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Role of Cyclic AMP in Idiopathic Nephrotic Syndrome: A Pathway Involving a Decrease in Glomerular Cell Heparan Sulfates?

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Abstract The physiopathological mechanisms of idiopathic nephrotic syndrome involve a circulating plasma factor and a decrease in HS in the glomerular basement membrane. Previous studies have demonstrated that plasma from patients with INS decreases glomerular cell HS in vitro. We examined the involvement of cyclic adenosine monophosphate (cAMP) in this interaction. We studied the effect of plasma from patients with INS on mesangial cell cAMP. We also determined mesangial cell HS when cAMP levels were modified using a cationic membrane after metabolic labeling. Cellular cAMP levels increased significantly when mesangial cells were incubated with plasma from patients with INS in comparison with control plasma (+77%, $P = 0.01$). Forskolin and IBMX, which increased cellular cAMP, decreased HS levels ($-21 \pm 9\%$ and $-15 \pm 6\%$ respectively, $P < 0.05$ for both), whereas dideoxyadenosine, which decreased cellular cAMP, increased HS levels ($+24 \pm 7\%$, $P < 0.05$). Plasma from patients with INS decreased glomerular cell HS in comparison with control plasma ($-34 \pm 8\%$, $P < 0,05$). This effect was abolished when cells were preincubated with ddAdo to prevent an increase in cAMP levels. We conclude that in mesangial cells, plasma from patients with INS increases cAMP levels, and that cAMP mediates a decrease in HS levels. Moreover, the action of plasma from patients on HS was inhibited when an increase in cAMP was prevented. cAMP may therefore be instrumental in the negative effect of the plasma factor on mesangial cell HS. J. Cell. Biochem. 78: 363–370, 2000.

Key words: idiopathic nephrotic syndrome; heparan sulfate; cyclic AMP

INS is the most common glomerular disease in childhood [Churg et al., 1970]. In the vast majority of cases of INS in childhood, minimal change glomerulopathy is found, characterized by changes in the morphology of podocytes seen by electron microscopy. In some cases, focal segmental glomerulosclerosis is observed. Experimental and clinical data favor the view that a humoral factor is involved in patients with INS with minimal change nephrotic syndrome and with focal and segmental glomerulosclerosis. Transient proteinuria in newborns from women with INS [Lagrue et al., 1991], and a successful transplantation of idiopathic minimal change nephrotic syndrome kidneys into recipients without persistence or recurrence of minimal change nephrotic syndrome [Ali et al., 1994], suggest that a circulating factor may be present in patients with INS with minimal change nephrotic syndrome. Proteinuria in rats or rabbits after injection of sera from patients with INS [Boulton Jones et al., 1983; Zimmermann, 1984; Wilkinson et al., 1989] would favor the view that a humoral factor is involved in patients with INS with minimal change nephrotic syndrome and with focal segmental glomerulosclerosis. The relapse of proteinuria after renal transplantation and the remission after immunoadsorption of plasma from patients support this hypothesis [Dantal et al., 1994]. Moreover, serum from

Abbreviations used: CS, chondroitin sulfates; ddAdo, dideoxyadenosine; GAG, glycosaminoglycans; HS, heparan sulfates; IBMX, isobutyl-methylxanthine; INS, idiopathic nephrotic syndrome; NSAID, nonsteroidal anti-inflammatory drugs.

