Demonstration of Antithrombotic Activity of Glomerular Adenosine Diphosphatase

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We have demonstrated that reduced glomerular adenosine diphosphatase (ADPase) activity within the rat kidney is associated with an increased thrombotic tendency. To establish a possible causal relationship between these intraglomerular events, experiments were conducted to inhibit adenosine diphosphatase (ADP) degradation without influencing other glomerular prothrombotic or antithrombotic mechanisms. Concurrently, we studied intraglomerular platelet aggregation. Two ways of selective inhibition of glomerular ADPase activity were applied: (1) by competitive substrates (ie, uridine diphosphate [UDP]), and (2) by the nondegradable ADP analogue ADP-β-S. Both strategies were used during ex vivo alternate perfusion of kidneys with platelets and ADP (to test intraglomerular thrombotic tendency). Each group (n = 6) received different substrates or a combination of substrates. A significant increase in platelet aggregation was observed in kidneys after perfusion with platelets and ADP together with the competitive substrate UDP as compared to perfusions with platelets and ADP alone (78.5% ± 9.8% vs 27.9% ± 11.4% glomeruli staining positive for platelets, P < .005). In contrast, UDP alone had no effect on platelet aggregation. Other nucleoside polyphosphates (guanosine diphosphate and inosine triphosphate) were also effective as competitive substrates in the ex vivo perfusion model (n = 4). None of these substrates was capable of increasing ADP-induced aggregation when studied in vitro. In addition, ADP-β-S also increased platelet aggregation in the perfusion model as compared with native ADP (P < .005). These results show that selective reduction of ADP degradation in intact kidneys strongly promotes the intraglomerular proaggregatory condition. It can be concluded that glomerular ADPase exerts potent antithrombotic activity within the normal rat kidney.

BLOOD VESEL walls possess the capacity to interfere directly with platelet aggregation, in addition to the anticoagulatory and anti-inflammatory mechanisms.1 Endothelial cells produce prostacyclin (PGI2) with potent antithrombotic activity,2 whereas the amplification signal adenosine diphosphate (ADP)3 is removed by membrane-associated ADPase activity.4 Although in vitro studies have demonstrated the potent antiaggregatory activity of ADPase,5,6 this antithrombotic mechanism has received relatively little attention. Recently, however, ADPase activity has been demonstrated in the glomerular basement membrane (GBM) of the rat kidney,6 and it was suggested that this enzyme subserves an important antithrombotic role. Thus, in rat kidneys with reduced ADPase activity (elicited within 24 hours by local radiation or adriamycin injection), platelet aggregation could be easily induced with alternate ex vivo perfusion of platelets and ADP.6

However, in the models described, it is difficult to prove a causal relationship between reduced ADPase activity on the one hand and increased thrombotic tendency on the other hand, as other prothrombotic or antithrombotic systems may also be affected by X-irradiation or adriamycin (ADR) treatment. It has been shown for instance that ADR-induced nephropathy is also associated with an increased intraglomerular thromboxane A2 (TXA2) production.8 Therefore, we now design experiments that enable us to study the antithrombotic role of glomerular ADPase, without influencing other vessel wall-associated hemostatic mechanisms, using two strategies. The first strategy to inhibit glomerular ADP degradation selectively was based on the observation that the enzyme exhibits nucleoside polyphosphatase activity; in addition to ADP, adenosine triphosphate (ATP), inosine triphosphate (ITP), or uridine diphosphate (UDP) can also serve as substrate for the enzyme.2 This observation opened the possibility of applying these as competitive substrates for ADPase activity in an ex vivo kidney perfusion system. An excess of UDP, for instance, supplemented to the ADP solution in the alternate perfusion system, would be expected to inhibit ADP degradation in intact kidneys due to the fact that excess of UDP rather than ADP is converted by glomerular phosphatases.

A second approach to inhibit ADP degradation selectively deals with the application of an ADP analogue (ADP-β-S) in the alternate kidney perfusion system. ADP-β-S is able to activate platelets but inhibits phosphatase activity by steric inhibition of the active site.9 In this case also enhanced thrombus formation can be expected in intact rat kidneys following alternate perfusion with platelets and ADP-β-S because, in contrast to perfusions with native ADP, the aggregation stimulus cannot be removed by the glomerular enzyme.

