Addendum and Errata

Page14, line3: “dietary excesses” for “dietary excuses”

End Page 14/top page 15: “estradiol :estrone ratios result in the development of obesity related diseases.” for “are the”

Page 17, line 12: delete “s”

Page18, penultimate line: “the majority” for “majority”

Page19, line 16: delete “why”

Page 23, line 1: “and” for “a”

Page 23, line 4: delete “,”

Page 24, penultimate line: delete “shows” and “glucose”

Page 26, last line: insert “.”

Page 28, line 6: “signalling” for “signally”

Page 33-38, Chapter 1 References: Insert line between references: 7 and 8, 23 and 24, 76 and 77, 91 and 92 and 106 and 105

Page 42, Thesis Aims: Format double space, Aim 4

Page 45, line 4: “study.” for “study”

Page 55, last line: “SD.” for “SD”

Page 57, line 17: “an” for “a”

Page 58: Comment: Food intake was monitored by housing mice for 24 hours in metabolic cages. Further to the results stated in the second paragraph, the decreased consumption of kilojoules per day was in correspondence to a decreased net weight amount of food. This detail was taken out of the final version of the manuscript as suggested by a *Kidney International* reviewer.

Page 70, Table 4: “RAGE-/-” for “BAKE-/-”

Page 58 and 71: Comment:

Page 80, line 7: “increased ratio” for “increased”

Page 90, line 7: delete “(Figure 3A)” and “(Figure 3C)”

Page 90, line 9: delete “(Figures 3B & 3D)”

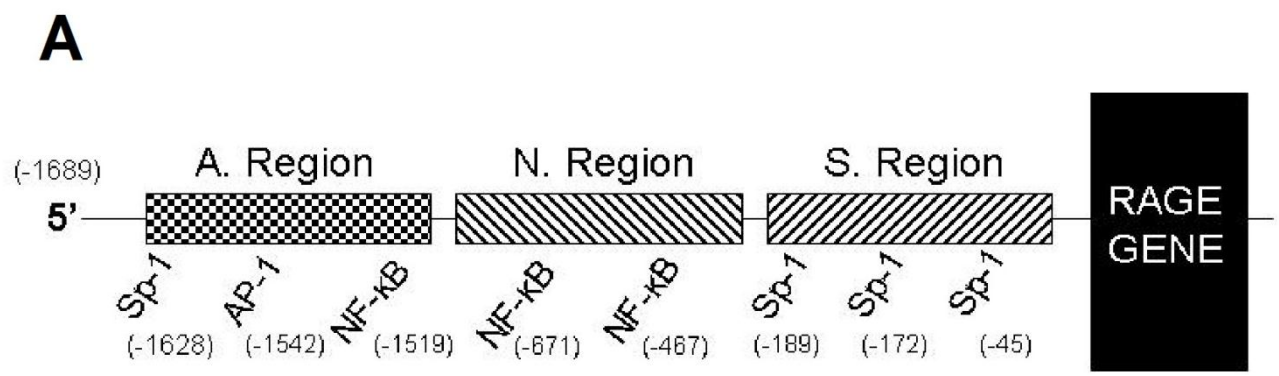
Page 90, line 11: delete “(Figure 3E-H)”

Page90, line 14: delete “(Figure 3E-H)”

Page 102: delete “TTT”

Page 105: delete “Statistical analysis performed via Student’s t-test and presented as Mean (±SD)

Page 106: Figure 5A



Page 108/109, last line: add "ł p<0.05 v WT HFF"

Page 122: "HCl" for "HCL"

Page 122: "Sodium Butyrate" for "NaB"

Page 124, line 7: add "as determined by fasting plasma glucose concentrations as outlined by the standard operating procedure requirements of the AMREP Animal Ethics Committee.

Page 125, line 18: "decrease" for "decline"

Page 125, last line: Comment: There was a statistically significant fold decrease in UAER in female db/db mice compared to male db/db mice.

Page 126, line 3: "(Figure 3D)" for "(Figure 3C)"

Page 126, line 6: "compared to sex"

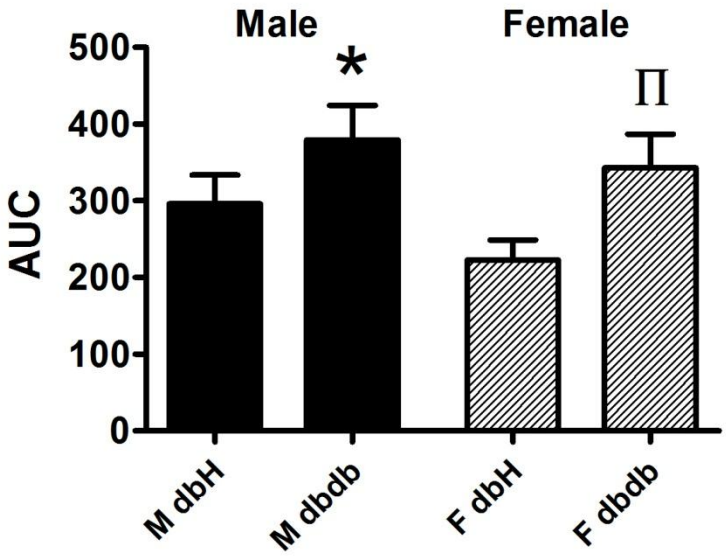
Page 127, 3rd paragraph, line 1: "reflects the total pool" for "reflects the pool"

Page 129, 2nd paragraph, last line: delete and add, This is the first time that this has been shown in a mouse model of type 2 diabetes.

Page 130, line 5: "a result" for "resultant"

Page 130, line 9: "which has a" for "consisting of"

Page 137, Figure 1B: * p<0.05 v M dbH, π p<0.05 v F dbH



Page 139: last line: delete * p<0.05 v M dbH, **p<0.01 v M dbH

Page 146, line 2: “to investigate if this was the case we utilised” for “if this we utilised”

Page 154, heading 2: TGF-β_1

Page 154, 3rd paragraph, line 6: delete second comma

Page 167, line 13: “examined” for “examine”

Page 168, line 1: “in” for “within”

Page 169, line 1: “estrogens to influence” for “estrogens influence”

Page 169, line 4: “interact” for “interacted”

Page 169, line 9: “binding” for “biding”

Page 169, line 10: “that are produced both in” for “are able to be produced both”

Page 169, line 12: delete comma after “that”

Page 171, 2nd paragraph, line 9: delete second “that”

Page 171 penultimate line: “were not mediated by inhibition of” for “were not inhibition”

Page 172, line 2: delete “as a consequence of X”

Page 172: line 3: “this thesis has demonstrated” for “this thesis demonstrated

"Pressure makes diamonds"

George S. Patton

"If you can make a girl laugh- you can make her do anything"

"If I observed all the rules, I'd never have gotten anywhere"

"It's all make believe, isn't it?"

"I don't mind living I a man's world as long as I can be a woman in it"

Marilyn Monroe

"I don't want to be a pie. I don't like gravy"

Babs, Chicken Run

RAGE, Adipose Derived Estrogens and Dietary AGEs; Interactions in Obesity Induced Kidney Disease

Brooke Elyse Harcourt

A thesis submitted in total fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Medicine (Immunology)

Monash University

Completed at Baker IDI Heart and Diabetes Institute

Division of Diabetes Complications

Department of Diabetes and Advanced Glycation

Melbourne, Australia

2011

i. Table of Contents

ii. Part A: General Declaration

Monash University

Monash Research Graduate School

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes three original papers published in and submitted to peer reviewed journals and one unpublished publication. The core theme of the thesis is the interactions between receptor for advanced gycation end product and adipose secreted estrogens in the development of diabetic nephropathy. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Diabetes Complications; Advanced Glycation laboratory at Baker IDI Heart and Diabetes Institute under the supervision of Josephine M. Forbes.

[The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.]

In the case of Chapters; 2, 3, 4 and 5, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	Targeting Advanced Glycation in Obesity Related Renal Dysfunction	Published	I completed the preparation of the manuscript, and completion of the animal experiments.. This publication was completed as a joint first author manuscript with Dr Karly C. Sourris; whom was responsible for the human study.
3	Nuclear Expression of Receptor for Advance Glycation End Products Perpetuates Obesity related Kidney Damage via Adipose Derived Estrogens	Submitted for publication in Diabetologia Journal; October 2011	I completed preparation of the manuscript, and all the laboratory experiments.
4	Premenopausal Renoprotection in Females is Overcome by Modulation of RAGE via Adipose Derived Estrogens in Obesity and Type 2 Diabetes	Manuscript in preparation	
5	Obesity Induced Renal Impairment in Exacerbated in Interleukin-6 Knockout Mice	In Press; September 2011	I completed preparation of the manuscript and experiments

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: .....

Date: 24-10-2011.....

iii. Acknowledgements

First and foremost Josie; thank-you for taking on the very young scientist I was and helping me to achieve all I have. All this would not have been possible without your guidance, support and understanding. Thanks for seeing something in me all those years ago and for taking up the challenge.

Melinda Coughlan and Karly Sourris, my 'surrogate supervisors'. Karly; whilst often annoying; the early morning phone calls and check-ups made this possible! Melinda; thanks for all your advice and guidance over the years, you have been an invaluable mentor.

Glycation Ladies, Sally, Felicia, Anna, Maryann, Amy, Vi and Vicki; thanks for all the coffee, cakes, laughs, and antics.

My candidature was generously supported by the Baker IDI 'Bright Sparks' Program, and the Baker IDI Foundation. I also received many travel opportunities and bursaries, from other organisations such as the Howard Mitchell Foundation and Monash University. With special thanks to, Eric and June Ross, Howard Mitchell and Bobbie Renard and your team of angels for this support.

Thank-you to all my family and friends for the copious amounts of understanding and support. If I named you all, I'd write my thesis twice over! I'm expecting you all to read this! (there'll be a questionnaire at the end!)

Special thanks to; Mum and Dad, Jason, Leesa, Nanna, Ma and Pa, Lyn, and Maddie, you all provided love and support, and endured all my absences and time between phone calls and visits. You all at one time or another helped fill my cupboard with bread, milk, baked beans and dog food when I needed you most (staples for my thesis completion!). Last but not least, thank-you Nick, for all your love and support.

iv. Publications

The following publications appear as Chapters in this thesis;

Chapter 2:

BE Harcourt*, KC Sourris*, B De Courten, AM Morley, SA Penfold, FYT Yap, MT Thomas, ME Cooper, JM Forbes. Controlled consumption of dietary AGEs in an obese population improves renal parameters. ALA and RAGE gene deletion in diet induced obesity improve renal function. Kidney International (2011) 80, 190-198.

Chapter 3:

BE Harcourt, ME Coughlan, KC Sourris, AM Morley, J Pete, MT Thomas, P Kantharidis A Bierhaus, ME Cooper, JM Forbes. Peri-renal Adipose derived estrogens activate RAGE in Type 2 diabetic nephropathy. Submitted to Diabetologia.

Chapter 4:

BE Harcourt, AM Morley, SA Penfold, A Gasser, MT Coughlan, KC Sourris, FYT Yap, ME Cooper, JM Forbes. Premenopause Renopreotection in Females is overcome by modulation of RAGE via adipose derived estrogens in obesity**.** Manuscript In Preparation.

Chapter 5:

BE Harcourt, JM Forbes and VB Matthews. Obesity induced renal impairment is exacerbated in interleukin-6 knockout mice. Nephrology, In Press, September 2011

I contributed to the following publications during my PhD Candidature;

Original Research

Sourris KC, **Harcourt BE**, Tang PH, Morley AL, Huynh K, Penfold SA, Coughlan MT, Cooper ME, Nguyen TV, Ritchie RH, Forbes JM. Ubiquinone (Coenzyme Q10) prevents renal mitochondrial dysfunction in an experimental model of type 2 diabetes. Free Radical Biology & Medicine. In Press, 4th August 2011

Sourris KC, **Harcourt BE**, Penfold SA, Yap FY, Morley AL, Morgan PE, Davis MJ, Baker ST, Jerums G, Forbes JM. Modulation of the cellular expression of circulating advanced glycation end-product receptor in type 2 diabetic nephropathy. Experimental Diabetes Research 2010.ID:974681

Tan AL, Sourris KC, **Harcourt BE**, Thallas-Bonke V, Penfold S, Andrikopoulos S, Thomas MC, O'Brien RC, Bierhaus A, Cooper ME, Forbes JM, Coughlan MT. Disparate effects on renal and oxidative parameters following RAGE deletion, AGE accumulation inhibition, or dietary AGE control in experimental diabetic nephropathy. Am J Physiol Renal Physiol. 2010 Mar;298(3):F763-70

Coughlan MT, Thorburn DR, Penfold SA, Laskowski A, **Harcourt BE**, Sourris KC, Tan AL, Fukami K, Thallas-Bonke V, Nawroth PP, Brownlee M, Bierhaus A, Cooper ME, Forbes JM. RAGE-induced cytosolic ROS promote mitochondrial superoxide generation in diabetes. J Am Soc Nephrol. 2009 Apr;20(4):742-52

Tikellis C, Thomas MC, **Harcourt BE**, Coughlan MT, Pete J, Bialkowski K, Tan A, Bierhaus A, Cooper ME, Forbes JM. Cardiac inflammation associated with a Western diet is mediated via activation of RAGE by AGEs. Am J Physiol Endocrinol Metab. 2008 Aug;295(2):E323-30

Published Abstracts

Coughlan MT, Mibus AL, Tan AL, **Harcourt BE**, Sourris KC, Penfold SA, Thallas-Bonke V, Yap FY, Gasser A, Thomas M, Su Q, Bierhaus A, Cooper ME, Forbes JM. Diabetes. 2008 Jun;57:A634

Sourris KC, Mibus AL, Kokita A, **Harcourt BE**, Tan ALY, Cooper ME, Forbes JM. Interaction between the renin-angiotensin system (RAS) and advanced glycatio end products (AGEs) in the development of diabetic nephropathy. Diabetologia. 2008, 51:S73-S73

Sourris KC, **Harcourt BE**, Morley AL, Penfold SA, Coughlan MT, Nugyen TV, Cooper ME, Ritchie RH, Forbes JM. Ubiquinone is renoprotective in an experimental model of type 2 diabetes. Nephrology. 2011.16:31-31

Review Papers

Harcourt BE, Forbes JM. Coming full circle in diabetes: from complications to initiation. Nature Reviews Endocrinology. In Press, October 2011

Harcourt BE. Decreasing the AGE burden of the 'western diet' as a therapy for obesity related renal disease. The International Maillard Reaction Society. 15th July 2010

Sourris KC, **Harcourt BE**, Forbes JM. A new perspective on therapeutic inhibition of advanced glycation in diabetic microvascular complications: common downstream endpoints achieved through disparate therapeutic approaches? Am J Nephrol. 2009;30(4):323-35

v. Abstracts, Presentations and Awards

Published Abstracts

Oral Presentations

BE Harcourt, MT Coughlan, KC Sourris, AL Morely, A Gasser, A Bierhaus, ME Cooper, JM Forbes. Nuclear Expression of RAGE perpetuates obesity related kidney damage via adipose derived estrogens. Nephrology. 16:65-65.
Australian New Zealand Society of Nephrology, 2011. Adelaide, Australia. Oral presentation

Poster Presentations

BE Harcourt, A Mibus, A Gasser, S Andikopoulos, M Arnstein, A Blair, M Thomas, MT Coughlan, KC Sourris, G Rice, A Bierhaus, ME Cooper, JM Forbes. Interaction between RAGE and Estrone in the development of Type 2 Diabetic Nephropathy. Diabetologia. 50:S454-S454.
43rd EASD Annual Meeting, Amsterdam 2007. Poster Presentation

Conference Abstracts

Oral Presentations

BE Harcourt, MT Coughlan, KC Sourris, AL Morley, A Gasser, A Bierhaus, ME Cooper, JM Forbes. Nuclear Expression of RAGE perpetuates obesity related kidney damage via adipose derived estrogens. *Australian Diabetes Society, 2011. Perth, Australia.* Oral presentation

BE Harcourt, KC Sourris, MT Coughlan, KZ Walker, SL Dougherty, S Andrikopoulos, AL Morley, V Chand, SA Penfold, J Pete, MC Thomas, BA Kingwell, A Bierhaus, ME Cooper, B de Courten, JM Forbes. Targeting advanced glycation in obesity related renal disease. *Victorian Obesity Consortium, November 2010*. Invited Oral Presentation

BE Harcourt, KC Sourris, MT Coughlan, KZ Walker, SL Dougherty, S Andrikopoulos, AL Morley, V Chand, SA Penfold, J Pete, MC Thomas, BA Kingwell, A Bierhaus, ME Cooper, B de Courten, JM Forbes. Targeting advanced glycation in obesity related renal disease. *Australian New Zealand Obesity Society*, October 2010. Oral Presentation

BE Harcourt, KC Sourris, AL Morley, MT Coughlan, A Gasser, A Bierhaus, ME Cooper, JM Forbes. Estrone activates RAGE expression in type 2 diabetic nephropathy. *Cardiovascular Health Symposium (CVH) 'Best of the Best', Monash University, 2010, Clayton VIC, Australia.* Oral Presentation

BE Harcourt, KC Sourris, AL Morely, MT Coughlan, A Gasser, A Bierhaus, ME Cooper, JM Forbes. Estrone activates RAGE expression in type 2 diabetic nephropathy.
Diabetes Complications. 10th International Symposium on the Maillard Reaction, 2009, Palm Cove Qld, Australia. Oral Presentation

Poster Presentations

BE Harcourt, MT Coughlan, KC Sourris, AL Morley, A Gasser, A Bierhaus, ME Cooper, JM Forbes. Regulation of renal receptor for advanced glycation end products (RAGE) via paracrine estrogens. *ISN-Nexus,2010. Kyoto, Japan.* Poster presentation

BE Harcourt, MT Coughlan, KC Sourris, AL Morely, A Gasser, A Bierhaus, ME Cooper, JM Forbes. Regulation of RAGE via paracrine estrogens.
American Society of Nephrology meeting, November 2010. Poster presentation, published abstract

BE Harcourt, KC Sourris, AL Morley, MT Coughlan, A Gasser, A Bierhaus, ME Cooper, JM Forbes. Estrone activates RAGE expression in type 2 diabetic nephropathy. *Diabetes Complications, Metabolism, and Obesity Keystone Symposium, Vancouver, Canada 2009.* Poster presentation

BE Harcourt, AL Mibus, MT Coughlan, KC Sourris A Gasser, A Bierhaus, ME Cooper, JM Forbes. Does Estrone influence the expression on RAGE in DN. *Australian Diabetes Society meeting, Melbourne Convention Centre*, September 2008. Poster Presentation

BE Harcourt, A Mibus, A Gasser, S Andikopoulos, M Arnstein, A Blair, M Thomas, MT Coughlan, K Sourris, G Rice, A Bierhaus, ME Cooper, JM Forbes. Interaction between RAGE and Estrone in the development of Type 2 Diabetic Nephropathy. *9th International Symposium on the Maillard Reaction, Munich, Germany 2007.* Poster presentation

Harcourt BE, Penfold SA, Tan A, Yap F, Gasser A, Fukami K, Cooper ME, Forbes JM. Interactions between Advanced Glycation End Products (AGEs) and their Receptors in Primary Renal Cells and their Role in the Progression of Diabetic Nephropathy. *Australian New Zealand Society of Nephropathy, Annual Scientific Conference*, Melbourne, 2006. Poster presentation

Awards and Scholarships

2011: Baker IDI 'Bright Sparks' Travel Scholarship: $400

2010: Australian New Zealand Obesity Society Travel Scholarship: $500

2009: NIH- National Institute of Diabetes, Digestive and Kidney Disease Travelling Fellowship: $2000

2009: IMARS Travelling Fellowship: $1000

2009: Monash University Travelling Scholarship: $2000

2007: Howard Mitchell Travelling Fellowship: $5000

2006-2009: 'Sportscolour' PhD Stipend, Baker IDI Heart and Diabetes Institute

2006-2009: Monash University, Department of Medicine PhD Scholarship

Also presented work at; Alfred Hospital Science Week 2005-2010, Baker IDI Research In-progress Seminars and Competed in 2007-2009 Rod Andrews Prize, and Paul Korner Award 2009

vi. Abbreviations

AGEs- advanced glycation end products

AP-1- activator protein 1

ChIP- chromatin immunoprecipitation

Db/db- homozygous leptin receptor genetic deletion

Db/H- heterozygous leptin receptor genetic deletion

DN- diabetic nephropathy

E1- Estrone

E2- Estradiol/ 17-β-estradiol

EIA- enzyme immuno-assay

ELISA- enzyme linked immunosorbent assay

ER-α- estrogen receptor alpha

ER-β- estrogen receptor beta

ESRD- end stage renal disease

IL-6- interleukin-6

IL-6-/- - interleukin-6 homozygous genetic deletion

IR- insulin resistance

MCP-1- monocyte chemoattractant protein

MIF- macrophage inhibitory factor

NF-κB- nuclear factor- kappa B

RAGE- receptor for advance glycation end products

RAGE-/- - mice deficient in the receptor for advanced glycation end products

RT-PCR- reverse transcriptase- polymerase chain reaction

Sp-1- specific protein 1

T2D- type 2 diabetes mellitus

Chapter 1

Chapter 1: Literature Review

General Introduction

Obesity is now commonly seen worldwide and across all age groups. Obesity is the result of excessive nutrient intake often from sources which are highly processed which have a high energy-to-nutrient ratio. These dietary excuses are then compounded by decreased physical activity levels. Whilst these aetiological influences are thought to be easily modifiable, the reality is that individuals are becoming increasingly over-weight and obesity related diseases are posing more problems than ever before.

Obesity has many metabolic consequences including, insulin resistance and predisposition for the development of chronic diseases such as cancers, autoimmune diseases, and type 2 diabetes (T2D). Diabetes then incurs further vascular complications in organs that have intricate microvascular networks such as those within, neurons, eyes and kidneys, leading to gangrenous limbs and amputations, cataracts and blindness, renal failure. Of course the ultimate complication of diabetes is early death, normally as a result of cardiac disease and heart failure. Receptor for advanced glycation end products (RAGE) and its modulation of downstream pathways are recognised pathological contributors to the vascular complications of diabetes including renal disease.

There is increasing evidence that obesity is an independent risk factor for kidney disease, in particular in the context of type 2 diabetes. However, the mechanisms by which obesity contributes to kidney disease remain to be determined. Estradiol (17-β-estradiol) has been shown to confer protection against renal and cardiovascular disease when it is the major biologically active estrogen. In obesity, white adipose tissue deposits secrete another estrogen isoform called estrone, which alters the balance of estrogens. Imbalances in

estradiol to estrone are the development of obesity related diseases. At the time of beginning this thesis, there were no drugs available for the specific treatment of obesity related renal diseases. Some treatments are borrowed from type 2 diabetes and chronic kidney disease and have modest effects on excretion of albumin or target blood pressure lowering pathways. Others directly target obesity, leading to weight loss although these are often temporary.

Therefore this thesis examines the development of obesity related renal disease. I have examined the role of excessive adipose tissue deposition and the ability of estrogens to modulate the receptor for advanced glycation end products (RAGE), to discover potential therapeutic targets to control the growing problem of obesity related kidney disease.

Diabetes Mellitus

Diabetes Mellitus is a group of heterogeneous disorders with common elements of hyperglycaemia and impaired glucose tolerance. Diabetes mellitus can be classified into four categories as outlined in Table 1. T2D, which comprises some 85% of the global diabetes burden, is thought to be increasing in incidence due to a number of factors that include population growth, aging, urbanisation, increasing prevalence of obesity and a decline in physical activity.

Table 1: Classification of Diabetes Mellitus Diseases		
Type 1 Diabetes	T1D	• Accounts for 10% of cases of diabetes • Predominant form seen in younger age groups in high income countries • Increasing in incidence in both rich and poor countries
Type 2 Diabetes	T2D	• 85-95% of diabetes in high income countries • 11% of diabetes in Low income countries
Gestational Diabetes	GDM	• Impaired glucose tolerance and impaired β-cell function that occurs during pregnancy
Other		• Genetic defects affecting β-cell function • Diseases of the exocrine pancreas such as cystic fibrosis and pancreatitis • Drug or chemically induced diabetes

Table 1: Classification of Diabetes Mellitus Diseases

Insulin Resistance and Type 2 Diabetes- Metabolic Disease

Obesity, insulin resistance or impaired glucose tolerance (IR) and T2D continue to rise in incidence not only in western societies, but more recently in eastern societies (1). Where once T2D was mostly restricted to the elderly, as it is classically considered a disease of the

aging population, additional environmental influences have led to persons of any age being diagnosed (2, 3). Diagnosis of T2D parallels the increases in obesity in western societies, and an estimated 7% of the world population are affected (1-3).

IR is broadly defined as the failure of the most insulin sensitive organs; white adipose tissue, skeletal muscle and the liver to recognise insulin thereby inducing adequate insulin signalling. This results in impaired uptake of circulating glucose. IR transitions to T2D when the pancreas tires of hyper production of insulin and blood glucose concentrations continue to rise to clinically relevant levels. Further contribution to increased blood glucose occurs throughout the body as IR results in increased hydrolysis of stored triglycerides from adipose cells, which consequents in increased free fatty acids (FFA) in circulation. In liver cells IR results in impaired glucagon synthesis and a failure to clear hepatic glucose production via gluconeogenesis. Skeletal muscle is also s unable to respond to insulin effectively and is unable to take up adequate glucose resulting in excess glucagon mediated storage.

T2D is a disease of elevated blood glucose and the body's inability to produce adequate biologically recognisable insulin.

Risk factors for the development of Type 2 Diabetes

Sedentary lifestyle, decreased physical activity, increased body mass index (BMI) and adiposity, poor diet or a combination of any of these, contributes to the development of 'metabolic syndrome' and are major risk factors for T2D. A BMI higher than 25kg/m^2 carries the definition of 'overweight', whilst a BMI >30kg/m^2 is defined as 'obese'. Unfortunately approximately 67.4% of Australian's are thought to fit within these categories (4). In other westernised countries; USA, New Zealand, the United Kingdom and Canada, the percentage of overweight and obese persons also constitutes the majority of their population (4, 5).

The high occurrence of obesity in westernised populations may in part be attributed to nutritional imbalance, as there is a trend to opt for over processed nutrient-poor food choices (6). A study on typical meals in USA/Australia also found that quality of consumed food correlates with household socioeconomic status (7). There also appears to be genetic risk factors for predisposing some individuals to developing T2D (8). Indeed studies have also suggested that T2D, may be associated with changes in the innate immune response(9, 10), and it is now well recognised that levels of C-reactive proteins predict the occurrence of T2D (11, 12).

It is recognised that prevention of overt T2D with strategies such as weight loss followed by strict glycaemic control, are the most effective ways to prevent vascular complications after diagnosis of the disorder. Once complications arise, treatments are limited in their efficacy and progression can be rapid.

Complications of Type 2 Diabetes

When the body first experiences hyperglycaemia it mounts an acute stress response, involving increased filtration by the kidneys to remove excess glucose via urination. Stores of adenosine-tri- phosphate (ATP) from adipose tissue fuel the body's energy needs and at first these compensatory responses combat hyperglycaemia. Symptoms experienced by patients at this stage include fatigue and increased thirst and urination. Patients with T1D will quickly develop pronounced symptoms, whereas T2D may persist for many years without diagnosis. As the hyperglycaemia chronically persists, the capacity of the kidneys to filter the excess glucose eventually fails. It is the prolonged exposure to hyperglycaemia that is now recognised as the primary causal factor in majority of diabetic complications (13, 14).

Micro- and macrovascular complications

Complications of diabetes manifest as both micro- and macrovascular complications due to prolonged elevated blood sugar levels. As well as sugar molecules directly affecting vasculature and organs due to insulin independent glucose uptake, high sugar levels also activate hemodynamic pathways; aldose reductase and polyol pathways, promote excessive generation of reactive oxygen species (ROS) producing oxidative stress and the action and formation of advanced glycation end products (AGEs)(15, 16).

Vascular injury is often seen in the context of hypertension, increased vascular permeability or ischemic injury. Macrovascular disease is the biggest cause of morbidity and mortality in both type 1 and T2D individuals, with a substantial amount of sufferers experiencing early heart attacks, strokes and premature death (16).

Retinopathy, neuropathy and nephropathy

Microvascular disease in diabetes typically manifests in three main pathological categories, retinopathy, neuropathy and nephropathy. Whilst each organ performs a distinctly different role in the body, research has shown that diabetic pathologies generally follow similar pathways. Increased inflammation and oxidative stress both in tissues and circulation lead way to pathological cascades that inhibit the normal function of the organs. In neuropathy, inflammation and free radicals, damage nerve endings of peripheral limbs, with the end stage result being amputation, most commonly of the lower limbs or extremities (17). The retina, being a highly vascularised tissue is also highly sensitive to the effects of poor glycemic control. An approximate 1 in 12 persons diagnosed with T2D over the age of 40 years will present with a form of retinopathy (18). This thesis will focus on the diabetic complication; nephropathy, and aims to contribute further knowledge to the disease that affects a large percentage of type 2 diabetics.

Diabetic nephropathy

During the normal course of diabetes, the kidneys go through several clinical stages; initial hyperfiltration, progresses to microproteinuria (microalbuminuria), followed by macroproteinuria in concert with a steady progressive decline in renal filtration, eventuating in end stage renal disease (ESRD)(19). Approximately 30-40% of patients with diabetes mellitus will sustain damage to the cells of the kidney and progress to ESRD (20) and a proportion of the remaining will present with normal renal structure. The current aim of most research is to investigate possible triggers and accelerators which determine whether a person with T2D will develop nephropathy (21).

The cells of the glomeruli are initially damaged during hyperfiltration which occurs in response to hyperglycaemia. The hyperfiltration causes hypertrophy, which for a prolonged period promotes the growth and accumulation of extracellular proteins within the glomerular structure. The basement membrane of the Bowman's capsule thickens with the extracellular matrix accumulation and consequently decreases the area of the Bowman's Space. The mesangium also accumulates excessive matrix proteins and condenses after the initial hypertrophy. The pedicle processes of the podocytes spread further apart and there is a loss of these cells allowing larger proteins to escape into the urine, causing microalbuminuria at first and then macroproteinuria.

Accounting for 90% of the total volume of the kidney is the tubulointerstitium; encompassing the tubular epithelium, vascular structures and the interstitium (22). The literature also suggests that the pathology of tubular epithelium is just as important as the pathological changes of the glomeruli (23). In fact, one-third of people with diabetes will have absent glomerular damage and disproportional and severe tubulointerstitial lesions (23-25). Pathological changes that have been described within the tubulointerstitium include; thickening of the tubule basement membrane, tubular atrophy, interstitial fibrosis, and arteriosclerosis (25). Fibroblasts are the major cell type present within the interstitium,

where they interconnect with tubules, vessels and each other to make a cellular scaffold. The other constituents of the interstitium are immune cells; dendritic cells, macrophages and lymphocytes (25).

Tubular epithelial cells are direct targets for damage by the high glucose levels present in the diabetic milieu. In tubular epithelial cells the uptake of glucose occurs independently of insulin, resulting in equilibrium of extracellular and intracellular glucose (26, 27). On presentation of glucose, tubule cells secrete vasoactive hormones such as angiotensin II, transforming growth factor β (TGFβ) and matrix proteins (23). The vasoactive hormones can entice neighbouring cells to undergo activation (28). Fibroblasts in particular will differentiate to become activated, express α- smooth muscle actin (myofibroblasts), and synthesise fibrillar collagens (25). Trans-differentiation can also occur when cellular contact between the myofibroblast cells and tubule cells, causes tubule cells to become 'fibroblast-like' (23), however the consequences of this transformation are yet to be described.

Like the glomerulus, the proximal tubule cells are exposed to high levels of advanced glycation end products (AGEs). The proximal tubule has been described as a site of re-absorption and catabolism of circulating AGEs. The AGEs are taken into the lysosomal apparatus of the cell and cause hypertrophy due to accumulating proteins and reduced lysosomal protease activity (29). The AGEs activate intracellular signal transduction that generates ROS, and activates NF-κB (23).

Gender specific predisposition to diabetes and its complications

Males are generally more susceptible to T2D diabetes, as are women that are post-menopausal. However, what renders males and females that develop a more 'male phenotype' susceptible? We and others (30, 31), hypothesise that this is most likely the result of an imbalance in estrogens and how they signal. There are three main types of estrogens;

estrone (E1), estradiol (E2) and estriol. E1 is secreted from adipose tissue and is rapidly synthesised via aromatase (CPY19) or estrone sulfatase (E1-sulfatase) from androgenic precursors. In males and post-menopausal women, E1 (and its more biologically active form estrone sulphate, E1S) is thought to be the main signalling estrogen isoform, and positively correlates with BMI and adiposity (32, 33). E2 is commonly thought of as a 'protective' estrogen isoform (34) and is the main signalling estrogen in menstrual women. A decline in circulating E2 correlates with pathogenesis of many diseases, which include diabetes vascular complications. E2 is secreted from the ovaries and more recently it has been discovered to be produced by the testis. It is now recognised that presence and activity of E2 in both males and female is of great biological importance throughout life and not just at embryogenesis. Estriol is the main estrogen form in pregnant women, and while estriol plays an important part in development of gestational diabetes, it is yet to be identified as having a role in T2D, and therefore will not be discussed further in this thesis.

Obesity and increased adiposity result in increased circulatory E1 and E1S and a decline in E2, in both males and females. Pre-menopausal women with increased adiposity, also have an imbalance in their E1:E2 ratio, altering the balance between these two isoforms (35). This may account for the loss of protection in women against IR, T2D and its associated complications seen with obesity.

It has further been demonstrated that increased serum levels of AGEs predict CVD mortality in nondiabetic women but not in nondiabetic men (36). In hyperthyroidism, a non-diabetic condition in which patients exhibit hyperglycaemia and hyperlipidaemias, levels of AGEs are increased in males versus females, and are therefore subjected to an increased chance of complications (37). Furthermore, low soluble RAGE concentrations are associated with increased risk of progression to complications and the severity of diabetic complications in

both men a women (38, 39).It begets the question; why are AGEs and RAGE differentially influenced in males and females?

