The effects of A_{2B} receptor modulators on vascular endothelial growth factor and nitric oxide axis in chronic cyclosporine nephropathy

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ABSTRACT

Introduction: To investigate the actions of adenosine A_{2B} receptor modulators on VEGF and NO levels in CsA nephropathy. **Materials and Methods:** Nephropathy was induced by administrating 25 mg/kg (s.c) of CsA for 5 weeks. The VEGF and NO levels were measured in kidney tissue. Serum creatinine, creatinine clearance, urinary albumin excretion, blood urea nitrogen, kidney pathology score were measured to assess renal function. The analysis of mRNA expression of A_{2B} receptor and VEGF was performed. **Results:** Administration of CsA for 5 weeks induced adverse renal function. The mRNA expression of VEGF was reduced in renal tissue after 5 weeks of CsA treatment. The renal VEGF and NO levels were also reduced in these animals. *In vivo* administration of A_{2B} adenosine receptor agonist increased renal VEGF which was inhibited by a selective A_{2B}AR antagonist (MRS1754) in CsA-treated animals. The increase in VEGF was associated with reversal of adverse renal functions. The effects of A_{2B}AR modulators were prominent in CsA-treated animals compared with control animals suggesting CsA treatment may upregulate A_{2B}ARs. The mRNA expression of A_{2B}AR was increased after 5 weeks of CsA. **Conclusions:** A_{2B}AR modulators may provide new therapeutic options to retard CsA nephropathy by mediating renal VEGF and NO.

Key words: A_{2B} adenosine receptor, CsA nephropathy, nitric oxide, VEGF

INTRODUCTION

Cyclosporine A (CsA) is a potent immunosuppressive agent with definite efficacy to prevent organ allograft rejection. However, CsA causes significant nephrotoxicity that might contribute to long-term kidney graft loss.[1] Acute CsA nephrotoxicity is characterized by renal vasoconstriction, which is dose-related and reversible with dose reduction. In contrast, chronic CsA nephrotoxicity is progressive and irreversible; the histological lesion includes afferent arteriolar hyalinosis, tubular atrophy, and striped interstitial fibrosis with mononuclear infiltration.[2]

A line of evidence has demonstrated alteration in vascular endothelial growth factor (VEGF) and nitric oxide (NO) in chronic nephropathy.[3-5] VEGF is an endothelial cell mitogen that increases angiogenesis and vascular permeability. Endogenous VEGF has a relevant role in the renal tubular defense against CsA toxicity. Blockade of the VEGF by α-VEGF results in intensification of the tubular injury and

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appearance of regenerative anemia in the CsA nephropathy.[6] The occurrence of both *in-vivo* and *in-vitro* effects of VEGF blockade provides evidence of a direct protective effect of VEGF on the tubular cell. The protective actions of VEGF in renal disease have been attributed to its ability to stimulate NO production in endothelial cells.[7] In the late phase of CsA nephropathy, eNOS (NO synthase) activation is reduced.[8] Numerous studies point to a critical role of NO in mediating the effects of VEGF on angiogenesis, vascular permeability, and blood pressure regulation.[7,9] The VEGF and NO interaction has been explained as a critical event in causing paradoxical effects of VEGF in renal diseases.[10]

One of the important stimulants of VEGF is hypoxia. Chronic nephropathy has been found to be associated with hypoxia. A purine nucleoside adenosine is a critical mediator released in extracellular space during hypoxia. It interacts with cell surface adenosine receptors (AR). Presently, four subtypes of ARs exist, designated A_1, A_{2A}, A_{2B}, and A_3. A_{2B}ARs regulate various pathological processes, one of which is angiogenesis. A_{2B}ARs induce angiogenesis via VEGF in different tissues.[11] A_{2B}ARs also protect kidney from ischemia.[12] A_{2B}ARs have been known to mediate NO release in various pathological settings.[13,14] The effects of A_{2B}AR modulation on the VEGF–NO axis in diabetic nephropathy have been recently studied.[15]