The results confirm the presence of nucleoside phosphatase activity, ie, ADPase and UDPase, cytochemically as well as biochemically within rat glomeruli and demonstrate the inhibitory effect of ADP-β-S on both ADPase and UDPase activity. Furthermore, data obtained by perfusion studies prove the antiaggregatory action of glomerular ADPase and suggest, in view of the importance of ADP as a mediator of platelet aggregation in vivo,2,10,11 a major role for this enzyme in preventing platelet aggregation and subsequent thrombus formation in the rat kidney in vivo.

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Supported by grants of the Dutch Kidney Foundation (C86-062).

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MATERIALS AND METHODS

**Animals**

Female PVG/c rats (200 to 220 g), 3 months old, were used throughout the study.

**Preparation of Platelet Suspensions**

Platelet-rich plasma (PRP) was prepared from citrated blood drawn from healthy volunteers as described previously. Platelets were kept in polyolefin bags (PL732; Baxter, Maarssen, The Netherlands) for a maximum of 5 days at 22°C and in a shaker to prevent aggregation. Before perfusion, PRP was centrifuged at 1,000g (15 minutes) and volumes were adjusted to a platelet number of 2.7 x 10^9 cells/mL. Platelet aggregability was routinely tested using an aggregometer. Batches not showing a standard response on ristocetin were discarded. Each batch of PRP was used for four ex vivo perfusion studies.

**Ex Vivo Perfusion**

Left kidneys of PVG/c rats were perfused at 37°C according to the method of Hoyer et al. Under halothane, O2/N2O, anesthesia. After removal of the blood with saline, kidneys were perfused with 4 mL endotoxin (1 μg/mL), 1 mL substrate in saline (see “Experimental Design”), 4 mL human PRP (2.7 x 10^9 cells/mL), and again with 1 mL substrate and 4 mL PRP using a peristaltic pump (flow rate 2 mL/min). Total perfusion time was 8 minutes and after perfusion kidney specimens were processed for histochemistry as described below.

**Histochemistry and Cytochemistry**

Tissue processing. Immediately after ex vivo perfusion with platelets and ADP, kidney specimens were fixed in 1% paraformaldehyde and embedded in plastic according to standard procedures. For cytochemical demonstration of ADPase and UDPase activity, kidneys were prepared according to standard procedures described elsewhere.

Enzyme cytochemistry. Phosphatase activity was demonstrated at the electron-microscopical level by staining of 30-μm vibratome sections using the cerium-based method. Sections were subsequently postfixed with OsO4 and embedded in Epon 812 (Serva, Heidelberg, Germany) according to standard procedures.

Platelet aggregation. Platelet aggregation was detected in 2-μm plastic sections by demonstrating fibrinogen binding to human platelets perfused through the rat kidney. On activation platelets expose membrane receptors for fibrinogen, leading to the binding of fibrinogen present in PRP. Subsequent conversion of fibrinogen is inhibited by citrate. Fibrinogen is demonstrated using indirect immunoperoxidase methods with rabbit-antihuman fibrinogen (Behringwerke AG, Marburg, Germany) according to standard methods.

**Biochemistry**

Glomerular nucleoside polyphosphatase activity was assayed in suspensions of glomeruli of normal rats. Kidneys were perfused with saline for 1 minute using a peristaltic pump (140 mm Hg pressure) and immediately snap frozen in freon (−80°C) for storage. Glomeruli were subsequently isolated from the cortex using standard sieving techniques with sieves of 180, 150, and 200 mesh, respectively. The whole isolation procedure was performed in 0.15 mol/L NaCl and at 4°C to preserve viability of glomeruli. Suspensions purified up to 90% as tested by lightmicroscopy were used for determination of enzyme activity. Phosphatase activity was assessed by measuring the amount of phosphate released by glomeruli after incubation with 2.5 mmol/L ADP, UDP, adenosine-5'-O-(2-thiodiphosphate) (ADP-β-S; Boehringer Mannheim GmbH, Mannheim, Germany) or a combination of substrates. Inorganic phosphate was subsequently measured according to the method of Chandrarajan and Klein. Phosphate release was related to the protein content of the suspension, measured according to the method of Lowry et al.