White adipose tissue

White adipose tissue (WAT), most likely evolved, to account for variance in nutrient supply, and act as a store of energy when available food was low. Excessive adiposity or obesity develops in response to an over-nutrient supply, and carries with it increased risk of developing chronic diseases that include T2D and cardiovascular disease to name only two consequences. WAT is not inert connective tissue, but is a highly functional endocrine organ that is important in the maintenance of peripheral insulin sensitivity, as well as having the ability to secrete various cytokines, fatty acids, adiponectin, leptin and estrogens (40). A further role for WAT is demonstrated in transgenic mice with lipodystrophy that contain little WAT, which develop hyperglycaemia and hyperinsulinemia (41).

In healthy humans WAT accounts for between 15-20% of body weight in men and 20-25% of body weight in women. The sexual dimorphism of body contour in humans can be attributed to differences in subcutaneous WAT distribution, as a result of hormonal influences. Females demonstrate a *gynoid* distribution of WAT, with larger accumulations in the breast, lower abdomen, buttocks and thigh regions until menopause when distribution tends to alter to an *android* distribution with larger accumulations in central locations (42). It is excessive central and specifically intra-abdominal adiposity, which combined with hyperglycaemia and hyperlipidemia, predisposes individuals to metabolic syndrome and cardiovascular disease (42, 43).

Many have discussed the notion that (40, 44) obesity is a state of chronic inflammation which stimulates induction of inflammatory signalling pathways linked with insulin resistance and T2D. Whilst the exact mechanisms are still in investigation, it is established that adipocyte

precursors that exist within the stromovascular of WAT in the context of obesity, are able to activate phagocytic cells, and promote infiltration of the organ by macrophages (45, 46). Immune cell infiltration is mediated through secretion of cytokines by WAT such as TNF-α and IL-6, which via communication with pre-adipocytes also promote other cytokines such as MCP-1. Insulin resistance begins at a cellular level, with a decline in autophosphorylation of the insulin receptor substrate family (IRS) and other substrates, thereby reducing the ability of insulin to act intracellularly (47-49). Insulin resistance (IR) and obesity activate multiple intracellular organelle stress mechanisms. The endoplasmic reticulum (ER) (50) and mitochondria (51), upon being exposed to hyperglycaemia have defects in protein folding and energy generation respectively. In the ER, the functional overload caused by obesity, results in misfolding of proteins and activation of 'unfolded response proteins' and further cytokines which further contribute to IR (52). The mitochondria upon exposure to free fatty acids and excessive glucose undergo oxidative stress via excessive reactive oxygen species (ROS) production and dysfunction, creating a loop of oxidative stress production.

Interestingly, the anatomical location of adipose plays an important role in the development of insulin resistance and bears much more importance than global BMI calculations. This is due to the different biological function of various fat pads, which further highlights the importance of adipose as a secretory organ and not purely an excess storage tissue. Central obesity, characterised by increased growth of intra-abdominal adipose, is associated with increased insulin resistance and type 2 diabetes (53, 54). Conversely increased subcutaneous adipose and peripheral obesity is associated with decreased risk of developing insulin resistance, and type 2 diabetes (55-57). Diet and exercise result in improved insulin sensitivity as a result or greater loss of visceral rather than subcutaneous adipose (58, 59). Further supporting evidence for the differences in adipose biology and function is that removal of visceral adipose shows results in decreased glucose levels of insulin and glucose (60) and that removal of subcutaneous fat via liposuction bares no improvement on insulin resistance or

diabetes risk (61). Hocking *et al* (62), designed elegant experiments that showed the different biological actions of adipose tissue depots by transplanting grafts of adipose from one deposit to another, for example subcutaneous to visceral adipose deposits. Interestingly mice receiving subcutaneous adipose tissue into their intra-abdominal cavity demonstrated significant decreases in inguinal, epididymal and retroperitoneal fat beds (62). Similar experiments were repeated at around the same time by Tran *et al* (63). These further highlighted that there is cross talk between adipose tissue depots and surrounding organs, and that the benefits observed are not the result of classic inflammatory pathways such as IL-6 and TNF-α, as these remained unchanged between groups. These experiments provided new novel insights into the treatment of obesity and highlighted the different actions of specific fat pads.

Of particular interest to nephropathy research is the adipose depot surrounding the kidney capsule of the kidney the, retroperitoneal or peri-renal fat pad. The peri-renal fat pad's main function is one of energy conservation, with publications showing that its properties are at times consistent with that of brown adipose tissue (BAT), giving it a role in homeostatic thermogenic regulation (64, 65). Given the close proximity of peri-renal adipose to the kidney, and previous research showing that WAT acts as a paracrine organ, factors secreted by peri-renal adipose are likely to effect the kidney, and may play an important role in the development of obesity related kidney disease and T2D nephropathy.

AGEs

Advanced glycation end products (AGEs) are non-enzymatically formed adducts of proteins, carbohydrates and DNA, that occur more rapidly with heat application to foods during cooking, and in the body when hyperglycaemia and oxidative stress are exacerbated. The formation of AGEs within cells is detrimental to protein function hence excessive accumulation of AGEs promote disease progression. AGEs are normally cleared from the body

by the kidneys, therefore obese persons who exhibit abnormalities in renal filtration, combined with excessive consumption of western foods, expose their kidneys to large amounts of AGEs (29, 66, 67).

Advanced glycation end products (AGEs) are abundant in highly processed foods and foods cooked at high temperatures. Excessive dietary intake of AGEs has been shown to contribute to renal and cardiovascular diseases, especially in combination with high fat and high AGEs (68-70). AGEs enter the bloodstream following consumption and likely contribute to chronic inflammation in adipose tissue and the activation of macrophages via RAGE (71-73). Furthermore inhibition of NF-κB, a transcription factor for RAGE via salicylate, improved glucose regulation (74). RAGE and NF-κB have also been associated with the development of IR in other glucose and insulin sensitive organs; pancreas, liver and muscle, as noted via their increased expression in IR and pre-diabetes.

Indeed accumulation of AGEs within the kidney, circulation and other tissues is a prominent marker of disease progression in diabetes and the target outcome for AGE therapeutics is to reduce tissue AGE pools (75). We and others have consistently shown that lowering of the body's AGE burden with pharmacological agents significantly improves kidney complications in diabetes (75-79). We further demonstrated that a diet low in AGEs or therapeutic intervention with an AGE inhibitor; alagebrium chloride can decrease obesity related abnormalities in the myocardium (69).

RAGE

RAGE is a multiligand receptor of the immunoglobulin superfamily. It is located within the major histocompatibility class III region on chromosome 6, along with numerous other genes that are involved in inflammatory and immune responses, and several complement responses (80, 81)

The elementary description of RAGE was as a receptor for AGEs, hence its name, however in the years since its discovery, it has become apparent that its multiple affinities include other ligands such as high mobility group box protein-1(HMGB1), S-100 calcium binding protein calgranulins, amyloid-β-peptides and the family of β-sheet fibrils, all of which are known to be elevated in chronic metabolic and inflammatory diseases. For the purpose of this thesis we will focus on the consequence of RAGE/AGE interactions. It is important to highlight that RAGE does not facilitate the uptake and removal of AGEs from the biological system, but rather, AGE/RAGE ligand interaction induces inflammation via the incessant activation of nuclear factor kappa-B (NF-κB)(82).

Understanding of the role of RAGE in the pathogenesis of diabetic complications was facilitated by the development of the RAGE-/- mouse model. To date the RAGE-/- mice have been used to show the role of RAGE in the pathogenesis of micro and macrovascular diseases which include, diabetic retinopathy, neuropathy, and nephropathy, cancer, and Alzheimer's disease (77, 80, 83-87). RAGE overexpressing mice, which develop advanced renal disease, further elucidated the role of RAGE in the development of diabetic nephropathy, as AGE therapeutics ameliorated renal injury, thereby highlighting both the role of the AGE/RAGE axis and the importance of RAGE signalling in diabetic nephropathy (88).

RAGE expression is regulated at a number of cellular levels. Diversity of ligand recognition is facilitated by the large extracellular domain, quaternary structure and post-transcriptional modifications of the primary structure. Glycosylation modifications of RAGE further influences affinity for different ligands, in that when N-glycolsylated affinity is increased for S100 and HMGB1 (89, 90), whereas when RAGE is de-glycosylated, affinity for AGEs is increased (91).

There are 2 isoforms of circulatory RAGE, both soluble RAGE (sRAGE) and endogenous secretory RAGE (esRAGE). sRAGE is likely a product of metalloproteinase digestion by the

protein ADAM 10 at the cellular surface of full length RAGE (92), whilst esRAGE is a product of RAGE mRNA processing (93). Both circulating RAGE variants have been correlated with glycaemic control, metabolic syndrome and vascular diseases in T1D (94, 95) and T2D (96-98). Therefore they have been considered as possible therapeutic targets (99), as their action is anti-inflammatory as they interact with circulating AGEs, thereby preventing action and signally cascade of cellular bound RAGE. It is however unknown how the *AGER* gene is regulated to transcribe each of the RAGE isoforms.

Hudson, *et al* (100), identified that 14 splice variants of RAGE are produced via splicing events in human lung tissue and vascular smooth muscle cells, which is 10 more than previously identified or characterised, with a further six isoforms not identified in the human tissue but produced with alternative splicing experiments (100). It was further identified that the 5' end of the *AGER* gene has multiple transcriptional start sites and that action of binding elements within these transcription start sites, initiates differential gene transcription (101). Kalea, *et al* (102), also identified that in mouse tissue, 20 splice variants of RAGE are produced from differential gene splicing, and that two of these new and uncharacterised splice variants are present within renal tissue. Indeed differential activation of the *AGER* via alternate transcriptional regulation in the 5' transcriptional domain influences the differing splice variant transcription, and warrants further research.

Within the 5' transcriptional regulatory domain of the *AGER* gene it is know that there are active DNA binding sites for NF-κB, Sp-1, AP-1 and ER-α (101), therefore it is action of these and their activating elements that may determine how gene splicing and differential transcription of RAGE isoforms occurs.

Do adipose derived Estrogens interact with RAGE?

Two different forms of estrogen receptors are currently known to exist; estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β). These both belong to the superfamily of nuclear hormone receptors, and mediate estrogen signalling. Both isoforms are known to be present in the kidney.

Certain genes have estrogen responsive elements (ERE), and therefore estrogens acting via estrogen receptors are able to mediate genetic expression in disease states. ER-α has been shown to mediate events that promote sex specific differences in mediating glomerular hypertrophy, via TGF-β (103). In addition, in hepatocytes, ER-α has been shown to mediate glucose homeostasis through mediation of the leptin receptor gene *Lep,* resulting in its down-regulation and a loss of hepatic glucose control in ER knockout mice (104). Interestingly, the RAGE gene, *AGER*, has also been shown to have ERE, whereby expression of RAGE was increased via interaction between E2 and ER-α interaction in endothelial cells (101). This may be why the deletion of ER-α in mice demonstrates a beneficial phenotype and decreased development of renal hypertrophy than littermate controls (103).

Less is known about the renal functions of ER-β as its discovery is more recent (105). Like ER-α, it is vital for normal physiological development. Upon stimulation by E2, ER-α and ER-β form homodimers and heterodimers that interact with specific DNA sequences and instigate transcription of estrogen responsive genes (106).

It has been demonstrated that estrogens and more specifically, an imbalance of estrogens, is able to influence a global inflammatory state, however a mechanism is yet to be identified. As mentioned previously, the promoter of RAGE's gene, *AGER* has an estrogen responsive element allowing for transcriptional regulation, through activation and translocation of ER-α (101). As well as RAGE inducible transcription factor; NF-κB mentioned previously, the 5' transcriptional binding domain of the *AGER* gene, is known to also contain transcriptional binding sites for specific protein 1 (Sp-1), activator protein 1 (AP-1), and ER-α, which consequently are all also directly or indirectly inducible by estrogens (101). Indeed, since the

initial manuscript describing 17-β-estradiol/ER-α dependent transcription of RAGE (101, 107), it has been elucidated that the 17-β-estradiol/ER-α complex described (101), acted via SP-1 and not NF-κB. Mukherjee *et al* (107), performed their analysis of the *AGER* promoter region in an immortalised vascular cell line and did not examine the effect of E1 in this system.

Hamilton *et al*, ovariectomised rats thereby rendering them estrogen-deficient and then analysed cardio-myocytes for induction of inflammatory pathways including RAGE (108). They demonstrated that in the absence of 'protective' E2, RAGE gene expression is decreased, and the rats were more susceptible to cardiac inflammation (108).

Selective estrogen receptor modulators in type 2 diabetic nephropathy

Treatment with estradiol in some animal models has been found to be renoprotective and even reverse renal injury such as fibrosis (109). However in obesity, hormone replacement therapy (HRT) is not a viable treatment as there is much evidence that indicated HRT as causing cancer.

Selective estrogen receptor modulators (SERMs) have fewer of the harmful side effects seen with hormone replacement therapy. Their action is through selective modulation of estrogen receptors and some bind with higher affinity than both estradiol and estrone, causing in the case of some SERMs activation of different gene target cascades than their natural ligands (110). Their action can be both agonistic and antagonistic depending on the tissue and their dosages. For this reason it is important that actions of SERMs are investigated in renal tissue and specifically in an obesity milieu. Whilst the discovery that estrogen receptors are expressed in multiple organs including the lungs, kidneys, bone and gut some 30 years ago, the role that estrogen receptors play in other diseases is recent work, as is the administration of tamoxifen for the treatment of other diseases.

Tamoxifen is a non-steroidal estrogen antagonist, a SERM developed initially as a contraceptive but proved more useful as a treatment for estrogen positive breast cancers, with an equal affinity to ER-α and ER-β (110, 111). Encapsulating peritoneal sclerosis is a complication that arises in renal failure patients that receive peritoneal dialysis (PD). It was discovered that the autoclave treatment of the dialysis solution, which has a high glucose content was forming AGEs, and activating a epithelial-to- mesenchymal (EMT) transition of the mesothelial cells causing them to become fibroblasts (112). Administration of tamoxifen in PD patients has proven useful, though in the contexts of this disease Tamoxifen is described as an antifibrotic agent because of the decline in TGF-β levels seen in patients on treatment. Complimentary research in diabetic nephropathy discovered that EMT causing renal epithelial cells to transition to mesangial cells, was due to AGEs and RAGE activation also and that in kidneys RAGE antagonism with neutralising RAGE antibodies was effective (113). RAGE has also been identified in the peritoneal cavity of PD patients and its hypothesised that it is the driver of fibrosis and more specifically we suggest it drives EMT. As direct RAGE antagonism is not a likely viable therapy due to its larger role in the innate immune system as an antigen presenting molecule, the administration of Tamoxifen in diabetic nephropathy may also prove effective.

Raloxifene another SERM, developed after Tamoxifen to more specifically antagonise ER-β (110), has proven more successful and carries less side effects. Most commonly administered to post-menopausal women who are at high risk of developing breast cancers and as a treatment for osteoporosis, its use in chronic renal failure patients has also been beneficial (114). Menopause, as discussed previously renders women more susceptible to chronic diseases than women with normal menstrual cycles because of the imbalance of estrogens; uremic patients and those on renal dialysis are therefore more susceptible to metabolic diseases (115). Administration of raloxifene in large scale clinical trials has been effective in not only decreasing the rate of bone fractures in these patients but has the added benefit of

renoprotection (116, 117) and decreasing nitric oxide and serum malondialdehyde (118). In addition, those patients also had an improvement in their lipid profiles, which further decreased their risk of progression to cardiovascular related mortality with raloxifene.

Other SERMs are available but there has been even less investigation as to their effectiveness in obesity related renal disease. Of importance is that obese males have an imbalance of estrogen levels combined with an imbalance in testosterone, further exacerbating their susceptibility to obesity related diseases. SERMs have been successful in reducing prostate cancer mortality, and hence there is precedent for treatment in other estrogen related diseases also.

This thesis examines the reasons why gender specific protection against kidney and cardiovascular disease in women is overcome in obesity and type 2 diabetes. Specifically interactions between adipose derived estrogens and the receptor for advanced glycation end products in the kidney have been studied.

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Thesis Aims

Based on the above literature review it is my hypothesis that estrogens secreted from adipose tissue may via activation of RAGE be an important mediator of kidney damage in diabetes, and that sexual differences observed in the development of obesity related kidney diseases may in part be via these mechanisms.

Therefore the aims of my thesis are:

1. To study the expression and action of estrogens and their receptors in the kidney, with relation to RAGE and diabetic nephropathy in a model of Type 2 Diabetes.

2. To study the expression of RAGE in a high fat feeding model of obesity.

3. To utilise the therapeutic Alagebrium chloride, and investigate its efficacy in a high fat feed model of obesity

4. To study the genetic regulation of RAGE, via adipose derived estogens in a high fat feeding model of obesity

Chapter 2

Chapter 2: Introduction

This publication is comprised of two separate studies that complemented each other and were hence published together. I was experimentally responsible for the mouse study portion of the paper, in addition to analysing the data obtained from the human study

In this publication we utilised mice deficient in the RAGE gene (RAGE-/-) deletion mouse (RAGE-/-) and wild-type littermates which were made obese via high fat feeding for 16 weeks. We studied the inflammatory profile and renal disease development in this model. We also utilised the AGE inhibitor; alagebrium chloride, in a model of high fat feeding and analysed renal function.

This paper was published in the journal, Kidney International, 16th March 2011, and is included as **Supplement 1.**

Monash University

Declaration for Thesis Chapter Two

Declaration by candidate

In the case of Chapter Three, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design, experimental protocol, experimental analysis- murine study. Preparation of manuscript	70%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
KC Sourris	Co-ordinated human study, experimental protocol and analysis- human study	
MT Coughlan	Revision of manuscript	
KZ Walker	Design of diets- human study	
SL Dougherty	Sample collection- human study	
S Andrikopoulos	Revision of manuscript	
AL Morley	Sample collection- murine study	
V Chand	Sample collection- human study	2%
SA Penfold	Sample collection- human study	
J Pete	Design of RT-PCR probes	
MC Thomas	Revision of manuscript	
BA Kingwell	Revision of manuscript, statistical analysis, supplied funding- human study	
A Bierhaus	Revision of manuscript, provided mouse model	
ME Cooper	Revision of manuscript	
B de Courten	Study design, sample collection, supplied - human	

	study	
JM Forbes	Study design, preparation of manuscript, supplied funding- murine and human study	

Candidate's Signature		**Date** 24/10/2011

Declaration by co-authors

The undersigned hereby certify that:

(7) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(8) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(9) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(10) there are no other authors of the publication according to these criteria;
(11) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(12) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Baker IDI Heart and Diabetes Institute, Melbourne, Australia**

		Date
Signature 1		12/10/2011
Signature 2		18/10/2011
Signature 3		18/10/2011
Signature 4		12/10/11
Signature 5		17/10/11
Signature 6		13/10/11
Signature 7		18/10/2011
Signature 8		10/10/11
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Signature 14		10/10/11
Signature 15		10/10/11

Targeting Advanced Glycation in Obesity Related Renal Dysfunction

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Abbreviated title: AGEs, RAGE and obesity related renal impairment

Keywords: obesity, nephropathy, RAGE, alagebrium chloride

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Abbreviations: AGEs; advanced glycation end products, RAGE; receptor for advanced glycation end products, ALA; alagebrium chloride, MCP-1; Monocyte chemoattractant protein-1, MIF; Macrophage migration inhibitory factor.

Abstract

Obesity, which is highly prevalent in western populations, is considered a risk factor for the development of renal impairment. Interventions that reduce the tissue burden of advanced glycation end products (AGEs) have shown promise in stemming chronic disease progression. We therefore aimed to test if therapeutic treatments that lower tissue AGE burden in humans and mice would improve obesity related renal dysfunction. Overweight and obese individuals (BMI 26-39) were recruited to a randomised, cross-over clinical trial involving two weeks of consumption of each of a low and high AGE containing diet. Renal functional parameters and inflammatory profile were improved following consumption of the low AGE diet. Mechanisms of advanced glycation related renal damage, were investigated in a mouse model of obesity, using the AGE lowering therapy, alagebrium (ALA) and RAGE deficient (RAGE-/-) mice. Obesity resulting from a high fat high AGE diet induced renal impairment; concomitant treatment with ALA and RAGE-/- improved urinary albumin excretion, creatinine clearance, inflammatory profiles (MCP-1 and MIF), in addition to renal oxidative stress. ALA treatment however resulted in decreased weight gain and improved glycaemic control compared to WT Obese mice. We have demonstrated improved renal function in obesity by targeting the advanced glycation pathway.

Introduction

Obesity is an important risk factor for type 2 diabetes and its subsequent complications including renal and cardiovascular disease. Between 2010 and 2030 it is estimated that worldwide numbers of diabetes cases will increase by 54% [1]. As such the International Diabetes Federation has proposed lifestyle changes as a cost-effective method of preventing or delaying the onset of type 2 diabetes [2], which would likely extend to manifestations of obesity such as an increased risk of chronic kidney disease [3]. Current figures show that 30-50% of diabetic patients will develop diabetic nephropathy [4].

It is well known that certain lifestyle choices such as diets high in saturated fat and processed foods contribute to obesity and the development of type 2 diabetes, although the exact mechanisms involved have not been fully defined. Dietary fat and processed foods are extremely high in a group of sugar modifications known as advanced glycation end products (AGEs). These molecules improve taste, reduce food spoilage and promote longer shelf life. Excessive dietary intake of AGEs has recently been shown to contribute to renal [5] and cardiovascular [6] disease and the development of type 2 diabetes, especially in the context of a high fat diet in animal models [7]. Once in circulation [8-10] dietary AGEs may cause inflammation and free oxygen radical production by modulation of specific receptors, including the receptor for AGEs, RAGE. Interestingly, the kidney is the main organ responsible for the removal of AGEs from the bloodstream [11]. This high exposure of the kidney to AGEs is likely to make the organ particularly susceptible to AGE-mediated damage. The potential for reduction in dietary AGEs to improve renal function in non-obese, renal failure patients has been demonstrated after a 4 week low AGE diet which reduced serum creatinine concentrations by 30-40% [12].

The present study, investigated the effects of lowering the accumulation of AGEs or interrupting RAGE downstream signalling pathways using a model of obesity related renal

disease in mice. The efficacy of a reducing dietary AGE intake to improve renal function in obese humans was also examined.

Methods

Clinical Study

Participant Selection: This study was approved by the Alfred Hospital Ethics Committee and conducted according to the principles expressed in the Declaration of Helsinki. All individuals gave written informed consent prior to commencement of the study. Participants were males, aged between 18-50 years with stable body weight (weight change <5 kg in last year), BMI $\geq$ 25 kg/m^2, normal glucose tolerance (OGTT) and healthy according to medical history, examination and basic blood screening. Exclusion criteria included morbid obesity (BMI $\geq$ 40 kg/m^2), current smoking habit, high alcohol use or a positive urine drug screening test, any medication taken within one month prior to commencing the study, presence of acute inflammation (by history, physical or laboratory examination) or highly unusual dietary habits or vegan diet.

Clinical Study Design, Anthropometric and Metabolic Measurements: Eleven healthy overweight males participated in a clinical dietary intervention study involving two weeks each of low and high AGE diet separated by a four week wash-out period. Participants kept a three-day diet record (two weekdays, one weekend day) based on household measures. Nutrient content was analysed with SERVE (SERVE Nutrition Systems, St Ives, NSW), based on Australian Food Composition tables plus US data for food AGE content [13]. Results guided food selection and indicated the approximate habitual AGE intake. A menu of carefully matched alternative food choices (Table 1), each similar in macronutrients and total kilojoules but differing in total AGE content were prepared for each meal of the day, including snacks and beverages according to previously described guidelines [13]. All foodstuffs for the low and high AGE diets were provided to the individuals, in addition to instructions for storage and preparation of meals (method, temperature and duration of cooking). Participants were instructed to eat to appetite, and maintain normal physical activity as measured by

International Physical Activity Questionaire (IPAQ, [14] and by accelerometer (Respirotics Minimitter Co Inc, OR, USA). Participants had a six-week run in period of the High AGE diet since this was generally similar to their normal dietary habits, and were then randomised to either the Low AGE or High AGE diet for two weeks. At the commencement and conclusion of each two week dietary test period, bodyweight, waste-hip ratio and adiposity by 4-point bioimpedence analysis (Body Composition Analyser, Model BC-418MA, Tanita UK Ltd) were measured and body mass index calculated. A 24 hour urine collection and fasting plasma sample were taken at the commencement of the study and further spot urine and fasting plasma samples were taken prior to and post dietary interventions. Fasting plasma samples were analysed for glucose (Radiometer, Copenhagen, Denmark) and insulin via ELISA.

Renal function: Before and after each dietary period, spot urines and plasma samples were taken to assess serum creatinine and urinary albumin/creatinine ratios. Creatinine clearance was estimated via the Cockcroft-Gault formula [15] and albumin excretion rates assayed in twenty four hour urine collections at baseline.

N-carboxymethyllysine (CML) Indirect ELISA: CML was measured in human serum (1:8000) and urine samples (1:4) before and after each diet at their respective dilutions, using an in-house indirect CML ELISA that has been previously described [16]. CML was also measured in mouse chow, murine plasma, urine, and tissue using the previously described methods [17, 18].

***Immunohistochemistry*:** Immunohistochemistry analysis for CML and Collagen IV was performed on paraffin embedded neutral buffered formalin fixed murine kidneys as previously described [19].

Cystatin C, MIF and MCP-1 ELISAs: Cystatin C was measured in human plasma samples according to the manufacture's direction at a 1:1000 dilution (Human Cystatin C, BioVendor,

Mordice, Czech Republic). MIF was measured in human plasma (R&D Systems, MN, USA) and murine plasma and renal cytosolic fractions (USCN Life, Wuhan, China) according to the manufacturer's guilelines. MCP-1 was assayed in human plasma (R&D Systems, MN, USA) and murine renal cortex cytosolic protein fractions (Raybiotech, Georgia, USA).

Murine Study

Study Design: Male wild type, C57BL/6J (WT) and RAGE deficient mice (RAGE-/- [20]) on a C57BL/6J background were housed in a temperature controlled environment with a 12 hour light-dark cycle (Alfred Medical Research and Education (AMREP) Precinct Animal Centre, Melbourne, Australia). At 8 weeks of age, groups of C57BL/6J mice (*n*=10/group) were randomised to either (i) a high AGE, high fat diet Western diet, (Obese; SF05-031, Specialty Feeds, Perth, Australia, baked for 1 hour at 160^{o}C, 101.9 nmol mol^{-1} lysine^{-1} of CML/100mg) (ii) a High AGE high fat western diet plus the AGE lowering therapy alagebrium chloride, (Obese ALA, 1mg^{-1}kg^{-1}day^{-1} oral gavage; Synvista Therapeutics, New Jersey, USA) or (iii) a low AGE standard fat diet, (Lean, AIN-93G, Specialty Feeds, Perth Australia, unbaked; 20.9 nmol mol^{-1} lysine^{-1} of CML/100mg). Food intake and water access was *ad libitum* with diets matched for vitamin and amino acid content. However 40% of total energy in the Western diet was derived from animal fat (Ghee; 210g/kg) versus 16% of total energy in the low AGE diet. One further group of RAGE-/- mice consuming the western diet were also studied (n=10; Obese RAGE-/-). All animal studies were performed in accordance with guidelines from the AMREP Animal Ethics Committee and the National Health and Medical Research Council of Australia.

Murine Physiological and Biochemical Parameters: Body weight, fasting plasma glucose and fasting plasma insulin were measured at 16 weeks as previously described [21]. Twenty four hour metabolic caging to collect urine and measure food and water intake was performed at weeks 8 and 16 of the study. Albumin excretion rate (AER) was assessed using a mouse albumin ELISA kit according to manufacturer's instructions (Bethyl Laboratories, Montgomery,

TX, USA). Creatinine clearance (CrCl) was determined following HPLC (Agilent HP1100 system, Hewlett Packard, Germany) measurement of creatinine content in timed plasma and urine samples as previously described and in accordance with AMDCC guidelines [22]. Frozen renal cortex was processed via ultracentrifugation as previously described [18] in order to generate membrane, cytosol and nuclear protein fractions.

Urinary Isoprostane Concentrations: As a non-invasive measure of oxidative stress, 8-isoprostane F_2 was measured in 24 hour human urine samples collected before and after each diet by competitive ELISA (Oxford Biomedical Research, Oxford, MI, USA). Human urine samples were assayed neat and the assay was conducted as per manufacturer's instructions. Murine urine samples were also analysed neat for 8-isoprostane, according to the manufacturer's instructions (8-isporostane EIA, Cayman Chemical, Michigan, USA).

Superoxide Production: Renal superoxide was measured in fresh murine renal cortical tissue as previously described via chemiluminescence of lucigenin [23, 24].

Renal RAGE expression: Murine renal cytosolic protein fractions were assayed for RAGE protein using an ELISA specific for mouse (R&D Systems, MN, USA). Unknown values were calculated relative to a 4 parameter logistic standard curve generated using the Graph Pad Prism program. All assays were run according to manufacturer's instructions.

Statistical Analyses: Human Data are expressed as mean ± (SEM) unless otherwise stated and were analysed using paired student t-test analysis. Urinary Albumin/Creatinine values were non-parametric and therefore logged prior to analysis. Order effect of the diets was analysed via repeated measure ANOVA with order as a between subject factor. Human statistical analyses were performed using SPSS (SPSS Statistics 17.0).

Murine study analyses were performed by one way ANOVA followed by Tukey's post-hoc analysis (GraphPad Prism, 5.2, SD, USA). Mouse data are presented as mean ± SD Mouse

albuminuria data were not normally distributed and were therefore log transformed prior to analysis. A $p<0.05$ was considered to be statistically significant.

Results

Clinical Study

The baseline characteristics of the eleven participants are shown in Table 2. While diets were isocaloric and matched for macronutrient content, on a 9 MJ/day diet individuals are calculated to consume 14,090 kU CML on the high AGE diet and 3,302 kU CML on the low AGE diet. There was no effect of the dietary interventions on body weight, BMI or adiposity, which remained elevated.

Renal Function and Inflammatory Makers

Urinary albumin/creatinine ratios were significantly lower following the low AGE dietary period in obese individuals (Low v High AGE diet: p=0.02, Figure 1A). Plasma cystatin C concentrations at baseline were above normal levels in both groups due to the increased BMI of participants. Plasma cystatin C levels were further increased following consumption of a high AGE diet for 2 weeks (Low v High: p=0.02, Figure 1B). Plasma CML concentrations following high AGE consumption declined (Low v High: p=0.01, Figure 1C), whilst urinary CML concentrations increased following consumption of the high AGE diet (Low v High: p=0.03, Figure 1D). The high AGE diet increased urinary 8-isoprostanes (Low v High: p=0.02, Figure 1E). Plasma MCP-1 also known as chemokine (C-C motif) ligand 2 (CCL2) showed a increased as a result of high AGE dietary consumption (Low v High: p=0.04, Figure 1F). Conversely, however, plasma MIF significantly declined after consumption of the high AGE diet (Low v High: p=0.04, Figure 1G). There was no significant effect of the order that the diets were received on any of the parameters when we analysed via repeated ANOVA with order as a between subjects factor. There were no differences in other circulating cytokines and transcription factors including esRAGE, sRAGE, NF-κB, IL-6 and hsCRP between diets (data not shown).

Murine Study

Biochemical and metabolic parameters

Both WT and RAGE-/- mice consuming the Western style diet, high in AGEs and fat content, were obese by week 16 (ΔBW; Table 4) with significant increases in epididymal and omental adipose depots (Table 4). Increases in body weight and fat deposition after the Western diet, were prevented using the AGE lowering therapy, alagebrium (ALA, Table 4). Kidney size was unaffected by dietary consumption of a western style diet (Table 4). Fasting plasma glucose and insulin concentrations were increased in obese mice following the consumption of the western diet, in both WT and RAGE-/- mouse strains (Table 4), and the parameters were significantly improved in the mice treated with ALA.

Renal Functional Parameters

Renal function was assessed by AER and CrCl. Obese WT mice consuming the Western style diet had albuminuria (Figure 2A), which was reduced in obese RAGE-/- mice fed a Western diet but not with alagebrium. CrCl was elevated in obese WT mice and significantly improved by ALA (Figure 2B). Furthermore a western diet did not induce hyperfiltration in RAGE-/- mice (Figure 2B). All obese mice had lower plasma CML concentrations (Figure 2C), despite consuming more dietary AGEs than lean low AGE fed mice (Figure 2D). Urinary CML excretion was below detectable limits (5.6 nmol/mol lysine) in all mice. Also of interest was that obese WT (16.8 ± 11.2 kJ/day) and RAGE-/- mice (29.7 ± 6.9 kJ/day) consumed less kilojoules per day than both lean low AGE fed mice (50.3 ±3.4kJ/day; $p<0.05$ vs Obese WT) or obese mice treated with alagebrium (34.9 ± 9.4 kJ/day; $p<0.05$ vs Obese WT).

RAGE Protein Expression and Inflammation

Membranous RAGE protein concentrations in renal cortices taken from obese WT mice were significantly higher than those in lean mice consuming a low AGE diet (Figure 3A).

This parameter was not affected by treatment with alagebrium (Figure 3A). Circulating levels of soluble RAGE (sRAGE), measured via ELISA, tended to be higher in obese mice, although they were significantly lower after alagebrium therapy (Lean Low AGE (216.6±65.98) v Obese (352.2±172.4) pg/ml RAGE, $p<0.05$. Obese v Obese ALA (152.2±55.67 pg/ml RAGE, $p<0.05$). As expected there was no expression of membranous or soluble RAGE protein detected in RAGE-/- mice (data not shown). Renal MCP-1 levels were significantly lower in obese ALA treated animals and obese RAGE-/- mice (Figure 3B) when compared with untreated obese WT mice. Plasma MIF concentrations in mice were decreased with obesity and significantly increased by ALA treatment or in obese RAGE-/- mice (Figure 3C). Kidney MIF levels were increased in obese mice, which were not affected by ALA treatment, however, deletion of RAGE significantly decreased renal MIF concentrations (Figure 3D).

Obesity induced excess cortical superoxide production in the mitochondrial (Figure 4A) and cytosolic compartments (Figure 4B). Treatment with ALA and the deletion of the RAGE gene significantly decreased renal superoxide levels (Figure 4A and B). Urinary 8-isoprostane concentrations were increased in obese mice; however this was attenuated with alagebrium therapy (Figure 4B).