However, it is necessary to determine whether or not A_{2B}AR modulators affect VEGF and NO in chronic nephropathy induced by CsA. Accordingly, it was hypothesized that A_{2B}AR agonists induce expression of key angiogenic factors such as VEGF in CsA-induced chronic nephropathy. Such an increase in renal VEGF expression and NO by A_{2B}AR activators may initiate the angiogenic response at the site of renal injury. The present study was designed to investigate whether A_{2B}AR modulators may produce a favorable change in VEGF and NO and improve adverse renal functions in CsA nephropathy.

MATERIALS AND METHODS

Animal model and experimental protocol

All animal experiments were conducted in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiment on Animals, India. Male C57BL/6 mice (body weight: 22 ± 2 g) were a kind gift from Zydus Research Centre, Ahmedabad. Animals were maintained at room temperature in a light (12 h light/12 h dark)-controlled environment with access to food and water ad libitum. One week after the acclimatization animals were divided into six different treatment groups (with two main subgroups: Control and CsA treated) as depicted in Table 1. A total of three groups (CsA treated) of animals were subjected to and maintained on low-salt diet throughout the trial. The salt depletion has been reported to enhance sensitivity to CsA-induced nephrotoxicity. After 1 week

on a low-salt diet, the mice were injected daily with 25 mg/kg/day CsA subcutaneously for 5 weeks. The olive oil was used as vehicle for CsA; hence, in the vehicle control group, animals were injected equivalent amount of olive oil. Following 5 weeks, the A_{2B}AR modulators were administered intraperitoneally for 2 weeks. The dose of MRS1754 (Abcam plc. UK) was 1 mg/kg. The dose of NECA (Abcam plc.UK) was 50 µg/kg of body weight. The control groups of animals were also treated with A_{2B}AR modulators.

Collection of urine, blood and tissue samples

Metabolic cages were used to collect 24-hour urine samples. Blood was drawn from the retro orbital tract. The food-derived creatinine clearance was avoided by setting up the metabolic cages between 15:00 and 16:00. At the end of treatment all the animals were sacrificed and whole kidneys were removed. The kidneys were weighed and frozen in liquid nitrogen in RNA later™ for the isolation of total RNA. The samples were stored at –80°C until used for biochemical analysis.

Renal function parameters

Plasma and urine creatinine (modified Jaffe's kinetic method), and BUN (GLDH kinetic method) were measured by commercially available kits following manufacturer`s instructions (Crest Biosystem, Goa, India). Urine albumin was measured by bromocresol green method taking mouse albumin as standard. Urinary albumin excretion (UAE) was calculated from 24-hour urine samples. Creatinine clearance was considered according to the $U/P \times V$ principle for the matching plasma and urine samples.

Quantitative VEGF determinations

Kidney tissues were washed with cold phosphate buffered saline (PBS) and homogenized (10% w/v) in ice bath. The homogenate was then centrifuged at 20,000 rpm at 4°C. The supernatant was stored at –80°C till further analysis. VEGF was measured in plasma and homogenate by ELISA following the manufacturer's protocols (Ray Biotech Inc., Norcross, GA, USA).

Quantitative NO determinations

As an indicator of NO bioavailability, nitrite and nitrate were estimated in urine and kidney homogenate by specrofluorimetric analysis. The method was adopted from the literature and modified slightly.[16] Briefly, all the samples were treated with 10 µl of nitrate reductase (0.5 U/ml, Sigma Chemical Co., MO, USA) and 10 µl of 0.05 mM NADPH. After incubation for about 60 minutes, 20 µl of DAN (0.05 mg/ml) and 130 µl HCl (1.5N) were added. After 10 minutes, the reaction was stopped by 130 µl of NaOH (2N). The resultant solution was diluted to 2 ml and the emission scan was recorded by a spectrofluorimeter (LS 55 Fluorescence spectrometer, PerkinElmer) exciting at 365 nm and reading at 415 nm. Sodium nitrite was used as the reference standard.

