Regulation of renal 12(S)-hydroxyeicosatetraenoic acid in diabetes by angiotensin AT$_1$ and AT$_2$ receptors

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Running head: Angiotensin receptors regulate 12(S)-HETE in diabetes

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Abstract

Diabetes is associated with increased production of 12(S)-Hydroxyeicosatetraenoic Acid [12(S)-HETE]. The mechanisms involved in this process remain unclear. We hypothesized that hyperglycemia and angiotensin II (Ang) regulate renal 12(S)-HETE production via a balance between angiotensin AT₁ and AT₂ receptors activities. Using microdialysis technique, renal interstitial fluid (RIF) levels of angiotensin II and 12(S)-HETE were monitored in normal control and streptozotocin induced diabetes rats at baseline, then weekly thereafter for 12 weeks. In a second group of normal and diabetic rats, 3 weeks after development of diabetes, we monitored RIF 12(S)-HETE levels in response to acute AT₁ receptor blockade with valsartan or AT₂ receptor blockade with PD 123319 individually or combined. Two weeks after induction of diabetes there were 404% increase in Ang II (p<0.05), 149% increase in 12S-HETE (p<0.05) and a 649% increase in urinary albumin excretion (p<0.05). These levels remained elevated throughout the study. PD 123319 given alone had no effect on 12(S)-HETE. Valsartan decreased 12(S)-HETE by 61.6% (p<0.0001), a response that was abrogated when PD 123319 was given with valsartan. These data demonstrate that hyperglycemia increases renal Ang II and 12(S)-HETE levels. The increase in 12(S)-HETE is mediated via AT₁ receptor. The attenuation of the effects of AT₁ receptor blockade by PD 123319 suggests that AT₂ receptor contributes to the down regulation of renal 12(S)-HETE production.

Key Words: Diabetes Mellitus, Angiotensin II, 12(S)-HETE, Kidneys, Urinary Albumin Excretion
Introduction

Diabetic nephropathy is one of the most serious complications of diabetes mellitus (DM). The US Renal Data System reports a continuous increase in the incidence of end stage renal disease (ESRD) in diabetic patients over the past two decades (10). The pathophysiology of diabetic nephropathy is not well understood. It is well established that increased activity of the renin angiotensin system (RAS) contributes to the development of this disease (7,19,31). Angiotensin II (Ang II) is the most effector hormone of the RAS and most of its effects are mediated by its AT1 and AT2 subtype receptors. Previous studies demonstrated the correlation between elevated blood glucose and RAS activity. Hyperglycemia stimulates the expression of angiotensinogen gene (45) through synthesis of diacylglycerol and the protein kinase C signal transduction pathway, increases kidney renin mRNA and ACE activities (47), enhances the expression of AT1 receptors (48) and increases renal Ang II production (37). The involvement of the AT1 receptor in the development of diabetic nephropathy is well established based on trials demonstrating reduction in albuminuria and slowing the progression to end stage renal disease by blocking this receptor activity of (7,19,31). Despite of this knowledge, the role of the AT2 receptors in diabetic kidney disease is not known.

12(S)-HETE is the lipid product of 12-lipoxygenase (12-LO) and is produced in the kidney, vascular smooth muscle (VSM) and adrenal cells (18,22). Previous studies demonstrated involvement of 12 LO and its metabolite 12(S)-HETE in vascular disease, hypertension and diabetes (8,17,30) yet, there are very limited studies describing its role in the development of diabetic nephropathy (2,15). Rat mesangial cells exposed to high glucose levels demonstrated increase in 12-LO m-RNA and protein expression, suggesting a role for 12-LO pathway in the pathogenesis of diabetic nephropathy (15). The mechanisms through which 12(S)-HETE may contribute to the pathogenesis of diabetic nephropathy are not well
established. Previous studies suggested that 12(S)-HETE plays a role in angiogenesis (28),
atherosclerosis (24), inflammation (4), modulation of Ang II-induced aldosterone secretion (27) and
activation of PKC (21). Enhancing 12-LO pathway and 12(S)-HETE activities augment the arteriolar
vasoconstrictor effect of Ang II (16,23), and facilitate its stimulatory actions on cellular calcium entry
into cultured VSM cells (25), while inhibition of 12-LO activities attenuates the vasoconstrictor action
of Ang II (52).