Discussion

This study has provided evidence that intervention using diets low in AGE content, may attenuate renal changes seen with obesity. While our current human study did not encourage weight loss in obese participants due to matching of caloric intake and the short duration of dietary intervention (two weeks), we were able to demonstrate that altering dietary AGE content alone is sufficient to improve inflammatory profiles and early renal disease. These findings are consistent with a previous study of patients with advanced end stage renal disease [12]. To complement these findings we performed studies in mice to further define potential mechanisms liking the AGE/RAGE axis to renal functional changes in the context of obesity. Indeed, our studies in obese mice highlighted that interfering with the AGE/RAGE axis by either preventing AGE tissue accumulation with the AGE lowering therapy, alagebrium or via RAGE deletion in RAGE-/- mice is protective against obesity related renal dysfunction. These findings are consistent with previous evidence that AGE formation is important in the pathogenesis of other chronic kidney diseases [18, 25-28].

The increases in the expression of the pro-inflammatory protein RAGE in kidney cortices taken from obese mice, and its contributory role to obesity related renal dysfunction in this model, was further suggested in obese RAGE-/- mice who had better renal function and less inflammation. Elevations in the circulating concentrations of soluble RAGE, sRAGE, were also seen in obese mice, consistent with findings in type 2 diabetic individuals with nephropathy who are generally obese [29, 30]. Although sRAGE was not changed after a low AGE diet in our human study, this was most likely due to the short duration of the dietary intervention. It is possible that a longer dietary duration would have ultimately led to lower circulating sRAGE concentrations. This hypothesis is supported by the improved inflammatory profile seen with consumption of a low AGE diet as reflected by decreased MCP-1 and MIF

concentrations which in the context of previous studies which have associated increases in sRAGE with systemic inflammation [30, 31].

Given the findings of the present study and the previously reported roles of RAGE, it is possible that inflammation plays a role in modulating the changes seen in this study. In both obese humans and mice there was evidence of low grade inflammation, which was enhanced by consumption of a high AGE diet. This increased plasma MCP-1 and lowered MIF concentrations attenuated by interrupting the AGE/RAGE axis, either by lowering the tissue AGE burden using dietary means, the AGE lowering therapy alagebrium or by deletion of RAGE. Activation of RAGE has already been reported to be crucial for macrophage recruitment, as highlighted by its role in host-pathogen defence [32]. Therefore, it is likely that RAGE activation as a result of AGE stimulation is a modulator of MCP-1 and MIF secretion in the present study. However, obesity related changes in circulating insulin concentrations seen in both humans and mice, may also be indirectly modulating the expression of MIF (localised in the pancreatic islets; [33]) and MCP-1 (from white adipose tissue;[34]) which are known to affect insulin secretion and action respectively.

AGEs and RAGE are also known to contribute to renal dysfunction via excess generation of reactive oxygen species [35-37]. High AGE diets in both obese humans and mice appear to influence oxidative stress as reflected by increases in urinary isoprostanes and renal superoxide production. This pro-oxidant effect of AGEs is further suggested by the findings in obese mice that received alagebrium which appeared to have less oxidative stress. RAGE deficiency did not improve obesity related increases in urinary isoprostane excretion, which was interesting given that this group also had a lack of effect on adiposity and obesity related abnormalities in glycaemic control. This suggests that the benefits afforded by low AGE diets and alagebrium on oxidative stress may be partly independent of RAGE. This is not totally surprising since AGEs can interact with other receptors in addition to RAGE and alagebrium is

likely to have additive actions that may be relevant including a modest effect as an anti-oxidant [18, 23].

In conclusion, this study suggests that a low AGE diet has an impact on modulating renal function in healthy obese individuals. Studies in murine models suggest that the mechanism responsible for AGE effects on renal function is likely to involve its receptor RAGE and include improvements in inflammation, oxidative stress and glycaemic control.

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Chapter 2: Tables and Figures

Table 1: **Representative example of isocaloric meal selections for high AGE versus low AGE diets consumed by obese individuals.**

[1] **Approximate values only as calculated from available American data for the carboxymethyl-lysine (CML) content of** foods (12). While Australian foods may differ in AGE content, all foods prepared for the high AGE diet were subjected to a high level of browning. [2] Coke a rich source of methylglyoxal in addition to CML. [3] %E: percent of total energy

High AGE diet	AGE (kU) [1]	**Low AGE diet**	AGE (kU)
Breakfast:		***Breakfast:***	
2 scrambled eggs	2,749	2 lightly poached eggs	628
1.5 slices toasted white bread (with crusts)	310	2 slices of fresh white bread (without crusts)	12
Commercial orange juice	9	Juice from a orange	0
Lunch:		***Lunch:***	
One apple	19	One apple	19
One toasted bacon sandwich (with crusts)	4,026	One avocado and ham sandwich (without crusts)	1,217
One glass Coke[2]	16	One glass diet lemonade	2
Dinner:		***Dinner:***	
Pan-fried chicken breast	5,387	Steamed chicken breast	989
Vegetables	391	Steamed vegetables	36
(fried in olive oil)	300	Olive oil dressing	300
Fried white rice	66	Boiled white rice	18
One apple	19	One apple	19
One glass Coke	16	One glass diet lemonade	2
Evening snack:		***Evening snack:***	
One glass of heated skim milk	138	One glass cold full-cream milk	48
Shortbread biscuits	644	Angel food cake	11
Total AGE content (kU)	**14,090**	**Total AGE content (kU)**	**3,302**
Total energy (MJ)	9.0	Total energy (MJ)	9.0
Protein (%E) [3]	16	Protein (%E)	16
Total fat (%E)	30	Total fat (%E)	30
Carbohydrate (%E)	54	Carbohydrate (%E)	55
Saturated fat (g)	10	Saturated fat (g)	10

	Mean (±SD)	Range
N	11	
Age (years)	30. (±9)	21 - 50
Body Mass Index (kg/m^2)	31.8 (±4.8)	27 - 36
Waist Circumference (cm)	96.9 (±18.4)	78.5 – 115.3
Waist/ Hip ratio	0.91(±0.12)	0.78 – 1.30
24hr Creatinine Clearance (ml/s)	2.4 (±1.1)	1.3 - 4.2
Urinary CML (nmol/mol lysine)	11.5 (±14.4)	0.35 – 44.4
Serum CML (umol/mol lysine)	224.5 (±166.9)	122.9 – 859.8
Fasting Plasma Glucose (mmol/L)	4.7 (±0.4)	4.1 – 5.5
Fasting Plasma Insulin (mU/mL)	10.2 (±4.1)	6.3-19.1
Insulin sensitivity (mg glucose /kg/min)	7.8 (±3.4)	2.5 – 17.1

Table 2: Baseline anthropometric and biochemical data in obese individuals recruited for the dietary intervention study (*n*=11 patients).

Table 3: Anthropometric and biochemical data at the completion of 2 weeks of dietary consumption of either a low or high AGE diet (*n*=11 patients). This clinical study in obese individuals (BMI 31.8 (±4.8)) was performed as a single blinded, randomised, cross-over dietary intervention study.

ns; not significant (*p>0.05*), *; A non-parametric analysis was performed

	Following Low AGE Diet	**Following High AGE Diet**	
	Mean (±SD)	**Mean (±SD)**	***P* value for Change**
Weight (kg)	93.2(±15.9)	93.9(±15.8)	ns
BMI (kg/m^2)	31.5(±4.2)	31.4(±4.2)	ns
Body Fat (%)	29.3(±6.4)	29.2(±6.8)	ns
Urine Albumin (mg/d) *	20.27(±34.8)	16.05(±20.9)	ns
Serum Creatinine (µmol/L)	72.3(±18.3)	70.2(±13.5)	ns
Total Cholesterol (mmol/L)	4.2(±0.9)	4.2(±1.0)	ns
Fasting Plasma Glucose (mmol/L)	5.1(±0.3)	4.8(±0.3)	ns

Table 4. Murine physiological and metabolic parameters at study completion (week 16).

Data are presented as Mean (±SD). Obese (High AGE/High Fat diet), ALA (AGE lowering therapy, alagebrium chloride 1mg/kg/day), RAGE-/- (RAGE deletion).

* $p<0.05$ v Lean Low AGE, † $p<0.05$ v Obese

		Δ Body Weight	Left Kidney Weight	Omental Adipose Tissue	Epididymal Adipose	KW/BW Ratio	Plasma Glucose	Plasma Insulin
		(g)	(g)	(g)	(g)	(x 10^3)	(mmol/L)	(ng/mL)
C57BL/6J	Lean	2.9 (±2.4)	0.19 (±0.02)	33.5 (±1.8)	0.89 (±0.3)	11.35 (±1.2)	5.3 (±1.8)	0.24 (±0.21)
	Obese	11.0 (±1.6) *	0.19 (±0.01)	40.8 (±2.6) *	1.59 (±0.2) *	9.35 (±1.2) *	8.5 (±1.5) *	1.69 (±0.68) *
	Obese ALA	8.1 (±2.5) *†	0.17 (±0.02)	37.8 (±3.8) *	1.31 (±0.3) *†	9.74 (±0.8)	6.6 (±1.5)	0.66 (±0.5) *†
BAKE-/-	Obese	15.4 (±2.2) *†	0.19 (±0.01)	39.7 (±2.6) †	1.94 (±0.3)†	9.8 (±0.6)	10.3 (±3.1)	3.36 (±0.97) *†

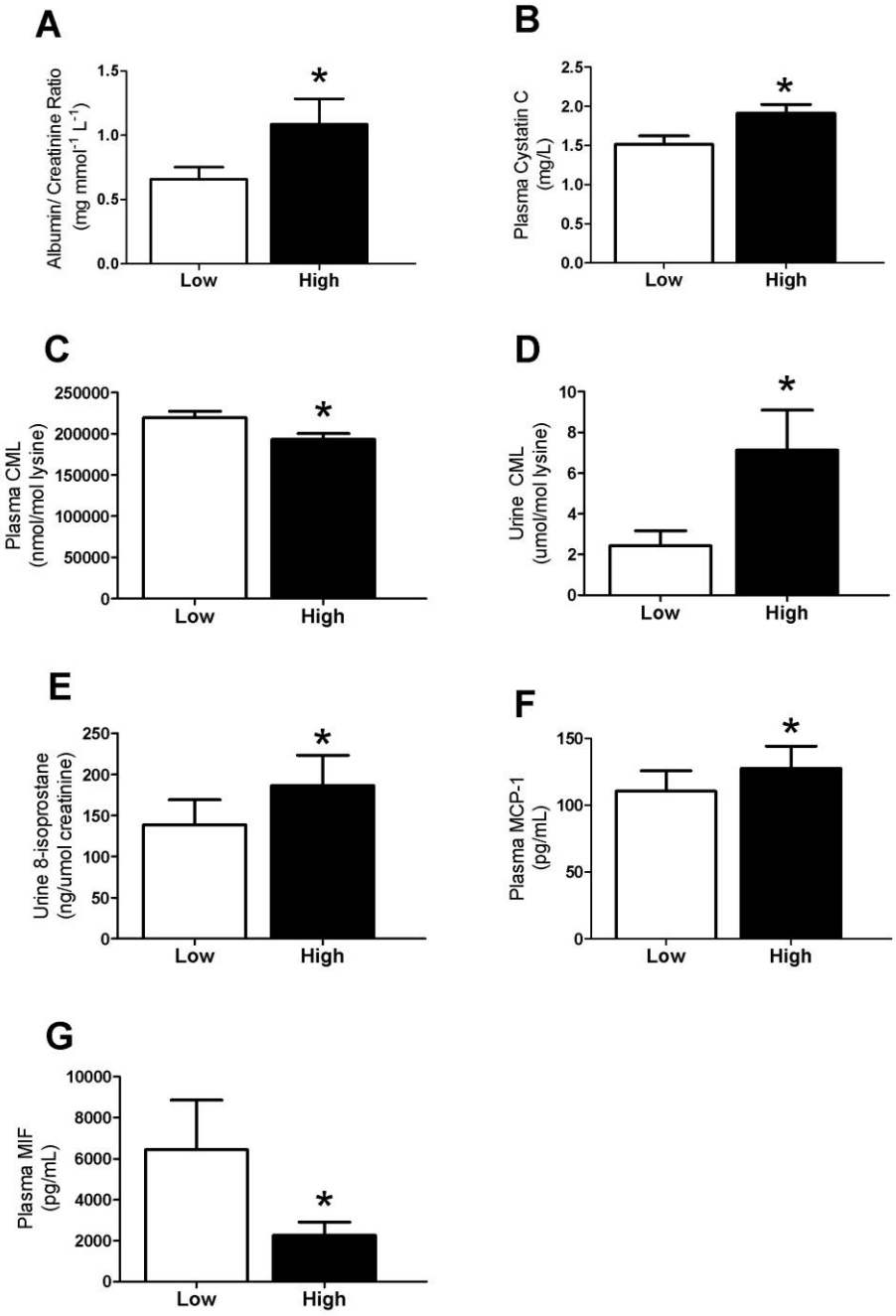

Figure 1: Renal and Inflammatory Parameters in Obese Humans Following Dietary Interventions

Assays were performed in samples collected from the same obese individuals following consumption of a diet either low or high in AGE content for two weeks. A) Urinary Albumin/Creatinine ratios,), B) Plasma Cystatin C concentration, C) Plasma concentrations of the AGE, CML, D) Urinary CML concentrations, E) Urinary 8-isoprostanes F) Plasma monocyte chemotactic protein-1 (MCP-1) concentrations, G) Plasma macrophage migration inhibitory factor (MIF) concentrations.

* $p<0.05$ Low versus High AGE diet, Student's paired t-test.

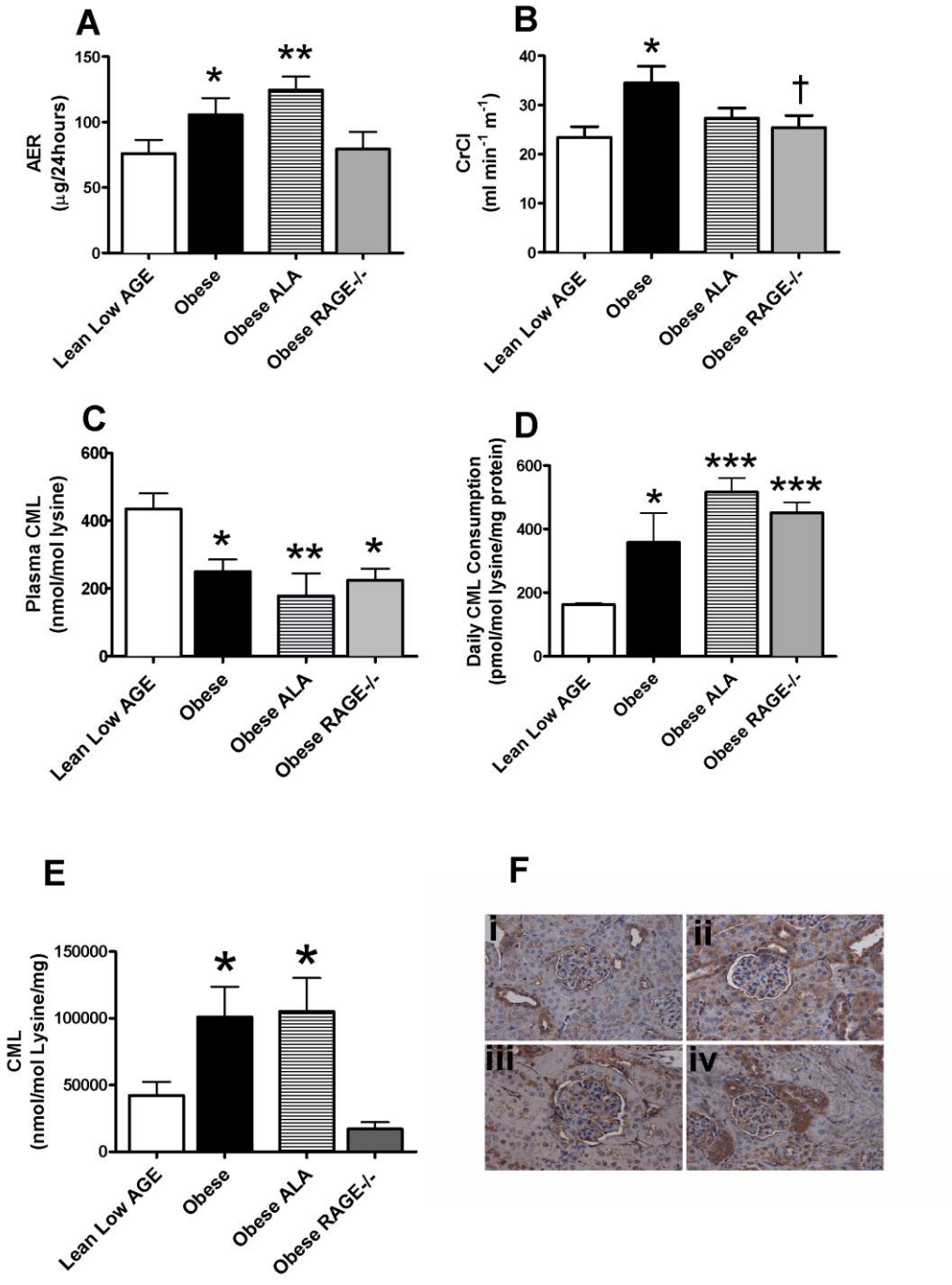

Figure 2: Murine renal and biochemical parameters at study completion

Groups of mice were followed for 16 weeks. A) Urinary albumin excretion rate (AER) over 24 hours measured by ELISA. B) Creatinine clearance (CrCl) as determined by HPLC following correction for body surface area. **C-E)** CML analysed by ELISA, in Plasma (C), Dietary CML consumption over 24 hours (D) and kidney cortex membrane protein (E). (F) CML immunohistochemistry staining on paraffin fixed kidney sections from; (i) Lean Low AGE (ii) Obese, (iii) Obese ALA and (iv) Obese RAGE-/-. Obese (High AGE/High Fat diet), ALA (AGE lowering therapy, alagebrium chloride 1mg/kg/day), RAGE-/- (RAGE deletion). Data for AER were logarithmically transformed as these were not normally distributed. Other data are presented as Mean ±SD.

* p<0.05 v Lean Low AGE, ** p<0.01 v Lean Low AGE, *** p<0.001 v Lean Low AGE, † p<0.05 v Obese

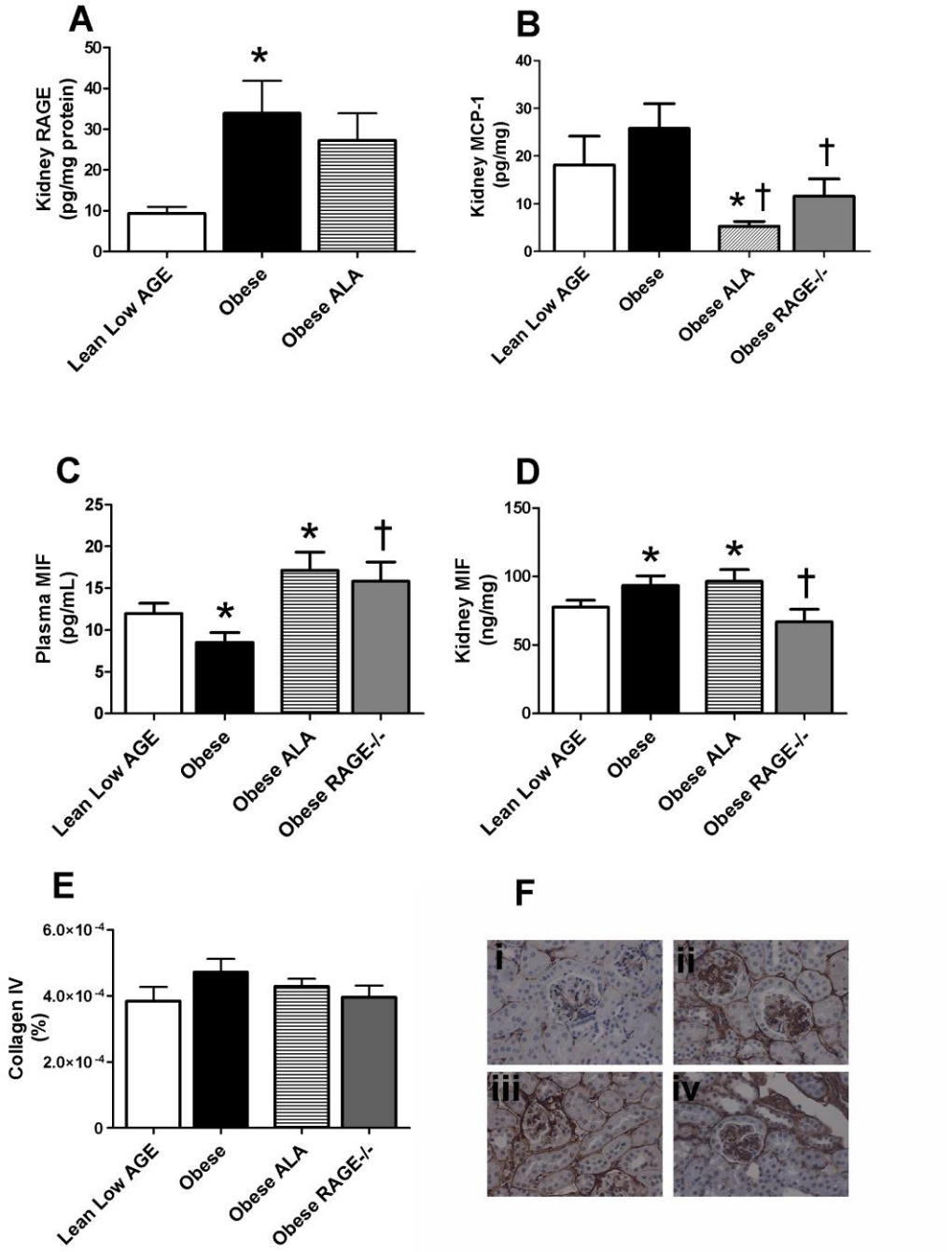

Figure 3. Murine inflammatory parameters following 16 weeks of High AGE dietary feeding.

A) RAGE protein content in kidney cortices measured by mouse specific ELISA. Kidney cortices from obese RAGE-/- did not have measurable membrane RAGE. B) Renal cytosolic MCP-1 assayed by ELISA. C) Plasma MIF concentration assayed by ELISA. D) Renal cytosolic MIF content assayed by ELISA. E) Semi quantification of Collagen IV in glomeruli (F) Representative collagen IV immunohisochemistry staining used for semiquantification (i)Lean Low AGE (ii) Obese (iii) Obese ALA and (iv) Obese RAGE-/-. Obese (High AGE/High Fat diet), ALA (AGE lowering therapy, alagebrium chloride 1mg/kg/day), RAGE-/- (RAGE deletion).

* $p<0.05$ v Lean Low AGE, † $p<0.05$ v Obese

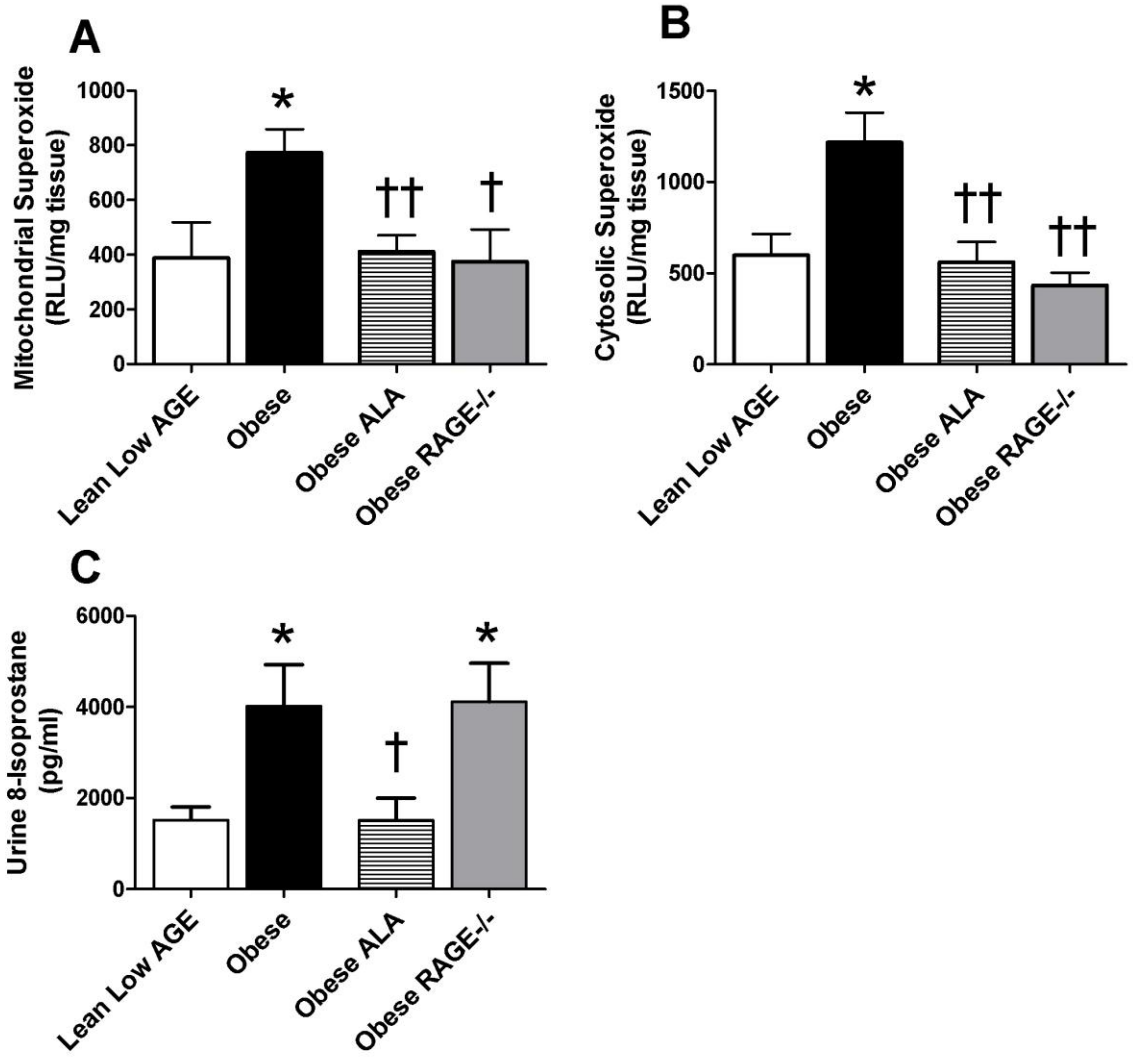

Figure 4. Murine oxidative parameters following 16 weeks of dietary intervention.

A) Mitochondrial NADH-dependent superoxide production in fresh kidney cortices, measured via lucigenin enhanced chemiluminescence. B) Cytosolic NADPH-dependent superoxide production in fresh kidney tissue. C) 8-Isoprostane measured via ELISA in urine. Obese (High AGE/High Fat diet), ALA (AGE lowering therapy, alagebrium chloride 1mg/kg/day), RAGE-/- (RAGE deletion).

* $p<0.05$ v Lean Low AGE, † $p<0.05$ v Obese, †† $p<0.01$ v Obese

Chapter 3

Chapter 3: Introduction

In this publication we wanted to study the effect of estrogens secreted from white adipose tissue depots on the expression of RAGE. To do this we again use male RAGE deficient (RAGE-/-) mice and littermate controls were high fat fed for a period of 16 weeks, rendering them obese. We further analysed the transcriptional binding domain of the RAGE gene via chromatin immunoprecipitation, to investigate if estrogen responsive elements played a role in the development of obesity related renal disease.

This manuscript was submitted to the journal, Diabetologia, in October 2011.

Monash University

Declaration for Thesis Chapter Three

Declaration by candidate

In the case of Chapter Four; the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental procedure/protocol, experimental design, preparation of manuscript	75%

The following co-authors contributed to the work. Co-authors who are students at Monash

University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
MT Coughlan	Collection of samples	
S Andrikopoulos	Performed IPGTT	
MC Thomas	Performed HPLC	
MA Febbraio	Reviewed manuscript, provided CLAMS analysis	
P Nawroth	Provided model, reviewed manuscript	
P Kantharidis	Review of Manuscript, Experimental design	
ME Cooper	Review of manuscript	
A Bierhaus	Review of manuscript, provided model	
JM Forbes	Experimental design, preparation of manuscript	

Candidate's Signature		**Date** 24/10/2011

Declaration by co-authors

The undersigned hereby certify that:

(13) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(14) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(15) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
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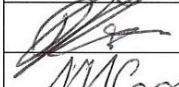
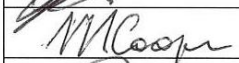
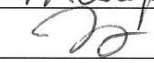
Signature 6		10/10/2011
Signature 7		10.10.11
Signature 8		10/10/11
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Nuclear Expression of Receptor For Advanced Glycation End Products Perpetuates Obesity Related Kidney Damage Via Adipose Derived Estrogens

Running Title: ESTROGEN EFFECT ON RAGE EXPRESSION

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Abstract

Dietary habits and a sedentary lifestyle are major contributors to type 2 diabetes and its complications, as are risk factors such as male gender or menopause in females. In these studies we examined the effects of peri-renal adipose derived estrogens on the renal expression of the receptor for advanced glycation end products (RAGE), in high fat fed (HFF) WT (wild type) and RAGE deficient (RAGE-/-) mice. First, we identified that HFF associated renal dysfunction observed in obese WT mice, was not seen in obese RAGE-/- mice. Obese WT mice also had increased of estrone to 17β-estradiol as compared with obese RAGE-/- mice. Chromatin immunoprecipitation (ChIP) revealed that the estrogen receptor β (ER-β), was interacting with the RAGE promoter at binding sites for both RelA and Sp-1 in obese WT kidney cortices. This produced an increase in both renal RAGE membrane expression and circulating soluble RAGE in obese WT mice. Obesity also increased nuclear expression of RAGE in WT mice and chromatin immunoprecipitation assays (ChIP) confirmed that nuclear RAGE/ER-β complexes were interacting with SP-1 sites within the RAGE promoter. This novel finding requires further investigation, but suggests that not only can adipose derived estrogens modulate the expression of RAGE, contributing to renal dysfunction in obesity, but that this may be further exacerbated via the binding of nuclear RAGE to the 5' upstream regulatory sequence of the *RAGE* gene.

Introduction

Dietary habits and a sedentary lifestyle are major risk factors for obesity, diabetes and its complications. Activation of the receptor for advanced glycation end products (RAGE) is also implicated in the pathogenesis of both diabetic micro [1, 2] and macrovascular [3-6] disease and recently as a contributor to obesity related cardiac [7] and vascular [8] abnormalities. It is postulated that RAGE expression is mediated via both ligand activation and transcriptional regulation, via factors such as nuclear factor kappa B (RelA) and specific protein 1 (Sp-1) [9]. In addition, previous studies in vascular endothelial cells have shown that estrogens such as 17β-estradiol (E2) can also modulate the gene expression of RAGE via activation of estrogen receptor-α (ER-α [10]). The *AGER* gene encodes for two major protein isoforms of RAGE, full length (RAGE) and soluble RAGE (sRAGE), though the transcriptional events leading to the ultimate expression each isoform are yet to be determined [11].

There are also other major risk factors for the development of type 2 diabetes, such as being male or post-menopause for women [12]. This suggests that estrogen, or likely the balance between the different major isoforms, estrone (E1), estradiol (E2) and estriol, may be an important determinant of the vascular complications of diabetes, including nephropathy [13]. E1 is secreted from white adipose tissue and is the primary estrogen isoform seen in obese individuals. In addition, the protection against cardiovascular and renal disease afforded by high levels of E2 in menstrual women is abrogated by obesity, most likely due to an increase in E1 levels, altering estrogen balance [14]. Indeed, previous studies have identified that decreases in E2 levels are associated with declining renal function in diabetes, leading to changes in estrogen signalling in target tissues primarily from estrogen receptor α (ER-α) to estrogen receptor β (ER-β) [15-17]. Furthermore,

renoprotection is granted by exogenous E2 administration in experimental models [17-20], or by selective estrogen receptor modulation in human diabetic nephropathy (DN, [21]).

Hence, in the present study, we investigated if changes in local peri-renal white adipose depots and the estrogens that they secrete altered renal function in obese RAGE deficient and wild type mice. The specific regulation of renal RAGE gene expression via ER-α and for the first time ER-β, in addition to RAGE itself, were examined.

Methods

Animal models

Male wild type, C57BL/6J (WT) and RAGE deficient mice (RAGE-/- [22],[23]) on a C57BL/6J background were housed in a temperature controlled environment with a 12 hour light-dark cycle (Alfred Medical Research and Education (AMREP) Precinct Animal Centre, Melbourne, Australia). Mice were plug-mated and at exactly 8 weeks of age, groups of mice (n=10/group) were randomised to either a standard growth chow diet (Control, AIN-93G, Specialty Feeds, Perth, Australia) or an identical diet high in fat content (HFF, SF05-031, Specialty Feeds, Perth, Australia) and followed for 16 weeks. Both diets were matched for vitamin and total calorie content, and available *ad libitum*. However 40% of total energy in the high fat diet was derived from animal fats (Ghee; 210g/kg) versus 16% in the control diet. All animal studies were performed in accordance with guidelines from the AMREP Ethics Committee and the National Health and Medical Research Council of Australia.

Measurements of Physiological and Biochemical Parameters

Body composition analyses were performed as described previously [24]. Total cholesterol and triglyceride concentrations were measured in plasma using a standard commercial enzymatic assay using a Beckman Coulter LX20PRO Analyser (Beckman Coulter Diagnostics Australia). Fasting plasma glucose and fasting plasma insulin were measured at 16 weeks as previously described [25]. Body Composition Analysis, the total body fat and lean muscle mass was measured using an Echo MRI 4-in-1 TM System (Echo Medical Systems, Houston, TX).

