

# Angiotensin (1-7)/Mas receptor axis activation ameliorates the changes in fatty acid composition in diabetic rats with nephropathy

Kulwinder Singh\*

Tajinder Singh\*

PL Sharma

Department of Pharmacology,  
ISF College of Pharmacy,  
Moga India; \*These authors  
contributed equally to this work

**Abstract:** Diabetes mellitus is often associated with altered fatty acids composition. This study was designed to investigate the role of angiotensin (Ang) (1-7)/Mas receptor in improving fatty acids composition in streptozotocin (STZ)-induced diabetic nephropathy (DN) in rats. Rats treated with STZ (50 mg/kg, i.p. once) developed DN after 8 weeks. Fatty acid composition was estimated in renal cortical tissue by gas chromatography. Treatment with Ang (1-7), A-779, and Ang (1-7) plus A-779 was given from week 4 to week 8. Diabetic rats exhibited a significant increase in levels of saturated fatty acids and a significant decrease in levels of polyunsaturated fatty acids (PUFAs). Treatment with Ang (1-7) significantly attenuated these diabetes-induced changes. In diabetic rats, prior administration of A-779 significantly attenuated the increase in PUFAs produced by Ang (1-7); however, for saturated fatty acids, A-779 significantly blocked the decrease in palmitic acid only. Our study, for the first time, documented that endogenous Ang (1-7) modulates fatty acid composition in rats. Further, treatment with Ang (1-7) significantly attenuated diabetes-induced changes in fatty acids composition. This may be an additional mechanism implying the renoprotective role of Ang (1-7) in diabetic rats.

**Keywords:** diabetic nephropathy, fatty acids, Mas receptor

## Introduction

Diabetic nephropathy (DN) is a leading cause of morbidity and mortality in diabetic patients, and its prevalence is increasing steadily in developing countries.<sup>1</sup> Diabetes is associated with altered fatty acid composition.<sup>2</sup> In diabetes mellitus, activity of  $\Delta^9$  desaturase and  $\Delta^6$  desaturase declines; consequently, enzymatic conversion of saturated fatty acids into beneficial polyunsaturated fatty acids (PUFAs) is decreased leading to subsequent increase in saturated fatty acid.<sup>2-4</sup> Nonessential saturated fatty acids, specifically palmitic acid, by increasing nuclear factor kappa B (NF $\kappa$ B) activity induce insulin resistance and releases inflammatory cytokines in diabetic patients with nephropathy.<sup>5-8</sup> Various evidence suggests that renin-angiotensin-aldosterone system overactivation is a possible molecular mechanism responsible for impaired free fatty acid metabolism, as treatment with the angiotensin-converting enzyme (ACE) inhibitor (captopril) has shown reduction in free fatty acids in mice fed with high-fat diet.<sup>9</sup> Further, free fatty acids are reported to potentiate angiotensin (Ang) II-mediated renal damage.<sup>10</sup> Moreover, increased expression of lipolytic enzymes was documented in ACE<sup>-/-</sup> animals leading to increased metabolism of fatty acids in liver.<sup>11</sup> It has been reported that beneficial effects of ACE inhibitors and angiotensin receptor blockers may be due to activation of Ang (1-7)/Mas receptor axis.<sup>12,13</sup> Further, Mas<sup>-/-</sup> mice have shown

Correspondence: PL Sharma  
Professor Emeritus, Department  
of Pharmacology, ISF College of  
Pharmacy, Moga 142 001, India  
Mobile +9109888036775  
Fax +9101636236564  
Email ksajjal77@rediffmail.com

development of dyslipidemia and insulin resistance.<sup>14</sup> Furthermore, PUFAs and Ang (1-7) have many overlapping actions such as stimulation of nitric oxide (NO) production,<sup>15,16</sup> inhibition of ACE/Ang II axis,<sup>17</sup> and inhibition of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and NF $\kappa$ B activity.<sup>18,19</sup> Therefore, the present study was undertaken to investigate the effect, if any, of Ang (1-7)/Mas receptor axis on the fatty acids composition in normal and in diabetic rats with nephropathy.

## Materials and methods

The experimental protocol used in the present study was approved by the Institutional Animal Ethical Committee. All experiments were carried out according to Indian National Science Academy guidelines for use and care of animals in scientific research. Age-matched young Wistar rats of either sex, weighing 200–250 g, were fed on standard chow diet and water ad libitum. They were acclimatized in an animal house and were exposed to a normal cycle of light and darkness.

## Induction of experimental DN

Diabetes was induced by a single injection of streptozotocin (STZ) (50 mg/kg, i.p.) dissolved in freshly prepared ice-cold citrate buffer (pH 4.5). After 1 week of STZ administration, animals having random serum glucose more than 240 mg/dL were considered as diabetic. DN was noted to develop after 8 weeks of STZ administration as reported in our earlier study.<sup>20</sup>

## Experimental protocol

Seven groups were employed in the present study, and each group comprised 6–8 rats. Ang (1-7) and A-779 were dissolved in normal saline (0.9% w/v).