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TABLE I. Clinical Data

Patient no.	Diagnosis	Treatment	Urinary proteins/ creatinine (g/mmol)	Urinary proteins (g/l)	Plasma albumin (g/l)
1	INS–Rel	WT	0.52	3.33	9.43
2	INS–Init	WT	1.33	4.80	14.60
	INS–Rem	WT	0.0	NA	51.80
3	INS–Init	WT	0.98	3.50	12.60
4	INS–Rel	WT	2.45	NA	NA
5	INS–Rel	Predn.	0.80	NA	NA
6	INS–Rel	Predn.	0.14	1.10	32.00
7	INS–Rel	Predn.	0.60	3.00	15.10
8	INS–Rel	Predn.	0.80	1.12	NA
9	INS–Init	WT	NA	3.0	7.0
	INS–Rem	Predn.	NA	0.0	NA
10	INS–Init	WT	NA	3.0	16.0
	INS–Rem	Predn.	NA	0.0	NA
11	INS–Init	WT	NA	16.2	13.2
	INS–Rem	Predn.	NA	0.0	NA
12	INS–Init	WT	NA	12.8	13.2
	INS–Rem	Predn.	NA	0.0	NA

INS, idiopathic nephrotic syndrome; Init, initial episode; Rel, relapse; Rem, remission; WT, without treatment; Predn., treated with prednisolone; NA, not available.

patients increases glomerular permeability to albumin in an in vitro model [Savin et al., 1996]. Animal and in vitro models have shown that HS are involved in the glomerular permeability to albumin [Rosenzweig and Kanwar, 1982; van den Born et al., 1992]. Moreover, HS are decreased in the glomerular basal membrane of patients with INS [van den Born et al., 1993]. We have recently demonstrated that plasma from children with INS is able to decrease in vitro glomerular epithelial cell and mesangial cell GAG, particularly HS [Girardin et al., 1998]. A circulating factor in the plasma of patients with INS might reduce glomerular HS by its effect on glomerular cells. The cellular mechanism leading to this decrease in HS has not yet been identified.

Cyclic adenosine monophosphate (cAMP) and agents increasing intracellular cAMP decreased GAG production in different cell types, particularly in bovine aortic endothelial cells [Kaji et al., 1996]. Furthermore, cAMP regulates basement membrane HS proteoglycan metabolism in glomerular epithelial cells [Ko et al., 1996]. cAMP might be involved in the cellular regulation of GAG metabolism.

The aim of this study was to determine whether cAMP mediates the decreased synthesis of HS by glomerular cells incubated with plasma from patients with INS. We investigated in vitro the effects of plasma from children with INS on cAMP levels in mesangial cells as well as the effects of cAMP on mesangial cell GAG, particularly HS. Finally, we examined the effects of plasma from children with INS on mesangial cell HS when variations in cellular cAMP were prevented.

METHODS

Study Subjects

The definition of INS and of the response to steroid therapy were those used by the International Study for Kidney Disease in Children [1981]. Plasma was obtained from 12 children with INS—6 in the initial episode, 6 in relapse, with or without treatment, and 5 in remission of the nephrotic syndrome. They all had a typical presentation and were steroid sensitive. The clinical data of these patients are shown in Table I. Blood samples were collected on EDTA and plasma was stored at −20°C until processed. The controls consisted of a pool of plasma obtained from 6 adults without proteinuria or medication (median age 35 years; range, 28–40 years). Preliminary experiments showed minor variations in GAG and HS production by mesangial cells in contact with these plasma [Girardin et al., 1998]. They were then pooled and the same pool of control

plasma was used throughout the study. Preliminary studies showed that the age of controls and study subjects had no influence on GAG production by glomerular cells within the group of controls and within each group studied [Girardin et al., 1998]. Plasma from patients with INS and the pooled plasma from normal donors were processed in the same conditions. The study was approved by the Ethics Committee of our institution.

Cell Culture

Confluent monolayers of mesangial cells were obtained from Sprague-Dawley rat kidneys as previously described [Harper et al., 1984].

The cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco), supplemented with 10% fetal calf serum at 37°C and in an atmosphere of 5% CO_2. Subcultures from the 5th to the 12th passages were used.

