

RESEARCH ARTICLE

Reno-protective effect of NECA in diabetic nephropathy: implication of IL-18 and ICAM-1

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ABSTRACT. Diabetic nephropathy (DN) remains the most common cause of end-stage renal disease. Although, adenosine acts as a local modulator with a cytoprotective function, extracellular adenosine usually disappears quickly due to a rapid uptake into adjacent cells. Therefore, we investigated the effect of 5'-(N-ethylcarboxamido)-adenosine (NECA), a stable, nonselective adenosine receptor agonist, on diabetes-induced increases in inflammatory cytokines and adhesion molecules. The enhancement of adenosine receptor action by NECA was examined in the renal tissues of rats with streptozotocin-induced diabetes. Daily i.p. injections of NECA at 0.3 mg/kg/day were given to rats, over a two-week period, six weeks after the induction of diabetes. Morphological changes were assessed in kidney sections. Oxidative stress was examined by measuring tissue malondialdehyde. Gene expression of interleukin (IL)-18, tumor necrosis factor (TNF)- α and intercellular adhesion molecule (ICAM)-1 was measured by real-time PCR. Activation of cellular, proapoptotic pathways was demonstrated by measuring the activation of c-Jun NH₂-terminal kinases (JNK)-mitogen-activated-protein kinase (MAPK). We found that diabetes-induced malondialdehyde formation activated the production of IL-18, TNF- α and ICAM-1, which, in turn, activated proapoptotic pathways in diabetic rats. Treatment with NECA protected diabetic rats by exerting hypoglycemic and antioxidant effects as well as reducing gene expression of proinflammatory cytokines. These effects were associated with deactivation of JNK-MAPK. In addition, diabetic rats treated with NECA showed mild glomerular effects and vacuolation of tubular epithelium. We can conclude that activation of adenosine receptors is a potential therapeutic target in DN. NECA acts via multiple mechanisms including: reducing diabetes-induced oxidative stress, inhibiting gene expression of IL-18, TNF- α and ICAM-1, and blocking activation of the JNK-MAPK pathway.

Key words: diabetic nephropathy, 5'-(N-ethylcarboxamido)-adenosine, oxidative stress, IL-18, I-CAM, TNF- α , JNK

Diabetic nephropathy (DN) is defined as a progressive increase in urinary albumin excretion, coupled with increasing blood pressure, leading to declined glomerular filtration and eventually end-stage renal failure. DN remains the most common cause of end-stage renal disease. It is found that about 25-40% of type 1 or type 2 diabetic patients develop DN within 20-25 year of the onset of diabetes [1].

Hyperglycemia and hypertension, systemic and/or intraglomerular, are established causal factors for DN. Nonetheless, there is growing evidence that activated innate immunity and inflammation are factors that contribute to the pathogenesis of DN. Therefore, diverse cells, including leukocytes, monocytes and macrophages, as well as other molecules such as chemokines, adhesion molecules, enzymes such as cyclo-oxygenase-2 and nitric oxide synthase, growth factors and nuclear factors such as nuclear factor (NF) κ B are implicated in processes related to DN [2]. Many studies supporting the contribution of inflammation in diabetes involve immunosuppressive

strategies that reduce renal macrophage accumulation and attenuate the development of DN [3, 4]. However, the stimulus for the inflammation in diabetes is still under investigation.

Cytokines are secreted proteins that exert a myriad of effects including regulation and determination of the nature of immune responses, trafficking of immune cells and cellular arrangements in immune organs [5]. Cytokines are produced following an immune stimulus; in turn, they regulate proliferation of immune cells and their differentiation. Distinct from their role as mediators of immunological reactions and inflammatory processes, inflammatory cytokines have been associated with significant renal effects, which play a major role in the development of renal injury in diabetes. These renal effects are related to the expression of a variety of molecules, intraglomerular hemodynamic abnormalities, alteration of the extracellular matrix and glomerular basement membranes, apoptosis and necrosis, endothelial permeability and oxidative stress [6].

Adenosine is an endogenous purine nucleoside released from various tissues and organs. It controls the supply and demand of energy and modulates a variety of physiological responses. It is a potent, autocrine, anti-inflammatory and immunosuppressive molecule that is released from cells into the extracellular space at sites of inflammation and tissue injury [7]. It acts as a local modulator with a generally cytoprotective function by increasing the ratio of oxygen supply to demand, protecting against ischemic damage by cell conditioning, triggering anti-inflammatory responses, and the promotion of angiogenesis [8].

Higher levels of endogenous adenosine do not exert pharmacological effects because extracellular adenosine usually disappears quickly due to its rapid uptake into adjacent cells, such as erythrocytes and endothelial cells, and subsequent metabolism. Therefore, we used NECA, an adenosine analog, which is 22,900 times more potent than adenosine as a vasodilator [9]. We investigated the effect of NECA on the diabetes-induced increase in inflammatory cytokines and adhesion molecules with the subsequent development of renal injury.

