such, this membrane progesterone receptor was reported to be

coupled to the Gi/o class of G-proteins. Based upon this observation, we postulated that the inhibitory effects of DHT-BSA on ERK and Akt phosphorylation could also be regulated through Gi/o. To test this hypothesis, we evaluated whether pertussis toxin (a Gi/o inhibitor) prevented the effect of DHT-BSA. Pertussis toxin (50 μM) failed to prevent DHT-BSA – mediated suppression of either ERK or Akt phosphorylation (data not shown), leading to our conclusion that this novel membrane androgen receptor was not coupled to the Gi/o class of G-proteins, at least in the C6 glial cell model.

Activation of the ERK/MAPK pathway is associated with various cellular responses, including the induction of cell differentiation, increased cell growth/proliferation, as well as the regulation of cell viability. (27, 28). Similarly, the activation of the PI-3K/Akt signaling pathway results in numerous effects on the cell, including the regulation of cell growth, motility, and survival (29). Various studies have shown that, depending on the cell type, androgens can either cause a decrease or an increase in phospho-ERK levels, (5, 22-24, 30, 31), and as a consequence, may result in varied cellular responses. In the brain, this variability in androgen function has also been observed such that depending on the experimental model used or region of the brain evaluated, androgens can exert either protective influences (32, 33), or be damage-promoting (34). For example, in a kainic acid model of hippocampal injury, DHT was found to reduce the amount of hippocampal neuron damage (33), whereas in a middle cerebral artery occlusion (MCAO) model of stroke, elevated androgen levels were associated with greater amounts of cortical cell death (34). This discrepancy may be

related to the relative abundance of the classical intracellular/intranuclear androgen receptor and the membrane androgen receptor identified here. Specifically, the protective effects of androgens may be seen only under conditions where the classical androgen receptor predominates, resulting in an increased activation of ERK and/or Akt, which in turn, would favor the promotion of cell survival. In contrast, if the membrane androgen receptor predominates, one might predict that elevated androgens may result in increased vulnerability to insult or injury due to the suppression of neuroprotective signaling pathways.

Another critical aspect in understanding the neurobiology of androgens is the possibility that glia may respond differently to androgens than do neurons. Of importance is that the androgen receptor is present not only in neurons, but in glial cells as well. In fact, brain astrocytes express high amounts of the AR (35). The functional significance of this expression in glia may be inferred from the observation that following stroke, AR levels are up-regulated in glial cells within the hippocampus and parietal cortex (36). Thus, androgens and the androgen receptor may play an important role in regulating brain vulnerability to injury, both by acting at androgen receptors on neurons as well as glia.

In summary, our data support the existence of a novel membrane-associated AR in glial cells, in addition to the classical androgen receptor. Further, the data argue for the existence of two, potentially competing, pathways in a given cell or tissue. Thus, the ratio of one receptor type over another may be predictive of whether androgens are beneficial or detrimental, and as such, could help explain existing discrepancies as to whether

androgens are protective or damage-inducing. Such information may also be instrumental in helping design appropriate therapeutic regimens that employ androgens for the treatment of various diseases of the brain.

ACKNOWLEDGMENTS

This work was supported by funds from the National Institutes on Aging grant (AG22550 and AG23330), NARSAD-sponsored Young Investigator Awarded to M.S., and the National Science Foundation SCORE (School's Cooperative Opportunities for Resources and Education) grant.

Figure 1. The classical androgen receptor is expressed in C6 cells. Total protein from C6 cells was isolated and evaluated for the expression of the AR using Western blot analysis. This analysis revealed two bands corresponding to the predicted molecular weights of AR-B (110 kDa) and AR-A (87 kDa). These bands co-migrated with the AR bands observed in the prostate cancer cell line, LNCaP, serving as the positive control.

Figure 1.

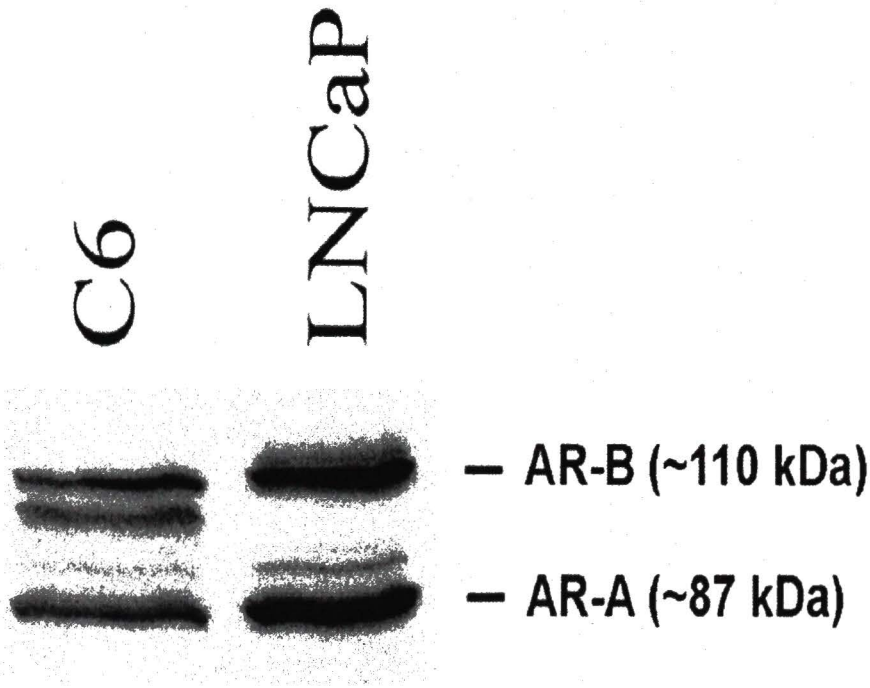


Figure 2. DHT-induced ERK phosphorylation is mediated by the classical AR. C6 cells were treated with DHT (0.01, 0.1, 1 μM) for 30 min in the presence/absence of the AR antagonist Flutamide (Flut., 3 μM). The upper blot and lower blot depicts ERK phosphorylation and total ERK protein, respectively, from a single independent experiment. The bar graph (upper panel), however, represents the densitometric analysis and statistical evaluation of data from three independent experiments, and is presented as signal intensity relative to that seen in the sham (vehicle-treated) control. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (* = $p < 0.01$). Data are expressed as the mean ± S.E.M.

Figure 2.

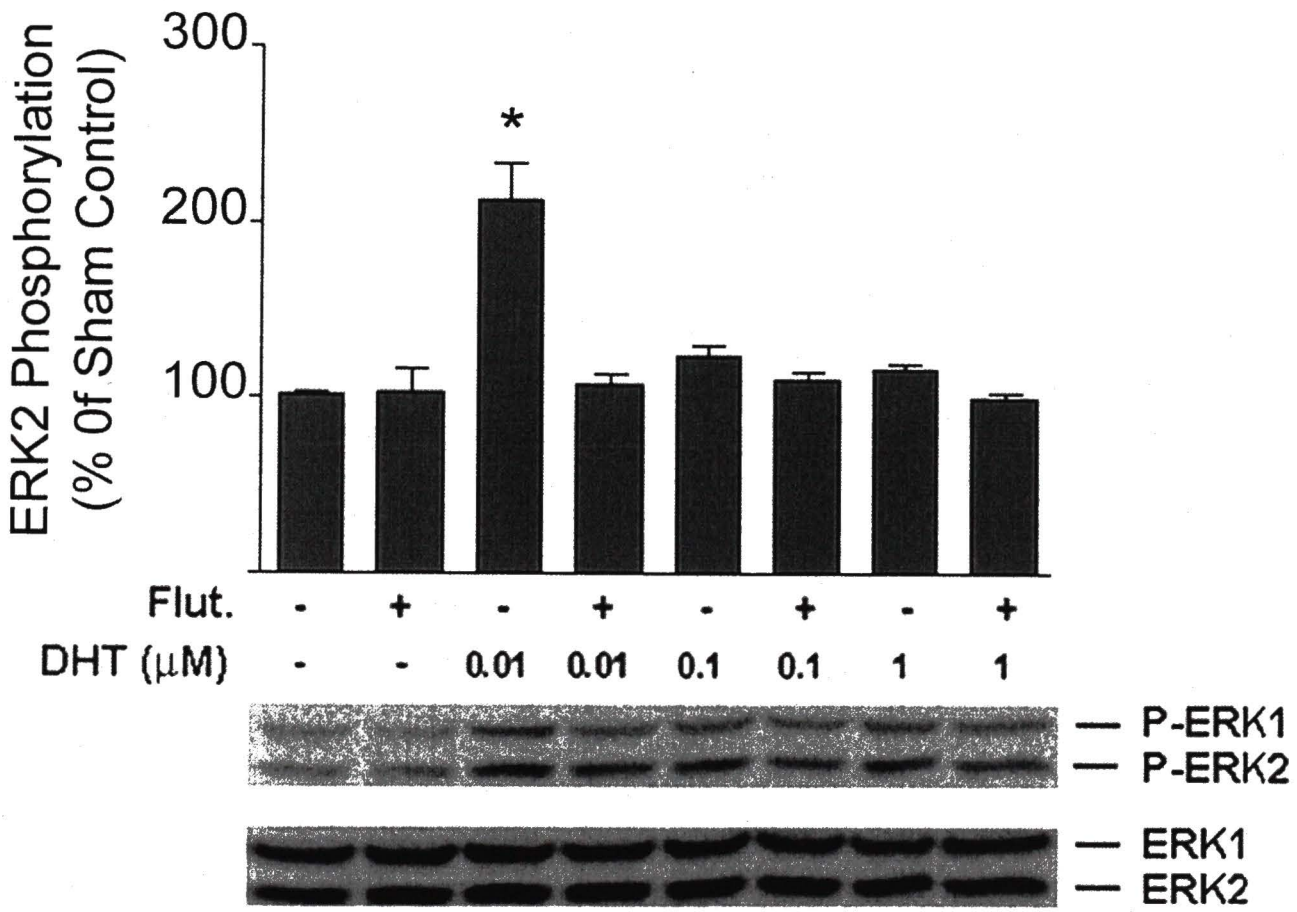

ERK2 Phosphorylation
(% Of Sham Control)
300
200
100
*
Flut. - + - + - + - +
DHT (μM) - - 0.01 0.01 0.1 0.1 1 1
P-ERK1
P-ERK2
ERK1
ERK2

Figure 3. DHT elicits an increase in phospho-Akt levels in C6 cells. Rat glioma cells were treated with DHT (0.01, 0.1, 1 μM) for 30 min in the presence/absence of the AR antagonist Flutamide (Flut., 3 μM). The results show that DHT elicited an increase in Akt phosphorylation, but was not inhibited by the AR antagonist, flutamide. The upper blot and lower blot depicts Akt phosphorylation and total Akt protein, respectively, from a single independent experiment. The bar graph (upper panel), however, represents the densitometric analysis from two independent experiments and is presented as signal intensity relative to that seen in the sham (vehicle-treated) control.

Figure 3.

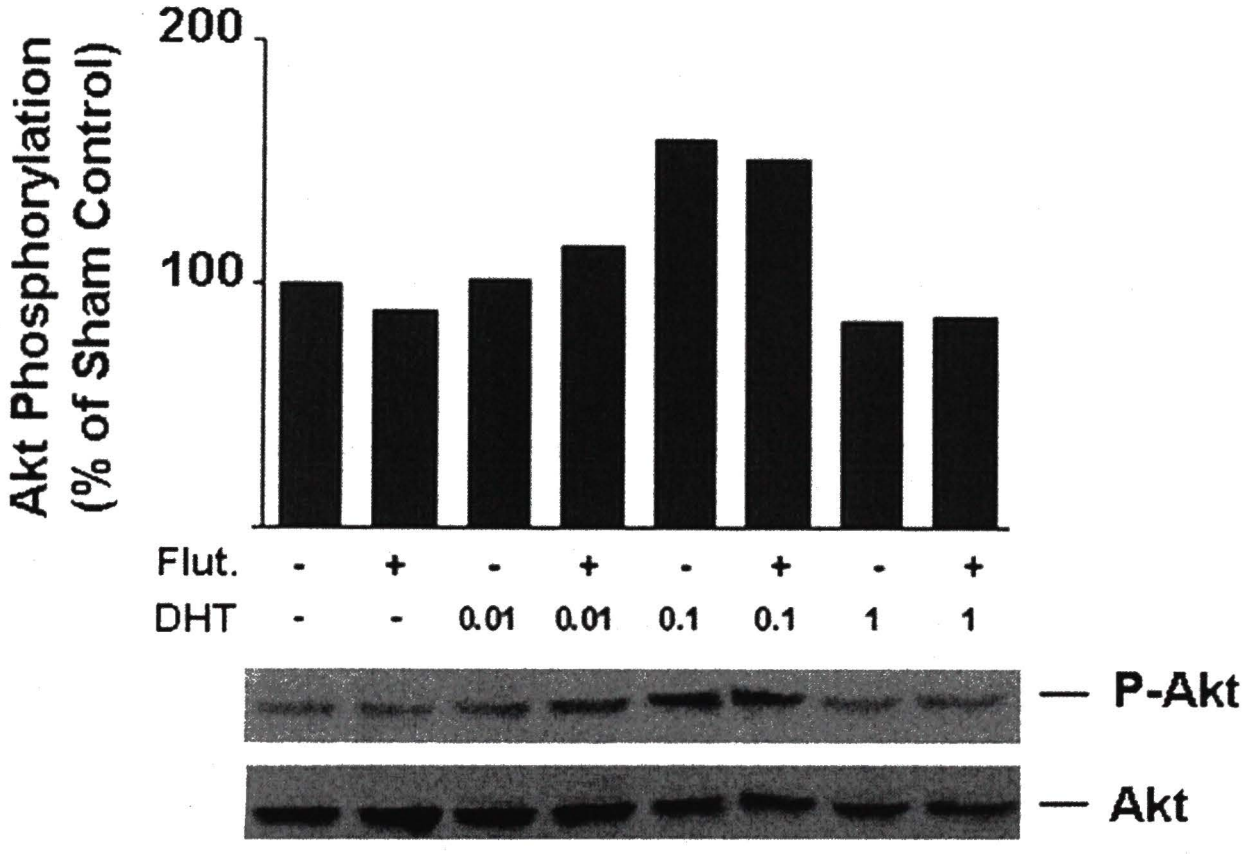

Akt Phosphorylation
(% of Sham Control)
200
100
Flut. - + - + - + - +
DHT - - 0.01 0.01 0.1 0.1 1 1
P-Akt
Akt

Figure 4. DHT-BSA treatment suppresses phospho-ERK levels in C6 cells. C6 cells were treated with increasing concentrations of DHT-BSA (0.01, 0.1, and 1.0 μM) for 30 min. DHT-BSA resulted in a dose-dependent decrease in phospho-ERK levels. The data revealed that DHT-BSA inhibited basal ERK phosphorylation levels in a dose-dependent manner. The upper blot and lower blot depict ERK phosphorylation and total ERK protein, respectively, from a single independent experiment. The bar graph (upper panel), however, represents the densitometric analysis and statistical evaluation of data from three independent experiments, and is presented as signal intensity relative to that seen in the sham (vehicle-treated) control. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (* = $p < 0.001$; # = $p < 0.01$). Data are expressed as the mean $\pm$ S.E.M.

Figure 4.

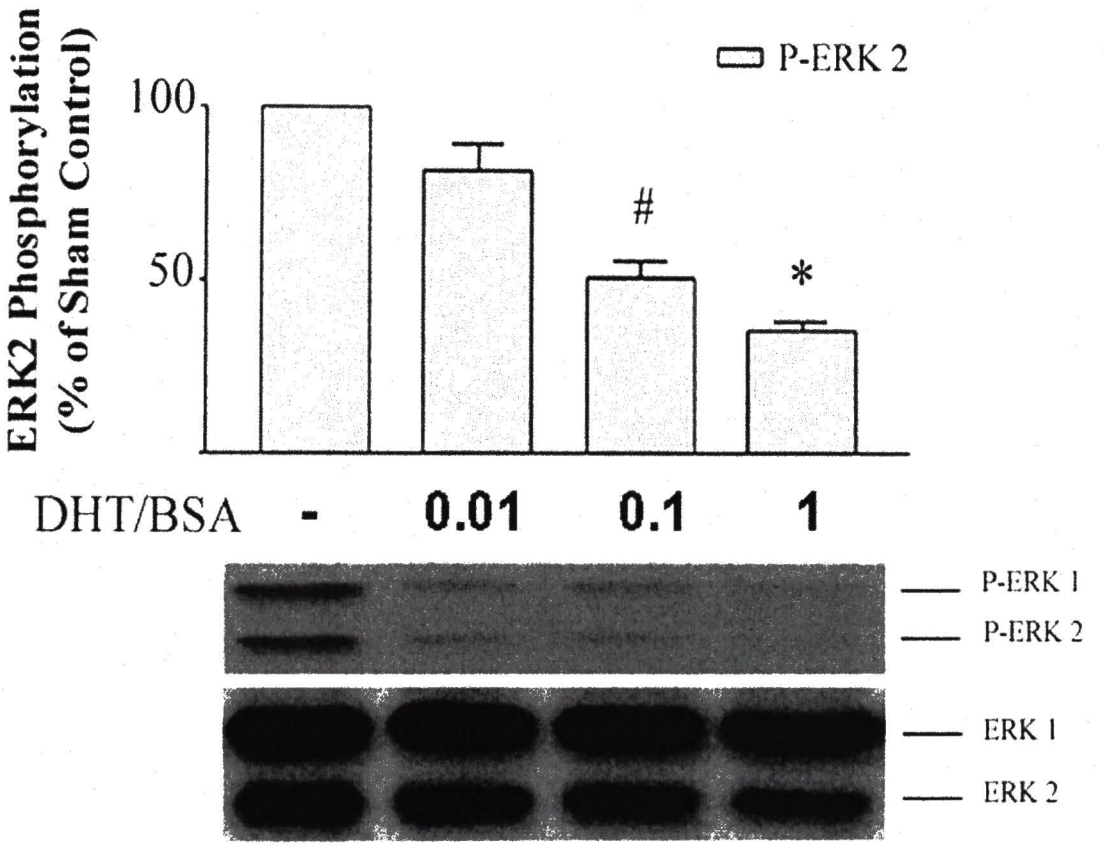

ERK2 Phosphorylation
(% of Sham Control)
P-ERK 2
100
50
#
*
DHT/BSA
-
0.01
0.1
1
P-ERK 1
P-ERK 2
ERK 1
ERK 2

Figure 5. DHT-BSA suppression of phospho-ERK levels in C6 cells is Flutamide insensitive. C6 cells were treated with increasing concentrations of DHT-BSA (0.01, 0.1, and 1.0 μM) for 30 min. in the presence/absence of the AR antagonist flutamide (Flut., 3 μM). The results demonstrated that the dose-dependent inhibition of ERK phosphorylation by DHT-BSA was not prevented by the AR antagonist, flutamide.The upper blot and lower blot depict ERK phosphorylation and total ERK protein, respectively, from a single independent experiment. The bar graph (upper panel), however, represents the densitometric analysis from two independent experiments, and is presented as signal intensity relative to that seen in the sham (vehicle-treated) control.

Figure 5.

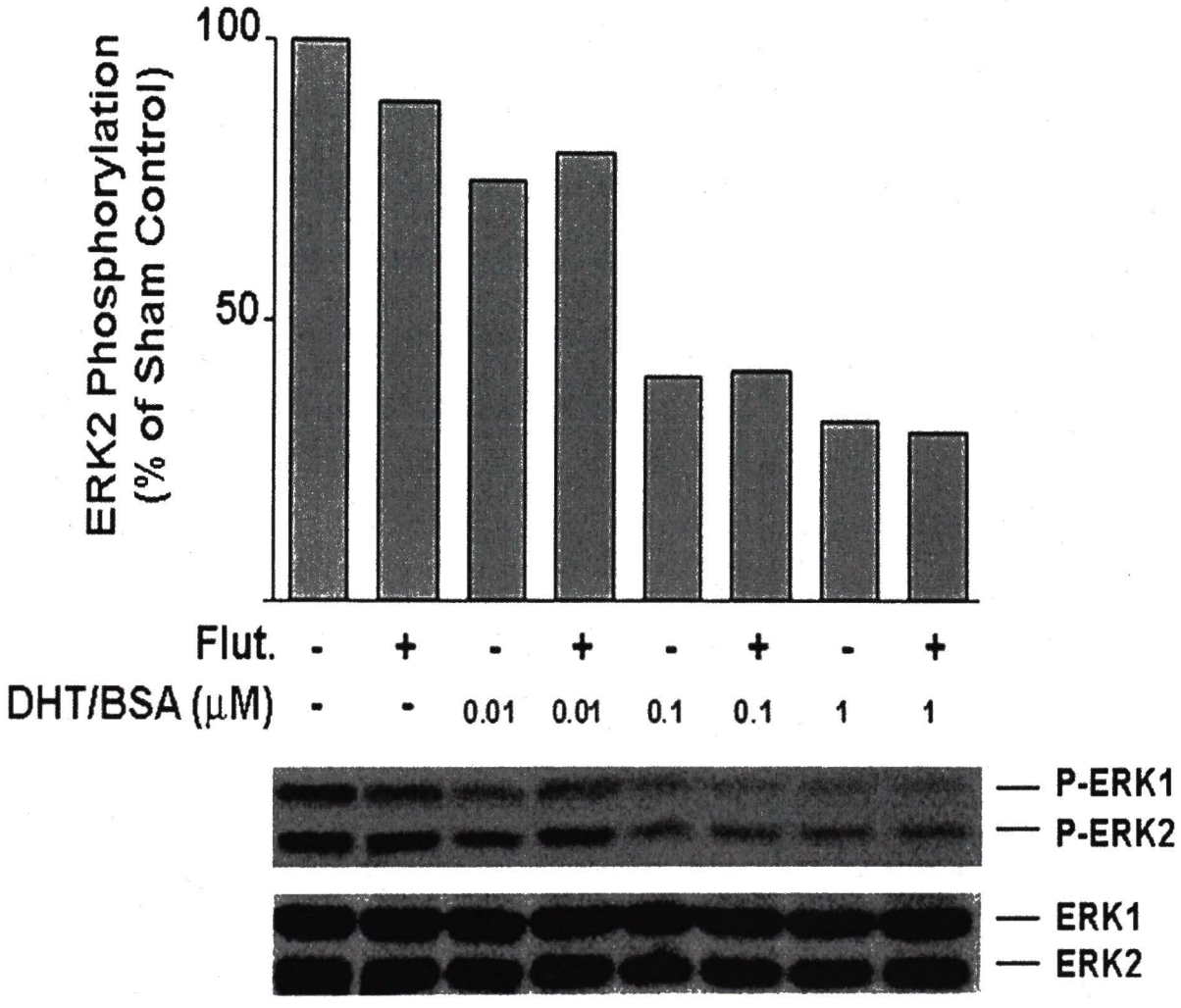

ERK2 Phosphorylation
(% of Sham Control)
100
50
Flut. - + - + - + - +
DHT/BSA (μM) - - 0.01 0.01 0.1 0.1 1 1
P-ERK1
P-ERK2
ERK1
ERK2

Figure 6. Carboxymethyloxime (CMO) conjugation to DHT does not alter DHT's ability to elicit ERK phosphorylation. C6 cells were treated with DHT:CMO (.01, .1, 1 μM) for 30 min in the presence/absence of the AR antagonist Flutamide (3 μM). DHT:CMO was included in these studies as a control group for the BSA-conjugated DHT (DHT-BSA). The upper blot and lower blot depicts ERK phosphorylation and total ERK protein, respectively, from a single independent experiment. The bar graph (upper panel), however, represents the densitometric analysis and statistical evaluation of data from three independent experiments, and is presented as signal intensity relative to that seen in the sham (vehicle-treated) control. Statistical significance was determined using a one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (* = $p < 0.01$). Data are expressed as the mean $\pm$ S.E.M.

Figure 6.

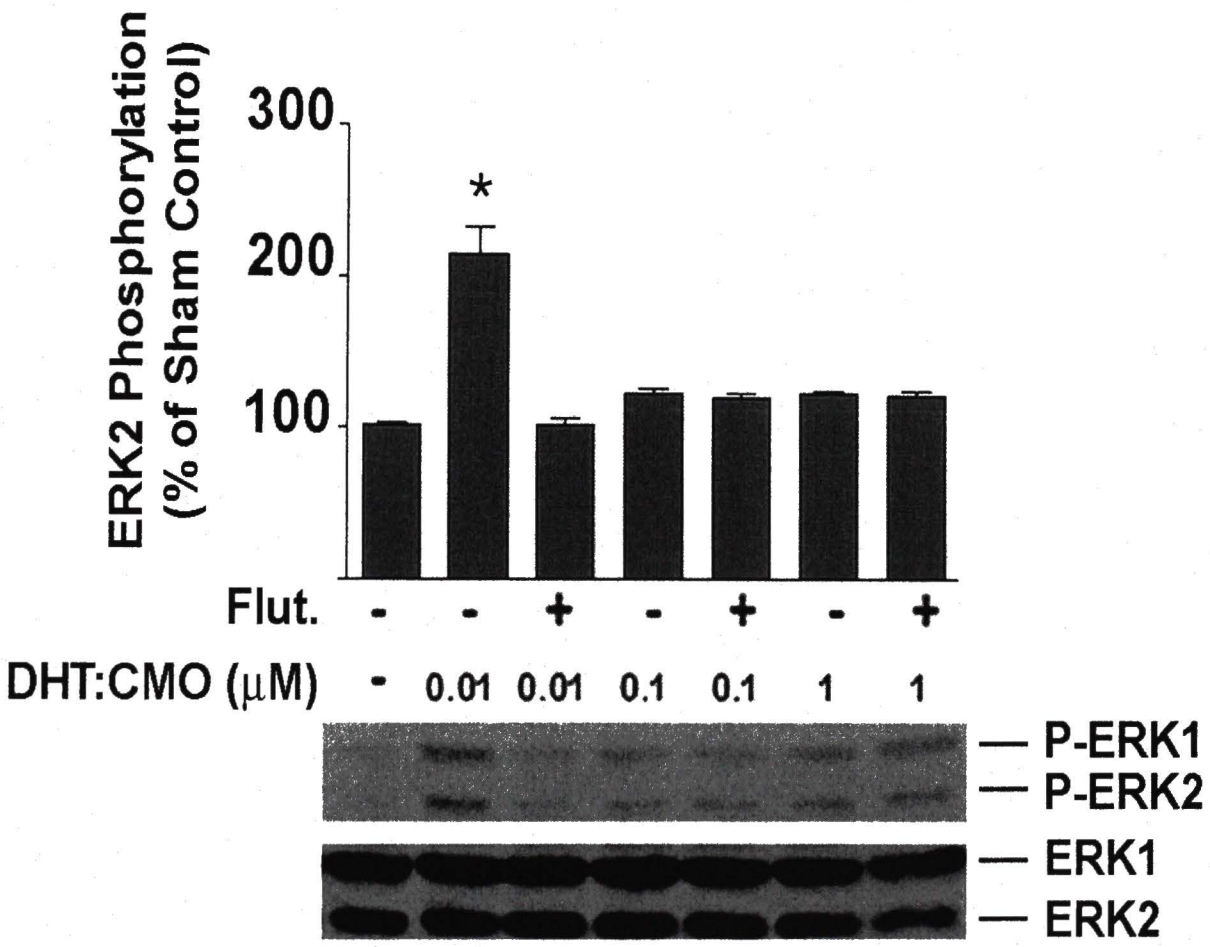

ERK2 Phosphorylation
(% of Sham Control)
300
200
100
*
Flut. - - + - + - +
DHT:CMO (μM) - 0.01 0.01 0.1 0.1 1 1
P-ERK1
P-ERK2
ERK1
ERK2

Figure 7. BSA does not elicit ERK phosphorylation. C6 cells were treated with increasing concentrations of BSA alone for 30 min. The data reveal that BSA treatment by itself failed to alter ERK phosphorylation levels. The upper blot and lower blot depicts ERK phosphorylation and total ERK protein, respectively, from a single independent experiment. The bar graph (upper panel), however, represents the densitometric analysis from two independent experiments, and is presented as signal intensity relative to that seen in the sham (vehicle-treated) control.