Quantification of cAMP in Cell Monolayers

Mesangial cells were cultured to confluent monolayers in 25 cm^2 culture flasks. They were rinsed with Hanks' balanced salt solution (Gibco), preincubated with 0.1 mM IBMX (Sigma) in DMEM medium for 30 min at 37°C, 5% CO_2 and further incubated for various times with DMEM medium containing 5% plasma from patients or from a pooled plasma from normal donors. Immediately after incubation, flasks were put on ice for 10 min. The medium was eliminated, and 0.5 ml of 1N cold perchloric acid was added. Cell monolayers were scraped in perchloric acid and sonicated for 3 × 10 s, before being centrifuged for 10 min at 2,000 *g*, at 4°C. The supernatant was neutralized with KOH 9N to obtain pH = 7. An aliquot of each of these samples was prepared for determination of cAMP concentrations following the instructions of the cAMP RIA kit (Immunotech).

Protein levels of the supernatant and of the pellet were measured for each test condition using the Biorad protein assay based on the Bradford dye-binding procedure (BioRad). Each amount of cAMP (in nanomolar) was expressed in relation to the amount of protein (in milligrams) of the same sample.

Quantification of GAG and HS

Mesangial cells were cultured to confluent monolayers in 24-well plates. They were incubated with agents increasing cellular cAMP: 0.1 mM forskolin (Sigma) or 0.05 mM IBMX; or agents decreasing cellular cAMP: 0.1 mM ddAdo (Sigma) for 12 or 24 h. Cells were incubated with these agents in SO_4-free medium containing 50 μCi/ml $Na_2\ ^{35}SO_4$ (Amersham, specific activity: 1,050–1,600 Ci/mmol) for a metabolic labeling of GAG. Each test condition was studied in duplicate. The mesangial cell GAG, HS, and chondroitin/dermatan sulfates (CS) were quantified as previously described [Rapraeger and Yeaman, 1989; Girardin et al., 1998]. Briefly, cellular and in the culture medium-secreted GAG were extracted in Tris buffer containing 8 M urea and 0.1% Triton X-100, heated at 90°C for 20 min, and centrifuged for 15 min at 5,000 *g*. GAG were then bound to a cationic nylon membrane (Zetaprobe, Bio-Rad Laboratories) using a dot-blot technique. Duplicates were pulled through the blot by vacuum. One sample was incubated in Tris buffer containing 0.65 M NaCl for one hour, washed and dried. The other sample was washed and incubated for 2 × 90 min with nitrous acid to depolymerize and release heparan sulfate chains [Lindahl et al., 1973], and then treated as the first fraction. In these test conditions, only GAG bearing CS remained bound to the membrane. The blots were exposed overnight to X-OMAT AR film (Eastman Kodak) for autoradiography and then cut for scintillation counting. The amount of HS was evaluated by the difference between total cpm and cpm obtained after nitrous acid treatment. The method was validated by comparison with diethylaminoethyl-Sephacel chromatography and gel filtration [Shworak et al., 1994; Girardin et al., 1998]. For all experiments, control cells were grown in parallel in exactly the same experimental conditions. These cells were scraped and dissolved in Tris buffer containing 2 M urea, and the amounts of cell protein in each well were measured using the Bio-Rad protein assay.

The result of each well was given in cpm/μg of cell protein for each test or control situation. Results were expressed as the mean of the duplicate. To minimize the variations induced by the half-life of ^{35}S for GAG, data were also expressed by the ratio of each test condition divided by the corresponding control condition.