MATERIALS AND METHODS

Animals and their treatment outlines

The animal protocol was approved by the ethical committee of the Faculty of Pharmacy, University of Mansoura. Sixty, adult male, Sprague Dawley rats weighing 180-230 g were used. They were purchased from and kept in the Urology and Nephrology Center, Mansoura University, Egypt. All animals in the study were maintained under standard conditions of temperature, about 25°C, with a regular 12-hour light/12-hour dark cycle and allowed free access to food and water. Rats were fed with standard rat food. They were classified into the following groups with 12 rats in each group, apart from the diabetic rats group, which contained 24 rats (*table 1*):

Control group: rats received 0.5% of CMC in citrate buffer (0.01 mol/L, pH 4.5) by i.p. injection and served as the negative control group throughout the study.

Treated control group: rats received a daily i.p. injection of NECA at 0.3 mg/kg/day in citrate buffer (0.01 mol/L, pH 4.4) for two weeks; this served as the treated, control group.

Diabetic group: rats were rendered diabetic by a single i.p. injection of 50 mg/kg of streptozotocin (STZ) (Sigma Aldrich Chemicals Co., St. Louis, MO, USA) after fasting for 12 hours. STZ was freshly dissolved in citrate buf-

fer (0.01 mol/L, pH 4.4) and immediately injected into rats. After 48 hours, tail vein blood glucose levels were measured using a glucometer (OneTouch® System, LifeScan, USA). Rats with blood glucose levels greater than 250 mg/dl were considered diabetic and were kept for six and eight weeks for further studies.

NECA-treated group: after six weeks of induction of diabetes in rats, they received a daily, i.p. injection of 0.3 mg/kg/day of NECA in citrate buffer (0.01 mol/L, pH 4.4) for two weeks. The doses and time course of the experiments used for NECA were within the range of those used in other studies [10]. In addition, the dose was determined after appropriate preliminary experiments.

Collection of samples

At the end of the study period, rats in each group were weighed and individually housed in metabolic cages (Nalgene; Nalge Company, Rochester, NY, USA) for 24h urine collection and continued to have free access to water and food. Total urine volume was measured. The animals were sacrificed by decapitation. Rat blood was collected and centrifuged at 3,000 rpm for five minutes and serum samples were separated and stored at -20°C. Rat kidneys were removed and weighed. The right kidney was fixed in 10% buffered formalin for subsequent morphological analysis and the left one was immediately immersed in liquid nitrogen and stored at -80°C for further analyses.

Measuring renal glomerular function

This was estimated through the following parameters.

- **Serum creatinine:** creatinine was measured in rat serum kinetically [11]. A kit from Dp International Co. was used.
- **Blood urea nitrogen (BUN):** urea was measured enzymatically in rat serum using a kit purchased from Stanbio Co., according to the reported procedure of Tabacco [12].
- **Urinary albumin excretion:** an aliquot of urine was collected from the 24h urine sample, and used for determination of microalbuminuria according to the method of Schosinsky [13] using a kit from ABC Diagnostics.

Biochemical analyses in renal tissue

Renal tissue preparation: renal tissue homogenate was prepared according to de Cavanagh [14]. One hundred milligrams of renal tissues and four volumes of 120 mM potassium chloride, 30 mM potassium phosphate, pH 7.4 buffer were sonicated for one minute and then centrifuged at 600g, at 4°C, for 10 minutes. The supernatant was referred to as homogenate and stored at -80°C until used.

Table 1
Effects of streptozotocin-induced diabetes on body weight and blood glucose levels in rat groups (Mean \pm SE):

Group	Number of rats	Start weight	End weight	Blood glucose level
Control	12	194.3 \pm 4.2	249.1 \pm 2.5	127.8 \pm 6.4
Control+NECA	12	192.4 \pm 5.1	239.6 \pm 3.9	121.1 \pm 5.3
Diabetes (6 wks)	12	197.8 \pm 4.7	183.1 \pm 0.7	480.3 \pm 15.9*
Diabetes (8 wks)	12	197.3 \pm 5.3	175.6 \pm 1.8	496 \pm 20.5*
Diabetes+NECA	12	196 \pm 5.6	206.8 \pm 3.1	367.5 \pm 7.6#*

* Significant difference as compared with the control groups at $p < 0.05$

Significant difference as compared with the diabetic groups at $p < 0.05$

Lipid peroxides, measured as thiobarbituric acid reactive species (TBARs): renal homogenate levels of TBARs, mainly malondialdehyde (MDA), were determined according to the reported method of Satoh [15], using a kit from Biodiagnostic Company. MDA was assayed on the same day.

Cell activation of pro-apoptotic pathways, measured as pJNK/JNK MAPK: renal tissue activation of JNK MAPK, measured as the ratio of pJNK/JNK, was determined according to Fleming [16], using a commercially available ELISA kit from RayBio® Company.

