Androgen Receptor Mediated Activity In The Ovary: Implications For Polycystic Ovary Syndrome.

Theresa E. Hickey

BA (Science)
Masters Qualifying Year

Research Centre For Reproductive Health
Department of Obstetrics and Gynaecology
Faculty of Health Sciences
University of Adelaide
The Queen Elizabeth Hospital
South Australia

Thesis submitted for the degree of *Doctor of Philosophy in Medical Science*

January 2006

TABLE OF CONTENTS

THESIS ABSTRACT

Context

The expression of androgen receptors (AR) in follicular granulosa cells (GC) of mammals suggests a role for direct AR-mediated androgen activity in the regulation of folliculogenesis, however this role and the mechanistic pathways involved have not been fully characterised. In women, excess androgen is a characteristic feature of polycystic ovary syndrome (PCOS), but AR-mediated activity has not been widely investigated in relation to the pathophysiology of this disorder.

Hypotheses

The current thesis tested two general hypotheses related to AR activity in PCOS: 1) The polymorphic (CAG)n repeat region in the AR gene, which has functional implications for receptor activity, influences the manifestation of PCOS and 2) AR signalling is disrupted in GC from women with PCOS.

Results

In a cross-sectional population analysis, this thesis reports an association between PCOS and long CAG repeat tracts in the AR gene, which functionally represent reduced androgen sensitivity. The association was further enhanced by compensating for the influence of X chromosome inactivation (XCI) on expression of specific AR alleles. Preferential expression of long CAG repeat tracts positively correlated with serum testosterone levels in PCOS patients. In an analysis of sister pairs with the same CAG repeat genotype at the AR locus, different patterns of XCI were evident when sisters had a different clinical manifestation of PCOS. Collectively, these results provide evidence that supports the hypothesis that the (CAG)n polymorphism in the AR influences the manifestation of PCOS, the effects of which are modulated by variable allele expression via a mechanism involving XCI. These findings accord with the concept that both genetic and environmental factors are determinants of this disorder.

At the level of the ovary, AR-mediated signalling in follicular GC was influenced by proximity to the oocyte in both pigs and humans. In particular, the ability of androgen to directly induce porcine GC proliferation in vitro was dependent upon presence of the oocyte or the oocyte mitogen, growth differentiation 9 (GDF9). This finding provides a potential mechanism to explain how androgens may enhance early follicle growth. Granulosa cells from women with PCOS had normal mRNA expression for AR signalling molecules, but GC surrounding the oocyte in vivo had reduced AR protein content and diminished responses to androgen in culture as compared to those from normal ovaries. GC from women with PCOS also expressed mRNA for an androgen-regulated serine protease (hKLK3), which did not occur in normal GC. Therefore, follicular GC from women with PCOS have evidence of perturbed AR-mediated signalling which is likely to contribute to the pathophysiology of this disorder. As AR-mediated signalling is influenced by the oocyte, the differences in AR-mediated signalling in GC from women with PCOS may be indicative of dysregulated signals emanating from the oocyte.

Conclusion

The results of this thesis indicate that abnormal AR action occurs in PCOS, but further investigation is required to determine whether this phenomenon represents a primary disruption or a secondary consequence of another primary disruption in the sequence of events that leads to aberrant folliculogenesis in this disorder.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made.

I give consent for this thesis, when deposited in the University of Adelaide library, to be available for photocopying and loan.

The research contained within this thesis was funded in part by the following sources: NH&MRC Program Grant, Australian Research Council, University of Adelaide Small Research Grant Scheme, and the Canadian Institutes for Health Research. To my knowledge, there are no intellectual property issues or conflicts of interest with other persons or organisations with respect to the data presented in this thesis.

Theresa E. Hickey

January 2006

ACKNOWLEDGEMENTS

Thank you to Professor Robert Norman, my principle supervisor, who encouraged me to pursue a higher degree and provided financial support as well as understanding of family issues, which enabled me to do so. Rob's belief in my capabilities, which he frequently voiced, provided the necessary boosts to my self-esteem that kept me going. I feel enormously grateful to all the various staff and students in the Department of Obstetrics and Gynaecology at the University of Adelaide, particularly those located at The Queen Elizabeth Hospital, who I have had the pleasure to be associated with and work (and/or have a cup of tea) beside over the years. For the most part, it has been fun working here because of all of you. In particular, I would like to thank Professor Dave Armstrong, Dr. Robert Gilchrist, Dr. Rebecca Robker, and Dr. Megan Mitchell for their collaborations and the many stimulating conversations we have had. I acknowledge Lesley Ritter as a friend and admirable work colleague, who has participated in the execution of some of the work presented in this thesis and has listened to me complain and crow over experimental data, manuscript preparations, the fact of being a PhD student, and life in general. She is a true model of laboratory skill and organisation. Thanks also to Clyde Milner, another friend and colleague, whose assistance in all things computational I highly valued. I acknowledge the contribution of others in separate chapters of this thesis, and I apologise to anyone I may have missed. In addition, I'd like to say thanks to the staff at Repromed who have assisted in recruiting patients, collecting samples, and gathering information for this study. In particular, I am grateful to the efforts of Helen Alvino and Regan Jeffries, who have been there all the way through. Thanks too to all of those patients who agreed to participate!