Figure 7.

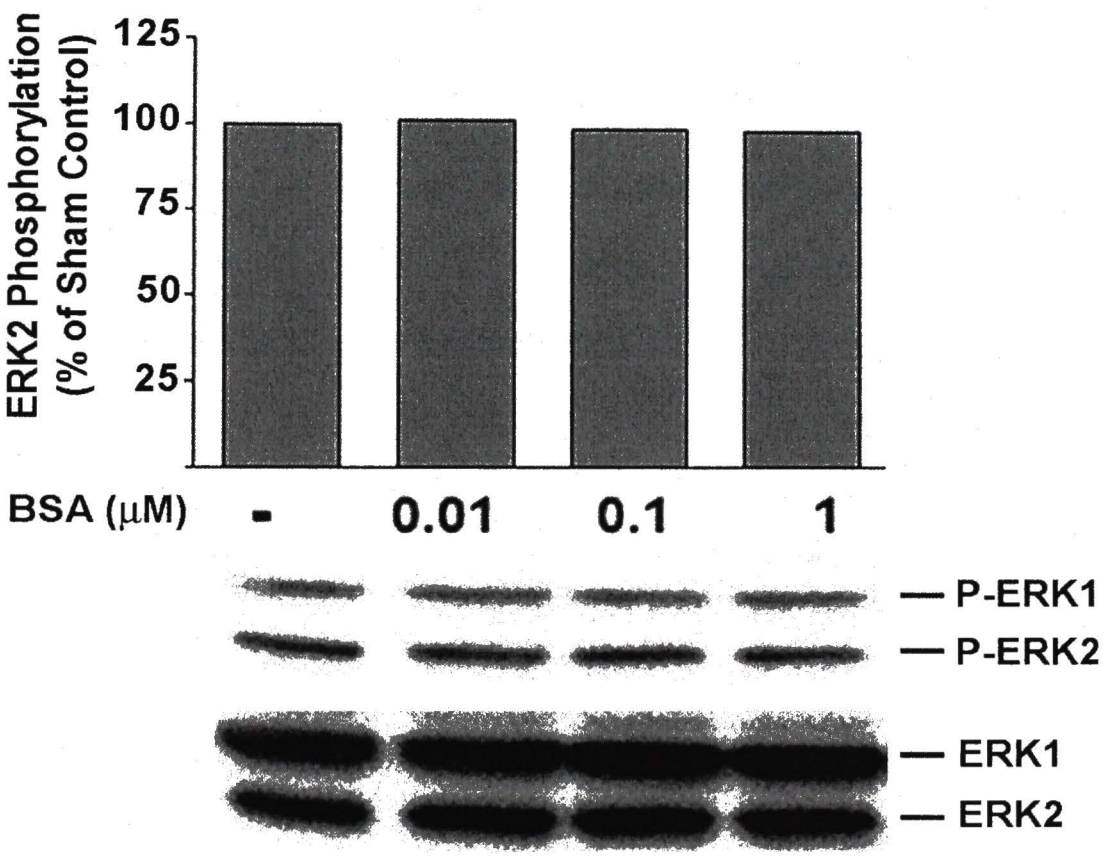

ERK2 Phosphorylation
(% of Sham Control)
125
100
75
50
25
BSA (μM)
-
0.01
0.1
1
P-ERK1
P-ERK2
ERK1
ERK2

Figure 8. DHT-BSA treatment suppresses phospho-Akt levels in C6 cells. C6 cells were treated with DHT/BSA (0.01, 0.10, and 1 μM) for 30 min. Treatment of C6 cells with DHT-BSA resulted in a dose dependent decrease in phospho-Akt levels. The data demonstrate that DHT-BSA inhibits basal Akt phosphorylation in a dose-dependent manner. The upper blot and lower blot depict Akt phosphorylation and total Akt protein, respectively, from a single independent experiment. The bar graph (upper panel), however, represents the densitometric analysis and statistical evaluation of data from three independent experiments, and is presented as signal intensity relative to that seen in the sham (vehicle-treated) control. Statistical significance was determined using one-way ANOVA, followed by Tukey's post-hoc analysis for group differences (* = $p < 0.001$; # = $p < 0.01$). Data are expressed as the mean ± S.E.M.

Figure 8.

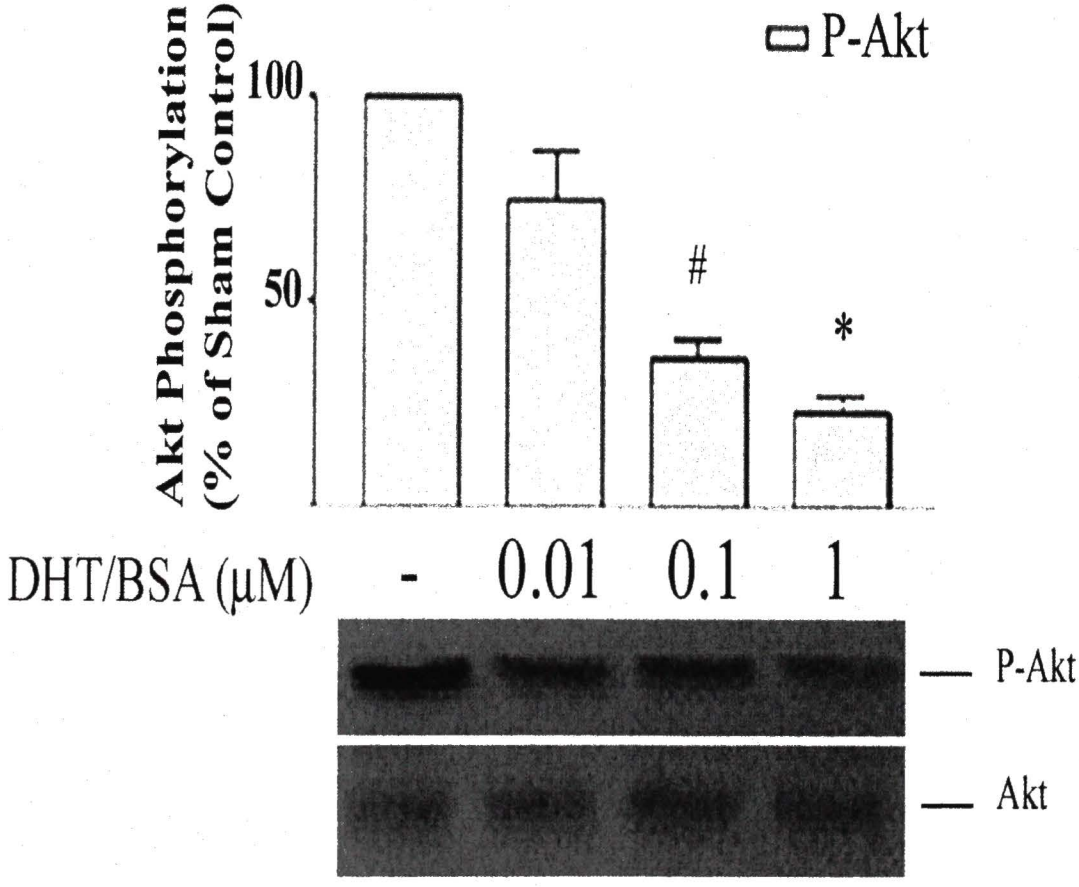

P-Akt
Akt Phosphorylation (% of Sham Control)
100
50
#
*
DHT/BSA (μM)
-
0.01
0.1
1
P-Akt
Akt

Figure 9. DHT-BSA blocks DHT-induced ERK phosphorylation. To determine if DHT/BSA competitively blocks DHT's effect on the MAPK, C6 cells were treated with DHT-BSA at various concentrations (0.01, 0.1, and 1.0 μM) for 30 min. in the presence/absence of DHT (10 nM, a concentration that effectively and reproducibly elicits ERK phosphorylation). As a control, BSA (1 μM) was included. The data revealed that DHT-BSA effectively prevented the effect of DHT on ERK phosphorylation. The upper blot and lower blot depict ERK phosphorylation and total ERK protein, respectively, from a single independent experiment. The bar graph (upper panel), however, represents the densitometric analysis from two independent experiments, and is presented as signal intensity relative to that seen in the sham (vehicle-treated) control.

Figure 9.

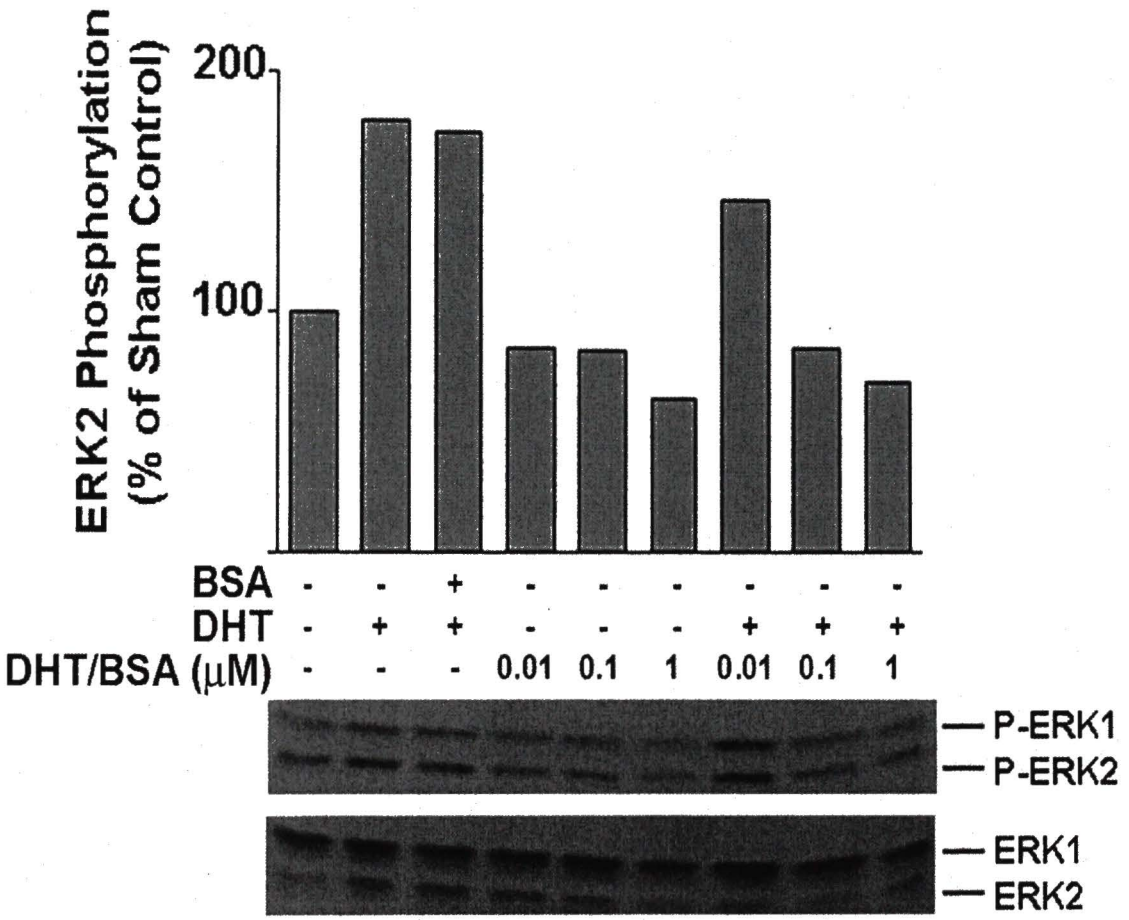
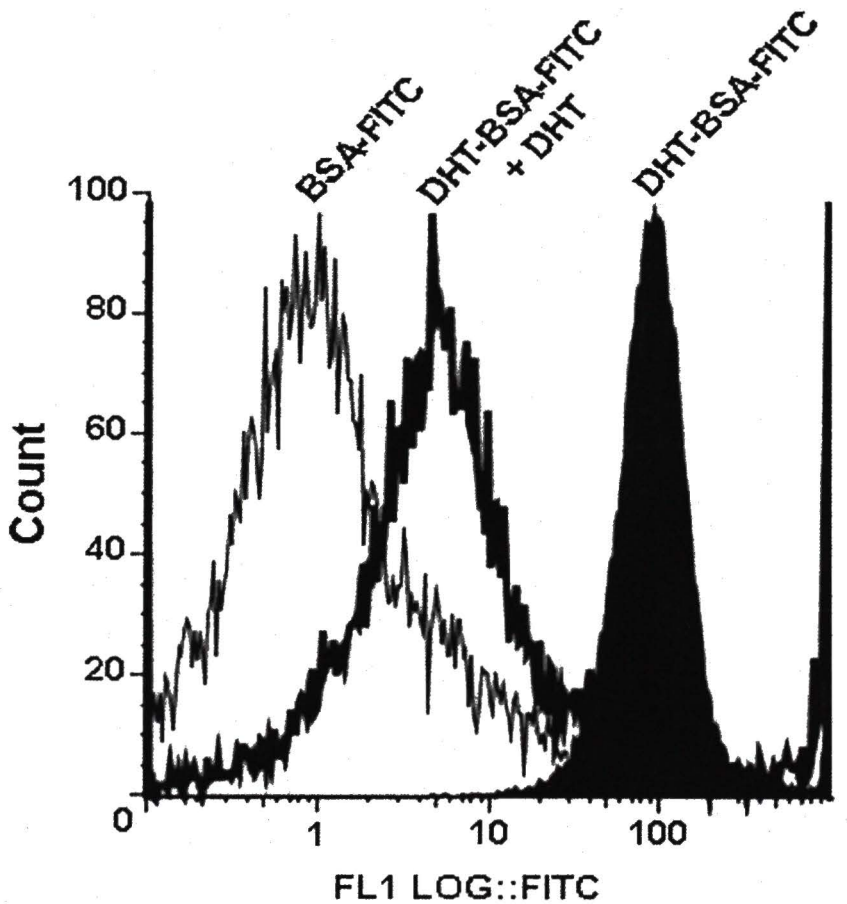

ERK2 Phosphorylation
(% of Sham Control)
200
100
BSA - - + - - - - - -
DHT - + + - - - + + +
DHT/BSA (μM) - - - 0.01 0.1 1 0.01 0.1 1
P-ERK1
P-ERK2
ERK1
ERK2

Figure 10. DHT-BSA binds to specific sites on the cell surface of C6 cells. To determine if DHT-BSA binds to surface (plasma membrane) receptors, C6 cells were treated with DHT-BSA-FITC (50 μM) for 30 min. in the presence/absence of a 20-fold molar excess of DHT (1 mM). Samples were washed and analyzed using flow cytometric analysis. The fluorescence histogram, depicting increasing fluorescence intensity on the x-axis, and cell number on the y-axis, describes significant labeling of cells with DHT-BSA-FITC. This labeling appeared to be displaced by DHT. The peak on the extreme left reflects the amount of fluorescence signal obtained when cells were incubated with BSA-FITC alone, representing non-specific binding. The data are representative of three independent experiments.

Figure 10.


BSA-FITC
DHT-BSA-FITC + DHT
DHT-BSA-FITC
Count
100
80
60
40
20
0
1
10
100
FL1 LOG::FITC

REFERENCES

1. **Mooradian AD, Morley JE, Korenman SG** 1987 Biological actions of androgens. Endocr Rev 8:1-28

2. **Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM.** 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-9

3. **Baron S, Manin M, Beaudoin C, Leotoing L, Communal Y, Veyssiere G, Morel L.** 2004 Androgen receptor mediates non-genomic activation of phosphatidylinositol 3-OH kinase in androgen-sensitive epithelial cells. J Biol Chem 279:14579-86

4. **Sun M, Yang L, Feldman RI, Sun XM, Bhalla KN, Jove R, Nicosia SV, Cheng JQ.** 2003 Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85alpha, androgen receptor, and Src. J Biol Chem 278:42992-3000

5. **Peterziel H, Mink S, Schonert A, Becker M, Klocker H, Cato AC** 1999 Rapid signalling by androgen receptor in prostate cancer cells. Oncogene 18:6322-9

6. **Wilson CM, McPhaul MJ** 1994 A and B forms of the androgen receptor are present in human genital skin fibroblasts. Proc Natl Acad Sci U S A 91:1234-8

7. **Wilson CM, McPhaul MJ** 1996 A and B forms of the androgen receptor are expressed in a variety of human tissues. Mol Cell Endocrinol 120:51-7

8. **Gao T, McPhaul MJ** 1998 Functional activities of the A and B forms of the human androgen receptor in response to androgen receptor agonists and antagonists. Mol Endocrinol 12:654-63

9. **Liegibel UM, Sommer U, Boercsoek I, Hilscher U, Bierhaus A, Schweikert HU, Nawroth P, Kasperk C.** 2003 Androgen receptor isoforms AR-A and AR-B display functional differences in cultured human bone cells and genital skin fibroblasts. Steroids 68:1179-87

10. **Papakonstanti EA, Kampa M, Castanas E, Stournaras C** 2003 A rapid, nongenomic, signaling pathway regulates the actin reorganization induced by activation of membrane testosterone receptors. Mol Endocrinol 17:870-81

11. **Shah C, Modi D, Gadkar S, Sachdeva G, Puri C** 2003 Progesterone receptors on human spermatozoa. Indian J Exp Biol 41:773-80

12. **Toran-Allerand CD, Guan X, MacLusky NJ, Horvath TL, Diano S, Singh M, Connolly ES Jr., Nethrapalli IS, Tinnikov AA.** 2002 ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. J Neurosci 22:8391-401

13. **Sakamoto H, Ukena K, Takemori H, Okamoto M, Kawata M, Tsutsui K** 2004 Expression and localization of 25-Dx, a membrane-associated putative progesterone-binding protein, in the developing Purkinje cell. Neuroscience 126:325-34

14. **Pappas TC, Gametchu B, Watson CS** 1995 Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. Faseb J 9:404-10

15. **Marquez DC, Pietras RJ** 2001 Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. Oncogene 20:5420-30

16. **Rambo CO, Szego CM** 1983 Estrogen action at endometrial membranes: alterations in luminal surface detectable within seconds. J Cell Biol 97:679-85

17. **Zhu Y, Rice CD, Pang Y, Pace M, Thomas P** 2003 Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci U S A 100:2231-6

18. **Heinlein CA, Chang C** 2002 The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. Mol Endocrinol 16:2181-7

19. **Benten WP, Lieberherr M, Stamm O, Wrehlke C, Guo Z, Wunderlich F** 1999 Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Mol Biol Cell 10:3113-23

20. **Benten WP, Becker A, Schmitt-Wrede HP, Wunderlich F** 2002 Developmental regulation of intracellular and surface androgen receptors in T cells. Steroids 67:925-31

21. **Braun AM, Thomas P** 2004 Biochemical characterization of a membrane androgen receptor in the ovary of the atlantic croaker (Micropogonias undulatus). Biol Reprod 71:146-55

22. **Somjen D, Kohen F, Gayer B, Kulik T, Knoll E, Stern N** 2004 Role of putative membrane receptors in the effect of androgens on human vascular cell growth. J Endocrinol 180:97-106

23. **Benten WP, Guo Z, Krucken J, Wunderlich F** 2004 Rapid effects of androgens in macrophages. Steroids 69:585-90

24. **Estrada M, Espinosa A, Muller M, Jaimovich E** 2003 Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. Endocrinology 144:3586-97

25. **Singh M, Setalo G, Jr., Guan X, Warren M, Toran-Allerand CD** 1999 Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. J Neurosci 19:1179-88

26. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-75

27. **Marshall CJ** 1995 Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179-85

28. **Stanciu M, DeFranco DB** 2002 Prolonged nuclear retention of activated extracellular signal-regulated protein kinase promotes cell death generated by

oxidative toxicity or proteasome inhibition in a neuronal cell line. J Biol Chem 277:4010-7

29. **Cantley LC** 2002 The phosphoinositide 3-kinase pathway. Science 296:1655-7

30. **Kayampilly PP, Menon KM** 2004 Inhibition of extracellular signal-regulated protein kinase-2 phosphorylation by dihydrotestosterone reduces follicle-stimulating hormone-mediated cyclin D2 messenger ribonucleic acid expression in rat granulosa cells. Endocrinology 145:1786-93

31. **Bell WC, Myers RB, Hosein TO, Oelschlager DK, Grizzle WE** 2003 The response of extracellular signal-regulated kinase (ERK) to androgen-induced proliferation in the androgen-sensitive prostate cancer cell line, LNCaP. Biotech Histochem 78:11-6

32. **Ramsden M, Nyborg AC, Murphy MP, Chang L, Stanczyk FK, Golde TE, Pike CJ.** 2003 Androgens modulate beta-amyloid levels in male rat brain. J Neurochem 87:1052-5

33. **Ramsden M, Shin TM, Pike CJ** 2003 Androgens modulate neuronal vulnerability to kainate lesion. Neuroscience 122:573-8

34. **Yang SH, Liu R, Wen Y, Perez E, Cutright J, Brun-Zinkernagel AM, Singh M, Day AL, Simpkins JW.** 2005 Neuroendocrine mechanism for tolerance to cerebral ischemia-reperfusion injury in male rats. J Neurobiol 62:341-51

35. **Finley SK, Kritzer MF** 1999 Immunoreactivity for intracellular androgen receptors in identified subpopulations of neurons, astrocytes and oligodendrocytes in primate prefrontal cortex. J Neurobiol 40:446-57

36. **Garcia-Ovejero D, Veiga S, Garcia-Segura LM, Doncarlos LL** 2002 Glial expression of estrogen and androgen receptors after rat brain injury. J Comp Neurol 450:256-71

ACTIVATION OF A MEMBRANE-ASSOCIATED AR IN C6 CELLS PROMOTES CELL DEATH

JOSHUA W. GATSON AND MEHARVAN SINGH[#*]

Department of Pharmacology and Neuroscience, The Institute for Aging & Alzheimer's Disease Research, and The Center FOR HER (Focused On Resources for her Health, Education and Research), University of North Texas Health Science, Fort Worth, Texas

Running Title: Membrane androgen AR promotes cell death

Corresponding author:

Meharvan Singh, Ph.D.
Department of Pharmacology and Neuroscience
University of North Texas Health Science Center at Fort Worth
3400 Camp Bowie Blvd.
Fort Worth, Texas 76107-2699
Phone: (817) 735-5429
Fax: (817) 735-0408
E-mail: msingh@hsc.unt.edu

CHAPTER III

ACTIVATION OF A MEMBRANE-ASSOCIATED AR IN C6 CELLS PROMOTES CELL DEATH

ABSTRACT

In the central nervous system androgens have been shown to protect as well cause an increase in cell damage, depending on the insult type. Within the hippocampus and cortex, testosterone was shown to protect from β-amyloid toxicity but increased lesion size in various stroke models, respectively. Previously, we reported that a BSA-conjugated androgen suppressed the activity of two important survival pathways, ERK and Akt, in rat glioma cells. In contrast, DHT increased ERK and Akt phosphorylation. Here, using C6 glial cells, we found that in the presence of a metabolic toxin and oxidative stress inducer, iodoacetic acid (IAA), DHT protected from IAA-induced toxicity. In contrast, DHT-BSA treatment led to an increase in cell death in the presence of a sublethal concentration of IAA. In addition, DHT-BSA blocked DHT and estrogen-induced protection. Collectively, these data support the existence of two, potentially competing, pathways for androgens in the C6 cells. Thus, depending on the predominance of one receptor mechanism over another, the outcome of androgen treatment may be very different, and as such, could help explain existing discrepancies as to whether androgens are protective or damage-inducing.

INTRODUCTION

Dihydrotestosterone (DHT), a major metabolite of testosterone, mediates cellular growth, differentiation, survival or death by the classical or non-genomic signaling pathways (Mooradian et al., 1987; Mangelsdorf et al., 1995; Sun et al., 2003). Androgens such as testosterone and DHT have been shown to protect neurons in the brain from various insults in a receptor dependent manner (Nguyen et al., 2005). Also, in recent studies, DHT has been shown to rapidly activate the ERK pathway and protect primary hippocampal neurons from kainic acid and β-amyloid toxicity (Ramsden et al., 2003b; Ramsden et al., 2003a; Nguyen et al., 2005). In addition, testosterone was shown to protect from serum deprivation in primary neuronal cultures (Hammond et al., 2001). In contrast, using the middle cerebral artery occlusion (Li et al., 2005) stroke model, testosterone was shown to cause an increase in lesion size following stroke. This increase in lesion size was due to an increase in glutamate toxicity in the presence of testosterone (Yang et al., 2002; Yang et al., 2005). Also, testosterone treatment led to an increase in cell death in primary oligodendrocytes in the presence of the excitoxin, kainic acid (Caruso et al., 2004). Additionally, in T47D breast cancer cells and LNCap cells, DHT-BSA was shown to induce apoptosis by binding to a membrane androgen receptor during serum deprivation (Hatzoglou et al., 2005; Kampa et al., 2005). These studies suggest that in the presence of various insults, androgens can protect or be damage inducing.

Recently, in C6 cells, we found that DHT-BSA bound to membrane sites and treatment with this steroid led to a decrease in the activity of the ERK and PI-3 kinase/Akt signaling pathways, which are important survival promoting pathways (Gatson et al., 2006). Thus, we hypothesize that activation of the membrane AR in the presence of IAA will exacerbate the damaging effects of this metabolic and oxidative insult.

To determine the role of DHT in the survival of glia, we treated the C6 cells with IAA in the presence and absence of DHT and DHT-BSA. We found that DHT protected from IAA-induced toxicity, while DHT-BSA treatment led to a significant increase in cell death. Since androgens have been shown to mediate negative effects in the brain during various disease states, we hypothesize that androgens acting through the membrane receptor increases cell death in glia. We conclude that DHT may act through two competing pathways, resulting in cell survival or death.

MATERIALS AND METHODS

Cell culture. Rat glioma cells (C6; American Type Culture Collection, Manassas, VA) were propagated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% charcoal-stripped fetal bovine serum (FBS; Hyclone, Logan, UT), and maintained at 37°C in a humidified environment containing 5% CO_2 until 90% confluent. Following treatment of the cells with the appropriate duration and dose of hormone, the cells were harvested, homogenized, and centrifuged

and the supernatant was subsequently collected and analyzed for total protein concentration using the Biorad DC protein assay kit.