Morphological analysis of renal tissue

The kidney was cut longitudinally; one half was fixed in 10% buffered formalin and embedded in paraffin. Five micrometer-thickness sections were cut and stained with Mayer's hematoxylin and eosin (H&E) for examination of cell structure by light microscope [17]. Renal specimens were anonymously coded and examined in a blinded manner. The morphological changes were photographed using a digital camera-aided computer system (Nikon digital camera, Japan).

Quantitative, real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from euthanized rat kidney using a RNeasy Mini kit (Qiagen, USA). The yield and quality of total RNA were determined spectrophotometrically using an absorbance of a 260 and 260/280 nm ratio, respectively. The amount of RNA was quantified using a Maxima® SYBR Green/Fluorescein qPCR Master Mix (Fermentas, USA). One microgram of total RNA was reverse-transcribed into single-stranded complementary DNA (cDNA) using QuantiTect® Reverse Transcription Kit (Qiagen, USA) with a random primer hexamer in a two-step RT-PCR reaction in which any genomic DNA contamination was first eliminated using gDNA wipeout buffer. The ICAM-1, TNF- α and IL-18 mRNA levels in different rat kidney tissues were determined using Maxima® SYBR Green/Fluorescein qPCR Master Mix by Rotor-Gene Q (Qiagen, USA). Meanwhile, rat GAPDH was used as a housekeeping gene and an internal reference control. Gene-specific PCR primers (table 2) were designed using Primer Express 3.0 (Applied Biosystems, USA) according to the nucleotide sequence obtained from the Gene Bank. Thermal cycling conditions included an initial activation step at 95°C for 10 min followed by 40-50 cycles

of 94°C for 15 seconds, 55°C or 58°C for 30 seconds and 72°C for one minute. Data acquisition was performed during the extension step. Melting curve analysis of the PCR product(s) was performed to verify their identity and specificity. Rotor-Gene Q (Qiagen, USA) collected data automatically and analyzed the value of Threshold Cycle (Ct). Rat ICAM-1, TNF- α , IL-18 and GAPDH mRNA relative expression was determined by using the $2^{-\Delta\Delta Ct}$ method. PCR products were confirmed by 1.2% agarose gel electrophoresis.

Data management and statistical analysis

For descriptive statistics, the frequency and percentage were calculated for qualitative variables, the mean values \pm standard error and range were used for quantitative variables. For comparison between two groups Student's t-test was used. Statistical computations were performed on a personal computer using the computer software SPSS version 13 (Chicago, IL, USA). Statistical significance was predefined as $P \leq 0.05$.

RESULTS

NECA reduced diabetes-induced oxidative damage in rat renal tissue

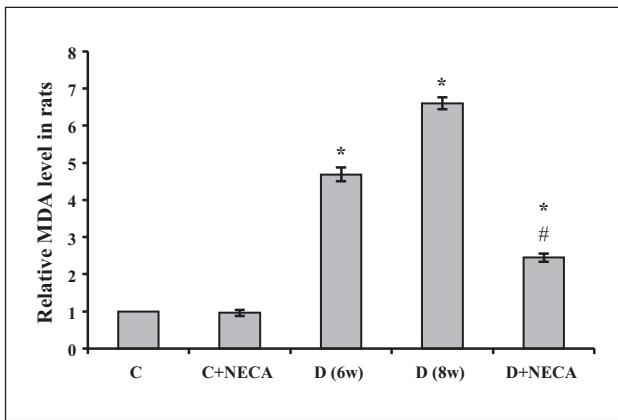
Chronic hyperglycemia in diabetes leads to free radical overproduction, which contributes to the development of diabetic nephropathy [18-20]. The present study investigated the effects of NECA, an adenosine analog, on diabetes-induced, oxidative renal damage. Statistical analysis of spectrophotometric measurement of MDA, a lipid peroxide product, in rat kidney tissue showed 4.7- and 6.5-fold increases in MDA formation over six and eight weeks respectively, in diabetic rats, as compared with the control group. Daily i.p. injection of NECA at 0.3 mg/kg/day for two weeks reduced MDA levels in diabetic rats, but did not affect control rats (figure 1).

NECA blocked diabetes-induced gene expression of inflammatory mediators in rat renal tissue

DN is increasingly considered to be an inflammatory process characterized by leukocyte infiltration at every stage of renal involvement. [21-23]. In addition, increased oxidative stress can stimulate the formation of pro-inflammatory cytokines [24]. Therefore, we aimed to measure the effect

Table 2
The primer sets used for the detection of rat GAPDH, ICAM-1, IL-18 and TNF- α .