Outside of the academic arena, I'd like to acknowledge my children, who had and will continue to have less of me in their lives as a result of this study: I'm sorry for all the things I've missed and hope that in terms of the B*ig Picture*, it has been for the best. I am also

grateful to my partner Kingsley, who does many of the domestic duties in our house, including the cooking of dinner. It is wonderful to come home after a long day and have a delicious meal already prepared. He also stays at home when I travel to conferences, often taking leave from his work to do so. To my family overseas, thanks for your enthusiasm for what I do even though you don’t understand it! I acknowledge the kind support, enthusiasm, and intellectual stimulation I get from the Intersections group: Dr. Pamela Lyon, Dr. David Scrimgeour, and Dr. Reginald Cahill. More recently, El Group (Di Sutton, Lesley Jenkins, and Anne Jurisevic) has provided another avenue of personal development that I find fascinating and very useful.

Finally, I thank the University of Adelaide for scholarship funding and acknowledge funds from the NHMRC Program Grant and funds from the University of Adelaide Small Research Grant Scheme, which supported this work. Lesley Jenkins compiled the abbreviations list and Dr. Sarah List aided in figure formatting.

PUBLICATIONS ARISING FROM THIS THESIS

2005

Hickey TE, Marrocco DL, Amato F, Ritter LJ, Norman RJ, Gilchrist RB, and Armstrong DT. Androgens augment the mitogenic effects of oocyte-secreted factors and growth differentiation factor 9 on porcine granulosa cells. **Biology of Reproduction** 73 (4):825-32, 2005.

Hickey TE, Legro RS, and Norman RJ. Epigenetic modification of the X chromosome influences susceptibility to polycystic ovary syndrome. **Journal of Clinical Endocrinology and Metabolism** (submitted 12 January 2006)

2004

Hickey TE, Marrocco DL, Gilchrist RB, Norman RJ, and Armstrong DT. Interactions between androgen and growth factors in granulosa cell subtypes of porcine antral follicles. **Biology of Reproduction** 71 (1): 45-52, 2004.

Norman RJ, **Hickey T**, Moran L, Boyle J, Wang J, and Davies M. Polycystic Ovary Syndrome - Diagnosis and Etiology. In: Daya SH and Kempers R, editors. **Advances in Fertility and Reproductive Medicine**. Montreal: Elsevier, 2004:225-32, 2004.

2002

Hickey TE, Chandy A, Norman RJ. The androgen receptor CAG repeat polymorphism and X-chromosome inactivation in Australian Caucasian women with infertility related to polycystic ovary syndrome**. J Clinical Endocrinology & Metabolism** 87(1): 161-165, 2002.

Hickey, TE, Norman, RJ. Polycystic ovary syndrome (PCOS): Genes and Environment. **Human Reproduction, II Edition**, Chapter 20:161-169, 2002. Eds. J. Remohi, A. Pellicer, C. Simon, J. Navarro.

Hickey TE, Norman RJ. Molecular genetics of polycystic ovary syndrome. **Journal of Reproductive Medicine** 8(2): 165-185, 2002.

ABSTRACTS ARISING FROM THIS THESIS

2005

Hickey TE, Alvino HJ, Gilchrist RB, and Norman RJ. Granulosa cells from polycystic ovaries have increased expression of androgen receptor regulated kallikrein 3, but normal expression of androgen receptor and two androgen receptor co-activators. Proc 87th Annual Meeting of the Endocrine Society, San Diego, CA, USA. Abstract # P2-242, pg 415. **(Winner, Australian Women in Endocrinology Award)**

Hickey TE, Milner CR, Sheetz B, Legro RS, and Norman RJ. Differential patterns of X-inactivation among sisters in family groups with polycystic ovary syndrome. Annual Meeting of the Androgen Excess Society, San Diego, CA, USA. **(Winner, New Investigator Award)**

Hickey TE, Marrocco DL, Amato F, Ritter LJ, Norman RJ, Gilchrist RB, and Armstrong DT. Androgens augment the mitogenic effects of oocyte-secreted factors and growth differentiation factor 9 on porcine granulosa cells. Annual Meeting of the Society for Reproductive Biology, Perth, Australia. **(Winner, New Investigator Award)**

Hickey TE, Milner CR, Sheetz B, Legro RS, and Norman RJ. Differential patterns of X-inactivation among sisters in family groups with polycystic ovary syndrome. 48th Annual Meeting of the Endocrine Society of Australia, Perth, Australia.

