Diabetic nephropathy (DN) is recognized as a major contributor to the overall morbidity and mortality in diabetic patients. This condition is characterized by excessive accumulation of extracellular matrix (ECM), albuminuria, and ultimately, organ failure. Prolonged exposure to hyperglycemia drives the recruitment of inflammatory cells and production of inflammatory cytokines and growth factors. This causes significant disruption to the regulatory processes that control the equilibrium between ECM synthesis and degradation, resulting in excessive matrix accumulation (fibrosis) and ultimately leading to renal failure.

Angiotensin (Ang) II is well-recognized as a key driver for the development and progression of DN. Reducing Ang II formation with an angiotensin-converting enzyme inhibitor or its activity with an angiotensin receptor blocker remains as a first-line therapy for patients with this condition. However, both disparate approaches have had limited success in improving diabetic renal disease. Ang II stimulates the production and secretion of inflammatory and fibrotic molecules, including monocyte-chemoattractant protein (MCP)-1, osteopontin, tumor necrosis factor-α, transforming growth factor (TGF)-β1, and connective tissue growth factor (CTGF), and activates NADPH oxidase–mediated reactive oxygen species production to induce oxidative stress. These actions of Ang II together with its ability to increase blood pressure and vasoconstriction are considered to be mediated primarily through the AT1 receptor (AT1R). Interestingly, Ang II negatively regulates its classical actions by signaling through the AT2 receptor (AT2R). AT2R activation has been shown to produce anti-inflammatory, antioxidative, vasodilatory, and antifibrotic responses in several organs, including the kidney. Indeed, studies have demonstrated that accelerated collagen deposition occurs in AT1R knockout mice and when the AT2R is blocked by a specific AT2R antagonist, PD123319. Taken together, these studies are consistent with the possibility that AT2R activation maybe a suitable therapeutic approach to antagonize the pathophysiological effects mediated by the AT1R. Although AT2R activation has been shown to be protective in numerous diseased models, including its effect on diabetes mellitus per se, such an approach in type 1 DN has yet to be fully characterized. This study sought to investigate the efficacy and the mechanisms whereby the novel, selective, nonpeptide AT2R agonist, Compound 21 (C21), could modulate DN using the well-validated experimental model of streptozotocin-induced type 1 diabetes mellitus.

AT2R Agonist, Compound 21, Is Reno-Protective Against Type 1 Diabetic Nephropathy

Christine Koulis,* Bryna S.M. Chow,* Maria McKelvey, Ulrike M. Steckelings, Thomas Unger, Vicki Thallas-Bonke, Merlin C. Thomas, Mark E. Cooper, Karin A. Jandeleit-Dahm, Terri J. Allen

Abstract—The hemodynamic and nonhemodynamic effects of angiotensin II on diabetic complications are considered to be primarily mediated by the angiotensin II type 1 receptor subtype. However, its biological and functional effect mediated through the angiotensin II type 2 receptor subtype is still unclear. Activation of the angiotensin II type 2 receptors has been postulated to oppose angiotensin II type 1 receptor–mediated actions and thus attenuate fibrosis. This study aimed to elucidate the reno-protective role of the novel selective angiotensin II type 2 receptor agonist, Compound 21, in an experimental model of type 1 diabetic nephropathy. Compound 21 treatment significantly attenuated diabetes mellitus–induced elevated levels of cystatin C, albuminuria, mesangial expansion, and glomerulosclerosis in diabetic mice. Moreover, Compound 21 markedly inhibited the expression of various proteins implicated in oxidative stress, inflammation, and fibrosis, in association with decreased extracellular matrix production. These findings demonstrate that monotherapy of Compound 21 is protective against the progression of experimental diabetic nephropathy by inhibiting renal oxidative stress, inflammation, and fibrosis. (Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.115.05204.)

Key Words: angiotensin II ■ AT2 receptor ■ compound 21 ■ diabetic nephropathy ■ kidney
Materials and Methods

Refer to Materials and Methods in the online-only Data Supplement for greater details.

In Vivo Experimental Procedure

Six-week-old ApoE−/− mice were rendered diabetic via 5-daily intraperitoneal injection of streptozotocin at 55 mg/kg/d (Sigma-Aldrich, St. Louis, MO). Diabetic animals were subjected to either a vehicle (0.1 mol/L citrate buffer) or C21 (1 mg/kg/d; Vicore Pharma AB, Göteborg, Sweden) treatment via daily gavaging, over a 20-week period. Additional subgroups of nondiabetic mice subjected to similar treatments were also studied concurrently.

In Vitro Experimental Procedure

Mouse mesangial cells cultured in normal (5 mmol/L) and high (25 mmol/L) glucose conditions were treated with C21 (0.1–1 μmol/L) in the absence or presence of Ang II (3 μmol/L; Sigma-Aldrich) for 72 hours. RNA and proteins were then extracted from cell layers using Trizol reagent (Life Technologies, Rockville, MD) according to the manufacturer’s instructions.

Metabolic and Renal Functional Measurements

Assessment of body weight, metabolic parameters, and blood pressure were performed as described. 19 Collected serum and urine samples were assayed for cystatin C and albuminuria level, respectively, by a kit-based enzyme-linked immunosorbent assay (ELISA).