Name	Sequence	Reference sequence	Amplicon size	Annealing temperature
Rat GAPDH 165 F	5'- CCATCAACGACCCCTTCATT-3'	NM_017008.3	193	58°C
Rat GAPDH 358 R	5'- CACGACATACTCAGCACCAGC -3'			
Rat ICAM-1 1396F	5'- GTGAAGTCTGTCAAACGGGAG -3'	NM_012967.1	152	55°C
Rat ICAM-1 1548R	5'- CGCAATGATCAGTACCAACAC -3'			
Rat IL-18 175F	5'- GACAAAAGAAACCCGCCTG -3'	NM_019165.1	147	55°C
Rat IL-18 322R	5'- ACATCCTTCCATCCTTCACAG -3'			
Rat TNF- α 318F	5'- CAACAAGGAGGAGAAGTTCC-3'	NM_012675.3	255	55°C
Rat TNF- α 573R	5'- GAAGAGAACCTGGAGTAGATAAG-3'			

**Figure 1**

NECA reduced diabetes-induced oxidative damage in rat renal tissue. Statistical analysis of MDA, a lipid peroxide product, in rat renal tissue showing about 4.7- and 6.5-fold increases in MDA formation after six and eight weeks in diabetic rats, respectively as compared with the control group. Daily treatment with NECA (0.3 mg/kg/day, i.p. for two weeks) reduced MDA levels in diabetic rats, but did not affect control rats. * Significant difference as compared with the control groups at $p<0.05$.

Significant difference as compared with the diabetic groups at $p<0.05$. C: control; D: diabetic; MDA: malondialdehyde; NECA: 5'-*N*-ethylcarboxamido)-adenosine.

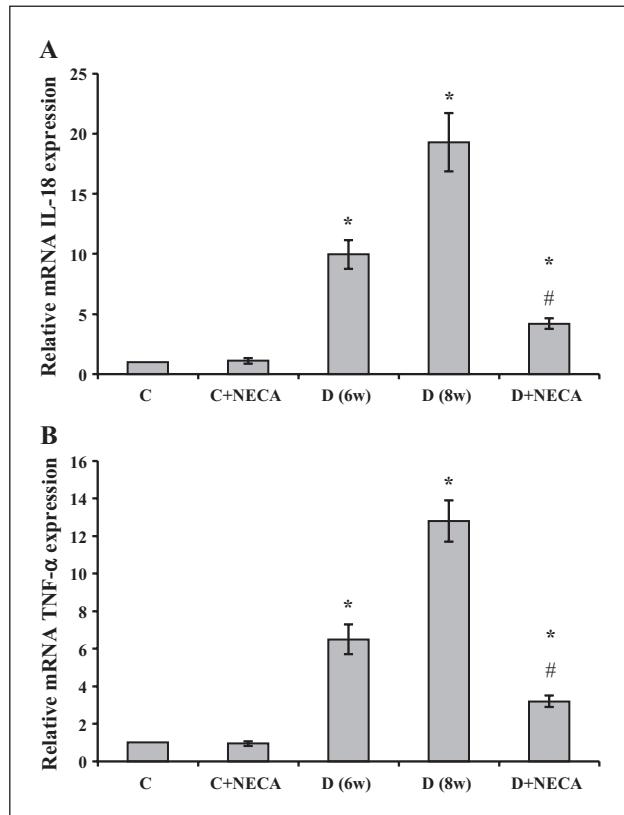
of NECA on diabetes-induced production of TNF- α and IL-18. We found 10- and 19-fold increases in the gene expression of IL-18 after six and eight weeks respectively, in diabetic rat renal tissue, as compared with the control group. In addition, 6- and 13-fold increases in gene expression of TNF- α were found after six and eight weeks respectively, in diabetic rat renal tissue. Daily treatment with NECA (0.3 mg/kg/day, i.p. for two weeks) reduced diabetes-induced gene expression of TNF- α and IL-18 in diabetic rats, but did not affect control rats (figure 2).

NECA blocked diabetes-induced gene expression of intracellular adhesion molecule in rat renal tissue

Experimental and clinical evidence has consistently demonstrated that renal macrophage infiltration is one of the most important events for the progression of DN [25-27]. However, the purpose of this study was to investigate whether the reno-protective effect of NECA is through suppression of renal expression of ICAM-1. As shown in figure 3, rat renal tissues showed 3.4- and 6.6-fold increases in the gene expression of ICAM-1 after six and eight weeks respectively, in diabetic rats, as compared with the control group. Daily i.p. injection of NECA at 0.3 mg/kg/day for two weeks blocked the increase in gene expression of ICAM-1 in diabetic rats, but did not affect control rats.

NECA inhibited diabetes-induced activation of the JNK MAPK pathway in rat renal tissue

Oxidative stress has been suggested to play a role as a common mediator of apoptosis and kidney damage in diabetes [28]. Therefore, we investigated the activation of the JNK MAPK apoptotic pathway in diabetic rat renal tissue, and the effect of NECA on apoptosis. Statistical analysis of rat renal tissue showed 1.6- and 1.8-fold elevations in the activation of JNK MAPK after six and eight weeks respectively, in diabetic rats, compared with the control group. Daily i.p. injection of NECA at 0.3 mg/kg/day for two

**Figure 2**

NECA blocked diabetes-induced gene expression of inflammatory mediators in rat renal tissue.