Mice were housed in metabolic cages to collect urine and measure food and water intake, for a 24 hour period. Albumin excretion rate (AER) was assessed using a mouse albumin ELISA kit according to manufacturer's instructions (Bethyl Laboratories, Montgomery, TX, USA). Creatinine clearance (CrCl) was determined following HPLC (Agilent HP1100 system, Hewlett Packard, Germany) measurement of creatinine content in timed plasma and urine samples as previously described and in accordance with AMDCC guidelines [26]. Frozen renal cortex was processed via ultracentrifugation as previously described [27] in order to generate membrane, cytosol and nuclear protein fractions.

Real Time RT-PCR

Two micrograms of total RNA was extracted from the left kidney cortex and genomic DNA removed using a DNA-Free^{TM} kit (Ambion Inc., Austin, Texas, USA). cDNA was synthesized using the Superscript First-Strand Synthesis kit for RT-PCR (Gibco BRL, Grand Island, New York, USA). Gene expression of AGER (RAGE) was analysed by real-time reverse transcription polymerase chain reaction (RT-PCR) performed with the Taqman system based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer, CA, USA). Sequences used for AGER (RAGE) were, forward primer GCTGTAGCTGGTGGTCAGAACA, reverse primer CCCCTTACAGCTTAGCACAAGTG and probe 6-FAM CACAGCCCGGATTG (NM_007425). The amplification protocol was 50˚C for 2 min, 95˚C for 10 min, 40 cycles of 95˚C for 20 seconds and 60˚C for 1 min. Relative quantification of gene expression was performed by the comparative C_T ($\Delta\Delta C_T$) method, with ribosomal 18S (18S rRNA Taqman Control Reagent kit, ABI Prism 7700) as the endogenous control. All results were expressed relative to values from WT control mice, which were assigned an arbitrary value of 1.

Processing of Adipose Tissues

Renal and omental adipose deposits were weighed and stored at -80^{o}C. Adipose protein was precipitated with iso-propanol and then incubated with ethanol-guanidine (0.3M guanidine-HCl in 95% EtOH). Samples were then washed with 100% EtOH. After the pellet was air dried urea/DTT (10M urea, 50mM DTT in water) solution was added. Samples were placed at 95^{o}C for 3 min and then placed on ice. Samples were then sonicated using a probe sonicator.

Endogenous Sex Steroid Analysis

Estrone (E1) and 17β-estradiol (E2) concentrations were measured via EIA (Estradiol and Estrone EIA Kits, Catalogue No. 582251 and No. 582301 respectively, Cayman Chemical, Ann Arbor, MI). The Estrone assay specifically measured estrone sulfatase and estrone glucocorticoids, which represents the entire Estrone content in sample. The E2 assay specifically identified total 17β-estradiol content. Assays were performed according to the manufacture's instructions. E1 and E2 expression was corrected for adipocyte lipid-binding protein (aP2) content, to control for the amount of adipose tissue within the protein sample. aP2 ELISA was performed on renal and omental adipose samples according to the manufacturers' instructions (BioVendor, Mordice, Czech Republic).

RAGE ELISA

Renal cytosolic and membrane protein fractions, and plasma were assayed for RAGE via ELISA according to the manufacturer's instructions (R and D Systems, Minneapolis, MN). Unknown values were calculated relative to a 4 parameter logistic standard curve generated using Graph Pad Prism (Version 5, Graphpad Software, L Jolla, CA).

Western Immunoblotting

Kidney cortical nuclear fractions was used for western immunoblotting. Protein (15 μg) was incubated for 5 minutes at 95˚C and separated on a 12% TEO-CL SDS-PAGE gel (Expedeon, CA, USA) and transferred onto polyvinyl dine fluoride membranes. Non-specific binding sites were blocked for an hour with 0.4% (w/v) skim milk powder in PBS-T, followed by incubation with primary antibodies; mouse anti- ER-α; 1:100 (Millipore, MA, USA), polyclonal rabbit anti- ER-β 1:200 (Millipore, USA) or polyclonal rabbit anti- Histone 3 1:1000 (Millipore, MA, USA) for 10-15 minutes on the Snap i.d protein detection system (Millipore, MA, USA). Bound antibodies were amplified and detected using Amersham ECL kit (Amersham Biosciences, Piscataway, NJ). Band intensity was quantified using a microcomputer imaging program and expressed relative to background and histone-3 expression.

Immunohistochemistry

Four micron sections were cut from paraffin embedded kidney. Following de-waxing and rehydration, sections were blocked in 3% H_2O_2/TBST for 20 minutes and then in PBA for 10 minutes. Primary antibodies; rabbit anti- ER-α 1:100, rabbit anti ER-β 1:25, or goat anti-RAGE 1:100 (Millipore, MA, USA) were incubated at 4^oC overnight. Sections were then developed with DAB and counterstained with haematoxylin. Quantification of renal nuclear staining for RAGE was performed as previously described [28], using Image pro-Plus (Media Cybernetics, Bethesda, MD).

Chromatin Immunoprecipitation

Kidney cortices were analysed for activity in the 5’ flanking region of the RAGE gene. Probes were designed using BLAST and were created to allow specific analysis of whether ER-α or

ER-β activity was able to directly influence the up-regulation of the RAGE gene. The primers designed also allowed for specific identification of the exact region of RAGE promoter that was activated. Region A. Probe; TCTGGAGATGTCAGCCC, forward primer; GTTCCCCACCCCACTTATATACTCT, reverse primer; TCCCCATTTTTGGCATCTCT. Region N. Probe; CCCTCAGACACATCCTC, forward primer; CAGCCCTGAACCCTTCATCTG, reverse primer; CCCATGGTGACAGTCTTGAAGA. Region S. Probe; ACCTGAAGGACTCTTG, forward primer; GGTCGGGTGAGATTGCTTCTAG, reverse primer; TGCCAGGAATCTGTGCTTCTG. The method used for chromatin immunoprecipitation (ChIP) is described in detail in the supplementary information.

All results were expressed relative to values from WT Control, which were assigned an arbitrary value of 1. Statistical analysis was performed, and reported on Log-transformed data (Y=Log(Y)) as data was found to be non-parametric.

Statistical Analysis

Results are expressed as Mean (±SD) unless otherwise specified. Analyses were performed by ANOVA followed by, with Tukey's post-hoc analysis, unpaired Student's t-test analysis (Version 5, Graphpad Software, L Jolla, CA). A $p<0.05$ was considered to be statistically significant.

Data for albuminuria were not normally distributed and therefore were analysed following logarithmic transformation.

Results

Body Composition, metabolic and biochemical analysis following 16 weeks of High fat feeding (HFF)

At baseline (Week 0) WT mice were significantly heavier than RAGE-/- mice (WT; 29.93±1.6 v RAGE-/-; 23.29±1.9 g; $p<0.001$). Both RAGE-/- and WT mice had a significant increases in body weight over the study duration when fed a high fat diet (Δ BW; Table 1). Magnetic resonance imaging revealed that high fat feeding significantly increased body fat mass and % body fat to the same degree in both RAGE -/- and WT mice (Table 1). However, obese RAGE-/- had lower lean body mass as compared with obese WT mice (Table 1).

Fasting plasma glucose and fasting plasma insulin levels were increased following the consumption of a high fat diet, in both mouse strains (Table 1). Plasma insulin levels were further increased in RAGE-/- HFF mice when compared to WT HFF mice.

HFF increased the daily caloric intake in mice. This coincided with significantly increased plasma cholesterol and plasma triglyceride levels in HFF mice (Table 1). Interestingly RAGE-/- Control mice had increased plasma triglyceride levels compared to WT Control mice (Table 1).

Renal Functional Parameters are worsened by HFF

Obesity has been previously associated with renal abnormalities [29-32]. We observed a decline in renal function in our study with obesity. Following 16 weeks on a high fat diet, WT HFF mice had elevated urinary albumin excretion rates (AER; Figure 1A), when compared with WT Control mice. WT HFF mice exhibited renal hyperfiltration as compared to WT Control, evident by an increase in creatinine clearance (CrCl; Figure 1B), when

corrected for body weight. This finding remained significant when adjusted for lean body mass, demonstrating hyperfiltration in WT HFF mice as compared with RAGE -/- HFF mice (Figure 1C). Importantly, these increases in urinary AER or CrCl seen in obese WT mice, were not evident in RAGE-/- mice fed a high fat diet, despite their increase in adiposity compared to WT Control mice (Figure 1A & B).

Adipose Tissue Depots and Estrogen Content following 16 weeks of HFF

Adipose tissue is a source of systemic estrogen in obesity [33,34]. We therefore measured estrone (E1) and 17β-estradiol (E2) concentrations in plasma, urine and white adipose tissue homogenates. The majority of plasma samples for both E1 and E2, had concentrations which were below assay detection limits (lower assay limit concentrations; E1: 3.3 pg/ml and E2; 7.8 pg/ml, data not shown). There were however, significant increases in E1 concentrations in omental fat pads from WT HFF mice, which were not seen in omental fat pads taken from RAGE-/- HFF mice (Figure 2C). Peri-renal adipose tissue E1 concentrations, corrected for the adipocyte lipid-binding protein (aP2) content, were also lower in RAGE-/- HFF mice as compared with WT HFF mice (Figure 2D). In addition, RAGE-/- Control mice had lower peri-renal adipose tissue E1 concentrations when compared with WT Control mice (Figure 2D). There were no changes in E2 concentrations within omental fat, amongst groups (Figure 2E). However, mice with an *AGER (RAGE)* gene deletion showed a decline in E2 concentrations in peri-renal fat, with both Control and high fat feeding (HFF, Figure 2F).

In omental adipose tissue, the ratio of estrogens (E2:E1; 17β estradiol:estrone) was increased by high fat feeding, however this was elevated to a lesser extent in RAGE-/- HFF mice (Figure 2G). E2/E1 ratio was decreased in RAGE-/- Control mice compared to WT

Control mice (Figure 2G). In peri-renal adipose tissue, the ratio of estrogens was increased in WT HFF mice compared to WT Control mice (Figure 2H), further highlighting the differential biological findings among the various white adipose tissue deposits. We did not observe an increased E2/E1 ratio in peri-renal fat deposits from RAGE-/- mice (Figure 2H).

Renal Cortical Estrogen Receptor Expression

Following evaluation of localised omental, peri-renal and systemic concentrations of E1 and E2, we next assessed the expression of the estrogen receptors (ER-α and ER-β) in renal tissue. Within paraffin embedded kidney sections, staining for both cytosolic and nuclear expression of ER-α were evident in WT (Figure 3A) and RAGE-/- (Figure 3C) mice fed control diets. High fat feeding enhanced nuclear immunohistochemical staining for ER-α, which was not seen in RAGE-/- HFF mice (Figures 3B & 3D).

ER-β expression in paraffin embedded kidney sections was localised within the renal tubules, but was not evident within glomeruli (Figures 3E-H). In RAGE-/- mice there was expression of ER-β on the basolateral surface of tubular epithelium, however this expression was not evident in WT mice. Nuclear expression of ER-β was also observed in HFF mice, which was not evident in Control fed mouse groups (Figures 3E-H).

We quantified renal ER-α and ER-β expression in nuclear fractions using western immunoblot analysis (Figure 4A). Both ER-α and ER-β expression tended to increase in nuclear fractions but this did not reach significance (Figure 4B & 4C).

Interactions between ER-α and ER-β and the promoter region of RAGE

The promoter region of the RAGE gene; *AGER* (encoding for the proteins RAGE, soluble RAGE and N-RAGE [35]), was separated into three regions of interest, namely; the A.

Region, N. Region, or the S. Region, which contained a majority of specific transcription factor binding sites for; activator protein 1 (AP-1), nuclear transcription factor-κB (NF-κB) and Sp-1 respectively (outlined in Figure 5A). ChIP analysis performed using antibodies to either ER-α or ER-β, demonstrated no significant differences in DNA binding activity within the A. Region of the RAGE promoter (data not shown). Furthermore, no significant differences were detected within the N. Region or within the S. Region of the RAGE promoter using an ER-α antibody (Figure 5B & C), indicative of a lack of ER-α association with AP-1, NF-κB or Sp-1 binding within the RAGE promoter in chromatin extracted from renal cortices.

When chromatin from renal cortices was immunoprecipitated using an ER-β antibody, we observed a significant increase in DNA binding within the N. Region of the RAGE promoter in WT HFF mice (Figure 5D), which was not seen in RAGE-/- HFF mice. Furthermore, these changes were also mirrored within the S. Region of the RAGE promoter, where WT HFF mice had an increase in ER-β binding and *AGER* promoter activity, when compared to WT Control mice or to RAGE-/- HFF mice (Figure 5E).

RAGE acts as a transcription factor within its own promoter region

Following from our observation that RAGE protein is present within the nucleus of renal cortices, we investigated whether RAGE protein could act as a transcription factor within the promoter of the *AGER* (RAGE) gene, thereby self-perpetuating its own expression. Hence chromatin was also immunoprecipitated with an antibody to RAGE and binding to the flanking region of the RAGE gene was analysed as described above for ER-α and ER-β. Firstly, there is a significant increase in RAGE binding to the A. Region (Figure 5F), although fold induction within the N. Region and S. Region did not significantly differ between groups (Figure 5G and 5H).

RAGE Gene and Protein Expression

We assessed both mRNA and protein expression of RAGE and noted that renal cortical gene expression of *AGER* (RAGE) was decreased with high fat feeding (Figure 6A). There was an increase in the expression of renal cortical RAGE with high fat feeding, which included nuclear expression in both glomeruli and tubules (Figure 6B & 6C). Kidney membranous expression of RAGE protein in cortices from WT HFF mice was increased when compared to WT Control mice (Figure 6D). As expected there was no membranous RAGE expression seen in RAGE-/- mice (Data not shown). RAGE protein expression within cytosolic fractions was decreased with high fat feeding in WT mice (Figure 6E). For the first time, we have identified that RAGE protein expression is localised to the nuclear compartment within renal cortices and we observed a significant increase in nuclear RAGE expression with high fat feeding (Figure 6F). Plasma concentrations of soluble RAGE (sRAGE) were also increased in WT HFF mice (Figure 6G), when compared with WT mice.

The DNA binding activity of the transcription factor RelA, was increased in renal nuclear fractions taken from cortices of WT HFF mice as compared to control fed mice. (Figure 6H). There were no changes in RelA activity, however, seen in RAGE-/- mice (Figure 6H). Nuclear RelB activity, representative of the non-canonical pathway for induction of RAGE expression, was increased with high fat feeding in WT but not RAGE-/- mice, when compared to control fed mice (Figure 6I).

Discussion

This group of studies demonstrates for the first time the novel action of RAGE as a regulator that may act to self-perpetuate its own transcription, thereby contributing to obesity related kidney abnormalities. Given that we also demonstrate evidence for the modulation of *AGER* (RAGE) expression via ER-β secreted from localised white adipose tissue, there may be multiple triggers which contribute to this self-perpetuating cycle offering a number of potential sites for therapeutic intervention.

We demonstrated differences in paracrine estrogen ratios contributed to the development of renal disease in obese mice, independent of changes in glycaemic control. Specifically, estrone secreted by peri-renal fat bound to renal ER-β receptors and this modulated the expression of the pro-inflammatory receptor RAGE. It is possible, given that a number of transcripts have been shown for the AGER gene [36,37], that these combinations of transcription factors (such as AP-1, ER-β, ER-α, NF-κB and Sp-1) may encode for different RAGE proteins. It is likely in renal disease that this contributes to a feed forward loop of sustained RAGE activation and signalling which further exacerbates end organ injury, since mice deficient in RAGE were protected against renal disease and downstream RAGE signalling events seen with obesity in WT mice within this study.

Hudson *et al* [36] identified that there are a multiple isoforms of RAGE encoded for by the *AGER* gene, and that isoforms of RAGE differ in diabetic and wild-type mice. Previously only three of these have been studied in detail, membrane bound full length-RAGE, endogenous secretary RAGE and N truncated-RAGE. In the present study, we have identified a novel role for RAGE as a transcriptional regulator (tr-RAGE) for the first time. In our chromatin analysis we demonstrated that tr-RAGE associates with DNA in the A. Region of the *AGER* gene promoter region. The mechanism behind this interaction is yet to be

established, but may be via direct binding to the *AGER* promoter region, or by associating with a transcriptional complex either Sp-1, AP-1 or NF-κB. Indeed, we demonstrated that *AGER* promoter bound ER-β/SP-1 complexes, perhaps in association with nuclear RAGE in the context of an overall decrease in transcription of the AGER gene, in the kidneys of WT high fat fed mice. Interestingly, in these animals increased protein expression of membranous and soluble RAGE was observed and was associated with a pro-inflammatory state, which was not seen in high fat fed RAGE-/- mice. This is consistent with previous studies which have shown decreased expression of AGER gene in response to 17-β-estradiol (E2) deficiency, in ovariectomised rat, in association with inflammation and cardiac disease [38].

Previously, *in vitro* studies have shown increases in RAGE gene expression in vascular endothelial cells treated with 17β-estradiol, via ER-α/Sp-1 DNA binding of the 5' flanking region of the RAGE gene [10]. In the present model, it is likely that increased local estrone secretion from peri-renal adipose tissue was responsible for the formation of the ER-β/Sp-1 complex seen in kidneys from obese WT mice. This suggests that localised autocrine or paracrine production of other gene promoters, such as tr-RAGE, which contain independent estrogen responsive elements in their own promoters (eg. Sp-1 and AP-1), may be modulated by excesses of locally secreted estrone from white adipose tissue.

Males are generally at greater risk of developing renal disease, particularly in the context of metabolic abnormalities, [39, 40]. Estrogens may hold the key to this sexual dimorphism. Within the present study, increases in estrogen receptor ER-β expression in renal cortex were associated with higher estrone content of peri-renal adipose tissue, as well as declining renal function. Therefore one could postulate that modulation of ER-β expression may be a potential therapeutic target for the treatment of renal disease as the

result of obesity and other metabolic abnormalities, since there are a number of undesirable side effects associated with modulation of estrogen (E1:E2) ratios in humans, such as the appearance of female characteristics in men [41, 42] and increased risk of breast [43] and endometrial cancer in women [44]. In support of this, selective estrogen receptor modulator (SERM) targeted therapeutics such as tamoxifen and raloxifene, protect against renal disease in experimental models of both type 1 and type 2 diabetes [18, 45, 46] and already have widespread use in humans albeit for other implications [47, 48]. Their effects on ER-β however, remain unknown. Furthermore, when these therapeutics selective estrogen receptor modulators (SERMs) bind to estrogen receptors in the same ligand-binding pocket as estrogens, they result in conformational changes which prevent access to the activation region of the receptor, which can either activate or repress transcriptional co-activators necessary to facilitate the activation of estrogen-responsive genes [49].

This group of studies may demonstrate an important new isoform of RAGE, 'tr-RAGE' that in the context of obesity, may contribute to a self-perpetuating cycle of kidney damage. Importantly, it was evident that excesses of locally rather than systemically derived estrone (E1) via ligation with renal ER-β, resulted in increases in renal RAGE protein expression and activation of a pro-inflammatory state leading to kidney disease. Therefore, we suggest that the local paracrine environment may be of greater pathological relevance in the development of end organ injury as a result of chronic diseases associated with obesity. However, the full role of RAGE as a transcription factor in the development of chronic renal disease such as that which results from obesity needs to be further investigated.

Abbreviations

AGEs, Advanced glycation end products; ER-α, estrogen receptor alpha; ER-β, estrogen receptor beta; E1, estrone/oestrone; E2, estradiol/oestradiol; DN, diabetic nephropathy; NF-κB, nuclear factor kappa B; RAGE, receptor for advanced glycation end products; Sp-1, specific protein 1; T2D, type 2 diabetes; activator protein 1 (AP-1).

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Chapter 3 Tables and Figures

		WT		RAGE-/-	
		Control	**HFF**	**Control**	**HFF**
Δ Body	***(g)***	2.9 (±2.4)	11.0 (±1.6) *	8.3 (±2.1) *	15.4 (±2.2) *†‡
Lean Body Mass	***(g)***	25.25 (±1.3)	27.83 (±1.2) *	25.00 (±2.9)	23.79 (±1.6) †
Fat Mass	***(g)***	5.74 (±1.9)	13.01 (±1.5) *	8.37 (±1.2)	11.67 (±1.4) ‡
% Body Fat	***(%)***	18.24 (±5.3)	31.78 (±2.3) *	25.14 (±6.6)	32.79 (±3.8) ‡
Fasting Plasma Glucose	***(mmol/L)***	5.26 (±1.8)	8.53 (±1.5) *	6.55 (±1.5)	10.28 (±3.1) *
Fasting Plasma Insulin	***(ng/ml)***	0.24 (±0.2)	1.69 (±0.7) *	0.66 (±0.5)	3.36 (±0.9) * † ‡
Caloric Intake	***(KJ/24h)***	0.24 (±0.2)	1.82 (±0.6) *	0.37 (±0.4)	3.22 (±0.9) * † ‡
Plasma Cholesterol	***(mmol/L)***	1.6 (±0.8)	2.9 (±0.7) *	1.8 (±0.7)	3.9 (±1.6) * †
Plasma Triglycerides	***(mmol/L)***	0.64 (±0.2)	0.96 (±0.2) *	0.95 (±0.2) *	0.98 (±0.6) *

Table 1. Physiological, Biochemical and Metabolic parameters at week 16.

Data are presented as Mean (± SD). WT (Wild-type), HFF (high fat fed), RAGE-/- (RAGE gene deletion).

* p<0.05 v WT Control, † p<0.05 v WT HFF, ‡ p<0.05 v RAGE-/- Control

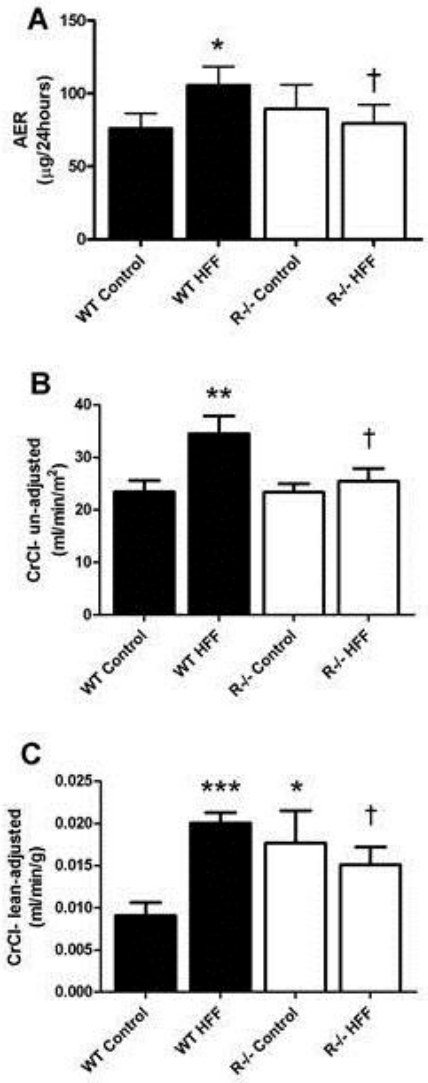


Figure 1. Renal impairment induced by high fat feeding is ameliorated in RAGE-/- mice.

A) Urinary albumin excretion rate (AER) over 24 hours measured by ELISA. B) Creatinine clearance (Cr/Cl) as determined by HPLC following correction for total body surface area. C) Creatinine clearance (Cr/Cl) as determined by HPLC following correction for lean body mass. WT (Wild-type), HFF (high fat fed), R-/- (RAGE deletion). Data were logarithmically transformed for albumin excretion rate analysis, as it was found to be not normally distributed. Other data were statistically analysed using Student's t-test and presented as Mean (±SD).

* p<0.05 v WT Control, † p<0.05 v WT HFF, ** p<0.01 v WT Control, *** p<0.001 v WT Control, ††† p<0.001 v WT HFF

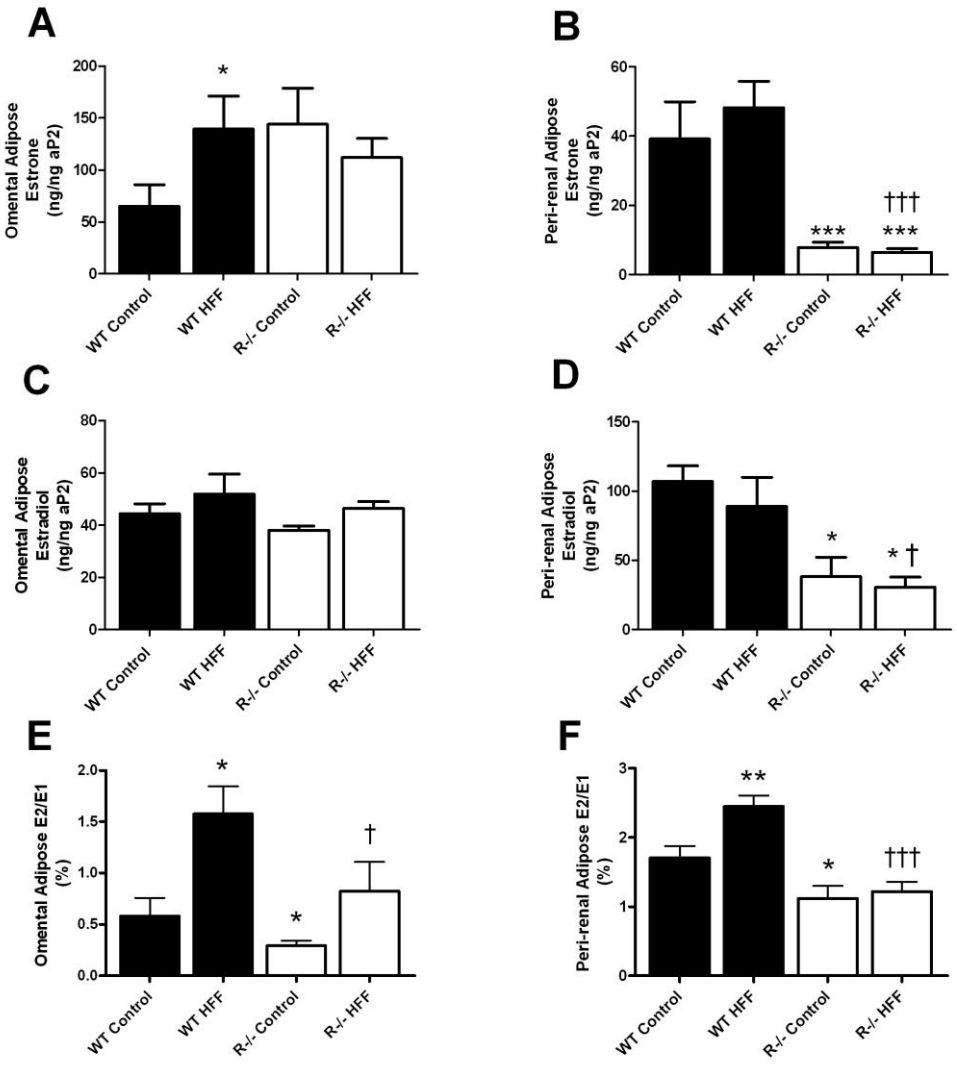


Figure 2. White adipose tissue estrogen content following high fat feeding.

A-B Estrone content within adipose tissue depots corrected for adipocyte lipid-binding protein (P2) concentration A) Omental adipose tissue E1 concentrations measured by EIA. B) Peri-renal adipose tissue E1 content measured by EIA. **C-D** E2 content within adipose tissue deposits. C) Omental adipose tissue 17β-Estradiol (E2) concentrations measured by EIA corrected for adipocyte lipid-binding protein (P2) concentration. D) Peri-renal adipose tissue E2 concentrations measured by EIA. E) Ratio of E2/E1 concentrations in omental adipose tissue. F) Ratio of E2/E1 concentrations in peri-renal adipose tissue. WT (wild-type), HFF (high fat fed), R-/- (RAGE gene deletion).

* p<0.05 v WT Control, † p<0.05 v WT HFF, *** p<0.001 v WT Control, ††† p<0.001 v WT HFF

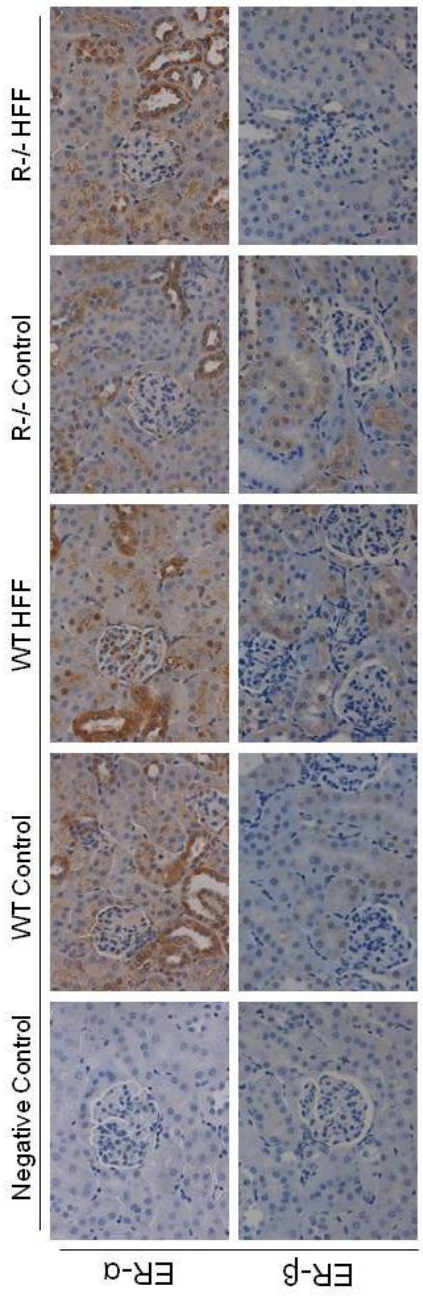


Figure 3. Photomicrographs of ER-α and ER-β in renal tissue.

Immunohistochemistry for renal cortical ER- α and ER-β expression

(X40 magnification)

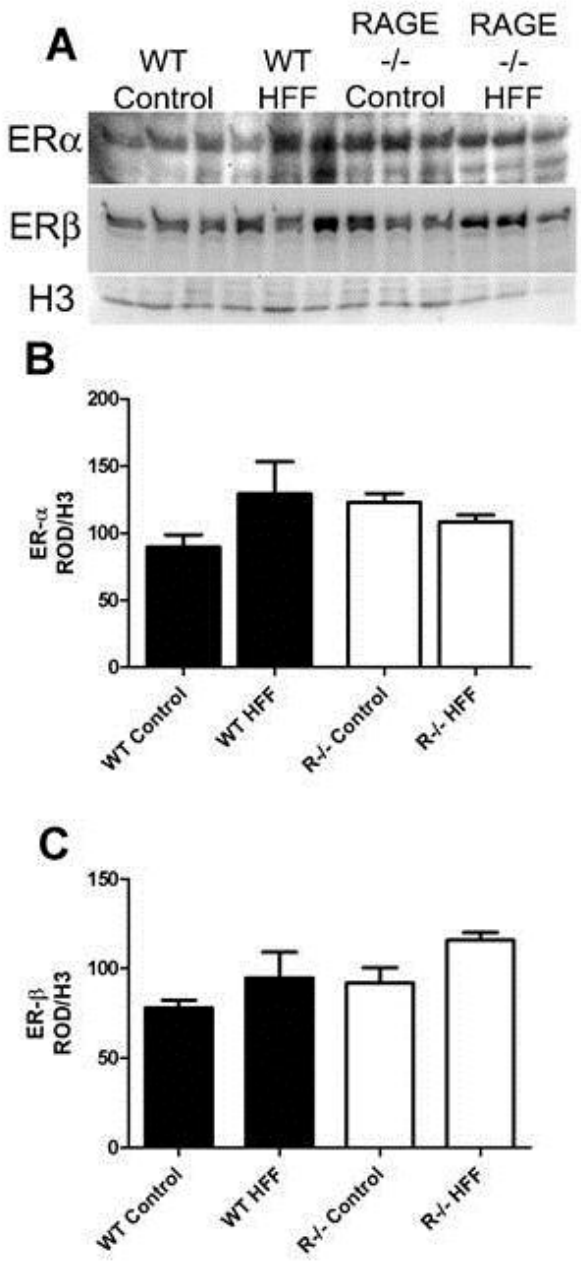


Figure 4. Renal estrogen receptor protein expression at 16 weeks of control or high fat feeding.

A) Representative micrographs of western immunoblots, analysed in B-C. B) Protein expression of ER-α in nuclear protein of kidney cortex as determined via western imunoblotting analysis. B) Quantified expression of ER-β in nuclear fractions from renal cortex. ROD (Relative Optical Density), WT (wild-type), HFF (high fat fed), R-/- (RAGE gene deletion). Statistical analysis performed via Student's t-test and presented as Mean (±SD).