In this study, we hypothesized that in the presence of diabetic nephropathy, enhanced renal Ang II levels
increases 12(S)-HETE production via AT1 receptor, a process that is attenuated by the AT2 receptor.
Materials and Methods

In Vivo Microdialysis Technique.

For the determination of renal interstitial fluid (RIF) ANG II and 12(S)-HETE, we constructed a microdialysis probe as previously described (38,44). Substances with a molecular mass >40,000 Da cannot cross the dialysis membrane. This molecular mass cutoff allows free passage of Ang II and 12(S)-HETE. In vitro best recoveries were observed with a perfusion rate of 3 µl/min and were 47% for Ang II and 52% for 12(S)-HETE (39,40,44). Negligible amounts of these substances stick to polyethylene tubes (39).

Blood Pressure Measurements

Systolic blood pressure (SBP) was measured in conscious animals by the tail cuff method as previously described (38,40) using an automated sphygmomanometer (Model 679, IITC/Life Sciences Instruments, Colorado springs, CO). Blood pressures were recorded at 10-min intervals for 30 min in each experimental day and values were averaged for each day.

Animal Preparation

All protocols were approved by the University of Virginia Animal Care Committee. Male Sprague-Dawley rats (Harlan Sprague Dawley Inc, Harlan Teklad, Madison, WI) weighing 350-400 gm were studied. For in vivo determinations of RIF 12(S)-HETE and Ang II, the rats were placed under general anesthesia with ketamine (80 mg/kg IM) and xylazine (8 mg/kg IM), and the left kidneys were exposed by a left lateral abdominal incision. A microdialysis probe was placed in the cortex of each rat left kidney. All RIF measurements were made 7 days after implanting the probes. For collection of RIF, the inflow tube of the dialysis probe was connected to a gas-tight syringe filled with lactated Ringer’s solution and perfused at 3 µL/min. The effluent was collected from the outflow tube of the dialysis probe for 60-minute sample periods in plastic non-heparinized tubes and stored in -80º C, until assayed.
for Ang II and 12(S)-HETE. Diabetes was induced in 16 animals by a single intravenous injection of Streptozotocin (30mg/kg body wt, Sigma Co., St. Louis, MO) 7 days after insertion of the Microdialysis tubes. Blood glucose from tail vein was monitored 2 days after STZ injection, then once weekly using Glucose measuring strips (Accu-Check, Boehringer Mannheim Corporation). Body weight and 24h urine collections for measurement of albumin excretion (UAE) were obtained at the beginning of the study, then once weekly. Nondiabetic animals (n=16) were used as normal control.

Renal 12(S)-HETE and Ang II Levels in Normal and Diabetic Rats

RIF Ang II and 12(S)-HETE levels were monitored in diabetic (n=8) and normal control (n=8) animals while on normal sodium intake. RIF collections were made at the beginning of the study, then weekly thereafter up to 12 weeks.

Renal Ang II and 12(S)-HETE in Response to Insulin Administration

To test whether elevated blood glucose plays a role in regulation of renal production of Ang II and 12(S)-HETE, normal (n=8) and diabetic (n=8) rats were treated with i.v. regular insulin 0.4 U/kg/hr for 5 hrs (Eli Lilly Co., Indianapolis, Indiana) at day 21 after onset of diabetes. Blood glucose levels were monitored hourly during the study. Insulin treatment was stopped if blood glucose decreases to 70 mg/dl. RIF Ang II and 12(S)-HETE were monitored before and at the end of insulin infusion. Blood glucose levels were maintained between 60 and 100 mg/dl.