Glomerulosclerotic Injury Assessment

Paraffin-embedded kidney sections were subjected to periodic acid-schiff staining for glomerulosclerotic injury assessment, as described. 20 Nuclei free area of periodic acid-schiff-positively stained glomeruli were expressed as a percentage of total glomerular area for quantification of mesangial area. 21

Quantitative Real-time PCR

Gene expression was analyzed by quantitative real-time PCR (qRT-PCR) using the Taqman System on an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA) as described. 22 Primer sequences for the analyzed gene of interest are detailed in Table in the online-only Data Supplement.

Immunohistochemistry

Paraffin-embedded kidney sections were stained for p47phox (Millipore, Bedford, MA), nitrotyrosine (Millipore), collagen I (Abcam, Cambridge, MA), collagen IV (Abcam), osteopontin (Santa Cruz, Biotechnology, Santa Cruz, CA), and TGF-β1 (Santa Cruz), as described. 21 Twenty glomeruli were assessed in each section using Image-Pro Analyser 7.0. Positive staining for each marker was expressed as a percentage of the area of the glomerulus.

Statistical Analysis

Results were analyzed by 1-way ANOVA using SPSS 20.0 software. Post hoc comparisons were performed among the various groups using Fisher’s least significant difference method. Data are expressed as mean±SEM, with P<0.05 considered as statistically significant.

Results

Metabolic Parameters and Systolic Blood Pressure

Diabetic mice demonstrated elevated levels of glycated hemoglobin and plasma glucose when compared with their nondiabetics counterparts, with no significant effect of C21 treatment on glycemic control. Diabetic mice had increased kidney/body weight ratio, urine volume, total cholesterol concentration, and triglyceride levels (Table). C21 treatment had no effect in altering any of these metabolic parameters in diabetic animals. Systolic blood pressure remained unaltered between the diabetic and nondiabetic animal group. C21 reduced systolic blood pressure modestly in nondiabetic mice (P<0.05 versus vehicle-treated nondiabetic mice) but not in the diabetic C21-treated group (Table).

Renal Injury and Albuminuria

Plasma cystatin C (Figure 1A) and albuminuria (Figure 1B) levels were elevated in diabetic mice (P<0.05 versus nondiabetic mice). There was also evidence of glomerular injury in the diabetic mice as reflected by an increased mesangial area and glomerulosclerotic injury (Figure 1C; P<0.05 versus nondiabetic mice). These diabetes mellitus–associated parameters were found to be significantly ameliorated by C21 treatment (all P<0.05 diabetic mice). In contrast, C21 had no effect on any of the above parameters in nondiabetic mice.

AT,R and AT,R Expression in Nondiabetic and Diabetic Mice

qRT-PCR analysis confirmed AT,R and AT,R expression in mouse renal cortex. Renal AT,R and AT,R were significantly

| Table. Characteristics of Nondiabetic and Diabetic ApoE−/− Mice After 20 Weeks of Study, Treated in the Absence or Presence of the AT,R Agonist, Compound 21 (C21) |
|---|---|---|---|---|
| Parameters | Control | Control+C21 | Diabetic | Diabetic+C21 |
| Number | 15 | 10 | 15 | 14 |
| Body weight, g | 31±1 | 31±1 | 26±1* | 25±1* |
| Kidney weight/body weight ratio | 0.66±0.02 | 0.62±0.02 | 0.85±0.03* | 0.75±0.04† |
| Systolic BP, mmHg | 107±1 | 103±1* | 108±1 | 108±1 |
| Glycated hemoglobin (HbA1c), % | 3.8±0.2 | 4.1±0.3 | 12.9±0.7* | 11.0±1.4* |
| Plasma glucose, mmol/L | 10.4±0.5 | 9.5±1.2 | 24.8±2.0* | 29.0±1.7* |
| Urine volume, mL/24 h | 1.1±0.2 | 0.9±0.1 | 19.9±0.6* | 16.8±1.4* |
| Total cholesterol, mmol/L | 9.5±0.6 | 9.1±0.8 | 14.0±0.9* | 13.9±1.6* |
| Triglycerides, mmol/L | 1.6±0.6 | 1.1±0.3 | 2.6±1.3* | 2.0±1.0* |

Data are shown as mean±SEM. BP indicates blood pressure.

*P<0.05 vs nondiabetic (control) mice.
†P<0.05 vs diabetic mice.
upregulated in diabetic mice (P<0.05 versus nondiabetic mice). Interestingly, C21 had no effect in modulating AT₂R and AT₁R mRNA levels in both normoglycemic and hyperglycemic animal groups (Figure S1).

Effect of C21 Treatment on Oxidative Stress

p47phox expression was determined for assessment of effects on the pro-oxidant enzyme (NADPH oxidase), whereas changes in nitrotyrosine level was quantitated for measurement of reactive oxygen species. qRT-PCR and immunohistochemical analysis demonstrated a diabetes mellitus–associated increase in renal p47phox (Figures 2 and 3A) and nitrotyrosine staining (Figure 3B), which was attenuated in C21-treated mice (P<0.05 versus diabetic mice). Importantly, C21 did not influence any of these oxidative stress markers in nondiabetic mice.