A) Statistical analysis showing 10 and 19-fold increases in the gene expression of IL-18 after six and eight week in diabetic rat renal tissue, respectively as compared with the control group. **B**) Statistical analysis showing 6- and 13-fold increases in the gene expression of TNF- α after six and eight weeks in diabetic rat renal tissue, respectively as compared with the control group. Daily i.p. injection of NECA at 0.3 mg/kg/day for two weeks blocked the increase in gene expression of IL-18 and TNF- α in diabetic rats, but did not affect control rats.

* Significant difference as compared with the control groups at $p<0.05$. # Significant difference as compared with the diabetic groups at $p<0.05$. C: control; D: diabetic; NECA: 5'-*N*-ethylcarboxamido)-adenosine; IL-18: interleukin 18; TNF- α : tumor necrosis factor- α .

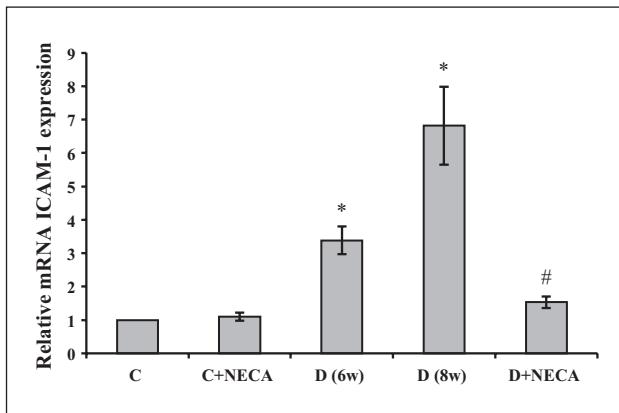
weeks blocked the activation of JNK MAPK in diabetic rats, but did not affect control rats (figure 4).

NECA reduced diabetes-induced impairment of renal glomerular function in rats

Next we evaluated the effect of NECA on renal glomerular function in diabetic rats. Daily i.p. injection of NECA at 0.3 mg/kg/day for two weeks reduced the diabetes-induced increases in albuminuria and serum levels of urea nitrogen and creatinine (figure 5).

NECA prevents diabetes-induced renal cell damage

The reno-protective effect of NECA was examined in kidney sections stained with H&E. As shown in (figure 6), sections of kidney from diabetic rats showed focal glomerulosclerosis with marked shrinkage of some glomerular tufts and tubular vacuolation with hyaline droplets. On the other hand, rats receiving 0.3 mg/kg/day of NECA i.p. for two weeks after six weeks of induction of diabetes showed mild glomerular impairment and vacuolation of tubular epithelium.

**Figure 3**

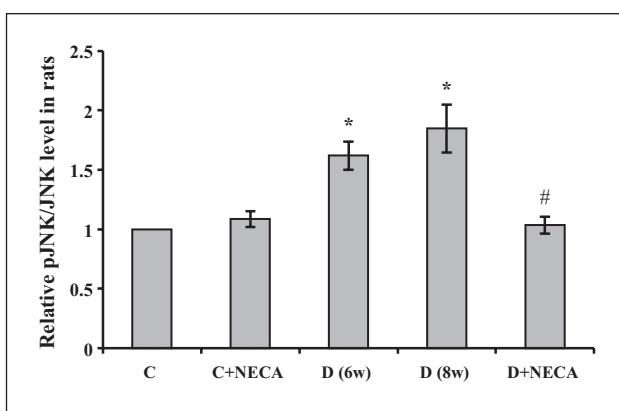
NECA blocked diabetes-induced gene expression of ICAM-1 in rat renal tissue.

Statistical analysis of rat renal tissues showing 3.4- and 6.6-fold increases in the gene expression of ICAM-1 after six and eight weeks in diabetic rats, respectively as compared with the control group. Daily i.p. injection of NECA at 0.3 mg/kg/day for two weeks blocked the increase in gene expression of ICAM-1 in diabetic rats, but did not affect control rats. * Significant difference as compared with the control groups at $p < 0.05$.

Significant difference as compared with the diabetic groups at $p < 0.05$. C: control; D: diabetic; NECA: 5'-*(N*-ethylcarboxamido)-adenosine; ICAM: intracellular adhesion molecule.