1. *Group I (normal control)*: Rats were maintained on a standard food and water regime, and no treatment was given.
2. *Group II*: Rats were administered Ang (1-7) (576  $\mu$ g/kg/day, i.p.)<sup>21</sup> for 4 weeks.
3. *Group III*: Rats were administered A-779, a specific Ang-(1-7)/Mas receptor antagonist (744  $\mu$ g/kg/day, i.p.)<sup>19</sup> for 4 weeks.
4. *Group IV (diabetic control)*: Rats were administered STZ (50 mg/kg, i.p., once) dissolved in citrate buffer (pH 4.5).
5. *Group V*: Rats after 4 weeks of STZ administration were treated with Ang (1-7) (576  $\mu$ g/kg/day, i.p.) for 4 weeks.

6. *Group VI*: Rats after 4 weeks of STZ administration were treated with A-779 (744  $\mu$ g/kg/day, i.p.) for 4 weeks.
7. *Group VII*: Rats after 4 weeks of STZ administration were treated with A-779 (744  $\mu$ g/kg/day, i.p.) 30 min before treatment with Ang (1-7) (576  $\mu$ g/kg/day, i.p.) for 4 weeks.

All parameters were assessed at the end of 8 weeks in both normal and STZ-treated rats.

## Extraction of total renal cortical lipids

Renal lipids were extracted according to procedure of Folch et al.<sup>22</sup>

## Extraction of renal lipids

The tissue was washed with saline and dried between filter paper. A weighed amount of tissue (250 mg) was homogenized with 1.5 mL of water, and then chloroform and methanol were added in a ratio of 2:1 v/v. The homogenate with biphasic system was shaken for 10 min. The homogenate was centrifuged for 10 min at 1500 g. The chloroform layer containing total tissue lipids was separated and evaporated to dryness.

## Washing of lipid extract

Lipid residue after evaporation was dissolved in 2.5 mL of chloroform–methanol mixture. The redissolved lipid extract was mixed with 1 mL of 0.1 N KCl, and contents were shaken well. The chloroform layer was separated and again washed thrice with 1 mL of Folch's reagent (0.1 N KCl:methanol:chloroform mixed in the ratio of 10:10:1), and the upper aqueous phase was aspirated. The lower chloroform layer was evaporated to dryness, and dried extract was used for estimations.

## Assessment of fatty acid composition

### Methylation of fatty acids

A modification of the method described by Metcalf and Schmitz was used.<sup>23</sup> To the lipid extracted from 250 mg of renal cortex was added 0.5 mL of a solution of 0.5 mol of sodium hydroxide/liter of methanol which was heated in a boiling water bath for 10 min in a screw-capped tube. To this, we added 0.5 mL of boron trifluoride in methanol (140 g/L) and then heated for an additional 5 min. The reaction mixture was then cooled, extracted with 2.5 mL of petroleum ether (30°–60°), and evaporated to dryness at 37°C. The dried extract was redissolved in n-heptane. To prepare samples for injection, fatty acid ester residue was dissolved in 500  $\mu$ L of n-heptane, of which 1  $\mu$ L was introduced into the injection

port.<sup>24</sup> Results were expressed in terms of percentage peak area.

### Instrumentation

A NUCON gas chromatograph was performed with an EC-wax fused silica capillary column (30 m × 0.25 mm internal diameter × 0.25 mm film thickness). The injector and flame ionization detector temperature was maintained at 250°C. The column temperature program was started from 150°C and held for 1 min, then raised up to 200°C with a heating rate of 25°C/min and held for 3 min, and final temperature was increased to 230°C with a rate of 15°C/min and held for 5 min. The pressure of nitrogen carrier gas was 100 KPa.<sup>25</sup>

### Statistical analysis

All values were expressed as mean ± SD. The data obtained from various groups were statistically analyzed using one-way ANOVA followed by Tukey's multiple range test. The *P* value < 0.05 was considered to be statistically significant.

### Drugs and chemicals

STZ was obtained from Sigma Aldrich Ltd (St. Louis, MO). Ang (1-7) peptide and A-779 were obtained from BACHEM, (Bubendorf, Switzerland). All other chemicals used in present study were of analytical grade.

## Results

All the animals survived till the end of the study. Administration of Ang (1-7) to normal rats significantly reduced saturated fatty acids (myristic acid, palmitic acid, and stearic acid). The changes in PUFAs were not statistically significant. Further, A-779 treatment normal rats significantly increased saturated fatty acids and significantly decreased linoleic acid and arachidonic acid (Table 1).