Treatment of cultures. C6 cells were treated with either vehicle control (DMSO, 0.1%), dihydrotestosterone (DHT; Steraloids Inc., Newport, RI), 5α-androstan-17β-ol-3-one-3-o-carboxymethyloxime:BSA (DHT-BSA; Steraloids Inc.), 17 β-estradiol (Sigma-Aldrich, St. Louis, MO), Triton-X (10%; Sigma), or iodoacetic acid (IAA; Sigma) for 12 hours. Controls for DHT-BSA treatment included bovine serum albumin (BSA) alone (Fisher Scientific, Fair Lawn, NJ). Inhibition of the classical androgen receptor was achieved using the androgen receptor antagonist, Flutamide (Sigma), which was applied 30 minutes prior to treatment with the hormone and/or insult.

LDH Assay. The LDH assay was carried out according to the manufacturer (Roche Molecular Biochemicals). This assay detects extracellular levels of LDH that is released by the cell and is indicative of dying or dead cells. In brief, 100 µl from each sample was pipetted into a 96-well plate. To each well, 100 µl of the assay reagent was added for 10 min. at 27°C. Following the 10 min. incubation, 50 µl of stop solution was added and the plates were analyzed using a Viktor3 ELISA plate reader (Perkin Elmer, Boston, MA). The 560 nm excitation and 590 nm emission wavelengths were used to measure extracellular LDH levels. Values were normalized and expressed as a percent of maximal LDH release.

Statistical Analysis. Data points from at least three independent experiments was subjected to an analysis of variance (ANOVA), followed by a Tukey's *post hoc* analysis

for the assessment of group differences, and presented as a bar graph depicting the average ± S.E.M., using the GraphPad Software (San Diego, CA).

RESULTS

Activation of the membrane AR in C6 cells exacerbates IAA-induced toxicity.

Our previous studies demonstrated that in C6 cells, DHT treatment increases ERK and Akt phosphorylation, while DHT-BSA suppressed the activity of these two key survival pathways (Gatson et al., 2006). To determine if the effect of androgens on cell survival during metabolic and oxidative damage in glia, we treated the C6 cells with IAA (10 μM) in the presence or absence of DHT (10 μM) or DHT-BSA (10 μM). Using the LDH assay, to measure LDH levels in the media, we found that DHT protected the C6 cells from IAA, consistent with the increased ERK and Akt phosphorylation (Fig. 1A). In contrast, after 12 hourrs, DHT-BSA caused an increase in cell death compared to the IAA only group. Treatment with DHT-BSA alone did not result in an increase in LDH release (Fig. 1B). The effect of DHT-BSA on survival in the C6 cells was flutamide insensitive, indicating that these effects are not mediated by the intracellular AR (Fig. 2).

DHT-induced protection is blocked by DHT-BSA.

To assess whether DHT-BSA could block the survival-promoting effects of other steroid hormones, we co-treated the C6 cells with DHT-BSA (10 μM) and DHT (10 μM) in the presence of IAA (10 μM) for 12 hours. We found that in the presence of IAA,

DHT protected against IAA-induced toxicity. In contrast, activation of the membrane-associated receptor by DHT-BSA blocked DHT's protective effects and even caused a significant increase in LDH release compared to the IAA only group (Fig. 3).

DHT-BSA blocks estradiol-induced protection.

To assess whether DHT-BSA could block the survival-promoting effects of other steroid hormones such as 17-β estradiol, we co-treated the C6 cells with DHT-BSA (10 μM) and estrogen (10 μM) in the presence of IAA (10 μM) for 12 hours. Our results showed that in the presence of IAA, estrogen protected against IAA-induced toxicity. In contrast, activation of the membrane-associated receptor by DHT-BSA blocked estrogen's protective effects and caused a significant increase in LDH release compared to the IAA only group (Fig. 4).

DISCUSSION

In plasma membrane fractions isolated from the ovary of the Atlantic Croaker, and using the radioligand binding assay and Scatchard analysis, it was shown that androgens bind to membrane receptors in the ovary. Androgen binding was also detected in the kidney, liver, heart, and brain tissue of the Atlantic Croaker (Braun and Thomas, 2004), indicating that the membrane AR exists in many different tissue types and may regulate the activity of many important pathways. Membrane androgen receptors have been shown to mediate the effect of androgens on cell signaling, growth, and survival in

T cells, smooth muscle, ovary, macrophage, prostate, breast cancer cells, and glial cells (Benten et al., 1999; Benten et al., 2002; Braun and Thomas, 2004; Hatzoglou et al., 2005; Kampa et al., 2005; Gatson et al., 2006). In T cells, testosterone-BSA (T-BSA) treatment led to an increase in calcium influx from the extracellular space. This influx of calcium by T-BSA was not blocked by the classical androgen receptor antagonist, cyproterone (Benten et al., 1997). With respect to cell survival, the activation of the membrane androgen receptor by T-BSA was shown to lead to an increase in apoptotic cell death during serum deprivation in the LNCap and T47D cancer cell lines (Hatzoglou et al., 2005; Kampa et al., 2005).

We show here that in C6 cells, DHT-BSA promotes cell death in the presence of the metabolic toxin and oxidative stress-inducer, IAA, while DHT protects from IAA-toxicity (Fig. 1). Previously, we showed that a 30 min treatment with DHT-BSA in C6 cells led to a suppression of the PI-3 kinase/Akt and ERK pathways (Gatson et al., 2006). Thus, activation of this membrane receptor and consequent suppression of protective signaling during various disease states may predispose a cell to the damaging effects of testosterone. The effect of DHT-BSA on survival is flutamide insensitive (Fig. 2), indicating that the binding properties of this novel membrane AR as well as the receptor itself may be different than the "classical" intracellular AR.

Based on our data, we suggest that during stroke, the damaging effects of androgens may be due to increased expression of the membrane AR relative to the intracellular AR. In individuals with low membrane AR, but high intracellular AR,

androgens may be protective in incidences of stroke or in diseases such as Alzheimer's disease. In the brain, estradiol has been shown to protect from oxidative stress and excitotoxicity (Fitzpatrick et al., 2002; Fan et al., 2003; Simpkins et al., 2004). In this study, co-treatment with DHT-BSA and estradiol, led to a significant decrease in the protective effects of estradiol in C6 cells (Fig. 4). These results indicate that steroid hormone type, mAR expression levels, and androgen levels may predict the amount of damage following insult. For instance, high androgen and mAR levels, may lead to a decrease in estrogen-induced protection, as a result of a shift towards a death inducing pathway. This shift would result in more glial cell death during acute brain injury such as stroke.

Within the brain, glia outnumber neurons approximately 10:1. These cells are important for the myelination of the neuronal axons, neurotransmission, buffering of excito-toxins, and neuronal guidance. Since glia are an important entity in the brain, here we show that the negative effects of androgens may occur in the glia, which ultimately lead to the demise of the neuronal populations following stroke and reperfusion injury.

In conclusion, our data shows that androgens can regulate cell survival through two competing pathways, intracellular AR and membrane AR. Activation of the intracellular AR pathway leads to cell survival, while activation of a membrane AR by DHT-BSA leads to cell death in the presence of insult. In addition, upon activation of the membrane AR in the presence of a metabolic and oxidative insult, the survival promoting effects of other hormones such as DHT and estradiol are blocked. These findings will

further our understanding of how androgens mediate negative effects during injury in the brain.

ACKNOWLEDGMENTS

This work was supported by funds from the National Institutes on Aging grant (AG22550 and AG23330), NARSAD-sponsored Young Investigator Awarded to M.S., and the National Science Foundation SCORE (School's Cooperative Opportunities for Resources and Education) grant.

Figure 1. Activation of a membrane AR in C6 cells exacerbates IAA-induced cell death. C6 cells were treated with IAA (10 μM) for 12 hours in the presence of DHT (A; 10 μM) or DHT-BSA (B; 10 μM). Culture media was analyzed for the release of LDH as an indicator of cell death. The values were normalized to percent of maximal LDH release (elicited by treating cultures with 0.1% Triton X-100). The graph represents three independent experiments. *$p<0.01$ vs. sham control, DHT-BSA only, or IAA only. Data are expressed as the mean ± S.E.M.

Figure 1.

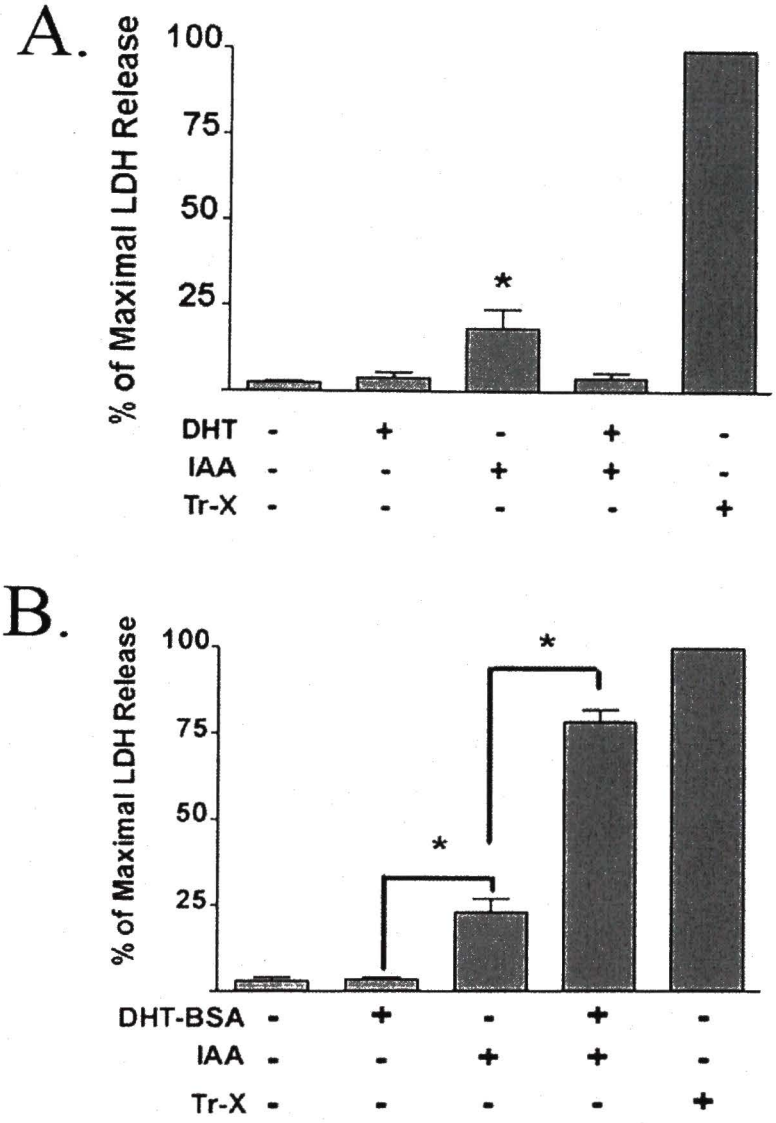

A.
% of Maximal LDH Release
100
75
50
25
*
DHT - + - + -
IAA - - + + -
Tr-X - - - - +
B.
% of Maximal LDH Release
100
75
50
25
*
*
DHT-BSA - + - + -
IAA - - + + -
Tr-X - - - - +

Figure 2. The damaging effects of DHT-BSA in C6 cells is not mediated by the intracellular AR. C6 cells were treated with IAA (10 μM) for 12 hours in the presence of fluatmide (3 μM), and/or DHT-BSA (10 μM). Culture media was analyzed for the release of LDH as an indicator of cell death. The values were normalized to percent of maximal LDH release (elicited by treating cultures with 0.1% Triton X-100). The graph represents three independent experiments. *$p<0.001$ vs. untreated control and IAA only groups. Data are expressed as the mean ± S.E.M.

Figure 2.

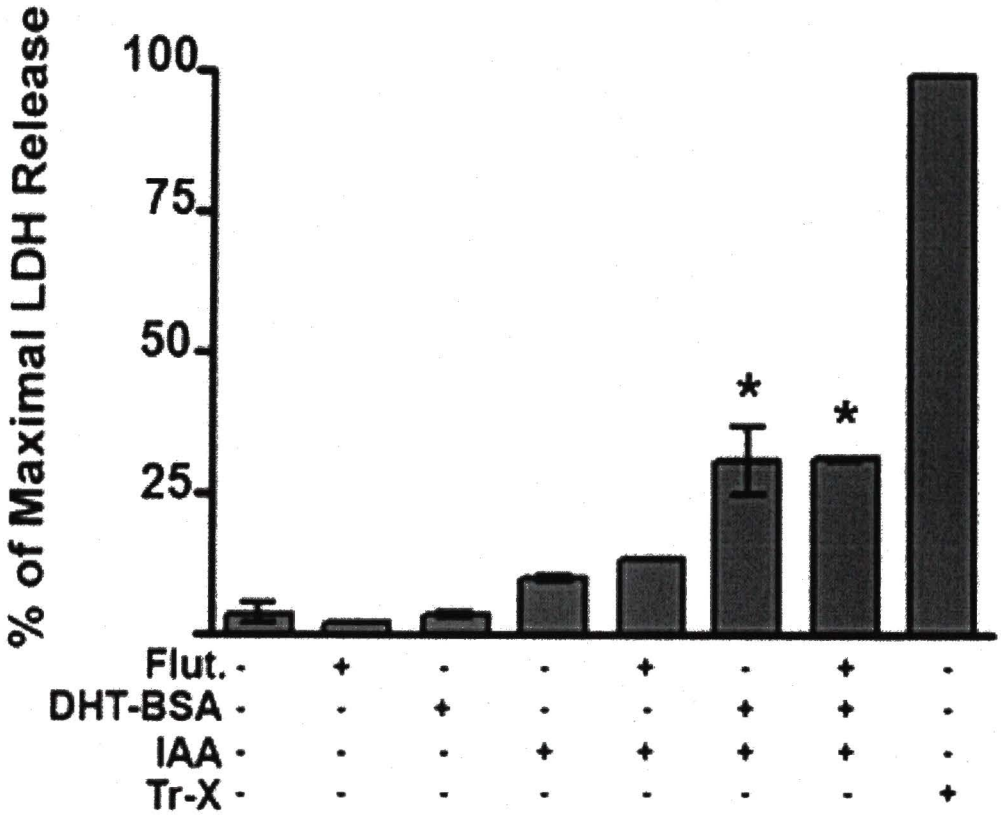


Figure 3. DHT-BSA blocks DHT-induced protection. C6 cells were treated with IAA (10 μM) for 12 hours in the presence of DHT (10 μM) and/or DHT-BSA (10 μM). Following the 12 hour incubation, the media was analyzed for the levels of LDH as an indicator of cell death. The protective effects of DHT were blocked by DHT-BSA. The values were normalized to percent of maximal LDH release. The graph represents three independent experiments. *$p<0.01$ vs. untreated control. Data are expressed as the mean ± S.E.M.

Figure 3.

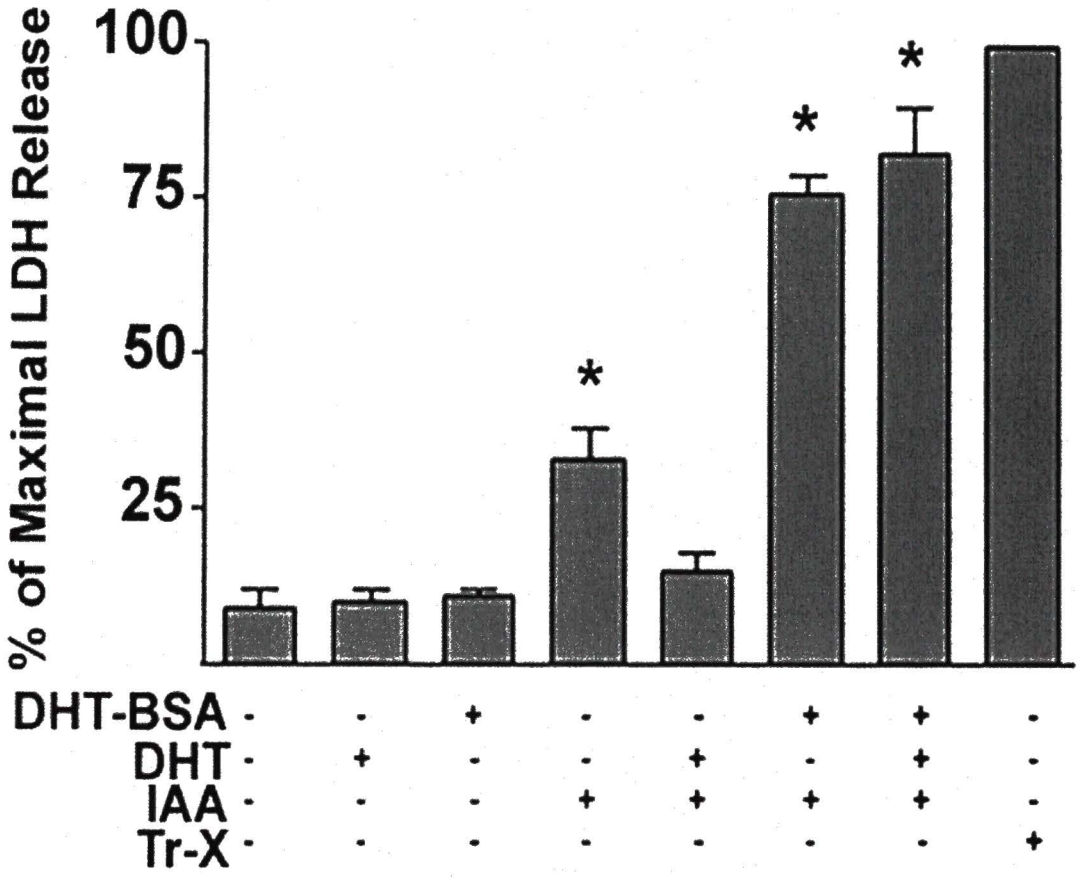
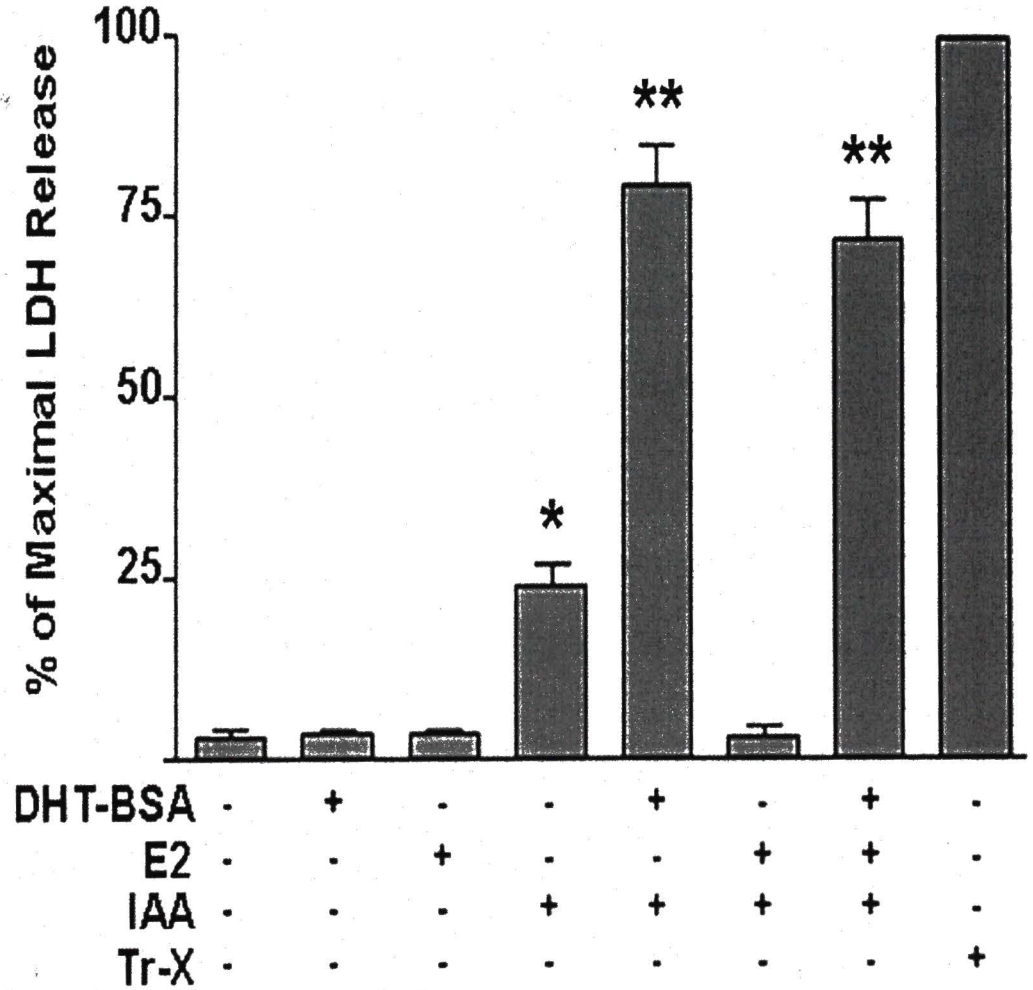


Figure 4. DHT-BSA blocks estradiol-induced protection. C6 cells were treated with IAA (10 μM) for 12 hours in the presence of 17β-estradiol (10 μM) and/or DHT-BSA (10 μM). Following the 12 hour incubation, the media was analyzed for the levels of LDH as an indicator of cell death. The protective effects of estradiol were blocked by DHT-BSA. The values were normalized to percent of maximal LDH release. The graph represents three independent experiments. *$p<0.01$ and ** $p<0.001$ vs. untreated control. Data are expressed as the mean $\pm$ S.E.M.

Figure 4.



REFERENCES

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol 7:261-269.

Bell WC, Myers RB, Hosein TO, Oelschlager DK, Grizzle WE (2003) The response of extracellular signal-regulated kinase (ERK) to androgen-induced proliferation in the androgen-sensitive prostate cancer cell line, LNCaP. Biotech Histochem 78:11-16.

Benten WP, Lieberherr M, Sekeris CE, Wunderlich F (1997) Testosterone induces Ca2+ influx via non-genomic surface receptors in activated T cells. FEBS Lett 407:211-214.

Benten WP, Becker A, Schmitt-Wrede HP, Wunderlich F (2002) Developmental regulation of intracellular and surface androgen receptors in T cells. Steroids 67:925-931.

Benten WP, Lieberherr M, Stamm O, Wrehlke C, Guo Z, Wunderlich F (1999) Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Mol Biol Cell 10:3113-3123.

Braun AM, Thomas P (2004) Biochemical characterization of a membrane androgen receptor in the ovary of the atlantic croaker (Micropogonias undulatus). Biol Reprod 71:146-155.

Caruso A, Di Giorgi Gerevini V, Castiglione M, Marinelli F, Tomassini V, Pozzilli C, Caricasole A, Bruno V, Caciagli F, Moretti A, Nicoletti F, Melchiorri D (2004) Testosterone amplifies excitotoxic damage of cultured oligodendrocytes. J Neurochem 88:1179-1185.

Fan T, Yang SH, Johnson E, Osteen B, Hayes R, Day AL, Simpkins JW (2003) 17beta-Estradiol extends ischemic thresholds and exerts neuroprotective effects in cerebral subcortex against transient focal cerebral ischemia in rats. Brain Res 993:10-17.

Fitzpatrick JL, Mize AL, Wade CB, Harris JA, Shapiro RA, Dorsa DM (2002) Estrogen-mediated neuroprotection against beta-amyloid toxicity requires expression of estrogen receptor alpha or beta and activation of the MAPK pathway. J Neurochem 82:674-682.

Gatson JW, Kaur P, Singh M (2006) Dihydrotestosterone Differentially Modulates the MAPK and the PI-3 Kinase/Akt Pathways Through the Nuclear and Novel Membrane Androgen Receptor in C6 Cells. Endocrinology 147:2028-2034

Hammond J, Le Q, Goodyer C, Gelfand M, Trifiro M, LeBlanc A (2001) Testosterone-mediated neuroprotection through the androgen receptor in human primary neurons. J Neurochem 77:1319-1326.

Hatzoglou A, Kampa M, Kogia C, Charalampopoulos I, Theodoropoulos PA, Anezinis P, Dambaki C, Papakonstanti EA, Stathopoulos EN, Stournaras C,

Gravanis A, Castanas E (2005) Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo. J Clin Endocrinol Metab 90:893-903.

Kampa M, Nifli AP, Charalampopoulos I, Alexaki VI, Theodoropoulos PA, Stathopoulos EN, Gravanis A, Castanas E (2005) Opposing effects of estradiol- and testosterone-membrane binding sites on T47D breast cancer cell apoptosis. Exp Cell Res 307:41-51.

Li J, Henman MC, Tatlisumak T, Shaw GG, Doyle KM (2005) The pre-ischaemic neuroprotective effects of N(1)-dansyl-spermine in a transient focal cerebral ischaemia model in mice. Brain Res 1055:180-185.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P (1995) The nuclear receptor superfamily: the second decade. Cell 83:835-839.

Mooradian AD, Morley JE, Korenman SG (1987) Biological actions of androgens. Endocr Rev 8:1-28.

Nguyen TV, Yao M, Pike CJ (2005) Androgens activate mitogen-activated protein kinase signaling: Role in neuroprotection. J Neurochem 94:1639-1651.

Ramsden M, Shin TM, Pike CJ (2003a) Androgens modulate neuronal vulnerability to kainate lesion. Neuroscience 122:573-578.

Ramsden M, Nyborg AC, Murphy MP, Chang L, Stanczyk FZ, Golde TE, Pike CJ (2003b) Androgens modulate beta-amyloid levels in male rat brain. J Neurochem 87:1052-1055.

Simpkins JW, Yang SH, Liu R, Perez E, Cai ZY, Covey DF, Green PS (2004) Estrogen-like compounds for ischemic neuroprotection. Stroke 35:2648-2651.

Sun M, Yang L, Feldman RI, Sun XM, Bhalla KN, Jove R, Nicosia SV, Cheng JQ (2003) Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85alpha, androgen receptor, and Src. J Biol Chem 278:42992-43000.

Yang SH, Perez E, Cutright J, Liu R, He Z, Day AL, Simpkins JW (2002) Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model. J Appl Physiol 92:195-201.

Yang SH, Liu R, Wen Y, Perez E, Cutright J, Brun-Zinkernagel AM, Singh M, Day AL, Simpkins JW (2005) Neuroendocrine mechanism for tolerance to cerebral ischemia-reperfusion injury in male rats. J Neurobiol 62:341-351.

