

Adrenomedullin mediates early phase angiogenesis induced diabetic nephropathy in STZ diabetic rats

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Abstract. – OBJECTIVE: The implication of pro-angiogenic factors including vascular endothelial growth factor (VEGF) and its receptor flk-1 has been reported in diabetic nephropathy as early event. Adrenomedullin (AM), a potent vasodilator peptide, enhances angiogenesis and high levels were seen in diabetic animals and humans. However, its exact role in diabetic nephropathy is unclear. The present study investigated the effects of adrenomedullin receptor antagonist (ADM-52-22) on the early phase angiogenesis-induced diabetic nephropathy.

MATERIALS AND METHODS: 28 male Wistar rats were divided into: 1) Control non-diabetic, 2) Streptozotocin (STZ)-induced diabetic rats (55 mg/kg, i.p.), 3) Control non-diabetic + ADM-52-22, and 4) STZ-diabetes + ADM-52-22 (7 per group). ADM-52-22 was infused for two weeks (250 µg/rat/day, i.p.).

RESULTS: Diabetes caused an increase in kidney weight, renal VEGF levels, 24 hr urinary protein and nitric oxide excretion and hyperfiltration indicated by creatinine clearance (CrCl). ADM-22-52 reduced the rise in CrCl, total urinary protein and renal hypertrophy in diabetic rats, and attenuated early angiogenic response to diabetes: CD31 staining, flk1 protein and VEGF renal levels.

CONCLUSIONS: These results show that AM through its receptor mediates early angiogenesis-induced diabetic nephropathy which attributes to the early changes as hyperfiltration and hypertrophy.

Key Words:

Diabetic Nephropathy, AM-22-52, Adrenomedullin, Angiogenesis, Hyperfiltration.

DN is characterized by glomerular hyperfiltration, nephromegally, glomerular and epithelial hypertrophy, and proteinuria¹. Interestingly, abnormal angiogenesis is an evolving mechanism proposed to contribute to the early phase of DN similar to that observed in diabetic retinopathy². Abnormal glomerular capillaries were identified in Streptozotocin (STZ) induced-diabetic rats³ and db/db mice⁴. Guo et al⁵ reported capillary changes in glomerular hypertrophy. Of importance, structural changes of the newly formed vessels, enhance vascular permeability⁶ and possibly contribute to the early lesions of DN. One of the major contributors of angiogenesis during the early phase of DN is vascular endothelial growth factor (VEGF) which was up-regulated by the high blood sugar (BS) level⁷. VEGF is designated as a potent stimulator of angiogenesis whereby it promotes endothelial cell proliferation, migration and tube formation⁸. In addition, VEGF induces vascular permeability⁹ and, therefore, may be implicated in hyperfiltration noticed during the early phase of DN¹⁰. VEGF functions through its receptors VEGFR1 (flt-1) and 2 (flk-1). Expression of VEGF and its receptor flk-1 was seen in short term STZ-induced diabetes¹¹ and the administration of neutralizing anti-VEGF antibody improved early renal dysfunction in diabetic rats¹². Targeting angiogenesis during the early phase of DN ameliorates alterations in the early stage of type I DN models^{4,12} may halt progression to end stage kidney disease. The present study searches new grounds for understanding the patho-physiology of early derangements of DN.

Adrenomedullin (AM), a 52 amino acid peptide originally isolated from human pheochromocytoma¹³, acts as a multifunctional regulatory peptide¹⁴. It is a member of a family of peptides with sequence homology to calcitonin

Introduction

Diabetic nephropathy (DN) is among the major microvascular complications of diabetes and contributes to approximately 40% of end-stage renal disease in diabetic patients. Early phase of

gene-related peptide (CGRP)¹⁵. Both peptides are multifunctional and have overlapping biological activities concerning their potent vasodilating properties¹⁴. These peptides along with their receptors are widely distributed in peripheral tissues including the kidney¹⁶. The receptor system involved in mediating the actions of CGRP and AM comprises a calcitonin receptor (CL) together with one of three receptor activity modifying proteins (RAMP1, 2 or 3)¹⁷. The CL/RAMP1 complex forms a CGRP receptor which is blocked by the fragment CGRP₈₋₃₇, whereas CL/RAMP2 and 3 complexes form AM₁ and AM₂ receptors, respectively. The former being blocked more effectively than the latter by the AM fragment AM-22-52¹⁸. Although high levels of AM were reported in kidneys of diabetic rats¹⁹ and plasma of diabetic patients²⁰, its potential role in the pathogenesis of microvascular complications in the diabetic kidneys is not known. Therefore, targeting AM or its receptor would be a tool to elucidate its exact role in DN.

The current study aimed to evaluate the implication of adrenomedullin receptor in the early phase of diabetic nephropathy particularly in relation to angiogenesis STZ-induced diabetes in the rat.