Effect of C21 Treatment on Renal Inflammation

Osteopontin, MCP-1, and tumor necrosis factor-α gene expression were determined for assessment of inflammation. qRT-PCR gene analyses revealed a significant upregulation in osteopontin, MCP-1, and tumor necrosis factor-α level in diabetic mice (all P<0.05 versus nondiabetic mice; Figure 2). Immunohistochemical staining of osteopontin expression and ELISA analysis of MCP-1 (Figure S2) also demonstrated significant upregulation of both markers in the kidneys of diabetic mice (by 0.8- to 1.5-fold, all P<0.05 versus nondiabetic mice). These diabetes mellitus–induced mediators of renal injury were markedly attenuated by C21 at both the mRNA and protein level (P<0.05 versus diabetic mice; Figure 2; Figure S2). No significant difference was observed for all analyzed parameters between the vehicle- and C21-treated nondiabetic animals.

Effect of C21 Treatment on Fibrosis

TGF-β1, CTGF, alpha smooth muscle actin, and matrix metalloproteinase (MMP)-2 and -9 expressions were assessed as a measure of fibrogenesis. qRT-PCR analysis demonstrated elevated mRNA levels of TGF-β1, CTGF, alpha smooth muscle actin, and MMP-2 in diabetic mice (P<0.05 versus nondiabetic mice), but no significant change in MMP-9 gene expression (Figure 2). C21 treatment to diabetic mice markedly inhibited TGF-β1, CTGF, and alpha smooth muscle actin gene expression, although increasing MMP-2 and MMP-9 levels (all P<0.05 versus diabetic mice). Similar findings were also observed at the protein level by immunohistochemistry, Western blotting, and gelatin zymography analyses (Figure S3). No significant changes in the above analyzed markers were detected between vehicle- and C21-treated nondiabetic animals.

Effect of C21 Treatment on ECM (Collagen) Content

Collagen I and IV (the predominant collagen isotypes involved in renal fibrosis) staining were significantly upregulated in diabetic mice (Figure 4; all P<0.05 versus nondiabetic mice). This increase in collagen (I and IV) level was further reflected by a marked increase in total collagen content (Figure S4). C21 treatment to diabetic mice markedly reduced collagen I (by 51%) and IV (by 42%) levels and total collagen concentration...
Hypertension May 2015

(by 92%; all \( P < 0.05 \) versus respective measurements from diabetic mice). Again, no significant difference was observed for any of the above analyzed markers between the vehicle- and C21-treated nondiabetic animal group. Similar findings were also observed by Western blot analysis of collagen I and IV (Figure S5).

**AT2R and AT1R Expression in Mouse Mesangial Cells**

Western blot and qRT-PCR analysis confirmed the expression of AT2R and AT1R in primary mouse mesangial cells. Both receptors were found to be markedly upregulated in cells cultured in high glucose condition and when stimulated by Ang II (3 \( \mu \text{mol/L} \)). Co-administration of high glucose and Ang II further increased both AT2R (Figures S6 and S7) and AT1R expression (Figures S8 and S9). Importantly, C21 had no effect in modulating the expression of both receptors in all cases.

**Effect of High Glucose and Ang II Treatment in Mouse Mesangial Cells**

Incubation of mesangial cells in high glucose-containing medium resulted in increased levels of collagen I and IV (Figure 5A), p47phox, and MCP-1 (Figure S10). These high–glucose induced effects were significantly attenuated by C21 dose-dependently (\( P < 0.05 \) versus normal glucose-treated [control] cells), with its optimal inhibitory actions being mediated at 0.5 \( \mu \text{mol/L} \). Furthermore, at this dose, C21 was able to inhibit Ang II–stimulated collagen levels (Figure 5B), p47phox, and MCP-1 (Figure S10B). Although higher concentration of C21 (1 \( \mu \text{mol/L} \)) alone appeared to affect the expression of the measured parameters in normal glucose-treated cells, at lower concentrations (0.1–0.5 \( \mu \text{mol/L} \)), C21 did not affect any of the analyzed markers, further demonstrating the specificity and ability of C21 in reversing the pathological changes associated with type 1 diabetes mellitus.

**Discussion**

This study is the first to specifically investigate the effect and potential mechanism of action of the novel AT2R agonist, C21, in an ApoE– mouse model of streptozotocin-induced type 1 DN. This is a well-characterized model of diabetes mellitus–induced renal injury,\(^2^0\) which demonstrates prominent renal fibrosis, as well as macrophage infiltration,
Consistent with our previous studies, an increase in serum cystatin C was observed in diabetic mice. Importantly, C21 treatment normalized serum cystatin C levels in these diabetic mice, consistent with the renal benefits seen with this agent with respect to renal structure, albuminuria, and ECM gene expression. This study has demonstrated that C21 ameliorated diabetes mellitus–associated renal changes by reducing the expression/levels of several inflammatory and profibrotic mediators and upregulating gelatinases (MMP-2 and MMP-9)-induced collagen degradation. This net effect of C21 in the diabetic kidney is a decrease in ECM content, which correlated with improved renal function and glomerular structure. In this study, it seems that gelatinases play a crucial role in the renal damage seen in this model and that the MMPs are differentially regulated, as seen in previous studies. Decreased MMP-9 levels correlate with the development of tubulointerstitial fibrosis and glomerulosclerosis in both rat and mouse models of renal injury, whereas the absence of MMP-2 accelerates the progression of diabetes mellitus–induced renal fibrosis. Taken together, these combined findings imply that gelatinases exhibit potent antifibrotic actions and that an increased level/activity of these enzymes could be a potential mechanism for the reduced fibrosis seen in C21-treated mice.

