

# Suppression of Mesangial Proliferative Glomerulonephritis Development in Rats by Inhibitors of cAMP Phosphodiesterase Isozymes Types III and IV

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## Abstract

Excessive mesangial cell (MC) proliferation is a hallmark of many glomerulopathies. In our recent study on cultured rat MC (Matousovich, K., J.P. Grande, C.C.S. Chini, E.N. Chini, and T.P. Dousa. 1995. *J. Clin. Invest.* 96:401–410) we found that inhibition of isozyme cyclic-3',5'-nucleotide phosphodiesterase (PDE) type III (PDE-III) suppressed MC mitogenesis by activating cAMP-dependent protein kinase (PKA) and by decreasing activity of mitogen-activated protein kinase (MAPK). We also found that inhibition of another PDE isozyme, PDE-IV, suppresses superoxide generation in glomeruli (Chini, C.C.S., E.N. Chini, J.M. Williams, K. Matousovich, and T.P. Dousa. 1994. *Kidney Int.* 46:28–36). We thus explored whether administration in vivo of the selective PDE-III antagonist, lixazinone (LX), together with the specific PDE-IV antagonist, rolipram (RP), can attenuate development of mesangioproliferative glomerulonephritis (MSGN) induced in rats by anti-rat thymocyte serum (ATS). Unlike the vehicle-treated MSGN rats, rats with MSGN treated with LX and RP did not develop proteinuria and maintained normal renal function when examined 5 d after injection of ATS. In PAS-stained kidneys from PDE-antagonists-treated MSGN-rats the morphology of glomeruli showed a reduction in cellularity compared with control rats with ATS. Compared with MSGN rats receiving vehicle, the MSGN rats receiving PDE-antagonists had less glomerular cell proliferation (PCNA  $\delta$  –65%), a significantly lesser macrophage infiltration ( $\delta$  –36% ED-1) and a significant reduction of  $\alpha$ -smooth muscle actin expression by activated MC; in contrast, immunostaining for platelet antigens and laminin were not different. The beneficial effect of PDE inhibitors was not due to a moderate decrease ( $\sim$  –20%) in systolic blood pressure (SBP); as a similar decrease in SBP due to administration of hydralazine, a drug devoid of PDE inhibitory effect, did not reduce severity of MSGN in ATS-injected rats. We conclude that antagonists of PDE-III and PDE-IV administered in submicromolar concentrations in vivo to ATS-injected rats can decrease the

activation and proliferation of MC, inhibit the macrophage accumulation, and prevent proteinuria in the acute phase of MSGN. We propose that PDE isozyme inhibitors act to block (negative “crosstalk”) the mitogen-stimulated intracellular signaling pathway which controls MC proliferation due to activating of the cAMP-PKA pathway. These results suggest that antagonists of PDE-III and IV may have a suppressive effect in acute phases or relapses of glomerulopathies associated with MC proliferation. (*J. Clin. Invest.* 1996. 98:262–270). Key words: cAMP phosphodiesterase isozymes • mesangial cells • proliferation • anti-Thy-1.1 nephritis • macrophages

## Introduction

Excessive proliferation of mesangial cells (MC)<sup>1</sup> is a frequent characteristic of many glomerular diseases caused by immunologic or other mechanisms of glomerular cell injury (1, 2). In particular, IgA nephropathy (Berger's disease) and the nephritis associated with Schönlein-Henoch purpura (3, 4), are characterized by active proliferation of MC as compared with other cell types endogenous to glomerulus (1, 3, 4). IgA nephropathy is presently recognized as the most common form of acute glomerulonephritis worldwide with as many as 50% eventually progressing to end-stage renal disease (3, 4). Effective and specific treatment of IgA nephropathy is lacking, although a recent report suggests that treatment with fish oil may slow progression (5). In view of the perceived importance of MC proliferation in IgA-nephropathy as well as other proliferative glomerular diseases, considerable effort has been devoted to clarifying the cellular mechanisms of MC proliferation with the aim of designing new specific and effective pharmacotherapies.

An experimental model of mesangial proliferative nephritis (MSGN) can be induced in rats with monoclonal or polyclonal (ATS) antibodies to the Thy-1.1 antigen, which is expressed on the rat mesangial cell membrane (6, 7). In this model, after single ATS injection, an initial complement-dependent mesangiolysis is followed by a rapid proliferation of the MC population, the acquisition of a phenotype by the MC characteristic of “myofibroblasts,” and a matrix expansion

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Received for publication 26 October 1995 and accepted in revised form 26 April 1996.

1. Abbreviations used in this paper: ATS, anti-thymocyte serum; LX, lixazinone; MC, mesangial cell(s); MSGN, mesangial proliferative nephritis; PAS, periodic acid Schiff stain; PCNA, proliferating cell nuclear antigen; PDE, cyclic-3',5'-nucleotide phosphodiesterase; RP, rolipram; Rx, medication (LX + RP); SBP, systolic blood pressure.

within 4–6 d (1, 8). Indeed, similar proliferative and phenotypic changes of the MC population have been documented in human glomerular diseases, including IgA nephropathy (1, 3).

In our recent study of rat MC in vitro (9) we observed that the mitogen-stimulated signaling pathway in MC can be inhibited by “negative crosstalk” with the cAMP-signaling pathway (10–12). MC proliferation in vitro under ambient growth conditions, (i.e., only under influence of mitogens synthesized endogenously in MC) (13), as well as when maximally stimulated by exogenous EGF or PDGF, is strongly suppressed by the addition of cyclic-3',5'-nucleotide phosphodiesterase (PDE) isozyme-selective antagonists which inhibit PDE isozyme type-III (PDE-III). Selective inhibitors of other types of PDE isozyme types (gene families) (14, 15) had no such inhibitory effect, except that antagonists of isozyme PDE-IV enhanced the effect of the PDE-III antagonists and also had marginal inhibitory effects upon MC mitogenesis alone (9). The PDE-III isozyme antagonists suppressed mitogenesis in MC without addition of stimulant(s) of adenylate cyclase and without measurable increase in MC content of cAMP (9). However, in situ activity of cAMP-protein kinase (PKA) was increased and the activity of mitogen-activated protein kinase (MAPK) was inhibited (9). These results indicate that PDE-III antagonists suppressed MC proliferation by interfering with a signaling step of the mitogen-stimulated pathway upstream to MAPK (10–12), probably by inhibiting the activity of protein kinase *Raf-1*, an enzyme which initiates the mitogen kinase phosphorylating cascade (10–12). The inhibition of *Raf-1* protein kinase activity was reported to be due to decreased phosphorylation of Ser<sup>43</sup> by activated PKA (12).