Materials and Methods

Animals

Male Wistar rats (300-350 g body weight) obtained from King Khalid University Hospital (KKUH) Animal House, were recruited in the present study. Diabetes was induced by intraperitoneal injection (i.p.) of 55 mg/kg of Streptozotocin (STZ) (Sigma, Chemical Co, St. Louis, MO, USA) in 10 mmol/L citrate buffer (pH 4.5). 48 hrs later, the diabetic state was confirmed by measuring blood sugar (BS) from rat tail, using One Touch Basic Blood Glucose Monitoring System (Lifescan Canada Ltd, Burnaby, BC, Canada) and One Touch strips (range = 0-600 mg/dl). Rats with BS level below 300 mg/dl were excluded from the study. The diabetic rats were randomly selected and divided into 4 groups (7 rats/group): a) Control rats; injected with citrate buffer; b) Control + AM-22-52, c) STZ-induced diabetic rats and d) STZ + AM receptor antagonist (AM-22-52). Throughout the study, all animals were provided standard free access laboratory rat chow and water ad libitum. The rats were kept at ambient

temperature of 22°C and 12 hrs night/day cycles. All animal experiments were performed in accordance with the national laws for the use of animals in research and approved by the local Ethical Committee at KKUH Health guidelines.

AM-22-52 is an AM receptor antagonist¹⁸, purchased from Phoenix, AZ, USA, as a lyophilized powder. After reconstitution in distilled water, it was given at a dose of 25 µg/rat/day and 250 µg/rat/day, i.p., to control (non-diabetic) or to diabetic rats, two days after induction of diabetes, for two weeks. The 25 µg dose was found ineffective in the context of creatinine clearance, urinary protein and immunostaining. The 250 µg/rat/day dose was selected and was found by previous reports to inhibit angiogenesis *in vivo*²¹.

On weekly basis BS level was checked and urine was monitored for ketone bodies. Two weeks after receiving AM-22-52, all rats were sacrificed under urethane anesthesia (125 mg/kg, i.p.). Final body weight was determined and heparinized blood samples were collected, plasma separated, and stored at -70°C. Kidneys were immediately removed, and kidney weight to final body weight was calculated. Then kidneys were snap frozen in liquid nitrogen and used for AM and VEGF measurement using ELISA, or for western blotting of flk-1 receptor. Renal tissue protein concentration was measured using standard Bradford method (BioRad Protein Assay Kit, BioRad, Hercules, CA, USA). Some slices of the kidney were kept in 10% buffered formalin, then paraffin embedded for histological and immunostaining studies.

Assessment of Glomerular Filtration Rate and Urinary Protein Excretion

Just prior to termination of the study, rats were kept separately in metabolic cages and 24 hr urine samples were collected. Creatinine was measured in urine and plasma and then creatinine clearance (CrCl), a measure of glomerular filtration rate (GFR) was calculated according to the standard equation:

$$\text{CrCl (mL/min/kg)} = \left[\frac{\text{urinary Cr (mg/dL)} \times \text{urinary volume (mL)}}{\text{serum Cr (mg/dL)}} \right] \times \left[\frac{1000}{\text{body weight (g)}} \right] \times (1/1440 \text{ (min)})$$

24 hr total urinary protein excretion was also measured using BioRad Proteinuria Assay Kit (BioRad, Hercules, CA, USA) and expressed as mg/24 hr. Urine was kept at -20°C for subsequent assay of nitric oxide metabolic products.

Measurement of VEGF in Renal Tissues

Kidney tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing 10% proteinase inhibitor cocktail (Sigma Aldrich, St Louis, MO, USA). The homogenate was centrifuged at 14,000 rpm for 10 min, and total protein measured in the supernatant. VEGF was determined using a rat ELISA kit (R&D-Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Histological Assessment and Immunohistochemistry

At time of sacrifice, kidneys were removed, fixed in 10% buffered formalin, and embedded in Paraffin. Sections (4 μ m) were stained with Hematoxylin and Eosin (H&E), examined under light microscope and assessed histologically. Other sections were immune stained with CD31 antibodies for detection of neovascularization in kidney tissues. Briefly, paraffin sections were deparaffinized, rehydrated and rinsed in water and 10 mM Tris hydrochloride. To stop endogenous peroxidase activity, sections were immersed in endogenous enzyme block solution (Dako, Glostrup, Denmark) for 15 min. Sections were then incubated with 1% goat serum for 30 min. Subsequently, sections were incubated with mouse anti-human CD31 monoclonal antibody (1:20 dilution, Dako). The sections were incubated in 3,3'-diaminobenzidine (DAB) to detect antibody binding and then counterstained with hematoxylin.

CD31 stained sections were analyzed using image analyzer to measure glomerular area. Briefly, a high-resolution whole-slide digital scan of all slides was created with a Scan Scope scanner (Aperio Technologies, Inc., Vista, CA, USA). The digital slide images of thirty glomeruli per section were viewed and analyzed using Aperio's viewing and image analysis tools. The total analysis area was used to represent the glomerular area and expressed as mm².