**Aggregometer Tests**

Substrates were tested in vitro for their potential to induce platelet aggregation using an aggregometer (Biodata Corp, Hatboro, PA; model PAP-4) according to standard procedures. Platelet suspensions were prepared by centrifugation (180g, 10 minutes) of fresh citrated blood drawn from healthy volunteers. ADP and ADP-β-S were used in concentration of 5 μmol/L, whereas UDP, guanosine diphosphate (GDP), and IPT were added in a final concentration of 50 or 300 μmol/L as indicated in the Results section.

**Experimental Design**

There were three different sets of experiments. Experiment A was performed to demonstrate glomerular phosphatase activity cytochemically as well as biochemically and to demonstrate the applicability of competitive substrates and ADP-β-S as inhibitors of glomerular ADPase activity. Experiments B and C were conducted to study intraglomerular platelet aggregation ex vivo during selective inhibition of glomerular ADP degradation by competitive substrates and ADP-β-S, respectively.

Experiment A. Glomeruli isolated from intact kidneys were incubated with the substrates ADP, UDP, ADP-β-S, or a combination of substrates. During the latter incubations ADP-β-S functioned as a steric inhibitor for the active site of glomerular ADPase and UDPase. For each isolation procedure (n = 5) six kidneys were used, harvested from three rats. Additionally, three rats were used for cytochemical demonstration of phosphatase activity.

Experiment B. The effect of ADPase inhibition by competitive substrates on intraglomerular thrombotic tendency was assessed in two groups of rats; a group with normal and a group with reduced glomerular ADPase activity induced by a single adriamycin injection (Adriblastina, Farmitalia; Belgium; 7.5 mg/kg body weight intravenously [IV]), 48 hours before the perfusion studies.

The thrombotic tendency was assessed by alternate kidney perfusion ex vivo with platelets and substrates. Each platelet batch was used for four perfusion studies using different substrates, excluding variations in platelet responsiveness between experimental groups. Kidneys were perfused with either 10 μg/mL ADP (group I; n = 6), 10 μg/mL ADP plus 100 μg/mL UDP (group II; n = 6), or 100 μg/mL UDP (group III; n = 6) as substrates or no substrate at all (group IV; n = 6). In an additional set of experiments (n = 4), the effect of other nucleoside diphosphates and triphosphates (GDP and ITP) was tested in the ex vivo perfusion system.

Moreover, direct effects of UDP, GDP, and ITP on platelet aggregation were also studied using an aggregometer (n = 5).

Experiment C. The effect of the ADP analogue ADP-β-S on platelet aggregation was studied in vitro using an aggregometer, whereas the effect of ADP-β-S on intraglomerular thrombotic tendency was studied in intact kidneys during alternate perfusion studies. Rats (n = 6) received standard alternate perfusion with either 10 μg/mL ADP-β-S or 10 μg/mL native ADP (n = 6).

**Quantitative Analysis**

Results of ex vivo perfusion studies were evaluated by counting the percentage of glomeruli per kidney, positive for platelet aggregates, which are readily detectable in plastic embedded rat tissue after staining for human fibrinogen. We scored the total number of glomeruli in each section containing aggregates, i.e,
more than one fibrinogen positive platelet clump, irrespective of their size, rather than to apply an arbitrary scale. This scoring resulted in a highly reproducible scoring procedure, although it overestimates the overall aggregation in kidneys with small aggregates. From each kidney, 75 glomeruli from three kidney sections were evaluated and the results expressed as the arithmetic means (±SD) of six kidneys per group. Data were analyzed by Wilcoxon’s test and considered significant at $P < .05$.

RESULTS

Experiment A

Photomicrographs of cytochemical staining for glomerular nucleoside polyphosphatase activity using the cerium-based method are shown in Fig 1. After incubation with ADP, reaction product can be demonstrated in normal rat kidneys along epithelial and endothelial plasma membranes as well as throughout the GBM. Incubation of kidney sections with UDP gives rise to the same localization of reaction product as compared to incubations with ADP (Fig 1B). In contrast, when ADP-β-S is used as the substrate, no reaction product at all can be demonstrated in kidney sections of normal rats (Fig 1C).