In another preceding study (16) we found that the antagonist of PDE-IV, rolipram, inhibits generation of reactive oxygen metabolites (ROM) in glomeruli (16), and more recently also in MC (19). ROM are known not only to inflict cell damage (18, 19) but also, as recently proposed, can serve as intracellular signals—“second messengers” (20). In MC an increase in generation of ROM is known to stimulate synthesis of monocyte chemoattractant protein MCP-1 and colony stimulating factor CSF-1 (21).

We hypothesized that antagonists of PDE-III and PDE-IV will exert in vivo effects upon MC (and perhaps other glomerular cells) similar to those which we observed in vitro (9, 16, 17) and will suppress MC proliferation and other pathogenic processes in development of MSGN. In view of these considerations, we investigated whether antagonists of PDE-III and PDE-IV exhibit similar effects in vivo as in vitro; namely to inhibit MC proliferation. In experiments reported herein we examined the effect of PDE-III and PDE-IV antagonists on rats developing MSGN induced by ATS and examined renal tissue on the fifth day after ATS, at the height of the disease and MC proliferation (1, 8). We found that after injection of ATS continuous administration of the PDE-III antagonist, lixazinone (22) and the PDE-IV antagonist, rolipram (23), prevented development of proteinuria and greatly suppressed the histopathologic changes, namely cell proliferation and phenotypic alterations, found in rats with anti Thy-1.1 MSGN.

## Methods

Male Wistar rats (180–220 grams; Simonsen Inc., Gilroy, CA) were housed in individual metabolic cages during the course of the experiment. As in previous studies (24, 25), the observation baseline before

administration of the anti-thymocyte antiserum (ATS) was 2 d (day –2, –1) (see Fig. 1). On day 0 the ATS and PDE isozyme antagonists were injected and osmotic minipumps (Alzet<sup>®</sup>) implanted under ketamine-xylazine anesthesia; the following days are numbered and designated +1–+5. In some rats during the course of the experiment the daily urine volume (U·V), fluid intake, and U·V<sub>Cr</sub> were measured; systolic blood pressure (SBP) was monitored daily by the tail-cuff sphygmomanometric method, using a Doppler ultrasound detector. On days –2, +2 and +5, the urinary excretion of protein per 24 h was determined. At the end of the experiment on day +5, animals were anesthetized, an aortic blood sample was collected for determination of plasma chemistries, and the kidneys were removed and cortical biopsies fixed in methyl Carnoy's solution, formaldehyde, or quickly snap-frozen and stored in liquid nitrogen.

**General experimental design (See Fig. 1).** Three groups of rats were studied. The control group (group A) received only nonimmune goat serum on day 0 ( $n = 5$ ). Rats with experimental MSGN ( $n = 9$ ) without treatment (group B) had two osmotic minipumps containing solvents in the same amount and concentration but without the tested drugs (placebo) implanted into the peritoneal cavity under anesthesia (day 0) and then injected i.v. with the solvent of priming dose of PDE isozyme antagonists and 10 min later i.v. with 4 ml/kg of goat ATS. The amount of solvents administered to group B was identical to group C. Rats with MSGN treated by PDE isozyme antagonists ( $n = 7$ ) (group C) were implanted i.p. with two osmotic minipumps with drugs and injected with 4 ml/kg ATS i.v. at the same time sequence as animals of group B (10 min after receiving i.v. injection of the priming dose of lixazinone and rolipram) in the same solvents as group B. Drugs for maintenance delivery (group C) or vehicle (group B) were loaded to two osmotic minipumps which were implanted i.p. under anesthesia. Rats were anesthetized by a mixture of ketamine 100 mg/ml and xylazine 20 mg/ml mixed (1:1) in dose 0.2 ml/200 g b.wt. The Alzet<sup>®</sup> minipumps (Model 2 ml, 10  $\mu$ l/h delivery 7 d) were filled, one with 6.5 mg/ml lixazinone (LX) solubilized in a solvent containing 10% ascorbic acid and *tert*-butanol (1:1), pH  $\sim$  2.6; another with 8.9 mg/ml rolipram (RP) dissolved in 50% DMSO. The minipumps were prewarmed in isotonic saline for 4 h before implantation to assure immediate delivery of the drugs. Priming dose of LX was 1 mg/kg body weight and rolipram 0.55 mg/kg body weight. Priming and maintenance doses were estimated (22, 23, 26) to achieve plasma levels of LX  $\approx$  0.4  $\mu$ M and RP  $\approx$  1  $\mu$ M, assuming total body fluid distribution (26). At the end of experiments, the pumps were weighed to confirm that the expected amount of fluid was delivered during the period of implantation, as designed. LX (22) and RP (23) were selected as the most potent and specific inhibitors of PDE-III and PDE-IV now available, which were also used in our in vitro study on cultured MC (9).

In a separate group of rats we examined the effect of lowering of SBP upon development of MSGN, and the design is depicted in Fig. 1. The two groups of rats were injected on day 0 with ATS and implanted with Alzet<sup>®</sup> minipumps containing either (3 mg/kg per day) hydralazine dissolved in 0.9% NaCl, or control group received solvent only. The rats received hydralazine in a dose aimed to achieve a plasma concentration of  $\sim$  0.4  $\mu$ M which, as we determined in preliminary studies, decreased SBP about  $\delta = 20\%$ , i.e., comparable with PDE isozyme antagonists. Measurement of SBP and other parameters were similar to studies with PDE isozyme antagonists. Whether hydralazine has any effect upon PDE was tested using the same PDE assay as in the preceding study (9, 32); extract (32) from renal cortical tissue was used as a source of PDE. We observed that 0.4 or 4  $\mu$ M hydralazine had no effect upon cAMP or cGMP hydrolysis while LX, RP, and other PDE isozyme antagonists showed typical inhibition.