Table 1: Experimental group of animals						
Groups	Control			CsA treated		
Name of group	Control (Vehicle control)	Control NECA	Control NECA + MRS	CsA	CsA + NECA	CsA + NECA + MRS
Treatment	Olive oil	NECA	NECA + MRS	CsA	CsA + NECA	CsA + NECA + MRS

N=6 in each group. NECA=An adenosine receptor agonist (5'-N-Ethylcarboxamidoadenosine).

Real time quantitative PCR for A_{2B}AR and VEGF mRNA

The RNeasy mini kit (Qiagen, Valencia, CA, USA) was used for the total RNA extraction according to the manufacturer's instructions. Reverse transcription of total RNA to cDNA was performed with the Verso cDNA synthesis kit (Thermo Scientific, ABgene, and Surrey, UK) in a DNA thermal cycler (Perkin-Elmer Applied Biosystems, Foster city, CA, USA) with random hexamers as primers. The quality of DNA and total RNA was checked by a bioanalyzer (2100 Bioanalyzer Instrument, Agilent Technologies, CA, USA). The real-time PCR was performed (7500 Fast, Applied Biosystems) using the Quanti Tect SYBR green PCR kit (Qiagen Valencia, CA, USA), with the cDNA synthesized above as a template in a reaction, following manufacturer's instructions. Specific primers used for the mouse VEGF were adopted from the earlier literature.[17,18] The gene for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference. Fold change in gene expression was calculated for each gene. The absence of nonspecific products was assured by analyzing melt curves upon completion of cycles.

Histochemistry

Kidney tissue was fixed in formalin and embedded in paraffin. The 5-μm sections were taken and stained with hematoxylin–eosin and masson trichrome reagent. Kidney pathology scores were determined using a semi-quantitative scoring system. Ten fields per kidney were examined. The histopathologic changes were assessed on the basis of interstitial infiltrates, tubular damage, thickening of arterioles, fibrosis and tubulointerstitial expansion. The semi-quantitative score was assigned by counting the percentage of the diseased area per kidney section. The score was as follows: 0, none; 1, <10% of diseased area; 2, 11% to 25% of diseased area; 3, 26% to 45% of diseased area; 4, 46% to 75% of diseased area; and 5, >76% of diseased area. The analysis was done by a blind observer.

Statistical analysis

Data are presented as the mean ± SEM (Standard Error of Mean). Statistical analysis was performed using Systat13. For comparisons of continuous variables, a test of normality was performed (Shapiro–Wilk test) prior to assessing statistical significance using either a *t*-test (parametric) or Fligner-Wolfe test (nonparametric) when comparing two groups. An association between the expressions of VEGF and kidney pathology score were analyzed by Pearson's correlation coefficient. The data were analyzed using ANOVA for a comparison between more than two groups.

RESULTS

Renal function parameters

The administration of CsA for 5 weeks adversely affected renal function parameters. Serum creatinine [Figure 1a] was increased and creatinine clearance [Figure 1b] was reduced after 5 weeks of CsA treatment. Similarly, BUN [Figure 1c] and UAE [Figure 1d] were increased in CsA-treated animals compared with control animals. Urine albumin levels in control animals were below the detection limit of the method employed for analysis. The treatment with NECA recovered BUN, serum creatinine, creatinine clearance and UAE in CsA-treated mice. MRS1754 inhibited the effects of NECA on renal function parameters, but interestingly it did not completely inhibit the action of NECA on serum creatinine clearance. Moreover, no change in renal functions was found in control animals after 2 weeks of treatment with A_{2B} receptor modulators, NECA and MRS1754.