Western Blotting

Immunoblot assay was performed as previously described²². Briefly, kidney homogenates were prepared on ice using 150-200 mg frozen kidney tissue/ml of homogenization buffer (20mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 5 mM EDTA, and protease inhibitor cocktail). Protein concentration in recovered protein extract was determined using BioRad Protein Assay Dye Reagent Concentrate (BioRad Labo-

ratories, Hercules, CA, USA). 50 μ g of total protein were separated by electrophoresis using 7.5% Tris-SDS polyacrylamide gels, at 200 V for 40 min, utilizing a Mini PROTEAN Tetra System (Bio-Rad Laboratories) and transferred to a nitrocellulose membrane. After reversible Ponceau S staining (Ponceau S Solution, Sigma Aldrich, St. Louis, MO, USA), the membrane was blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Twee-20 (TBST) for 1hr at room temperature. This was followed by incubation, overnight at 4°C, with flk-1 monoclonal mouse IgG (1:1000) in blocking buffer, recognizing protein at 200 kDa (sc 6251, Santa Cruz Biotech Inc., Santa Cruz, CA, USA). Blots were washed in blocking buffer for three times for 5 min at room temperature, then, incubated with horseradish peroxidase-conjugated goat anti-mouse antibodies (1:5000, Santa Cruz Biotech Inc.) in blocking buffer for 45 min at room temperature. Thereafter, membranes were washed three times for 5 min in TBST. The membranes were stripped for 40 min, followed by a single 10 min wash in phosphate buffered saline (PBS), and three 10 min washes in blocking buffer. The membranes were re-probed with polyclonal anti β -actin goat IgG antibodies (1:1000, Santa Cruz Biotech Inc.) and then incubated with horse radish peroxidase (HRP) anti-goat IgG. The signal was detected by chemiluminescence using the ECL Detection kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) followed by autoradiography. The density of the bands measured by densitometry (Bio-Rad, USA), analyzed and expressed with respect to β -actin.

Urinary Excretion of Stable Nitric Oxide end Products ($\text{NO}_2^-/\text{NO}_3^-$)

24hr urine samples were assayed for $\text{NO}_2^-/\text{NO}_3^-$ using Nitrate/Nitrite Assay kit (Cayman Chemical, Ann Arbor, MI, USA).

Reagents

All chemicals were supplied by Sigma unless otherwise specified.

Statistical Analysis

Data are presented as mean \pm SD. The data were analyzed using Prism GraphPad Software (GraphPad Software Inc., San Diego, CA, USA) using appropriate statistical tools. Means of different groups were analyzed by one-way ANOVA and subjected to Tukey's posttests for multi-

Table I. Biochemical characteristics of the experimental groups.

	Blood sugar (mg/dl)	Cr. CL (ml/min/kg)	Urinary protein (mg/24 h)
Control	98.9 ± 9.3	0.1338 ± 0.022	20.9 ± 2.1
Control + AM-22-52	84 ± 7.8	0.1556 ± 0.0312	19.5 ± 3.7
Diabetic	367 ± 18.7*	0.4436 ± 0.09*	46.8 ± 4.5*
Diabetic+ AM-22-52	146 ± 31**.#	0.205 ± 0.057	26.2 ± 5.1#
<i>p</i> -value	<i>p</i> < 0.001	<i>p</i> < 0.01	<i>p</i> < 0.01

Data are presented as mean ± SD. **p* < 0.05: vs control, control + AM-22-52, diabetic+AM-22-52; ***p* < 0.05: vs control. #*p* < 0.05: vs control + AM-22-52.

ple comparisons. *p* < 0.05 was considered statistically significant.

Results

Effect of AM-22-52 on Blood Sugar, Creatinine Clearance and Urinary Protein Excretion in STZ-Induced Diabetic Rats

Injection of STZ (55 mg/kg) led to hyperglycemia throughout the experimental period of two weeks compared to control non-diabetic rats (*p* < 0.001). Administration of AM-22-52 to diabetic rats reduced BS significantly (*p* < 0.01). However, in non-diabetic rats the decrease noticed in BS did not reach a significant level with the administration of AM-22-52 (Table I).

STZ-induced diabetes resulted in a three fold increase in CrCl, reflecting hyperfiltration, compared to non-diabetic rats. AM-22-52 treatment of the diabetic animals markedly reduced hyperfiltration by some 50%. However, no effect was reported in the non-diabetic rats receiving AM-22-52 as compared to non-diabetic rats receiving vehicle (Table I).

Urinary protein excretion was nearly doubled in diabetic animals compared to non-diabetic ones (*p* < 0.05). AM-22-52 administration reduced urinary protein excretion in the diabetic rats by some 40% (*p* < 0.05) (Table I).

Effect of AM-22-52 on Body Weight and Absolute and Relative Kidney Weights and Total Kidney Protein

Body weight was significantly reduced in diabetic group compared with non-diabetic animals (*p* < 0.05). Diabetic rats receiving AM-22-52 showed a normal gain in body weight similar to non-diabetic rats. No effect was detected on body weight of control rats treated with AM-22-52 (Table II).