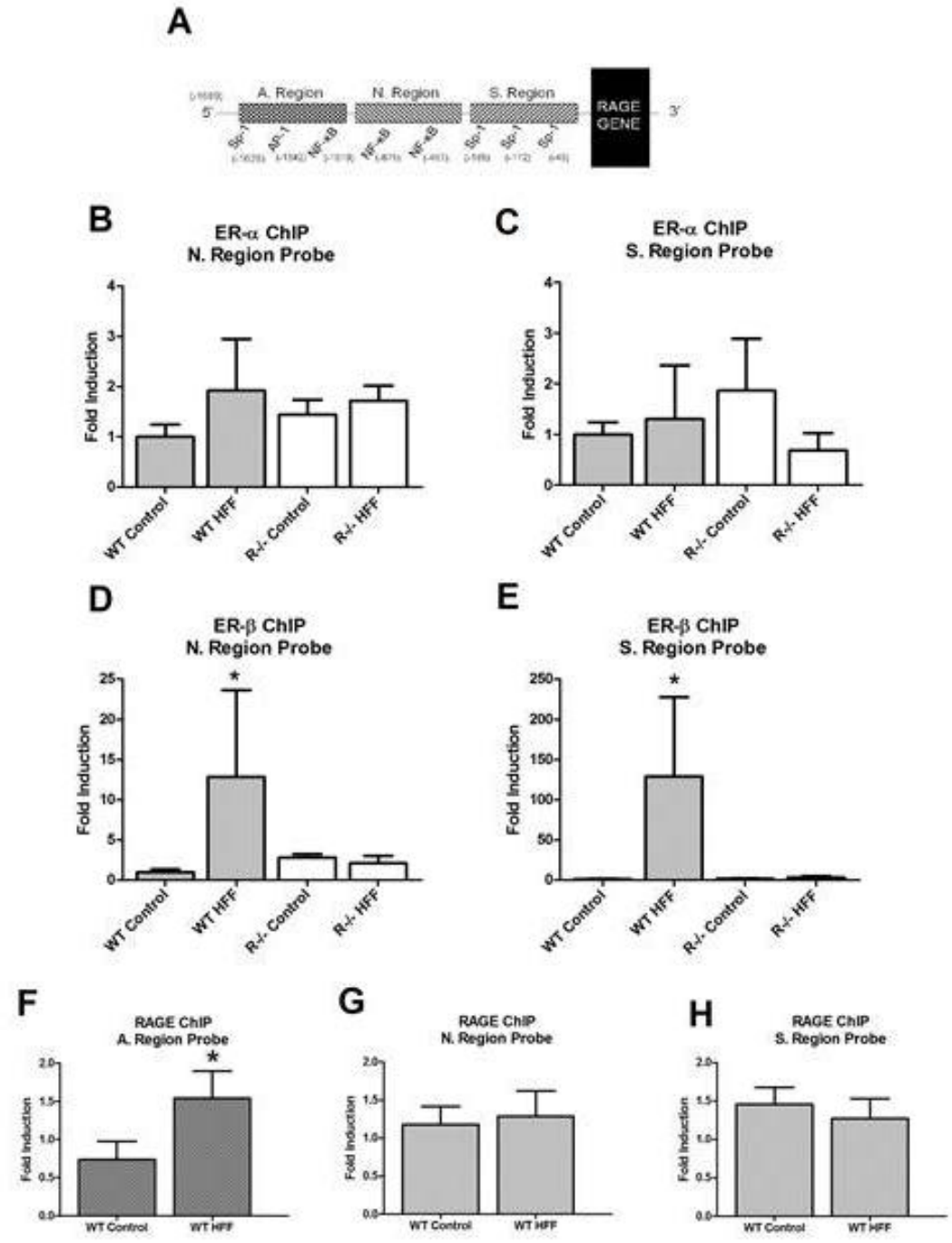


Figure 5. ChIP of ER-α, ER-β and RAGE, and analysis for upstream binding of the AGER gene.

Figure 5. ChIP of ER-α, ER-β and RAGE, and analysis for upstream binding of the AGER gene.

A) Representation of gene binding sites in 5' region of the RAGE gene (base pairs from RAGE gene). B) Analysis of 300bp fractionated chromatin segments immunoprecipitated with ER-α antibody and analysed for N. Region binding activity using real time RT-PCR. C) Analysis of 300bp fractionated chromatin segments immune-precipitated with ER-α antibody and analysed for S. Region binding activity using real time RT-PCR. D) Analysis of 300bp fractionated chromatin segments immunoprecipitated with ER-β antibody and analysed for N. Region binding activity using real time RT-PCR. E) Analysis of 300bp fractionated chromatin segments immune-precipitated with ER-β antibody and analysed for S. Region binding activity using real time RT PCR. F) Analysis of 300bp fractionated chromatin segments immune-precipitated with RAGE antibody and analysed for A. Region binding activity using real time RT PCR. G) Analysis of 300bp fractionated chromatin segments immune-precipitated with RAGE antibody and analysed for N. Region binding activity using real time RT PCR. H) Analysis of 300bp fractionated chromatin segments immune-precipitated with RAGE antibody and analysed for S. Region binding activity using real time RT PCR. WT (wild-type), HFF (high fat fed), R-/- (RAGE gene deletion). Significant values expressed are Mean (±SD) of Y=Log(Y) transformed data.

* $p<0.05$ v WT Control

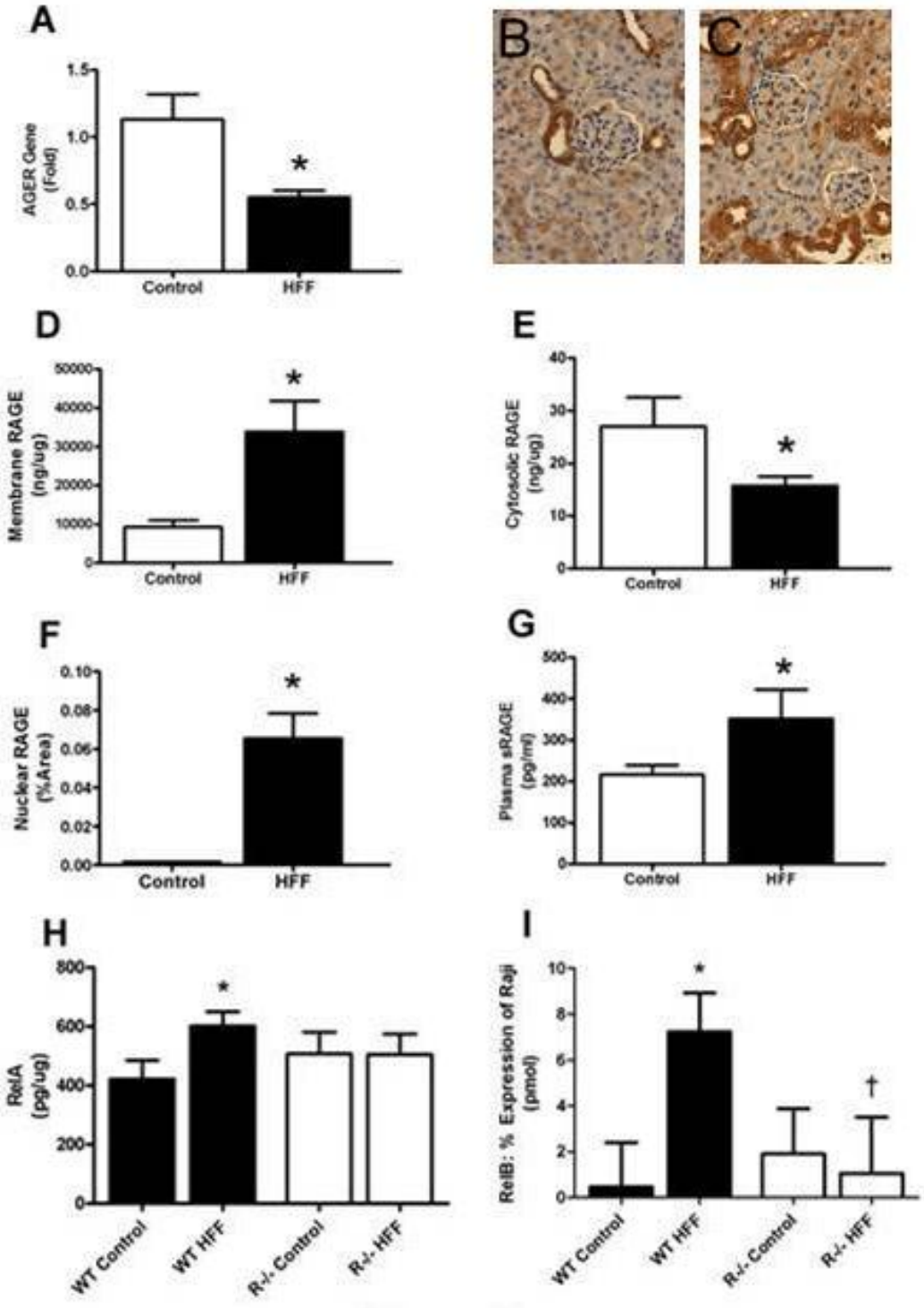


Figure 6. RAGE expression in wild type mice following 16 weeks of high fat feeding

Figure 6. RAGE expression in wild type mice following 16 weeks of high fat feeding

A) Real time RT-PCR analysis for mRNA of AGER (RAGE) gene in kidney cortex of wild type mice, presented as fold induction relative to Control. B) Immunohistochemistry for RAGE in WT Control mice C) Immunohistochemistry analysis for RAGE in WT HFF mice. D) RAGE protein in cellular membrane protein extracted from kidney cortex measured via ELISA. E) RAGE protein in cell cytosolic fractions extracted from kidney cortex measured via ELISA. F) RAGE protein in nuclei quantified from photomicrographs. G) Soluble RAGE (sRAGE) protein in plasma measured via ELISA. H) RelA expression as measured by DNA binding transcription factor assay. I) RelB expression, expressed as a percentage of control Raji measured by DNA binding transcription factor assay. HFF (high fat fed). Statistical analysis performed via Student's t-test and presented as Mean (±SD).

* $p<0.05$ v Control

Chapter 4

Chapter 4: Introduction

Mice with a genetic mutation within the leptin receptor, develop obesity, and are a widely accepted model of Type 2 Diabetes (T2D). Female gender protects against the development of T2D and associated complications. This protection by the female gender is removed however by obesity. This chapter aims to investigate interactions that may occur between local adipose derived estrogens, their receptors and RAGE, to further elucidate the differences between males and females in the development of T2D and nephropathy in the context of obesity.

It is intended that this manuscript will be submitted for publication by the end of December, 2011, when it has been combined with further studies that were not able to be completed within the timeframe of this thesis. These experiments are 100% being completed by myself, and the authorship will remain as dictated on page 109.

Monash University

Declaration for Thesis Chapter Four

Declaration by Candidate

In the case of Chapter Two, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design, experimental procedure, preparation of manuscript	82%

The following co-authors contributed to the work. Co-authors who are students at Monash

University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
KC Sourris	Assist with harvesting of animal tissue	
SA Penfold	Assist with harvesting of animal tissue	
AL Morely	Assist with harvesting of animal tissue	
MT Coughlan	Assist with harvesting of animal tissue	
JM Forbes	Preparation/review of manuscript, experimental design, funding	

Candidate's Signature		**Date** 24/10/2011

Declaration by co-authors

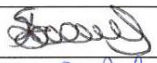
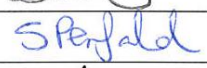
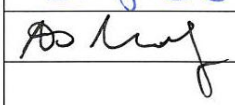
The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Baker IDI Heart and Diabetes Institute, Melbourne, Australia**

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

		Date
Signature 1		12/10/11
Signature 2		10/10/11
Signature 3		13/10/11
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Premenopausal Renoprotection in Females is Overcome by Modulation of RAGE via Adipose Derived Estrogens in Obesity and Type 2 Diabetes

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Running Head: *Female renoprotection in type 2 diabetes via RAGE*

Abstract: 280

Word Count: 4329

Figure Count: 3 Tables, 8 Figures

Keywords: Type 2 Diabetes, Renal, Estradiol, Estrone, RAGE, db/db

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Abstract

Background and aim: Receptor for advanced glycation end products (RAGE) is involved in the pathogenesis of nephropathy as a result of type 2 diabetes. Adipose derived estrogens are able to modulate the expression of RAGE via the transcription factors Sp-1, AP-1 and NF-κB. The aim of this study was to therefore investigate if differences between males and female susceptibility to diabetic nephropathy are the result of modulation of RAGE by adipose derived estrogens.

Methods: We randomised 10 week old mice with a mutation in the leptin receptor gene (*Lepr*$^{(+/+)}$*C57BL/KsJ:* db/db, n=10/group) and their appropriate littermate controls (*Lepr* $^{(+/-)}$*C57BL/KsJ:* db/H, n=10/group). Renal dysfunction, glucose tolerance, hormone profile and biochemical indices were analysed in mice at week 20 of age. The renal RAGE profile was analysed via real time RT-PCR, ELISA and chromatin immunoprecipitation (ChIP).

Discussion of results: Renal function was decreased in db/db mice. Urinary albumin excretion rate (AER) and Cystatin C, were increased in db/db vs. db/H mice (p<0.05), and this was more prominent in male vs. female db/db mice (p<0.05). Glycaemic control, indicative of type 2 diabetes, was poorer in db/db vs. db/H mice (p<0.05), but this was not affected by sex. Renal RAGE expression was significantly increased in male db/db vs. db/H (p<0.05)), which corresponded with disruptions in estrone:estradiol ratios. In male db/db mice, transcriptional regulation within the promoter of RAGE occurred via novel regulation by RAGE itself acting in the nucleus as a transcriptional regulator and via nuclear ER-α and ER-β complexes. Conversely, in female db/db mice nuclear RAGE complexed with NF-κB.

Conclusion: This study demonstrates females have differential regulation of renal RAGE which slows the development of nephropathy, which can be overcome by obesity in type 2 diabetes.

Introduction

Type 2 diabetes (T2D) is a global health epidemic resulting in a number of vascular complications including nephropathy, retinopathy and neuropathy, as well as cardiovascular disease. There is a predisposition for males to develop albuminuria and chronic kidney disease in greater numbers than premenopausal females. This is likely the result of protection afforded by estrogens (17-β-estradiol/ E2) premenopause, which is overwritten by obesity and increased in adiposity [1, 2]. As the result of obesity, estrogen balances are altered, since there is increased secretion of estrone, an estrogen secreted from white adipose tissue [1, 2]. Adipose tissue is a source of inflammatory cytokines in obesity [3, 4]. It is also known the anatomical positioning of adipose tissue is of great importance given that central, rather than subcutaneous accumulation is associated with greater risk of developing T2D [5, 6].

We have demonstrated previously, that the expression of a pro-inflammatory gene, the receptor for advanced glycation end products (RAGE) within the kidney is in part regulated by estrogen secreted in a paracrine manner from local peri-renal adipose deposits in obese mice (**Chapter 3**). In addition, RAGE gene expression in that study was directly modulated by DNA binding of estrogen receptors (ER-α and ER-β), suggesting direct modulation of RAGE by estrogens derived from local white adipose tissue. Furthermore, a novel isoform of RAGE found within the nucleus, transcriptional regulator RAGE (trRAGE), was also shown to modulate the expression of the RAGE gene via interactions with ER-β. It has been identified that 17-β-estradiol (E2) can regulate RAGE gene transcription [7] in endothelial cells via Sp-1 and NF-κB. Studies in a non-obese mouse model also show that E2 ligation with ER-α leads to nuclear translocation and binding to Sp-1 to induce transcription [8]. It is unknown however, whether estrogens can regulate renal RAGE expression in type 2 diabetes, or if there are different regulatory events which occur in male versus female mice and whether this is altered by obesity. This is particularly important given that increases in

membrane expression and signalling via RAGE within the diabetic kidney and obesity are known pathogenic contributors to the development of nephropathy [9-11].

The development of T2D in humans requires insulin resistance in the context of deficiencies in pancreatic islet β-cell insulin secretion. These defects in the db/db mouse are due to a point mutation in the leptin receptor [12, 13]. Consequently the db/db mouse on the KsJ background, develops progressive T2D characterised by common comorbidities seen in humans such as hyperglycaemia, hyperinsulinaemia, hypertension and obesity by 6-8 weeks of age [14] resulting in nephropathy and cardiovascular disease. The aim of this chapter was to investigate the differences between male and female mice in a model of T2D nephropathy and the role that estrogens derived from local white adipose tissue depots play in the renal regulation of RAGE. We will also investigate the role that RAGE may play in self-regulation within its *AGER* 5' promoter region, in males versus female db/db mice. The premise of these studies was based on those findings within **Chapter 3.**

Methods

Experimental Mouse Model of Diabetes: Male and female *Lepr* (+/+) *C57BL/KsJ (db/db*) mice were originally purchased from Jackson Laboratories and randomised at 10 weeks of age. This mouse strain develops diabetic nephropathy in the context of severe metabolic defects (hypertension, hyperlipidaemia, obesity, insulin abnormalities) similar to that seen in type 2 diabetes in humans. Male and female *Lepr* (+/−)*C57BL/KsJ (db/H)* littermates were followed concurrently (*n =* 10/group) and served as the appropriate control. All groups were followed for 10 weeks, with the study completed at week 20 of age. At the completion of the study, a blood sample was collected via cardiac puncture to perform biochemical analyses. All procedures were performed in accordance with the guidelines set out by the Alfred Medical Research and Education Precinct Animal Ethics Committee and the National Health and Medical Research Council of Australia.

Measurements of Physiological and Biochemical Parameters

Total cholesterol and triglyceride concentrations were measured in plasma using a standard commercial enzymatic assay using a Beckman Coulter LX20PRO Analyser (Beckman Coulter Diagnostics Australia). Fasting plasma glucose and fasting plasma insulin were measured at 20 weeks as previously described [15].

Mice were housed in metabolic cages to collect urine and to measure food and water intake at week 20 of age, for a 24 hour period. Albumin excretion rate (AER) was assessed using a mouse albumin ELISA kit according to manufacturer's instructions (Bethyl Laboratories, Montgomery, TX, USA). Creatinine clearance (CrCl) was determined following HPLC (Agilent HP1100 system, Hewlett Packard, Germany) measurement of creatinine content in timed plasma and urine samples as previously described and in accordance with the animal models of diabetes complications consortium (AMDCC) guidelines [16]. Plasma cystatin-C concentrations were determined via ELISA according to the manufacturer's instructions (BioVendor, Heidelberg, Germany). Frozen renal

cortices were separated into cellular compartments using ultracentrifugation as previously described [10] to generate membrane, cytosol and nuclear protein fractions.

Real Time RT-PCR

Two micrograms of total RNA were extracted from the left kidney cortex and genomic DNA removed using a DNA-Free^{TM} kit (Ambion Inc., Austin, Texas, USA). cDNA was synthesized using the Superscript First-Strand Synthesis kit for RT-PCR (Gibco BRL, Grand Island, New York, USA). Gene expression of RAGE was analysed by real-time reverse transcription polymerase chain reaction (RT-PCR) performed with the Taqman system based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer, CA, USA). Sequences used for *AGER* (RAGE) were, designed to exon 2 of the *AGER* gene, which has been shown to be highly conserved between isoforms; forward primer GCTGTAGCTGGTGGTCAGAACA, reverse primer CCCCTTACAGCTTAGCACAAGTG and probe 6-FAM CACAGCCCGGATTG (NM_007425). The amplification protocol was 50˚C for 2 min, 95˚C for 10 min, 40 cycles of 95˚C for 20 seconds and 60˚C for 1 min. Relative quantification of gene expression was performed by the comparative C_T ($\Delta\Delta C_T$) method, with ribosomal 18S (18S rRNA Taqman Control Reagent kit, ABI Prism 7700) as the endogenous control. All results were expressed relative to values from WT control mice, which were assigned an arbitrary value of 1.

Processing of Adipose Tissues

Peri-renal and omental adipose deposits were weighed, snap frozen and then stored at -80^{o}C upon extraction. Messenger RNA (mRNA) and protein was extracted according to the manufacturer's instructions (RNeasy Lipid Tissue kit, Qiagen, Victoria, Australia).

Endogenous Sex Steroid Analysis

Estrone (E1) and 17β-estradiol (E2) were measured via EIA (Estradiol and Estrone EIA Kits, Catalogue No. 582251 and No. 582301 respectively, Cayman Chemical, MI, USA). The Estrone assay specifically measured estrone sulfatase and estrone glucocorticoids, which represents the entire

Estrone content in sample. The E2 assay specifically identified total 17β-estradiol content. Assays were performed according to the manufacturer's instructions. E1 and E2 expression was corrected for adipocyte lipid-binding protein (aP2) content, to control for the amount of protein within the adipose tissue sample (aP2 ELISA, BioVendor, Mordice, Czech Republic).

RAGE, MCP-1, IL-6 and MIF ELISAs

Renal nuclear, cytosolic and membrane fractions and plasma were assayed for RAGE via ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Monocyte chemottractant protein (MCP-1), macrophage inhibiting factor (MIF) and interleukin-6 (IL-6) were analysed in renal cytosolic fractions in accordance with the manufacturers' instructions (Quantikine ELISA Systems, R&D Systems, Minneapolis, MN).

Renal Histology

Glomerulosclerotic index (GSI) was assessed in a blinded manner by a semi-quantitative method in PAS-stained sections as previously described [17].

Chromatin Immunoprecipitation

Kidney cortices were analysed for DNA binding activity within the 5' flanking region of the *AGER* (RAGE) gene which encodes for a number of isoforms of RAGE including membrane bound and soluble RAGE. Probes were designed using the BLAST program and were created to examine whether ER-α or ER-β DNA activity could be detected within the promoter region of the *AGER* gene. The primers designed also allowed for specific localisation of ER binding region within the AGER promoter to one of three binding regions; A. Region, with binding sites for Sp-1, AP-1 and NF-κB, N.Region which has majority of binding sites for NF-κB and S.Region with majority binding sites for Sp-1 as previously described (**Chapter 3**, [7]).

Frozen mouse kidney (100mg) was finely minced in a petri dish over dry ice using a scalpel. Low glucose (0.5% D-glucose) DMEM (Gibco by Invitrogen), with 2% parafomaldehyde (PFA, Sigma-Aldrich), was added and incubated with slow agitation for 10 minutes at RT. The cross-linking reaction was stopped with the addition of 2.5M glycine. Tissue was pelleted in an eppendorf tube at 1000rpm for 8 min at 4^{o}C. Following washing with PBS, pelleted cells were re-suspended in cell lysis buffer (10mM Tris-HCL pH 8.0, 10mM NaCl, 0.2% NP40, 10mM NaB, 300uM PMSF), and incubated for 10 min on ice. Following this the now lysed cells were centrifuged for 5 min, 1500rpm at 4^{o}C. Pellets now containing nuclei were incubated in cold nuclear lysis buffer (50mM Tris-HCL pH 8.0, 10mM EDTA pH 8.0, 1% SDS, 10mM NaB, 300uM PMSF), at 4^{o}C for 2 hours. IP dilution buffer (IPD Buffer; 20mM Tris-HCL pH 8.0, 2mM EDTA pH 8.0, 150mM NaCl, 0.01% SDS, 1% Triton X, 10mM NaB, 300uM PMSF) was added to the samples. Chromatin was then fragmented in a water bath sonicator for 40 min at 70% pulse at 4^{o}C. Insoluble material was cleared and removed by spinning for 10 min at 15,000rpm, 4^{o}C. A small amount of soluble chromatin was used to verify chromatin fragment size, and remaining used for immunoprecipitation. Size of chromatin fragments were then verified after reverse crosslinking at 55^{o}C overnight, DNA purification with phenol/chloroform, and precipitation with cold EtOH, on a 1% Agarose gel, with 0.1%EtBr. Chromatin size was verified at 300bp. Previous fragmented chromatin was diluted with IPD buffer at a ratio of 1:8. 100µl of diluted chromatin was stored as the In-put Sample at -80^{o}C. Salmon sperm/Protein A agarose slurry (Amersham) was added to each sample to be immunoprecipitated, at 1:12 ratio and incubated on a rotating carousel, 1 hour at 4^{o}C. Samples were centrifuged for 5 min at 5000rmp and supernatant collected, then divided into primary antibody lots. Primary antibodies, ER-α (rabbit anti-ER-α, Millipore, USA; 1:100) ER-β (rabbit anti-ER-β, Millipore, USA; 4µg/ul) and a No-antibody control, were incubated with samples overnight at 4^{o}C on a rotary carousel. Additional salmon sperm/protein A slurry was added to each sample at a ratio of 1:6, and incubated for a further hour at 4^{o}C on the rotary. Samples were centrifuged for 10-20 seconds at 12,000rpm and 200µl of each sample was aliquoted to a new tube as the Un-bound Sample. Remaining supernatant was discarded and pellets were washed with 500µl of each of the

following for 3-5 min at RT on the rotary carousel then pelleted at 12,000rpm for 10-20 seconds; 4x IP wash buffer #1 (20mM Tris-HCL, 2mM EDTA, 50mM NaCl, 0.1%SDS, 1% Triton X), 1x IP wash buffer #2 (10mM Tris-HCL, 1mM EDTA, 250mM LiCl, 1% NP40, 1% Deoxycholic Acid) and 3x TE buffer (10mM Tris-HCL pH 8.0, 1mM EDTA pH 8.0). The chromatin was pelleted at 4,000rpm for 2 min and left on ice for 1 min. Supernatant was then removed from the beads and proteins were eluted for 15 min in elution buffer (100mM Sodium Bicarbonate, 1% SDS). Samples were incubated overnight with 8% NaCl at 65^{o}C. Following phenol/chloroform (Sigma-Aldrich) extraction, DNA was precipitated and washed in EtOH then air dried and dissolved in water. Samples were assayed for ER-α and ER-β binding of transcription factors were analysed by real-time RT-PCR performed with the Taqman system based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer, CA, USA). The amplification protocol was 50˚C for 2 min, 95˚C for 10 min, 50 cycles of 95˚C for 20 seconds and 60˚C for 1 min. Relative quantification of gene expression was performed by the comparative C_T ($\Delta\Delta C_T$) method, with the Input control for each sample accounting for total DNA used as the endogenous control, and correction for non-specific activity with a No-antibody sample.

Statistical Analysis

Results are expressed as Mean ± Standard Deviation (SD) unless otherwise specified. Analyses were performed by ANOVA followed by, with Tukey's post-hoc analysis, unpaired Student's t-test analysis (Graph Pad Prism). A $p<0.05$ was considered to be statistically significant. Data for albuminuria were not normally distributed and were therefore analysed following logarithmic transformation.

Results

Body composition and biochemistry analysis at week 20 of age

Progression to renal disease, in particular albuminuria in T2D is more commonly seen in males than females [18]. We therefore investigated the differences between male and female mice with type 2 diabetes from week 10 to week 20 of age. Both male and female db/db mice progressed in parallel to require daily injections of insulin (1-2U/kg) from approximately week 14-16 of age, following destruction of pancreatic β-cells.

We followed changes in bodyweight over the duration of the study. At the study conclusion (week 20) there were no significant differences in bodyweight between male db/db and male db/H mice (Table 1), however the growth curves of these two male groups were significantly different when area under the curve was examined from week 10 to 20 of age (Figure 1B. Male db/H: 296.2 (±37.5) vs. male db/db: 379.5 (±45.1); AUC, $p<0.05$), indicative of male db/db mice being obese for a longer period of time. In contrast, female db/db and db/H mice had significantly different body weights at week 20 of age (Table 1), and these differences were evident over the duration of the study (Figure 1A and 1B). At the end of the study, female db/db mice were significantly heavier in body weight than male db/db mice (Table 1; Figure 1A).

Visceral adipose depot weights were increased in female db/db mice compared to female db/H mice (Table 1). There were no differences between male db/db and db/H mouse and visceral adipose tissue weights (Table 1).

Both male and female diabetic db/db mice consumed greater amounts of water as compared with respective db/H control mice over a twenty-four period as assessed during metabolic caging (Table 2). Urine output was also increased in all db/db mice irrespective of sex when compared with sex matched db/H control mice. More calories were consumed by db/db mice during metabolic caging at week 20, than db/H sex matched litter controls over a twenty-four hour period (Table 2).

Circulating high density lipoprotein (HDL) levels were significantly increased in all T2D mice (db/db) when compared to age and sex matched db/H mice (Table 3). HDL levels were also further increased in female db/db as compared with male db/db mice. HDL levels were significantly lower in female db/H mice compared to male db/H mice (Table 3). Plasma triglyceride concentrations did not differ between male db/H and male db/db mice (Table 3). By contrast, female db/db mice had significantly higher triglyceride concentrations compared with female db/H mice (Table 3).

Fasting plasma glucose concentrations were significantly elevated in all db/db mice when compared with sex matched littermate control db/H mice (Figure 2A). Plasma glycated haemoglobin concentrations were also significantly increased in both male and female db/db mice, in contrast to sex matched littermate control db/H mice (Figure 2B).

Renal parameters at 20 weeks of age

Total kidney weight was increased in male and female db/db mice compared to sex matched db/H controls, and further increased in male db/db compared to female db/db mice (Table 1). Renal hypertrophy as depicted by kidney weight/body weight (KW:BW) ratio was evident in male db/db versus male db/H mice (Table 1). Renal hypertrophy was also seen in female db/db versus db/H mice (Table 1).

Renal function has been shown previously to decline with both obesity and T2D. Indeed, in male db/db mice there was a decline in the glomerular filtration as shown by the decrease in creatinine clearance (Cr/Cl; Figure 3A) and the retention of cystatin C (Figure 3B) in the circulation of these mice when compared with db/H male mice. Conversely, in female db/db mice we observed an increase in Cr/Cl (compared to male db/db mice, Figure 3A), in conjunction with an increase in retention of plasma cystatin C (Figure 3B) indicative of hyperfiltration in this group. Furthermore, all db/db mice had elevations in twenty-four hour urinary albumin excretion rates (UAER) which were not seen in db/H mice, but UAERs were more significantly elevated in male as compared with female

db/db mice (Figure 3C). Urinary excretion of AGEs (CML) was also greatest in male db/db mice when compared with female db/db mice (Figure 3D). Urinary AGEs were also significantly increased in female db/db versus female db/H mice (Figure 3C).

The degree of renal structural damage was assessed by quantification of glomerulosclerosis which was scored in fixed and stained histological sections. Glomerulosclerosis (GSI) was increased in male and female db/db mice compared sex matched db/H littermate mice (Figure 4A, Figure 4B-4E, representative micrographs). There was also a worsened degree of glomerulosclerosis in male db/db mice compared to female db/db mice (Figure 4A).

Estrogen content of fat pads at 20 weeks of age

Concentrations of the estrogens Estrone (E1) and Estradiol (E2) were measured in peri-renal and omental adipose tissue pads. Within peri-renal adipose tissue, the content of E1 (Figure 5C) and E2 (Figure 5D) seen in male db/db mice, was significantly increased as compared with sex and age matched db/H mice (Figure 5A and 5B). E2 expression within peri-renal adipose tissue was significantly decreased in female db/db mice compared to both male db/db mice and female db/H mice (Figure 5B). No changes in E1 content were seen in peri-renal adipose tissue in female mice.

Within omental adipose tissue, the concentration of E1 was significantly increased in male db/db mice compared to male db/H mice (Figure 5C). No changes in omental E1 concentration were seen in female mice (Figure 5C).

RAGE gene and protein expression

We have previously shown that deletion of RAGE in mice protects against renal dysfunction as the result of obesity (**Chapter 3**). Within the present study the expression of RAGE within the renal cortex was assessed. In db/db mice, the expression of *AGER*, the gene encoding for all RAGE isoforms was elevated by diabetes and this was five-fold higher in females as compared with males

(Figure 6A). *AGER* mRNA levels were also significantly increased in female db/H mice compared to male db/H mice (Figure 6A).

Increases in the renal expression of the membrane bound isoform of RAGE have been shown on previous occasions to coincide with progressive renal disease in diabetes [9, 16, 19]. Within renal cortices, membrane bound RAGE concentrations in our study were significantly increased in all mice as compared to male db/H mice (Figure 6B). Female db/db did not show elevations in renal membrane bound RAGE concentrations as compared with female db/H mice.

Within the cytosol, which likely reflects of pool of RAGE able to be secreted from cells (therefore mostly soluble RAGE), RAGE concentrations were significantly increased by up to twelve-fold in female mice as compared to male mice (Figure 6C). Interestingly cytosolic RAGE concentrations did not differ between the db/H and db/db genotype within the same sex (Figure 6C).

Inflammatory parameters at week 20 of age

Interleukin-6 (IL-6) concentrations in renal cortices were increased in female db/H mice compared to male db/H mice (Figure 7A). Levels of IL-6 in male db/db tended to be higher than male db-H mice although this did not reach significance (p=0.053, Figure 7A). Renal IL-6 was also elevated in female db/H and db/db mice when compared with male db/H. Renal cortical concentrations of monocyte chemoattractant protein 1 (MCP-1) in male db/db mice were increased compared to male db/H mice (Figure 7B), but this was not seen in female mice. Macrophage migration inhibitory factor (MIF) concentrations in renal cortices were significantly increased in female db/db mice compared to female db/H mice (Figure 7C). All male mice had an increase in renal MIF concentrations when compared with female db/H mice.

Estrogen and RAGE activity in the 5' transcriptional domain of the AGER gene promoter

The *AGER* gene is multi-isomeric and encodes membrane bound RAGE, soluble RAGE and a further 12 uncharacterised RAGE splice variants [19, 20]. We have recently identified a new

functional RAGE isoform within the nucleus, which plays a role in the transcriptional modulation of the AGER gene (**Chapter 3**). The 5' transcriptional domain contains active binding sites for the transcription factors Sp-1, AP-1 and NF-κB (Figure 8A). Within the present study, the AGER promoter was separated into three regions as described by Tanaka *et al* [7] to analyse DNA binding activity (Figure 8A).

DNA binding activity of the nuclear transcriptional regulator RAGE (trRAGE) within the N.Region of the AGER promoter, which contains a majority of NF-κB binding domains, was increased in renal cortices from both male and female db/db mice (Figure 8B). Within the A.Region of the AGER promoter, which contains binding sites for Sp-1, AP-1 and NF-κB, there was increased RAGE (Figure 8C), ER-α (Figure 8D) and ER-β (Figure 8E) binding to DNA, seen in male db/db mice. This selective binding in the A.Region of the AGER promoter was not seen in female db/db mice. There were no significant differences in binding activity of RAGE, ER-α or ER-β, within the S.Region, which contain majority of Sp-1 binding sites, among mouse groups.

Discussion

This study demonstrated differences between male and female mice in the development of nephropathy in an experimental model of T2D. This may in part be attributed to the self-perpetuating transcriptional regulation of RAGE via adipose derived estrogen activation of ER-α and ER-β, seen within these studies.

Importantly we demonstrated that with the development of obesity, the protection usually afforded to females against the development of renal pathologies was reduced. This is also seen in human studies, where the risk for developing renal disease in obese individuals is the same for both males and females, whereas in T1D where obesity is not a common manifestation, males are more likely than females to progress to diabetic nephropathy [21]. In the present study, obese diabetic male and female mice had increased evidence of renal impairment as compared to db/H controls. However, worsened renal disease was evident in male db/db and compared with female db/db mice which included a decline in glomerular filtration rate, increased urinary excretion of albumin and CML, in addition to more glomerulosclerosis. Please say if this is consistent with previous studies or if not then state that “this is the first time that this has been shown in a mouse model of type 2 diabetes”.

There were sex specific differences observed in the estrogen production by specific adipose tissue deposits. Obese diabetic male mice had increased content of both estrone and 17-β-estradiol (E2) in peri-renal adipose deposits. In addition, female db/db mice had lower peri-renal derived 17-β-estradiol (E2) than db/db males, but this was still elevated as compared with non-obese db/H female mice. This is consistent with previous studies where decreased circulating E2 levels have been associated with an increase in cardiovascular disease risk in women [22], particularly given that declining renal function is an important risk factor for this condition [23].