Change in VEGF gene expression

To evaluate the impact of CsA on the expression level of VEGF in mice kidney, we examined mRNA levels in kidneys of control and CsA-treated animals. The changes in mRNA level were evaluated based on results from real-time PCR performed on cDNA transcribed from total RNA isolated from whole kidney. There was a significant reduction in the mRNA level of VEGF in kidney, 5 weeks after cyclosporine administration [Figure 2a]. Administration of NECA for 2 weeks raised the mRNA expression of VEGF in CsA-treated animals significantly, compared with control animals wherein, a partial increase was observed. The increase in VEGF mRNA expression by NECA was blocked by the treatment of MRS1754.

VEGF protein levels

VEGF protein measurement by ELISA supported results obtained by gene expression study [Figure 2b]. A comparison of VEGF protein level in kidneys of control and cyclosporine-treated mice indicated significant reduction in the level of VEGF in CsA-treated kidney. Administration of NECA for 2 weeks raised the level of VEGF in control and CsA-treated animals significantly. The rise was significantly greater in CsA-treated animals compared with control animals ($P < 0.05$). The increase in VEGF levels by NECA was blocked by the treatment with MRS1754. A_{2B} receptor modulators, NECA and MRS1754, did not produce any change in VEGF levels in control groups.

Nitrite levels

A comparison of nitrite level in kidney homogenate [Figure 3a] and urine [Figure 3b], of control and cyclosporine-treated mice,
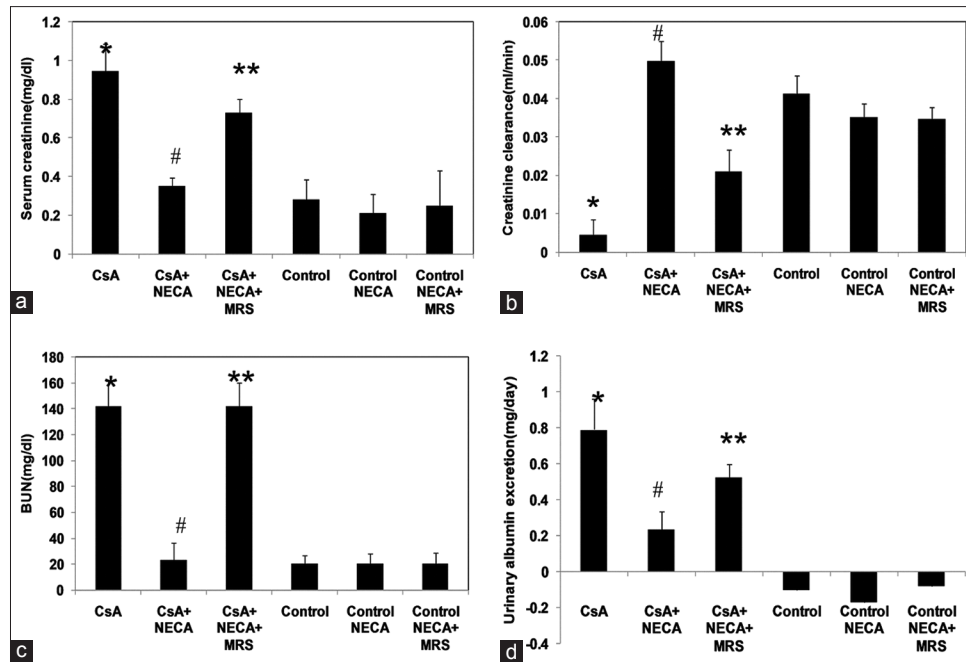
Figure 1: Effect of adenosine receptor modulators on (a) serum creatinine, (b) creatinine clearance, (c) BUN and (d) UAE in control and CsA-treated animals. Data are means (±sem) *P < 0.05 vs. control group, #P < 0.05 vs. CsA group, **P < 0.05 vs. CsA + NECA group, n = 6

indicated significant reduction of nitrite level in CsA-treated animals. Administration of NECA for 2 weeks increased the nitrite levels in CsA-treated animals significantly. The increase in nitrite by NECA was blocked by the treatment of MRS1754. A_{2B} receptor modulators did not produce any change in nitrite levels in control groups.