Given the importance of mesangial cells in driving the pathology of DN and glomerular ECM accumulation, this study further sought to explore the role of C21 in primary mouse mesangial cells, which endogenously expressed the AT2R and AT1R. Consistent with our in vivo observations, aberrant collagen accumulation and increased levels of oxidative stress, inflammation, and fibrosis markers were observed in these cells in response to high glucose and were further amplified in the presence of exogenous Ang II. Strikingly, C21 markedly ameliorated these pathological markers of DN, whereas having no effect in modulating any of the analyzed markers in normal glucose conditions, further highlighting its potential safety as a therapeutic agent. As AT2Rs are expressed at low levels in healthy tissues during physiologically quiescent states but can be markedly elevated under pathological conditions, including diabetes mellitus, the increase in AT2R levels in injured/diseased condition, such as nephropathy, may lead to improvement in the functional response to its agonist, C21. This may explain why C21 only displays its antioxidant, anti-inflammatory, and antifibrotic effects under pathological conditions, without affecting normal ECM, as seen in this study. Importantly, C21 did not afford these
renal effects by altering AT$_R$ expression/levels, consistent with its putative mode of action as a specific receptor agonist.

Although C21 has been shown to be reno-protective in several experimental models of renal disease,$^{32-35}$ its functional role in diabetes mellitus is yet to be extensively explored. In contrast to our present findings that C21 as a monotherapy exhibits potent reno-protective activity in type 1 DN, a recent study has demonstrated that C21 had only a modest effect in an experimental model of type 2 DN.$^{36}$ C21 was reportedly shown to be only able to reduce inflammation and fibrosis (by inhibiting tumor necrosis factor-$\alpha$ and interluekin-6) when coadministered with the AT$_1$R blocker, losartan. This less impressive effect of C21 in the type 2 diabetes mellitus setting cannot be fully explained, but maybe as a result of the lower dose of C21 been used or it could reflect a difference in efficacy of this agent between different types of diabetes mellitus. Because type 2 diabetes mellitus is associated with obesity and an increase in blood pressure, it is possible that these factors could confound the potential responsiveness of the kidney to C21. Nonetheless, our findings that C21 had no effect in influencing metabolic control and any of the analyzed markers in the normal context demonstrate the specificity and safety of C21 as a therapeutic agent. Thus, these findings provide strong evidence to support the potential of C21 as a therapy for type 1 DN.

Given that the affinity of C21 for the AT$_2$R is 25 000-fold higher than for the AT$_1$R$^{37}$ and its ability to inhibit inflammatory markers was markedly abolished by the AT$_2$R antagonist, PD123319,$^{38}$ consistently demonstrates that the effects of C21 are specifically mediated through the AT$_2$R. Future studies are warranted to further elucidate the signaling transduction pathway of the AT$_2$R, in particular its involvement with the nitric oxide pathway$^{32,39}$ and its interaction with the AT$_1$R because these receptors have been previously reported to act as heterodimers.$^{17,40}$ Potential interaction between the 2 Ang II receptor subtypes may involve the ability of either receptor to alter the expression of the other receptor. Indeed, this possibility is supported by previous experiments demonstrating that activation of the AT$_2$R decreases AT$_2$R expression$^{41}$ and antagonizes AT$_1$R-mediated effects.$^{17,42}$ In this study, C21 did not alter AT$_1$R expression but rather it is likely to inhibit well-described biological effects of the AT$_1$R by targeting downstream effectors of AT$_1$R, such as TGF-$\beta$, to mediate its reno-protective effects.$^{39}$ Taken together, it is likely that a complex interaction exists between the 2 Ang II receptor subtypes, both of which are significantly upregulated in diabetic kidney.$^{33,44}$

Figure 4. Immunohistochemical analysis of collagen I (as a % of the glomerulus; A) and collagen IV (as a % of the glomerulus; B) staining from nondiabetic (control), control+C21, diabetic, and diabetic+C21 mice. **P<0.01 vs nondiabetic (control) group; #P<0.05 vs diabetic mice. N=5 to 7 animals per group.
In conclusion, this study has provided insights into the potential mechanism of action of C21, in particular, its ability as a monotherapy in ameliorating the functional and structural changes of a model of insulin-deficient DN. Nonetheless, future studies are still warranted to determine whether C21 is protective against other diabetic complications, as well as assessing its reno-protective activity in comparison to the current first-line therapy of DN.