ACTIVATION OF A MEMBRANE-ASSOCIATED AR PROMOTES CELL DEATH IN PRIMARY CORTICAL ASTROCYTES

JOSHUA W. GATSON AND MEHARVAN SINGH[#*]

Department of Pharmacology and Neuroscience, The Institute for Aging & Alzheimer's Disease Research, and The Center FOR HER (Focused On Resources for her Health, Education and Research), University of North Texas Health Science, Fort Worth, Texas

Running Title: Membrane androgen receptor promotes cell death

Corresponding author:

Meharvan Singh, Ph.D.
Department of Pharmacology and Neuroscience
University of North Texas Health Science Center at Fort Worth
3400 Camp Bowie Blvd.
Fort Worth, Texas 76107-2699
Phone: (817) 735-5429
Fax: (817) 735-0408
E-mail: msingh@hsc.unt.edu

CHAPTER IV

ACTIVATION OF A MEMBRANE-ASSOCIATED AR PROMOTES CELL DEATH IN PRIMARY CORTICAL ASTROCYTES

ABSTRACT

In the central nervous system, androgens can exert either protective or damage-promoting effects. For example, testosterone protects neurons against β-amyloid toxicity while in other studies, testosterone exacerbated stroke-induced lesion size. The mechanism underlying this duality of androgens is still unclear. Recently, our laboratory reported that androgens elicit opposite effects on the ERK/MAPK and Akt signaling pathways depending on whether a membrane androgen receptor (AR) or intracellular AR was activated. By extension, we hypothesized that androgens may affect cell viability differently depending on which receptor was activated. Here, we found that dihydrotestosterone (DHT) protected primary cortical astrocytes from the metabolic and oxidative insult associated with iodoacetic acid (IAA)-induced toxicity, whereas DHT-BSA, a cell impermeable analog of DHT that preferentially targets the membrane AR, suppressed Akt signaling, increased caspase 3/7 activity and enhanced IAA-induced cell death. Interestingly, DHT-BSA also blocked the protective effects of DHT and estradiol. Collectively, these data support the existence of two, potentially competing, pathways for androgens in a given cell or tissue that may provide insight into the controversy of whether androgen therapy is beneficial or detrimental.

INTRODUCTION

Dihydrotestosterone (DHT), a major metabolite of testosterone, can regulate cellular growth, differentiation, survival or death through both the classical "genomic" mechanism of action, or through "non-genomic" signaling pathways (1). Androgens, including testosterone and DHT, can protect neurons from various insults, including kainic acid toxicity (2), β-amyloid toxicity (3, 4) and serum deprivation (5), and have been shown to rapidly activate the cytoprotection-associated ERK/MAPK pathway (2, 4). In terms of the receptor(s) involved in these protective effects, the classical androgen receptor has been implicated (4, 5). In contrast, supraphysiological levels of testosterone were found to increase neuronal apoptosis (6). In fact, the damage-promoting effects of testosterone have been observed in several experimental models. For example, testosterone was shown to exacerbate middle cerebral artery occlusion (MCAO)-induced lesion size in male rats (7, 8). Further, testosterone treatment increased kainic acid-induced cell death of cultured oligodendrocytes (9). And at least in one study, albeit in non-neuronal models (T47D breast cancer cells and LNCap cells), the cell death promoting effects of androgens were attributed to a membrane androgen receptor as supported by the death-promoting effects of the BSA-conjugated testosterone (T-BSA) (10, 11). Thus, androgens can be protective or damage promoting, but the mechanism underlying this duality is still unclear.

Recently, our laboratory reported the presence of displaceable membrane binding sites for androgens in C6 glial cells. Interestingly, activation of these putative membrane androgen receptors resulted in a decrease in the phosphorylation of ERK and Akt (12), two key effectors of the cytoprotection-associated MAPK and PI-3 kinase signaling pathways, respectively. Here, we built on our previous observations and hypothesized that activation of the membrane androgen receptor would result in enhanced cell death. In fact, here we report that activation of the putative membrane androgen receptor with BSA-conjugated DHT (DHT-BSA) resulted in a decrease in Akt phosphorylation and expression, an increase in caspase 3/7 activity, increased TUNEL staining, and ultimately, an increased vulnerability to a metabolic and oxidative insult resulting from iodoacetic acid (IAA) treatment of primary astroglial cultures. Further our results suggest that the cascade of events initiated by activation of the putative membrane androgen receptor can antagonize the protective effects of other hormones.

MATERIALS AND METHODS

Primary cortical astrocytes. Primary cortical astrocytes were derived from the cerebral cortex of postnatal day (P) 3 C57Bl/6 mice. Briefly, following removal of the brain from the cranial cavity, the meninges were removed and the cerebral cortex was dissected and placed into a sterile vacutainer tube containing 2 ml of the Dissociation solution (1.9 ml PBS 1X pH 7.2 + 0.25% Trypsin [final concentration]). The tube was incubated at 37°C for 10 min. Following this incubation, the tube was centrifuged at 400

x *g* for 5 min. The resulting pellet was then re-suspended in 2 ml of plating media (DMEM with sodium pyruvate/10% FBS + 1% Penicillin/Streptomycin) and dissociated further with the aid of a fire-polished Pasteur pipet. After this dissociation, the sample was centrifuged and re-suspended in 2ml of plating media. These re-suspension/centrifugation steps were repeated twice and the cell suspension was filtered through a 70μm cell strainer into a 50ml conical tube. The filtrate was diluted by adding 5 ml of plating media. The cells were counted and plated at the desired density (~200,000 cells/well, 24-well plate; ~400,000 cells/well, 6-well plate). To establish pure astrocyte cultures, the media was changed every 48 hrs such that just prior to the media change, the tissue culture plate/flask was shaken at 350 rpm for 5 min. The cultures were treated with hormone or drug on the 14 day *in vitro* (d.i.v.).

All procedures involving the use of animals was approved the UNT Health Science Center's Institutional Animal Care and Use Committee (IACUC).

Treatment of cultures. Primary astrocytes were treated with either vehicle control (DMSO, 0.1%), dihydrotestosterone (DHT; Steraloids Inc., Newport, RI), 5α-androstan-17β-ol-3-one-3-o-carboxymethyloxime:BSA (DHT-BSA; Steraloids Inc.), 17 β-estradiol (Sigma-Aldrich, St. Louis, MO), Triton-X (Sigma, 1% final concentration), or iodoacetic acid (IAA; Sigma) for 12 hrs. IAA is an inhibitor of glycolysis (13) and also has been shown to promote oxidative stress (14). Controls for DHT-BSA treatment included equimolar concentrations of bovine serum albumin (BSA) alone (Fisher Scientific, Fair Lawn, NJ). To avoid the potential confound of having "free" or

unconjugated DHT in the DHT-BSA preparation, the DHT-BSA solution was pre-filtered using a 30 kDa nominal cut-off column to remove any unconjugated steroid. The retentate (consisting of conjugated DHT-BSA) was then reconstituted in the original volume. Inhibition of the classical androgen receptor was achieved using the androgen receptor antagonist, flutamide (Sigma), which was applied 30 min prior to treatment with the hormone and/or insult.

Western blot analysis. Primary glial cultures were treated with hormone and/or inhibitor, in the presence or absence of IAA. Following the appropriate duration of treatment, the cultures were harvested into lysis buffer containing protease and phosphatase inhibitors as described previously (15). Following homogenization, samples were centrifuged at 99,000 x g for 15 min at 4°C, and the resulting supernatants were evaluated for total protein concentrations using the BioRad DC (Biorad Labs, Hercules, CA) protein assay kit [based on the method of Lowry; (16)]. Sample lysates were loaded onto a sodium dodecyl sulfate (SDS), 10% polyacrylamide gel (PAGE), subjected to electrophoresis, and subsequently transferred onto a polyvinylidene difluoride membrane (PVDF; 0.22 μm pore size, BioRad). The membrane was blocked for 6 hrs with a 3% BSA in 0.2% Tween-containing TBS (TBS-T) solution prior to application of the primary antibody. The following primary antibodies were used: for the detection of rabbit anti-phospho-Akt (Ser473; 1:1000; Cell Signaling, Beverly, MA); for the detection of total Akt, rabbit anti Akt (1:1000; Cell Signaling); and for the detection of actin, goat anti-actin (I-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibody binding to the

membrane was detected using a secondary antibody (either goat anti-rabbit or rabbit anti-goat) conjugated to horseradish peroxidase (1:20,000; Pierce, Rockford, IL.) and visualized using enzyme-linked chemiluminescence (SuperSignal West Pico, Pierce) with the aid of the UVP imaging system.

Immunocytochemistry. Primary cortical astrocytes were fixed with 4% formaldehyde in PBS (pH 7.4) for 10 min at room temperature. The coverslips were washed with PBS and blocked with 3% BSA in PBS for 15 min. Following the blocking step, the coverslips were treated with the primary glial fibrillary acidic protein antibody (GFAP; Chemicon, Temecula, CA) for 2 hrs at room temperature. To detect the binding of the primary antibody, the Alexa Fluor 488-conjugated secondary antibody (1:500; Molecular Probes, Eugene, OR) was applied to the coverslips and incubated for 1 hr. The coverslips were subsequently washed three times with PBS for 10min. Coverslips were then mounted onto microscope slides using the Aqua Poly/Mount solution (Polysciences, Warrington, PA). The slides were viewed with the aid of a confocal laser scanning microscope (Zeiss) and analyzed using the Zeiss LSM imaging software.

Caspase 3/7 Assay. The Apo-ONE Homogeneous Caspase-3/7 Assay was carried out as recommended by the manufacturer (Promega). This assay is based on the ability of caspase 3/7 present in the experimental sample to cleave the non-fluorescent caspase 3/7 substrate, Z-DEVD conjugated to Rhodamine 110 (Z-DEVD-R110). The greater the activity of caspase3/7 in the sample, the more cleavage of the substrate, resulting in the liberation of the intensely fluorescent Rhodamine 110. Briefly, primary cortical

astrocytes were plated onto 48-well plates at a density of 50,000 cells/well and maintained for 10 d.i.v. On the 10th d.i.v., the samples cells were treated for 12 hrs with hormone or test compound. After this incubation period, 200 µl of the Apo-ONE Caspase-3/7 reagent (containing Z-DEVD-R110) was added to each well. The plate was then placed on a plate shaker (500 rpm) for 30 min and incubated for 1 hr. Following the 1 hr treatment, the active caspase-3/7 levels were determined by measuring the level of fluorescence (wavelengths for excitation and emission were 485nm and 530nm, respectively).

LDH Assay. A fluorometric assay (CytoTox-One Homogenous Membrane Integrity Assay Kit, Promega) was used for the measurement of lactate dehydrogenase (LDH) released from damaged or dying cells. Briefly, 100 µL of conditioned media was aliquoted into a black 96-well plate. To each well, 100 µl of the CytoTox-One reagent was added for 10 min. Following termination of the enzymatic reaction, resulting fluorescence was measured [560 nm (excitation)/590 nm (emission)] using a Viktor3 ELISA plate reader (Perkin Elmer, Boston, MA). Relative fluorescent units (RFU) were normalized to the amount of protein in the culture (assessed using BIORAD DC protein assay kit) from which the conditioned media was derived. These values were subsequently normalized and expressed as a percentage of total LDH release (assessed by treating cultures with 1% Triton X-100)

TUNEL Staining. The DeadEnd Colorimetric Apoptosis Detection System was used as recommended by the manufacturer (Promega). Briefly, hormone or test

compound-treated primary astroglial cultures (on glass coverslips) were fixed with 4% formaldehyde in PBS (pH 7.4) for 25 min at 4°C. After a series of washes in PBS, the cells were permeabilized in 0.2% Triton-X-100 solution for 5 min, re-washed and incubated in equilibration buffer (200mM potassium cacodylate, pH 6.6; 25mM Tris-HCl, pH 6.6; 0.2mM DTT; 0.25mg/ml BSA; 2.5mM cobalt chloride) for 10 min at room temperature. Then, cultures were incubated with 50 µl of the TdT reaction mix for 60 min at 37°C. The reaction was terminated by immersing the cultures in 2X SSC for 15 min. After a series of washes, 0.3% hydrogen peroxide (5 min) was used to block endogenous peroxidase activity. Then, Streptavidin HRP was applied (30 min), after which DNA strand breaks were visualized using the chromogen, diaminobenzidine (10 min incubation), as a substrate for HRP. Cells undergoing apoptosis were defined as those exhibiting condensed nuclei with dark brown staining. Cell viability was assessed by counting the number of GFAP+ cells that did not exhibit such morphology and staining. Such cell counts were obtained from three separate fields of view per coverslip and averaged. This averaged value was deemed an "n" of 1. Such analysis was repeated for each experimental group four times.

Statistical Analysis. Data obtained from no fewer than three independent experiments were analyzed using an analysis of variance (ANOVA), followed by a single degree of freedom F test to assess differences between experimental groups (SYSTAT ver. 7, Systat Software Inc., San Jose, CA). The data are presented as a bar graph depicting the mean ± S.E.M., using the GraphPad Software (San Diego, CA).

RESULTS

DHT-BSA enhances cell death in primary cortical astrocytes.

Previously, our laboratory has shown that DHT treatment increases ERK and Akt phosphorylation, while DHT-BSA suppresses the phosphorylation of these two key survival-related signaling proteins (12). In order to determine if DHT-BSA-induced suppression of survival promoting signaling is correlated to an increase in cell death, we extended our previous work in C6 cells and assessed the effects of DHT-BSA on the viability of primary cortical astrocytes, in the presence or absence of a metabolic/oxidative insult. To this end, we evaluated the effect of increasing concentrations of DHT-BSA (0.01, 0.1, 1, 10 μM) on IAA-induced LDH release. The concentration (10μM) and duration of IAA treatment (12hr) was chosen so that IAA resulted in no more than a 25 – 30% increase in LDH release. Thus, while IAA alone resulted in an approximately 25% increase in LDH release, DHT-BSA (10 μM) greatly sensitized the cells to IAA-induced cell death, such that the combined treatment with DHT-BSA and IAA resulted in a nearly maximal increase in LDH levels (Fig. 1). Lower concentrations of DHT-BSA were without effect. Interestingly, DHT-BSA by itself, did not result in an increase in LDH release at any concentration tested. BSA alone (10μM) was without effect when administered by itself and did not enhance IAA-induced toxicity, suggesting that the death-enhancing effects of DHT-BSA were not due to non-specific effects of BSA.

DHT protects primary cortical astrocytes from IAA-induced toxicity in an AR-dependent manner.

In order to distinguish the effects of activating a putative membrane androgen receptor from the effects of activating the intracellular AR, we compared the effects of DHT with DHT-BSA on cell viability. We explored the role of the two receptor mechanisms further using the pharmacological antagonist to the classical AR, flutamide. Our data show that unlike DHT-BSA (Fig. 1 and 2), DHT protected primary cortical astrocytes from IAA-induced toxicity (Fig. 2). Further, the protective effect of DHT was blocked by the classical AR antagonist, flutamide (Fig. 3A), while the death-enhancing effects of DHT-BSA was not (Fig. 3B). Neither DHT, DHT-BSA nor Flutamide when administered alone elicited an increase LDH release.

DHT-BSA treatment results in suppression of Akt signaling and an increase in active caspase-3/7.

Our recent data showed that DHT-BSA suppresses Akt phosphorylation in C6 cells whereas DHT elicited its phosphorylation (12). Given the well documented importance of the PI-3 kinase/Akt pathway with regard to its ability to prevent or reduce apoptosis (17-19), we wanted to determine if the enhanced cell death seen with DHT-BSA was associated with alterations in the expression or activation (phosphorylation) of Akt. We determined that IAA (10 μM) resulted in a modest, but statistically significant ($p = 0.046$) decrease in Akt phosphorylation. However, in the presence of IAA, DHT-BSA

(10 μM) greatly exacerbated the decline in levels of phosphorylated Akt and resulted in a decrease in total Akt expression as well (Fig. 4). To determine if such reduced Akt phosphorylation and expression was associated with indices of apoptotic cell death, we assessed the levels of active caspase 3/7 in cultures treated with IAA, in the presence or absence of DHT-BSA. We found that DHT-BSA greatly enhanced the levels of active caspase-3/7 in cells that had been treated with IAA (Fig. 5). While IAA alone resulted in a small (but statistically significant) increase in caspase 3/7 activity ($p = 0.017$), the effect of DHT-BSA alone was not different relative to control ($p = 0.059$) (Fig. 5).

DHT-BSA treatment increases apoptotic cell death in IAA-treated primary cortical astrocytes.

To determine if the effect of DHT-BSA on caspase-3/7 activity was indeed reflecting an increase in apoptosis, we assessed TUNEL staining in primary cortical astrocytes treated with DHT-BSA in the presence or absence of IAA. Simultaneous immunostaining for GFAP was also performed and confirmed the identity of astrocytes in our glial cultures. Visually, neither DHT-BSA or IAA alone resulted in an appreciable increase in TUNEL positive cells. However, DHT-BSA in the presence of IAA resulted in a significant appearance of TUNEL-positive cells, whose morphology was also consistent with dead or dying cells (Fig. 6A). Figure 6B provides quantitation of the amount of cell death by assessing the number of GFAP-positive cells. While IAA treatment alone resulted in an approximately 25% reduction in the number of GFAP-

positive astrocytes, cultures treated with DHT-BSA (10 μM) in the presence of IAA resulted in a significantly greater decrease in cell viability.

DHT-BSA blocks the survival promoting effects of DHT and estradiol.

In order to assess if the cell death promoting mechanisms activated through the putative membrane androgen receptor alter the protective effects of DHT or other known neuroprotective steroid hormones, we assessed the effect of DHT-BSA on DHT- or estradiol-induced cytoprotection. Using a concentration and duration of IAA treatment that resulted in an approximately 75% increase in LDH release, we found that, as expected and consistent with prior reports, both DHT (10 μM) and 17-β estradiol (10 μM) were protective. However, co-application of DHT-BSA with DHT, or estradiol, significantly attenuated the ability of either DHT or estradiol to reduce the IAA-induced LDH release (Fig. 7 and 8, respectively).

DISCUSSION

The presence of functional membrane androgen receptors have been suggested for a variety of cell types, including T cells (20), ovary (21), macrophages (22), prostate cells (11), breast cancer cells (10), and most recently, glial cells (12). Though the precise identity of this membrane receptor is still unknown, the pharmacology of putative membrane receptors has been addressed to some degree. For example, testosterone has been shown to exhibit specific binding to purified plasma membrane preparations derived

from the teleost ovary (21). In addition, using flow cytometry it was determined that C6 glial cells express DHT-displaceable DHT-BSA binding sites on the cell surface (12). Further, while testosterone-BSA (T-BSA), acting via the putative membrane AR of T-cells led to an increase in calcium influx, a classical androgen receptor antagonist, cyproterone, failed to block this effect (20). Similarly, data from our laboratory showed that the stimulatory effects of DHT on intracellular signaling and cell survival were blocked by the AR antagonist, flutamide, while the DHT-BSA-induced suppression of ERK and Akt phosphorylation (12), or the DHT-BSA – induced increase in cell death, was insensitive to flutamide (Fig. 3B). Together, these data suggest that the pharmacological profile of the membrane androgen receptor is distinct from the classical androgen receptor.

With respect to cell survival, the role of this putative membrane AR has only been assessed in non-CNS tissue where the activation of the membrane androgen receptor by BSA-conjugated testosterone promoted apoptotic cell death in both the LNCaP prostate cell line and in T47D breast cancer cells (10, 11). Based on our recent work that described the ability of DHT-BSA to suppress the phosphorylation of two key effectors of the cell survival-promoting MAPK and PI-3K/Akt pathways (12), and by extension, enhance cell death in rat C6 glioma cells (data not shown). Here, we sought to determine if these effects on cell signaling were relevant to the regulation of cell viability in primary cortical astrocytes. It was determined that activation of the membrane AR by DHT-BSA enhances cell death of primary cortical astrocytes (Fig. 1, 2, 3B, 6 - 8), as we have

observed in C6 cells (data not shown). Interestingly, DHT-BSA when administered alone did not promote cell death, but rather, exacerbated IAA-induced toxicity, suggesting that activation of the putative membrane androgen receptor sensitizes cells to toxic insult. Moreover, the death promoting effects of DHT-BSA were in sharp contrast to the survival promoting effects of DHT (Fig. 3A).

As a possible mechanism underlying this enhanced vulnerability to IAA, we determined if activation of the membrane AR with DHT-BSA altered the phosphorylation (and activation) or expression of Akt. In fact, DHT-BSA treatment, when co-administered with a sub-lethal concentration of IAA, resulted in an apparent reduction in not only the phosphorylation, but also the expression, of total Akt. Given that total Akt expression also decreased with DHT-BSA treatment, it is difficult to ascertain if the decrease in Akt phosphorylation is a true reflection of decreased kinase activity of Akt, or is merely the consequence of reduced expression of the protein. The reduced phosphorylation and/or expression of Akt as a mechanism underlying the increased vulnerability of cells is consistent with previous reports showing that over-expression of a dominant negative form of Akt interferes with growth factor-induced neuronal survival (17).

In order to explore the nature of cell death elicited by activation of the putative membrane androgen receptor, we determined if biochemical hallmarks of apoptosis, specifically, activation of caspases were evident following treatment with DHT-BSA. In fact, DHT-BSA, when co-applied with IAA, resulted in a significant increase in caspase

3/7 activity. This effect of DHT-BSA on caspase-3/7 activity was correlated with an increase in TUNEL staining, strengthening our conclusion that DHT-BSA promoted apoptotic cell death. And like we observed in earlier experiments, treatment with DHT-BSA or IAA alone were insufficient to increase caspase 3/7 activity, further supporting the fact that activation of the membrane androgen receptor does not itself lead to cell death, but instead, increases the vulnerability of the cell to insult.

It is worth mentioning that the cell death-promoting effects of DHT-BSA were not observed at nanomolar concentrations, but instead were seen at micromolar concentrations. The negative consequences of androgens at higher concentrations are not without precedent. In fact, Estrada and colleagues (6) found that in contrast to nanomolar concentrations of testosterone (up to 100 nM), low micromolar concentrations of testosterone (1 – 10 μM) resulted in apoptotic cell death, as evidenced by an increase in Annexin V staining, caspase activation and DNA fragmentation. This effect did not appear to be non-specific, as it could be prevented with both caspase inhibitors and either inhibition or knock-down of intracellular calcium channels (IP3 receptors) (6). Another important point to consider is that the effective concentration of steroids *in vitro* cannot be readily translated to the *in vivo* situation, since exogenously applied androgens may bind to several sites other than its biologically relevant target, i.e., the classical AR or the putative membrane androgen receptor discussed here. Further, the use of BSA-conjugated steroids in estimating an "EC50" value is problematic given the reported lower binding efficiency of these conjugated steroids (23), the interpretation of which is that higher

concentrations of DHT-BSA are likely required to maximally occupy the membrane androgen receptor. Therefore, even though the cell death-promoting effects were observed at micromolar concentrations of DHT-BSA in vitro, we cannot rule out the possibility that its target, the putative membrane androgen receptor, is a physiologically relevant target of androgens.

Interestingly, our data also suggest that activation of the putative membrane androgen receptor not only promotes cell death, but reduces the beneficial effects of another protective hormone, estradiol as well. Estradiol, at a wide range of concentrations, has been shown by numerous groups to exert protective effects against a wide variety of insults, including IAA-induced toxicity (24), β-amyloid toxicity (25-29), excitotoxicity (27, 30-32) and serum deprivation (33), to name a few. Here, we found that DHT-BSA prevented both DHT and estradiol-induced protection against IAA-mediated toxicity. Since androgens do not decline precipitously following the menopause, as do estradiol and progesterone levels (34), the persistence of androgen, coupled with the possibility of having a relative abundance of this putative membrane receptor expressed, may influence the response of postmenopausal women to estrogen therapy. Such a mechanism may be relevant when interpreting the results of the Women's Health Initiative (35-38).

Collectively, the data presented here argue for the presence of two competing pathways for androgens, one that is associated with brain protection and the other which is associated with the promotion of cell death. Though our data are derived from glial

cells, we believe that this may be equally important as data derived from neuronal cultures in that glia play an important role in helping to maintain neuronal function. In addition to being the dominant population of cells in the brain, glia are an important source of trophic factors (39), can buffer "excitotoxins" (such as glutamate, which at higher concentrations can lead to excitotoxicity) ((39, 40) for review), and can reduce amyloid burden (41, 42). As such, a mechanism that compromises glial viability (such as through activation of the putative membrane androgen receptor) may also contribute to an increase vulnerability of neurons. Given the existing controversy surrounding the use of androgen therapy, rooted in part to equivocal reports on whether androgens are protective or damage-promoting, our data may offer novel insight into the inconsistent consequences of androgen treatment. That is, depending on the relative ratio of the putative membrane androgen receptor and the classical intracellular receptor, androgens may result in different effects, and may even alter the efficacy of other neuroprotective steroid hormones.

ACKNOWLEDGMENTS

This work was supported by funds from the National Institutes on Aging (NIA) grant (AG22550 and AG23330), a NIA-sponsored Research Supplement to Promote Diversity in Health Related Research, the National Science Foundation SCORE (School's Cooperative Opportunities for Resources and Education) grant (NSF 0440334) and a Grass Foundation Fellowship awarded to JWG.

Figure 1. DHT-BSA enhances cell death in primary cortical astrocytes. Primary cortical astrocytes were treated with IAA (10 μM) for 12 hrs in the presence/absence of DHT-BSA (0 to 10 μM). Culture media was analyzed for the release of LDH as an indicator of cell death. The data are presented as a percentage of maximal LDH release, as defined by treatment of the cultures with Triton X-100 (Tr-X). The data are representative of four independent experiments and expressed as the mean ± S.E.M. Following an Analysis of Variance (ANOVA), individual comparisons were made using a single degree of freedom F-test within the main effect of the ANOVA ($F(11,34) = 89.086$, $p < 0.001$). Symbols denoting individual group differences: * $p<0.001$ vs. BSA-treated control; $^{\#}$ $p<0.001$ vs. IAA alone.

Figure 1.