Kidney pathology scores

CsA administration induced striped renal injury that included damage to tubular epithelial cells, inflammatory infiltrates, and tubulointerstitial expansion accompanied by fibrosis which was calculated as a kidney pathology score [Figure 4a]. The score was increased in animals treated with CsA. NECA inhibited the pathological changes. Administration of antagonist inhibited the effect of NECA. The expression of VEGF and kidney pathology score were positively correlated [Figure 4b]. The characteristic features of CsA nephropathy are presented in Figure 5b-e.

A_{2B}AR mRNA level

To confirm the state of A2BAR expression in CsA-treated animals, we performed quantitative real-time PCR from the same cDNA that was prepared for VEGF. We could find a significant increase in the A_{2B} receptor mRNA after 5 weeks of CsA administration [Figure 5a].

DISCUSSION

Since CsA is considered as an excellent immunosuppressive drug, several therapeutic agents have been investigated in animal models of chronic CsA nephrotoxicity in an attempt to inhibit or prevent CsA-induced toxic renal effects.

The vasoconstricting effect of CsA on the renal vasculature can induce hypoxia producing injuries to endothelial cells. Hypoxia has been found to play a critical role in the pathogenesis of chronic CsA nephrotoxicity.[19,20] VEGF may become upregulated in response to hypoxic endothelial injury. The upregulation of VEGF repairs and maintains the damaged endothelium. This is evidenced in the early phase of CsA nephropathy; however, in the late phase, VEGF decreases. In the present study, the kidney VEGF levels were decreased after 5 weeks of cyclosporine treatment which is in consistence with the earlier literature.[6]

Adenosine is an important mediator in the renal system.[21] Microarray analyses of cDNA derived from endothelial cells subjected to various periods of hypoxia revealed significant changes in the AR profile, wherein the prominent phenotypic change favored A_{2B}AR expression, with concomitant downregulation of A_1AR and A_3AR.[22] Different ARs were investigated as a vasoactive mediator of protective effects in cyclosporine nephropathy.[3] A_1ARs have been investigated in radiocontrast media-induced nephropathy.[23] In an acute model of CsA nephrotoxicity, concomitant administration of theophylline, a nonselective adenosine antagonist, did not improve renal functions.[24] The mRNA expressions of A_1AR and A_{2A}AR were decreased by chronic administration of CsA.[25] We also observed correlation between VEGF
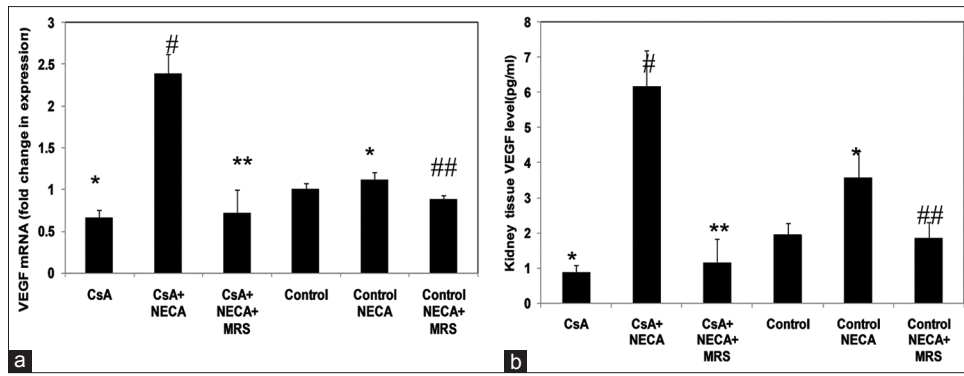
Figure 2: Effect of adenosine receptor modulators on (a) VEGF mRNA and (b) kidney tissue VEGF levels in control and CsA-treated animals. Data are means (±sem) *$P < 0.05$ vs. control group, #$P < 0.05$ vs. CsA group, **$P < 0.05$ vs. CsA + NECA group, ##$P < 0.05$ vs. control NECA group, $n = 6$
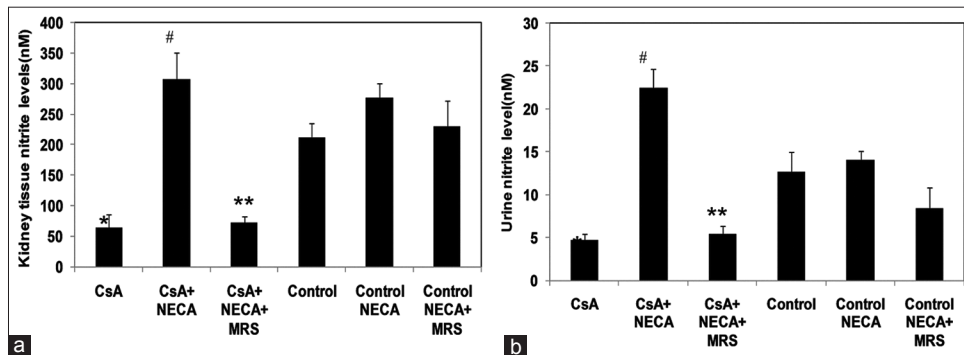