To assess the effect of diabetic state and AM-22-52 treatment on renal hypertrophy parameters; kidney weight (absolute and relative) and total kidney protein content were measured. Diabetic rats developed marked increase in absolute and relative kidney weight compared with non-diabetic animals by some 30% and 40% respectively. Administration of AM-22-52 over two weeks period, led to a decrease in absolute and relative kidney weight by 23% and 29% with respect to untreated diabetic animals. No effect of AM-22-52 was detected on kidney weight (relative or absolute) of non-diabetic rats compared with their control (vehicle treated) (Table II).

Total kidney protein was measured and expressed as mg/kidney. Diabetic rats exhibited a significant increase in kidney protein content compared to non-diabetic rats by some 32%. Treatment of diabetic animals with AM-22-52 significantly reduced total kidney protein to near

Table II. Changes of renal hypertrophy related parameters.

	Final body weight (g)	Absolute kidney weight (g)	Relative kidney/body	Kidney protein (mg/kidney)
Control	326 ± 20	1.047 ± 0.09	–	86.32 ± 12.94
Control+AM-22-52	314 ± 10	1.054 ± 0.07	0.336 ± 0.023	86.43 ± 9.013
Diabetic	280 ± 19*.#	1.471 ± 0.12*.#	0.524 ± 0.028*.#	127.1 ± 25.61*.#
Diabetic + AM-22-52	305 ± 10	1.129 ± 0.14	0.37 ± 0.047	90.22 ± 11.03
<i>p</i> -value	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001

Data are means ± SD, n = 7 in each group; **p* < 0.05: vs control, control + AM-22-52; #*p* < 0.05: vs diabetic + AM22-52.

control level while non-diabetic rats were not affected with AM-22-52 administration. These results imply that AM-22-52 has an impact on renal hypertrophy in diabetic rats (Table II).

Histology

Histological examination of H&E stained kidney sections revealed, increased cellularity and mild expansion of the mesangial area induced by STZ (Figure 1 a-d). After two weeks treatment with AM-22-52, these changes caused by STZ-induced diabetes were attenuated.

CD31 Immunostaining and Immunohistochemical Analysis

To evaluate the therapeutic effect of AM-22-52 on the markers of angiogenesis in the DN induced by STZ, kidney sections were stained with CD31 antibodies. Enhanced immune-reactivity was detected in diabetic kidneys, mainly in the glomerular capillaries. Treated diabetic rats with

AM-22-52 showed attenuation in immune-reactivity for this antibody. The expression of CD31 was very minimal in non diabetic kidneys receiving citrate buffer or AM-22-52 (Figure 2 a-d). Quantitative analysis revealed that the STZ-induced increase in glomerular capillary area, was markedly suppressed by AM-22-52. These results reveal that AM-22-52 administration led to attenuation of the increase in glomerular CD31⁺ endothelial area induced by hyperglycemia probably by its anti-angiogenic effect (Figure 2 e).

Renal Tissue Concentration of VEGF

VEGF concentration was estimated in renal tissue homogenate. VEGF levels increased by 40% in kidneys of STZ-diabetic rats compared with non-diabetic control rats (1957 ± 224 vs 1116 ± 352 pg/100 mg protein, $p < 0.01$). Treatment with AM-22-52 resulted in a significant suppression of VEGF in kidney homogenate compared with vehicle treated diabetic rats