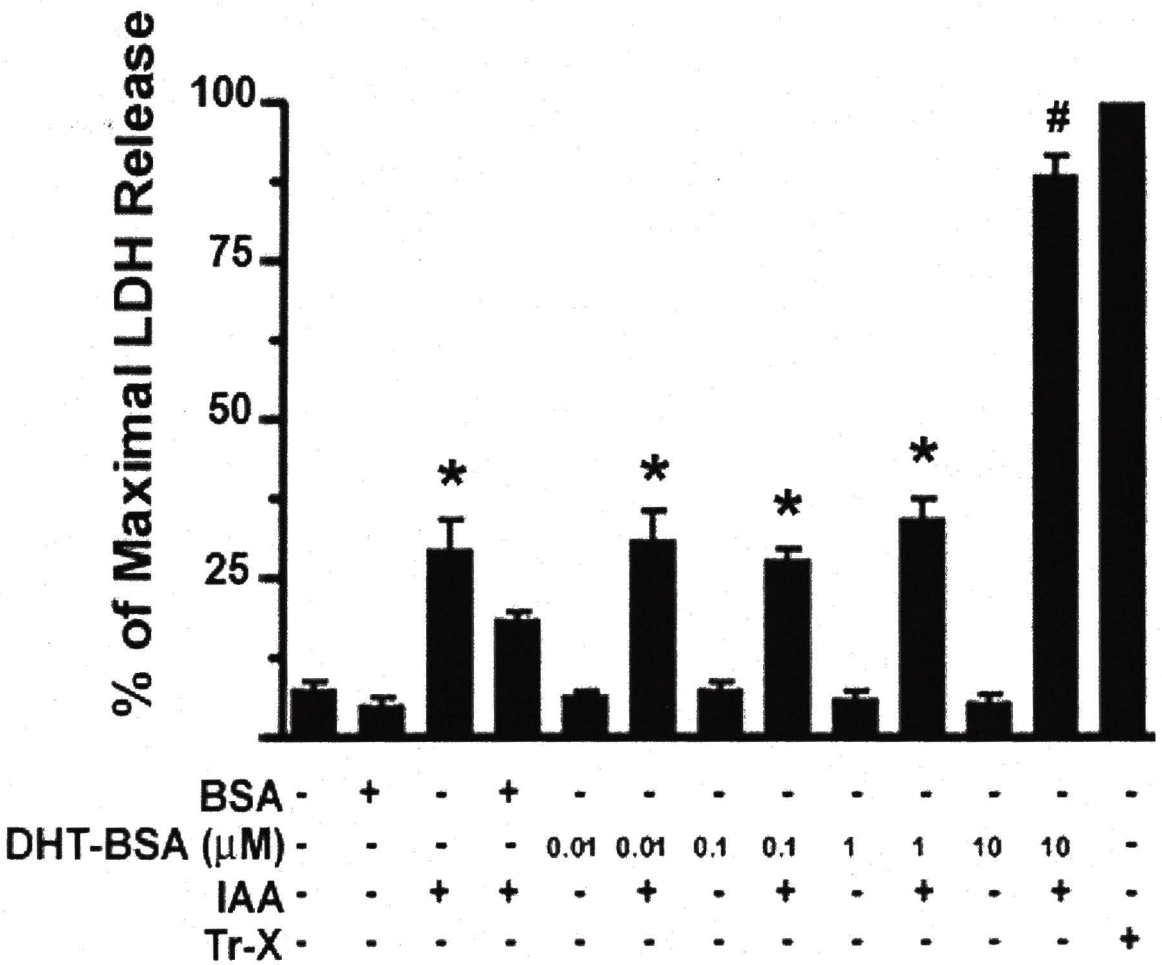

% of Maximal LDH Release
100
75
50
25
*
*
*
*
#
BSA - + - + - - - - - - - - -
DHT-BSA (μM) - - - - 0.01 0.01 0.1 0.1 1 1 10 10 -
IAA - - + + - + - + - + - + -
Tr-X - - - - - - - - - - - - +

Figure 2. DHT protects astrocytes from IAA-induced cell death. In the presence of a minimally toxic concentration of IAA (10 μM), primary cortical astrocytes were treated with DHT (10 μM) or DHT-BSA (10 μM) for 12 hours. The data are presented as a percentage of maximal LDH release. The data are representative of four independent experiments and expressed as the mean ± S.E.M. Following an Analysis of Variance (ANOVA), individual comparisons were made using a single degree of freedom F-test within the main effect of the ANOVA ($F(5,12) = 283.564$, $p < 0.001$). Symbols denoting individual group differences: * $p<0.001$ vs. control; # $p<0.001$ vs. IAA alone.

Figure 2.

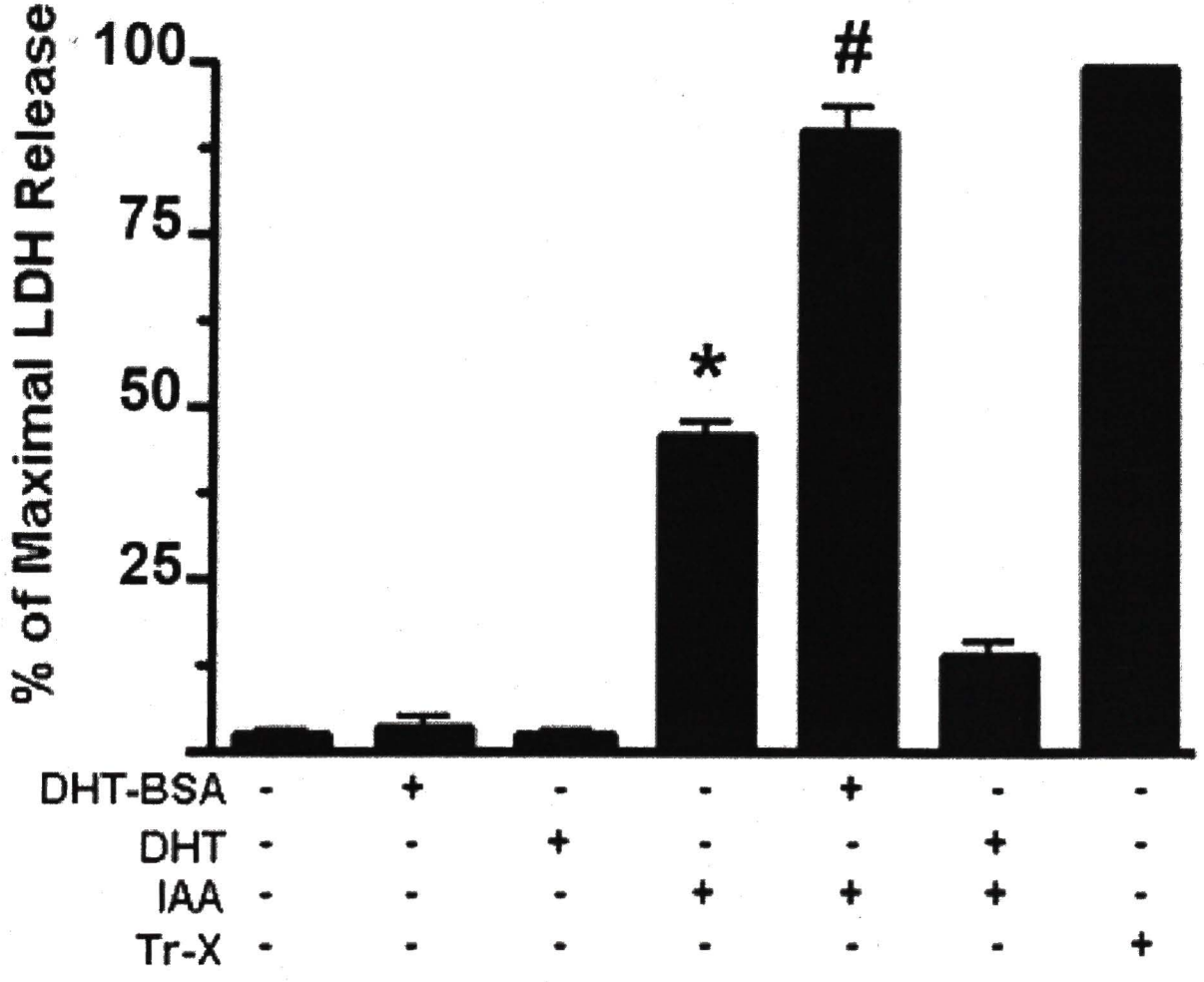


Figure 3. The effect of DHT-BSA on cell survival is flutamide insensitive. Primary cortical astrocytes were treated with IAA (10 μM), Flut. (3 μM), DHT (A; 10 μM), and DHT-BSA (B; 10 μM) for 12 hours. Following treatment, the media was analyzed for LDH levels as an indicator of cell death. The ability of DHT to protect against IAA-induced toxicity was blocked by the AR antagonist, flutamide. In contrast, flutamide did not prevent the ability of DHT-BSA to promote cell death. The data are presented as a percentage of maximal LDH release. The data are representative of three independent experiments and expressed as the mean ± S.E.M. Following an Analysis of Variance (ANOVA), individual comparisons were made using a single degree of freedom F-test within the main effect of the ANOVA (For Figure 3A: $F(5,12) = 86.306$, $p < 0.001$; For Figure 3B: $F(5,12) = 39.175$, $p<0.001$). Symbols denoting individual group differences: * $p<0.001$ vs. control; $^{\#}$ $p<0.001$ vs. IAA alone; $^{@}$ $p<0.001$ vs. DHT + IAA.

Figure 3.

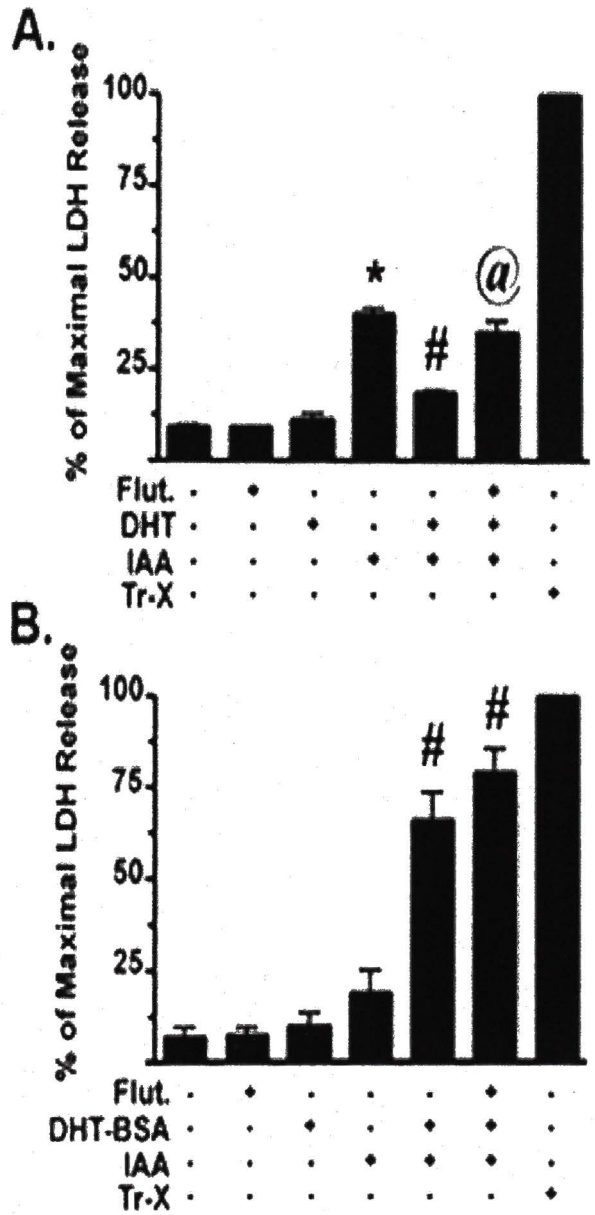

A.
% of Maximal LDH Release
100
75
50
25
*
#
@
Flut.
DHT
IAA
Tr-X
B.
% of Maximal LDH Release
100
75
50
25
#
#
Flut.
DHT-BSA
IAA
Tr-X

Figure 4. In the presence of IAA, DHT-BSA suppresses Akt phosphorylation and expression. Primary cortical astrocytes were treated with IAA (10 μM) in the presence or absence of DHT-BSA (10 μM) for 8 hours. Following treatment, sample lysates (60μg/lane) were then subjected to SDS-PAGE and Western blot analysis for the evaluation of Akt phosphorylation. The upper, middle, and lower blots of panel A, depict Akt phosphorylation, total Akt, and actin protein, respectively, from the same samples (i.e., a single independent experiment). The bar graphs, however, represents the densitometric analysis from four independent experiments. The densitometry from the phospho-Akt (B) and total Akt blots (C) were normalized and presented as signal intensity relative to that seen in the BSA-treated control. Data are expressed as the mean ± S.E.M. Following an Analysis of Variance (ANOVA), individual comparisons were made using a single degree of freedom F-test within the main effect of the ANOVA ($F(3,12) = 31.182$, $p < 0.001$). Symbols denoting individual group differences: * $p<0.05$ vs. BSA-treated control; $^{\#}$ $p<0.001$ vs. IAA alone.

Figure 4.

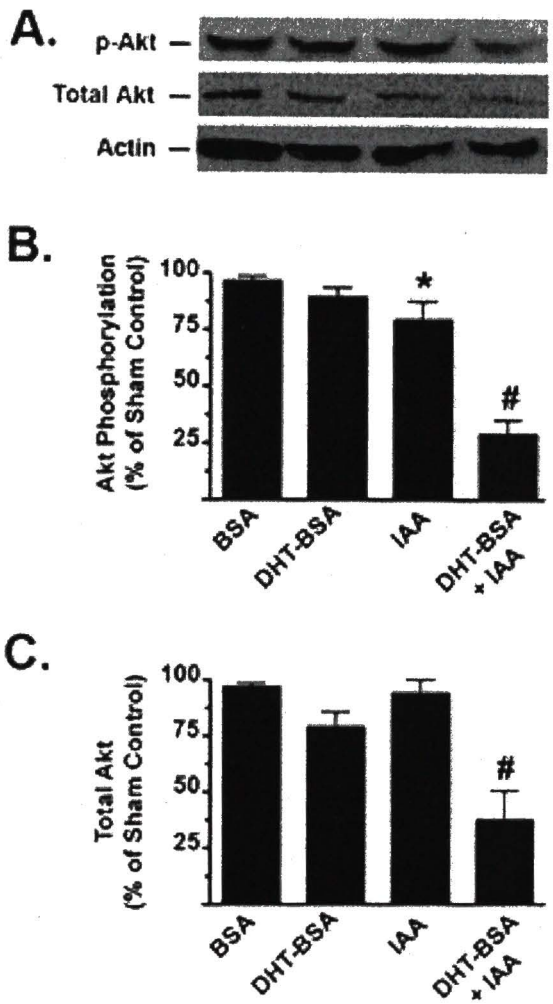

A.
p-Akt
Total Akt
Actin
B.
Akt Phosphorylation (% of Sham Control)
100
75
50
25
*
#
BSA
DHT-BSA
IAA
DHT-BSA + IAA
C.
Total Akt (% of Sham Control)
100
75
50
25
#
BSA
DHT-BSA
IAA
DHT-BSA + IAA

Figure 5. DHT-BSA increases caspase 3/7 activation in primary astrocytes during metabolic inhibition. The primary glia were treated with IAA (10 μM) in the presence of estrogen (10 μM) and DHT-BSA (10 μM) for 8 hours. Values represent caspase 3/7 activity and are presented as a percent of the vehicle control. The data are representative of three independent experiments and expressed as the mean ± S.E.M. Following an Analysis of Variance (ANOVA), individual comparisons were made using a single degree of freedom F-test within the main effect of the ANOVA ($F(3,8) = 114.364$, $p < 0.001$). Symbols denoting individual group differences: * $p<0.05$ vs. control; # $p<0.001$ vs. IAA alone.

Figure 5.

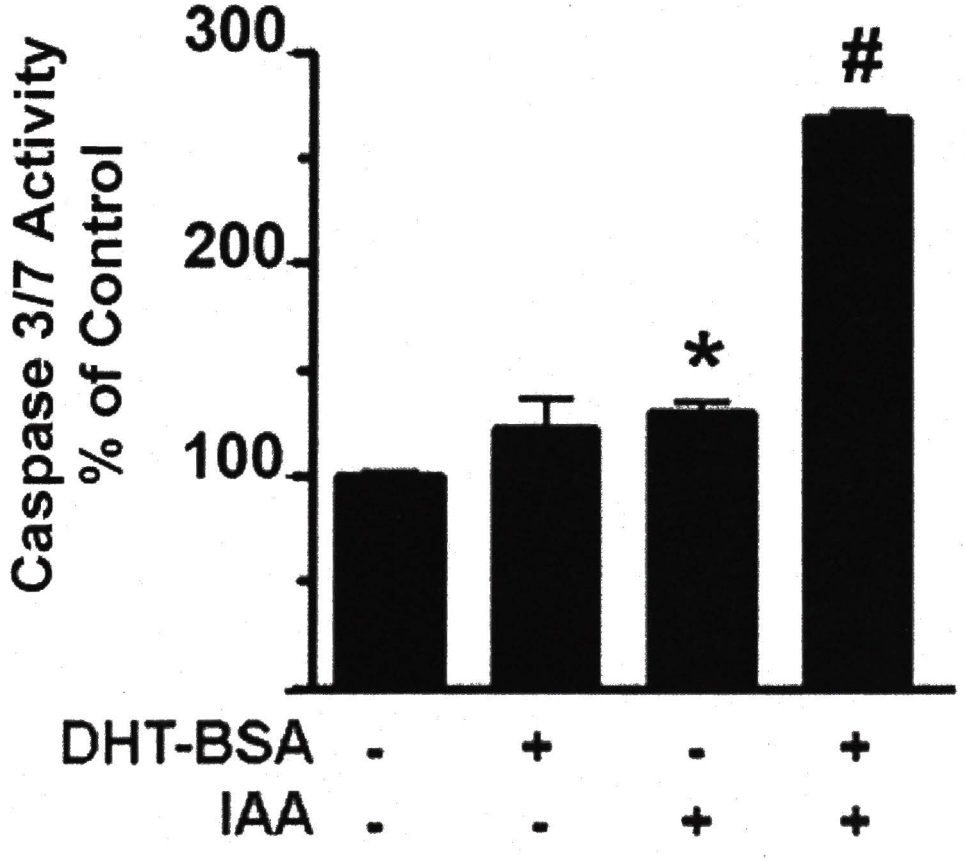


Figure 6. DHT-BSA treatment results in an increase in TUNEL staining and reduces the number of GFAP-positive astrocytes. Primary cortical astrocytes were treated with DHT-BSA (10 μM) for 8 hours in the presence or absence of IAA (10 μM). Following treatment, the cells were stained for glial fibrillary acidic protein (GFAP) and fragmented DNA using immunocytochemistry and the TUNEL assay, respectively. Panel (A) depicts individual optical slices taken with a confocal microscope. *Bar*, 20 μm. Panel (B) shows the graphical representation of the number of GFAP-positive astrocytes in each treatment group. The data in Panel (B) are representative of four independent experiments and expressed as the mean ± S.E.M. Following an Analysis of Variance (ANOVA), individual comparisons were made using a single degree of freedom F-test within the main effect of the ANOVA ($F(4,15) = 49.053$, $p < 0.001$). Symbols denoting individual group differences: * $p<0.001$ vs. BSA-treated control; $^{\#}$ $p<0.001$ vs. IAA alone.

Figure 6.

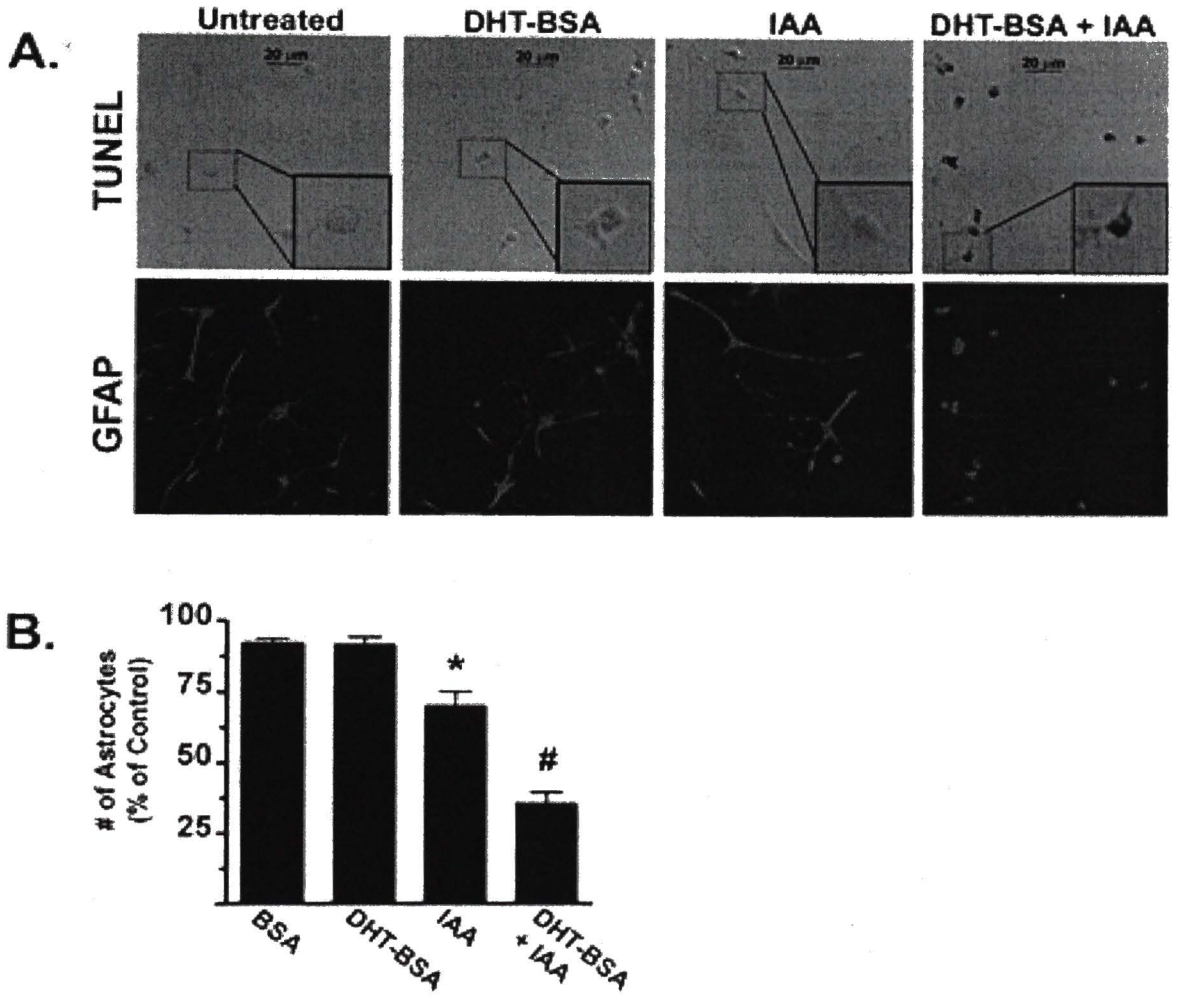

A.
Untreated
DHT-BSA
IAA
DHT-BSA + IAA
TUNEL
GFAP
B.
# of Astrocytes (% of Control)
100
75
50
25
*
#
BSA
DHT-BSA
IAA
DHT-BSA + IAA

Figure 7. DHT-BSA blocks DHT-induced protection from IAA-mediated toxicity. Primary astrocytes were treated with IAA (10 μM) for 24 hours in the presence of DHT (10 μM) and/or DHT-BSA (10 μM). Following the 24 hour incubation, the media was analyzed for the levels of LDH as an indicator of cell death. DHT-BSA completely prevented the ability of DHT to protect against IAA-induced toxicity. The data are presented as a percentage of maximal LDH release. The data are representative of three independent experiments and expressed as the mean ± S.E.M. Following an Analysis of Variance (ANOVA), individual comparisons were made using a single degree of freedom F-test within the main effect of the ANOVA ($F(6,21) = 116.772$, $p < 0.001$). Symbols denoting individual group differences: * $p<0.001$ vs. control; $^{\#}$ $p<0.001$ vs. IAA alone; $^{@}$ $p<0.05$ vs. IAA alone; $^{+}$ $p<0.001$ vs. DHT + IAA.

Figure 7.

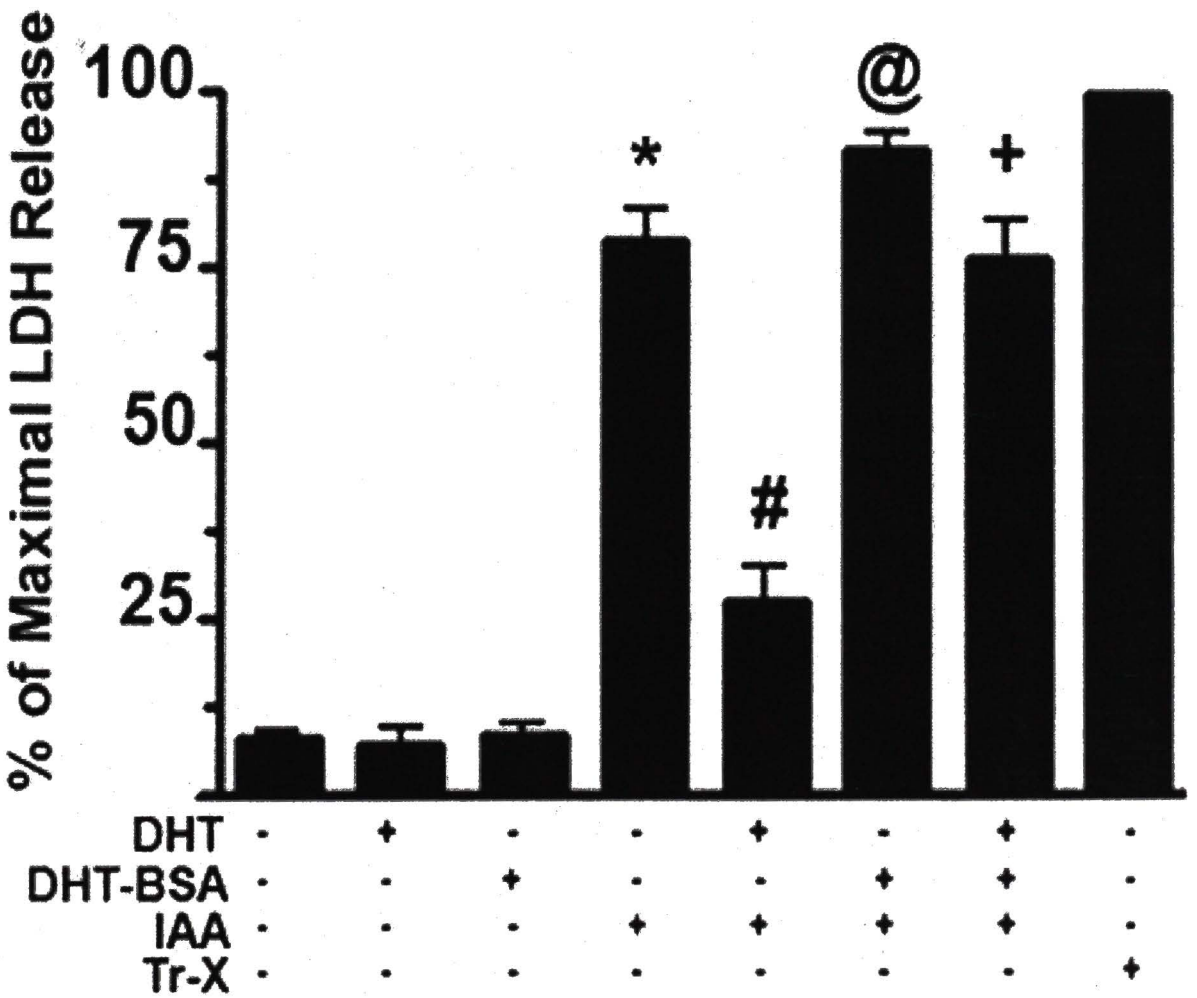
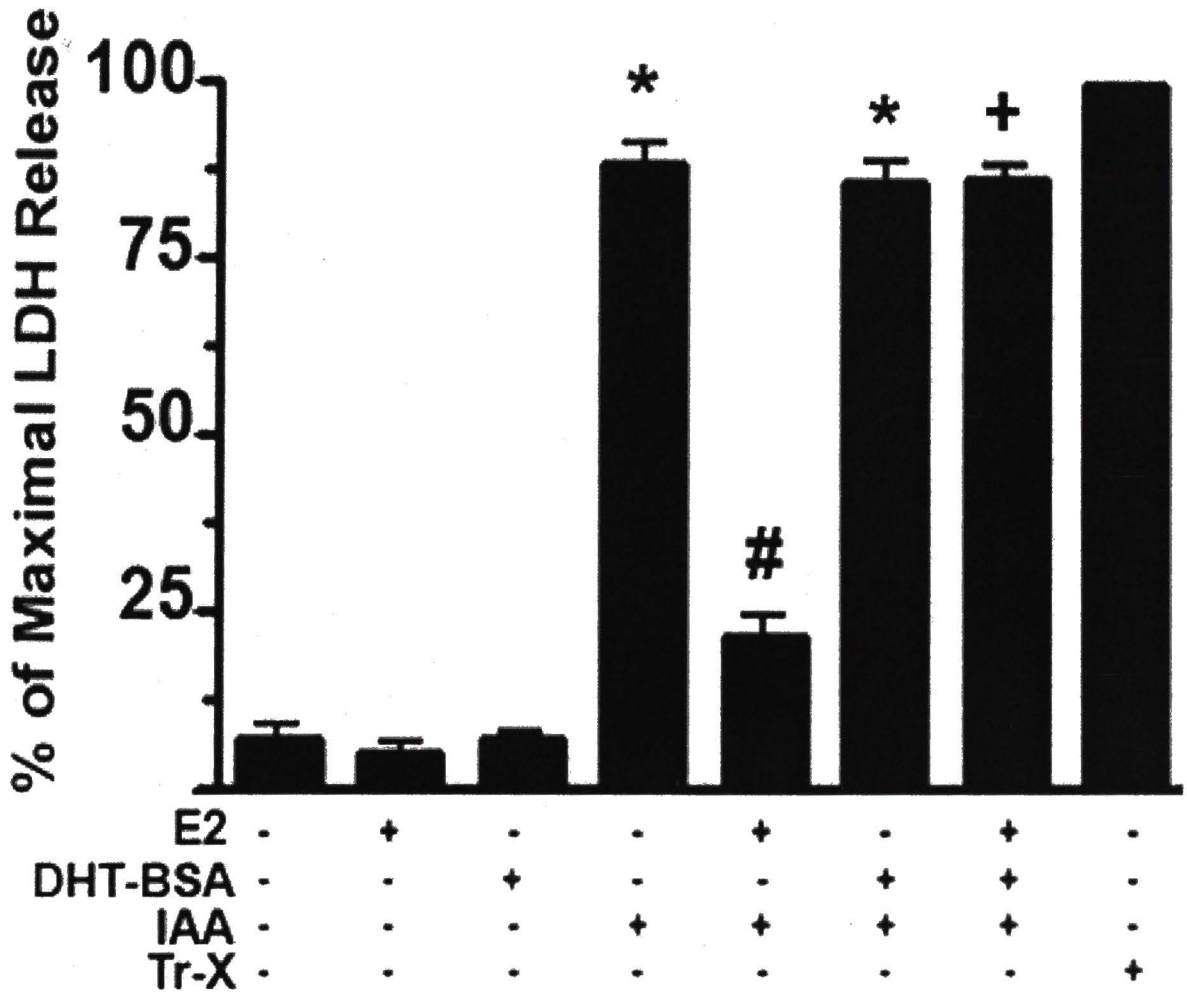