**Analytical procedures and materials.** Plasma and urine creatinine were measured using standard calorimetric methods, plasma proteins by Lowry's method (28), calcium by atomic absorption spectrometry, and phosphate by the method of Chen et al. (32). For determination of urinary protein the urine collected in 24 h was filtered, precipitated by 5% (final concentration) trichloroacetic acid (TCA), collected by centrifugation, and measured by the method of Lowry et al. (28). The

content of AMP and cGMP in plasma were determined in pre-acylated samples by radioimmunoassays, as used in our previous studies (30).

**Immunostaining.** Renal biopsies were placed in methyl Carnoy's solution, embedded in paraffin, and cut in 5- $\mu$ m sections for indirect immunoperoxidase staining as previously described (24, 25) using diaminobenzidine with nickel as a chromogen. Renal histology was evaluated by routine PAS staining.

To determine the number of proliferating glomerular cells, we used antibody to PCNA, which is expressed from late G<sub>1</sub> to M phase of cell cycle. A murine monoclonal IgM antibody against PCNA (1:1000 dilution; 19A2, Coulter Immunology, Hialeah, FL) was incubated overnight at 4°C followed by a peroxidase-conjugated rat anti-mouse IgM (Zymed Laboratories, San Francisco, CA). Black nuclear staining was detected using diaminobenzidine (Sigma Chemical Co., St. Louis, MO) with nickel as a chromogen. To determine the number of proliferating cells in individual animals, 50 glomerular profiles from each renal biopsy was analyzed, and positive staining was defined as black nuclear staining.

To determine phenotypic change of MC (26), we used monoclonal antibody (IgG2A; Sigma Chemical Co.) to  $\alpha$ -smooth muscle actin ( $\alpha$ -sm actin).

A semiquantitative evaluation was used (24, 25) where each glomerulus was graded (25) on scale of +1→+4 using the following criteria: +1 denotes < 25% positive stains per glomerulus; +2 denotes 25–50% positive stains; +3 denotes 50–75% positives; and +4 is grade where > 75% of glomerulus is positive. The score is based on 50 glomeruli in renal tissue samples from each rat; the mean score was computed to obtain mean±SEM for each experimental group (A, B, and C).

Macrophages were immuno-stained with ED-1 mouse monoclonal IgG (ScroTel, Washington, DC) directed against cytoplasmic antigen present in monocyte macrophages and dendritic cells. Values denote the number of macrophages from 20 glomeruli in kidneys of each animal, similarly as for PCNA stain.

Staining for platelet antigens was performed with a murine monoclonal antibody against rat platelets, (PL-1, a gift of Dr. W.W. Becker, Groningen, The Netherlands). The method of scoring was +1→+4 as described for  $\alpha$ -sm-actin (see above), the average is reported.

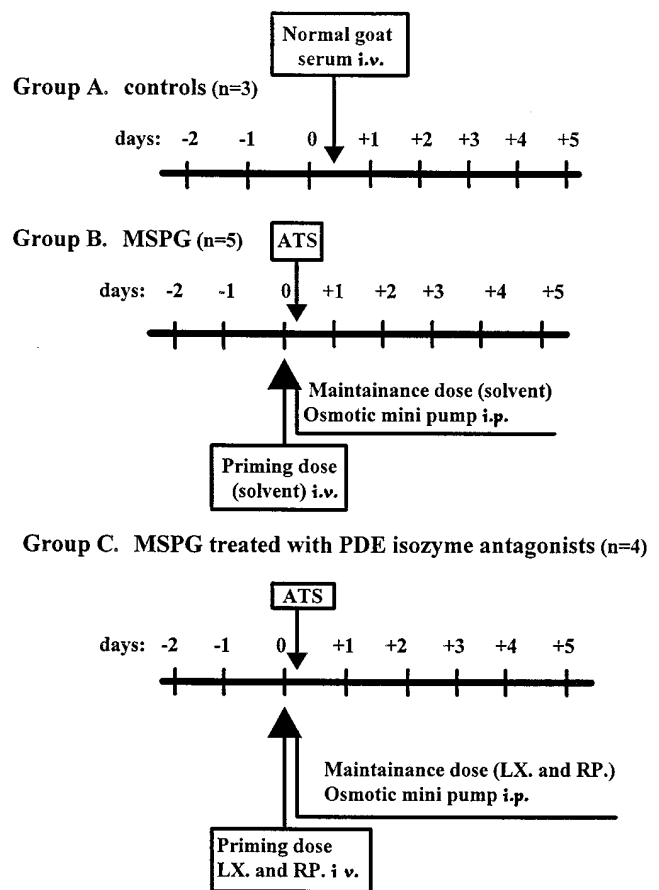
Laminin was detected using a rabbit anti-rat laminin polyclonal antibody (Chemicon International, El Segundo, CA). We used a scoring system graded 0→+4, where 0 denotes absence of any staining and scores +1→+4. In each rat kidney 50 glomeruli were examined.

**Materials.** Lixazinone (LX), *N*-cyclohexyl-*N*-methyl-4 (7-oxy-1,2,3,5-tetrahydroimide 20 [2,1-b] quinazolin-2-one) butyramide (RS-82856) was a gift from Dr. R. Alvarez (Syntex Research, Palo Alto, CA). Rolipram (RP), 4-(3-cyclopentyl-4-methylphenyl)-2-pyridine (ZK 62711) was a gift from Berlex Laboratories (Cedar Knolls, NJ) and Schering A.G. Pharmaceutical (Berlin, Germany). The cAMP-RIA kits were purchased from Biomedical Technologies Inc. (Stoughton, MA); cGMP-RIA kits from Du-Pont NEN (Boston, MA). Osmotic minipumps (Alzet®) were from Alza Co. (Palo Alto, CA). All other chemicals, all of the highest purity grade available, were purchased from Sigma Chemical Co., or other standard suppliers.

All analyses were performed in a blinded fashion using coded samples, an identity of the samples was not revealed until the analysis was complete. The results were evaluated by Student's *t* test for group or paired comparison; *P* < 0.05 was considered statistically significant.