Effects of AT₁ and AT₂ Receptors Blockade on RIF Ang II and 12(S)-HETE
To investigate the relationship between Ang II receptor subtypes and 12(S)-HETE, the angiotensin AT₁ receptor blocker, valsartan 10mg/kg I.V. (Novartis Pharmaceuticals, East Hanover, USA), or the AT₂ receptor blocker, PD-123319 50 µg/kg/min I.V. infusion (Parke-Davis, Ann Arbor, MI) were administered individually or combined for 8 hrs. This treatment was given to normal (n=8) and diabetic (n=8) animals (n=8) at day 21 of the study.

Specimen Collection, Storage, and Assays
Specimens of RIF Ang II and 12(S)-HETE were collected, and stored at -80°C until they were assayed. The RIF Ang II samples were collected in tubes containing a mixed inhibitor solution containing 5 mmol/L EDTA, 10nmol/L pepstatin, 20 nmol/L enalaprilate, and 1.25 mmol/L 1, 10-phenanthroline and kept on ice, which is known to prevent Ang II formation or degradation, and evaporated to dryness (Speed Vac, Savant Instruments, Holbrook, NY). Measurement of RIF Ang II was done as previously described (44) using enzyme immunoassay kit (SPI-BIO, France). This assay sensitivity is 0.5 pg/ml. RIF 12(S)-HETE was measured by ELISA (Assay Design, Ann Arbor, MI). The assay sensitivity for 12(S)-HETE is 146.3 pg/mL. UAE was measured (14) by ELISA using Nephrat™ (Exocell, Inc., Philadelphia, PA). The assay sensitivity for urinary albumin is 0.3 µg /ml.

Statistical analysis of data. Comparison among different treatment and control groups were examined by one-way ANOVA. Comparisons between before and after treatment groups were examined using t test using SPSS program (SPSS Inc., Chicago, IL). Data are expressed as mean ±SEM. Differences of P<0.05 were considered significant.
Results

Blood glucose, blood pressure, body weight and urine albumin excretion

Blood glucose ranged between 78.5 ± 3.1 to 80 ± 3.3 mg/dl in normal control rats. DM was induced by STZ treatment as demonstrated by the rise of baseline mean blood glucose (BG) level from 81.7 ± 1.6 mg/dl to 356.1 ± 41.3 mg/dl 2 days after STZ injection (p < 0.0001). The elevated BG level was maintained throughout the 12 weeks study period ranging from 332.9 ± 28.3 mg/dl to 452.7 ± 16.8 mg/dl. There was no significant difference in systolic blood pressure between the diabetic and the control groups throughout the study, ranging from 113.2 ± 1.5 mmHg and 111.8 ± 1.3 mmHg at baseline to 112.2.8 ± 0.7 mmHg and 110.1 ± 0.7 mmHg by week 12, respectively. There were no significant changes in systolic blood pressure with individual treatment with valsartan or PD123319 or their combination. At baseline the average body weight of the normal control group was 398 ± 4.8 gm and progressively increased to 423 ± 10.2 gm at week 12. In the diabetic animals, the average body weight at baseline was 395 ± 11.3 gm and increased to 404 ± 12.4 and 398.2 ± 3.1 gm at weeks 6 and 12 respectively. There was no significant difference in body weights between normal and diabetic rats.

UAE in normal control rats was low ranging from 3.68 ± 1.12 to 3.90 ± 0.94 µg/24hr throughout the study period. In contrast, UAE in diabetic rats (Figure 1) increased from 3.96 ± 0.93 ug/24hr at baseline to 25.7 ± 2.3 µg/24hr (p < 0.05) by the end of the second week and continued to increase progressively thereafter up to week 12 reaching 933.6 ± 88.7 µg/24hrs (p < 0.0001).

RIF Ang II Levels in the normal control and diabetic groups
There were no significant changes in RIF Ang II in normal control rats throughout the study ranging from 3.6 ± 0.5 pg/ml to 3.4 ± 0.8 pg/ml (Figure 2A). In contrast, in the diabetic group there was a progressive significant increase in RIF Ang II levels (Figure 2A) from 3.3 ± 0.9 at baseline to 13.4 ± 3.1 pg/ml by the second week and continued to be elevated throughout the duration of the study peaking at day 63 to 24.8 ± 3.1 pg/ml.