Kalea *et al* [19], previously described 15 isoforms of RAGE that are transcribed when the 5' transcriptional promoter region of the RAGE gene is alternatively spliced in mice. Kalea also demonstrated two novel isoforms of RAGE that are produced in nine month old male db/db mice but not in age and sex matched db/H mice [19]. These novel isoforms associated with diabetes were found to be resultant of deletion of part of exon 8 or exon 10. Future research is necessary to discover which isoforms are being transcribed in our model. We observed an increase in RAGE mRNA transcription in female mice compared to male mice, which was exacerbated by obesity. This was reflected in RAGE binding activity within the 5' transcriptional domain of the *AGER* gene, specifically within the N.Region (Figure 8A), consisting on a majority of NF-κB sites. This may suggest that binding of RAGE and NF-κB within this region of the *AGER* promoter is essential for cellular RAGE protein expression. However it is yet to be fully determined if RAGE forms a complex with NF-κB or if it has the capacity to directly bind within the AGER promoter to alter gene transcription. In addition, we do not know if males and female mice have differential expression at different renal cell types within the cortex. Interestingly, the results which were seen for DNA binding within the A.Region of the AGER promoter, would suggest that RAGE, ER-α and ER-β form a complex to regulate AGER transcription in db/db male mice, which is contributing to worse renal function. However, the exact nature of this interaction remains to be fully elucidated.

Inflammatory markers present in the kidney, differed between male and female mice. The expression of IL-6 did not change with the onset of T2D in this study, and interestingly tended to be increased in female db/H versus male db/H mice. Whereas, the expression of MCP-1, was only significantly increased in male diabetic mice that also had overt nephropathy. MCP-1, IL-6 and macrophage infiltration have previously been shown to be partially regulated by 17-β-estradiol in both *in vivo* and *in vitro* studies [24-26]. In post menopausal women circulating MCP-1 levels naturally increase with the loss of estradiol protection and are then decreased with hormone replacement therapies thereby decreasing vascular dysfunction [26]. However it remains to be determined if estrone has the same direct effect as obesity derived factors do in our study.

Furthermore the expression profile of MIF in our study appeared to coincide with the incidence of obesity rather that diabetes, as expression was lowest in lean non-diabetic female db/H mice.

In summary, we have demonstrated that obesity can override the protection afforded females against the development of T2D nephropathy. This is likely via alternate regulation of RAGE transcriptional activity by nuclear RAGE itself and estrogen receptors which appears to be different between males and females, which may in part explain some of the gender differences in susceptibility to CVD seen in type 2 diabetes. The association of nuclear RAGE with ER-α, ER-β and NF-κB and these complexes in regulating renal impairment in type 2 diabetes remains to be fully elucidated.

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Chapter 4: Tables and Figures

Table 1. Physiological parameters taken from male and female mice at 20 weeks of age

Data are presented as Mean (± SD). KW/BW (renal hypertrophy). * p<0.05 v Male db/H, ** p<0.01 v Male db/H, *** p<0.001 v Male db/H, † p<0.05 v Male db/db, †† p<0.01 v Male db/db, ‡ p<0.05 v Female db/H

		Body Weight (g)	KW/BW Ratio (x103)	Visceral Adipose (g)	Peri-renal Adipose (g)
Male	**db/H**	37.5 (±2.5)	1.2 (±0.2)	0.9 (±0.3)	0.6 (±0.2)
	db/db	38.7 (±3.8)	1.6 (±0.2)**	0.9 (±0.2)	0.5 (±0.3)
Female	**db/H**	27.1 (±1.7)*	0.8 (±0.5)***	0.5 (±0.1)***	0.2 (±0.1)***
	db/db	40.2 (±11.9)††	1.1 (±0.4)† ‡	1.0 (±0.5)‡	0.7 (±0.5)‡

Table 2. Metabolic caging analysis of male and female mice at 20 weeks of age

Data are presented as Mean (± SD). * p<0.05 v Male db/H, ** p<0.01 v Male db/H, *** p<0.001 v Male db/H, † p<0.05 v Male db/db, ‡‡ p<0.01 v Female db/H, ‡‡‡ p<0.001 v Female db/H

		Water (ml)	Food (g)	Urine Output (ml)
Male	**db/H**	3.6 (±2.4)	0.8 (±0.5)	0.9 (±0.3)
	db/db	18.0 (±9.8)**	7.2 (±4.4)**	16.5 (±8.9)***
Female	**db/H**	2.7 (±1.6)	1.6 (±0.5)*	0.6 (±0.2)
	db/db	6.9 (±7.2)† ‡‡	3.8 (±2.7)† ‡‡	3.2 (±2.5)† ‡‡‡

Table 3. Biochemical parameters in male and female mice at 20 weeks of age

Data are presented as Mean (± SD).

* p<0.05 v Male db/H, ** p<0.01 v Male db/H, *** p<0.001 v Male db/H, ‡‡ p<0.01 v Female db/H,

		HDL (mmol/L)	Chol (mmol/L)	Trig (mmol/L)
Male	**db/H**	1.1 (±0.2)	1.7 (±0.4)	0.5 (±0.1)
	db/db	1.7 (±0.4)*	1.9 (±0.7) *	0.5 (±0.2)
Female	**db/H**	0.5 (±0.2)**	0.7 (±0.2) ***	0.2 (±0.1)*
	db/db	2.1 (±0.7)‡‡	2.1 (±0.8)	0.5 (±0.2)‡‡

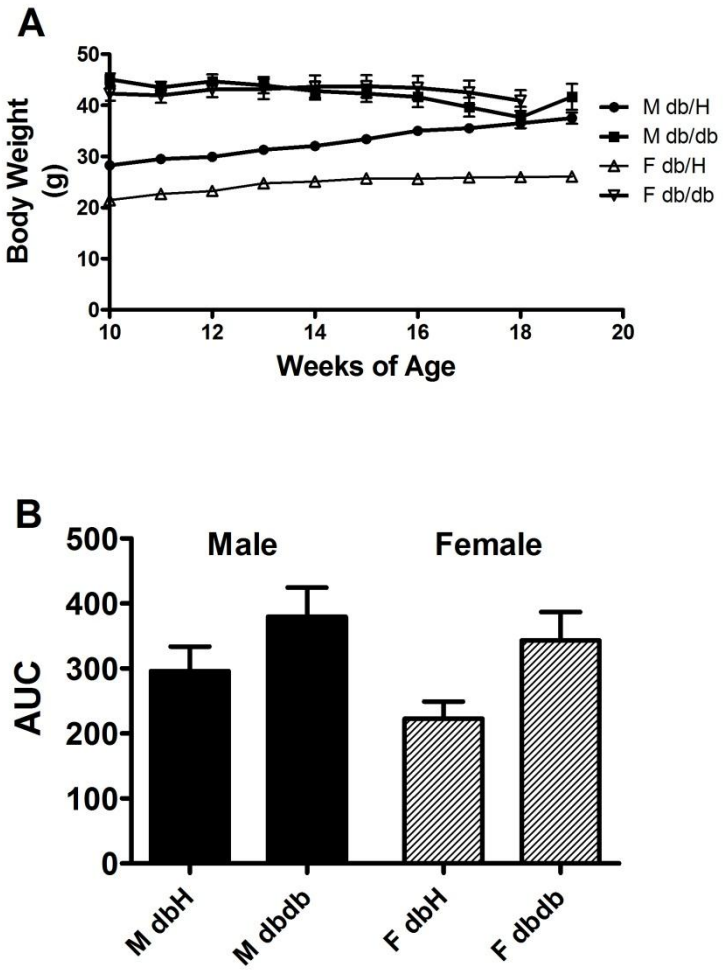

Figure 1. Body weight from 10 to 20 weeks of age

A) Body weight was measured weekly from week 10 to week 20 of age. B)The area under the curve (AUC) of the plotted weekly bodyweight (A), was calculated.

M dbH (male db/H), M dbdb (male db/db), F dbH (female db/H), F dbdb (female db/db)

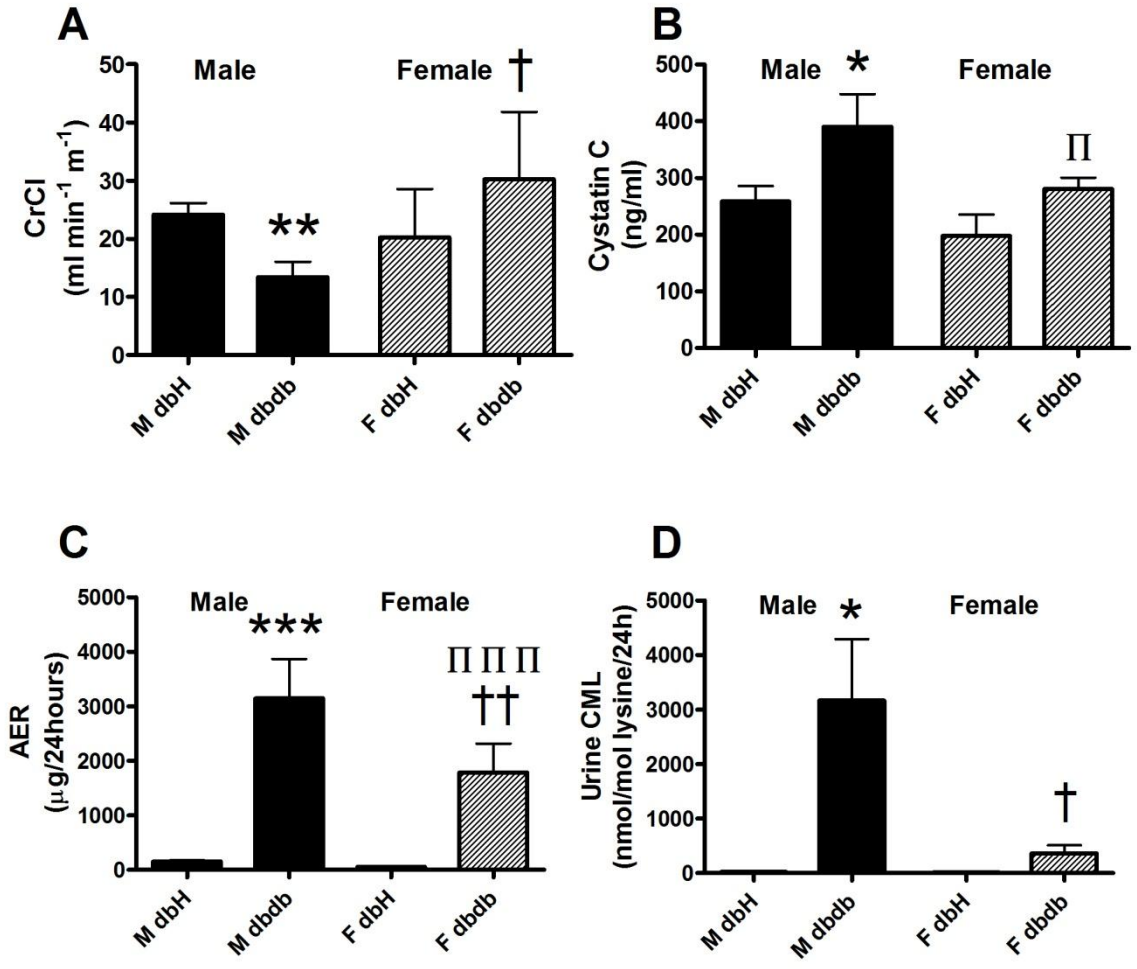

Figure 2. Renal parameters in type 2 diabetes in male and female mice at 20 weeks of age

A) Creatinine clearance (Cr/Cl) was detemined by HPLC following correction for total body surface area. B) Cystatin C concentrations were measured via ELISA C) Urinary CML concentrations were measured via ELISA D) Urinary albumin excretion rate (AER) was measured via ELISA.M dbH (male db/H), M dbdb (male db/db), F dbH (female db/H), F dbdb (female db/db)

* p<0.05 v M dbH, ** p<0.01 v M dbH, *** p<0.001 v M dbH, † p<0.05 v Male db/db, †† p<0.01 v Male db/db, Π p<0.05 v F dbH, Π Π Π p<0.001 v FdbH

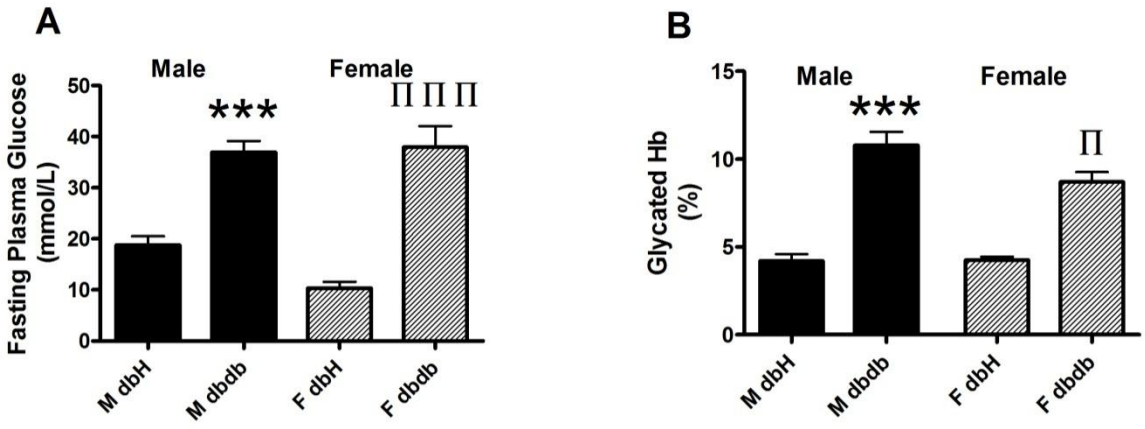

Figure 3. Glycaemic control is impaired in type 2 diabetic male and female mice at week 20

A) Fasting plasma glucose concentration B) Glycated haemoglobin (Hb). M dbH (male db/H), M dbdb (male db/db), F dbH (female db/H), F dbdb (female db/db)

* p<0.05 v M dbH, ** p<0.01 v M dbH, *** p<0.001 v M dbH, Π p<0.05 v F dbH, Π Π Π p<0.001 v FdbH

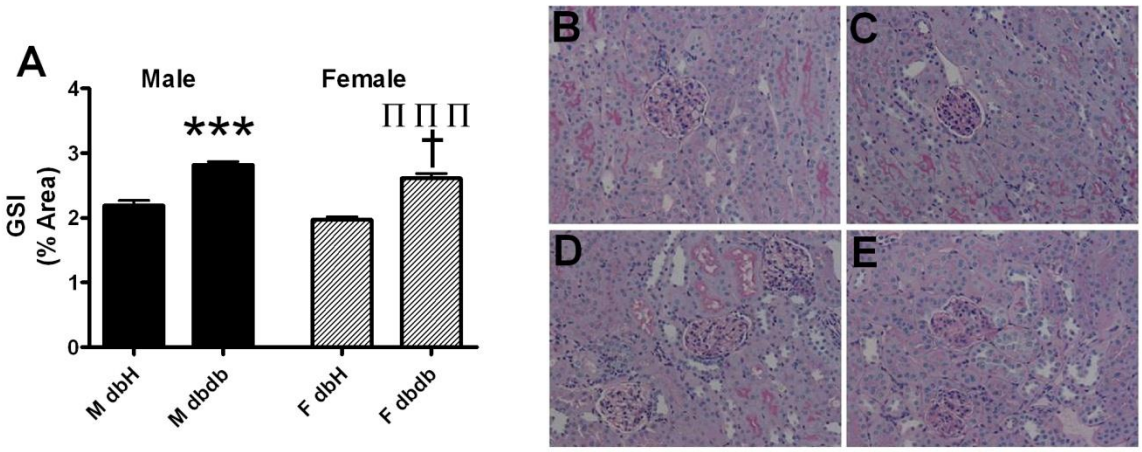

Figure 4. Renal glomerulosclerosis is increased by diabetes in male and female mice at week 20

A) Glomerulosclerotic Index (GSI) calculation from representative kidney sections **B-E.** B) Male db/H C) Male db/db D) Female db/H E) Female db/db. Magnification 40x. M dbH (male db/H), M dbdb (male db/db), F dbH (female db/H), F dbdb (female db/db)

*** $p<0.001$ v M dbH, † $p<0.05$ v Male db/db, Π Π Π $p<0.001$ v FdbH

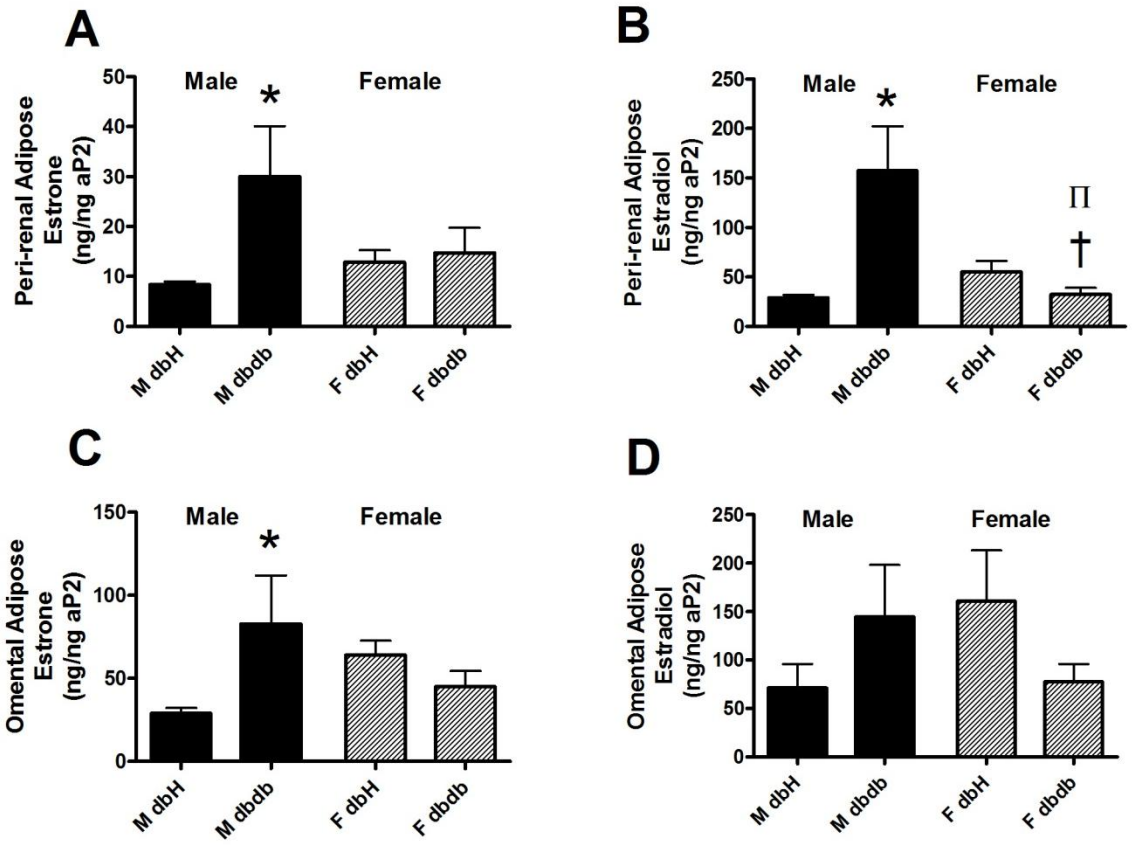

Figure 5. Estrogen concentrations in white adipose tissue deposits

Estrogen concentrations in adipose deposits are measured via enzyme linked immunoassay and corrected for aP2.A) Estrone (E1) concentration in peri-renal adipose B) Estradiol (E2) concentration in peri-renal adipose C) Estrone (E1) concentration in omental adipose C) Estradiol (E2) concentration in omental adipose.M dbH (male db/H), M dbdb (male db/db), F dbH (female db/H), F dbdb (female db/db)

* $p<0.05$ v M dbH, † $p<0.05$ v Male db/db, Π $p<0.05$ v F dbH

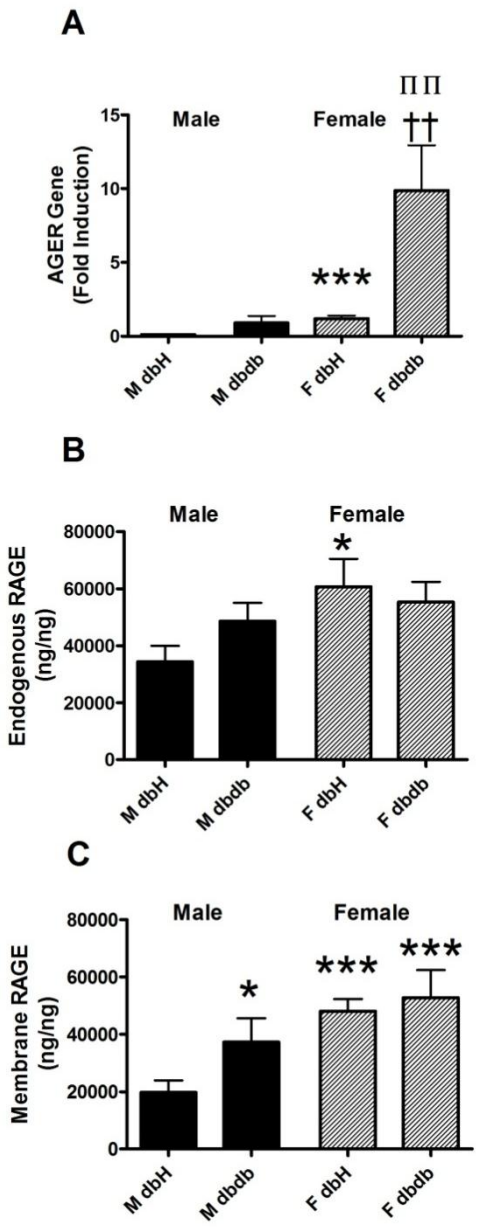

Figure 6. Renal RAGE expression in male and female type 2 diabetic mice

A) Real time RT-PCR analysis for AGER mRNA in renal cortex, presented as fold induction relative to male db/H mice. B) RAGE protein in renal cortex cytosolic fractions, measured via ELISA C) RAGE protein in renal cortex cellular membranes, measured by ELISA. M dbH (male db/H), M dbdb (male db/db), F dbH (female db/H), F dbdb (female db/db)

*** $p<0.001$ v M dbH, †† $p<0.01$ v Male db/db, Π Π $p<0.01$ v FdbH

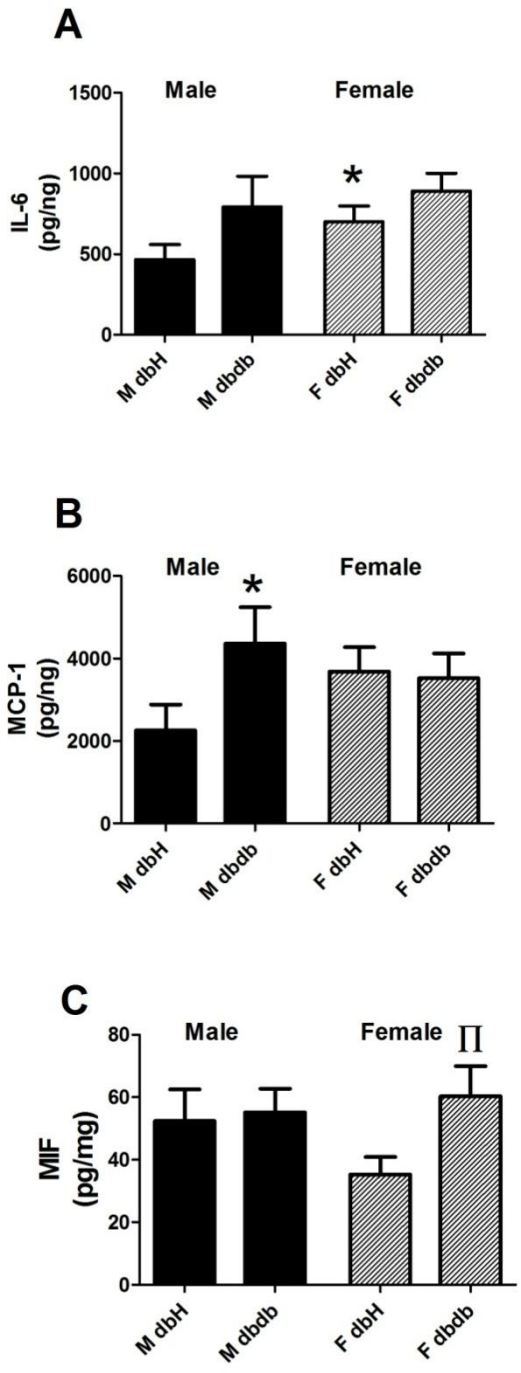

Figure 7. Inflammatory profile in male and female diabetic mice

A) IL-6 in renal cortex, as measured by ELISA B) MCP-1 in renal cortex as measured by ELISA C) MIF in renal cortex as measured by ELISA. M dbH (male db/H), M dbdb (male db/db), F dbH (female db/H), F dbdb (female db/db)

* $p<0.05$ v M dbH, Π $p<0.05$ v F dbH

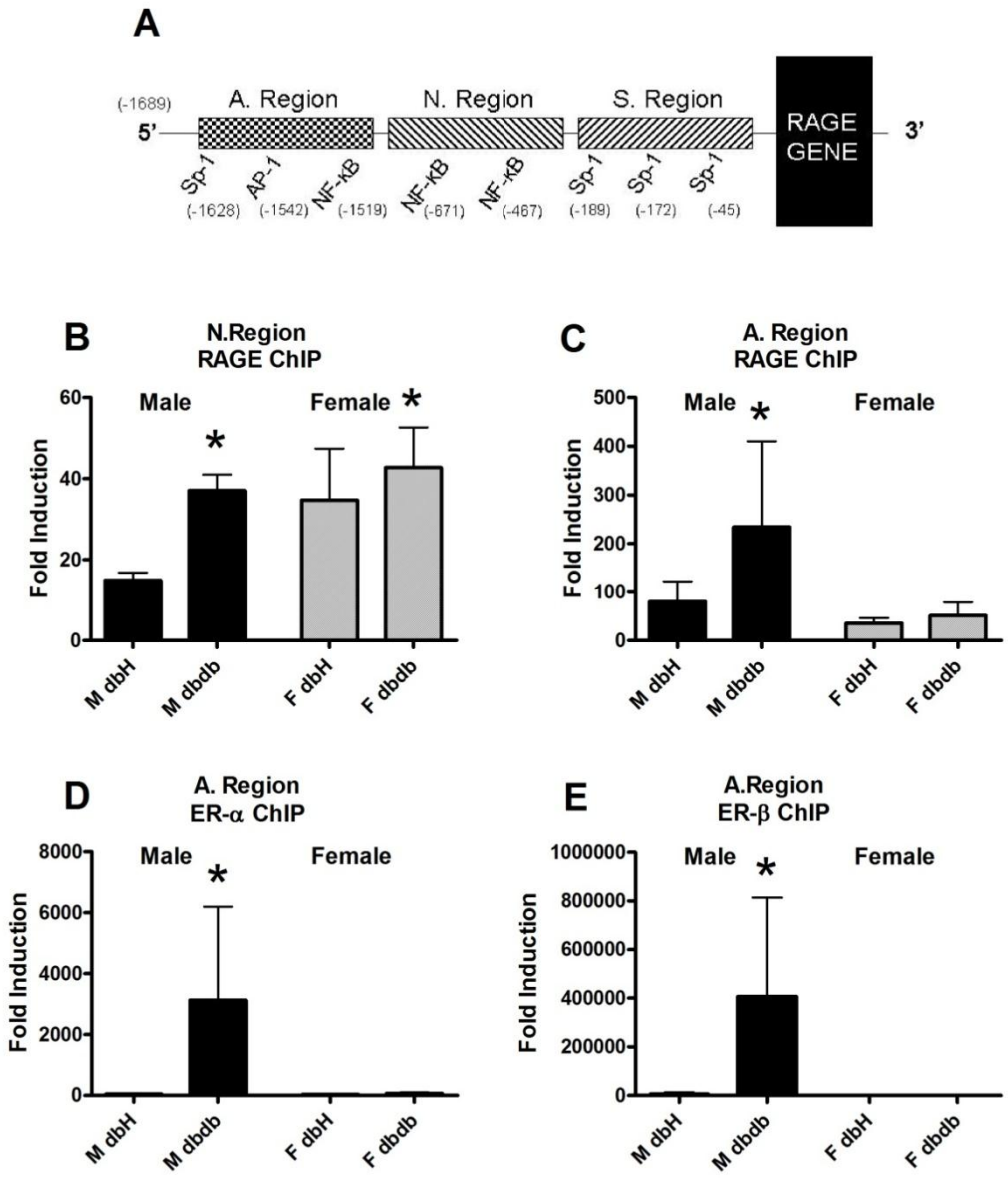

Figure 8. ChIP of ER-α, ER-β and RAGE, with analysis for transcriptional domain activity of the *AGER* gene

A) Representation of the transcriptional domain of the *AGER* gene (base pairs from gene). B) Analysis of 300bp fractionated chromatin fragments immune-precipitated with RAGE antibody and analysed for N.Region binding activity using RT-PCR C) Analysis of 300bp fractionated chromatin fragments immune-precipitated with RAGE antibody and analysed for A.Region binding activity using RT-PCR D) Analysis of 300bp fractionated chromatin fragments immune-precipitated with ER-α antibody and analysed for A.Region binding activity using RT-PCR E) Analysis of 300bp fractionated chromatin fragments immune-precipitated with ER-β antibody and analysed for A.Region binding activity using RT-PCR

M dbH (male db/H), M dbdb (male db/db), F dbH (female db/H), F dbdb (female db/db)

* $p<0.05$ v M dbH

Chapter 5

Chapter 5 Introduction

Interleukin-6 (IL-6), is a RAGE/NF-κB dependent gene, and is therefore likely affected by RAGE and estrogen interactions. To investigate if this we utilised the IL-6 deficient (IL-6-/-) mouse and littermate controls which were high fat fed for 16 weeks rendering them obese. We studied the onset of renal disease and the IL-6 dependent inflammatory pathways.

This manuscript is In Press in the journal, Nephrology.

Declaration for Thesis Chapter Five

Declaration by candidate

In the case of Chapter Five, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Performed and analysed all experiments on kidneys, prepared manuscript	90%

The following co-authors contributed to the work. Co-authors who are students at Monash

University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
JM Forbes	Preparation of manuscript	
VA Matthews	Provided animal model, Revision of manuscript, provided funding	

Candidate's Signature		**Date** 24/10/2011

Declaration by co-authors

The undersigned hereby certify that:

(19) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(20) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(21) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(22) there are no other authors of the publication according to these criteria;
(23) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(24) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Baker IDI Heart and Diabetes Institute**

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

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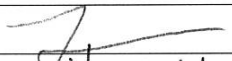
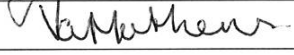
Signature 1		11TH OCT 2014
Signature 2		

OBESITY INDUCED RENAL IMPAIRMENT IS EXACERBATED IN INTERLEUKIN-6 KNOCKOUT (IL-6-/-) MICE

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Running Head: IL-6 KO worsens kidney injury in obesity

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Abstract

Background/Aims: Interleukin-6 is secreted from adipose tissue and thought to contribute to obesity related disorders. The aim of this study was to assess if IL-6 knockout (IL-6-/-) mice would develop obesity induced renal impairment.

Methods: WT and IL-6-/- mice were high fat fed (HFF) for 16 weeks to induce obesity. At the end of the study, renal function was measured via Albumin/Creatinine ratio and serum Creatinine levels, using ELISA and HPLC. Glomerulosclerotic index (GSI) was scored in periodic acid shift stained sections and collagen IV accumulation was assessed by immunohistochemistry. Renal cortical TGF-β_1 activity and MCP-1 levels were measured via ELISA.

Results: Renal IL-6 concentrations were increased with obesity. Although both WT HFF and IL-6-/- HFF mice exhibited renal impairment as measured by increased serum creatinine and urinary albumin/creatinine ratios, this was exacerbated in IL-6-/- mice. Obese mice had renal activation of cortical TGF-β_1 which was also higher in IL-6 -/- mice. Collagen IV staining was not affected by obesity. GSI was increased with obesity in both WT and IL-6-/- mice.

Conclusion: Obese IL-6-/- mice demonstrated renal functional and structural abnormalities above that seen in obese WT mice. We suggest that absence or low IL-6 levels may be an important accelerating factor implicated in the development and progression of obesity induced renal disease.

Key Words: IL-6, IL-6-/-, high fat feeding, obesity, nephropathy, collagen IV

Introduction

Both diabetes and obesity are known to be independent contributors to the development and progression of renal disease. Interleukin-6 (IL-6) has been implicated in the development of obesity related disorders, such as cancer (1) and hepatic disorders (2). However its role in obesity related renal disease has not been evaluated. IL-6 is considered pleiotrophic in nature due to its signalling threshold. Acute IL-6 signalling is considered beneficial and is mediated via IL-6 interacting with the membrane bound IL-6 receptor (IL-6R). However, chronic hyperactive IL-6 signalling is likely due to IL-6 interacting with the soluble IL-6 receptor (3, 4). Indeed, between 10 and 35% of the body's IL-6 pool is secreted from white adipose tissue, hence levels coincide with increased adiposity (5-7). Furthermore, elevated levels of IL-6 are seen in type 2 diabetic patients, particularly those with insulin resistance (5-7).