(Finalist, New Investigator Award)

Hickey TE, Alvino HJ, Gilchrist RB, and Norman RJ. Androgen receptor mediated activity in ovarian granulosa cells: Implications for polycystic ovary syndrome. Annual Meeting of the Australian Society for Medical Research, SA division, Adelaide.

Hickey T, Alvino H, Gilchrist R, and Norman, R. Androgen receptor associated signalling in ovarian granulosa cells from normal and polycystic ovaries. National Meeting of the Australian Society for Medical Research, Couran Cove, Queensland.

Hickey TE, Milner CR, Sheetz B, Legro RS, and Norman RJ. Genotype and epigenotype in Polycystic Ovary Syndrome: Evidence for a causal gene on the X chromosome. The Queen Elizabeth Hospital Research Day, Adelaide, Australia. **(Winner: Best Presentation; PhD student category)**

2004

Hickey TE, Alvino HJ, and Norman RJ. Expression of prostate specific antigen by ovarian granulosa cells. Proc 47th Annual Meeting of the Endocrine Society of Australia, Sydney. Abstract #129, pg 99.

Hickey TE, and Norman RJ. Expression of prostate specific antigen in the ovary. Annual Meeting of the Australian Society for Medical Research, SA division, Adelaide.

Hickey TE, Alvino HJ, Gilchrist RB, and Norman RJ. Androgen receptor mediated activity in ovarian granulosa cells: Implications for polycystic ovary syndrome. The Queen Elizabeth Hospital Research Day, Adelaide. Poster prize.

Armstrong DT, Marrocco DL, **Hickey TE**, Ritvos O, and Gilchrist RB. Growth and differentiation factor-9 (GDF-9) enhances mitogenic responses of pig granulosa cells to IGF-1 and inhibits steroidogenic responses to FSH. Proc 37th Annual Meeting of the Society for the Study of Reproduction, Vancouver, Canada**. Biology of Reproduction** (Special Supplement) Abstract # 763, pg 267.

Armstrong DT, Marrocco DL, **Hickey TE**, Gilchrist RB, and Norman RJ. Oocyte-secreted factors(s) augment mitogenic responses of porcine granulosa cells to insulin-like growth factor-1 and 5-α-dihydrotestosterone. Proc 86th Annual Meeting of the Endocrine Society, New Orleans, USA. Abstract # P3-87, pg 485.

2003

Hickey TE, and Norman, RJ. Expression of androgen receptor and enhancement of FSH signaling by dihydrotestosterone in a granulosa tumour cell line. Proc. 46th Annual Meeting of the Endocrine Society of Australia, Melbourne. Abstract # 169, pg 120.

Hickey TE, Nishi Y, Yanase T, Nawata H, Norman RJ. KGN: A new model for studying androgen activity in human ovarian granulosa cells. Annual Meeting of the Australian Society for Medical Research, SA division, Adelaide.

2002

Hickey TE, Gilchrist RB, Ritter LJ, Norman RJ, Armstrong DT. In vitro effects of 5-alpha-dihydrotestosterone on granulosa cells from gilts: differential response between mural granulosa cells and cumulus-oocyte complexes. Proc 36th Annual Meeting of the Society for the Study of Reproduction, Baltimore, USA. *Biology of Reproduction* 66 (Suppl 1): Abstract #11, pg. 102.

Hickey TE, Marrocco DL, Gilchrist RB, Ritter LJ, Norman RJ and Armstrong DT. Differential androgen response between mural and cumulus granulosa cells in the pig ovary. Proc 42nd National Scientific Conference of the Australian Society for Medical Research, Adelaide. Abstract # 11, pg 30.

2001

Hickey TE, Chandy A, Norman RJ, Androgen receptor CAG repeat polymorphism and X-chromosome inactivation in Australian Caucasian women with polycystic ovaries. Proc 83rd Annual Meeting of the Endocrine Society, Denver CO, USA. Abstract # 0R19-3, pg 102.

1999

Hickey TE, and Norman RJ. Variation of polymorphic trinucleotide repeat in the androgen receptor in Caucasian women with polycystic ovary syndrome. 42nd Annual Scientific Meeting of the Endocrine Society of Australia, Melbourne. Abstract #63, pg 91.