## Results

**Functional parameters.** During the course of the study no animals showed clinically adverse effects. At the end of the experiment (day +5) the three experimental groups of rats (Fig. 1), (i.e.; [group A] controls; [group B] ATS-injected rats receiving placebo; and ATS-injected rats receiving PDE isozyme antag-



**Figure 1.** Flow diagram of the experiments examining effects of PDE-III (lixazinone, LX) and PDE-IV (rolipram, RP) inhibitors, on rats with MSGN elicited by injection of ATS. The priming dose of LX = 1 mg/kg body weight; RP = 0.55/kg body weight. Maintenance doses (by osmotic minipumps Alzet®, flow rate 10  $\mu$ l/h) were LX = 6.5 mg/ml and RP = 8.9 mg/ml.

onists lixazinone [LX] and rolipram [RP] [group C]), did not differ in body weight or in plasma creatinine (data not shown). Likewise, plasma cAMP did not differ between control rats (group A: 63±3 nM; mean±SE, *n* = 5) and ATS-injected rats given placebo (group B: 56±3 nM; mean±SE, *n* = 9) while in ATS-injected rats treated with LX and RP the plasma cAMP was significantly (*P* < 0.01; *t* test) elevated (group C: 117±16 nM; mean±SE, *n* = 7). The three groups did not differ in plasma levels (in nM, mean±SE) of cGMP (group A: 12.7±2.4, *n* = 5; group B: 13.7±3.2, *n* = 9; group C: 12.1±2.8, *n* = 7).

In several rats from each group functional parameters were monitored during the course of experiment (from day -2 to day +5) (Fig. 1). Throughout the experiment (from day -2 to day +5) the three groups did not differ in urinary volume, fluid intake, and urinary excretion of creatinine (*U<sub>C</sub>V*) (data for the last day of experiment are shown in Table I, other days not shown). The systolic blood pressure (SBP) was identical in all three groups on day -2 and day -1 and in groups A and B the SBP remained unchanged for the rest of the study, i.e., up to day +5 (Fig. 2). In group C rats, after receiving a priming dose and implantation of the osmotic minipumps with LX and with RP for continuous delivery of maintaining dose on day 0, showed modest but significant ( $\delta$  = 19±2%, mean±SEM;

Table I. Functional Parameters on Day 5

	Controls (group A)	ATS (group B)	ATS + Rx (group C)
Creatinine ( $P_{cr}$ ) (mg/dl)	$0.37 \pm 0.03$	$0.27 \pm 0.05$	$0.31 \pm 0.04$
Proteins ( $P_{prot}$ ) (g/dl)	$6.6 \pm 0.4$	$5.5 \pm 0.3$	$6.3 \pm 0.2$
Phosphate ( $P_{pi}$ ) (mM)	$1.49 \pm 0.05$	$1.45 \pm 0.02$	$1.22 \pm 0.03$
Calcium ( $P_{ca}$ ) (mM)	$1.88 \pm 0.06$	$2.00 \pm 0.03$	$1.9 \pm 0.03$
$P_{cAMP}$ (nM)	$59 \pm 3$	$55 \pm 5$	$106 \pm 18^*$
$P_{cGMP}$ (nM)	$12 \pm 2$	$18 \pm 5$	$18 \pm 2$
Creatinine Clearance $C_{cr}$ (ml/min)	$1.55 \pm 0.27$	$1.74 \pm 0.3$	$1.60 \pm 0.51$

Plasma and excretory data at the end of the experiment in animals with functional parameters followed in course of experiment (Fig. 2): controls (group A;  $n = 3$ ); ATS (group B;  $n = 5$ ) and ATS + Rx (with PDE isozyme inhibitors) (group C;  $n = 4$ );  $n$  denotes number of animals. \*Value significantly ( $P < 0.05$ , test) higher than in group A and group B. There were no significant differences between the three groups in other parameters.

paired  $t$  test,  $n = 4$ ) drop in SBP compared with pretreatment days  $-1$  and  $-2$ . Thereafter, the SBP in group C remained constant at a significantly lower level ( $\sim 8 - 18\%$  difference from groups A and B) for the rest of the experiment, i.e., days  $+1 - +5$  (Fig. 2).

Before the injection of ATS, no difference in urinary protein excretion was present between the three groups (Fig. 2). Compared with control rats (group A), ATS-injected rats (group B) developed frank proteinuria ( $> 6\times$  higher than in controls) on the day  $+2$ , whereas ATS-injected rats treated with PDE isozyme antagonists (group C), in the same time,

showed no significant difference in protein excretion from control group A (Fig. 2). In non-treated ATS-injected rats (group B) the extent of proteinuria declined from  $+2$  d to  $+5$  d (Fig. 2), nevertheless, on the day  $+5$  the urinary protein excretion remained more than 4 fold higher in group B than in control group A and in ATS-injected rats treated with PDE isozyme antagonists; (group C) (Fig. 2). Plasma creatinine, calculated creatinine clearance, total plasma protein and plasma phosphate and calcium or cGMP obtained at day  $+5$  (Table I) were not different between the three groups A, B, and C. Only the plasma levels of cAMP were significantly higher ATS-injected