Perspectives
In summary, this study has demonstrated that C21 is protective against type 1 DN, with its actions independent of blood pressure and metabolic control. Elucidating the signal transduction pathways that are activated by C21 to mediate its protective actions is now required to determine the strengths and limitations of this agent as a therapeutic option. Moreover, these findings have provided valuable insights into verifying the therapeutic potential of C21, and it is now worth exploring if combination therapy with current treatment strategies, including blockade of the AT1R (sartans) and angiotensin-converting enzyme inhibition, may confer superior benefits in preventing and reversing DN.

Acknowledgments
We thank Maryann Arnstein, Pooja Krishnaswamy, Samantha Sacca, Elisha Lastavec, and Megan Haillay for their technical support. Special thanks to Anders Ljunggren, Vicore Pharma AB, Göteborg, Sweden, for his generosity in providing Compound 21.

Sources of Funding
This study was supported by a National Health and Medical Research Council of Australia (NHMRC) Project Grant. V. Thallas-Bonke is a recipient of an Advanced Postdoctoral Juvenile Diabetes Research Foundation (JDRF) grant. M.E. Cooper, M.C. Thomas, K.A. Jandeleit-Dahm, and T.J. Allen have been supported by NHMRC fellowships.

Disclosures
The authors declare that no competing interests exist. K.A. Jandeleit-Dahm and T.J. Allen received Compound 21 from Vicore Pharma, Mölndal, Sweden, as a gift.

References
2. Singh KP, Gerard HC, Hudson AP, Boros DL. Dynamics of collagen, MMP and TIMP gene expression during the granulomatous, fibrotic


25. Yang J, Chen C, Ren H, Han Y, He D, Zhou L, Hopfer U, Jose PA, Zeng C. Angiotensin II AT(2) receptor decreases AT(1) receptor...


Novelty and Significance

**What Is New?**
- This study is the first to demonstrate that C21 as a monotherapy is able to inhibit oxidative stress, inflammation, and the progression of fibrosis associated with type 1 diabetic nephropathy.

**What Is Relevant?**
- C21 was found to preserve renal structure and function in type 1 diabetic mice, independent of glycemic or blood pressure control.
- These reno-protective benefits of C21 are likely caused by its ability to inhibit the expression/activity of several markers of inflammation and fibrosis, as was identified at both the in vitro and in vivo level.

**Summary**
C21 is reno-protective against the progression of type 1 diabetic nephropathy via inhibition of renal oxidative stress, inflammation, and fibrosis. These effects are independent of blood pressure and glucose regulation.
AT2R Agonist, Compound 21, Is Reno-Protective Against Type 1 Diabetic Nephropathy
Christine Koulis, Bryna S.M. Chow, Maria McKelvey, Ulrike M. Steckelings, Thomas Unger,
Vicki Thallas-Bonke, Merlin C. Thomas, Mark E. Cooper, Karin A. Jandeleit-Dahm and Terri J.
Allen

Hypertension, published online March 16, 2015;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/early/2015/03/16/HYPERTENSIONAHA.115.05204

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2015/03/16/HYPERTENSIONAHA.115.05204.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial
Office. Once the online version of the published article for which permission is being requested is located,
click Request Permissions in the middle column of the Web page under Services. Further information about
this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
The AT2R agonist, Compound 21, is reno-protective against type 1 diabetic nephropathy

Christine Kouli1#, Bryna S.M. Chow1#, Maria McKelvey1, Ulrike M. Steckelings2, Thomas Unger3, Vicki Thallas-Bonke1, Merlin C. Thomas1, Mark E. Cooper1, Karin A. Jandeleit-Dahm1, Terri J. Allen1* #C.K and B.S.M.C contributed equally to the manuscript

1Diabetic Complications Group, Baker IDI Heart and Diabetes Research Institute, Melbourne, VIC, Australia; 2IMM-Department of Cardiovascular and Renal Research, University of Southern Denmark, Odense. Denmark; 3School for Cardiovascular Diseases Maastricht University, Maastricht, Netherlands.

*Future correspondence and reprint requests of this manuscript should be addressed to:

Dr Terri J Allen, PhD
Baker IDI Heart and Diabetes Research Institute
75 Commercial Road
PO BOX 6492, MELBOURNE VIC 3004, Australia
Tel: +613 8532 1453
Fax: +613 8532 1100
Email: terri.allen@bakeridi.edu.au
Expanded Material and Methods

Animals

Male apolipoprotein-E knockout (ApoE\(^{-/-}\)) mice (on C57BL6/J background, Jackson Laboratory, Sacramento, CA, USA) were used throughout the study. Animals were housed in a 12-hour light and dark cycle in a pathogen free environment with free access to water and rodent lab chow (Specialty Feeds, WA, Australia). These experiments were approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committees and National Health and Medical Research Council of Australia, which adhere to the Australian code of practice for the care and use of laboratory animals for scientific purposes.