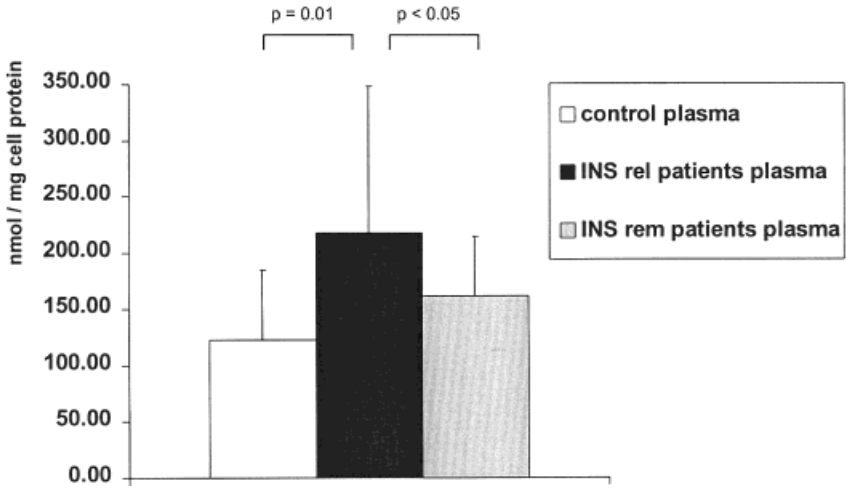

Fig. 1. cAMP levels of mesangial cell monolayers incubated with plasma from patients with INS or pooled plasma from normal donors. After preincubation with IBMX, confluent monolayers were incubated for 4 h with medium containing 5% plasma from pooled plasma from normal donors (control plasma) from patients with INS (INS rel patients plasma) or from patients with INS in remission from the disease (INS rem patients plasma). cAMP was extracted with perchloric acid, and the measurements were performed using a radioimmunoassay. Each concentration of cAMP (in nanomolar) was divided by the concentration of proteins (in milligrams) from the same cell monolayer. The results given are mean ± SE. n = 11 for control, n = 10 for INS rel patients, n = 5 for INS rem plasma. For abbreviations, see list.

Quantification of HS of Cells Incubated With Plasma After Preincubation With ddAdo

Confluent monolayers of mesangial cells were preincubated with 0.1 mM ddAdo in DMEM for 30 min at 37°C, 5% CO_2 to prevent an increase in cAMP levels. Each test condition was studied in duplicate. The supernatants were then removed and the monolayers were incubated with medium containing 0.1 mM ddAdo and 5% plasma from patients with INS or normal plasma in medium for 24 h. The quantification of HS was determined as described.

Mesangial cells produced the same amounts of HS in the presence of normal plasma with or without ddAdo.

The result of each well was given in cpm/μg of cell protein for each test condition divided by cpm/μg of cell protein for the control medium. Results were expressed as the mean of the duplicate.

Statistics

Data are expressed as the mean ± SE. n represents the number of experiments. The Wilcoxon signed-rank test was used to evaluate the significance of the results.

RESULTS

Effects of Plasma From Patients With INS on Mesangial Cell cAMP Levels

As shown in Figure 1, when mesangial cells were incubated for 4 h with 5% plasma from a pool of normal donors, the level of cAMP was 123.1 ± 74.5 nmol/mg protein (n = 11). The level increased significantly to 217.8 ± 163.5 nmol/mg protein (n = 10) when cells were incubated with 5% plasma from children with INS ($P = 0.01$). There was no difference between patients with INS treated by steroids and those who were untreated. cAMP increased in the first hour in the presence of plasma from patients with INS compared to control plasma, but this effect was maximal after 4 h. When mesangial cells were cultured with 5% plasma of children in remission of the nephrotic syndrome, we did not observe an increase in cAMP levels: 161.5 ± 48.3 nmol/mg protein, n = 5, NS in comparison with control plasma. Cyclic AMP levels were significantly different when cells were cultured with plasma from children in relapse in comparison with plasma from the same children but in remission of the disease ($P < 0.05$). When mesangial cells were cultured with 5% plasma from patients with membranous glomerulopathy and