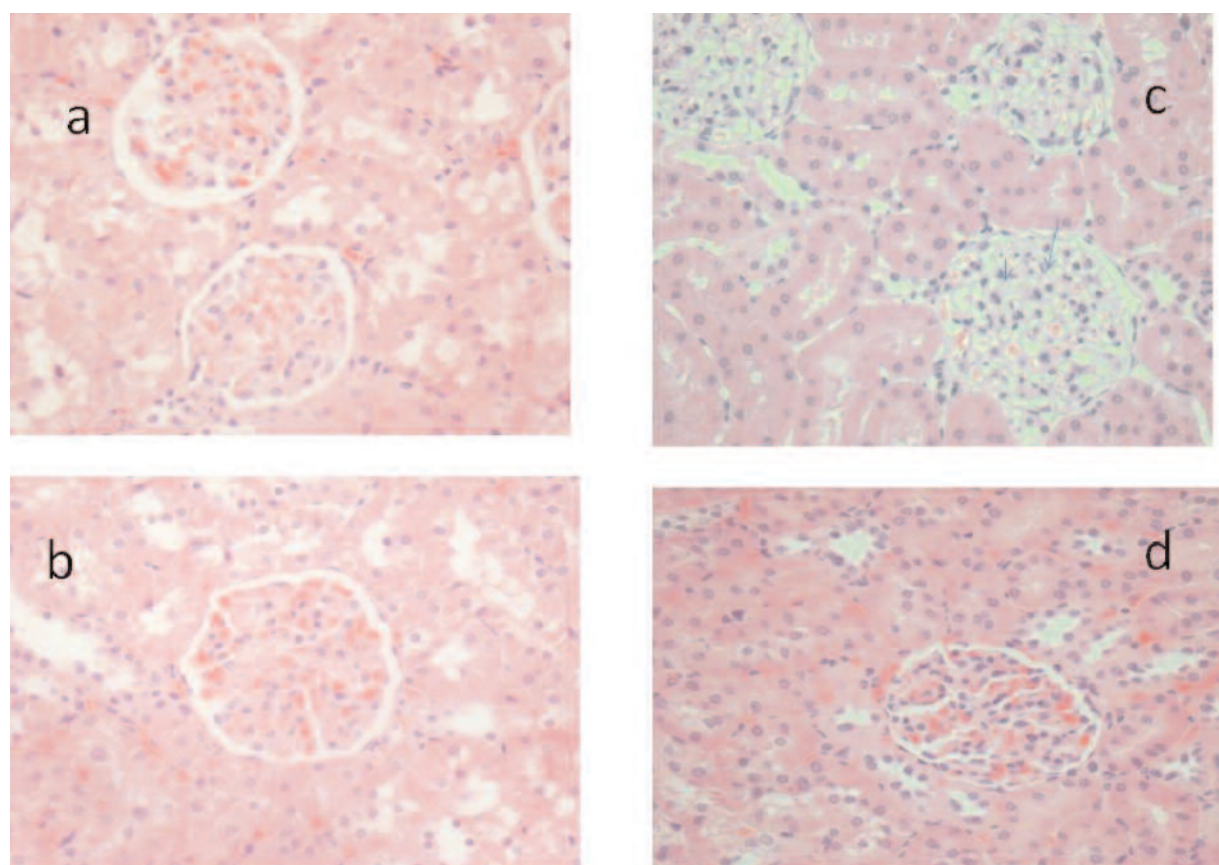


Figure 1. Representative photo micrographic appearance of H&E stained glomeruli of the studied groups; **(A)** non-diabetic control rats, **(B)** non-diabetic rats received AM-22-52, **(C)** diabetic rats, and **(D)** diabetic rats treated with AM-22-52 for two weeks ($250 \mu\text{g}/\text{rat}/\text{day}$, i.p.) (Magnification X400). Increased glomerular cellularity and mesangial expansion are seen in kidney sections of diabetic rats.