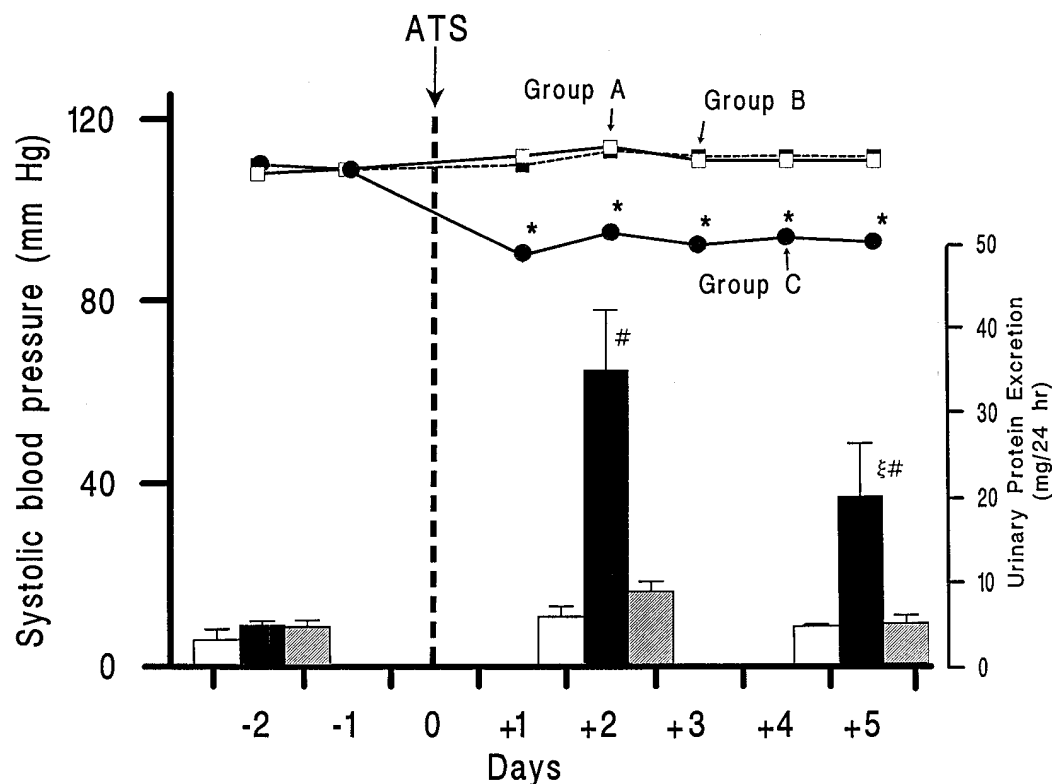
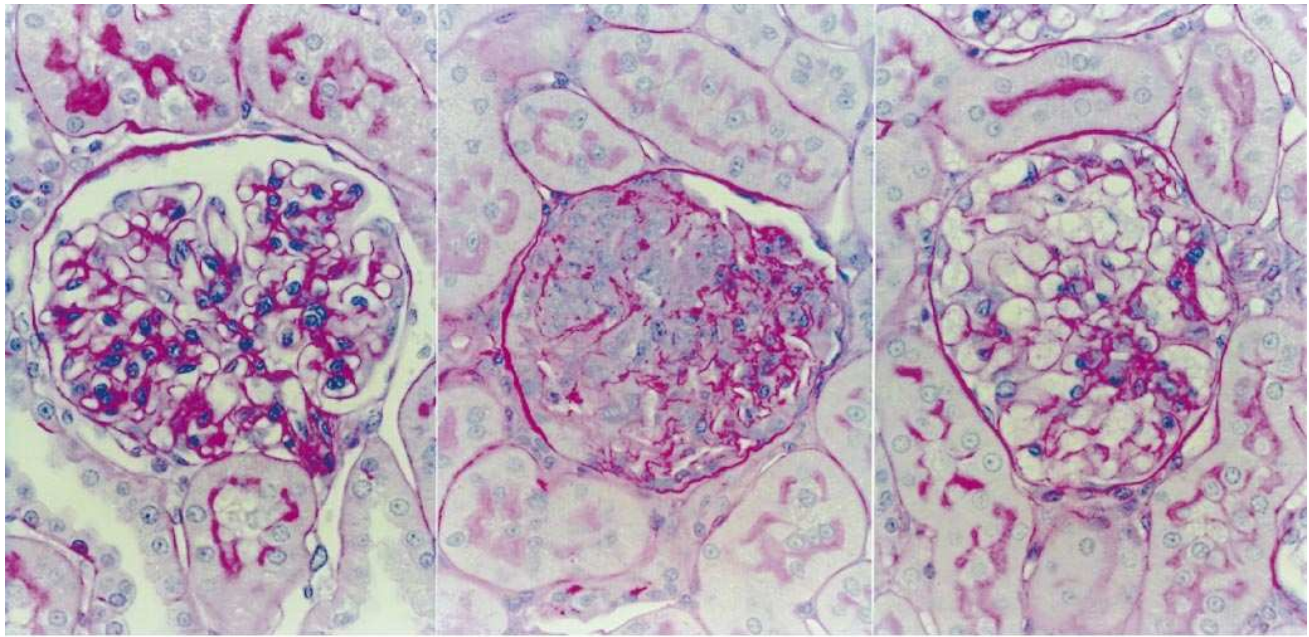


Figure 2. Systolic blood pressure (SBP) in the three groups of rats (see Fig. 1) in the course of experiment. Abscissa: time (days); vertical line denotes start of drug delivery. Ordinates: left side, SBP (mm Hg), right side, urinary protein excretion (mg/24 h). (—●—) denotes rats injected with ATS and PDE isozyme antagonists, group C ( $n = 4$ ); (—■—) rats injected with ATS and placebo, group B ( $n = 5$ ); (—□—) control rats group A ( $n = 3$ ). Each point denotes mean  $\pm$  SEM; SEM are small and hidden in graphical signs. Asterisk (\*) indicates values significantly ( $P < 0.05$  or higher degree of significance, paired  $t$  test) different from the value in the same group before initiation of administration of PDE isozyme inhibitors or the value significantly different from the other two

groups on the same day (group  $t$  test;  $P < 0.05$ );  $n$  denotes number of rats. Bars denote urinary protein excretion: (□) controls; (■) ATS-injected receiving placebo; (▨) ATS-injected, treated with LX and RP. Value significantly higher ( $P < 0.01$ , test) than in controls and in drug-treated groups. (ζ) Significantly ( $P < 0.05$ , paired  $t$  test) lower than corresponding value day  $+2$ .



**Group A  
(CONTROL)**

**Group B  
(MSGN)**

**Group C  
(MSGN + Rx)**

Figure 3. Histologic PAS stain of glomeruli from group A, controls (left); ATS-injected and untreated, group B (center); (right) ATS-injected and treated with PDE isozyme inhibitors, group C.  $\times 700$ .

group treated with PDE isozyme antagonists (group C), compared with other two groups, i.e., group A and group B (Table I).

**Histologic studies.** Renal cortical tissue evaluated by PAS stain (Fig. 3) from ATS-injected rats (group B) at day +5 showed mesangial hypercellularity, with occasional disintegration of mesangial architecture, an increased frequency of mitotic figures and marked expansion of extracellular matrix—changes typical (1, 24, 25) for this stage of the MSGN model (Fig. 3). Extraglomerular tissue was not affected (Fig. 3). In contrast, in ATS-injected rats treated with PDE isozyme inhibitors LX and RP (group C) the pathologic changes were mild and the glomerular morphology was quite comparable to controls (group A) (Fig. 3).

