Exogenously administered adenosine attenuates renal damage in streptozotocin-induced diabetic rats

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ABSTRACT

Background: Diabetic nephropathy (DNP) is one of the most serious complications of diabetes mellitus (DM). In the present study, we investigated the potential of adenosine as a therapeutic candidate for preventing DNP.

Methods: Twenty-one adult male rats were included in the study. Fourteen rats were administered a single dose of 60 mg/kg streptozotocin (STZ) to induce diabetes. Seven rats served as normal control group. Diabetic rats were randomly divided into two groups: one group was treated with 1 mL/kg saline/day (DM + saline) and the other group was treated with 5 mg/kg/day adenosine (DM + adenosine) for 6 weeks. After 6 weeks, biochemical parameters including urea, creatinine, blood urea nitrogen (BUN), kidney injury molecule-1 (KIM-1) and tumor necrosis factor-α (TNF-α) were measured in plasma samples. Also, kidneys were removed for histopathological assessment.

Results: Both of plasma KIM-1 and TNF-α levels were significantly higher in DM + saline group compared to controls. However, treatment of diabetic rats with adenosine significantly decreased the plasma KIM-1 and TNF-α levels compared to DM + saline group. Significant histopathological changes were observed in diabetic rats whereas adenosine treatment effectively prevented these changes.

Conclusions: The findings of the present study suggest that adenosine may be a useful therapeutic agent for preventing DNP.

ARTICLE HISTORY
Received 1 March 2016
Revised 20 May 2016
Accepted 24 June 2016
Published online 14 July 2016

KEYWORDS
Adenosine; diabetes mellitus; KIM-1; nephropathy; TNF-α

Introduction

Diabetic nephropathy (DNP) is the single most common cause of end-stage renal failure worldwide, and a major indication for renal replacement therapy. Approximately 20–40% of patients with diabetes develop DNP. Currently angiotensin converting enzyme (ACE) inhibitors are widely used to prevent proteinuria in DNP.

DNP is characterized by increased kidney size and glomerular volume, thickening of basement membranes and progressive accumulation of extracellular matrix. Several mechanisms contribute to the development and outcomes of DNP including an interaction between hyperglycemia-induced metabolic and hemodynamic changes, and genetic predisposition. The pathological factors playing a role in DNP include renin–angiotensin–aldosterone system (RAAS), hyperglycemia-induced increased glycation, increased protein kinase-C (PKC) activity, inflammation (cytokines), growth factors, metalloproteinases, activated polyol pathway, genetic susceptibilities, oxidative stress, transforming growth factor-β (TGF-β), plasminogen activator inhibitor 1 (PAI-1) and connective tissue growth factor (CTGF). Chronic low-grade inflammation and activation of the innate immune system are closely involved in the pathogenesis of diabetes and its microvascular complications. Proinflammatory cytokines, mainly IL-1, IL-6 and IL-18, as well as TNF-α, are involved in the development and progression of DNP.

Kidney injury molecule-1 (KIM-1) is a transmembrane glycoprotein whose expression is prominently up-regulated in various reason-caused renal tubular injury. Recently, several experimental and human studies suggest that KIM-1 is a sensitive and specific biomarker of early detection and monitoring of renal injury. As tubulointerstitial injury is present in all kinds of chronic kidney disease, measurement of KIM-1 as a tubular phase indicator of renal damage may help to detect
nephropathy before the development of albuminuria, the currently used marker of early DNP. Adenosine, a purine nucleoside, plays critical roles in several biochemical processes, such as energy transfer—as adenosine triphosphate (ATP) and adenosine diphosphate (ADP) as well as in signal transduction as cyclic adenosine monophosphate (cAMP). Adenosine also regulates blood flow to various organs through its vasodilatory effect. Earlier studies have suggested its significant role in normal and pathological states of kidney function. Clinically, adenosine is used as an antiarrhythmic agent in the treatment of a number of supraventricular tachycardia that does not improve with vagal maneuvers. In the present study, based on previous reports, we hypothesized that exogenously administered adenosine may lead to protection and restoration of the functions and architecture of kidneys in experimentally induced diabetes. To test the effects of adenosine, we evaluated histopathological and biochemical injury markers in a rat model of type I diabetes.