DISCUSSION

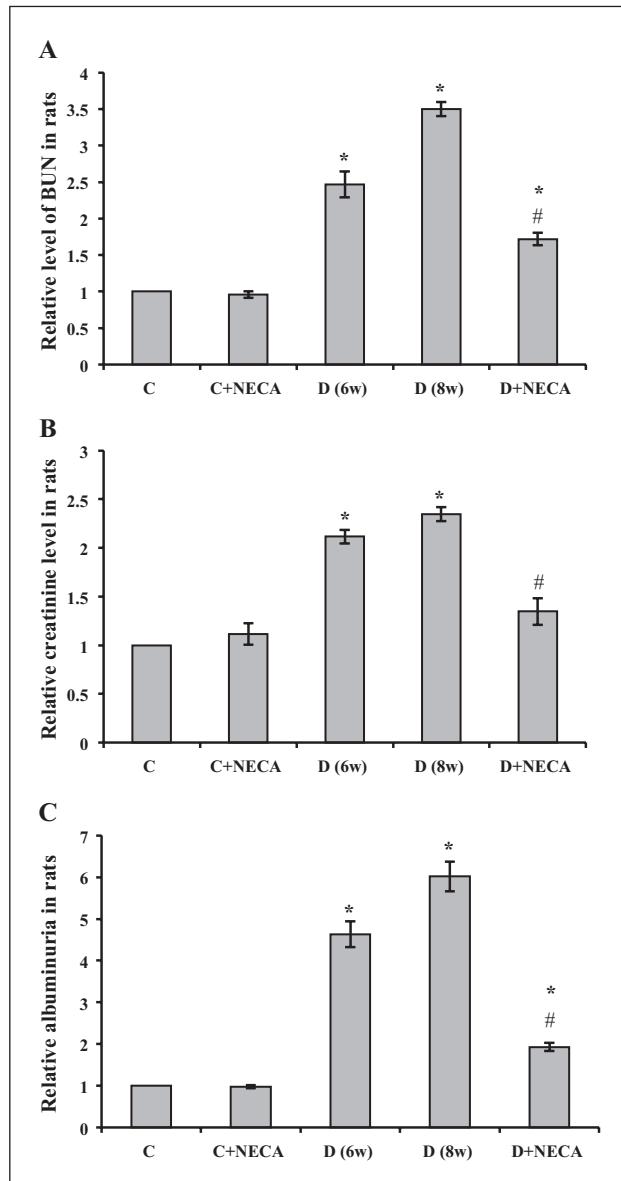
The main findings of the current study are that NECA, an adenosine analog, inhibits the impairment of renal glomerular function in diabetic rats via multiple mechanisms including: (1) reducing diabetes-induced oxidative stress, as indicated by inhibition of MDA in rat renal tissue; (2) inhibiting diabetes-induced, pro-inflammatory cytokine expression as demonstrated by the reduction of gene expression of TNF- α and IL-18; (3) blocking diabetes-induced gene expression of ICAM-1 in rat renal tissue and

**Figure 4**

NECA inhibited diabetes-induced activation of the JNK MAPK pathway in rat renal tissue.

Statistical analysis of rat renal tissue showing 1.6- and 1.8-fold increases in the activation of JNK MAPK after six and eight weeks in diabetic rats, respectively compared with the control group. Daily i.p. injection of NECA at 0.3 mg/kg/day for two weeks blocked the activation of JNK MAPK in diabetic rats, but did not affect control rats.

* Significant difference as compared with the control groups at $p < 0.05$. # Significant difference as compared with the diabetic groups at $p < 0.05$. C: control; D: diabetic; NECA: 5'-*(N*-ethylcarboxamido)-adenosine; JNK MAPK: c-Jun NH₂-terminal kinases mitogen-activated protein kinase.

**Figure 5**

NECA reduced diabetes-induced impairment of renal glomerular function in rats.

A) Statistical analysis showing 2.5- and 3.5-fold increases in BUN after six and eight weeks in diabetic rats, respectively compared with the control group. **B**) Statistical analysis showing 2.1- and 2.3-fold increases in serum creatinine in diabetic rats compared with the control group. **C**) Statistical analysis showing 4.6- and 6-fold increases in albuminuria in diabetic rats as compared with the control group. Daily treatment with NECA (0.3 mg/kg/day, i.p. for two weeks) reduced diabetes-induced impairment of renal glomerular function in diabetic rats, but did not affect control rats.

* Significant difference as compared with the control groups at $p < 0.05$. # Significant difference as compared with the diabetic groups at $p < 0.05$. C: control; D: diabetic; NECA: 5'-*(N*-ethylcarboxamido)-adenosine; BUN: serum urea nitrogen.

(4) blocking diabetes-induced activation of the proapoptotic JNK MAPK pathway. To the best of our knowledge, our study demonstrates, for the first time, a reno-protective role of NECA in rats with streptozotocin-induced diabetes. Despite the benefits derived from current treatments for diabetic nephropathy, these strategies provide less than perfect protection against renal damage. This highlights the need for new therapeutic agents that have the potential to influence the primary mechanisms contributing to the pathogenesis of diabetic nephropathy (DN). Under