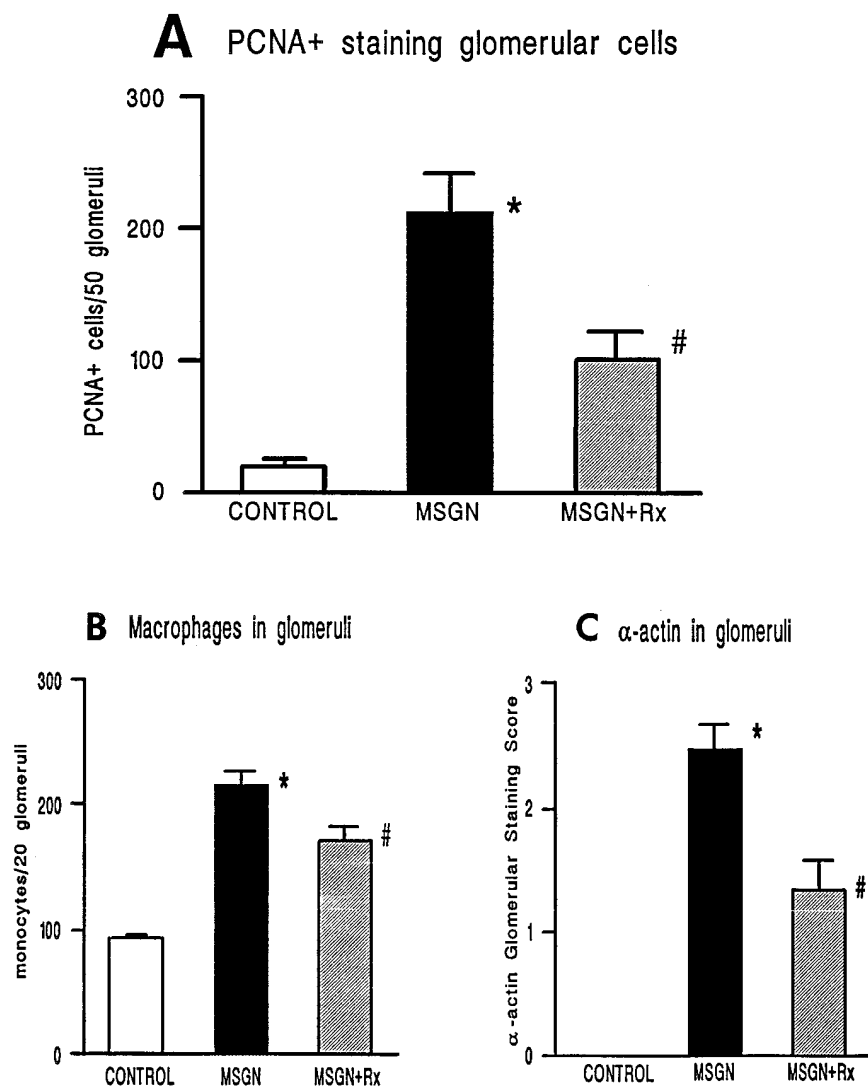
Quantitative evaluation of glomerular cell proliferation by measurement of PCNA-staining cells (Fig. 4 A) in glomeruli showed a 10-fold increase of PCNA-positive cells in untreated ATS-injected rats at day +5 (group B) compared with controls (group A). In contrast, in glomeruli of ATS-rats receiving PDE isozyme inhibitors (group C) the number of PCNA-positive cells was significantly lower ( $\delta - 58\%$ ) than in ATS-injected rats without treatment (group B) (Fig. 4 A). Immunostaining of glomeruli with ED-1, a specific antibody to macrophage/monocytes, also demonstrated a significant macrophage influx ( $\delta + 129\%$ ) in group B (Fig. 4 B). However, the influx of macrophages was significantly reduced ( $\delta - 36\%$ ) in rats treated with PDE isozyme antagonists (group C) (Fig. 4 B). The extent of staining for platelet-containing antigens (PL-1) was not different between untreated rats with MSGN (group B:  $1.48 \pm$

$0.12$ ,  $n = 9$ ) and MSGN rats receiving PDE isozyme inhibitors (group C:  $1.28 \pm 0.1$ ;  $n = 7$ ). Rats with MSGN (group B) showed de novo expression of  $\alpha$ -smooth muscle actin and this phenotypic change was significantly decreased ( $\delta - 46\%$ ) in glomeruli of rats undergoing treatment with the PDE isozyme antagonists (group C), (Fig. 4 C). Finally, we observed no statistically significant difference in content of laminin (laminin staining score) between ATS-injected rats with MSGN receiving placebo (group B:  $2.73 \pm 0.42$ ,  $n = 9$ ) and MSGN animals treated with LX and RP (group C:  $1.78 \pm 0.47$ ,  $n = 7$ ).

The effect of decrease in SBP per se upon development of MSGN was tested by lowering the SBP by hydralazine, a hypotensive drug which has no effect upon PDE activities. Hydralazine administration caused sustained decrease ( $\delta \approx -24\%$ ), in SBP (Fig. 5) in a similar manner and extent as administration of PDE isozyme antagonists (compare Figs. 2 and 5). There was no difference between hydralazine-treated MSGN rats and control MSGN animals in development of proteinuria (Fig. 5), or other urinary parameters, such as urine flow or  $U_{Cr}:V$  (not shown). Immunohistologic analysis (Table II) showed no significant differences between MSGN rats made hypotensive by hydralazine and the normotensive controls.

## Discussion

The present study was designed to determine whether the anti-mitogenic effect of PDE-III isozyme antagonists upon MC, discovered recently in MC cultured in vitro (9) can be elicited



**Figure 4.** Immunohistochemical analysis of glomeruli. (□) Controls ( $n = 5$ ); (■) MSGN rats receiving placebo ( $n = 9$ ); (▨) MSGN rats ( $n = 7$ ) treated (Rx) with antagonists of PDE isozymes III (lixazinone) and IV (rolipram). \*Value significantly ( $t$  test) higher than controls; (#) significantly lower than MSGN without treatment ( $t$  test). (A) PCNA-staining proliferating glomerular cells; (B) immunostaining for macrophages/monocytes; (C) Immunostaining for  $\alpha$ -actin.

also in vivo and thereby ameliorate the course of MSGN in rats, namely in the acute proliferative phase of anti-Thy1.1 model (1, 7, 24, 25). We used the potent PDE-III antagonist lixazinone (LX) together with rolipram (RP), an antagonist of PDE-IV, which by itself has little anti-mitogenic activity in MC but which enhances effects of PDE-III antagonists (9). More importantly, PDE-IV antagonist can also suppress ROM formation in glomeruli (16) and in MC (17) and ROM generated by infiltrating monocytes (18, 31). Generated ROM may not only inflict injury to glomerular cells and capillary wall in vivo (18, 19), but at lower subtoxic concentration may stimulate MCP-1 synthesis (21) in MC and possibly activate MAPK (32).