Materials and methods

Animals

Twenty-one, adult male Sprague–Dawley albino rats were used in the study. Animals were fed ad libitum and housed in pairs in steel cages with a temperature controlled environment (22 ± 2°C) and 12 h light/dark cycles. All experimental procedures were approved by the Committee for Animal Research of Ege University. All animal studies conformed strictly to the animal experiment guidelines of the Committee for Human Care.

Induction of DNP

Diabetes was induced by a single intraperitoneal (i.p.) injection of streptozotocin (STZ; 60 mg/kg, Sigma-Aldrich Inc., St. Louis, MO) following an overnight fast. STZ dissolved in 0.9% NaCl and adjusted to a pH 4.5 with citric acid. Briefly, while 14 rats were injected with STZ, no drug was administered to the remaining rats (n = 7), which served as controls (Group 1, n = 7). Diabetes was verified after 48 h by measuring blood glucose levels with glucose oxidase reagent strips (Boehringer-Mannheim, Indianapolis, IN). Rats with blood glucose levels > 250 mg/dL were considered as diabetic and included in the study. Diabetic rats were randomly distributed into two groups. DM + saline group (Group 2, n = 7) was treated 1 mL/kg/day saline while DM + adenosine group (Group 3, n = 6) was treated with 5 mg/kg/day adenosine (Abfen Farma, Ankara, Turkey) intramuscularly for 6 weeks. Control rats received no treatment or interaction. On the last day of Week 6, all animals were euthanized and blood samples were collected by cardiac puncture for biochemical analysis. The plasma was separated from cells by centrifugation at 3000 rpm for 10 min, and aliquots were frozen at −30°C for further use. Nephrectomies were performed for histopathological examination.

Biochemical assessment

Plasma levels of urea, creatinine and blood urea nitrogen (BUN) of all animals were measured by Olympus AU5800 auto-analyzer and expressed as milligram per deciliter. Measurements of plasma KIM-1 and TNF-α levels were carried out using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Boster Biological Technology Co., Ltd., Pleasanton, CA) specific for rats. All samples from each animal were measured in duplicate according to the manufacturer’s guidelines. The detection limit for each ELISA kit was 31.2 pg/mL and 7.8 pg/mL, respectively. Intraassay and interassay coefficients of variation were <10% in each determination.

Histopathological examination of renal tissue

For histolopathological studies, all animals were anesthetized by an i.p. injection of 40 mg/kg ketamine (Alfamine®, Ege Vet, Alfasan International B.V. Woerden, Holland) and 4 mg/kg xylazine (Alfazyne®), then perfused with 200 ml 4% formaldehyde in 0.1 M phosphate-buffered saline. Kidney sections were cut at 5 μm and stained with hematoxylin and eosin. All sections were photographed with an OlympusC-5050 digital camera mounted on an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan). A semiquantitative scoring system (sclerosis index) was used to evaluate the degree of glomerular sclerosis. Glomerular hypertrophy was calculated from the cross-sectional area of the glomerular tuft. The maximum diameter of a glomerulus that appeared in the cross section was photographed and converted to a digital image by an examiner blinded to the source of the tissue. The glomerular tuft and mesangial areas were measured using a computerized image analysis system (Image-Pro Express 1.4.5; Media Cybernetics, Inc., Silver Spring, MD). Fifty glomeruli were examined from each animal.

Statistical analysis

Data analyzes were performed using SPSS software (version 15.0, SPSS Inc., Chicago, IL). Non-parametric tests
were used for statistical analysis. Statistical differences among the groups were tested with the Kruskal–Wallis test. If the differences significant, Mann–Whitney U-test was used. Data are presented as means and standard error of the mean (SEM). Pearson's correlation analysis was conducted to examine the association between histological and biochemical parameters. p-Values <0.05 were considered statistically significant.