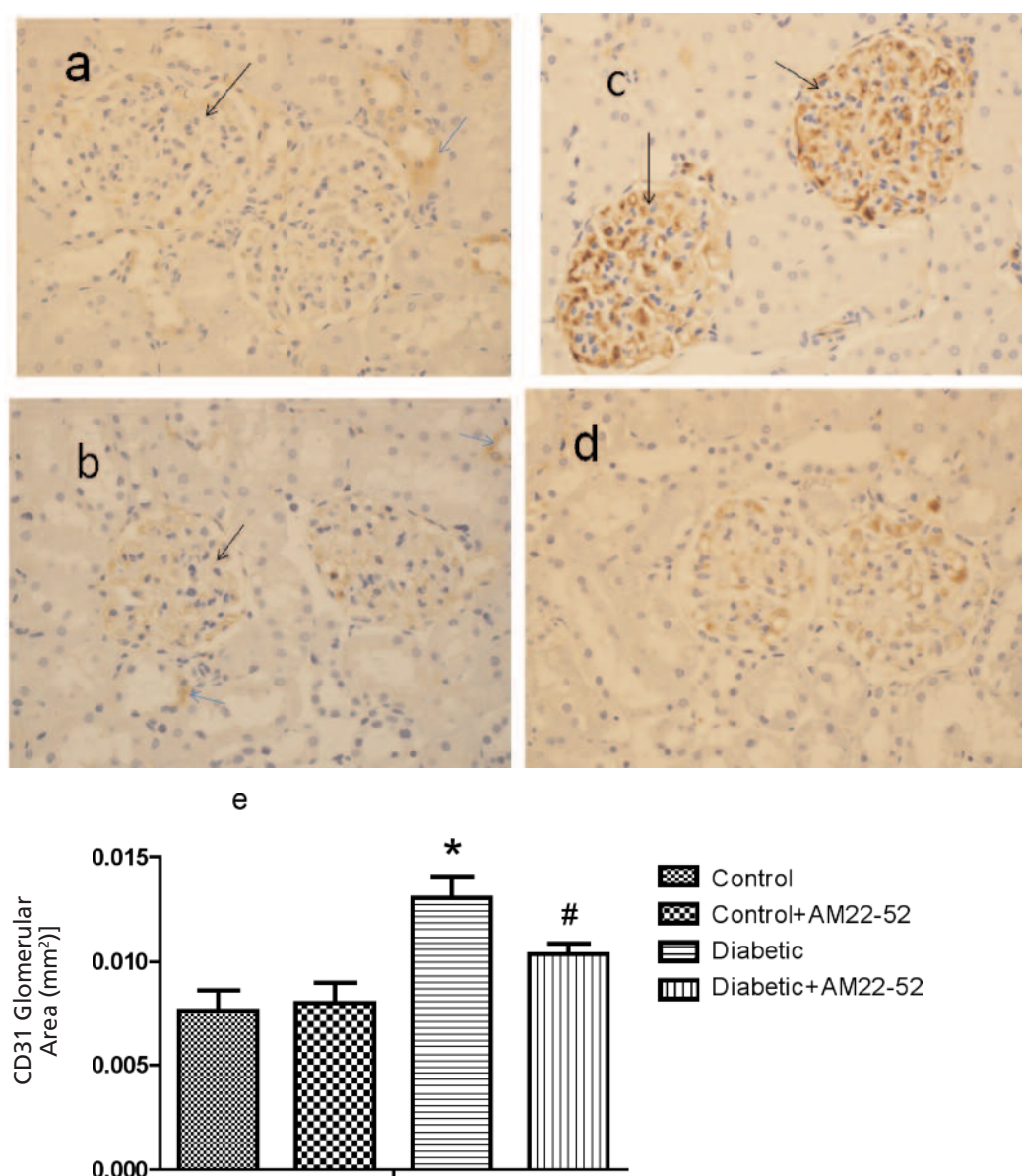


Figure 2. Immunostaining of CD31, an endothelial cell marker. Distribution of CD31 was determined by immunostaining technique in **A**, non-diabetic control rats, **B**, non-diabetic rats received AM-22-52, **C**, diabetic rats treated with vehicle or **D**, diabetic rats treated with AM-22-52. CD31 is intensely seen in the glomeruli of the diabetic rats compared with diabetic rats receiving AM-22-52 (250 μ g/rat/day). **E**, Glomerular capillary area is suppressed following AM-22-52 treatment in the diabetic rats (n=4/group) (Magnification x 400). * $p < 0.05$ vs control, control+AM-22-52, diabetic+AM-22-52, # $p < 0.05$ vs control, control+ AM-22-52.

(1468 \pm 250 vs 1957 \pm 224 pg/100 mg protein, $p < 0.05$). Control non-diabetic rats were not affected by AM-22-52 administration (Figure 3).

Protein Expression of flk-1 Receptor

The effect of AM-22-52 treatment on the expression of flk-1 receptor of the proangiogenic factor, VEGF, was studied by immunoblot assay. The level of flk-1 was higher in diabetic rats com-

pared to control non-diabetic animals ($p < 0.05$). Treatment with AM-22-52 markedly suppressed the STZ-induced increase of flk-1 (Figure 4 a,b).

Urinary Excretion of Stable Nitric Oxide end Products ($\text{NO}_2^-/\text{NO}_3^-$)

In STZ-induced diabetic rats, a marked urinary excretion of $\text{NO}_2^-/\text{NO}_3^-$ was detected compared to non-diabetic rats (2484 \pm 344 vs 1218 \pm 176

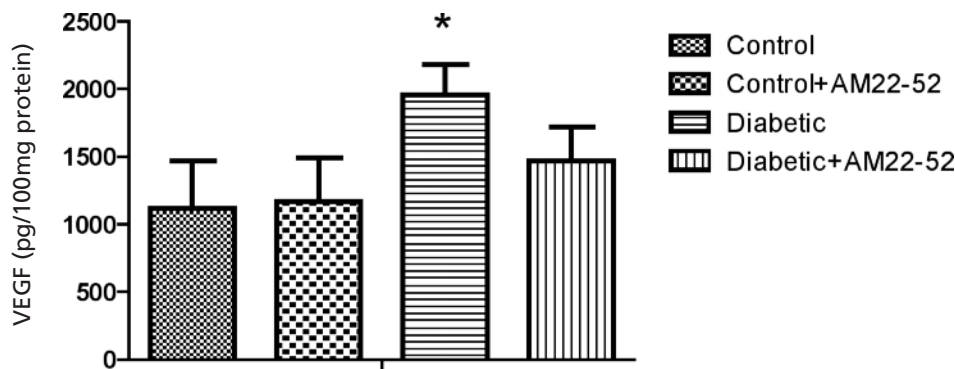


Figure 3. Levels of VEGF in renal tissue homogenate detected by ELISA. Data are represented as mean \pm SD, n=7/group. * $p < 0.05$ vs control, control+AM-22-52, diabetic+AM-22-52.

nmol/day, $p < 0.01$). Infusion of AM-22-52 significantly reduced urinary excretion of $\text{NO}_2^-/\text{NO}_3^-$ in treated diabetic rats compared with untreated diabetic animals (1832 ± 326 vs 2484 ± 344 nmol/day, $p < 0.01$). The excretory levels of these metabolites in control rats receiving AM-22-52 and non-diabetic animals were not comparable (933 ± 131 vs 1218 ± 176 nmol/day) (Figure 5).

Discussion

The present study investigated the implication of AM receptor on the early alterations of

STZ-induced DN in the rat with special emphasis on the angiogenesis process as an early event. Results showed that the administration of AM receptor antagonist, AM-22-52, attenuated the early-phase derangements of DN; hyperfiltration, proteinuria and renal hypertrophy, possibly by reducing the angiogenic response in the glomeruli. This was evidenced by down regulation of markers of angiogenesis; flk-1 receptor, VEGF and CD31 in renal tissue and by reducing nitric oxide metabolite excretion in the urine. The study provides a possible link between AM receptor and early phase DN. The normal kidney was not affected by the administration of AM-22-52.