ABBREVIATIONS

A4	Androstenedione
AMH	Anti-Mullerian Hormone
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
AR	Androgen Receptor
ARA	Androgen Receptor Associated protein
ARE	Androgen Response Element
ASRM	American Society for Reproductive Medicine
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
B-TCM	Bicarbonate-buffered Tissue Culture Medium
bFGF	Basic Fibroblast Growth Factor
BMI	Body Mass Index
BMP	Bone Morphogenetic Protein
C	Carbon
CAG	Cytosine Adenosine Guanine (nucleotide sequence)
cAMP	Cyclic Adenosine Monophosphate
CBP	CREB Binding Protein
CC	Cumulus Cells
CCG	Cytosine Cytosine Guanine (nucleotide sequence)
cDNA	Complementary DNA
CL	Corpus Luteum
CO_2	Carbon Dioxide
COC	Cumulus Oocyte Complex
COOH	Carboxyl group

CPM	Counts Per Minute
CREB	cAMP Response Element Binding Protein
CYP	Cytochrome P450
DBD	DNA Binding Domain
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
DO	Denuded Oocytes
DTT	Dithiothreitol
E1	Estrone
E2	Estradiol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
ERK	Extracellular Signal-Regulated Kinase
ESHRE	European Society for Human Reproduction and Embryology
FAI	Free Androgen Index
FCS	Fetal Calf Serum
FHL	Four and a half LIM domains
FSH	Follicle Stimulating Hormone
FSHR	FSH Receptor
GATA	Family of transcription factors
GC	Granulosa Cells
GDF	Growth Differentiation Factor
GnRH	Gonadotrophin Releasing Hormone
GEE	General Estimating Equation
GLC	Granulosa Lutein Cells

HA	Hyperandrogenic
hCG	Human Chorionic Gonadotrophin
HEPES	N-cyclohexyl-2-aminoethanesulfonic acid
HIC	Hydrophobic Interaction Chromatography
hKLK	Human Kallikrein
HPO	Hypothalamic Pituitary Ovarian Axis
HSD	Hydroxysteroid Dehydrogenase
HSP	Heat Shock Protein
H-TCM	HEPES-buffered Tissue Culture Medium
ICSI	Intracytoplasmic Sperm Injection
IGF	Insulin-like Growth Factor
IGFBP	IGF Binding Protein
IGFR	IGF Recptor
IHC	Immunohistochemistry
INS	Insulin gene
IRS	Insulin Receptor Substrate
IU	International Units
IVF	In Vitro Fertilization
kDa	Kilo-Daltons
KITL	Kit Ligand
LBD	Ligand Binding Domain
LDL	Low Density Lipoprotein
LH	Luteinizing Hormone
LHR	LH Receptor
LIM	A conserved cysteine-rich protein domain
MAP	Mitogen Activated Protein

MAPK	MAP Kinase
MGC	Mural Granulosa Cells
mg	Milligrams
min	Minutes
ml	Millilitres
mRNA	Messenger Ribonucleic Acid
mm	Millimetres
MWCO	Molecular Weight Cut-Off
NaCl	Sodium Chloride
ND	Not Determined
NH_2	Amino group
NICHD	National Institute of Child Health and Development
NIH	National Institutes of Health
nM	Nanomolar
OHF	OH-Flutamide (hydroxyflutamide)
OSF	Oocyte Secreted Factor(s)
P450arom	P450 Aromatase
P450scc	P450 Side chain cleavage
PAGE	Polyacrylamide Gel Electrophoresis
PBR	Peripheral-type Benzodiazepine Receptor
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PCO	Polycystic Ovaries
PCOS	Polycystic Ovary Syndrome
PDE	Phosphodiesterase
PI-3	Phospho-inosital-3

PKA	Protein Kinase A
pmol	Picomoles
PVA	Polyvinyl Alcohol
rhFSH	recombinant human FSH
rhIGF1	recombinant human IGF1
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitation Assay buffer
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
SAS	Statistical Analysis Software
SBMA	Spinobulbar Muscular Atrophy
SCF	Stem Cell Factor
SDS	Sodium Dodecylsulphate
SEM	Standard Error of the Mean
SHBG	Steroid Hormone Binding Globulin
SMAD	Mothers Against Decapentaplegic protein
SPSS	Scientific Products SYSTAT Software
SRC	Steroid Receptor Coactivator
StAR	Steroidogenic Acute Regulatory protein
T	Testosterone
TCM	Tissue Culture Medium
TET	Tetrachlorofluorescein
TGF	Transforming Growth Factor
TRAM	Thyroid Receptor Activator Molecule
TSX	Transsexual
µCi	Microcurie

ug	Micrograms
U	Units
URR	Upstream Regulatory Region
UTR	Untranslated Region
v/v	volume / volume
VNTR	Variable Nucleotide Triplicate Repeat
XCI	X Chromosome Inactivation