### Effect of various pharmacological interventions on saturated fatty acids in diabetic rats

A significant increase in saturated fatty acids (myristic acid, palmitic acid, and stearic acid) was noted in diabetic rats when compared with age-matched normal rats. Treatment with Ang (1-7) significantly decreased all three saturated fatty acids in the diabetic rats. However, administration of Ang (1-7) in presence of A-779 produced significant increase in palmitic acid without affecting myristic acid and stearic acid compared to the diabetic rats treated with Ang (1-7) alone (Table 1).

**Table 1** Effect of various pharmacological interventions on fatty acids composition in renal cortical tissue

Groups	Myristic acid (percentage peak area)	Palmitic acid (percentage peak area)	Stearic acid (percentage peak area)	Linoleic acid (percentage peak area)	Arachidonic acid (percentage peak area)
Normal control, n = 8	13.0 ± 2.8	11.8 ± 2.6	16.5 ± 3.5	7.5 ± 0.8	3.3 ± 0.6
Ang (1-7) in normal rats, n = 8	5.5 ± 2.0 <sup>1</sup>	0.6 ± 0.3 <sup>1</sup>	0.92 ± 0.2 <sup>1</sup>	8.1 ± 0.8	2.7 ± 0.5
A-779 in normal rats, n = 7	20.8 ± 2.9 <sup>1</sup>	18.2 ± 3.0 <sup>1</sup>	28.6 ± 4.7 <sup>1</sup>	1.1 ± 0.3 <sup>1</sup>	1.7 ± 0.3 <sup>1</sup>
Diabetic control, n = 6	24.6 ± 3.8 <sup>1</sup>	22.5 ± 3.1 <sup>1</sup>	27.4 ± 1.6 <sup>1</sup>	1.8 ± 0.2 <sup>1</sup>	1.0 ± 0.2 <sup>1</sup>
Ang (1-7)-treated diabetic rats, n = 7	12.6 ± 2.1 <sup>2</sup>	7.15 ± 1.0 <sup>2</sup>	3.5 ± 1.1 <sup>2</sup>	4.2 ± 0.8 <sup>2</sup>	13.3 ± 1.3 <sup>2</sup>
A-779-treated diabetic rats, n = 6	28.7 ± 4.7	31.1 ± 2.3 <sup>2</sup>	25.5 ± 2.2	0.7 ± 0.1 <sup>2</sup>	0.41 ± 0.20
Diabetic rats treated with A-779 + Ang (1-7), n = 6	11.1 ± 1.5	24.2 ± 3.2 <sup>3</sup>	5.5 ± 1.9	0.3 ± 0.2 <sup>3</sup>	2.1 ± 0.5 <sup>3</sup>

**Notes:** All values are expressed as mean ± SD. <sup>1</sup>*P* < 0.05 versus normal control; <sup>2</sup>*P* < 0.05 versus diabetic control; <sup>3</sup>*P* < 0.05 versus Ang (1-7)-treated diabetic rats.  
**Abbreviation:** Ang (1-7), Angiotensin (1-7).

## Effect of various pharmacological interventions on PUFAs in diabetic rats

A significant decrease in PUFAs (linoleic acid and arachidonic acid) was observed in diabetic rats compared to age-matched normal rats. Treatment with Ang (1-7) significantly increased PUFAs compared to diabetic rats. However, concurrent administration of Ang (1-7) in the presence of A-779 showed a significant decrease in PUFAs compared with diabetic rats treated with Ang (1-7) alone (Table 1).

## Discussion

Diabetes mellitus is a complex metabolic syndrome characterized by absolute insulin deficiency or development of insulin resistance that leads to hyperglycemia and an altered glucose, fat, and protein metabolism.<sup>26</sup> STZ (50 mg/kg, i.p.) has been documented to induce diabetes mellitus, and persistent hyperglycemia leads to development of nephropathy in 8 weeks.<sup>27</sup> Increased blood urea nitrogen (BUN), proteinuria, and renal collagen content and decreased creatinine clearance are well-documented markers of DN.<sup>28–30</sup> In our earlier study,<sup>20</sup> BUN, proteinuria, and renal collagen contents were noted to be increased and creatinine clearance was significantly decreased in diabetic rats. Further, Ang (1-7) administration had no effects on these markers, while A-779, a specific antagonist of Ang (1-7), significantly increased total urinary proteins and collagen content of renal cortex in the normal rats. Treatment with Ang (1-7) significantly attenuated the diabetes-induced changes, except creatinine clearance, while A-779 produced a significant increase in creatinine clearance only. This is not in agreement with Shao et al<sup>31</sup> who reported that continuous infusion of Ang (1-7) for 6 weeks accelerated progressive DN in the diabetic rat.