AM has complex physiological roles mediated by its actions on three receptor complexes CL/RAMP1, 2 and 3¹⁷ where CL/RAMP2 is blocked more effectively by the AM fragment AM-22-52¹⁸. There is a concern that systemic inhibition of AM may have significant and possibly harmful effects on normal physiology. For this reason, rather than targeting AM itself by using AM antibodies, the current study targeted one of its receptors; using AM-22-52. Since RAMP2 is up regulated in glomerular endothelial cells and mesangial cells and in the afferent arteriole smooth muscle and endothelial cells of STZ-rats¹⁹, it would be encouraging to use AM-22-52 as an AM receptor blocker in this model. In addition the specificity of AM-22-52 has been shown in renal blood vessels²³. The dose chosen for the present work, $250 \mu\text{g}/\text{rat}/\text{day}$, was found to be effective by previous reports whereby it inhibited vasculogenesis and fetoplacental growth in rats²¹. The current work, examined the role of AM receptor as a mediator of hyperfiltration, renal hypertrophy and abnormal angiogenesis in DN in

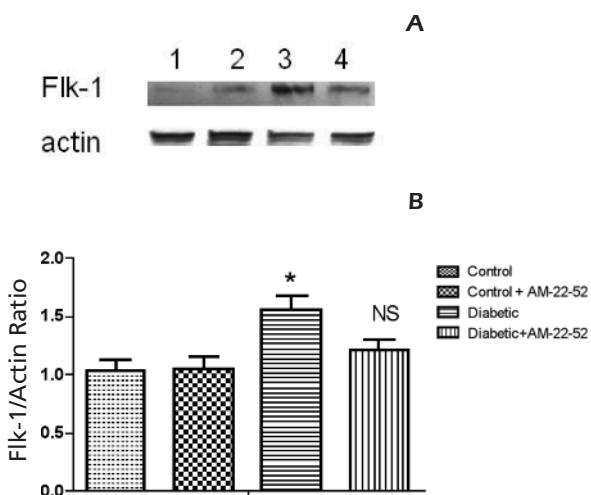
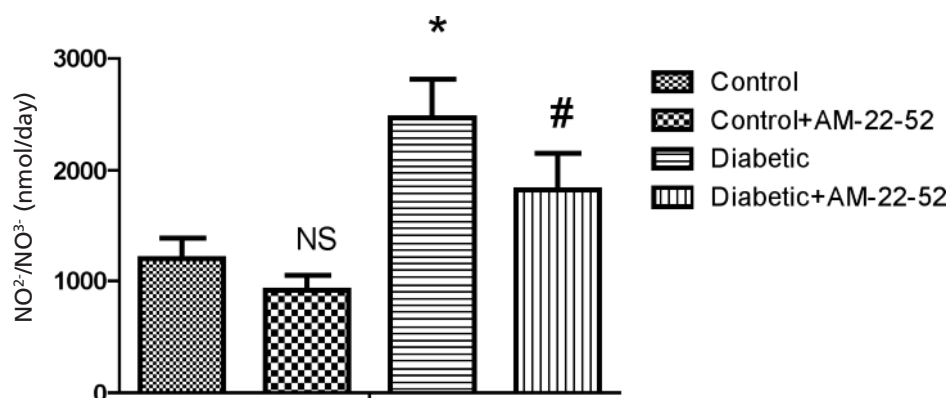


Figure 4. **A**, Immunoblots of flk-1 receptor. Each lane was loaded with $50 \mu\text{g}$ protein from each sample. **B**, Densitometric analysis showed significant expression of flk-1 in the kidneys of diabetic rats compared to non-diabetic. Treatment with AM-22-52 reduced flk-1 expression ($p < 0.05$) (n=4/group). Data are expressed as mean \pm SD. * $p < 0.05$ vs control, control+AM-22-52, diabetes+AM-22-52.

Figure 5. Urinary excretion of $\text{NO}^2\text{-NO}^3$ metabolites. Data are represented as mean \pm SD, $n=7/\text{group}$. * $p < 0.01$ vs control, control+AM-22-52, diabetic+AM-22-52. NS = non significant vs control. # $p < 0.01$ vs control, control+AM-22-52.



the rat and the effectiveness of blocking AM receptor as a potential therapeutic target.