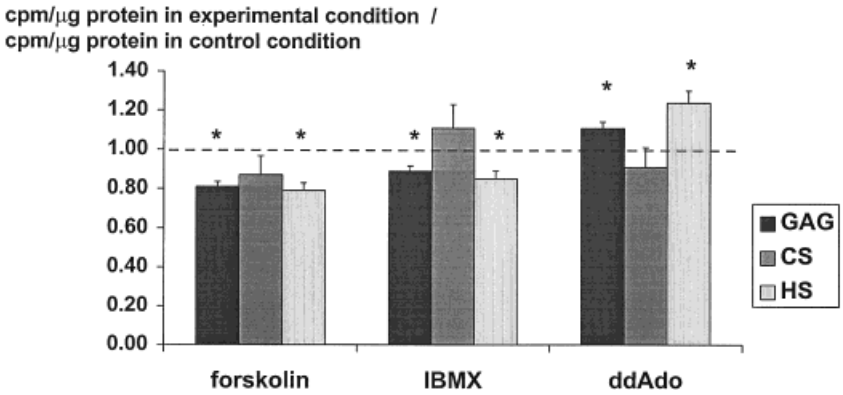

Fig. 2. Mesangial cell GAG, HS, and CS after incubation of cells with 0.1 mM forskolin, 0.05 mM IBMX, or 0.1 mM ddAdo. GAG, HS, and CS were quantified using a dot-blot technique after metabolic labeling, as described in the Methods section. The results are expressed as the mean ± SE of the ratios of the cpm/μg of proteins when cells were incubated with agents modifying cAMP levels divided by cpm/μg of proteins when cells were cultured in control medium. *$P < 0.05$ in comparison with control medium. n = 6. The horizontal line represents a ratio of 1, no change in GAG, HS, and CS levels. For abbreviations, see list.

IgA nephropathy, there was no modification of cAMP levels.

Effects of Modifications in cAMP Levels on Mesangial Cell GAG, HS and CS

Preliminary studies showed variations in cAMP levels of mesangial cell monolayers after 4 h incubation with forskolin (+77%, n = 2), with IBMX (+58%, n = 3), and with ddAdo (−20%, n = 2).

When mesangial cells were incubated with 0.05 mM IBMX or 0.1 mM forskolin for 12 h, GAG decreased, and the mean ratios were 0.89 ± 0.05 ($P < 0.05$) and 0.81 ± 0.06 ($P < 0.05$), respectively (Fig. 2). In the same conditions HS decreased, the mean ratios were 0.85 ± 0.06 ($P < 0.05$) with IBMX and 0.79 ± 0.09 ($P < 0.05$) with forskolin (Fig. 2). Primary data for HS were 156 ± 44 cpm/μg when cells were cultured in control medium versus 132 ± 53 cpm/μg with IBMX; 305 ± 203 cpm/μg with control medium versus 241 ± 167 cpm/μg with forskolin. In contrast, CS were unchanged, and the mean ratios were 1.11 ± 0.29 (NS) and 0.87 ± 0.15 (NS) with IBMX and forskolin, respectively (Fig. 2). These effects were transient and had disappeared when the cells were incubated with forskolin or IBMX in the same conditions but for 24 h.

When mesangial cells were cultured with 0.1 mM ddAdo for 24 h, we observed an increase in GAG and HS, the mean ratios being 1.11 ± 0.04 ($p < 0.05$) and 1.24 ± 0.07 ($p < 0.05$), respectively when compared to control medium (Fig. 2). Primary data for HS were 388 ± 220cpm/μg with control medium vs 481 ± 319 cpm/μg with ddAdo. CS were unchanged in these conditions, the mean ratio was 0.91 ± 0.11 (NS).

Effects of Plasma From Patients With INS on Mesangial Cell HS After Preincubation With ddAdo

The results of the effect of INS plasma on mesangial cell HS when cells were preincubated with 0.01 mM ddAdo are shown in Figure 3.

Plasma from patients with INS decreased significantly mesangial cell HS (0.28 ± 0.05 with INS plasma versus 0.42 ± 0.07 with plasma from a pool of normal donors, $P = 0.04$, n = 6). There was no difference between Patients with INS treated by steroids and those who were untreated. The decrease in HS induced by INS plasma was abolished in the presence of ddAdo (0.40 ± 0.09 with INS plasma versus 0.43 ± 0.06 with plasma from a pool of normal donors, NS, n = 6). The effect of plasma from patients with INS on mesangial cell HS was significantly different without ddAdo and after preincubation of cells with 0.1 mM ddAdo ($P = 0.03$).