Figure 3: Effect of adenosine receptor modulators on (a) kidney tissue nitrite level and (b) urine nitrite level in control and CsA-treated animals. Data are means (±sem) *$P < 0.05$ vs. control group, #$P < 0.05$ vs. CsA group, **$P < 0.05$ vs. CsA + NECA group, $n = 6$
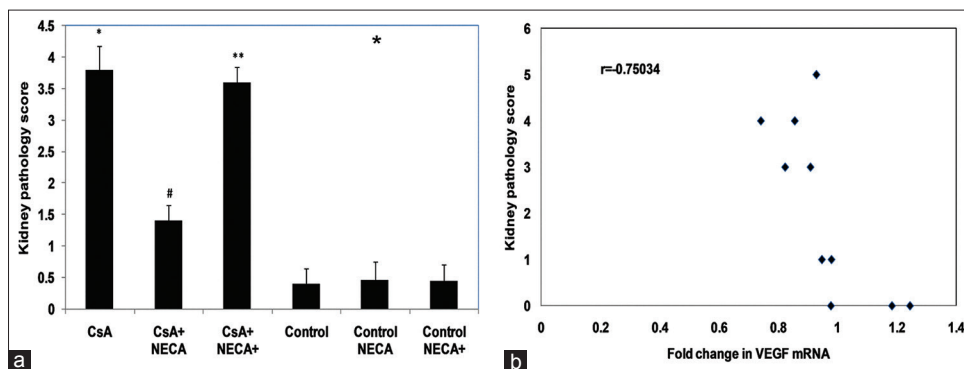

Figure 4: (a) Kidney pathology score in CsA-treated animals. (b) Correlation between VEGF and kidney pathology score in CsA-treated animals. Data are means (±sem) *$P < 0.05$ vs. control group, #$P < 0.05$ vs. CsA group, **$P < 0.05$ vs. CsA + NECA group, $n = 6$

and renal dysfunction. We could not find earlier report on expression of $A_{2B}AR$ in chronic CsA nephropathy; however, we found an increase in $A_{2B}AR$ expression in chronic CsA nephropathy. We also evaluated the effects of AR agonist (NECA) on VEGF-mediated renal functions in chronic CsA nephropathy. In the present study the mRNA expression and protein levels of VEGF were raised by NECA significantly, which was blocked by an antagonist. These effects were prominent in CsA-treated animals than in the control animals suggesting upregulation of $A_{2B}AR$ in the CsA

nephropathy, which are otherwise very less expressed. The reversal of albuminuria is greater in CsA-treated animals, when treated with NECA compared with the control group of animals. These effects were blocked by MRS1754 suggesting that $A_{2B}AR$ may be involved in regulating renal functions by modulating VEGF.