IL-6 levels in renal tissue are secreted in relatively large amounts by diabetic mesangial cells in response to high glucose along with tumour necrosis factor alpha (TNF-α) and monocyte chemotractive protein (MCP-1), and are thought to be crucial for the development of diabetic nephropathy (8). Lectins (9) and immune complexes such as receptor for advanced glycation end products (RAGE) (10) also interact with renal IL-6 contributing to nephropathy. IL-6 regulation of tumor growth factor- β may also be involved in nephropathy progression (11). Furthermore, circulating IL-6 levels are elevated in patients with chronic renal failure (12), and serve as a predictor of mortality in patients with end stage renal disease (13-15). Hence targeting the IL-6 pathway is being investigated as a potential therapeutic target in renal metabolic disease. However previous renal studies, which either utilised the IL-6 knockout mouse (IL-6-/-), or administered therapeutics that resulted in IL-6 antagonism demonstrate paradoxical results. In studies of acute kidney injury, gene deletion of IL-6 attenuated $HgCl_2$ induced acute kidney injury. Conversely, in sodium-arsenite induced renal injury, IL-6 gene deletion exacerbated immune cell autophagy resulting in increased renal injury compared with controls (16). In an in vivo model of mesangioproliferative disease, Etiner

et al (17) showed that IL-6 was not an important mediator of mesangial cell proliferation and glomerular matrix production, both vital processes in the development of renal pathologies. Furthermore, transgenic IL-6 mice that constitutively express IL-6 in the liver progress to develop considerable renal lesions that initiate in the proximal and distal tubules. Hence, this provides support for the use of IL-6 antagonists as potential therapeutics for renal disease.

Our previous utilisation of a mouse deficient in IL-6, demonstrated that when administered a high fat diet, IL-6-/- mice develop obesity and insulin resistance (18). This was also demonstrated by Wunderlich *et al* (19), confirming the role of liver IL-6R signalling in the prevention of insulin resistance and maintenance of glucose homeostasis. Previous studies suggest insulin resistance and decreased glucose control lead to a decline in renal function (5, 20). In this study, we aim to investigate for the first time if IL-6 plays a role in obesity related renal impairment.

Methods

Animal Model: Male C57BL6/J wild type (WT) and C57BL6/J mice with a global IL-6 gene deletion (IL-6-/-, Kopf and Kohler) were placed onto a standard chow (8% fat, Specialty Feeds, Perth, Australia) or high fat diet (HFD, 23% fat, SF04-001, Specialty Feeds, Perth, Australia) from 8 weeks of age for 16 weeks. All groups were comprised of 10 mice. Animals were housed in a temperature controlled environment with a 12 hour light dark cycle and the study was performed at Baker IDI Heart and Diabetes Institute, Melbourne, Australia, in accordance with guidelines from the AMREP Ethics Committee and the National Health and Medical Research Council of Australia.

We have previously published and characterised the mice used for this study (18). The effects of a high fat diet on body composition and metabolic parameters were reported; WT and IL-6-/- mice had significantly higher body weights at the study's conclusion when fed a high fat diet (18). In addition, IL-6-/- mice were more overweight and had increased body fat percentages than their respective wild type controls when fed a control diet (Chow, (18)). We have therefore previously assessed the suitability of using this model to study obesity related renal disease. We also demonstrated previously that fasting plasma glucose and insulin levels were increased following the consumption of a high fat diet, in both mouse strains (WT Chow v WT HFF and IL6-/- Chow v IL6-/- HFF $p<0.05$, (18)).

Measurements of Physiological, Biochemical and Metabolic Parameters: After 15 weeks of the study, mice were housed for 24 hours in metabolic cages (Comprehensive Laboratory Animal Monitoring System, Columbus Instruments, Columbus, OH) to measure food and water intake. Mice were weighed at killing following cardiac bleed, kidneys were harvested and frozen, and urine was collected from the bladder. Peri-renal fat pads were removed and then weighted.

Assessment of Renal Function: Urinary albumin concentration was assessed using a mouse albumin ELISA kit following manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). Urinary

creatinine and serum creatinine were determined following HPLC (Agilent HP1100 system, Hewlett Packard, Nuremburg, Germany) measurement of creatinine content in timed plasma and urine samples as previously described and in accordance with AMDCC guidelines (21). Glomerulosclerotic Index (GSI) was assessed in a blinded manner by a semi-quantitative method in paraffin embedded PAS-stained sections as previously described (22). Forty glomeruli per section were graded according to the severity of glomerular damage including mesangial matrix expansion and/or hyalinosis with focal adhesions, true glomerular tuft occlusion, sclerosis and capillary dilation. Specifically, grade 0 indicates a normal glomerulus; 1, <25% glomerular injury; 2, 26-50%; 3, 51-75%; 4, >75%. GSI was calculated using the formula: GSI = (1 x n1)+(2 x n2)+(3 X n3) + (4 x n4)/(n0+n1+n2+n3+n4), where n= number of glomeruli scored within each grade of glomerulosclerosis.

Collagen IV Immunohistochemistry: Immunohistochemistry analysis for Collagen IV was performed on paraffin embedded neutral buffered formalin fixed murine kidneys as previously described (22).

TGF-, IL-6 and MCP-1 ELISAs: IL-6, active TGF- β_1 and MCP-1 were measured in protein extracts from whole kidney cortex. 50mg of kidney cortex was homogenised in PBS and 0.5mm zirconiumoxide beads for 4 minutes using a bullet blender system (Next Advance, Averill Park, NY, USA). The IL-6 and MCP-1 ELISAs were performed according to the manufacturer's instructions (Quantikine, R&D Systems, Minneapolis, MN, USA). The TGF-β_1 ELISA was performed according to the manufacturer's instructions, which involved, acid treatment of the samples prior to performing the ELISA to ensure that active TGF-β_1 was measured (Promega, Sunnyvale, CA, USA).

Statistical Analysis: Results are expressed as Mean (±SD) unless otherwise specified. Analyses were performed by one way ANOVA followed by a Tukey's test post-hoc analysis (Graph Pad Prism, La Jolla, CA, USA). A $p<0.05$ was considered to be statistically significant.

Results

Renal Physiological and Functional Parameters and Inflammatory Marker Expression following 16 weeks of HFF

Renal function has previously been seen to decline with obesity (23, 24). Renal hypertrophy (KW/BW) was significantly increased following 16 weeks of HFF (WT Chow v WT HFF and IL6-/- Chow v IL6-/- HFF, $p<0.05$), however significant differences were not observed between Chow fed groups (Figure 1A). Peri-renal white adipose tissue deposits were significantly increased in HFF animals compared with Chow fed mice (Figure 1B).

Following 24 hours of metabolic caging, we saw no significant differences between the groups with regards to food or water intake (data not shown).

We assessed microalbuminuria by calculating urinary Albumin/Creatinine ratio (Figure 2A). Microalbuminuria was increased in mice that were HFF, compared to chow fed mice (WT Chow vs. WT HFF, $p<0.05$; IL-6-/- Chow vs. IL-6-/- HFF, $p<0.05$). Interestingly, microalbuminuria was further worsened in IL-6-/- HFF vs. WT HFF mice ($p<0.05$, Figure 2A). Serum creatinine concentrations significantly increased with HFF in IL-6-/- mice (IL-6-/- Chow vs. IL-6-/- HFF $p<0.05$) but not in WT HFF mice (WT Chow vs. WT HFF $p>0.05$, Figure 2B).

IL-6 concentrations in renal tissue were found to increase in WT mice following high fat feeding (Figure 2C) but were absent in IL-6-/- mice as expected (data not shown). Active TGF-β_1 in renal cortices increased as a consequence of both IL-6 gene deletion and high fat feeding (Figure 2D). Renal cortical monocyte chemoattractant protein, MCP-1 was increased, in WT HFF mice (Figure 2E). Renal expression of MCP-1, did not increase with high fat feeding in IL-6-/- mice (Figure 2E).

Histological analysis of renal tissue following 16 weeks of HFF

Semi- quantitative analysis of glomerulosclerosis (GSI) in PAS stained sections demonstrated an increase in focal segmental damage in glomeruli with high fat feeding in WT and IL6-/- mice. Significantly less damaged glomeruli were observed in IL-6-/- Chow fed mice compared with WT Chow fed mice (Figure 3A).

Quantified immunohistochemical staining for Collagen IV in renal cortex revealed a significant decrease in the percentage of glomerular area stained in IL-6-/- Chow fed mice (Figure 3F and 3I).

Discussion

This study highlights for the first time that deletion of the *IL-6* gene and therefore lack of IL-6 protein, does not afford protection against obesity induced renal impairment.

We demonstrated that IL-6 is not involved in adipose deposition in a HFF model of obesity, as IL-6-/- HFF mice possessed comparable bodyweight, body fat percentages, and fat pad weights as their WT counterparts. IL-6-/- mice on a chow diet have previously been shown to develop IR (19).

IL-6 has previously been shown to play a vital role in wound healing via modulation of collagen deposition (25). Here we saw both collagen IV deposition and glomerulosclerosis levels decrease in IL-6 mice, though not in HFF animals. Both the IL-6-/- Chow and HFF mice had increased activity of renal TGF-β_1, which may be a consequential compensatory effect of IL-6 deletion. We also observed a significant decreased expression of renal MCP-1 obese IL-6-/- mice compared to obese wild-type mice. This has been observed in previous studies, whereby MCP-1 promoted vascular inflammation and subsequent injury was prevented in IL-6-/- mice (26).

Whilst anti-IL-6 therapies have proven effective in the treatment of some inflammatory diseases (rheumatoid arthritis (27) etc.), and some cancers (28), the side-effects associated with the blockade or even the deletion of IL-6, is significant weight gain and insulin resistance. Both side-effects bring their own complications. Here we have assessed the effect of IL-6 gene deletion in a model of obesity, and find that it is ineffective in a condition of metabolic stress, and in fact exacerbates the pathogenesis of renal disease. In our hands and others, the IL-6-/- mouse model also develops obesity when fed a normal chow diet (18, 29).

Tomiyama-Hanayama *et al*, demonstrated renal protection in a high fat fed mouse model treated with the experimental IL-6 receptor antagonism therapeutic antibody; MR16-1 (30). This study concluded that there was renal protection with therapeutic MR16-1 administration in combination with a high fat diet, though they failed to report a non-high fat fed control group and

treatment was for only a four week period (30). These results contradict our own as we show that blockade of IL-6 signalling via *IL-6* gene deletion does not improve renal function in a metabolically stressed environment. The IL-6 transgenic mouse fed a normal chow diet develops proliferative glomerulonephritis (31), which can be alleviated with the administration of IL-6 receptor antagonistic therapeutics. These results and the findings that are presented in our current study highlight that the consequential binding of IL-6 to its receptor has a duel action that either provides protection or initiates pathological pathways. Although the differential activation of intracellular pathways and the intensity of ERK1/2 or STAT1/3 signalling in these conditions has not been fully defined as yet, it does suggest that attenuation of IL-6 signalling in a clinical setting should proceed with caution. We speculate that ERK1/2 and/or STAT1/3 signalling which stems from the IL-6 signal transducing receptor, gp130, is required to protect against obesity induced renal disease.

The differential response, between the actions of complete IL-6 removal, demonstrated here and IL-6 receptor antagonism results demonstrated by others (30), further highlights the pleiotrophic nature of IL-6. In obesity related diseases, it appears that IL-6 presence and action upon the IL-6 receptor is necessary for protection. Whilst as seen by Kopf et al, over expression of IL-6 resulted in disease progression, only alleviated by blockage of the receptor (32). We conclude that research in this area has not progressed far enough to administer with certainty therapeutics against IL-6 and the IL-6 receptor to alleviate renal disease.

Abbreviations: AGEs, Advanced glycation end products; IL-6, interleukin-6: IR, insulin resistance; DN, diabetic nephropathy; NF-κB, nuclear factor kappa B; RAGE, receptor for advanced glycation end products; T2D, type 2 diabetes; TGF-β_1, tumour growth factor beta TNF-α, tumour necrosis factor alpha.

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Chapter 5: Tables and Figures

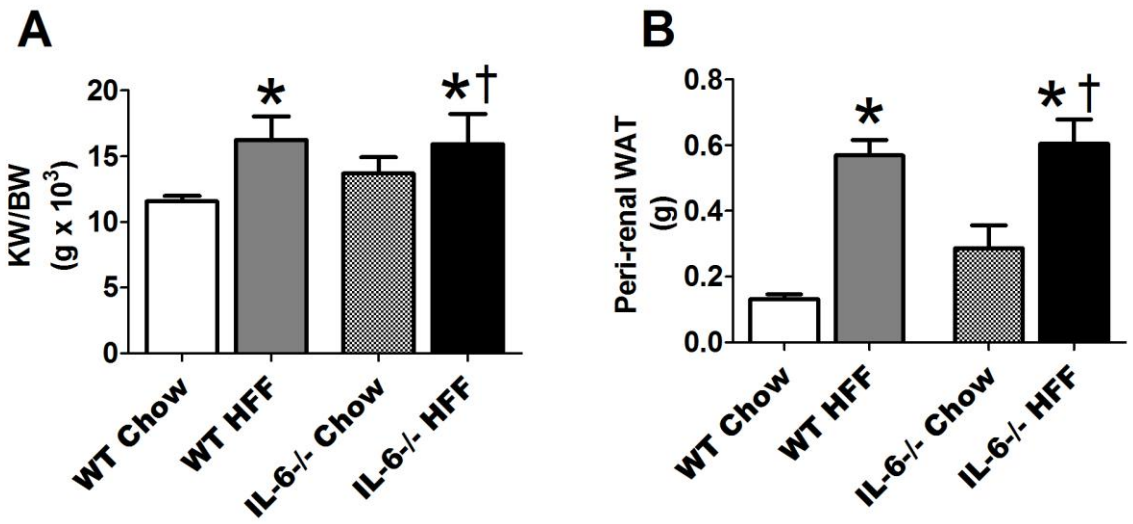

Figure 1: Physiological and Biochemical Characteristics

A) Renal Hypertrophy calculated by Kidney weight/Body weight B) Peri-renal WAT weight. Data are presented as Mean (±Standard Deviation). WT (Wild-type), IL-6-/- (IL-6 Gene deletion), HFF (high fat fed), BW (Body weight), KW (kidney weight). Data are presented as Mean ±SD.

* $p<0.0001$ v WT Chow; † $p<0.05$ v WT Chow

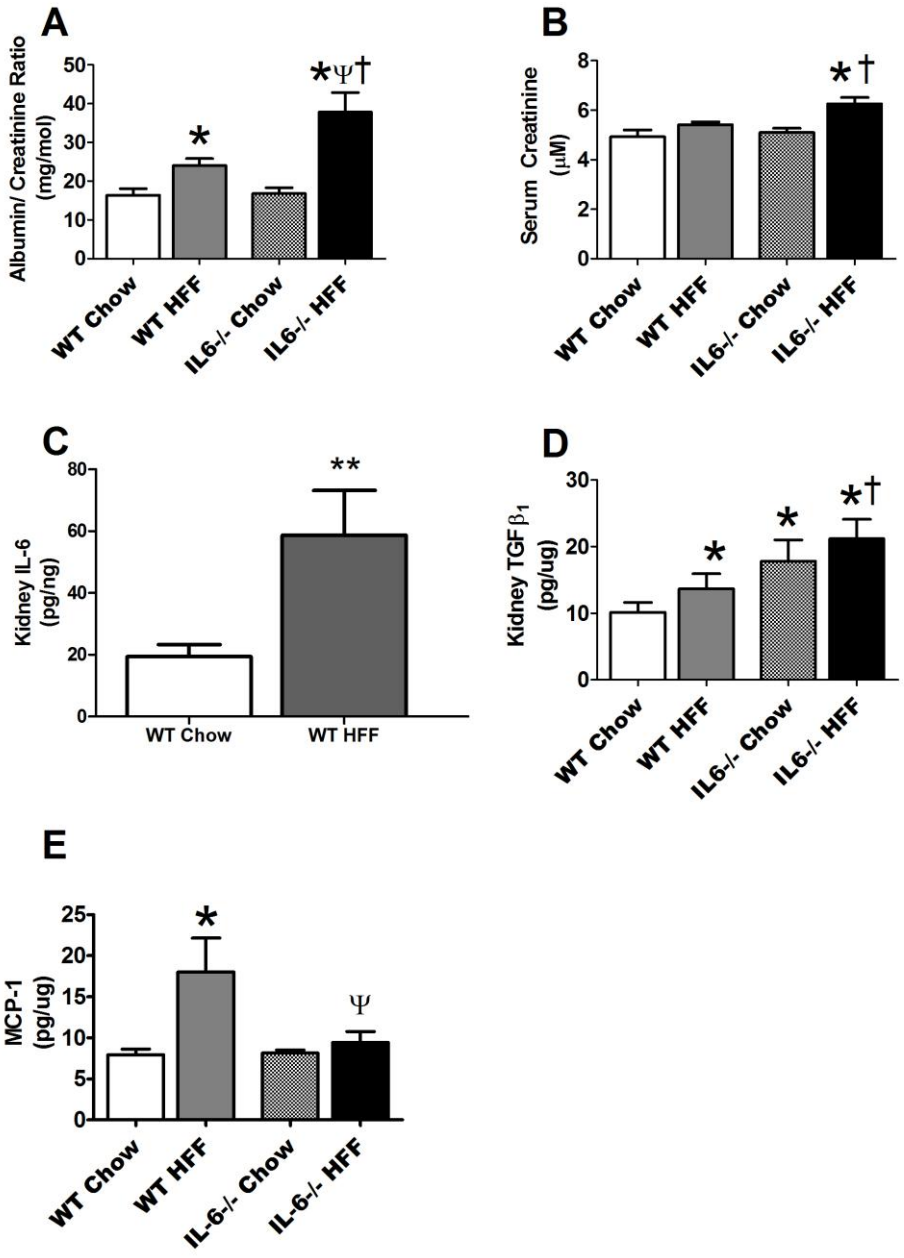

Figure 2: Renal Function Parameters and Inflammation Markers following 16 weeks of HFF A) Microalbuminuria in urine calculated via Albumin/Creatinine Ratio as measured by ELISA and HPLC respectively. B) Serum creatinine concentration as determined by HPLC. C) Kidney IL-6 concentrations in WT mice measured by ELISA. D) Kidney TGF-β_1 concentrations measured by ELISA. E) Kidney MCP-1 concentrations measured by ELISA. WT (Wild-type), HFF (high fat fed), IL-6-/- (IL-6 Gene deletion). Data are presented as Mean ±SD.

* p<0.05 v WT Chow, ** p<0.01 v WT Chow, ψ p<0.05 v WT HFF, † p<0.05 v IL-6-/- Chow

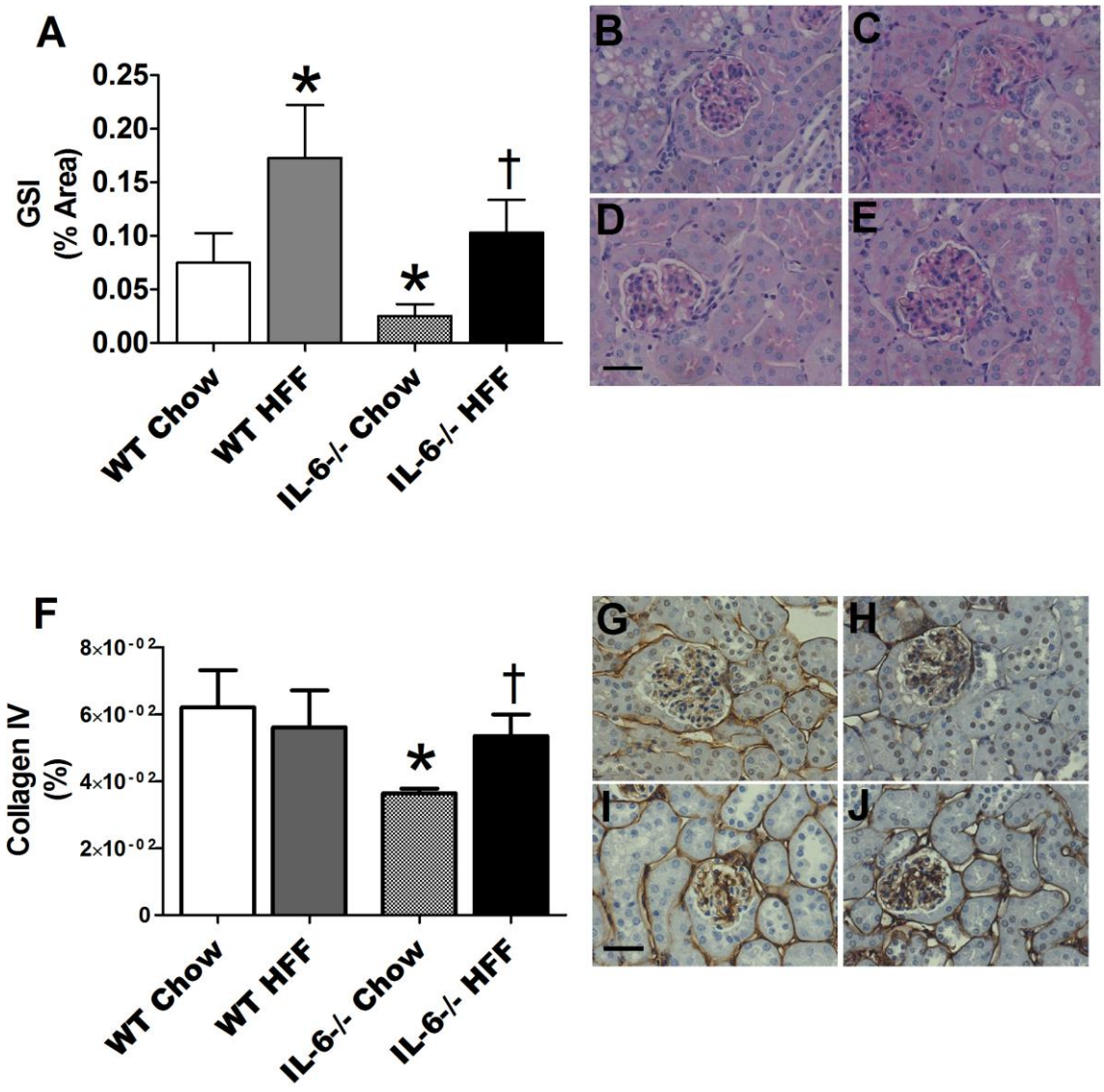

Figure 3: Glomerulosclerotic Index and Collagen IV following 16 weeks of HFF A) Semi-quantitative analysis of scored glomeruli. **B-E** Representative images of glomeruli from; B) WT Chow C) WT HFF D) IL6-/- Chow E) IL6-/- HFF. F) Quantified kidney collagen IV expression **G-J** Representative images of glomeruli from G) WT Chow, H) WT HFF, I) IL6-/- Chow, J) IL6-/- HFF. Data are presented as Mean ±SEM. 40 x magnification. Scale bar 15µm.

* $p<0.05$ v WT Chow, † $p<0.05$ v IL-6-/- Chow

Chapter 6

Conclusions and Future Directions

In contrast to females within healthy body weight ranges having a lower risk of developing chronic kidney disease, obese females and males are equally at risk of developing renal dysfunction, cardiovascular disease, and type 2 diabetes. Obesity is also thought to impair renal function. This suggests that obesity may be an important risk factor for the development of nephropathy in type 2 diabetes, given that a high proportion of individuals with T2D are overweight or obese. Within this thesis, I investigated if the loss of protection in females due to obesity thereby increasing their risk for renal disease, was mediated by the secretion of estrogens from local white adipose deposits altering normal tissue estrogen balance. Furthermore, I examined if obesity related renal damage as a result of estrogen imbalances, was mediated via the receptor for advanced glycation end products, a known mediator of renal damage in diabetes. Previously, others have demonstrated the importance of fat pads for the control of IR [1] and the influence they have on organ function, such as is seen within the pancreas in cancer [2]. Given this, and the previously reported secretion of the estrogen, estrone from white adipose tissue, I examine the effects of estrogen receptor binding to estrogen responsive domains within the 5' transcriptional domain of the RAGE gene (AGER). In addition, I studied the expression and actions of estrogens and their receptors in the kidney and related these to modulation and expression of the RAGE in renal disease in obesity and in type 2 diabetes. I also investigated the contribution of dietary AGEs, which are known RAGE ligands, to the development of nephropathy in obesity. These studies were performed in obese humans to assess if the beneficial effects of reduced dietary AGE consumption could improve renal function. Furthermore, high fat fed mice were studied following administration of the AGE lowering therapy, alagebrium chloride, or in mice with a genetic deficiency in RAGE. Lastly this thesis investigated the role of IL-6, a possible downstream mediator of the effects of RAGE in a high fat induced model of obesity, to delineate the contribution of inflammation to obesity related renal disease.

Firstly, I investigated the development of renal disease as a consequence of obesity within Chapter 2. Specifically, I examined if excessive dietary fat and AGE intake, contribute to obesity related renal disease via RAGE. I also tested the efficacy of an AGE lowering therapeutic, alagebrium chloride in mice and a low AGE diet in humans and mice. The human and the *in vivo* mouse data obtained were complementary and identified that reduction of dietary AGEs improved renal function in the context of obesity. There has previously been one study which suggests that a low AGE diet may improve renal function, although this was performed in individuals with renal failure who were on dialysis [3]. Furthermore, a rodent study has also shown that a low AGE diet can improve renal function in mouse models of type 1 and type 2 diabetes albeit over a short time time-period [4]. I further identified that a deficiency in the RAGE gene and therefore deletion of a membrane bound RAGE receptor, was beneficial and decreased the amount of AGE induced renal injury seen with obesity. We also demonstrated the beneficial effects of lowering AGE accumulation using alagebrium chloride, to combat renal dysfunction resultant of obesity. This is consistent with our previous studies where alagebrium chloride has shown significant effects on renal function in rodent models of type 1 diabetes [5, 6] and reduced cardiac abnormalities seen in obese mice [7]. Alagebrium chloride has previously been administered to patients for the treatment of isolated systolic hypertension, where it improved endothelial dysfunction [8], and to individuals with diastolic dysfunction [9] and heart failure [10, 11]. Within Chapter 2, I also demonstrated that exacerbation and activation of membrane bound renal RAGE is pertinent for the development of obesity related renal disease and that ligands derived from food sources are able to mediate this damage.

Therefore, Chapter 2 suggested that the AGE-RAGE axis warranted further investigation as precipitating agent for renal disease as a consequence of obesity. In Chapter 3, I studied the possible role of RAGE regulation via adipose derived estrogens and the effect on renal pathologies. A recent publication was the rationale for the development of the hypothesis as it had described the specific regulation of RAGE gene expression via 17-β-estradiol and ER-α [12]. In this chapter I firstly demonstrated the importance of adipose derived estrogens from the local peri-renal adipose tissue

depots, and examined the ability of these locally secreted estrogens influence the differential transcription of the *AGER* gene via interaction with estrogen receptors, ER-α and ER-β. In the renal tissues studied, *AGER* gene regulation by estrogens could occurred via both ER-α and ER-β, which interacted with DNA binding sites for AP-1 and Sp-1 respectively.

In Chapter 3, I also demonstrated the presence and DNA binding action of a novel nuclear RAGE isoform, which is referred to within this thesis as transcriptional regulator RAGE (tr-RAGE). This new isoform appears to have a self-regulatory role mediating RAGE gene transcription given that tr-RAGE demonstrated significant binding within the 5' promoter region of the *AGER* gene in the ChIP experiments, solely or in combination with NF-κB at a different biding site. Hudson *et al*, have previously reported the existence of multiple RAGE gene isoforms, are able to be produced both *in vitro* and *in vivo* via alternate splicing events [13, 14], and at least one of the splice variants had been identified in diabetic mouse kidneys. This thesis was limited in that, the MALDI-TOF sequencing of tr-RAGE was not completed. This will be required in the future to determine if tr-RAGE is a completely new isoform of RAGE, or whether we have identified a new function for 'membrane bound or cytosolic RAGE' as a nuclear transcription factor. Furthermore, in order to identify the specific binding sequence for RAGE within its own promoter region, a chromatin walk may be necessary within the regions identified in chapters 2 and 3.

The activation of RAGE is implicated in the pathogenesis of other chronic diseases both with and without obesity as a confounding factor, such as Alzheimer's disease [15], psoriasis, peripheral vascular disease, atherosclerosis [16], congestive heart failure [17] and diabetic microvascular complications [6, 18]. Therefore, the implication of identifying RAGE as a self-perpetuating transcriptional regulator may be applicable across other disciplines in medicine and further our understanding of the pathogenic mechanisms of other chronic inflammatory conditions. Furthermore, this thesis has also contributed further knowledge to the understanding of RAGE as an estrogen modulated gene, implicating that RAGE may also be involved in conditions where estrogen

balance is disrupted, including estrogen positive breast cancer [19] and encapsulating peritoneal sclerosis [20].

In Chapter 4, I utilised a mouse model of type 2 diabetes to determine whether males and females had differential regulation of renal RAGE in T2D, since they have different estrogen regulation and expression. As the male db/H mice also developed obesity without type 2 diabetes at the study endpoint, it allowed for the individual analysis of four separate study areas; (i) renal dysfunction as a result of obesity in the absence of diabetes (male db/H mice), (ii) overt renal disease in type 2 diabetes (male db/db mice) (iii) obesity and renal dysfunction in type 2 diabetes and the loss of renal protection due to adipose derived estrogens (female db/db mice) and (iv) a group that was afforded protection and did not develop obesity, renal disease or diabetes (female db/H mice). These experiments allowed me to assess which pathogenic pathways were mediated by hyperglycaemia, increased adiposity alone or via a combination of both of these defects. We demonstrated that whilst both male and female diabetic mice had the same degree of adiposity and glycaemic control, female mice did not develop the same degree of renal dysfunction seen in male db/db mice. In addition, male db/H mice also had worse renal dysfunction than was seen in female db/H mice. This was likely due to protective estrogens preventing the development of the same degree of renal dysfunction that we saw in male mice. These findings are consistent with previous studies suggesting that females are less susceptible to chronic kidney disease [21, 22].

In this study, I also investigated if the novel RAGE isoform discovered in chapter 3, tr-RAGE, is differentially regulated in male versus female mice and how this was affected by type 2 diabetes. The ChIP experiments demonstrated that RAGE binding to NF-κB region of the RAGE promoter is not estrogen or adipose dependent, but likely a result of hyperglycaemia, given that expression was increased equally in both male and female db/db mice. However, I demonstrated that binding within the most distal region of the RAGE promoter, which has binding sites for Sp-1, AP-1 and NF-

κB, is increased in obese diabetic male mice, corresponding with increased levels of imbalance of adipose derived estrogens, and this is not seen in female db/db mice.

These findings led to the conclusion in Chapter 4, that obesity ameliorates the protection normally afforded females, and they develop renal disease and type 2 diabetes at similar rates to that seen in male mice. However, renal disease in females did not progress to the same degree that was seen in males. This was likely a result of protection afforded by estrogens in female mice, which had lower estrone secreted from peri-renal adipose tissue depots than was seen in male db/db mice. Other previous studies have also shown that local adipose tissue depots can influence organ behaviour via paracrine secretion of mediators [2]. In addition, female db/db mice did not have any binding to the AGER promoter by RAGE within the A.region, which contains binding sites for Sp-1, AP-1 and NF-κB. Understanding that different pathways that are involved in the pathogenesis of chronic diseases between males and females is important for the development of better targeted future therapeutics, not only for obesity and diabetic renal disease, but also for other conditions where estrogens influence disease progression.

In Chapter 5, we utilised another novel *in vivo* model of high fat feeding; the interleukin-6 knockout mouse (IL-6-/-). IL-6 is also secreted from WAT, has a plieotrophic nature and is capable of being induced by RAGE via NF-κB. We utilised the IL-6-/- mice to delineate if the beneficial renal effects we had seen in the RAGE knockout mice were as a result of inhibition of IL-6 action, which was thought to be the case given our results in chapter 3 showing mediation of IL-6 expression by AGE lowering therapeutic approaches. My findings within this chapter, however, demonstrated that the beneficial effects on the development of renal injury seen via intervention to reduce AGEs or RAGE signalling in obesity, were not inhibition of IL-6 expression. This was apparent since perhaps surprisingly, IL-6-/- mice developed more pronounced real injury than their wild-type littermates

following high fat feeding. This however is in agreement with one previous study suggesting that IL-6 deficiency had no influence on the development of renal disease as a consequence of X [23].

In summary, this thesis demonstrated a potential role for local paracrine estrogen action in the modulation of kidney RAGE, via ERα and ER-β, Sp-1 and AP-1, in models of obesity and type 2 diabetes. I also showed obesity and renal injury as the result of a high fat high AGE diet, could be improved by targeting the AGE-RAGE axis in mice and via lowering dietary AGE intake in obese humans. Importantly, this thesis has contributed to understanding the genetic regulation of RAGE not only in obesity related renal injury and T2D, but likely other chronic diseases, by identifying a new novel transcriptional regulator of RAGE, trRAGE.

Given that an estimated 284 million persons worldwide by 2030 will be overweight or obese with insulin resistance or type 2 diabetes, it is hoped that the knowledge presented in this thesis will ultimately contribute to the development of therapies to combat the diabetic nephropathy epidemic that is manifest.

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Supplementary

original article http://www.kidney-international.org

see commentary on page 133

Targeted reduction of advanced glycation improves renal function in obesity

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Obesity is highly prevalent in Western populations and is considered a risk factor for the development of renal impairment. Interventions that reduce the tissue burden of advanced glycation end-products (AGEs) have shown promise in stemming the progression of chronic disease. Here we tested if treatments that lower tissue AGE burden in patients and mice would improve obesity-related renal dysfunction. Overweight and obese individuals (body mass index (BMI) 26–39 kg/m^2) were recruited to a randomized, crossover clinical trial involving 2 weeks each on a low- and a high-AGE-containing diet. Renal function and an inflammatory profile (monocyte chemoattractant protein-1 (MCP-1) and macrophage migration inhibitory factor (MIF)) were improved following the low-AGE diet. Mechanisms of advanced glycation-related renal damage were investigated in a mouse model of obesity using the AGE-lowering pharmaceutical, alagebrium, and mice in which the receptor for AGE (RAGE) was deleted. Obesity, resulting from a diet high in both fat and AGE, caused renal impairment; however, treatment of the RAGE knockout mice with alagebrium improved urinary albumin excretion, creatinine clearance, the inflammatory profile, and renal oxidative stress. Alagebrium treatment, however, resulted in decreased weight gain and improved glycemic control compared with wild-type mice on a high-fat Western diet. Thus, targeted reduction of the advanced glycation pathway improved renal function in obesity.