DISCUSSION

The involvement of cAMP in the physiopathological mechanism of INS is not known. Indeed, it has previously been suggested that prostaglandins decrease HS proteoglycans via

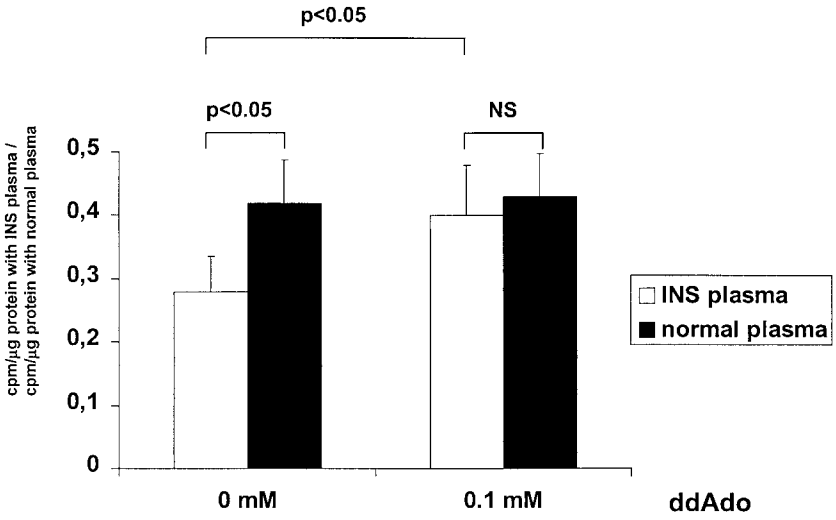

Fig. 3. Effects of plasma from patients with INS on mesangial cell HS after preincubation of cells with ddAdo to prevent an increase in cAMP levels. Cells were preincubated with 0.1 mM ddAdo for 30 min before incubation with 5% plasma from patients with INS or pooled plasma from normal donors for 24 h. HS were quantified using a dot-blot technique after metabolic labeling, as described in the Methods section. The results are expressed as the mean ± SE of the ratios of the cpm/µg of proteins when cells were incubated in the test condition divided by cpm/µg of proteins when cells were incubated with control medium and without ddAdo. n = 6. For abbreviations, see list.

a cAMP-dependent pathway, and prostaglandins might be involved in nephrotic syndrome [Dunn, 1990; Ko et al., 1996]. In this study, we demonstrated in vitro that plasma from patients with INS was able to increase cAMP levels in mesangial cells in comparison with pooled plasma from normal donors. In contrast, plasma from the same patients but in remission from the disease did not modify cAMP levels. The involvement of one or more plasma-born factors in the physiopathological mechanism in INS, minimal change nephrotic syndrome, and segmental and focal glomerulosclerosis has been suggested by various studies [Boulton Jones et al., 1983; Zimmermann, 1984; Wilkinson et al., 1989; Lagrue et al., 1991; Ali et al., 1994; Dantal et al., 1994; Savin et al., 1996]. This is the first study that emphasizes modifications in cAMP induced by plasma from patients with INS.

We have previously shown in vitro that plasma from patients with INS decreases glomerular cell GAG, especially HS, in mesangial cells and epithelial cells, and thus might increase glomerular permeability to albumin [Girardin et al., 1998]. One could hypothesize that cAMP might be a pathway involved in the effect of plasma from patients on glomerular cell HS. The effect of plasma from patients with INS was similar on human glomerular epithelial cell HS and on rat mesangial cell HS, so there is probably no species barrier concerning the plasma-born factor in INS, as a similar effect of human plasma on human and rat glomerular cells was observed [Girardin et al., 1998]. In preliminary studies, we observed that the increased cAMP levels induced by plasma from patients with INS was similar in human glomerular epithelial cells and in rat mesangial cells. For this reason, we examined the involvement of cAMP on rat mesangial cells.