Results

Evaluation of plasma glucose levels

Plasma glucose levels of the study groups were measured weekly throughout the study. The alterations in blood glucose levels represented in Figure 1. STZ-received rats showed higher blood glucose levels than those of controls (p < 0.0005). The increase in plasma glucose was measured as early as 48 h after STZ injection and was preserved throughout the study period. No significant difference was observed between saline-treated and adenosine-treated groups by means of plasma glucose levels.

Evaluation of renal histopathology

Figure 2 shows representative glomerular morphology observed in the study groups. Glomerular sclerosis index was measured in nephrectomy materials in each group by using a scoring system. Diffuse glomerular sclerotic lesions and diffuse expansion of mesangium were more prominent in saline-treated diabetic rats compared to the respective controls. Sclerosis score was significantly higher in saline-treated diabetic group when compared with control group (p < 0.05). In DM + adenosine group, sclerosis index was significantly decreased compared to saline-treated diabetic group (p < 0.05).

Evaluation of biochemical parameters

Table 1 represents the alterations in plasma concentrations of urea, creatinine and BUN determined at the end of experiments. Blood urea, creatinine and BUN levels were considerably elevated in DM + saline group (p < 0.005). Blood urea and BUN levels revealed a significant reduction in DM + adenosine group when compared to saline-treated group.

Figure 3 depicts the plasma KIM-1 and TNF-α levels in the study groups. Kruskal–Wallis test showed significant differences among the groups with regard to plasma KIM-1 and TNF-α levels (p < 0.005 and p < 0.0005, respectively). Both of plasma KIM-1 and TNF-α levels were significantly higher in DM + saline group compared to normal controls (p < 0.005). However, treatment of diabetic rats with adenosine significantly decreased the plasma KIM-1 and TNF-α levels compared to DM + saline group (p < 0.05 and p < 0.005, respectively).

When we analyzed the relationship between histopathological and biochemical parameters, we found a significant and positive correlation between the sclerosis score and KIM-1 level (r = 0.782, p < 0.0001) and TNF-α level (r = 0.634, p < 0.0001). Figure 4 shows the correlation analysis between histopathological and biochemical parameters.

Discussion

Advanced DNP is the leading cause of glomerulosclerosis and end-stage renal disease. The mechanisms leading to DNP are very complicated and involve the interaction of hemodynamic and metabolic factors. The early stage changes in kidney include increased renal blood flow, hypertrophy and glomerular hyperfiltration. These early changes are reversible; however, prolonged hyperglycemia for several years further causes irreversible structural and cellular changes in the kidney. The main structural changes in kidneys contain glomerular basement membrane thickening, mesangial expansion, nodular glomerulosclerosis (Kimmelstiel–Wilson lesions), tubulointerstitial fibrosis and diffuse glomerulosclerosis.19

A large body of evidence suggests the important role of proinflammatory cytokines in the development and progression of DNP. Experimental studies have confirmed the increased TNF-α expression and protein levels in glomerular and proximal tubule cells in diabetic rats. TNF-α activates several transcription factors,
synthesis of other cytokines, growth factors, cell adhesion molecules, secondary messengers and reactive oxygen species (ROS) in renal cells.20 TNF-α can alter the intraglomerular blood flow by disturbing the balance between vasoconstrictive and vasodilatory mediators, and also lead to injury of endothelial permeability by changing the distribution of adhesion receptors associated with cell-cell adhesion. Several clinical studies have suggested a significant relationship between clinical markers and plasma and urinary TNF-α levels from diabetic patients with nephropathy.21

