Urinary excretion of platelet-activating factor in human and experimental nephrosis

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Abstract

Background. Platelet-activating factor (PAF) is a phospholipid that has been implicated in the pathogenesis of glomerulonephritis and can be synthesized by glomerular cells in response to different stimuli. PAF increases glomerular permeability to proteins and urinary PAF has been determined to be of renal origin. In order to assess whether urinary PAF can be found augmented in situations of glomerular damage without glomerular leukocyte infiltration, urinary PAF was quantified in human and experimental nephrosis.

Methods. Urinary PAF was quantified by platelet bioassay and glomerular PAF by incorporation of $^{3}H$-acetate into PAF. PAF was characterized by its behaviour on thin-layer chromatography and high performance liquid chromatography and the blockade of its bioactivity by specific receptor antagonists.

Results. Urinary PAF excretion was significantly higher in patients with active idiopathic nephrotic syndrome than in controls (5.8±1.5 versus 1.7±0.75 ng/24 h; P<0.05) and patients in remission (1.63±0.75 ng/24h; P<0.02). In rats with nephrosis induced by puromycin aminonucleoside there was an early increase in urinary PAF excretion (138±19 versus 49±22 pg/24h in controls; P<0.035) that coincided with the augmented glomerular PAF synthesis (67±3.4 versus 36±1.2 DPM/mg protein in controls; P<0.003).

Conclusions. These results suggest that the synthesis of PAF in the kidney may be involved in the pathogenesis of the proteinuria in idiopathic nephrotic syndrome and that urinary PAF excretion may be a good marker of disease activity.

Key words: nephrosis; platelet-activating factor; urine

Introduction

Platelet-activating factor (PAF) is a phospholipid mediator of inflammation that can be synthesized, after appropriate stimulation, by a variety of cells including monocytes, macrophages, neutrophils, endothelial cells, isolated rat glomeruli and cultured glomerular mesangial and epithelial cells (reviewed in [1]).

This phospholipid exerts a wide range of biological activities: it induces aggregation of platelets, promotes chemotaxis of neutrophils and monocytes, causes rapid stimulation of prostaglandin and oxygen radicals synthesis, and provokes cell contraction in cultured glomerular mesangial cells [2]. Moreover, when injected into the abdominal aorta of rabbits or into isolated perfused rat kidneys, PAF enhances vascular permeability and proteinuria and modifies the size and charge selectivity of the glomerular filtration barrier [3].

Recent data strongly suggest that PAF is one of the mediators released early during glomerular injury, that induces the production of other mediators and expands the possibility of further tissue damage [4]. In this context we have documented that isolated glomeruli from experimental animals with adriamycin or puromycin aminonucleoside (PAN) nephrosis synthesize significantly more PAF than normal glomeruli, and the maximal PAF production precedes the peak of proteinuria [5]. Furthermore, treatment with PAF antagonists induced an improvement in proteinuria and in glomerular lesions in models of immune and nonimmune glomerulonephritis [6,7].

The idiopathic nephrotic syndrome (minimal change nephropathy and its variants) is characterized by massive proteinuria with fusion of epithelial cell processes and partial loss of sialoglyprotein staining. However, its pathogenesis is not completely understood. In recent years it has been speculated that a lymphokine released by circulating blood mononuclear cells could alter the permeability of the glomerular capillary wall. Our recent data show that patients with minimal change disease have an increase in tumour necrosis factor (TNFα) serum levels and production by monocytes when they are in activity [8]. TNFα can induce PAF release by a variety of cells including renal cells [4].

Our laboratory first reported the presence of PAF in urine from normotensive healthy controls and patients with lupus nephritis [9]. Recent studies have
demonstrated that urinary PAF comes mainly from the renal synthesis and that circulating PAF is not recovered in urine even in situations of increased glomerular permeability [10], and therefore urinary PAF seems to be a good marker of the renal synthesis of this mediator.

In proliferative glomerulonephritis infiltrating cells have often been implicated in the synthesis of mediators such as PAF. The present study was undertaken in patients with idiopathic nephrotic syndrome and in rats with PAN nephrosis with the aim to establish a potential relationship between urinary PAF excretion and clinical activity of the disease in situations of glomerular damage without glomerular leukocyte infiltration.