Figure 8. DHT-BSA inhibits estradiol-induced protection. Primary glial cultures were treated with IAA (10 μM) for 24 hours in the presence of 17-β estradiol (10 μM) and/or DHT-BSA (10 μM). Following the 24 hour incubation, the media was analyzed for the levels of LDH as an indicator of cell death. The protective effects of estrogen were blocked by DHT-BSA. The data are presented as a percentage of maximal LDH release. The data are representative of three independent experiments and expressed as the mean ± S.E.M. Following an Analysis of Variance (ANOVA), individual comparisons were made using a single degree of freedom F-test within the main effect of the ANOVA ($F(6,21) = 302.778$, $p < 0.001$). Symbols denoting individual group differences: * $p<0.001$ vs. control; $^{\#}$ $p<0.001$ vs. IAA alone; $^{+}$ $p<0.001$ vs. E2+IAA.

Figure 8.



REFERENCES

1. **Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, et al.** 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-9

2. **Ramsden M, Shin TM, Pike CJ** 2003 Androgens modulate neuronal vulnerability to kainate lesion. Neuroscience 122:573-8

3. **Ramsden M, Nyborg AC, Murphy MP, Chang L, Stanczyk FZ, Golde TE, Pike CJ** 2003 Androgens modulate beta-amyloid levels in male rat brain. J Neurochem 87:1052-5

4. **Nguyen TV, Yao M, Pike CJ** 2005 Androgens activate mitogen-activated protein kinase signaling: Role in neuroprotection. J Neurochem 94:1639-51

5. **Hammond J, Le Q, Goodyer C, Gelfand M, Trifiro M, LeBlanc A** 2001 Testosterone-mediated neuroprotection through the androgen receptor in human primary neurons. J Neurochem 77:1319-26

6. **Estrada M, Varshney A, Ehrlich BE** 2006 Elevated testosterone induces apoptosis in neuronal cells. J Biol Chem 281:25492-501

7. **Yang SH, Perez E, Cutright J, Liu R, He Z, Day AL, Simpkins JW** 2002 Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model. J Appl Physiol 92:195-201

8. **Yang SH, Liu R, Wen Y, Perez E, Cutright J, Brun-Zinkernagel AM, Singh M, Day AL, Simpkins JW** 2005 Neuroendocrine mechanism for tolerance to cerebral ischemia-reperfusion injury in male rats. J Neurobiol 62:341-51

9. **Caruso A, Di Giorgi Gerevini V, Castiglione M, Marinelli F, Tomassini V, Pozzilli C, Caricasole A, Bruno V, Caciagli F, Moretti A, Nicoletti F, Melchiorri D** 2004 Testosterone amplifies excitotoxic damage of cultured oligodendrocytes. J Neurochem 88:1179-85

10. **Kampa M, Nifli AP, Charalampopoulos I, Alexaki VI, Theodoropoulos PA, Stathopoulos EN, Gravanis A, Castanas E** 2005 Opposing effects of estradiol- and testosterone-membrane binding sites on T47D breast cancer cell apoptosis. Exp Cell Res 307:41-51

11. **Hatzoglou A, Kampa M, Kogia C, Charalampopoulos I, Theodoropoulos PA, Anezinis P, Dambaki C, Papakonstanti EA, Stathopoulos EN, Stournaras C, Gravanis A, Castanas E** 2005 Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo. J Clin Endocrinol Metab 90:893-903

12. **Gatson JW, Kaur P, Singh M** 2006 Dihydrotestosterone differentially modulates the mitogen-activated protein kinase and the phosphoinositide 3-kinase/Akt pathways through the nuclear and novel membrane androgen receptor in C6 cells. Endocrinology 147:2028-34

13. **Malcolm CS, Benwell KR, Lamb H, Bebbington D, Porter RH** 2000 Characterization of iodoacetate-mediated neurotoxicity in vitro using primary cultures of rat cerebellar granule cells. Free Radic Biol Med 28:102-7

14. **Matthews RT, Ferrante RJ, Jenkins BG, Browne SE, Goetz K, Berger S, Chen IY, Beal MF** 1997 Iodoacetate produces striatal excitotoxic lesions. J Neurochem 69:285-9

15. **Singh M, Setalo G, Jr., Guan X, Warren M, Toran-Allerand CD** 1999 Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. J Neurosci 19:1179-88

16. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-75

17. **Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME** 1997 Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661-5

18. **D'Mello SR, Borodezt K, Soltoff SP** 1997 Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI 3- kinase in IGF-1 signaling. J Neurosci 17:1548-60

19. **Klesse LJ, Meyers KA, Marshall CJ, Parada LF** 1999 Nerve growth factor induces survival and differentiation through two distinct signaling cascades in PC12 cells. Oncogene 18:2055-68

20. **Benten WP, Lieberherr M, Sekeris CE, Wunderlich F** 1997 Testosterone induces Ca2+ influx via non-genomic surface receptors in activated T cells. FEBS Lett 407:211-4

21. **Braun AM, Thomas P** 2004 Biochemical characterization of a membrane androgen receptor in the ovary of the atlantic croaker (Micropogonias undulatus). Biol Reprod 71:146-55

22. **Benten WP, Lieberherr M, Stamm O, Wrehlke C, Guo Z, Wunderlich F** 1999 Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Mol Biol Cell 10:3113-23

23. **De Goeij AF, van Zeeland JK, Beek CJ, Bosman FT** 1986 Steroid-bovine serum albumin conjugates: molecular characterization and their interaction with androgen and estrogen receptors. J Steroid Biochem 24:1017-31

24. **Perez E, Liu R, Yang SH, Cai ZY, Covey DF, Simpkins JW** 2005 Neuroprotective effects of an estratriene analog are estrogen receptor independent in vitro and in vivo. Brain Res 1038:216-22

25. **Behl C, Widmann M, Trapp T, Holsboer F** 1995 17-beta estradiol protects neurons from oxidative stress-induced cell death in vitro. Biochem Biophys Res Commun 216:473-82

26. **Green PS, Gridley KE, Simpkins JW** 1996 Estradiol protects against beta-amyloid (25-35)-induced toxicity in SK- N-SH human neuroblastoma cells. Neurosci Lett 218:165-8

27. **Goodman Y, Bruce AJ, Cheng B, Mattson MP** 1996 Estrogens attenuate and corticosterone exacerbates excitotoxicity, oxidative injury, and amyloid beta-peptide toxicity in hippocampal neurons. J Neurochem 66:1836-44

28. **Xu H, Gouras GK, Greenfield JP, Vincent B, Naslund J, Mazzarelli L, Fried G, Jovanovic JN, Seeger M, Relkin NR, Liao F, Checler F, Buxbaum JD, Chait BT, Thinakaran G, Sisodia SS, Wang R, Greengard P, Gandy S** 1998 Estrogen reduces neuronal generation of Alzheimer beta-amyloid peptides. Nat Med 4:447-51

29. **Fitzpatrick JL, Mize AL, Wade CB, Harris JA, Shapiro RA, Dorsa DM** 2002 Estrogen-mediated neuroprotection against beta-amyloid toxicity requires expression of estrogen receptor alpha or beta and activation of the MAPK pathway. J Neurochem 82:674-82

30. **Mendelowitsch A, Ritz MF, Ros J, Langemann H, Gratzl O** 2001 17beta-Estradiol reduces cortical lesion size in the glutamate excitotoxicity model by enhancing extracellular lactate: a new neuroprotective pathway. Brain Res 901:230-6

31. **Singer CA, Figueroa-Masot XA, Batchelor RH, Dorsa DM** 1999 The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. J Neurosci 19:2455-63

32. **Huang Y, Huang YL, Zhang S, Zhu YC, Yao T** 2004 Estradiol acutely attenuates glutamate-induced calcium overload in primarily cultured rat

hippocampal neurons through a membrane receptor-dependent mechanism. Brain Res 1026:254-60

33. **Green PS, Bishop J, Simpkins JW** 1997 17 alpha-estradiol exerts neuroprotective effects on SK-N-SH cells. J Neurosci 17:511-5

34. **Laughlin GA, Barrett-Connor E, Kritz-Silverstein D, von Muhlen D** 2000 Hysterectomy, oophorectomy, and endogenous sex hormone levels in older women: the Rancho Bernardo Study. J Clin Endocrinol Metab 85:645-51

35. **Wassertheil-Smoller S, Hendrix SL, Limacher M, Heiss G, Kooperberg C, Baird A, Kotchen T, Curb JD, Black H, Rossouw JE, Aragaki A, Safford M, Stein E, Laowattana S, Mysiw WJ** 2003 Effect of estrogen plus progestin on stroke in postmenopausal women: the Women's Health Initiative: a randomized trial. Jama 289:2673-84

36. **Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, Hendrix SL, Jones BN, 3rd, Assaf AR, Jackson RD, Kotchen JM, Wassertheil-Smoller S, Wactawski-Wende J** 2003 Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. Jama 289:2651-62

37. **Shumaker SA, Legault C, Kuller L, Rapp SR, Thal L, Lane DS, Fillit H, Stefanick ML, Hendrix SL, Lewis CE, Masaki K, Coker LH** 2004 Conjugated equine estrogens and incidence of probable dementia and mild cognitive

impairment in postmenopausal women: Women's Health Initiative Memory Study. Jama 291:2947-58

38. **Rapp SR, Espeland MA, Shumaker SA, Henderson VW, Brunner RL, Manson JE, Gass ML, Stefanick ML, Lane DS, Hays J, Johnson KC, Coker LH, Dailey M, Bowen D** 2003 Effect of estrogen plus progestin on global cognitive function in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. Jama 289:2663-72

39. **Aschner M, Sonnewald U, Tan KH** 2002 Astrocyte modulation of neurotoxic injury. Brain Pathol 12:475-81

40. **Anderson CM, Swanson RA** 2000 Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32:1-14

41. **Nagele RG, Wegiel J, Venkataraman V, Imaki H, Wang KC** 2004 Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. Neurobiol Aging 25:663-74

42. **Roth AD, Ramirez G, Alarcon R, Von Bernhardi R** 2005 Oligodendrocytes damage in Alzheimer's disease: beta amyloid toxicity and inflammation. Biol Res 38:381-7

THE DAMAGING EFFECTS OF DHT-BSA IN ASTROCYTES IS NOT MEDIATED BY cAMP

JOSHUA W. GATSON AND MEHARVAN SINGH

Department of Pharmacology and Neuroscience, The Institute for Aging & Alzheimer's Disease Research, and The Center FOR HER (Focused On Resources for her Health, Education and Research), University of North Texas Health Science, Fort Worth, Texas

Running Title: PKA is not involved in DHT-BSA-induced toxicity

Corresponding author:

Meharvan Singh, Ph.D.
Department of Pharmacology and Neuroscience
University of North Texas Health Science Center at Fort Worth
3400 Camp Bowie Blvd.
Fort Worth, Texas 76107-2699
Phone: (817) 735-5429
Fax: (817) 735-0408
E-mail: msingh@hsc.unt.edu

CHAPTER V

THE DAMAGING EFFECTS OF DHT-BSA IN ASTROCYTES IS NOT MEDIATED BY cAMP

ABSTRACT

In non-central nervous system (CNS) tissue, activation of a membrane-associated AR (mAR) has been shown to activate several signaling pathways. Recently, our laboratory has shown that activation of a mAR leads to a decrease in ERK and Akt signaling (two survival-promoting pathways) and exacerbated iodoacetic acid (IAA)-induced toxicity in rat glioma (C6) cells. Since other reports have shown that increased cAMP and PKA signaling suppresses the levels of these two survival-promoting pathways in C6 cells, we postulated that the mAR-mediated suppression of ERK and Akt is mediated by the cAMP/PKA pathway. Thus, contrary to our hypothesis, we found that activation of the mAR by DHT-BSA did not change basal levels of intracellular cAMP, but partially blocked forskolin-induced cAMP production. Additionally, the effects of DHT-BSA on survival were not blocked by the PKA inhibitors, H-89 and KT5720. Collectively, these data suggest that the damaging effects of DHT-BSA during metabolic and oxidative stress are not mediated by cAMP or PKA.

INTRODUCTION

In the cell, cAMP and PKA signaling can have different effects on cell growth, differentiation, and proliferation depending on the cell type. For example, in epithelial, pancreatic islet, and Schwann cells, increased cyclic AMP (cAMP) signaling leads to an increase in cellular growth and proliferation (1). In contrast, cAMP accumulation resulted in the inhibition of cellular growth and proliferation in both a PKA-dependent and independent manner in fibroblasts, neurons, muscle, and glia (2-5). With respect to glia, in C6 cells and astrocytes, it was determined that this effect consequent to cAMP accumulation was mediated by the inhibition of the ERK and Akt pathways (4).

We have previously found that activation of a membrane-associated AR (mAR) in C6 cells resulted in a decrease in the levels of phospho-ERK and Akt (10). This suppression of Akt resulted in a greater increase in cell death under conditions of metabolic and oxidative stress (Chapter 4). Since cAMP and PKA have been shown to decrease ERK and Akt signaling in glia, we hypothesized that the mAR-mediated suppression of ERK and Akt, along with the enhanced cell death, was mediated by cAMP-stimulated PKA. Here, we found that the damaging effects of androgens is not mediated by cAMP or PKA, since DHT-BSA did not alter cAMP levels and pharmacological inhibition of PKA did not block DHT-BSA-induced cell death. Also, forskolin blocked the damaging effects of DHT-BSA, further supporting the fact that increased cAMP and PKA signaling in astrocytes does not promote cell death.

MATERIALS AND METHODS

Primary cortical astrocytes. Primary cortical astrocytes were derived from the cerebral cortex of postnatal day (P) 3 C57Bl/6 mice. Briefly, following removal of the brain from the cranial cavity, the meninges were removed and the cerebral cortex was dissected and placed into a sterile vacutainer tube containing 2 ml of the Dissociation solution (1.9 ml PBS 1X pH 7.2 + 0.25% Trypsin [final concentration]). The tube was incubated at 37°C for 10 min. Following this incubation, the tube was centrifuged at 400 x *g* for 5 min. The resulting pellet was then re-suspended in 2 ml of plating media (DMEM with sodium pyruvate/10% FBS + 1% Penicillin/Streptomycin) and dissociated further with the aid of a fire-polished Pasteur pipet. After this dissociation, the sample was centrifuged and re-suspended in 2ml of plating media. These re-suspension/centrifugation steps were repeated twice and the cell suspension was filtered through a 70μm cell strainer into a 50ml conical tube. The filtrate was diluted by adding 5 ml of plating media. The cells were counted and plated at the desired density (~200,000 cells/well, 24-well plate; ~400,000 cells/well, 6-well plate). To establish pure astrocyte cultures, the media was changed every 48 hrs and just prior to the media change, the tissue culture plate/flask was shaken at 350 rpm for 5 min. The cultures were treated with hormone or drug on the 14 day *in vitro* (d.i.v.).

All procedures involving the use of animals was approved the UNT Health Science Center's Institutional Animal Care and Use Committee (IACUC).

Treatment of cultures. Primary astrocytes were treated with 5α-androstan-17β-ol-3-one-3-o-carboxymethyloxime:BSA (DHT-BSA; Steraloids Inc.), H-89 (Calbiochem), Forskolin (Calbiochem), KT5720 (Calbiochem), Triton-X (10%; Sigma), or iodoacetic acid (IAA; Sigma) at the indicated time points. Controls for DHT-BSA treatment included equimolar concentrations of bovine serum albumin (BSA) alone (Fisher Scientific, Fair Lawn, NJ). To avoid the potential confound of having "free" or unconjugated DHT in the DHT-BSA preparation, the DHT-BSA solution was pre-filtered using a 30 kDa nominal cut-off column to remove any unconjugated steroid. The retentate (consisting of conjugated DHT-BSA) was then reconstituted in the original volume.

cAMP Assay. To determine the amount of cellular cAMP produced following treatment with various drugs, the R & D Systems cAMP assay was used. In brief, the primary cortical glia were plated, maintained for 15 days and treated with DHT-BSA (0.01, 0.1, 1, 10 μM) and/or forskolin (30 μM) for 20 minutes at 37°C. Following treatment, total protein was isolated and 100 μl of the total lysate was loaded into a well on a 96-well plate. To each well, 50 μl of the cAMP primary antibody solution was added. In addition, 50 μl of the cAMP conjugate was added to each well. The plate was incubated at 27°C for 3 hours and each well was washed four times with 400 μl of wash buffer. To detect the levels of cAMP, 200 μl of substrate solution was added to each well for 30 minutes at 27°C. After the 30 minute incubation, 50 μl of stop solution was added

to each well and the plate was read at 450 nM using the Viktor3 ELISA plate reader (Perkin Elmer, Boston, MA).

LDH Assay. A fluorometric assay (CytoTox-One Homogenous Membrane Integrity Assay Kit, Promega) was used for the measurement of lactate dehydrogenase (LDH) released from damaged or dying cells. Briefly, 100 µL of conditioned media was aliquoted into a black 96-well plate. To each well, 100 µl of the CytoTox-One reagent was added for 10 min. Following termination of the enzymatic reaction, resulting fluorescence was measured [560 nm (excitation)/590 nm (emission)] using a Viktor3 ELISA plate reader (Perkin Elmer, Boston, MA). Relative fluorescent units (RFU) were normalized to the amount of protein in the culture (assessed using BIORAD DC protein assay kit) from which the conditioned media was derived. These values were subsequently normalized and expressed as a percentage of total LDH release (assessed by treating cultures with 1% Triton X-100).

Statistical Analysis. Data points from at least three independent experiments were subjected to an analysis of variance (ANOVA), followed by a Tukey's *post hoc* analysis for the assessment of group differences, and presented as a bar graph depicting the average ± S.E.M., using the GraphPad Software (San Diego, CA).

RESULTS

Activation of the membrane-associated AR by DHT-BSA did not increase cAMP levels.

Previously, we reported that activation of a mAR in astrocytes suppresses the ERK and Akt survival-promoting pathways (10). In another study, however, increased cAMP and PKA signaling led to the inhibition of these survival pathways (4, 6, 11). As such, we postulated that the mAR-mediated suppression of ERK and Akt was mediated by an increase in cAMP. To test this hypothesis, we first evaluated if activation of the putative mAR alters cAMP levels with increasing concentrations of DHT-BSA (0.01 to 10μM) and assessed cellular levels of cAMP. Figure 1 shows that DHT-BSA (0.01 to 10 μM) did not increase or decrease the basal levels of intracellular cAMP. In contrast, forskolin (30 μM) treatment resulted in a 2-fold increase in intracellular cAMP (Fig. 1). Suprisingly, DHT-BSA at the higher concentrations (0.1 to 10 μM) partially blocked forskolin-induced cAMP production (Fig. 2).

The damage-promoting effects of DHT-BSA are not mediated by PKA.

Various groups have reported that PKA signaling results in suppression of the ERK and Akt pathways in C6 cells (4, 7-9, 11). To determine if the PKA pathway was involved in the death-promoting effects of DHT-BSA, we treated the astroglia with DHT-BSA (10 μM) in the presence or absence of IAA (10 μM) and/or the PKA inhibitors, H-89 or KT5720. Neither DHT-BSA, H-89, KT5720, or IAA-alone caused an increase in cell death. In addition, H-89 and KT5720 did not alter the effects of LDH release (Fig. 3). Treatment with DHT-BSA and IAA for 12 hours led to a significant increase in cell damage as expected. However, the PKA inhibitors, H-89 and KT5720 failed to protect

the cortical glia from DHT-BSA-induced toxicity (Fig.3). These results suggest that PKA is not involved in the damaging-promoting effects of DHT-BSA.

Forskolin fails to exacerbate IAA-induced toxicity and instead, inhibits the death-promoting effects of DHT-BSA.

In order to further address the involvement of the cAMP/PKA pathway in the death-promoting effects of DHT-BSA, we determined if elevating the levels of cAMP would exacerbate IAA-induced toxicity. Primary astrocytes were treated with DHT-BSA (10 μM) in the presence or absence of IAA and/or forskolin (30 μM). As we have seen previously, DHT-BSA exacerbated the effect of IAA. However, forskolin not only failed to mimic the effects of DHT-BSA, but instead protected against the damaging effects of DHT-BSA in the presence of IAA (Fig. 4). Since the cAMP/PKA pathway promotes cell survival in these primary astrocytes, we conclude that the death-promoting effects of DHT-BSA were not mediated by the cAMP/PKA pathway.

DISCUSSION

Several recent reports support the existence of a mAR (10, 12-17), whose activation has been shown to influence cellular growth and survival. With respect to the regulation of cell viability, pharmacological activation of a mAR was found to increase serum deprivation-induced cell death in both prostate cancer cells as well as breast cancer cells (13, 19). Additionally, our laboratory has shown that suppression of Akt in the

primary cortical astrocytes by DHT-BSA results in an increase in caspase signaling, DNA damage, and cell death during injury (10). The mechanisms by which these effects are elicited, however, are not completely understood. Some studies, performed in muscle, heart, T-cells, and macrophages, suggest that the mAR is coupled to the $G\alpha_{i/o}$ class of G-proteins, based on the ability of pertussis toxin (PTX) to inhibit the consequences of activating the membrane androgen receptor (17-21). In glial cells, our previous work has implicated the suppression of ERK and Akt signaling (10) in mediating the effects of the mAR. Given that activation of cAMP/PKA signaling has been shown to lead to the suppression of both ERK and Akt in a glial cell line, C6 cells (4, 7), we hypothesized that the DHT-BSA – induced suppression of ERK and Akt signaling and the promotion of cell death we observed was mediated by the activation of the cAMP/PKA signaling pathway.

Contrary to our hypothesis, we found that DHT-BSA did not alter cAMP levels, and nor was the cell-death promoting effects of DHT-BSA altered by the PKA inhibitors, H-89 and KT5720. Additionally, the DHT-BSA – induced exacerbation of cell death was not mimicked by forskolin, further demonstrating in primary astrocytes that an increase in cAMP does not underlie the death-promoting effects of DHT-BSA. Interestingly, however, DHT-BSA did partially inhibit the forskolin-induced increase in cAMP levels. We believe that the data strongly argue against the involvement of the cAMP/PKA signaling pathway in mediating the suppression of signaling or death-promoting effects of DHT-BSA seen previously.

We considered the possibility that the effects of DHT-BSA, and thus, the mAR, was coupled to $G\alpha_{i/o}$ containing G-protein. As such, activation of the mAR would result in suppression of cAMP, and thus, may explain the ability of DHT-BSA to partially inhibit the forskolin-induced increase in cAMP. Such inhibition of cAMP accumulation would reduce the activation of its downstream effector, PKA, the consequence of which, has been shown to result in reduced activation of ERK in multiple systems. This coupling between the mAR and $G\alpha_{i/o}$ has, in fact, been supported in other cell models, based on the ability of PTX to inhibit the consequences of pharmacological activation of the mAR (18-21).

However, we have found that in primary astroglial cultures, the mAR is not sensitive to PTX, as it did not block DHT-BSA-induced suppression of Akt or the increased cell death (appendix Fig. 1, Fig. 2). These results suggest that the mAR in glia may not be G□$_{i/o}$-linked. Thus, other G-proteins may be involved in the damaging effects of DHT-BSA.

Clues as to how activation of the mAR leads to a suppression of ERK and Akt signaling may be discerned from Figure 4, where forskolin prevented the DHT-BSA – induced increase in cell death, and was, in fact, protective. Thus, the increase in cAMP induced by forskolin inhibited the pathway through which DHT-BSA promoted cell death. Additional studies will be needed to identify if known targets of cAMP and/or PKA (such as Rap1, Epac) are relevant components of the mAR "death promoting

cascade", and if such targets might be inhibited through an increase in cAMP or activation of PKA.