Because plasma from patients increased cAMP levels, we determined the effect of modifications in cAMP levels on mesangial cell GAG, HS and CS. We showed that IBMX and forskolin decreased mesangial cell GAG and HS, whereas ddAdo increased mesangial cell GAG and HS. These results suggested that higher levels of cAMP decreased GAG and HS, whereas lower cAMP levels raised GAG and HS. Mesangial cell CS were unchanged. These results were in agreement with previous reports, which showed that agents increasing cellular levels of cAMP decreased GAG production, particularly in endothelial cells [Kaji et al., 1996]. Moreover, when cAMP levels were increased after stimulation by prostaglandins, decreases in HS proteoglycan and in perlecan gene expression were observed in glomerular epithelial cells [Ko et al., 1996]. We showed not only a decrease in HS and GAG levels when cAMP levels were increased, but also an increase in HS and GAG levels when mesangial cells were incubated with ddAdo, indicating that variations in cAMP levels inversely affected HS levels.

Finally, we showed that when the synthesis of cAMP was prevented by the inhibition of adenylate cyclase, the decrease in mesangial cell HS induced by INS plasma was inhibited. These data demonstrated that the effect of INS

plasma on mesangial cell HS levels was transduced via a cAMP pathway.

cAMP might by this way be involved in the physiopathology of proteinuria in INS. Indeed, GAG, and especially HS, are major elements in glomerular permeability to albumin [Kanwar et al., 1980; Goode et al., 1991], and monoclonal antibodies against HS or a decrease in HS immediately induce proteinuria in rats and increase permeability to albumin [Rosenzweig and Kanwar, 1982, van den Born et al., 1992]. HS of the glomerular basement membrane are secreted by glomerular cells, particularly by mesangial cells [Thomas et al., 1995]. Plasma from patients with INS decreases glomerular epithelial cell and mesangial cell HS in vitro [Girardin et al., 1998]. These plasma might therefore increase glomerular permeability to albumin via a decrease in HS in the glomerular basement membrane. Also, cAMP might be involved in physiopathology of INS via its effect on glomerular HS.

However, cAMP could also be involved in INS independently of GAG and HS synthesis; indeed, plasma from patients with INS increased cAMP levels in mesangial cells. One pathway might be an interaction with phosphorylation mechanisms. Changes in the phosphorylation processes might be involved in podocyte foot process effacement in experimental nephrotic syndrome in rats [Smoyer and Mundel, 1998].

The involvement of cAMP in the mechanism of proteinuria could partially explain the beneficial effect of NSAID in INS [Dunn, 1990; Low et al., 1997], because prostaglandins increased cellular cAMP levels [Ko et al., 1996]. The inhibition of prostaglandins decreases proteinuria through vasoconstriction of glomerular capillaries and a decrease in filtration. It could be hypothesized that the inhibition of prostaglandins could also have a beneficial effect on proteinuria through a decrease in cAMP levels. The effects of NSAID on glomerular filtration of proteins are very intricate; indeed, on the one hand NSAID could reduce proteinuria in some cases, and on the other hand NSAID were able to induce proteinuria with minimal change nephropathy, membranous glomerulonephritis, focal sclerosis, and other glomerulonephritis [Ravnskov, 1999]. We pointed out that the involvement of cAMP in proteinuria could partially explain the beneficial effect of NSAID in INS through an inhibition of prostaglandins and consequently an inhibition of cAMP. However, this may only be a minor mechanism of the beneficial action of NSAID on proteinuria in INS; the major mechanism is a vasoconstriction of glomerular capillaries and consequently a decrease in filtration.

From our in vitro data, we conclude that cAMP may be involved in the physiopathological mechanisms of INS, in particular via a pathway including alterations of HS synthesis by glomerular cells.

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