**Subjects and methods**

**Patients**

Fourteen different children with idiopathic nephrotic syndrome (five of them were studied both during activity and during remission, which account for the total n = 19). A group of six controls of similar age was also studied. All patients were steroid responsive and they were divided into two groups according to the activity of the disease at the moment of the study: (1) Patients in activity (n = 9; proteinuria over 40 mg/h/m²); (2) Patients in remission (n = 10; proteinuria under 4 mg/h per m²).

The clinical data of the patients are shown in Table 1.

**Experimental nephrosis**

Female Wistar rats weighing 200–225 g were injected through the tail vein with a single dose of 150 mg/kg body weight of puromycin aminonucleoside (PAN) (Sigma, St Louis, MO). The increase in proteinuria and serum cholesterol and the decrease in serum proteins presented the maximum on day 8 after injection. A group of control rats was also studied in the same conditions along the whole experiment. In a previous work we demonstrated that in this model, glomerular PAF production is normal), after PAN injection.

The amount of PAF in each sample was determined by its ability to elicit the release of [3H]Serotonin (NEN, Boston, Ma, USA) from labelled rabbit platelets [13]. Briefly, platelets were incubated for 30 min at 37°C with 1 μCi/ml of [3H]serotonin and then washed twice in Tyrode's buffer, resuspended in a Tris-buffered medium containing 1 mM Ca²⁺ and Mg²⁺, and the PAF extracts were added. The percentage of release induced by each of the samples was then compared with a standard curve of the release elicited by known amounts of synthetic PAF (Sigma). The amount of PAF in each sample was expressed as nanogram-equivalents of the synthetic PAF.

**Glorimerular PAF quantification**

Rat glomeruli were isolated according to a previously published technique based on the ability of glomeruli to pass through a 105-μm sieve and to be retained in a 75 μm sieve [15]. The glomerular suspension was digested by 20 units/ml of collagenase type IA (290 U/mg)(Boehringer Mannheim, Germany) in 4 ml of HEPES buffer for 60 min at room temperature and then washed twice. Then, glomeruli were resuspended in HEPES 0.1% glucose, 0.25% bovine serum albumin (BSA) and PAF production was estimated from the ability to incorporate [3H]-acetate acid into [3H]-PAF via an acetyl transferase [15]. Glomeruli were incubated with 50 μCi of [3H]-acetate acid sodium salt (3.6 Ci/mmol, Dupont, Germany) for 30 min at 37°C. In each experiment some samples were incubated with 50 μM calcium ionophore A23187 (Sigma) as a positive control. The reaction was stopped by adding a mixture of chloroform-acidified methanol. Lipids were extracted with a 1:1:0.9 mixture of chloroform, methanol, and water (50:25:8:4, vol/vol). The area of the plates migrating with a standard of synthetic PAF (a mixture of C18 and C16, Sigma) was scraped off, eluted and resuspended in a HEPES-buffered medium for testing biological activities.

**Bioassay of PAF**

Twenty-four-hour urine samples were collected from normal volunteers and from patients with nephrotic syndrome. Rats were placed in metabolic cages and 24-h urine samples were collected on days 1 and 2. The samples were concentrated threefold and the extraction of the lipid fraction was then performed according to the procedure described by Pinckard et al. [11]. Briefly, 4 volumes of methanol were added to 1 volume of urine, and after standing at room temperature for 30 min the methanolic extracts were centrifuged to precipitate insoluble proteins. Chloroform and water were then added to obtain a solvent mixture containing chloroform, methanol, and water (1:1:0.9, vol/vol). The chloroform layer was removed and evaporated to dryness under nitrogen stream. Thin layer chromatography (TLC) was performed with the lipid extracts on analytical silica gel plates of 2 mm thickness (Merck, Darmstadt, Germany) as previously described [12], by using the following solvent system: chloroform, methanol, acetic acid, and water (50:25:8:4, vol/vol). The area of the plates migrating with a standard of synthetic PAF (a mixture of C18 and C16, Sigma) was scraped off, eluted and resuspended in a HEPES-buffered medium for testing biological activities.