Several studies have suggested the role of NO in the hemodynamic alterations seen with CsA treatment.[26] CsA-induced acute renal dysfunction was shown to improve
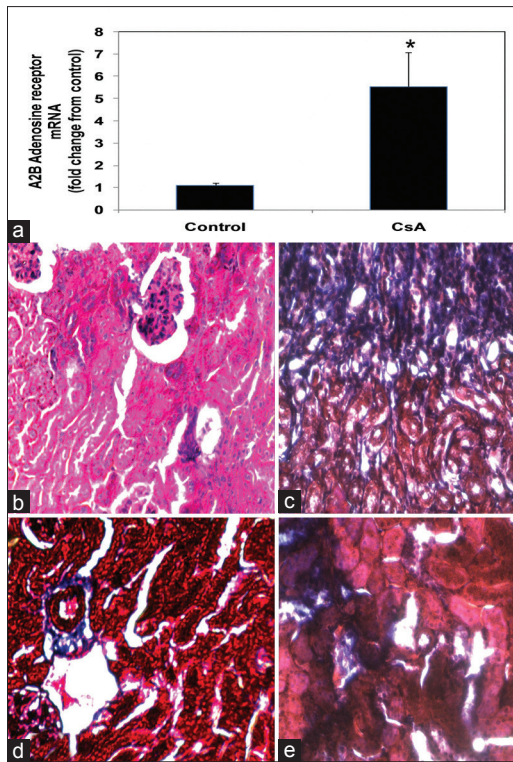
Figure 5: (a) Expression of A_{2B} receptor mRNA. *$P < 0.05$ vs. control group. (b) Histopathological characteristics of kidney tissue. The kidney tissues were stained by hematoxylin-eosin and Masson trichrome reagent. (b) Collapsing glomerulopathy. (c) Collagen deposition. (d) arterioplar hylinosis. (e) tubulointerstitial fibrosis seen in CsA nephropathy at original magnification of 400X

with l-Arginine administration and to worsen with NO blockade.[27] Exogenous NO augmentation is associated with a decrease in VEGF expression while NO blockade increased VEGF expression in chronic CsA nephrotoxicity.[28] A_{2B}ARs have been found to induce NO release.[13,14,29,30] In the present study we could find reduction in the NO levels after 5 weeks of CsA administration, which was improved after administration of NECA. The effect was blocked by MRS1754 suggesting a role of A2BAR in modulating the NO level in diseased kidney.

There is a considerable body of evidence for interaction between VEGF and NO.[31,32] CsA-induced nephropathy is characterized by arteriolopathy. NO is proposed to inhibit vascular smooth muscle cell (VSMC) proliferation and their migration. Hence, the activation of VSMC could be prevented by VEGF-induced endothelial NO release, leading to amelioration of arteriolar disease.[28] These studies suggest that NO–VEGF interaction regulates renal function in CsA nephropathy but the results obtained differed from the diabetic nephropathy.[33]

The current literature evidence indicates angiotensin II as the contributing factor to induce VEGF and to modulate ARs in kidney.[34] Intra-renal renin and angiotensinogen levels are increased in CsA nephropathy.[35] These studies suggest a common pathogenic pathway involving adenosine signaling in chronic kidney disease.

It is probable that VEGF plays a role, either independently or it is dependent on NO via A_{2B}AR, in CsA-induced nephropathy. Our studies do not exclude the possibility that A_{2B} receptor activation produces a favorable intraglomerular hemodynamic effect to reduce proteinuria. It is also possible that A_{2B} receptor activation mitigate proteinuria through direct effects by blocking vasoactive inflammatory mediators. Additional studies are necessary to address this issue.

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