**RIF 12(S)-HETE levels in the normal control and diabetic groups**

In the control normoglycemic rats, the RIF 12(S)-HETE was 1349.7 ± 178.9 pg/ml at baseline (not significantly different from the baseline of the diabetic group) and did not significantly change throughout the study (Figure 2B). RIF 12(S)-HETE increased in diabetic rats (Figure 2B) by the second week of the study from 1774.5 ± 61.5 pg/ml to 2636.7 ± 143.5 pg/ml (p < 0.05) and continued to be elevated throughout the duration of the study peaking at day 63 to 6039.1 ± 531.7 pg/ml (p < 0.001).

**Correlation between blood glucose, Ang II, 12(S)-HETE and albuminuria**

In the diabetic rats there was a positive correlation between blood glucose level and both RIF 12(S)-HETE (r =0.81, p<0.001) and RIF Ang II level (r =0.79, p<0.001). Similarly, urinary albumin excretion correlated positively with both RIF 12(S)-HETE (r=0.73, p<0.0001) and Ang II levels (r=0.85, p<0.0001). A positive correlation was also noted between RIF Ang II and RIF 12(S)-HETE (r= 0.83, p<0.0001).

**RIF Ang II and 12(S)-HETE response to insulin in diabetic rats**

In normal control rats blood glucose decreased during insulin administration from 78.2 ± 0.6 mg/dl to 66.8 ± 0.4 mg/dl (p<0.05). There was no significant changes in RIF Ang II or 12(S)-HETE levels in response to insulin treatment in control animals (Figure 3A and 3B). In diabetic rats, insulin treatment
decreased the blood glucose level from 358.3 ± 19.4 mg/dl to normal value of 88.4 ± 2.3 mg/dl (p<0.01). RIF Ang II decreased from 20.3 ± 0.4 pg/ml at baseline to 3.8 ± 0.6 pg/ml (p<0.001) by the end of insulin treatment (Figure 3A). Similarly, when blood glucose was reduced in the diabetic rats, there was significant reduction in 12(S)-HETE level (Figure 3B) from 2240 ± 149.8 pg/ml to 1051.3 ± 157.9 pg/ml (p<0.001).

Changes in RIF Ang II and 12(S)-HETE levels in response to valsartan, PD 123319 individually and combined

RIF Ang II levels in the non-diabetic control group increased slightly following individual treatment with valsartan or PD123319 or their combination, and were significantly lower than its levels in the diabetic group (Figure 4A).

In diabetic rats, RIF Ang II levels were elevated at baseline compared to normal control animals and increased further (p<0.001) during treatment with valsartan or PD123319 individually and combined (Figure 4A). Valsartan alone increased RIF Ang II levels from 18.5 ± 1.6 pg/ml to 30.1± 2.1 pg/ml (p<0.001). Similarly, PD 123319 alone or combined Valsartan and PD123319 treatment increased RIF Ang II from 20.9 ± 2.6 pg/ml and 18.7 ± 0.9 pg/ml to 30.1± 2.1 pg/ml (p<0.001) and 39.8 ± 1.5 pg/ml (p<0.0001) respectively (Figure 4A).

RIF 12(S)-HETE level changes in response to valsartan, PD 123319 individually and combined

In normoglycemic control rats, 12(S)-HETE levels did not show significant changes with different treatments (Figure 4B). Twenty-one days after onset of diabetes, RIF 12(S)-HETE was 2833.6 ± 277.5 pg/ml and decreased in response to valsartan to 1087.1 ± 269.3 pg/ml (p < 0.0001). There were no significant changes in 12(S)-HETE levels when PD123319 was given alone. However, the decrease in
12(S)-HETE level with valsartan alone (Figure 4B) was partially abrogated when PD 123319 was given together with valsartan (p <0.05).

**Discussion**

This study shows that 12(S)-HETE is present locally in the renal interstitial fluid. These levels are increased in STZ-induced diabetic rat model by the second week, and continue throughout the 12 weeks duration of the study. This rise in RIF 12(S)-HETE in diabetic rats was not mirrored by a similar rise in the control rats and correlated positively with blood glucose level. These results suggest that the increase in renal production of 12(S)-HETE level is related to the elevated blood glucose. This finding was confirmed by reduction in the level of renal 12(S)-HETE following insulin treatment and normalization of blood glucose.