**Table 1. Clinical data of patients**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Remission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Episodes studied</td>
<td>9</td>
</tr>
<tr>
<td>No treatment</td>
<td>9</td>
</tr>
<tr>
<td>Prednisone treated</td>
<td>—</td>
</tr>
<tr>
<td>CsA-treated</td>
<td>—</td>
</tr>
<tr>
<td>Age (years)</td>
<td>9.2 ± 4.3</td>
</tr>
<tr>
<td>Proteinuria (mg/kg per day)</td>
<td>160 ± 33</td>
</tr>
<tr>
<td>Serum albumin (g/dl)</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Total serum proteins (g/dl)</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.7 ± 0.25</td>
</tr>
<tr>
<td>Glomerular filtration rate (ml/min per 1.73 m²)</td>
<td>120 ± 29</td>
</tr>
</tbody>
</table>
for radioactivity. Results were expressed as DPM/mg glomerular protein.

Characterization of PAF

The characterization of PAF was based on: (1) the chromatographic behaviour of the samples on TLC. The Rf of the samples was the same as the standard PAF included in each experiment (Rf = 0.45); (2) high-performance liquid chromatography was performed in selected samples using a dual-pump Kontron Model 420 (Kontron Instruments, Zurich, Switzerland) with a 220 x 4.6-mm Spheri-5 Silica column as previously described [16]. The retention time of the glomerular 3H-PAF was 23 min and identical to a synthetic labeled PAF (C16-C18 mixture, Amersham, Buckinghamshire, UK). PAF bioactivity was determined in the urine fractions eluting at 20, 21, 22, 23, 24, and 25 min and the maximal bioactivity was also found at minute 23 (Figure 1). (3) The platelet activating ability of the samples was blocked by two specific PAF receptor antagonists: BN52021 10⁻⁶M (a gift from Institute Henri Beaufour, Les Plessis Robinson, France) blocked PAF activity by 80% and WEB2086 10⁻⁶M, a hetrazepine, (Boehringer Ingelheim, Germany) blocked PAF activity by 85%.

Statistical analysis

Results are expressed as the mean ± SEM. Significance was established using GraphPAD InStat (GraphPAD Software). Wilcoxon non-parametric test was used to compare the data, and differences were considered significant when the two-tailed P value was <0.05.

Results

PAF bioactivity has been shown to be present in the urine of normal subjects [9]. We determined the urinary PAF excretion in a group of patients with idiopathic nephrotic syndrome in activity or in remission, and in controls. Urinary PAF excretion was significantly elevated in patients in clinical activity (5.8 ± 1.5 ng/24 h) compared to patients in remission (1.63 ± 0.75 ng/24 h; P = 0.02) and to controls (1.7 ± 0.75 ng/24 h; P < 0.05) (Figure 2).

On the whole there was a close relationship between the urinary PAF excretion rate and the course of the disease. Thus, in a group of five patients in whom the urinary PAF excretion could be studied on two occasions, the maximal urinary excretion coincided in all of them with the disease relapse (Figure 3).

In order to quantify the amount of labelled PAF recovered at the end of the procedure, 150000 c.p.m. of ³H-labelled synthetic PAF (Amersham, UK) were added to some nephrotic and control urine samples. Recovery rates were 45 and 65% respectively. As the addition of BSA (40 mg/ml) to normal urine did not alter the recovery of labelled PAF, the lowest PAF recovery in nephrotic urine does not seem to be related to the presence of protein in urine.