Hyperfiltration was reported as one of the early manifestations of DN¹⁰. The increase in renal tissue AM in diabetic rats, as was demonstrated in this study, together with the localization of AM receptors in glomerular endothelial cells and afferent arteriole¹⁹, can cause dilation of glomerular capillaries and an increase in renal blood flow and GFR. The current investigation gives a strong evidence that AM receptors, at least partially mediated hyperfiltration during the early phase of DN whereby administration of AM-22-52 for two weeks reduced GFR (assessed by CrCl) in the diabetic rats by some 30%. This effect is possibly related to nitric oxide as we reported here; the increase in $\text{NO}^2\text{-NO}^3$ metabolites in the urine of diabetic rats was reduced by AM-22-52 treatment. It can be explained by damping the up regulation of endothelial nitric oxide synthase which occurs early in diabetes²⁴. This is supported by the fact that AM actions are mediated by nitric oxide²⁵ and that the deletion of endothelial nitric oxide synthase (eNOS) can lead to abrogation of AM actions²⁶. Therefore, blocking AM receptors possibly inhibits the downstream effectors; nitric oxide-cGMP pathway or the enzyme; eNOS, leading to reduction of the end-product metabolites, i.e. $\text{NO}^2\text{-NO}^3$. Another player implicated in hyperfiltration is VEGF. Suppression of VEGF protein level and its receptor flk-1 in the kidney by AM-22-52, possibly reduced vascular permeability and hyperfiltration. Considering that VEGF induces vascular permeability⁹, and that it is implicated in hyperfiltration noticed during the early phase of DN¹⁰, it would be more feasible to use agents that suppress VEGF rather than using VEGF antibodies.

Renal hypertrophy/nephromegaly, also occurs in the early phase of DN as reported in this study

and others (1). Nephromegaly possibly results from both glomerular and tubular hypertrophy. In the present study, renal growth was associated with the high levels of renal VEGF, and the endothelial glomerular proliferation detected by increased CD31 expression. In addition to its potent angiogenic effect, VEGF augments protein synthesis and hypertrophy in renal proximal tubular epithelial cells²⁷. The infusion of AM-22-52 led to a reduction in renal hypertrophy as evidenced by a decrease in absolute and relative kidney weights as well as kidney protein content. The inhibitory effects of AM-22-52 on renal growth are possibly mediated by abrogation of VEGF and its receptor flk-1 as demonstrated herein, in addition to attenuation of the growth promoting effects known for AM²⁸. Therefore, the suppressor effect of AM-22-52 on kidney growth is double fold; by blocking AM actions and attenuating VEGF effects resulting in a decrease in protein synthesis and hypertrophy of renal cells and glomeruli, as reported by this investigation. These observations are supported by a previous study showing that, AM-22-52 inhibited cell proliferation and/or induced apoptosis²¹.

Abnormal angiogenesis and micro-angiopathy are among the complications of diabetes and occurs early in DN (3). We demonstrated an increase in glomerular area, and expression of CD31 and flk-1 receptor protein and kidney VEGF in diabetic rats, in accordance with previous studies¹¹. The angiogenic effects of AM are likely mediated through direct stimulation of endothelial cell proliferation²⁸ and protection of endothelial cells from apoptosis²⁹. The current work provided evidence that AM receptor mediated angiogenesis induced diabetic glomerulopathy as was demonstrated by abrogation of markers of angiogenesis in kidneys of STZ-diabetic rats. An

interesting marker of angiogenesis studied in this investigation was CD31. In the kidney, CD31 is localized to rat glomeruli as well as peri-tubular capillaries. Our results showed up regulation of CD31 in endothelial glomerular area of diabetic rats with respect to control and were suppressed by AM-22-52 treatment. The inhibitory effect of AM-22-52 on VEGF and flk-1 receptor provides more evidences that AM mediates angiogenesis and acts through VEGF signaling in diabetic kidneys. Supporting our view, there is a good body of evidence provided by recently published reports. For example, intranasal administration of AM-22-52 reduced lung capillary density in neonatal rats. This effect was associated with decreased lung eNOS and VEGF mRNA expression³⁰. In the vascular endothelial cells the binding of AM to its receptor (CRL/RAMP2) could trigger a trans-activation of the flk-1 receptor, leading to a signaling cascade inducing proangiogenic events in the cells³¹. More recently, AM inclusion in Matrigel plugs induced angiogenesis while systemic administration of an anti-AM receptor antibody, reduced neo-vascularization of Matrigel plugs in a dose-dependent fashion³².

The current study investigated the role of AM receptor in the early phase of diabetic kidney. Interesting results were recently published by Hayashi et al³³, reporting that deletion of AM in STZ-mice, induced renal damage. That study highlighted AM as a protective agent via anti-oxidant mechanism, which looks conflicting with the current study. This can be explained by the study design, and duration. Heterozygous deletion of RAMP2 and AM, induced extensive renal damage in mice after 4 weeks of STZ administration³⁴. Nevertheless, blocking AM receptor would avoid blocking beneficial effects of AM that might be mediated by other AM receptors. On the other hand, genetic deletion of AM, its receptor or both may induce extensive effects in many organs.

Conclusions

The present investigation provides evidence that adrenomedullin mediates early phase events in diabetic nephropathy.

Acknowledgements

The authors appreciate the "Research Center of College of Medicine", Deanship of Scientific Research at King Saud

University for funding this project. The authors extend their appreciation to Mr. Jamal Abdulghani for animal care, monitoring and sampling. The authors are also thankful for Mr. James Chu assistance in western blotting experiments, for Mrs. Rahmatulnesa Lathief experimental work assistance, and for Ms. Afnan Alsuhaibani secretarial assistance.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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