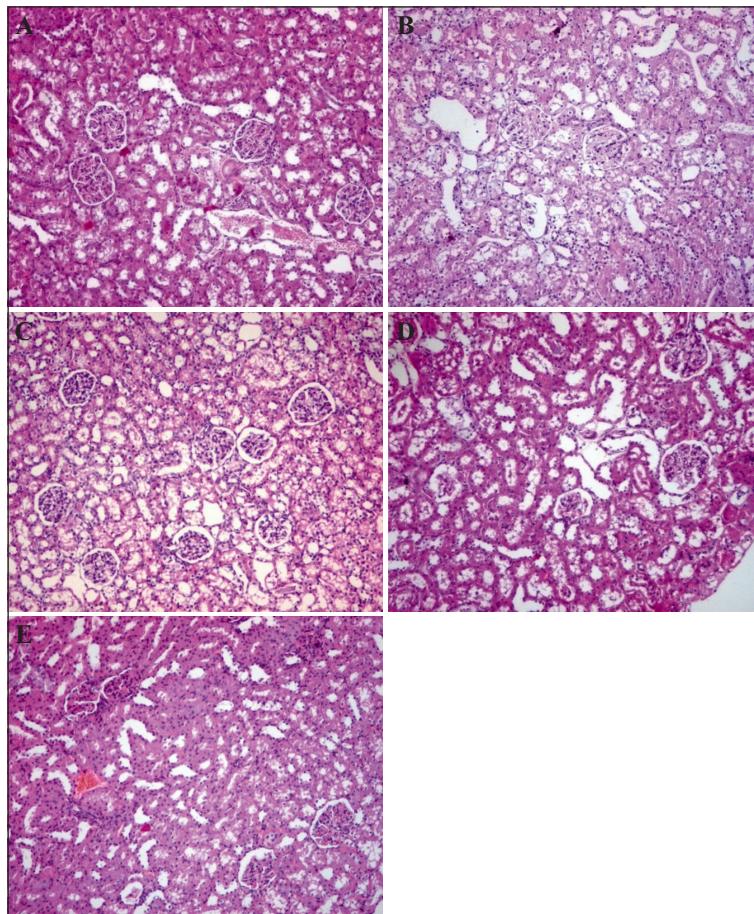


Figure 6

NECA prevents diabetes-induced renal damage.

A) Section of a control rat kidney stained with H&E showing normal glomerular tuft and tubules (100 \times magnification). **B**) Section of a control rat kidney treated with NECA (0.3 mg/kg/day, i.p. for two weeks) showing normal glomerular tuft and tubules (100 \times magnification). **C**) Section of a rat kidney after six weeks of diabetes showing focal glomerulosclerosis with marked shrinkage of some glomerular tufts. The tubules show vacuolation and hyaline droplets (100 \times magnification). **D**) Section of a rat kidney after eight weeks of diabetes showing marked glomerulosclerosis and shrinkage and tubular vacuolation (100 \times magnification). **E**) Section of rat kidney receiving 0.3 mg/kg/day i.p. of NECA for two weeks following six weeks of induction of diabetes showing mild glomerular affection and vacuolation of tubular epithelium (100 \times magnification).

several adverse conditions, including ischemia, hypoxia, trauma, stress, seizures, pain and inflammation, adenosine production is increased as a result of an increased demand for energy as supplied by ATP. The increased extracellular adenosine protects against excessive tissue damage or organ dysfunction by interacting with adenosine receptors [29]. However, the increase in endogenous adenosine levels is insufficient to exert pharmacological effects because extracellular adenosine usually disappears quickly due to its rapid uptake into adjacent cells, such as erythrocytes and endothelial cells, and subsequent metabolism. In fact, adenosine added to whole blood is taken up, with a half-life of less than 30 seconds, and disappears within one minute [30]. Therefore, NECA, a stable adenosine analog and nonselective receptor agonist, is 22,900 times more potent than adenosine as a vasodilator. NECA is agonist-specific for adenosine receptors, but has, however, little selectivity for the different receptor subtypes. The order of potency of NECA for the different adenosine receptor subtypes is (A1 \geq A3>A2A>A2B), which resembles that of adenosine, and for this reason NECA is a useful analog for studying adenosine receptors [9].

We found that NECA ameliorated the course of hyperglycemia and diabetes mellitus symptoms, as administration of 0.3 mg/kg/day for two weeks in diabetic rats resulted in

a 26% reduction in blood glucose levels as compared with diabetic rats after eight weeks. In addition, body weight loss, an index of diabetes mellitus, was improved in treated diabetic rats. The fact that NECA decreased the expression of several proinflammatory cytokines *in vivo* may explain the efficacy of this agent in improving the course of diabetes. It had been found previously that NECA can attenuate hyperglycemia in STZ-induced diabetes in rats [31] and mice [10]. It is less likely that NECA prevented the development of diabetes by directly protecting β -cells via adenosine receptor stimulation on these cells, because NECA failed to prevent cytokine-induced β -cell death *in vitro* [10]. In addition, our results indicate that NECA had no effect on blood glucose levels in non-diabetic rats.

Reactive oxygen species (ROS) are a primary suspect in the development of DN. Chronic hyperglycemia in diabetes leads to free radical overproduction, which contributes to the development of DN [19, 20]. We found that NECA reduced the increase in MDA production in diabetic rats and did not affect control rats. Oxygen derivatives, acting as second messengers, activate transcription factors such as NF κ B, leading to the transcription of genes encoding cytokines [32]. Expression of NF κ B is increased in kidneys of experimental diabetic animals [33]. In addition, enhanced macrophage migration in diabetes induces the

release of inflammatory and pro-fibrotic cytokines, which in turn stimulates further ROS production [34]. Therefore, oxidative stress-induced cytokine production is likely to further increase oxidative stress levels setting up a vicious cycle.