Recent studies have described elevated levels of urinary PAF in mice with lupus nephritis [17]. The fact that PAF injected into the renal artery is recovered...
Urinary PAF in nephrosis

![Graphs showing urinary and glomerular PAF levels in PAN injected and control animals.](image)

**Fig. 4.** Urinary excretion and glomerular production of PAF in rats with puromycin nephrosis. Urinary PAF is expressed as picograms of PAF excreted during 24 h and glomerular PAF as DPM per milligram of glomerular protein. Both studies were done on days 1 after PAN injection. Each bar represents the mean of four to six animals ± SEM. (*) $P<0.035$; (**) $P<0.003$.

in negligible amounts in the urine, even in animals with increased glomerular permeability, suggests that urinary PAF may be of renal origin [10]. In rats injected with PAN (150 mg/kg) urinary PAF excretion was significantly elevated on day 1 after PAN injection (138 ± 19 pg/24 h) compared to controls (49 ± 22 pg/24 h; $P<0.035$) coinciding with the maximal glomerular PAF production in this model (67 ± 3.4 versus 36 ± 1.2 DPM/mg protein; $P<0.003$) (Figure 4). On day 2 both urinary excretion and glomerular production were in the same range in PAN injected and control animals (not shown).

**Discussion**

The availability of specific PAF antagonists has made it possible to assess the role of PAF in several experimental models of glomerular injury associated with platelet and neutrophil infiltration [17,18]. It has been suggested that PAF antagonists may interfere with the PAF-induced release of cationic proteins from neutrophils and platelets, thus reducing proteinuria [4]. Furthermore we have previously observed a beneficial effect of PAF antagonists in PAN and adriamycin nephrosis [5], two experimental models of human idiopathic nephrotic syndrome characterized by the absence of leukocyte infiltration. This raises the question of a possible role of PAF in human nephrosis.

In this paper we have shown that urinary PAF excretion was significantly increased in paediatric patients with idiopathic nephrotic syndrome when they are in activity versus patients in remission and controls. Furthermore, when the same patients were studied during activity and remission, the latter was accompanied by decrements in urinary PAF excretion. The amount of PAF found by us in urine is similar to that previously reported by some authors (1.2–4 pg/ml) [19] although lower than the amount reported by others [20]. Differences may be ascribed to the paediatric age of our subjects and/or the quantification method used (bioassay versus RIA).

Increased urinary PAF excretion probably reflects increased renal PAF production. Previous studies in animals have shown that circulating PAF is excreted in negligible amounts in the urine even in situations of increased glomerular permeability [10]. In this sense the finding that in rats with PAN nephrosis the maximal urinary PAF excretion coincides with the peak of glomerular PAF production on day 1, while both are lower on day 2, is consistent with the glomerular origin of urinary PAF. Since experimental and human nephrosis are characterized by an absence of glomerular inflammation, our data support the idea that resident glomerular cells could be the source of PAF. In fact, both cultured glomerular mesangial and epithelial cells release PAF [5].

Glomerular PAF production may play a role in the genesis of proteinuria. PAF injection into isolated perfused rat kidney enhances proteinuria [3]. Recently we have noted that PAF decreases the *in vitro* production of proteoglycans by cultured glomerular epithelial cells [21]. This effect may be modulated by ROS (reactive oxygen species), since PAF induces the generation of ROS in glomerular cells [22], and ROS are able to diminish specifically the *in vitro* and *in vivo* proteoglycan synthesis by glomerular cells [23]. PAF may also induce proteinuria indirectly via the release of inflammatory mediators from resident glomerular cells. In this sense we have shown that the increased production of PAF in the glomeruli of rats with nephrosis was followed by an increase, not only in proteinuria, but also in glomerular TNFα levels [5]. Interestingly, in those animals cyclosporin administration did normalize the glomerular production and urinary excretion of PAF and TNFα coincidentally with a diminution in proteinuria.

In conclusion, the present study shows that increased urinary excretion of PAF occurs in human and experimental minimal change nephrosis. The absence of glomerular infiltrating cells in these conditions suggests that intrinsic glomerular cells could participate in the PAF generation. Our results further strengthen the previous data suggesting that human glomerular disease in activity is associated with an excessive renal synthesis and urinary PAF excretion [20]. However,
this is the first demonstration of increased urinary PAF in a glomerular disease not mediated by immune complexes. Since in the experimental nephrosis urinary PAF precedes the appearance of proteinuria, further studies are needed to assess the potential value of urinary PAF determination as an early index of glomerular damage.

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