It has been well documented that fatty acid composition is impaired in diabetic animals with nephropathy.<sup>2</sup> Further, lipids get deposited in the kidney of diabetic patients leading to glomerulosclerosis, tubulointerstitial fibrosis, and increased collagen expression, subsequently leading to proteinuria.<sup>32,33</sup> The inclusion of saturated fatty acids into the cell membrane phospholipids will make them rigid, while increased incorporation of PUFAs into the membrane will make it more fluid and upregulate receptors and their affinity to their respective hormones or growth factors.<sup>34</sup>

Saturated fatty acids are converted into unsaturated fatty acids via enzymes  $\Delta^5$  desaturase and  $\Delta^6$  desaturase. The activity of  $\Delta^5$  and  $\Delta^6$  desaturases is known to be decreased in diabetes mellitus, leading to decreased conversion of saturated fatty acids into PUFAs.<sup>2,3,35</sup> Diabetes-induced changes in composition of fatty acids in our study can thus

be explained on this basis. A significant increase in saturated fatty acids and decrease in PUFAs produced by A-779 in the normal rats indicate that endogenous Ang (1-7) has a role in maintenance of normal fatty acid composition. This is a new finding in our study. In addition, the changes in fatty acid composition in the diabetic rat mimicked the changes produced by A-779 in the normal rat. The significant attenuation of these changes by exogenously administered Ang (1-7) further support this viewpoint.

Prior administration of A-779 was unable to block the effect of exogenously administered Ang (1-7) on myristic acid and stearic acid; either the dose of A-779 used was not adequate or it may have been due to the presence of a Mas receptor subtype that is not blocked by A-779. It has been reported earlier that A-779 failed to block vasodilatory effects of Ang (1-7) at highest concentration in aorta, but another Mas-receptor antagonist, D-Pro<sup>7</sup>-Ang-(1-7), blocked vasodilatory response of Ang (1-7) at a very low concentration.<sup>36</sup>

It has been reported that ACE inhibitors decrease fatty acids in mice fed on a high-fat diet.<sup>9</sup> ACE2-Ang-(1-7)Mas receptor axis has been documented to play a role as an ACE-Ang II-AT1 receptor counter-regulatory axis.<sup>37</sup> An increase in glomerular Ang II levels and a decrease in expression of ACE2 mRNA protein and activity have been reported in STZ-treated diabetic rats.<sup>38,39</sup> Since ACE2 mediates the conversion of Ang II to Ang (1-7), the changes in fatty acid composition observed in our study may be due to decreased formation of endogenous Ang (1-7) and increased formation of endogenous Ang II. Further, free fatty acids have been reported to potentiate Ang II-mediated renal damage.<sup>10</sup> Thus, beneficial effects of Ang (1-7) on fatty acid composition in diabetic, as well as in normal, rats suggest an active role of ACE2-Ang (1-7)Mas receptor axis in maintenance of normal fatty acid composition. Moreover, even beneficial effects of ACE inhibitors on lipid composition may be due to an increase in the Ang (1-7) levels.<sup>19</sup>

The mechanism of renoprotective effects of Ang (1-7) in diabetic rats is not fully understood. It may be due to stimulation of NO production,<sup>16</sup> inhibition of TNF- $\alpha$  and NF $\kappa$ B activity, suppressed expression of inflammatory genes,<sup>19</sup> and increased levels of PUFAs as documented in our study. In addition, PUFAs have been reported to produce beneficial effects through inhibition of ACE activity and generation of Ang II,<sup>17</sup> inhibition of TNF- $\alpha$  and NF $\kappa$ B activity,<sup>18</sup> and consequent increase in NO release from endothelium.<sup>15</sup> Thus, Ang (1-7) and PUFAs have many overlapping actions.

It may be concluded that STZ-induced nephropathy is also associated with an increase in saturated fatty acids



and consequent decrease in PUFAs in the renal cortex of Wistar rats. Our study, for the first time, demonstrates that 1) endogenous Ang (1-7) plays a role in maintenance of normal fatty acid composition in the rat and 2) chronic treatment with Ang (1-7) in diabetic rats increased PUFAs and decreased saturated fatty acids. However, the mechanism of differential effect of A-779 in saturated fatty acids needs to be investigated further. Therefore, Ang (1-7) may have prevented the development of nephropathy possibly by ameliorating the changes in fatty acid composition in STZ-treated rats. This article proposes an additional mechanism that might be involved in the renoprotective effect of this peptide.

## Limitations of the present study

1. We have used only a single dose level of Ang (1-7). A dose response study of Ang (1-7) would have been more informative.
2. A study on Mas<sup>-/-</sup> mice would have further validated the result obtained with Mas receptor antagonist A-779.

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## Disclosure

Authors contributed equally to work. There is no real or perceived conflict of interest.

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