KIM-1 is a type 1 cell membrane glycoprotein and believed to be a good predictor of renal tubular damage.22 Renal expression of KIM-1 displays a significant and positive relationship with interstitial damage and glomerular pathology including focal and segmental glomerulosclerosis and mesangial matrix expansion.23 Although KIM-1 is not detectable in normal kidney tissue or urine it is highly expressed in proximal tubule epithelial cells in human and rodent kidneys following ischemic or toxic injury. The ectodomain of KIM-1 is released into the renal tubule following its cleavage by matrix metalloproteinases and its increased levels in the blood and urine samples reflect acute and chronic kidney injury in animals and humans.23–25 In an animal study, it has been reported that urinary KIM-1 in the spontaneous type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats significantly increased at 14 weeks of age, and the elevation continued up to 22 weeks of age.11 In a clinical study, Sabisetti et al.26 have found increased KIM-1 levels with increasing

Table 1. Effects of adenosine treatment on plasma concentrations of urea, creatinine and BUN.

| Treatment type   | Urea (mg/dL)   | Creatinine (mg/dL) | BUN (mg/dL)  |
|------------------|----------------|--------------------|--------------|
| Control          | 45.71 ± 1.49   | 0.39 ± 0.01        | 21.46 ± 0.68 |
| DM + saline      | 64.07 ± 5.67   | 0.55 ± 0.01        | 29.94 ± 2.62 |
| DM + adenosine   | 45.67 ± 1.33   | 0.53 ± 0.01        | 22.12 ± 0.76 |

Data are given as mean ± SEM.

*p < 0.05 versus control group.

*p < 0.05 versus diabetic group.

*p < 0.0005 versus control group.

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chronic kidney disease stage in patients with type 1 diabetes and proteinuria with longitudinal follow-up.

In the present study, we evaluated both histopathological and functional effects of adenosine on diabetic kidneys in a rat model. The results of the current study confirm the typical early histological changes caused by diabetes such as glomerular basement membrane thickening, mesangial matrix increase and glomerular volume increase. In addition to histological findings, plasma levels of urea, creatinine and BUN clearly demonstrated renal dysfunction in diabetic rats. To ascertain whether the protective effect of adenosine on renal dysfunction occurs due to an anti-inflammatory mechanism, we measured TNF-α levels in plasma samples of the animals. Our results demonstrated increased TNF-α levels in diabetic rats than those of controls whereas adenosine treatment significantly decreased TNF-α levels. Consistent with these results, plasma KIM-1 levels were significantly increased in saline-treated diabetic group. Adenosine treatment markedly attenuated nephropathy and improved renal dysfunction, as evidenced by reduced levels plasma of urea, creatinine, BUN and KIM-1 levels. The beneficial effects of adenosine are independent of blood glucose levels.

These results suggest that adenosine may elicit a protective role in DNP through its vasodilatory and anti-inflammatory effects.

Although there are limited studies regarding the therapeutic effects of exogenous adenosine on DNP, the results of the present study are supported by the results of previous reports. In the kidney, adenosine regulates GFR, renal blood flow and hormone and neurotransmitter release. Adenosine is also an important mediator of tubular glomerular feedback (TGF) response. Adenosine exerts its diverse effects through G protein-coupled purinergic receptors, namely A₁AR, A₂A AR, A₂B AR and A₃AR. Adenosine receptors are expressed on both tubules and renal vasculature. Several experimental studies have reported that ischemic, hypoxic or inflammatory conditions may change the expression level of AR subtypes. Increased expression of both A₁ and A₂ receptors and increased sensitivity to exogenous adenosine has been demonstrated in experimentally induced diabetes.²⁷,²⁸

Many beneficial effects of adenosine are thought to be through the A₂A or A₂B receptors. Adenosine can inhibit glomerular mesangial cell growth through its autocrine and paracrine effects on A₂B AR.²⁹ A recent literature report indicates that adenosine subtype A₂B receptors physiologically maintain GFR in the normal kidney by controlling efferent arteriolar tone, and thereby filtration fraction. However, this is abolished in the diabetic kidney, leading to vasoconstriction of efferent arteriole, elevation of filtration fraction and consequently causing glomerular hyperfiltration.³⁰