ACKNOWLEDGMENTS

This work was supported by funds from the National Institutes on Aging (NIA) grant (AG22550 and AG23330), and a NIA-sponsored Research Supplement to Promote Diversity in Health Related Research.

Figure 1. DHT-BSA does not increase cAMP levels. Primary cortical astrocytes were treated with DHT-BSA (0.01, 0.1, 1, 10 μM) or forskolin (30 μM) for 20 min. Following treatment, the cells were lysed evaluated for the levels of cAMP. The data are presented as a percentage of the untreated control. The data are representative of four independent experiments. *$p<0.001$ vs. untreated control. Data are expressed as the mean ± S.E.M.

Figure 1.

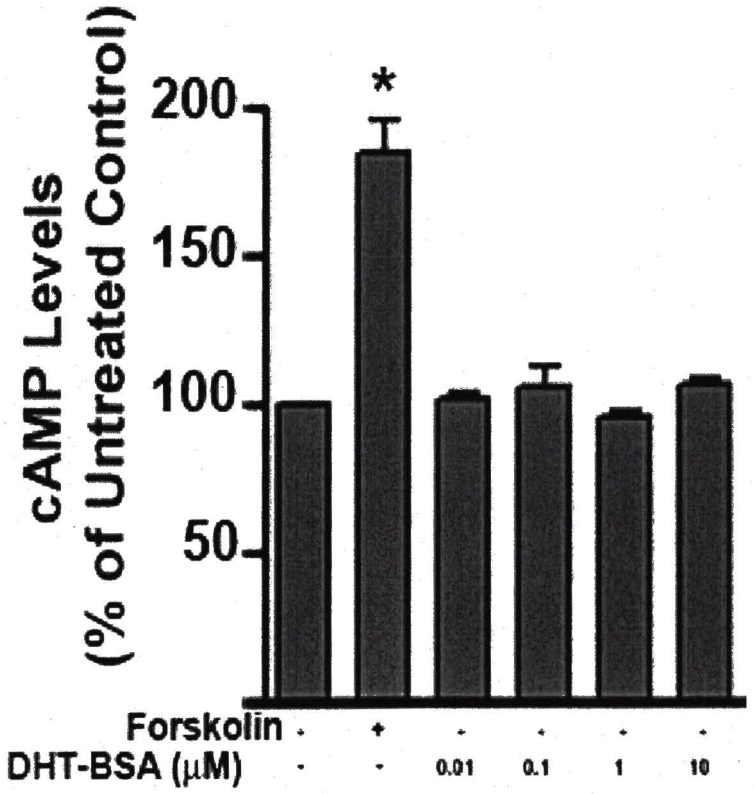

cAMP Levels
(% of Untreated Control)
200
150
100
50
*
Forskolin - + - - - -
DHT-BSA (μM) - - 0.01 0.1 1 10

Figure 2. DHT-BSA attenuates forskolin-induced cAMP production. Primary cortical astrocytes were treated with DHT-BSA (0.01, 0.1, 1, 10 μM) and/or forskolin (30 μM) for 20 min. Following treatment, the cells were lysed and assessed for the levels of cAMP. The data are presented as a percentage of the untreated control. The data are representative of four independent experiments. *$p<0.001$ vs. untreated control; %$p<0.01$ vs. forskolin alone; #$p<0.05$ vs. forskolin alone. Data are expressed as the mean $\pm$ S.E.M.

Figure 2.

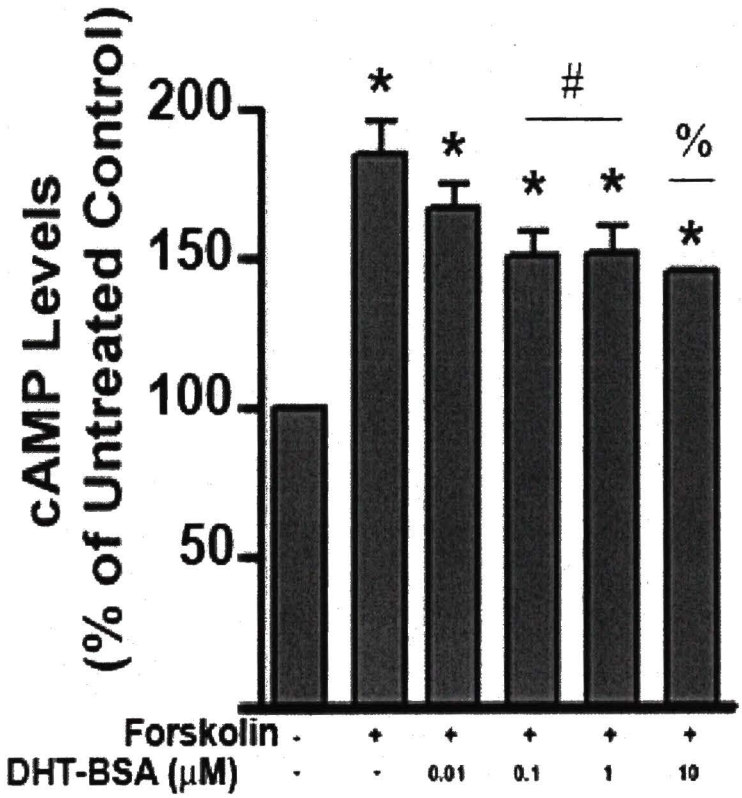

cAMP Levels
(% of Untreated Control)
200
150
100
50
*
*
*
*
*
#
%
Forskolin - + + + + +
DHT-BSA (μM) - - 0.01 0.1 1 10

Figure 3. PKA does not mediate the death promoting-effects of DHT-BSA. Primary cortical astrocytes were treated with IAA (10 μM), H-89 (100 nM), KT5720 (150 nM) and/or DHT-BSA (10 μM) for 12 hours. Following treatment, the media was analyzed for LDH levels as an indicator of cell death. The data are presented as a percentage of maximal LDH release. The data are representative of four independent experiments. *p<0.001 vs. the IAA-treated group and untreated control. Data are expressed as the mean ± S.E.M.

Figure 3.

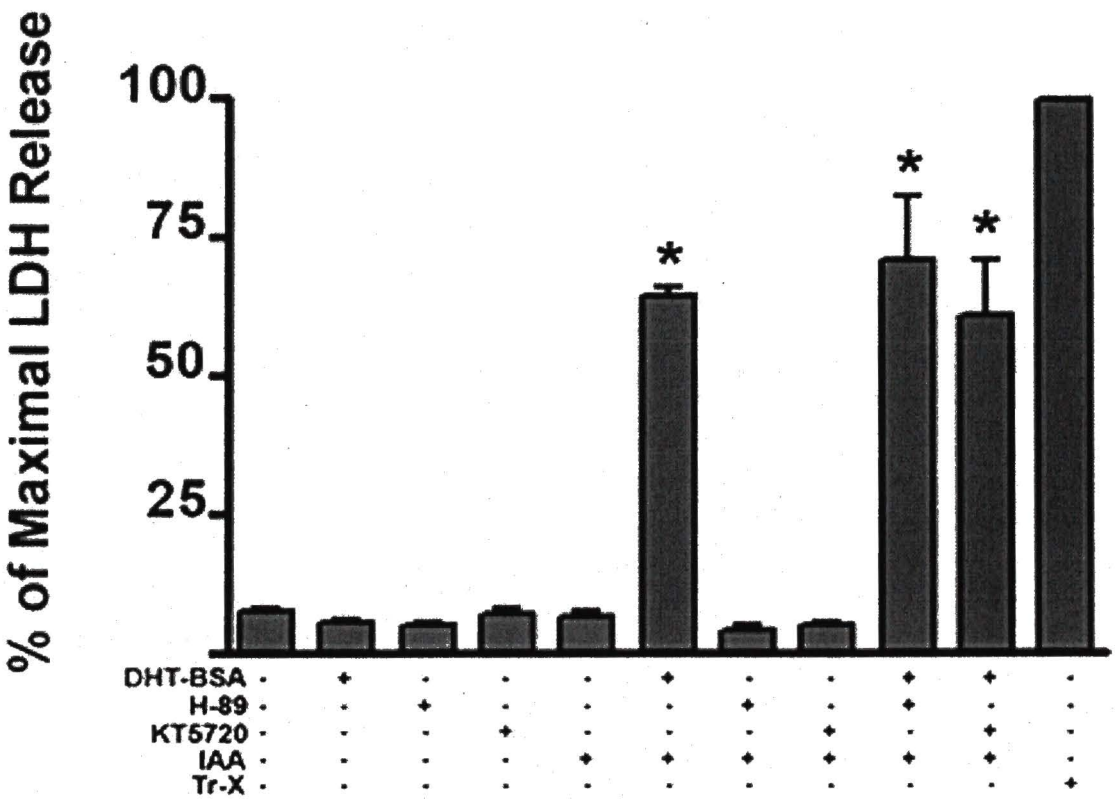
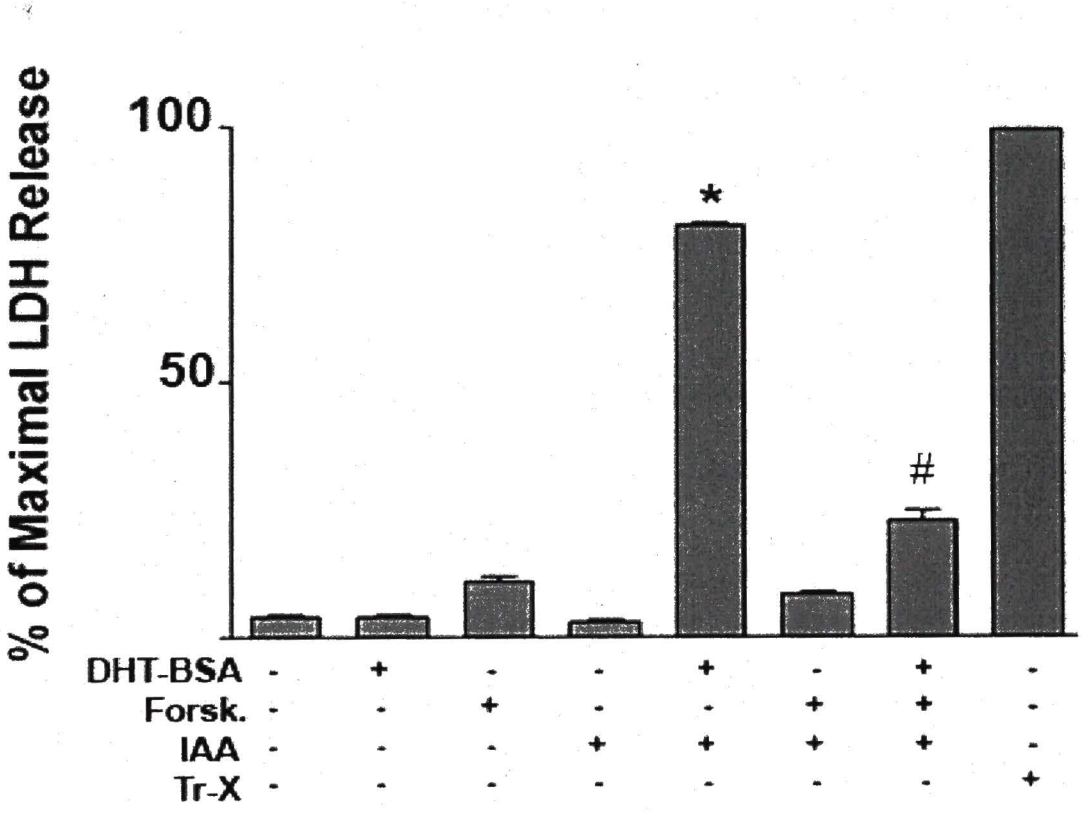


Figure 4. Forskolin fails to exacerbate IAA-induced cell death and instead protects against DHT-BSA-induced toxicity. Primary cortical astrocytes were treated with IAA (10 μM), forskolin (30 μM), and/or DHT-BSA (10 μM) for 12 hours. Following treatment, the media was analyzed for LDH levels as an indicator of cell death. The data are presented as a percentage of maximal LDH release. The data are representative of four independent experiments. *$p<0.001$ vs. IAA-treated group and untreated group. #$p<0.001$ vs. DHT-BSA + IAA. Data are expressed as the mean $\pm$ S.E.M.

Figure 4.



REFERENCES

1. **Dumont JE, Jauniaux JC, Roger PP** 1989 The cyclic AMP-mediated stimulation of cell proliferation. Trends Biochem Sci 14:67-71

2. **Blomhoff HK, Blomhoff R, Stokke T, deLange Davies C, Brevik K, Smeland EB, Funderud S, Godal T** 1988 cAMP-mediated growth inhibition of a B-lymphoid precursor cell line Reh is associated with an early transient delay in G2/M, followed by an accumulation of cells in G1. J Cell Physiol 137:583-7

3. **Mark MD, Storm DR** 1997 Coupling of epidermal growth factor (EGF) with the antiproliferative activity of cAMP induces neuronal differentiation. J Biol Chem 272:17238-44

4. **Wang L, Liu F, Adamo ML** 2001 Cyclic AMP inhibits extracellular signal-regulated kinase and phosphatidylinositol 3-kinase/Akt pathways by inhibiting Rap1. J Biol Chem 276:37242-9

5. **Dugan LL, Kim JS, Zhang Y, Bart RD, Sun Y, Holtzman DM, Gutmann DH** 1999 Differential effects of cAMP in neurons and astrocytes. Role of B-raf. J Biol Chem 274:25842-8

6. **Schmitt JM, Stork PJ** 2001 Cyclic AMP-mediated inhibition of cell growth requires the small G protein Rap1. Mol Cell Biol 21:3671-83

7. **Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ** 1997 cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell 89:73-82

8. **Qiu W, Zhuang S, von Lintig FC, Boss GR, Pilz RB** 2000 Cell type-specific regulation of B-Raf kinase by cAMP and 14-3-3 proteins. J Biol Chem 275:31921-9

9. **Mischak H, Seitz T, Janosch P, Eulitz M, Steen H, Schellerer M, Philipp A, Kolch W** 1996 Negative regulation of Raf-1 by phosphorylation of serine 621. Mol Cell Biol 16:5409-18

10. **Gatson JW, Kaur P, Singh M** 2006 Dihydrotestosterone differentially modulates the mitogen-activated protein kinase and the phosphoinositide 3-kinase/Akt pathways through the nuclear and novel membrane androgen receptor in C6 cells. Endocrinology 147:2028-34

11. **Hafner S, Adler HS, Mischak H, Janosch P, Heidecker G, Wolfman A, Pippig S, Lohse M, Ueffing M, Kolch W** 1994 Mechanism of inhibition of Raf-1 by protein kinase A. Mol Cell Biol 14:6696-703

12. **Braun AM, Thomas P** 2004 Biochemical characterization of a membrane androgen receptor in the ovary of the atlantic croaker (Micropogonias undulatus). Biol Reprod 71:146-55

13. **Hatzoglou A, Kampa M, Kogia C, Charalampopoulos I, Theodoropoulos PA, Anezinis P, Dambaki C, Papakonstanti EA, Stathopoulos EN, Stournaras C,**

Gravanis A, Castanas E 2005 Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo. J Clin Endocrinol Metab 90:893-903

14. **Papakonstanti EA, Kampa M, Castanas E, Stournaras C** 2003 A rapid, nongenomic, signaling pathway regulates the actin reorganization induced by activation of membrane testosterone receptors. Mol Endocrinol 17:870-81

15. **Somjen D, Kohen F, Gayer B, Kulik T, Knoll E, Stern N** 2004 Role of putative membrane receptors in the effect of androgens on human vascular cell growth. J Endocrinol 180:97-106

16. **Alexaki VI, Dermitzaki E, Charalampopoulos I, Kampa M, Nifli AP, Gravanis A, Margioris AN, Castanas E** 2006 Neuronal differentiation of PC12 cells abolishes the expression of membrane androgen receptors. Exp Cell Res 312:2745-56

17. **Benten WP, Lieberherr M, Giese G, Wrehlke C, Stamm O, Sekeris CE, Mossmann H, Wunderlich F** 1999 Functional testosterone receptors in plasma membranes of T cells. Faseb J 13:123-33

18. **Estrada M, Espinosa A, Muller M, Jaimovich E** 2003 Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. Endocrinology 144:3586-97

19. **Kampa M, Nifli AP, Charalampopoulos I, Alexaki VI, Theodoropoulos PA, Stathopoulos EN, Gravanis A, Castanas E** 2005 Opposing effects of estradiol-

and testosterone-membrane binding sites on T47D breast cancer cell apoptosis. Exp Cell Res 307:41-51

20. **Vicencio JM, Ibarra C, Estrada M, Chiong M, Soto D, Parra V, Diaz-Araya G, Jaimovich E, Lavandero S** 2006 Testosterone induces an intracellular calcium increase by a nongenomic mechanism in cultured rat cardiac myocytes. Endocrinology 147:1386-95

21. **Benten WP, Lieberherr M, Stamm O, Wrehlke C, Guo Z, Wunderlich F** 1999 Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. Mol Biol Cell 10:3113-23

CHAPTER VI

SUMMARY AND FUTURE DIRECTION

Summary

The main goal of my dissertation project was to elucidate the role of a novel membrane androgen receptor (mAR) in regulating cell viability. Taken as a whole, my data suggest that activation of this membrane receptor increases the vulnerability of cells to oxidative damage and metabolic insult by suppressing protective signaling pathways and turning on death signaling pathways, ultimately leading to an increase in cell death.

The data I have presented in the preceding chapters support the presence of a membrane binding site for dihydrotestosterone (DHT) and its membrane-impermeable analog, DHT-BSA. Further, the data suggest that activation of this putative membrane androgen receptor results in the suppression of the phosphorylation of ERK and Akt, two key effectors of the cytoprotection-association MAPK and PI3-K pathways, respectively. This suppression of signaling was correlated with an increase in caspase activity and cell death. Of note, treatment with the putative membrane androgen receptor activator (DHT-BSA) did not by itself promote cell death, but instead, rendered the cells more vulnerable to toxic insult. We interpreted, therefore, that the suppression of cytoprotection-associated signaling and increased caspase activation rendered the cells more vulnerable to cell death, such that in the presence of the metabolic and oxidative insult, IAA, the cells died. A working model, based on my results, is presented in Figure 1.

Though other studies have proposed the existence of a membrane androgen receptor, no such entity has been described in the brain prior to the studies described herein. We do not believe that the membrane androgen receptor described here is the "classical" intracellular androgen receptor that is simply localized to a distinct subcellular compartment (i.e., the plasma membrane) since the consequences of activating this putative membrane androgen receptor was insensitive to the classical androgen receptor antagonists, flutamide and nilutamide. Though we recognize that this is not conclusive evidence that the two receptor entities are distinct, they do appear to be, at the very least, pharmacologically distinct. For example, the stimulatory effect of DHT on ERK phosphorylation and the cell-survival-promoting effect of DHT were inhibited by flutamide, while the suppression of signaling and the promotion of cell death elicited by DHT-BSA were not. Thus, we argue that two distinct, and potentially competing androgen-stimulated pathways exist within the same cell population (glia, in this case) to regulate cell survival.

These data could explain some of the existing controversy as to whether androgens are beneficial or detrimental. For example, androgens such as testosterone and DHT have been shown to be protective as well as death-promoting. For example, testosterone protects from β-amyloid toxicity (1), but increases lesion size following stroke and reperfusion injury (2). So based on our data, we would infer that the outcome associated with androgen treatment could be quite different depending on which receptor system is dominant (membrane androgen receptor versus intracellular androgen receptor). Accordingly, an increase in the relative expression of the membrane androgen receptor as

well as persistent androgen levels with age may also render an individual more susceptible to specific insults associated with disease pathology in the brain (such as those that might cause a decrease in bioenergetics, or increase in oxidative stress). Thus, being able to define the relative ratio of the mAR versus the intracellular AR may be useful in predicting the vulnerability of an individual to the damaging effects androgens. And perhaps a more provocative theory, an increase in the expression of the membrane androgen receptor may render an individual more susceptible to certain disorders or degenerative diseases such as stroke and Alzheimer's disease. Thus, development of a screening assay to determine the relative abundance of the membrane androgen receptor and the classical receptor may help identify the appropriate population for which androgens are most likely to exert a benefit, and possibly, could serve as a surrogate marker to identify the propensity of other populations to develop certain degenerative diseases.

Thus, complete identification and characterization of this novel androgen receptor will be extremely valuable with regards to developing new tools for both the study and therapeutic targeting of the membrane androgen receptor. Such advances would include the development of antibodies and drugs (agonists and antagonists) that specifically target the membrane androgen receptor.

Given the proposed importance of the membrane androgen receptor in regulating cell viability, there may also be a potential use for drugs that target this receptor on cancer. For example, one could envision that selective mAR agonists may slow the

progression, or contribute to the eradication of cancers such as breast and prostate cancer. We already know that prostate cells have both the classical androgen receptor and a putative membrane androgen receptor. The latter is supported by the description of membrane binding sites for testosterone (3) as well as effects elicited by the membrane impermeable androgen, testosterone-BSA (T-BSA) (3). If the membrane androgen receptor in prostate cancer cells exhibits characteristics similar to what I have described for the glial membrane androgen receptor, it may be a useful addition to conventional therapy for prostate cancer (using the androgen receptor antagonist, flutamide), whose efficacy is based on reducing the proliferation of prostate cancer cells. This "theoretical" agonist of the membrane androgen receptor would therefore complement the growth arrest of the tumor elicited by flutamide by promoting the cancer cell's death.

We recognize that the data presented in Chapters 2 – 5 only scratch the surface with respect to the characterization of this novel membrane androgen receptor. As such, there are numerous important directions that the research must go to further characterize this receptor in order to lead us to new and important tools to treat specific diseases. One is to better understand the signaling pathways that mediate the death-promoting effects of activating the membrane androgen receptor. In chapter 5, we initiated this study by addressing the role of cAMP and/or PKA in the suppression of the signaling and death promoting effects of DHT-BSA. Our results appear to have disproved our hypothesis, and suggest that neither cAMP accumulation nor PKA activation was an important mediator of the effects of DHT-BSA.

Future Directions

PKC-Delta

To date, our knowledge as to the cellular mechanisms that mediate mAR-induced cell death is still incomplete. By identifying what signaling pathways are involved, we will further our knowledge of downstream mediators of the effects of androgens. The PKC pathway may be an important pathway involved in androgen-induced cell death, since it has been shown to play a role in worsening the damaging effects of oxidative and excitotoxic stressors. More specifically, the PKC-delta isoform has been shown to exacerbate damage caused by stroke and reperfusion injury (4).

Previously, PKC-delta was found to exacerbate the deleterious effects of ROS, excitotoxins, and caspase-induced program cell death during stroke and reperfusion injury. Various groups have demonstrated that a specific isoform of PKC (PKC-delta), is activated by caspase-3 (4). This increase in the activation of PKC-delta was found to be mediated by interleukin-1β converting enzyme (ICE)-like proteases (5). Following activation of PKC-delta, this kinase suppressed Akt and resulted in an increase in caspase activation and DNA damage (4). Since DHT-BSA also suppresses the Akt pathway and increases cell death in primary astrocytes, we hypothesized that the damaging effects of DHT-BSA during metabolic and oxidative stress is mediated by PKC-delta. Though incomplete at this stage, we believe we have generated sufficient preliminary data to support the involvement of PKC-delta in the DHT-BSA – mediated exacerbation of cell death. For example, we assessed the effects of rottlerin (a PKC-delta antagonist) on

DHT-BSA – induced exacerbation of IAA-induced cell death in primary cortical astrocytes. Results from these experiments showed that rottlerin blocks the damaging effects of DHT-BSA (Fig. 4), whereas a PKC-α and PKC-β1 blocker, GO6976, had no effect (Fig. 3). In addition, bisindolylamide, which blocks the α, β, γ, δ and ε isoforms at different affinities, also had no effect (Fig. 2), indicating that the effects of DHT-BSA may be mediated specifically by the PKC-delta isoform. As a future direction, silencing RNA will be used to specifically decrease PKC-delta protein levels. By silencing PKC-delta, we will be able to determine if this particular isoform is an important mediator of androgen-induced toxicity.

ERK Pathway

In addition to exploration of the PKC pathway, we assessed the consequence of inhibiting ERK signaling on the death promoting effects of DHT-BSA. In previous studies it has been shown that depending on the pattern of ERK activation, there may be an increase or decrease in the survival rate. A rapid onset (minutes) and sustained (hours) activation of ERK was found to be important for cell survival during injury. Whereas a protracted duration of activation is coincident with cell death (6-8). By influencing the activity of this pathway, steroid hormones such as estrogen, progesterone, and testosterone/DHT have been shown to influence cell growth, differentiation, or survival (1, 9-12).

In this preliminary study, we found that inhibition of MEK1/2 (upstream activator of ERK) with UO126 blocked the effect of DHT-BSA on cell death in C6 cells, indicating that ERK may also be involved in the mAR death-promoting pathway (Figure 5). We initially predicted that pharmacological inhibition of ERK would enhance the effects of DHT-BSA on IAA-induced toxicity, so the protective effects of UO126 were not expected. However, others have demonstrated that UO126 is also cytoprotective in the C6 cells. Alternatively, the Singh lab has found evidence for reciprocal inhibition between the ERK/MAPK pathway and the PI3-K/Akt pathway such that inhibition of the ERK signaling pathway can enhance the stimulus-induced Akt phosphorylation. Thus, UO126 treatment may have enhanced the activity of the well-known survival-promoting Akt signaling pathway. To further characterize the ERK pathway during injury, the Singh laboratory will use Western analysis to determine the ERK kinetics in the presence of IAA and DHT-BSA at various time points.

Other CNS Models

Another important direction that this research needs to go is to determine if the effects of activating the mAR has similar consequences in neurons as we have now characterized in glia. The data I generated here were all obtained from either the glial cell model, C6 glioma cells, or from primary cortical astrocytes. I chose to study the biology of androgens in glia for two primary reasons. First, I recognized that glia far out number neurons, and thus, I simplistically argue that the predominance of glia in the brain may

underscore its importance. Second, astroglia have several important regulatory roles. For example, glia buffer potential neurotoxins such as high levels of glutamate and β-amyloid. In addition, glia provide structure to the neurons, as well as providing important trophic support (i.e., they synthesize and release neurotrophic factors, such as NGF and BDNF) (13-16). In a preliminary study, I found that DHT-BSA also exacerbates IAA-toxicity in cerebral cortical explants (slice cultures that are comprised of both neurons and glia) (Fig. 6). This preliminary study suggests that this phenomenon is not only seen in dissociated cultures glial cultures, but is also seen in the intact brain. By extending this research and studying the role of the mAR in stroke and traumatic brain injury models, we can determine if the mAR mediates androgen-induced cell death following injury. As an extension of this project, the Singh laboratory will determine if the mAR is present in primary neurons and if activation of this receptor during injury, increases cell death.