Moreover, proteinuria is intimately related to inflammation. Briefly, proteinuria induces activation of renal tubular cells, which leads to expression of numerous chemokines, adhesion molecules and proinflammatory cytokines. In turn, these responses result in interstitial infiltration by monocytes, neutrophils and lymphocytes, which then contribute to renal cell injury, tubulointerstitial damage and fibrosis [35]. Of note, we found a significant increase in gene expression of IL-18 in diabetic rats. IL-18 is constitutively expressed in renal tubular epithelia, and recent studies demonstrated that infiltrating monocytes, macrophages and T cells, along with proximal tubular cells, are potential sources of this cytokine [36]. Moreover, elevated levels of IL-18 are a determinant of early renal dysfunction in patients with Type 2 DM [22, 37]. Indeed, IL-18 has been proposed to have a contributory role specific to the progression of DN, rather than other diabetic complications [38]. In addition, we found a significant increase in the gene expression of TNF- α in diabetic rats. The increase in renal TNF- α production in diabetes appears to be related to hyperglycemia [39]. Reported effects of TNF- α on renal cells include activation of second messenger systems, transcription factors, synthesis of cytokines, growth factors, receptors, cell adhesion molecules and enzymes involved in the synthesis of other inflammatory mediators (for review [2]). The variety of the biological activities of TNF- α results in diverse effects including a significant role in the development of renal damage in diabetes. In addition, other relevant TNF- α effects have been reported, such as induction of apoptosis and necrotic cell death [40], alterations of intraglomerular blood flow and glomerular filtration rate, as well as alterations of endothelial permeability [41]. However, daily i.p. injection of NECA at 0.3 mg/kg/day for two weeks blocked the increase in gene expression of TNF- α and IL-18 in diabetic rats, but did not affect control rats.

TNF- α and IL-18 induced production of other inflammatory cytokines, upregulation of ICAM-1, and apoptosis of endothelial cells [42]. ICAM-1 is a cell-surface protein with five immunoglobulin-like domains. ICAM-1 production is induced by inflammatory cytokines such as TNF- α , interleukin-1 and interferon- γ [43]. The expression of adhesion molecules including ICAM-1 constitutes the basis for leukocyte migration into endothelial cells preceding endothelial inflammation [27]. We found increased renal expression of ICAM-1 in diabetic rats during diabetic renal impairment. In diabetes, the induction of adhesion molecules is one of the first steps in hyperglycemia-mediated endothelial dysfunction. Increased expression of ICAM-1 in diabetic renal tissue may be induced by inflammatory cytokines [43] or oxidative stress [44]. Of note, we found that NECA reduced the expression of ICAM-1 in diabetic rats, which may be attributed to the ability of NECA to reduce levels of oxidative stress and proinflammatory cytokines.

MAPK cascades comprise one of the major signaling systems by which cells transduce and integrate diverse intracellular signals. The three subfamilies of MAPK are

the extracellular signal-regulated kinases (ERKs), the p38 MAPKs and the JNK. ERKs are activated primarily in response to proliferated stimuli, whereas the other MAPKs are activated primarily in response to inflammatory and stress-ful stimuli [45]. Oxidative stress has been suggested to play a role as a common mediator of cell activation and apoptosis, and kidney damage in diabetes [28, 46]. Although, oxidative stress is the most notable JNK activator, JNK MAPK can also be activated by hyperglycemia [45], cytokines and adhesion molecules [45, 47]. The activation of JNK has been shown to be involved in inflammation in tissues in diabetes, and in some proinflammatory responses of mesangial cells [48]. Indeed, treatment of diabetic rats with NECA reduced the activation of the JNK MAPK pathway. Next, we examined the reno-protective effect of NECA. We found that daily i.p. injection of NECA at 0.3 mg/kg/day for two weeks, blocked diabetes-induced impairment of renal glomerular function in rats as indicated by a reduction in albuminuria and serum levels of urea nitrogen and creatinine. In addition, morphological examination revealed that NECA attenuated diabetes-induced glomerular impairment and vacuolation of tubular epithelium. The reno-protective effect of NECA might possibly be attributed to its ability to reduce hyperglycemia in diabetic rats, nevertheless we found that NECA strongly inhibited oxidative stress and suppressed expression of the proinflammatory cytokines, such as IL-18 and ICAM-1 in diabetic rats, as well as deactivating the JNK MAPK apoptotic pathway. Moreover, it has been found previously that NECA suppressed the expression of TNF- α , MIP-1 MIP-1 α , IL-12 and IFN- γ in endotoxin-, or anti-CD3-stimulated splenic cells, and T helper 1 lymphocytes [10]. Therefore, activation of adenosine receptors is a potential therapeutic target in DN. To the best of our knowledge, our study demonstrates for the first time, a reno-protection role for NECA in STZ-induced diabetes in rats.

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