Experimental studies have provided evidence that A₂A AR activation significantly limits inflammation and tissue damage, therefore may play an anti-inflammatory role.³¹ In a recent study, it has been demonstrated that chronic adenosine A₂A receptor stimulation prevents proteinuria and glomerular damage in experimentally induced diabetes through an anti-inflammatory mechanism independent of oxidative stress and kidney hypoxia. The stimulation of A₂A reduces TNF-α levels, infiltration of macrophages, glomerular damage and basement membrane thickness and also increases anti-inflammatory IL-10 levels.¹⁵

Collectively, the present study demonstrates that treatment with adenosine prevents histopathological alterations in diabetic kidney, suggesting a protective role on the structure of glomerulus and mesangial matrix. The reduction in plasma TNF-α and KIM-1 levels in diabetic rats, as well as the decrease in glomerulosclerosis index evidently indicate that adenosine may prevent the development of renal fibrosis and tubulointerstitial injury associated with DNP. Although this is the first report that demonstrates the beneficial

Figure 3. Alterations in plasma TNF-α and KIM-1 levels. DM + saline group had significantly higher TNF-α and KIM-1 levels compared to saline group. Data are given as mean ± SEM. *p < 0.005 versus control group. #p < 0.05 versus DM + saline group. ##p < 0.05 versus DM + saline group.
effects of exogenous adenosine on DNP in a rat model of diabetes, lack of dose-response and time-course experiments are the main limitations of our study. Future studies are required to clarify the precise mechanisms involved in therapeutic effects of adenosine in DNP.

Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding
This study was supported by Turkish Kidney Disease and Hypertension Research Foundation.

References
1. Estacio RO, Schrier RW. Diabetic nephropathy: Pathogenesis, diagnosis, and prevention of progression. Adv Intern Med. 2001;46:359–408.
2. Alsaad KO, Herzenberg AM. Distinguishing diabetic nephropathy from other causes of glomerulosclerosis: An update. J Clin Pathol. 2007;60:18–26.
3. Nauta FL, Boertien WE, Bakker SJ, et al. Glomerular and tubular damage markers are elevated in patients with diabetes. Diabet Care. 2011;34:975–981.
4. Zatz R, Dunn BR, Meyer TW, et al. Prevention of diabetic glomerulopathy by pharmacological amelioration of glomerular capillary hypertension. J Clin Invest. 1986;77:1925–1930.
5. Ravid M, Savin H, Jutrin I, et al. Long-term stabilizing effect of angiotensin-converting enzyme inhibition on plasma creatinine and on proteinuria in normotensive type II diabetic patients. Ann Intern Med. 1993;118:577–581.
6. Kanwar YS, Wada J, Sun L, et al. Diabetic nephropathy: Mechanisms of renal disease progression. Exp Biol Med. 2008;233:4–11.

7. Ruggenenti P, Cravedi P, Remuzzi G. The RAAS in the pathogenesis and treatment of diabetic nephropathy. Nat Rev Nephrol. 2010;6:319–330.

8. Navarro-González JF, Mora-Fernández C. The role of inflammatory cytokines in diabetic nephropathy. J Am Soc Nephrol. 2008;19:433–3442.

9. Alkayyali S, Lyssenko V. Genetics of diabetes complications. Mamm Genome. 2014;25:384–400.

10. Garud MS, Kulkarni YA. Hyperglycemia to nephropathy via transforming growth factor beta. Curr Diabetes Rev. 2014;10:182–189.

11. Hosohata K, Ando H, Takeshita Y, et al. Urinary Kim-1 is a sensitive biomarker for the early stage of diabetic nephropathy in Otsuka Long-Evans Tokushima Fatty rats. Diab Vasc Dis Res. 2014;11:243–225.

12. Lin Q, Chen Y, Lv J, et al. Kidney injury molecule-1 expression in IgA nephropathy and its correlation with hypoxia and tubulointerstitial inflammation. Am J Physiol Renal Physiol. 2014;306:F885–F895.