Current knowledge of the mechanisms regulating renal production of 12(S)-HETE in diabetes is not well established. Furthermore, the status of in vivo 12(S)-HETE levels in diabetes is controversial with some studies showed increases (2,15,20), while others showing no change or decrease in its levels (34,46). Renal mesangial cells cultured in high glucose medium demonstrated an increase in 12(S)-HETE mRNA and protein (15). Ang II also was reported to increase 12(S)-HETE production by renal microvasculature in normoglycemic state (52).

Similar to 12(S)-HETE, our study shows increased renal Ang II levels in diabetic rats throughout the 12 weeks duration of the study. This finding is consistent with our previous finding (36) of increased renal Ang II production in diabetic rats. The mechanisms responsible for the increased renal Ang II in diabetes are still being elucidated. An increase in renal cortical renin mRNA (1), enhanced macula densa signaling pathway for renin production (6) or increased activity of plasma and tissue angiotensin
converting enzyme (13) are among the factors that may lead to the observed increase in renal Ang II production in diabetes.

In the current study we focused on monitoring changes in the kidney Ang II and 12(S)-HETE levels by renal microdialysis technique. This method has advantages over monitoring these substances in blood or urine. Ang II level in renal tissue is about 1,000 fold higher than its systemic (41) concentration, suggesting the importance of Ang II in the local regulation of renal physiology and pathophysiology. Other studies also confirmed that renal concentrations of Ang I and Ang II are substantially higher than the corresponding plasma concentrations (29). Similarly, the measurement of circulating 12(S)-HETE is not totally reliable since it can be formed in large amounts \textit{ex vivo} in collected blood samples (12).

In our study, we confirmed the presence of diabetic nephropathy by the presence of microalbuminuria as early as two weeks after induction of diabetes. The increase in albuminuria correlated positively with both renal Ang II and 12(S)-HETE levels, suggesting a role for both Ang II and 12(S)-HETE in the pathogenesis of diabetic nephropathy. The reduction in renal 12(S)-HETE levels during valsartan treatment confirms the involvement of Ang II in mediating renal production. We further confirmed that Ang II increases 12(S)-HETE levels in diabetes via AT\textsubscript{1} receptor stimulation. Several mechanisms were suggested to explain the contribution of AT1 receptor to the pathogenesis of diabetic nephropathy (9,32,35,53). Stimulation of renal 12(S)-HETE production by the AT1 receptor could be another mechanism that contributes to the deleterious effects of AT1 receptor in development of diabetic nephropathy. Our study supports the notion that there is a cross talk between the AT1 receptor and 12(S)-HETE. A recent report demonstrated increased AT1 receptor expression by 12(S)-HETE (51). Furthermore, 12(S)-HETE may contribute to development
of diabetic nephropathy through enhancing the renal vasoconstrictor effects of Ang II (52), TGFβ production (17), matrix formation (33) and COX-2 production (50).

In contrast to AT₁ receptor, the potential role of AT₂ receptor in diabetic nephropathy or its influence on 12(S)-HETE is not known. Abrogation of the effects of the AT1 receptor blockade on 12(S)-HETE by PD123319 supports the involvement of the AT2 receptor in regulation of this factor. Our study suggests that AT₂ receptor inhibits renal 12(S)-HETE production in diabetic rats and is in agreement with previous reports suggesting a protective role of AT₂ in the kidney (43). Several mechanisms could contribute to the inhibitory effect of the AT₂ receptor on 12(S)-HETE production. AT₂ receptor expression was reported to be reduced in diabetes (5,49), which is also associated with reduction in renal NO production (3). Reduction in renal NO enhances production of renal renin and Ang II (42,44) and increases 12-LO activities (20). Our study also shows that Ang II level is increased further when combined blockade of the AT₁ and AT₂ receptors. These results are consistent with recent reports of AT₂ receptor inhibition of renin and Ang II production (42,44). Taken together, the increase in Ang II levels and AT₁ receptor activity, combined with the reduction of AT₂ expression and NO activity could explain the marked increase in 12(S)-HETE levels in diabetic nephropathy.