**In vivo experimental procedure**

6-week old ApoE\(^{-/-}\) mice were rendered diabetic via 5-daily intraperitoneal injection of streptozotocin at 55mg/kg/day (Sigma-Aldrich, St Louise, MO, USA). Only animals with a blood glucose level >15 mmol/L after 5-days post streptozotocin in a fasting state were included in the study as diabetic. Diabetic animals were subjected to either a vehicle (0.1mol/L citrate buffer) or C21 (1mg/kg/day; Vicore Pharma AB, Göteborg, Sweden) treatment via daily gavaging, over a 20-week period. Additional subgroups of non-diabetic mice, subjected to similar treatments were also studied concurrently. The dose of C21 (at 1mg/kg/day) was chosen as it has been shown to potently activate rodent AT2R and attenuate fibrosis progression in numerous disease models [1,2]. 5-10 animals per treatment group were used in this study. At the end of the study, animals were culled by intraperitoneal injection of sodium pentobarbitone (100mg/kg; Virbac, NSW, Australia) and kidneys were collected for analysis, as previously described [3].

**In vitro experimental procedure**

To complement the in vivo studies, in vitro studies were performed. Specifically, primary mesangial cells were isolated from ApoE\(^{-/-}\) mice as previously described [4,5]. Mesangial cells cultured in normal (5mmol/L) and high (25mmol/L) glucose conditions were seeded at 100,000 cells/well in 12-well plates, followed by treating with increasing concentrations of C21 (0.1-1\(\mu\)mol/L) for 72hours. The optimal dose at which C21 inhibited high glucose-stimulated inflammatory and fibrotic markers was subsequently tested to determine if it also inhibits Ang II-induced renal fibrosis. Cells were treated with C21 (0.5mg/ml) in the absence or presence of Ang II (3\(\mu\)mol/L, Sigma-Aldrich) for 72hours before harvesting for RNA and protein content using Trizol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer’s instructions. Cells cultured in normal glucose (5mmol/L) condition were used as appropriate controls. All described experiments were performed at least 3-4 separate times in duplicate, with primary mouse mesangial cells used between passages 8-12.

**Metabolic and renal functional measurements**

Diabetic mice at week 20 were placed in metabolic cages (Iffa Credo, L’Arbresle, France) for 24hours for assessment of body weight, metabolic parameters and blood pressure, as described [6]. Collected serum was assayed for cystatin C level using a specific mouse cystatin C enzyme-linked immunosorbent assay (ELISA) (BioVendor Inc, Asheville, NC, USA), while urinary albuminuria level was assessed by a specific ELISA kit (Bethyl laboratories Inc, Montgomery, TX, USA) according to the manufacturer’s instructions.
Linear-regression and Boltzmann sigmoidal curve calculation of the data was performed for quantitation of cystatin C and albuminuria concentration, respectively, using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

**Glomerulosclerotic injury (GSI) assessment**

Paraffin embedded kidney sections from each of the animals studied were subjected to periodic acid-schiff (PAS)-staining for assessment of GSI and mesangial expansion, as described [7]. Nuclei free area of PAS-positively stained glomeruli expressed as a percentage of total glomerular area was determined for quantitation of mesangial area [3]. GSI was assessed according to the severity of glomerular damage; with a grade score of 0-4 assigned. A grade score of 0-4 was assigned; with grade 0 representing intact glomerulus and grade 1-4 representing injury involving >25%, 25-50%, 50-75% and 75-100% of the glomerulus, respectively. 20 glomeruli were assessed per kidney in a masked fashion using Capture-Pro software (Version 5, Media Cybermetics) and an Olympus BX50 microscope. GSI was calculated using the formula, with \( n_x \) represents the number of glomeruli in each grade of injury:

\[
\text{GSI} = \frac{(1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)}{n_0 + n_1 + n_2 + n_3 + n_4}
\]

**Quantitative Real-time PCR (qRT-PCR)**

Extracted RNA contents were subjected to DNA-free DNAase treatment (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. DNA-free RNA samples were then reverse transcribed into cDNA using the Superscript First Strand Synthesis System (Life Technologies). Expression of genes encoding markers of oxidative stress (p47phox), inflammation (MCP-1, TNF-\( \alpha \), osteopontin) and fibrosis (TGF-\( \beta \), CTGF, \( \alpha \)-SMA, MMP-2 and MMP-9) were analysed by qRT-PCR using Taqman System on an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Fluorescence for each cycle was analysed quantitatively and gene expression were normalised relative to the housekeeping gene (18S mRNA). Results were expressed relative to the control (either the non-diabetic animals or normal-glucose treated cells) group, which was assigned an arbitrary value of 1.

**Immunohistochemistry**

Paraffin embedded kidney sections from each of the animals studied were stained for p47phox (Millipore, Bedford, MA, USA), nitrotyrosine (Millipore), collagen I (Abcam, Cambridge, MA), collagen IV (Abcam), osteopontin (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) and TGF-\( \beta \)1 (Santa Cruz) as described [3]. Antibody binding was visualised with 3,3’-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and counterstained with Mayer’s haematoxylin, dehydrated and cover-slipped. All sections were examined under light microscopy (Olympus BX-50; Olympus Optical) and digitised with a high-resolution camera. Twenty glomeruli were assessed in each section using Image-Pro Analyzer 7.0. Positive staining for each marker was expressed as a percentage of the area of the glomerulus.

**MCP-1 Enzyme-linked immunosorbent assay (ELISA)**

Equal volume of protein homogenate from kidney cortex was used to determine MCP-1 concentration, according to the manufacturer’s instructions (R&D Systems, BioScientific, Kirrawee, NSW, Australia). Assays were performed in duplicate and optical density was measured. Results were expressed relative to the total protein concentration quantitated by a BCA-based protein assay (Pierce, Thermo Scientific, Scoresby, VIC, Australia).