Conclusion

In conclusion, I have characterized 2 pathways through which androgens can regulate cell survival in the presence of metabolic and oxidative stressors. In the first pathway, activation of the nuclear AR through an intracellular pathway, elicits protective signaling and protects against various insults. In contrast, activation of a mAR results in a decrease in the levels of ERK and Akt and a correlative increase in caspase activity, TUNEL staining and cell death (Fig. 1). These results support the existence of two, potentially competing pathways that may dictate whether androgens are beneficial or

damage producing. As such, my data may help explain why there is a discrepancy as to whether androgens are "good" or "bad". While these studies provide an important step toward better understanding hormone neurobiology, I recognize that more studies are necessary to fully characterize this system and to determine the role of the mAR in diseases such as stroke and Alzheimer's disease. Ultimately, these studies will help in the discovery of therapeutic treatments for a variety of disorders.

Figure 1. Proposed model of DHT and DHT-BSA action in astrocytes in the presence of metabolic and oxidative insults. In the presence of a metabolic and oxidative insult (IAA), DHT protects, whereas DHT-BSA suppresses Akt signaling, resulting in an increase in caspase signaling, DNA damage, and cell death.

Figure 1.

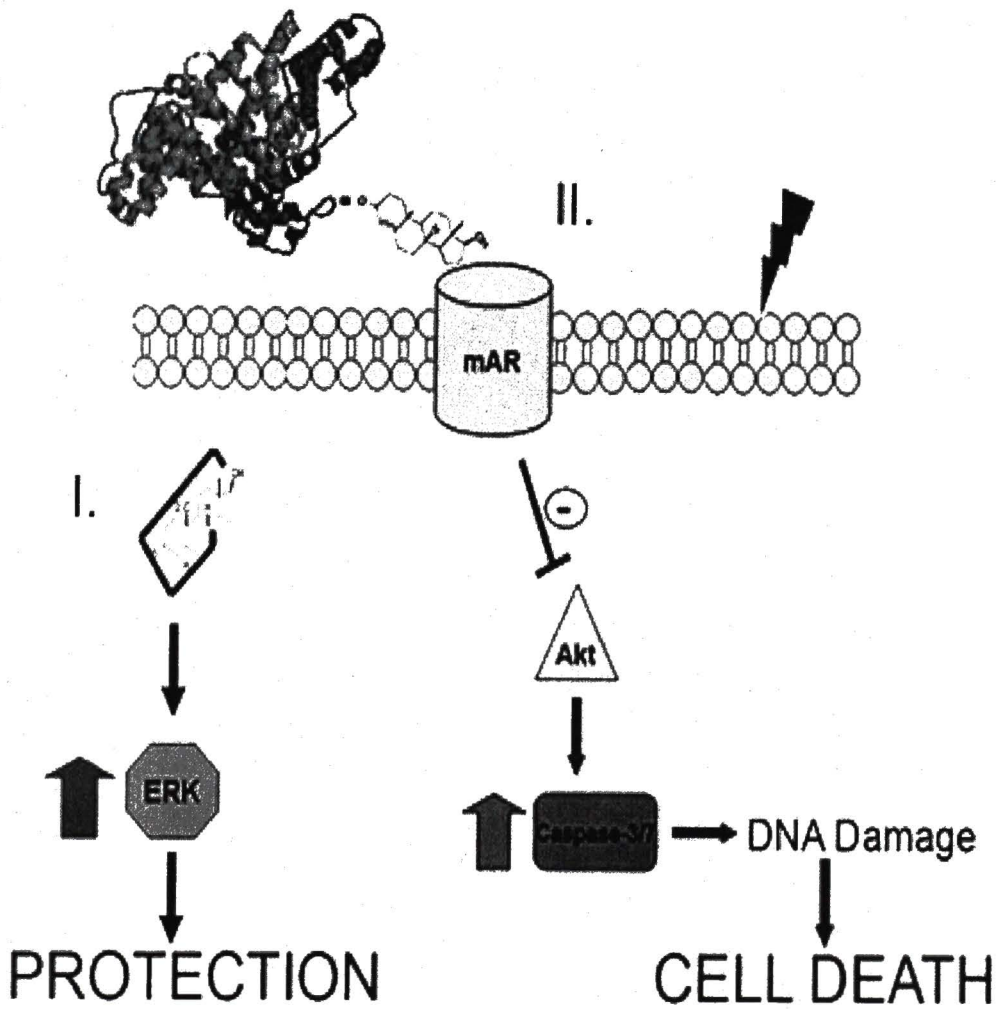


Figure 2. Bisindolylmaleimide does not block DHT-BSA-induced toxicity in astrocytes. Primary astro-glia were treated with IAA (10 μM) in the absence/presence of bisindolylamide (10 μM) and/or DHT-BSA (10 μM) for 12 hours. Following treatment, the media was analyzed for LDH levels as an indicator of cell death. The data are presented as a percentage of maximal LDH release. The data are representative of four independent experiments. $*P<0.001$ was compared to the IAA-treated group and untreated group. Data are expressed as the mean $\pm$ S.E.M.

Figure 2.

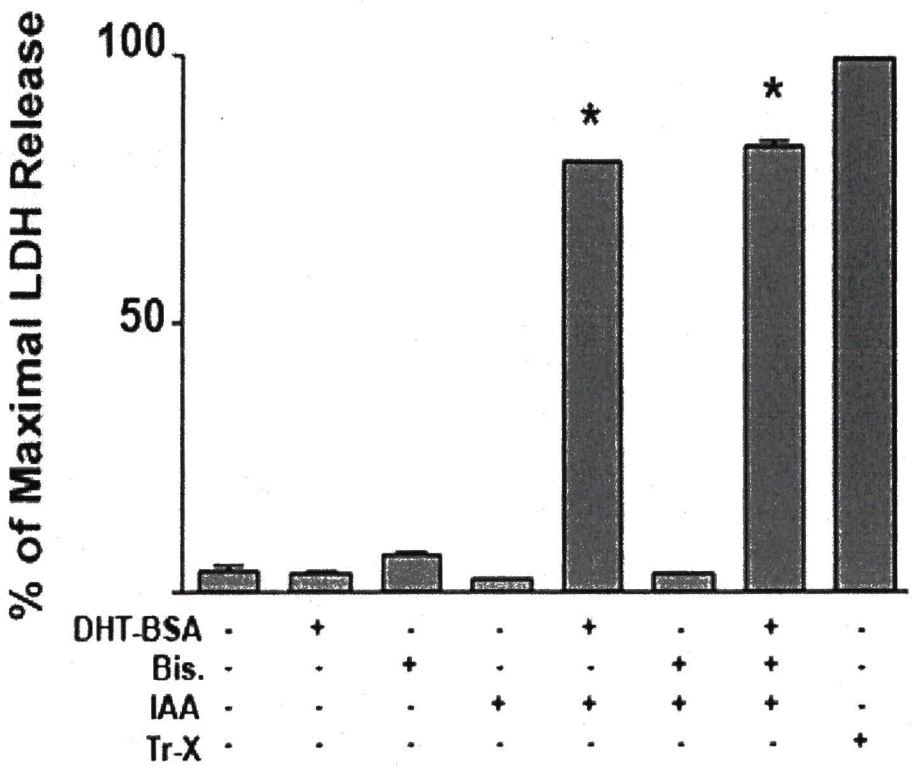


Figure 3. PKC-α and β does not mediate the damaging effects of DHT-BSA in the presence of IAA. The primary astroglia were treated with IAA (10 μM) in the absence/presence of GO6976 (30 nM) and/or DHT-BSA (10 μM) for 12 hours. Following treatment, the media was analyzed for LDH levels as an indicator of cell death. The data are presented as a percentage of maximal LDH release. The data are representative of four independent experiments. *P<0.001 was compared to the IAA-treated group and untreated group. Data are expressed as the mean ± S.E.M.

Figure 3.

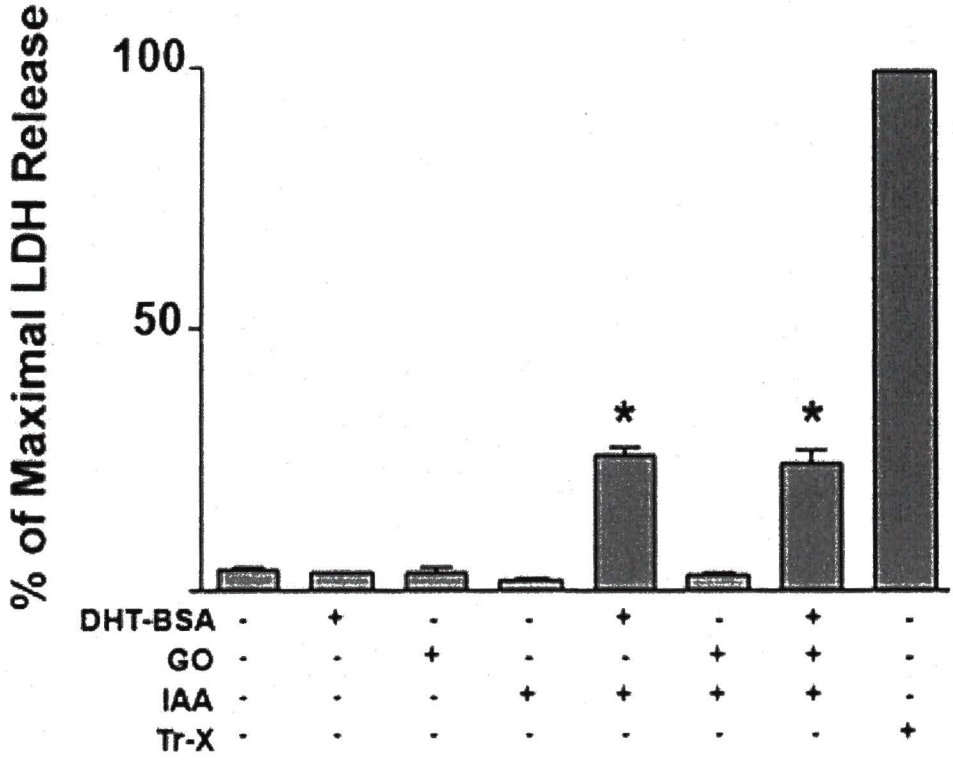


Figure 4. The damaging effects of DHT-BSA is mediated by PKC-delta. The primary astro-glia were treated with IAA (10 μM) in the absence/presence of rottlerin (10 μM) and/or DHT-BSA (10 μM) for 12 hours. Following treatment, the media was analyzed for LDH levels as an indicator of cell death. The data are presented as a percentage of maximal LDH release. The data are representative of four independent experiments. *P<0.001 vs. IAA-treated group and untreated group and #p<0.001 vs. DHT-BSA + IAA. Data are expressed as the mean ± S.E.M.

Figure 4.

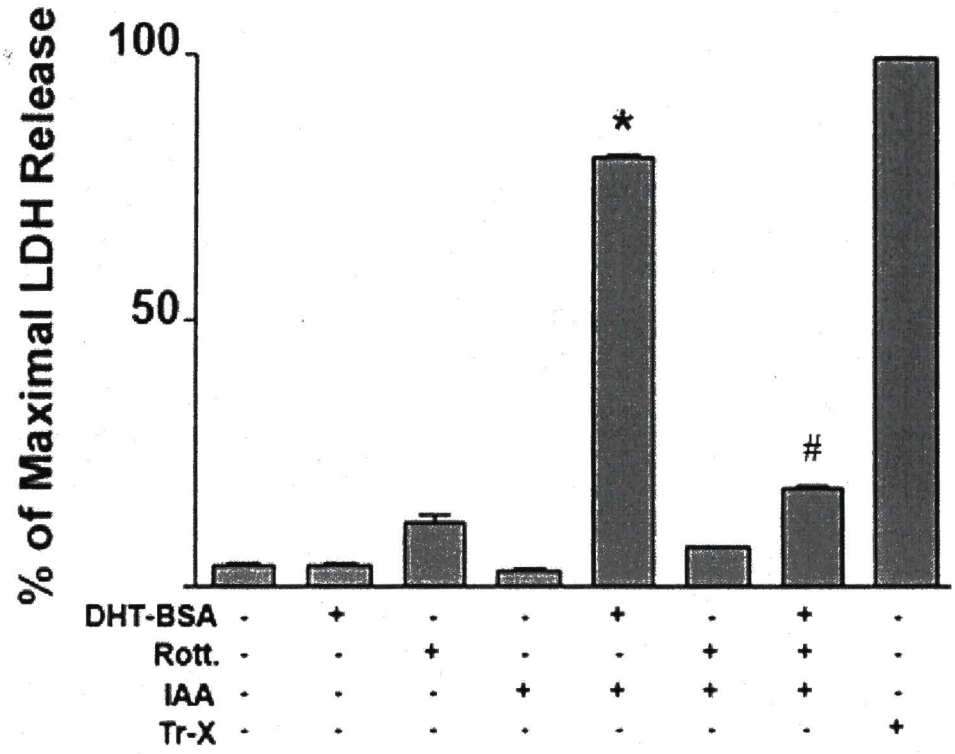


Figure 5. UO126 blocks DHT-BSA-induced toxicity in C6 cells. C6 cells were treated with IAA (10 μM) for 12 hrs in the presence/absence of DHT-BSA (10 μM) and UO126 (10 μM). Following treatment, the culture media was analyzed for the release of LDH as an indicator of cell death. The data are presented as a percentage of maximal LDH release.

Figure 5.

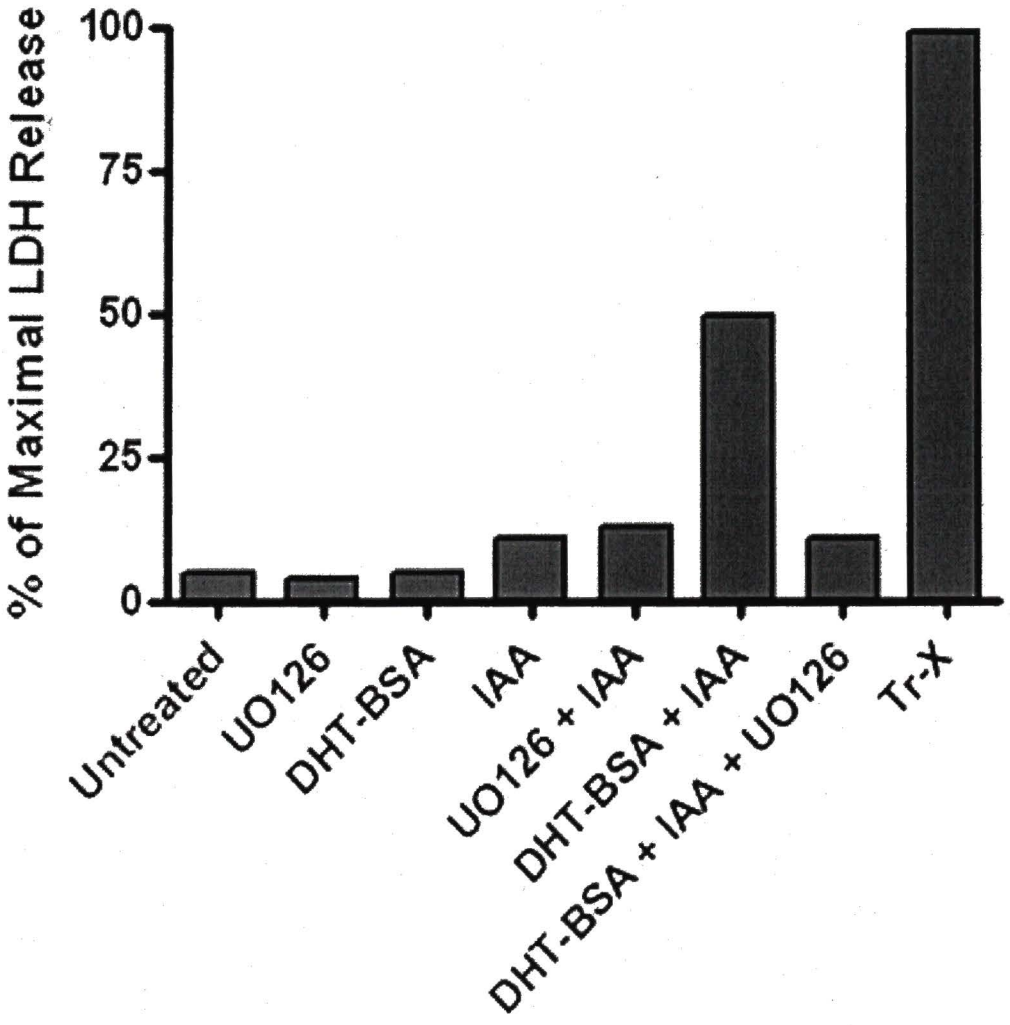
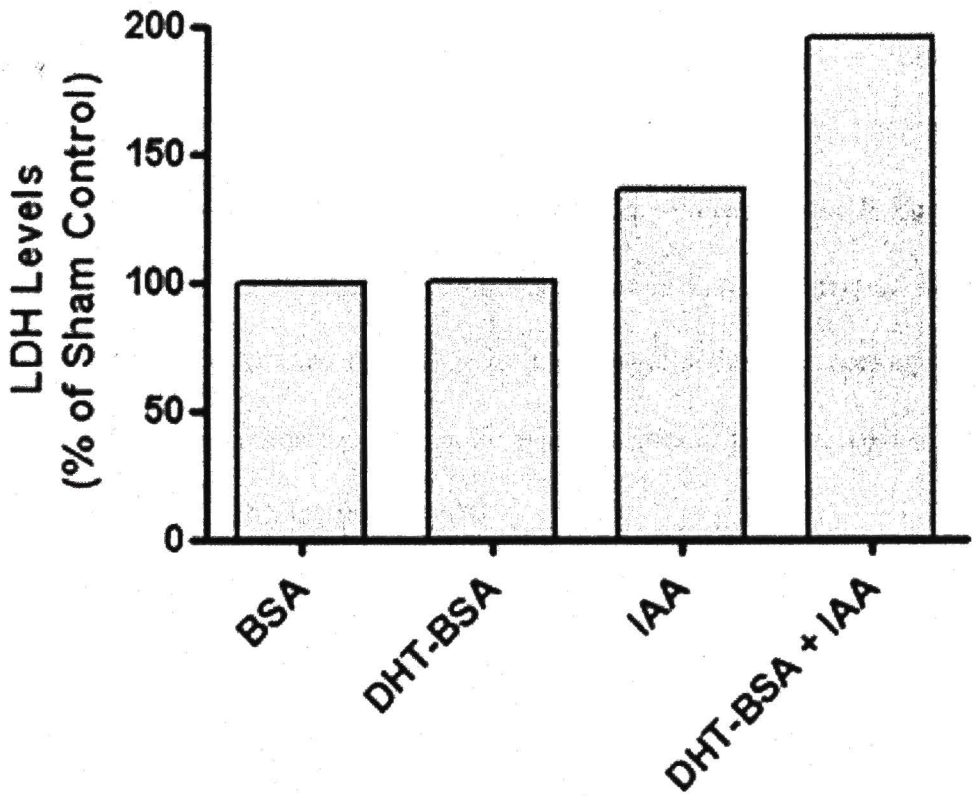

% of Maximal LDH Release
100
75
50
25
0
Untreated
UO126
DHT-BSA
IAA
UO126 + IAA
DHT-BSA + IAA
DHT-BSA + IAA + UO126
Tr-X

Figure 6. DHT-BSA enhances IAA toxicity in cerebral cortical explants. Cerebral cortical explants were treated with IAA (10 μM) for 12 hrs in the presence/absence of DHT-BSA (10 μM). Following treatment, the culture media was analyzed for the release of LDH as an indicator of cell death. The data are presented as a percentage of the sham-treated control.

Figure 6.


LDH Levels
(% of Sham Control)
200
150
100
50
0
BSA
DHT-BSA
IAA
DHT-BSA + IAA

REFERENCES

1. **Nguyen TV, Yao M, Pike CJ** 2005 Androgens activate mitogen-activated protein kinase signaling: Role in neuroprotection. J Neurochem 94:1639-51

2. **Yang SH, Perez E, Cutright J, Liu R, He Z, Day AL, Simpkins JW** 2002 Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model. J Appl Physiol 92:195-201

3. **Hatzoglou A, Kampa M, Kogia C, Charalampopoulos I, Theodoropoulos PA, Anezinis P, Dambaki C, Papakonstanti EA, Stathopoulos EN, Stournaras C, Gravanis A, Castanas E** 2005 Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo. J Clin Endocrinol Metab 90:893-903

4. **Bright R, Mochly-Rosen D** 2005 The role of protein kinase C in cerebral ischemic and reperfusion injury. Stroke 36:2781-90

5. **Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbanda S, Robertson M, Ghayur T, Wong WW, Kamen R, Weichselbaum R, Kufe D** 1995 Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells. Embo J 14:6148-56

6. **Marshall C** 1995 Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179-85

7. **Stanciu M, Wang Y, Kentor R, Burke N, Watkins S, Kress G, Reynolds I, Klann E, Angiolieri MR, Johnson JW, DeFranco DB** 2000 Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. J Biol Chem 275:12200-6

8. **Stanciu M, DeFranco DB** 2002 Prolonged nuclear retention of activated extracellular signal-regulated protein kinase promotes cell death generated by oxidative toxicity or proteasome inhibition in a neuronal cell line. J Biol Chem 277:4010-7.

9. **Singh M** 2001 Ovarian hormones elicit phosphorylation of Akt and extracellular-signal regulated kinase in explants of the cerebral cortex. Endocrine 14:407-15.

10. **Gatson JW, Kaur P, Singh M** 2006 Dihydrotestosterone differentially modulates the mitogen-activated protein kinase and the phosphoinositide 3-kinase/Akt pathways through the nuclear and novel membrane androgen receptor in C6 cells. Endocrinology 147:2028-34

11. **Singh M, Setalo G, Jr., Guan X, Warren M, Toran-Allerand CD** 1999 Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. J Neurosci 19:1179-88

12. **Peterziel H, Mink S, Schonert A, Becker M, Klocker H, Cato AC** 1999 Rapid signalling by androgen receptor in prostate cancer cells. Oncogene 18:6322-9

13. **Anderson CM, Swanson RA** 2000 Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32:1-14

14. **Aschner M, Sonnewald U, Tan KH** 2002 Astrocyte modulation of neurotoxic injury. Brain Pathol 12:475-81

15. **Nagele RG, Wegiel J, Venkataraman V, Imaki H, Wang KC** 2004 Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. Neurobiol Aging 25:663-74

16. **Roth AD, Ramirez G, Alarcon R, Von Bernhardi R** 2005 Oligodendrocytes damage in Alzheimer's disease: beta amyloid toxicity and inflammation. Biol Res 38:381-7

CHAPTER VII

APPENDIX

Figure 1. PTX does not block DHT-BSA-induced suppression of ERK. C6 cells were treated with DHT (10 nM) or DHT-BSA (0.01 to 10 μM) for 30 min. To block Gαi/o signaling, the cells were pretreated with pertussis toxin (PTX; 1μg/ml) for 18 hours. In this study, PTX failed to block DHT-BSA-induced suppression of ERK. In Addition, at all of the concentrations of DHT-BSA (0.01 to 10 μM), PTX treatment caused a greater decrease in the levels of ERK phosphorylation. These results suggest that Gαi/o does not mediate DHT-BSA-induced suppression of ERK. The bar graph represents the densitometric analysis from immunoblot data from one independent experiment, and is presented as signal intensity relative to that seen in the vehicle control.

Figure 1.

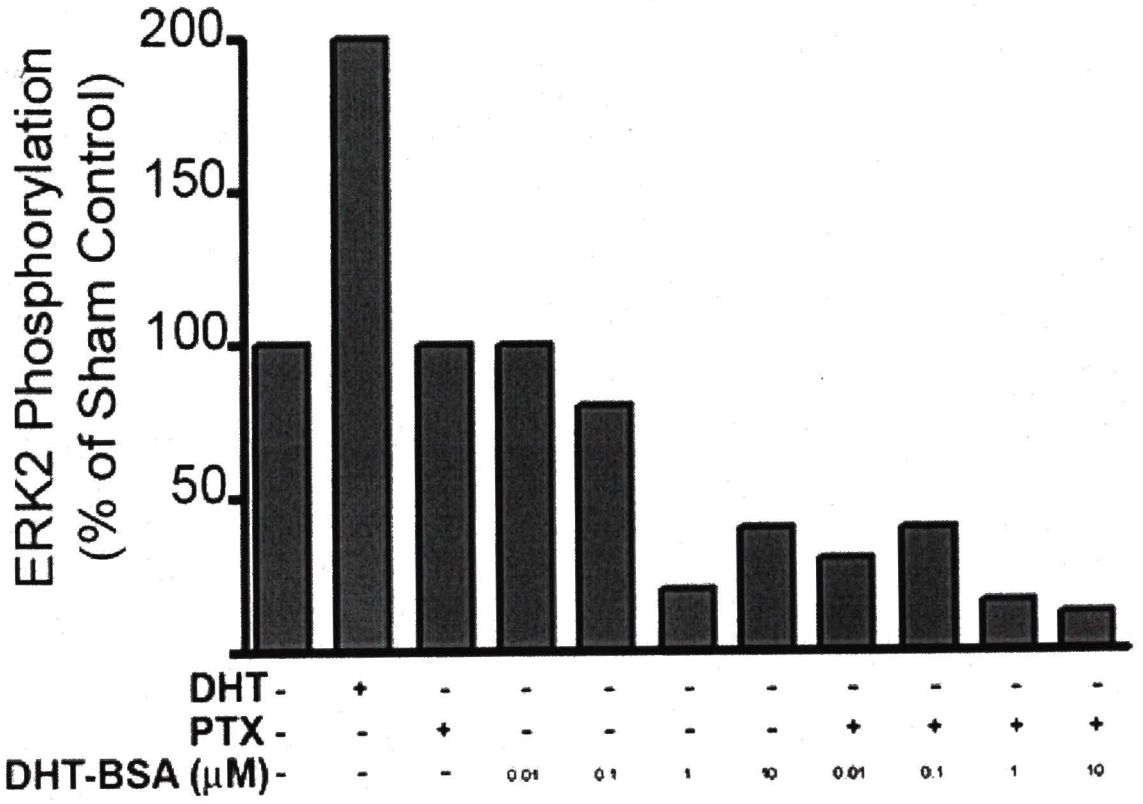

ERK2 Phosphorylation
(% of Sham Control)
200
150
100
50
DHT - + - - - - - - - - -
PTX - - + - - - - + + + +
DHT-BSA (μM) - - - 0.01 0.1 1 10 0.01 0.1 1 10

Figure 2. The effects of DHT-BSA on survival is not PTX sensitive. Primary cortical astrocytes were treated with IAA (10 μM) for 12 hrs in the presence/absence of DHT-BSA (10 μM). To determine if the membrane is coupled to Gαi/o, the cells were pretreated with pertussis toxin (PTX; 1 μg/ml) for 6 hours prior to treatment. Following treatment, the culture media was analyzed for the release of LDH as an indicator of cell death. From these studies it was determined that PTX does not block DHT-BSA-induced cell death, indicating that Gαi/o may not be involved in the damaging effects of DHT-BSA. The data are presented as a percentage of maximal LDH release, as defined by treatment of the cultures with Triton X-100 (Tr-X). The data are representative of four independent experiments. *$p<0.001$ vs. IAA treated group and untreated control. Data are expressed as the mean ± S.E.M.

Figure 2.