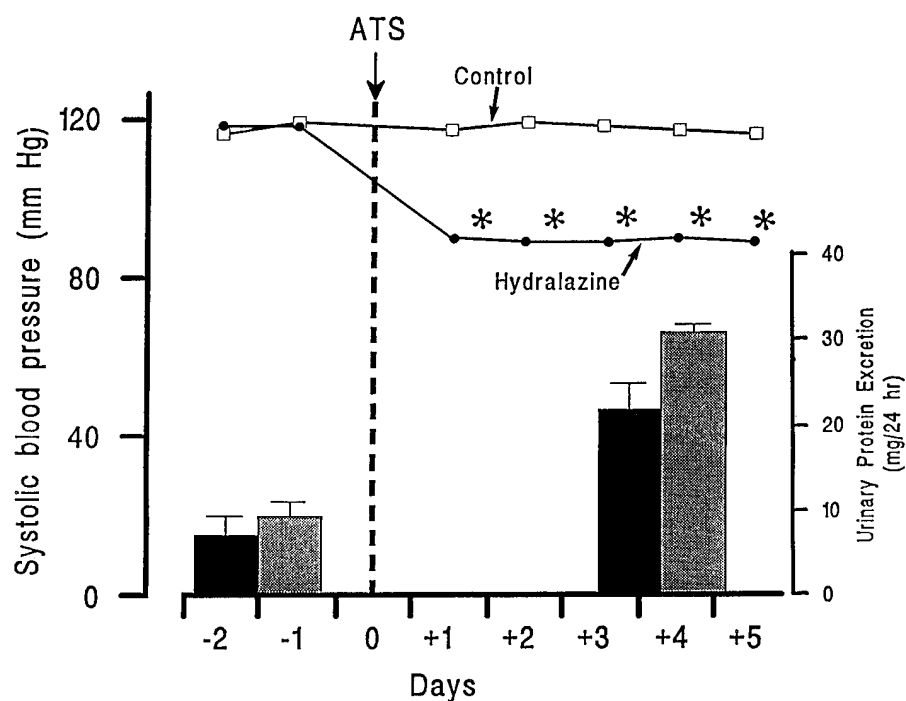
Since the available information on the pharmacokinetics of LX and RP in rats is very limited (22, 23, 27), in the present study the priming and maintaining doses delivered by osmotic minipumps were based mainly on the effectiveness of these agents in vitro (9) and on pilot in vivo studies. We also postulated that the in vivo activity of adenylate cyclase in MC will be under stimulatory action by circulating hormones and autacoids (33, 34). Therefore, lesser concentrations of PDE isozyme inhibitors would be needed in vivo to achieve the same degree of in situ PKA activation, inhibition of mitogenesis of MC (9), and to decrease ROM formation (16, 17).

The modest but significant increase of cAMP but not cGMP (Table I) in plasma determined at the end of experiment also confirmed that PDE isozyme antagonists indeed exhibited their expected effect in vivo, i.e. selective inhibition of cAMP hydrolysis (14, 15). It should be stressed that this measurement reflects the level of circulating extracellular cAMP and does not reflect the effect of LX or RP upon the cAMP and cGMP content within the glomeruli or MC.

The moderate ( $\sim 19\%$ ) drop in SBP observed in rats receiving PDE isozyme antagonists (Fig. 2) is most likely due to a decrease in peripheral resistance since PDE-III antagonists are well known to relax vascular smooth muscle (22). The fact that this decrease of SBP was of the same extent in +1–+5 suggests, indirectly, that the effect of PDE antagonists was steady, neither cumulative nor underdosed (Fig. 2). Injection of ATS and development of MSGN per se does not influence SBP (Fig. 2).

In view of sustained decrease in SBP of otherwise normotensive MSGN rats receiving LX and RP, we investigated whether such hypotension per se may not ameliorate the development of MSGN. The notion that the decrease in SBP per se does not play a role is supported by results of a control study (Fig. 5, Table II). Infusion of hydralazine, a vasodilator devoid





**Figure 5.** Effect of hydralazine upon systolic blood pressure (SBP) and urinary protein excretion in rats with MSGN due to injection of ATS. Abscissa: time (days); vertical dashed line denotes injection of ATS and start of hydralazine administration (day 0). Ordinates: left side, SBP (mm Hg), right side, urinary protein excretion (mg/24 h). (●—●) denotes MSGN rats receiving hydralazine ( $n = 6$ ); (□—□) denotes control MSGN rats receiving solvent only ( $n = 5$ ); each point denotes mean  $\pm$  SEM. Asterisk (\*) indicates values significantly ( $P < 0.05$  or higher degree of significance;  $t$  test) different from controls and from values prior to administration of hydralazine. Bars denote urinary protein excretion in (■) MSGN controls; (▨) and in MSGN rats treated with hydralazine. (#) Value significantly higher than corresponding value prior to ATS injection ( $P < 0.01$ ;  $t$  test). There was no statistically significant (NS) difference in protein excretion between the two groups either prior to or after ATS-injection.

of inhibitory effect upon PDE, decreased SBP in a similar manner as did PDE isozyme antagonists (Fig. 1), but did not prevent proteinuria, histologic changes, proliferation, and phenotype change resulting from ATS injection (Table II and Fig. 5). Thus, lowering of normal SBP per se did not suppress immuno-inflammatory injure of glomeruli.