We conclude that hyperglycemia increases renal Ang II, which in turn increases renal 12(S)-HETE levels via AT₁ receptor. To our knowledge, this is the first study demonstrating that AT₂ receptor plays a role in modulating renal 12(S)-HETE in diabetes. Future studies are needed to define the exact mechanisms by which AT2 receptor modulates renal 12(S)-HETE, and to further clarify how they contribute to the pathogenesis of diabetic nephropathy.
Perspectives and Significance

This study demonstrates that hyperglycemia increases renal Ang II, which in turn increases renal 12(S)-HETE levels via AT₁ receptor. To our knowledge, this is the first study demonstrating that AT₂ receptor plays a role in modulating renal 12(S)-HETE in diabetes. Future studies are needed to define the exact mechanisms by which AT₂ receptor modulates renal 12(S)-HETE, and to further clarify how they contribute to the pathogenesis of diabetic nephropathy.
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Disclosures

None.
References


interactions between TGF-β1 actions and the 12/15-lipoxygenase pathway in mesangial cells. J

monohydroxyeicosatetraenoic acids from arachidonic acid by cultured rabbit aortic smooth

19. Lewis EJ, Hunsicker LG, Clarke WR, Berl T, Pohl MA, Lewis JB, Ritz E, Atkins
RC, Rohde R, Raz I; Collaborative Study Group. Renoprotective effect of the angiotensin-

20. Lianos EA. Activation and potential interactions between the arachidonic acid and L-arginine:

21. Ma YH, Harder DR, Clark JE, Roman RJ. Effects of 12-HETE on isolated dog renal arcuate

22. Nadler JL, Natarajan R, Stern N. Specific action of the lipoxygenase pathway in mediating
angiotensin II-induced aldosterone synthesis in isolated adrenal glomerulosa cells. J Clin Invest

growth factor BB mediated regulation of 12-lipoxygenase in porcine aortic smooth muscle cells.

24. Natarajan R, Gerrity RG, Gu JL, Lanting L, Thomas L, Nadler JL. Role of 12-lipoxygenase and
oxidant stress in hyperglycaemia- induced acceleration of atherosclerosis in a diabetic pig model.


**Figure Legends**

Figure 1. 24h urinary albumin excretion rate in conscious normoglycemic control (square) and streptozotocin induced diabetes (diamond) rats (n=8 each group). WK=week, +p<0.05, *P < 0.0001 vs. control

Figure 2. Renal interstitial levels of angiotensin II (A) and 12(S)-HETE in conscious normoglycemic control (square) and streptozotocin induced diabetes (diamond) rats (n=8 each group). WK=week, +p<0.05, * P < 0.001 vs. control

Figure 3. Renal interstitial levels of angiotensin II (A) and 12(S)-HETE (B) in conscious normoglycemic control (open bars) and streptozotocin induced diabetes (solid bars) rats (n=8 each group) in response to insulin treatment (n=8 each group). * P < 0.001 vs. baseline of diabetic rats

Figure 4. Renal interstitial levels of angiotensin II (A) and 12(S)-HETE (B) in conscious normoglycemic control (C) and streptozotocin induced diabetes (DM) rats in response to treatment with valsartan or PD123319 and their combination. Open bars represent baseline and solid bars represent treatment (n=8 each treatment group). *p<0.05 from corresponding baseline group, **p<0.001 from control groups; +p<0.0001 and ++P<0.0001 from corresponding baseline of diabetic rats
Figure 1

Baseline
WK 1
WK 2
WK 3
WK 4
WK 5
WK 6
WK 7
WK 8
WK 9
WK 10
WK 11
WK 12

Urinary Albumin Excretion (µg/ml)
Figure 2

(A) Angiotensin II (pg/ml)

(B) 12(S)-HETE (pg/ml)
Figure 3
Figure 4