**Western blotting**

Ten-micrograms of protein was analysed by electrophoresis under either reducing (α-SMA, AT1R and AT2R) or non-reducing (collagen I and IV) conditions. Completed gels were subsequently transferred to PVDF membranes for 90 minutes at 100V. Western blot analyses were then performed with a primary antibody to either α-SMA (Dako), AT1R (Santa-Cruz), AT2R (Santa-Cruz), collagen I or collagen IV; and the appropriate secondary antibodies. α-tubulin levels (Sigma-Aldrich) were also assessed to demonstrate equal loading of samples. Blots were detected using the ECL detection kit (Sigma-Aldrich) before being quantified by densitometry using Quantity-One software (Bio-Rad Laboratories, Richmond, CA, USA).

**Gelatin zymography**

Ten-micrograms of total proteins were electrophoresed on zymogram gels consisting of 7.5% acrylamide and 1mg/ml gelatin, as described [8]. Clear bands indicating gelatinolytic activity and densitometry of the latent and active MMP-2 and MMP-9 bands from each treated group were performed as detailed above.

**Hydroxyproline assay**

A portion of kidney tissue was lyophilized to dry weight and hydrolysed in 6M hydrochloric acid for assessment of hydroxyproline content, as described [8]. Hydroxyproline values were then converted to total collagen content by multiplying by a factor of 6.94 (based on hydroxyproline representing approximately 14.4% of the amino acid composition of collagen in most mammalian tissues [9]). Collagen content was then expressed as a percentage of the tissue dry weight to correct for any discrepancies in the size of the tissue portion analysed.
REFERENCES


**Supplementary Table**  
List of Primer Sequences used for RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Primer Sequence</th>
</tr>
</thead>
</table>
| **p47phox**      | Forward primer: 5’ CCGGCTATTTCCCATCCAT 3’  
|                  | Reverse primer: 5’ TCGCTGGGCTGGTTAT 3’ |
| **Osteopontin**  | Forward primer: 5’ TCCAATCGTCCCTACAGTCGAT 3’  
|                  | Reverse primer: 5’ AGCCCTTCAACATGTCTGTTCA 3’ |
| **MCP-1**        | Forward primer: 5’ GTCTGTGCTGACCCCAAGAAG 3’  
|                  | Reverse primer: 5’ TGGTTCCCGATCCAGGTGTCA 3’ |
| **TNF-α**        | Forward primer: 5’ GGCTGCCCAGACTACGT 3’  
|                  | Reverse primer: 5’ TTCTCCTCTGATGATGCAACTC 3’ |
| **TGF-β**        | Forward primer: 5’ GCAGTGGCTGAACCAAGGA 3’  
|                  | Reverse primer: 5’ TGTGCTACTTCGTTGAACCTGTA 3’ |
| **CTGF**         | Forward primer: 5’ GCTGCCTACCGATGGAAGA 3’  
|                  | Reverse primer: 5’ CTTAGAACAGGCGCTCCACTCT 3’ |
| **α-SMA**        | Forward primer: 5’ GACCCTGAAGTATCCGATAGAACA 3’  
|                  | Reverse primer: 5’ GGCCACACGAAGCTCGTTAT 3’ |
| **MMP-9**        | Forward primer: 5’ TGAGTCCCGGAGACAATCCT3’  
|                  | Reverse primer: 5’ GCACCTGGATCTCAGAATA 3’ |
| **MMP-2**        | Forward primer: 5’ TCACCTTCTGGGCAACAAATGCTCCTGGTAT 3’  
|                  | Reverse primer: 5’ GCCACGAGGAATAGGCTATATCC 3’ |
| **AT2R**         | Forward primer: 5’ ATTAACCTGATGATGTAGGTAGGGGCA 3’  
|                  | Reverse primer: 5’ GGATCTCTTTCTTTGAGACAGAAA 3’ |
| **AT1R**         | Forward primer: 5’ CCATTGTCCACCGATGAA 3’  
|                  | Reverse primer: 5’ TGACTTTGGCCACCAAGCAT 3’ |
Supplementary Figures

S1:

A

B

Figure S1: Renal A) AT2R and B) AT1R mRNA expression in non-diabetic (control), control+C21, diabetic and diabetic+C21 mice. Gene expression was normalised to 18S mRNA and reported as ratio to that of the non-diabetic (control) mice, which was given an arbitrary value of 1. Data are shown as the mean ± SEM from 5-10 animals per group analysed. *p<0.05, **p<0.01 vs non-diabetic (control) group.
Figure S2: A) Immunohistochemical analysis of renal osteopontin staining (as a % of the glomerulus) and B) ELISA analysis of renal MCP-1 protein levels, in non-diabetic (control), control+C21, diabetic and diabetic+C21 mice. Data shown as mean ± SEM of MCP-1, corrected for equal protein concentration as determined by BCA protein assay. *p<0.05, **p<0.01 vs non-diabetic (control) group; #p<0.05, ##p<0.01 vs diabetic mice. N=5-9 animals per group.
Figure S3: A) Immunohistochemical analysis of renal TGF-β1 staining (as a % of the glomerulus) and B) Western blot of α-SMA and zymographs of latent and active MMP-9 and MMP-2, from non-diabetic (control), control+C21, diabetic, and diabetic+C21 mice. Additional blot of α-tubulin was used to demonstrate equivalent loading of protein samples. Shown is the relative mean ± SEM optical density (OD) levels of α-SMA, MMP-9 and MMP-2, corrected for α-tubulin levels, as determined by densitometry scanning, expressed in relative to that of the non-diabetic (control) mice, which was expressed as 1 in each case. *p<0.05, **p<0.01 vs non-diabetic (control) group; #p<0.05, ##p<0.01 vs diabetic mice. N=7-10 animals per group.
Figure S4: Hydroxyproline analysis of total renal collagen concentration (% collagen content/dry weight kidney tissue). Data shown as mean ± SEM from each of the animals group, *p<0.05 vs non-diabetic (control) group; #p<0.05 vs diabetic mice. N=5-7 animals per group.
Figure S5: Representative Western blots of collagen I and IV in non-diabetic (control), control+C21, diabetic and diabetic+C21 mice. Additional blot of α-tubulin was used to demonstrate equivalent loading of protein samples. Data shown as relative mean ± SEM optical density (OD) levels of total collagen I (α1 and α2) and collagen IV, corrected for α-tubulin levels as determined by densitometry scanning, expressed in relative to that of the non-diabetic (control) mice, which was expressed as 1. *p<0.05, **p<0.01 vs non-diabetic (control) group; #p<0.05, ##p<0.01 vs diabetic mice. N=5-7 animal per group.
Figure S6: qRT-PCR analysis of AT₁R mRNA levels in primary mouse mesangial cells cultured in either normal (5mmol/L) or high (25mmol/L) glucose, treated in the presence of C21 (0.1-1µmol/L) (A) and in the absence or presence of 3µmol/L Ang II (B). Data shown as mean ± SEM. Gene expression was normalised to 18S mRNA and reported as a ratio relative to that of the normal glucose (NG)-treated cells, which was given an arbitrary value of 1. Cells treated with mannitol served as osmotic control. *p<0.05, **p<0.01 vs NG-treated cells; #p<0.05 vs high glucose (HG)-treated cells. N=3-4 per treatment group, in duplicate.
Figure S7: Western blot analysis of AT1R in primary mouse mesangial cells cultured in either normal (5mmol/L) or high (25mmol/L) glucose, treated in the absence or presence of 3µmol/L Ang II. Additional blot of α-tubulin was used to demonstrate equivalent loading of protein samples. Data shown as relative mean ± SEM optical density (OD) levels of AT1R, corrected for α-tubulin levels as determined by densitometry scanning, expressed in relative to that of the normal glucose (NG)-treated cells, which was expressed as 1. *p<0.05; **p<0.01 vs NG-treated cells; ##p<0.01 vs high-glucose (HG)-treated cells. N=3-4 per treatment group, in duplicate.
Figure S8: Western blot analysis of AT$_2$R in primary mouse mesangial cells cultured in either normal (5mmol/L) or high (25mmol/L) glucose, treated with increasing concentrations of C21 (0.1-1µmol/L). Additional blot of α-tubulin was used to demonstrate equivalent loading of protein samples. Data shown as relative mean ± SEM optical density (OD) levels of AT$_2$R, corrected for α-tubulin levels as determined by densitometry scanning, expressed in relative to that of the normal glucose (NG)-treated cells, which was expressed as 1. **p<0.01 vs NG-treated cells. N=3-4 per treatment group, in duplicate.
**Figure S9:** Western blot analysis of AT$_2$R in primary mouse mesangial cells cultured in either normal (5mmol/L) or high (25mmol/L) glucose, treated in the absence or presence of 3µmol/L Ang II. Additional blot of α-tubulin was used to demonstrate equivalent loading of protein samples. Data shown as relative mean ± SEM optical density (OD) levels of AT$_2$R, corrected for α-tubulin levels as determined by densitometry scanning, expressed in relative to that of the normal glucose (NG)-treated cells, which was expressed as 1. **p<0.01 vs NG-treated cells; ##p<0.01 vs high-glucose (HG)-treated cells. N=3-4 per treatment group, in duplicate.
Figure S10: qRT-PCR analysis of renal p47phox and MCP-1 in primary mouse mesangial cells cultured in either normal (5mmol/L) or high (25mmol/L) glucose treated with C21 (0.1-1µmol/L) (A) and in the absence or presence of 3µmol/L Ang II (B). Data shown as mean ± SEM. Gene expression was normalised to 18S mRNA and reported as a ratio relative to that of the normal glucose (NG)-treated cells, which was given an arbitrary value of 1. Cells treated with mannitol served as osmotic control. *p<0.05, **p<0.01 vs NG-treated cells; #p<0.05, ##p<0.01 vs high glucose (HG)-treated cells; ‡p<0.05 vs NG+Ang II-treated cells; ¶p<0.05, ¶¶p<0.01 vs HG+Ang II-treated cells. N=3-4 per treatment group, in duplicate.