Of major interest was the observation that administration of LX and RP decreased the number of glomerular cells (Fig. 4 A). In previous studies of anti-Thy-1.1 nephritis double-labeling with antibodies against PCNA and Thy-1.1-antigen (1), showed that the majority ( $> 85\%$ ) of PCNA-positive cells in the present study are also proliferating MC (26). Concerning the cellular mechanism by which MC proliferation was arrested in vivo (Fig. 4 A), we surmise that it was caused by activation of PKA and inhibition of *Raf-1* protein kinase—and ultimately due to the suppression of MAPK activity (9, 10). It is now known that other, analogous signaling pathways which can regulate mitogenesis (35, 36) do not include *Raf-1* as a target for inhibitory action of PKA. In view of this, the present re-

sults strongly suggest that the proposed mitogen-activated signaling pathway and its “negative crosstalk” with cAMP signaling regulated by PDE-III, at the point of *Raf-1* is most likely a dominant mechanism in control of MC proliferation (9). Our findings are in agreement with the notion that PDGF, EGF, and related autocrine/paracrine growth factors play a key role in pathogenesis of anti-Thy-1.1 MSGN (1, 37). Since the PDE-III inhibitor ultimately blocks the signaling pathway at the *Raf-1* step, where different types of mitogenesis-activating signaling pathways converge (10, 38), the employed treatment is suited to block simultaneously the action of a number of mitogenic stimuli which all contribute to MC proliferation (10, 12, 38). The possibility that more than one regulatory pathway was blocked by antagonists of PDE-III and PDE-IV is also consistent with the observation that the treatment suppressed (8–58%) not only the proliferation of MC (Fig. 4 A), which is known to be PDGF-mediated (37), but also suppressed the activation of MC to express  $\alpha$ -actin (Fig. 4 C) (1).

Of importance is also the finding of a decreased accumulation of macrophages in glomeruli of MSGN rats (–36%) treated with PDE isozyme inhibitors (group C) (Fig. 4 B), a feature not observed in prior studies in which MC proliferation was inhibited by administration of interferon- $\gamma$  (24) or heparin (25). The influx of macrophages into diseased glomeruli is considered to be an unfavorable factor in the course of human MSGNs such as IgA nephropathy (31) and, other glomerular diseases (1). Possibly the decreased invasion of macrophages may be due to an inhibition of chemotactic factors such as MCP-1 (21). Synthesis of MCP-1 by MC is, according to a recent report, mediated by generation of ROM (21) and, as we have observed, RP antagonist of PDE-IV, inhibits ROM generation in glomeruli and MC (16, 17). Thus, administration of antagonists both of PDE-III and PDE-IV may, via decreasing synthesis of ROM and MCP-1, cause or contribute to the diminished influx of macrophages (Fig. 4 B). Further, it should be recalled that ROM were shown to activate MAPK (32).

**Table II.** Immunohistory in Hydralazine-treated Rats

	MSGN control rats	MSGN rats receiving hydralazine
PCNA <sup>+</sup> staining cells (cells per 40 glomeruli)	177 $\pm$ 49	221 $\pm$ 21
Macrophages (ED-1 <sup>+</sup> ) (cells per 20 glomeruli)	150 $\pm$ 6.2	154 $\pm$ 10
Laminin content (cells per 20 glomeruli)	57 $\pm$ 0.7	58 $\pm$ 0.4

Immunohistologic analysis of ATS-injected MSGN rats without (controls) or MSGN rats receiving hydralazine. \*Mean  $\pm$  SEM from 3–6 animals. None of the values from control MSGN rats were significantly ( $P < 0.05$ ,  $t$  test) different from MSGN rats receiving hydralazine.

Thus, reduction of ROM generation in glomeruli and MC, due to inhibition of PDE-IV (16, 17), may possibly contribute to the anti-proliferative effect of PDE-III antagonist. Needless to say, decreased synthesis of ROM is bound to diminish the extent of any direct oxidant injury (18, 19) of glomerular cells as well as blunt the synthesis of chemoattractants (21). Therefore, administration of antagonists of PDE-III and PDE-IV together probably inhibits two or more cellular pathogenic processes and thereby amplifies the efficacy of suppression of MSGN with PDE isozyme antagonists.

Our results suggest that the stage of mesangiolysis (day +2) and rapid proliferation of MC up to +5 d in this model of MSGN has profound impact upon the permselectivity barrier of capillary walls in glomeruli, as manifested as proteinuria—the only parameter of renal dysfunction detected in this model (Fig. 2 and 5), and this was completely prevented/suppressed by administration of PDE isozyme antagonists (Fig. 2). In many glomerular diseases associated with MC proliferation, proteinuria heralds activation of glomerulonephritis and eventually progression to renal insufficiency. We surmise that the novel type of pharmacotherapy with PDE isozyme antagonists that suppresses both development of proteinuria and accelerated MC proliferation as well as phenotypic transformation, deserves intense and detailed future studies ultimately for potential clinical use.

It would be indeed premature to conclude at the present, that the cellular mechanisms which apparently inhibit MC proliferation and decrease macrophage influx in the acute phase of MSGN administered with antagonists of PDE-III and IV in vivo, are identical to effects which these antagonists exhibit upon MC proliferation in vitro (9), generation of ROM (16, 17) and induction of MCP-1 (21). For example, the presence of platelets apparently plays an important role in very early phases (1, 8) of development of MSGN elicited by ATS injection (39) and PDE-III inhibitors are well known to antagonize platelet aggregation and adhesion (40). It should be thus considered that some of the observed suppressive effect(s) of PDE-III isozyme antagonist in vivo upon development of MSGN reported herein, may be due, at least in part, to its dampening influence upon platelet functions, although the finding that the degree of platelet deposition in glomeruli was not altered by this therapy seems to argue against it (Results). In any case, the cellular pharmacologic and pathophysiologic mechanism by which antagonists of PDE isozyme III and IV exhibited marked suppressive effects upon the development of the acute phase of MSGN is a subject which deserves carefully planned and intense future experimental studies, with the eventual aim to design targeted pharmacotherapy of various glomerular diseases. It should be stressed that it will be of major importance to determine whether PDE isozyme antagonists will be able to reverse pathologic changes in already established MSGN.

## Acknowledgments

This study was supported by U.S. Public Health Service, National Institute of Diabetes and Digestive and Kidney Diseases grants DK-16105 and DK-30795 (T.P. Dousa); DK-02918 and DK-43422 (R.J. Johnson), and by the Mayo Foundation. Ms. Mary E. Bennett provided excellent secretarial assistance. Dr. R. Alvarez (Syntex Research, Palo Alto, CA) kindly provided helpful advice concerning formulation of lixazinone.

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