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KEYWORDS: alagebrium chloride; nephropathy; obesity; RAGE

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Obesity is an important risk factor for type 2 diabetes and its subsequent complications including renal and cardiovascular diseases. Between 2010 and 2030, it is estimated that worldwide numbers of diabetes cases will increase by 54%.[1] As such, the International Diabetes Federation has proposed lifestyle changes as a cost-effective method of preventing or delaying the onset of type 2 diabetes,[2] which would likely extend to manifestations of obesity such as an increased risk of chronic kidney disease.[3] Current figures show that 30–50% of individuals with diabetes will develop nephropathy.[4]

It is well known that certain lifestyle choices such as diets high in saturated fat and processed foods contribute to obesity and the development of type 2 diabetes, although the exact mechanisms involved have not been fully defined. Dietary fat and processed foods are extremely high in a group of sugar modifications known as advanced glycation end-products (AGEs). These molecules improve taste, reduce food spoilage, and promote longer shelf life. Excessive dietary intake of AGEs has recently been shown to contribute to renal[5] and cardiovascular[6] diseases and the development of type 2 diabetes, especially in the context of a high-fat diet in animal models.[7] Once in circulation,[8–10] dietary AGEs may cause inflammation and free oxygen radical production by modulation of specific receptors, including the receptor for AGE (RAGE). Interestingly, the kidney is the main organ responsible for the removal of AGEs from the bloodstream.[11] This high exposure of the kidney to AGEs is likely to make the organ particularly susceptible to AGE-mediated damage. The potential for reduction in dietary AGEs to improve renal function in nonobese, renal failure patients has been demonstrated after a 4-week low-AGE diet that reduced serum creatinine concentrations by 30–40%.[9]

This study investigated the effects of lowering the accumulation of AGEs or interrupting RAGE downstream signaling pathways using a model of obesity-related renal disease in mice. The efficacy of a reducing dietary AGE intake

to improve renal function in obese humans was also examined.

RESULTS

Clinical study

The baseline characteristics of the 11 participants are shown in Table 1. Although diets were isocaloric and matched for macronutrient content, on a 9 MJ/day diet, individuals were calculated to consume 14,090 kU *N*-carboxymethyllysine (CML) on the high-AGE diet and 3302 kU CML on the low-AGE diet. There was no effect of the dietary interventions on body weight, body mass index (BMI), or adiposity, which remained elevated (Table 2).

Renal function and inflammatory makers. Urinary albumin/creatinine ratios were significantly better following the low AGE dietary period in obese individuals (low- vs high-AGE diet: $P = 0.02$, Figure 1a). Plasma cystatin C levels were elevated following consumption of a high-AGE diet for 2 weeks (low vs high: $P = 0.02$, Figure 1b). Plasma CML concentrations following high AGE consumption declined (low vs high: $P = 0.01$, Figure 1c), whereas urinary CML concentrations increased following consumption of the high-AGE diet (low vs high: $P = 0.03$, Figure 1d). The high-AGE diet increased urinary 8-isoprostanes (low vs high: $P = 0.02$, Figure 1e). Plasma monocyte chemoattractant protein-1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2), was increased as a result of high AGE dietary consumption (low vs high: $P = 0.04$, Figure 1f). Conversely, however, plasma macrophage migration inhibitory factor (MIF) significantly declined after consumption of the high-AGE diet (low vs high: $P = 0.04$, Figure 1g). There were no significant effects of the order in which the diets were consumed on any of the parameters, when these data were analyzed via repeated measures analysis of variance with order as a between-subject factor. There were no differences in other circulating cytokines and transcription factors including endogenous secreted form of RAGE, soluble RAGE (sRAGE), nuclear factor-κB, interleukin-6, and high-sensitivity C-reactive protein between diets (data not shown).

Table 1 | Baseline anthropometric and biochemical data in obese individuals recruited for the dietary intervention study (*n*=11 patients)

	Mean (±s.d.)	Range
N	11	
Age (years)	30 (±9)	21–50
BMI (kg/m^2)	31.8 (±4.8)	27–36
Waist circumference (cm)	96.9 (±18.4)	78.5–115.3
Waist/hip ratio	0.91 (±0.12)	0.78–1.30
24 h Creatinine clearance (ml/s)	2.4 (±1.1)	1.3–4.2
Urinary CML (nmol/mol lysine)	11.5 (±14.4)	0.35–44.4
Serum CML (μmol/mol lysine)	224.5 (±166.9)	122.9–859.8
Fasting plasma glucose (mmol/l)	4.7 (±0.4)	4.1–5.5
Fasting plasma insulin (mU/ml)	10.2 (±4.1)	6.3–19.1
Insulin sensitivity (mg glucose/kg/min)	7.8 (±3.4)	2.5–17.1

Abbreviations: BMI, body mass index; CML, *N*-carboxymethyllysine.

Murine study

Biochemical and metabolic parameters. Both wild-type (WT) and RAGE–/– mice consuming the Western-style diet, high in AGEs and fat content, were obese by week 16 (ΔBW; Table 3), with significant increases in epididymal and omental adipose depots (Table 3). Increases in body weight and fat deposition after the Western diet were prevented using the AGE-lowering therapy, alagebrium (ALA, Table 3). Kidney size was unaffected by dietary consumption of a western-style diet (Table 3). Fasting plasma glucose and insulin concentrations were increased in obese mice following the consumption of the western diet in both WT and RAGE–/– mouse strains (Table 3), and the parameters were significantly improved in the mice treated with ALA.

Renal functional parameters. Renal function was assessed by albumin excretion rate and creatinine clearance. Obese WT mice consuming the Western-style diet had albuminuria (Figure 2a), which was reduced in obese RAGE–/– mice fed a Western diet but not with ALA. Creatinine clearance was elevated in obese WT mice and significantly improved by ALA (Figure 2b). Furthermore, a western diet did not induce hyperfiltration in RAGE–/– mice (Figure 2b). All obese mice had lower plasma CML concentrations (Figure 2c) despite consuming more dietary AGEs than lean low-AGE-fed mice (Figure 2d). Urinary CML excretion was below detectable limits (5.6 nmol/mol lysine) in all mice. Also of interest was that obese WT (16.8 ± 11.2 kJ/day) and RAGE–/– mice (29.7 ± 6.9 kJ/day) consumed less kilojoules per day than both lean low-AGE-fed mice (50.3 ± 3.4 kJ/day; $P < 0.05$ vs obese WT) or obese mice treated with ALA (34.9 ± 9.4 kJ/day; $P < 0.05$ vs obese WT).

Concentrations of the AGE CML in renal cortices were significantly increased in obese and obese ALA-treated animals but not in obese RAGE knockout mice when measured via enzyme-linked immunosorbent assay (ELISA; Figure 2e). Immunohistochemistry confirmed that there were increases in CML in renal cortices taken from obese mice that were not seen in lean low-AGE-fed mice (Figure 2f).

RAGE protein expression and inflammation. Membranous RAGE protein concentrations in renal cortices taken from obese WT mice were significantly higher than those in lean mice consuming a low-AGE diet (Figure 3a). This parameter was not affected by treatment with ALA (Figure 3a). Circulating levels of sRAGE, measured via ELISA, tended to be higher in obese mice, although they were significantly lower after ALA therapy (lean low AGE (216.6 ± 65.98 pg/ml) vs obese (352.2 ± 172.4 pg/ml) RAGE, $P < 0.05$; obese vs obese ALA (152.2 ± 55.67 pg/ml RAGE, $P < 0.05$). As expected, there was no expression of membranous or soluble RAGE protein detected in RAGE–/– mice (data not shown). Renal MCP-1 levels were significantly lower in obese ALA-treated animals and obese RAGE–/– mice (Figure 3b) when compared with untreated obese WT mice. Plasma MIF concentrations in mice were decreased with obesity and significantly increased by ALA treatment or in obese

Table 2 | Anthropometric and biochemical data at the completion of 2 weeks of dietary consumption of either a low- or high-AGE diet (*n*=11 patients)

	Following low-AGE diet Mean (±s.d.)	Following high-AGE diet Mean (±s.d.)	*P*-value for change
Weight (kg)	93.2 (±15.9)	93.9 (±15.8)	NS
BMI (kg/m^2)	31.5 (±4.2)	31.4 (±4.2)	NS
Body fat (%)	29.3 (±6.4)	29.2 (±6.8)	NS
Urine albumin (mg/day)*	20.27 (±34.8)	16.05 (±20.9)	NS
Serum creatinine (μmol/l)	72.3 (±18.3)	70.2 (±13.5)	NS
Total cholesterol (mmol/l)	4.2 (±0.9)	4.2 (±1.0)	NS
Fasting plasma glucose (mmol/l)	5.1 (±0.3)	4.8 (±0.3)	NS

Abbreviations: AGE, advanced glycation end-product; BMI, body mass index; NS, not significant ($P>0.05$).
This clinical study in obese individuals (BMI 31.8 (±4.8)) was performed as a single-blinded, randomized, crossover dietary intervention study.
*A nonparametric analysis was performed.

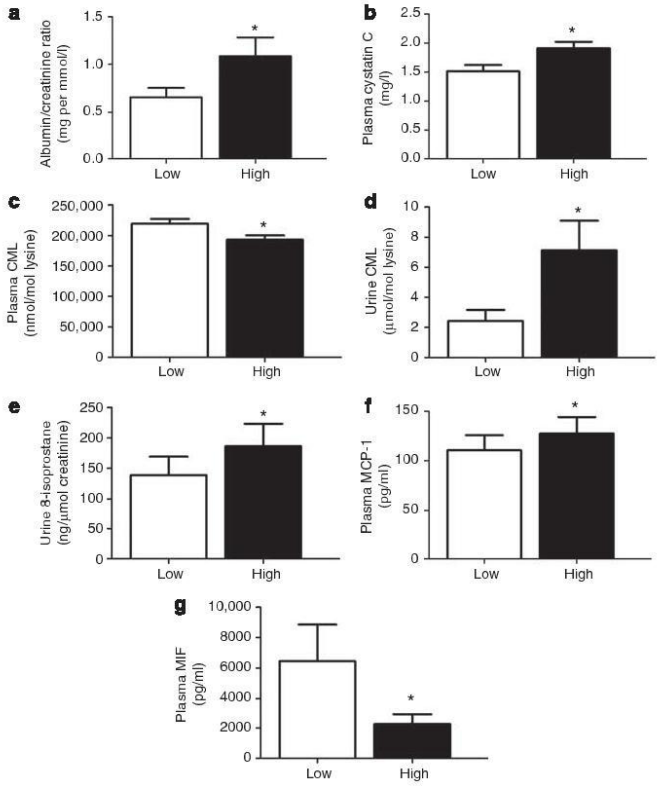

Figure 1 | Renal and inflammatory parameters in obese humans following dietary interventions. Assays were performed in samples collected from the same obese individuals following consumption of a diet either low or high in advanced glycation end-product (AGE) content for 2 weeks. (**a**) Urinary albumin/creatinine ratios, (**b**) plasma cystatin C concentration, (**c**) plasma concentrations of the AGE, *N*-carboxymethyllysine (CML), (**d**) urinary CML concentrations, (**e**) urinary 8-isoprostanes, (**f**) plasma monocyte chemotactic protein-1 (MCP-1) concentrations, and (**g**) plasma macrophage migration inhibitory factor (MIF) concentrations. *$P<0.05$ low- versus high-AGE diet, Student's paired *t*-test.

RAGE–/– mice (Figure 3c). Kidney MIF levels were increased in obese mice, which were not affected by ALA treatment; however, deletion of RAGE significantly decreased renal MIF concentrations (Figure 3d). Collagen IV deposition in glomerular cortices was not significantly different among treatment groups (Figure 3e and f).

Table 3 | Murine physiological and metabolic parameters at study completion (week 16)

	Δ Body weight (g)	Left kidney weight (g)	Omental adipose tissue (g)	Epididymal adipose (g)	KW/BW ratio ($\times 10^3$)	Plasma glucose (mmol/l)	Plasma insulin (ng/ml)
C57BL/6J							
Lean	2.9 (± 2.4)	0.19 (± 0.02)	33.5 (± 1.8)	0.89 (± 0.3)	11.35 (± 1.2)	5.3 (± 1.8)	0.24 (± 0.21)
Obese	11.0 (± 1.6)*	0.19 (± 0.01)	40.8 (± 2.6)*	1.59 (± 0.2)*	9.35 (± 1.2)*	8.5 (± 1.5)*	1.69 (± 0.68)*
Obese ALA	8.1 (± 2.5)*†	0.17 (± 0.02)	37.8 (± 3.8)*	1.31 (± 0.3)*†	9.74 (± 0.8)	6.6 (± 1.5)	0.66 (± 0.5)*†
RAGE–/–							
Obese	15.4 (± 2.2)*†	0.19 (± 0.01)	39.7 (± 2.6)†	1.94 (± 0.3)†	9.8 (± 0.6)	10.3 (± 3.1)	3.36 (± 0.97)*†

Abbreviations: AGE, advanced glycation end-product; ALA, alagebrium chloride; BW, body weight; KW, kidney weight; RAGE, receptor for AGE.
Data are presented as mean (± s.d.).
Obese (high AGE/high-fat diet), ALA (AGE-lowering therapy, alagebrium chloride 1 mg/kg/day), and RAGE–/– (RAGE deletion).
*$P<0.05$ vs lean low AGE, †$P<0.05$ vs obese.

Obesity induced excess cortical superoxide production in the mitochondrial (Figure 4a) and cytosolic compartments (Figure 4b). Treatment with ALA and the deletion of the *RAGE* gene significantly decreased renal superoxide levels (Figure 4a and b). Urinary 8-isoprostane concentrations were increased in obese mice; however, this was attenuated with ALA therapy (Figure 4b).

DISCUSSION

This study has provided evidence that intervention using diets low in AGE content may attenuate renal changes seen with obesity. Although our current human study did not encourage weight loss in obese participants because of matching of caloric intake and the short duration of dietary intervention (2 weeks), we were able to demonstrate that altering dietary AGE content alone is sufficient to improve inflammatory profiles and early renal disease. These findings are consistent with a previous study of patients with advanced end-stage renal disease.[12] To complement these findings, we performed studies in mice to further define potential mechanisms linking the AGE/RAGE axis to renal functional changes in the context of obesity. Indeed, our studies in obese mice highlighted that interfering with the AGE/RAGE axis by either preventing AGE tissue accumulation with the AGE-lowering therapy, ALA, or via *RAGE* deletion in RAGE–/– mice is protective against obesity-related renal dysfunction. These findings are consistent with previous evidence that AGE formation is important in the pathogenesis of other chronic kidney diseases.[13–17]

The increases in the expression of the proinflammatory protein RAGE in kidney cortices taken from obese mice, and its contributory role to obesity-related renal dysfunction in this model, was further suggested in obese RAGE–/– mice who had better renal function and less inflammation. Elevations in the circulating concentrations of sRAGE were also seen in obese mice, consistent with findings in type 2 diabetic individuals with nephropathy who are generally obese.[18,19] Although sRAGE was not changed after a low-AGE diet in our human study, this was most likely because of the short duration of the dietary intervention. It is possible that a longer dietary duration would have ultimately led to lower circulating sRAGE concentrations. This hypothesis is supported by the improved inflammatory profile seen with consumption of a low-AGE diet as reflected by decreased MCP-1 and MIF concentrations which in the context of previous studies which have associated increases in sRAGE with systemic inflammation.[19–21]

We have also demonstrated for the first time that total AGE burden is likely a combination of circulating, tissue and excreted AGE concentrations in obesity. Furthermore this can be modulated via alteration of diet or treatment with therapies, as was the case in our murine study. Previous other studies have reported that circulating AGE concentrations may be an indication of renal disease progression as they showed increases.[22] As a result of our findings, we therefore heed caution at this becoming a gold standard marker.

Given the findings of this study and the previously reported roles of RAGE, it is possible that inflammation plays a role in modulating the changes seen in this study. In both obese humans and mice, there was evidence of low-grade inflammation, which was enhanced by consumption of a high-AGE diet. This increased plasma MCP-1 and lowered MIF concentrations, attenuated by interrupting the AGE/RAGE axis, either by lowering the tissue AGE burden using dietary means, the AGE-lowering therapy ALA, or by deletion of *RAGE*. Activation of RAGE has already been reported to be crucial for macrophage recruitment, as highlighted by its role in host–pathogen defense.[23] Therefore, it is likely that RAGE activation as a result of AGE stimulation is a modulator of MCP-1 and MIF secretion in this study. However, obesity-related changes in circulating insulin concentrations seen in both humans and mice may also be indirectly modulating the expression of MIF (localized in the pancreatic islets[24]) and MCP-1 (from white adipose tissue[21,25]) that are known to affect insulin secretion and action, respectively.

AGEs and RAGE are also known to contribute to renal dysfunction via excess generation of reactive oxygen species.[26–28] High-AGE diets in both obese humans and mice appear to influence oxidative stress as reflected by increases in urinary isoprostanes and renal superoxide production. This pro-oxidant effect of AGEs is further suggested by the findings in obese mice that received ALA that appeared to

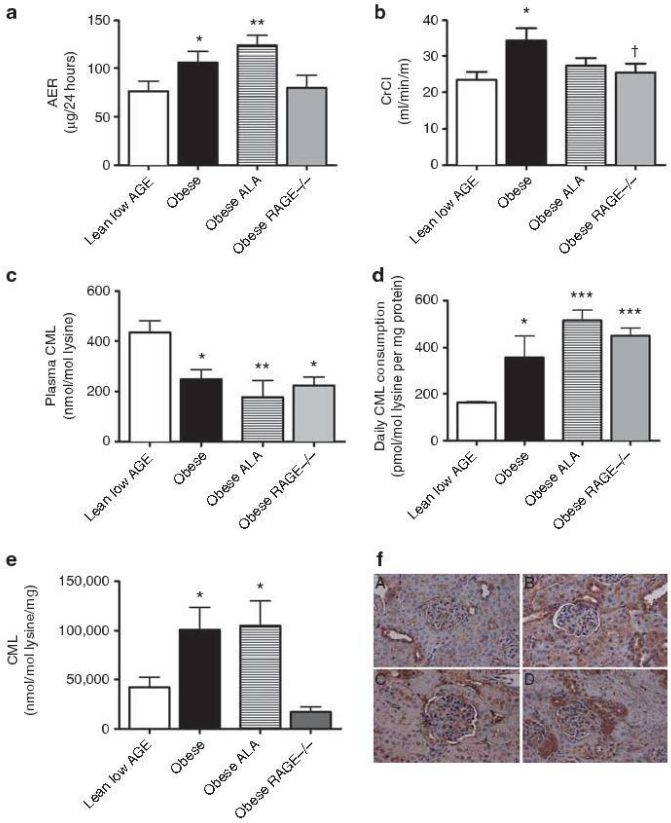

Figure 2 | Murine renal and biochemical parameters at study completion. Groups of mice were followed for 16 weeks. (**a**) Urinary albumin excretion rate (AER) over 24 h measured by enzyme-linked immunosorbent assay (ELISA). (**b**) Creatinine clearance (CrCl) as determined by high-performance liquid chromatography (HPLC) following correction for body surface area. (**c**–**e**) *N*-Carboxymethyllysine (CML) analyzed by ELISA in plasma (**c**), dietary CML consumption over 24 h (**d**), and kidney cortex protein (**e**). (**f**) CML immunohistochemistry staining on paraffin-fixed kidney sections from (A) lean low advanced glycation end-product (AGE), (B) obese, (C) obese alagebrium (ALA), and (D) obese RAGE–/–. Obese (high AGE/high-fat diet), ALA (AGE-lowering therapy, alagebrium chloride 1 mg/kg/day), and RAGE–/– (RAGE deletion). Data for AER were logarithmically transformed as these were not normally distributed. Other data are presented as mean ± s.d. $^{*}P<0.05$ vs lean low AGE, $^{**}P<0.01$ vs lean low AGE, $^{***}P<0.001$ vs lean low AGE, $^{\dagger}P<0.05$ vs obese.

have less oxidative stress. RAGE deficiency did not improve obesity-related increases in urinary isoprostane excretion, which was interesting given that this group also had a lack of effect on adiposity and obesity-related abnormalities in glycemic control. This suggests that the benefits afforded by low-AGE diets and ALA on oxidative stress may be partly independent of RAGE. This is not totally surprising as AGEs can interact with other receptors in addition to RAGE, and ALA is likely to have additive actions that may be relevant including a modest effect as an antioxidant.[15,29]

In conclusion, this study suggests that a low-AGE diet has an impact on modulating renal function in healthy obese individuals. Studies in murine models suggest that the mechanism responsible for AGE effects on renal function is likely to involve its receptor RAGE and include improvements in inflammation, oxidative stress, and glycemic control.

MATERIALS AND METHODS

Clinical study

Participant selection. This study was approved by the Alfred Hospital Ethics Committee and conducted according to the Declaration of Helsinki Principles. All individuals gave written informed consent before commencement of the study. Participants were males, aged between 18 and 50 years with stable body weight (weight change <5 kg in last year), BMI $\geqslant 25$ kg/m^2, normal glucose

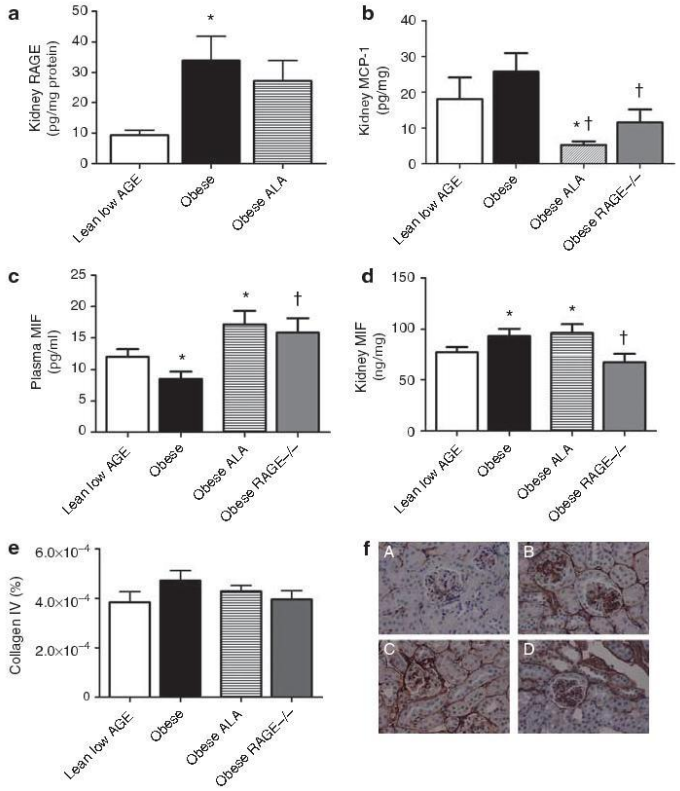

Figure 3 | Murine inflammatory parameters following 16 weeks of high advanced glycation end-product (AGE) dietary feeding. (**a**) The receptor for AGE (RAGE) protein content in kidney cortices measured by mouse-specific enzyme-linked immunosorbent assay (ELISA). Kidney cortices from obese RAGE–/– did not have measurable membrane RAGE. (**b**) Renal cytosolic monocyte chemotactic protein-1 (MCP-1) assayed by ELISA. (**c**) Plasma macrophage migration inhibitory factor (MIF) concentration assayed by ELISA. (**d**) Renal cytosolic MIF content assayed by ELISA. (**e**) Semiquantification of collagen IV in glomeruli. (**f**) Representative collagen IV immunohistochemistry staining used for semiquantification of (A) lean low AGE, (B) obese, (C) obese ALA, and (D) obese RAGE–/–. Obese (high AGE/high-fat diet), ALA (AGE-lowering therapy, alagebrium chloride 1 mg/kg/day), and RAGE–/– (RAGE deletion). $^{*}P<0.05$ vs lean low AGE, $^{\dagger}P<0.05$ vs obese.

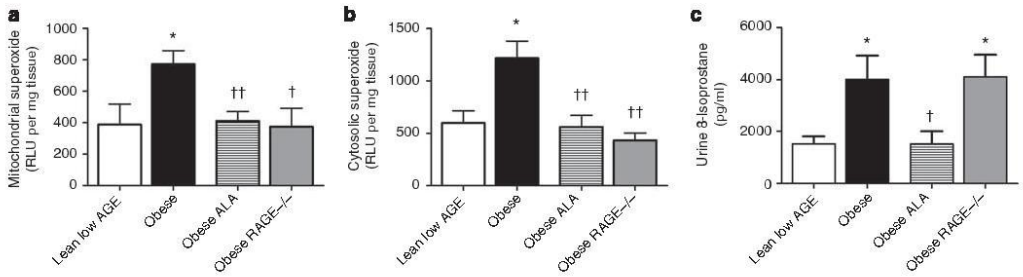

Figure 4 | Murine oxidative parameters following 16 weeks of dietary intervention. (**a**) Mitochondrial NADH-dependent superoxide production in fresh kidney cortices, measured via lucigenin-enhanced chemiluminescence. (**b**) Cytosolic NADPH-dependent superoxide production in fresh kidney tissue. (**c**) 8-Isoprostane measured via enzyme-linked immunosorbent assay (ELISA) in urine. Obese (high advanced glycation end-product (AGE)/high-fat diet), ALA (AGE-lowering therapy, alagebrium chloride 1 mg/kg/day), RAGE–/– (RAGE deletion). NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate. $^{*}P<0.05$ vs lean low AGE, $^{\dagger}P<0.05$ vs obese, $^{\dagger\dagger}P<0.01$ vs obese.

Table 4 | Representative example of isocaloric meal selections for high-AGE versus low-AGE diets consumed by obese individuals

High-AGE diet	AGE (kU)[a]	Low-AGE diet	AGE (kU)
Breakfast		*Breakfast*	
2 scrambled eggs	2749	2 lightly poached eggs	628
1.5 slices toasted white bread (with crusts)	310	2 slices of fresh white bread (without crusts)	12
Commercial orange juice	9	Juice from an orange	0
Lunch		*Lunch*	
One apple	19	One apple	19
One toasted bacon sandwich (with crusts)	4026	One avocado and ham sandwich (without crusts)	1217
One glass cola[b]	16	One glass diet lemonade	2
Dinner		*Dinner*	
Pan-fried chicken breast	5387	Steamed chicken breast	989
Vegetables	391	Steamed vegetables	36
(fried in olive oil)	300	Olive oil dressing	300
Fried white rice	66	Boiled white rice	18
One apple	19	One apple	19
One glass cola	16	One glass diet lemonade	2
Evening snack		*Evening snack*	
One glass of heated skim milk	138	One glass cold full-cream milk	48
Shortbread biscuits	644	Angel food cake	11
Total AGE content (kU)	*14,090*	*Total AGE content (kU)*	*3302*
Total energy (MJ)	9.0	Total energy (MJ)	9.0
Protein (%E)	16	Protein (%E)	16
Total fat (%E)	30	Total fat (%E)	30
Carbohydrate (%E)	54	Carbohydrate (%E)	55
Saturated fat (g)	10	Saturated fat (g)	10

Abbreviations: AGE, advanced glycation end-product; %E, percent of total energy.
[a]Approximate values only as calculated from available American data for the *N*-carboxymethyllysine (CML) content of foods.[12] While Australian foods may differ in AGE content, all foods prepared for the high-AGE diet were subjected to a high level of browning.
[b]Cola is a rich source of methylglyoxal in addition to CML.

tolerance (by oral glucose tolerance test), and healthy according to medical history, examination, and basic blood screening. Exclusion criteria included morbid obesity (BMI $\geqslant 40\,kg/m^2$), current smoking habit, high alcohol use or a positive urine drug screening test, any medication taken within 1 month before commencing the study, presence of acute inflammation (by history, physical, or laboratory examination), or highly unusual dietary habits or vegan diet.

Clinical study design and anthropometric and metabolic measurements. In all, 11 healthy overweight males participated in a clinical dietary intervention study involving 2 weeks each of low- and high-AGE diet separated by a 4-week wash-out period. Participants kept a 3-day diet record (two weekdays and one weekend day) based on household measures. Nutrient content was analyzed with SERVE (SERVE Nutrition Systems, St Ives, NSW, Australia), based on Australian Food Composition tables plus US data for food AGE content.[30] Results guided food selection and indicated the approximate habitual AGE intake. A menu of carefully matched alternative food choices (Table 4), each similar in macronutrients and total kilojoules but differing in total AGE content, were prepared for each meal of the day, including snacks and beverages, according to previously described guidelines.[30] All foodstuffs for the low- and high-AGE diets were provided to the individuals, in addition to instructions for storage and preparation of meals (method, temperature, and duration of cooking). Participants were instructed to eat to appetite, and maintain normal physical activity as measured by IPAQ (International Physical Activity Questionnaire)[31] and by accelerometer (Respirotics Minimitter, Bend, OR). Participants had a 6-week run-in period of the high-AGE diet as this was generally similar to their normal dietary habits, and were then randomized to either the low-AGE or high-AGE diet for 2 weeks. At the commencement and conclusion of each 2-week dietary test period, body weight, waist-hip ratio, and adiposity by four-point bioimpedence analysis (Body Composition Analyser, Model BC-418MA; Tanita, Middlesex, UK) were measured and BMI calculated. A 24-h urine collection and fasting plasma sample were taken at the commencement of the study and further spot urine and fasting plasma samples were taken before and after dietary interventions. Fasting plasma samples were analyzed for glucose (Radiometer, Copenhagen, Denmark) and insulin via ELISA.

Renal function. Before and after each dietary period, spot urines and plasma samples were taken to assess serum creatinine and urinary albumin/creatinine ratios. Creatinine clearance was estimated via the Cockcroft–Gault formula,[32] and albumin excretion rates assayed in 24 h urine collections at baseline.

CML indirect ELISA. CML was measured in human serum (1:8000) and urine samples (1:4) before and after each diet at their respective dilutions, using an in-house indirect CML ELISA that has been previously described.[33] CML was also measured in mouse chow, murine plasma, urine, and renal cortices using the previously described methods.[15,29]

Immunohistochemistry. Immunohistochemistry analysis for CML and collagen IV was performed on paraffin-embedded neutral buffered formalin-fixed murine kidneys as previously described.[34]

Cystatin C, MIF, and MCP-1 ELISAs. Cystatin C was measured in human plasma samples according to the manufacture's instructions in a 1:1000 dilution (Human Cystatin C; BioVendor,

Mordice, Czech Republic). MIF was measured in human plasma (R&D Systems, Minneapolis, MN) and murine plasma and renal cytosolic fractions (USCN Life, Wuhan, China) according to the manufacturer's guidelines. MCP-1 was assayed in human plasma (R&D Systems) and murine renal cortex cytosolic protein fractions (Raybiotech, Norcross, GA).

Murine study

Study design. Male WT, C57BL/6J (WT), and RAGE-deficient mice (RAGE–/–)[35] on a C57BL/6J background were housed in a temperature-controlled environment with a 12 h light–dark cycle (Alfred Medical Research and Education (AMREP) Precinct Animal Centre, Melbourne, Australia). At 8 weeks of age, groups of C57BL/6J mice ($n = 10$/group) were randomized to either (1) a high-AGE, high-fat, Western diet (obese; SF05-031, Specialty Feeds, Perth, Australia, baked for 1 h at 160 °C, 101.9 nmol/mol lysine of CML per 100 mg) (2) a high-AGE, high-fat Western diet plus the AGE-lowering therapy ALA (obese ALA; 1 mg/kg/day oral gavage; Synvista Therapeutics, Montvale, NJ), or (3) a low-AGE standard fat diet (lean; AIN-93G, Specialty Feeds, unbaked; 20.9 nmol/mol lysine of CML per 100 mg). Food intake and water access was *ad libitum* with diets matched for vitamin and amino-acid content. However, 40% of total energy in the Western diet was derived from animal fat (Ghee; 210 g/kg) versus 16% of total energy in the low-AGE diet. One further group of RAGE–/– mice consuming the Western diet were also studied ($n = 10$; obese RAGE–/–). All animal studies were performed in accordance with the guidelines from the AMREP Animal Ethics Committee and the National Health and Medical Research Council of Australia.

Murine physiological and biochemical parameters. Body weight, fasting plasma glucose, and fasting plasma insulin were measured at 16 weeks as previously described.[36] The 24 h metabolic caging to collect urine and measure food and water intake was performed at weeks 8 and 16 of the study. Albumin excretion rate was assessed using a mouse albumin ELISA kit according to the manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). Creatinine clearance was determined following HPLC (Agilent HP1100 system, Hewlett Packard, Nuremberg, Germany) measurement of creatinine content in timed plasma and urine samples as previously described and in accordance with AMDCC (Animal Models of Diabetic Complications Consortium) guidelines.[37] Frozen renal cortex was processed via ultracentrifugation as previously described[15] in order to generate membrane, cytosol, and nuclear protein fractions.

Urinary isoprostane concentrations. As a noninvasive measure of oxidative stress, 8-isoprostane F_2 was measured in 24 h human urine samples collected before and after each diet by competitive ELISA (Oxford Biomedical Research, Oxford, MI). Human urine samples were assayed neat and the assay was conducted as per the manufacturer's instructions. Murine urine samples were also analyzed neat for 8-isoprostane, according to the manufacturer's instructions (8-isporostane enzyme immunoassay; Cayman Chemical, Ann Arbor, MI).

Superoxide production. Renal superoxide was measured in fresh murine renal cortical tissue as previously described via chemiluminescence of lucigenin.[38,39]

Renal RAGE expression. Murine renal cytosolic protein fractions were assayed for RAGE protein using an ELISA specific for mouse (R&D Systems). Unknown values were calculated relative to a four-parameter logistic standard curve generated using the GraphPad Prism program (GraphPad Prism, San Diego, CA). All assays were run according to the manufacturer's instructions.

Statistical analyses. Human data were expressed as mean ± s.e.m. unless otherwise stated and were analyzed using paired Student's *t*-test analysis. Urinary albumin/creatinine values were nonparametric and were therefore logarithmically transformed before analysis. Order effect of the diets was analyzed via repeated measures analysis of variance with order as a between-subject factor. Human statistical analyses were performed using SPSS (SPSS Statistics 17.0, IBM, Somers, NY).

Murine study analyses were performed by one-way analysis of variance followed by Tukey's *post hoc* analysis (GraphPad Prism, 5.2). Mouse data are presented as mean ± s.d. Mouse albuminuria data were not normally distributed and were therefore logarithmically transformed before analysis. A $P < 0.05$ was considered to be statistically significant.

DISCLOSURE

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DISCLAIMER

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the article.

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