13. Wierema TK, Houben AJ, Kroon AA, et al. Mechanisms of adenosine-induced renal vasodilatation in hypertensive patients. J Hypertens. 2005;23:1731–1736.

14. Edlund A, Sollevi A. Renal effects of i.v. adenosine infusion in humans. Clin Physiol. 1993;13:361–371.

15. Persson P, Friederic-Persson M, Fasching A, et al. Adenosine A2 receptor stimulation prevents proteinuria in diabetic rats by promoting an anti-inflammatory phenotype without affecting oxidative stress. Acta Physiol. 2015;214:311–318.

16. Rankin AC, Brooks R, Ruskin JN, et al. Adenosine and the treatment of supraventricular tachycardia. Am J Med. 1992;92:655–664.

17. Aydin S, Kuloglu T, Aydin S, et al. Expression of adropin in rat brain, cerebellum, kidneys, heart, liver, and pancreas in streptozotocin-induced diabetes. Mol Cell Biochem. 2013;380:73–81.

18. Bob FR, Gluhovschi G, Herman D, et al. Histological changes and immunohistochemical markers in the assessment of glomerulosclerosis in patients with glomerulonephritis. Rom J Morphol Embryol. 2011;52:1027–1032.

19. Bloodworth JM Jr. A re-evaluation of diabetic glomerulosclerosis 50 years after the discovery of insulin. Hum Pathol. 1978;9:439–453.

20. Abdul-Aziz KK, Tuorkey MJ. Targeting tumor necrosis factor alpha (TNF-α) in diabetic rats could approve avenues for an efficient strategy for diabetic therapy. Diabetes Metab Syndr. 2012;6:77–84.

21. Macisaac RJ, Ekinci EI, Jerums G. Markers of and risk factors for the development and progression of diabetic kidney disease. Am J Kidney Dis. 2014;63:S39–S62.

22. Vaidya VS, Niewczas MA, Ficociello LH, et al. Regression of microalbuminuria in type 1 diabetes is associated with lower levels of urinary tubular injury biomarkers, kidney injury molecule-1, and N-acetyl-β-D-glucosaminidase. Kidney Int. 2011;79:464–470.

23. van Timmeren MM, van den Heuvel MC, Bailly V, Bakker SJ, et al. Tubular kidney injury molecule-1 (KIM-1) in human renal disease. J Pathol. 2007;212:209–217.

24. van Timmeren MM, Bakker SJ, Vaidya VS, et al. Tubular kidney injury molecule-1 in protein-overload nephropathy. Am J Physiol Renal Physiol. 2006;291:F456–F464.

25. Waanders F, van Timmeren MM, Stegeman CA, et al. Kidney injury molecule-1 in renal disease. J Pathol. 2010;220:7–16.

26. Sabbisetti VS, Waikar SS Antoine DJ, et al. Blood kidney injury molecule-1 is a biomarker of acute and chronic kidney injury and predicts progression to ESRD in type I diabetes. J Am Soc Nephrol. 2014;25:2177–2186.

27. Pawelczyk T, Grden M, Rzepko R, et al. Region-specific alterations of adenosine receptors expression level in kidney of diabetic rat. Am J Physiol. 2005;290:F325–F335.

28. Pflueger AC, Schenk F, Osswald H. Increased sensitivity of the renal vasculature to adenosine in streptozotocin-induced diabetes mellitus rats. Am J Physiol. 1995;269:F529–F535.

29. Jackson EK, Raghvendra DK. The extracellular cyclic AMP-adenosine pathway in renal physiology. Annu Rev Physiol. 2004;66:571–599.

30. Persson P, Hansell P, Palm F. Adenosine A2 receptor-mediated regulation of renal hemodynamics and glomerular filtration rate is abolished in diabetes. Adv Exp Med Biol. 2013;765:225–230.

31. Haskó G, Kuhel DG, Chen JF, et al. Adenosine inhibits IL-12 and TNF-[alpha] production via adenosine A2a receptor-dependent and independent mechanisms. FASEB J. 2000;14:2065–2074.