Figure 2 shows the results of biochemical studies of glomerular phosphatase activity. The results demonstrate a glomerular phosphate release of $2.63 \pm 0.22$ mmol/L of inorganic phosphate (Pi) per milligram of protein during incubation with ADP, which is similar to glomerular phosphate release after incubation with UDP ($2.64 \pm 0.53$ mmol/L Pi/mg protein). In contrast, after incubations with ADP-β-S as substrate no inorganic phosphate following a 30-minute incubation period can be detected ($0.00 \pm 0.00$ mmol/L Pi/mg protein). Moreover, after incubation of glomeruli with ADP-β-S in combination with ADP, a significant reduction in the amount of free phosphate is demonstrated as compared to incubations with ADP ($0.04 \pm 0.04$ mmol/L Pi/mg protein; $P < .005$, Wilcoxon). This effect of ADP-β-S is also observed when UDP is applied as substrate ($0.06 \pm 0.02$ mmol/L Pi/mg protein; $P < .005$, Wilcoxon).

Experiment B

Intraglomerular platelet aggregates after alternate perfusion experiments are demonstrated by staining for human fibrinogen (Fig 3). As can be seen in Fig 3B, perfusions with the combination of ADP with an excess of UDP lead to a strong increase in intraglomerular staining as compared to perfusions with ADP alone (Fig 3A). If present, intraglomerular platelet aggregates were small in the glomeruli of ADP-perfused kidneys, in contrast to the large aggregates found in kidneys perfused with the combination of ADP and UDP. Intraglomerular platelet aggregates in UDP and saline perfused kidneys (groups III and IV, respectively) were generally small, as in ADP-perfused kidneys.

Quantitative evaluation of intraglomerular platelet aggregation with UDP as the competitive substrate is demonstrated in Fig 4. Platelet aggregation was analyzed by scoring the percentage of glomeruli per kidney section positive for human fibrinogen (see Materials and Methods). As can be seen in Fig 4, supplementation of excess UDP to the ADP solution significantly increases intraglomerular...
platelet aggregation in intact kidneys as compared to perfusions with ADP alone (78.5% ± 9.8% positive glomeruli vs 27.9% ± 11.4%, respectively; P < .005, Wilcoxon), whereas the substrate UDP alone leads to a similar percentage of fibrinogen-positive glomeruli as in saline-perfused kidneys (25.1% ± 5.3% vs 21.7% ± 5.1%, respectively). In contrast, thrombotic tendency in kidneys of ADR-treated rats is significantly increased when ADP is used as the substrate as compared with kidneys of untreated rats (69.8% ± 9.1% vs P < .005, Wilcoxon). UDP supplementation to the perfusate also increases intraglomerular platelet aggregation in ADR-treated rats (87.9% ± 7.8%). However, the increased aggregation in these kidneys is not observed when UDP alone is used as the substrate: 16.0% ± 9.6%, which is comparable with the percentage of fibrinogen positive glomeruli in saline-perfused kidneys of ADR-treated rats (25.6% ± 2.9%).

To discriminate between effects of UDP as a nucleoside polyphosphatase substrate on the one hand and on platelets on the other hand, aggregation responses were also studied in vitro using an aggregometer. Typical aggregation responses on the stimuli are depicted in Fig 5. It is shown that UDP does not induce platelet aggregation nor does it interfere with ADP-induced platelet aggregation when the UDP concentration is 10 times the ADP concentration.

Additionally, other nucleoside diphosphates and triphosphates, ie, GDP and ITP, were also tested in the ex vivo kidney perfusion model (n = 4). Perfusion of these substrates in combination with ADP also induced an increase in intraglomerular platelet aggregation as compared to perfusion experiments with ADP alone (45.0% ± 13.4% and 36.5% ± 18.5% fibrinogen-positive glomeruli for GDP and ITP respectively, vs 26.4% ± 9.91% positive glomeruli after ADP perfusion (P < .025).

Results of experiments with nucleoside diphosphates and triphosphates in vitro are summarized in Table 1. In this table the maximal aggregation response of platelets on ADP is compared to aggregation responses on ADP in combination with either UDP, GDP, or ITP in vitro using an aggregometer. As can be seen, none of these substrates per se induces platelet aggregation in vitro. The aggregation response on ADP in combination with UDP or GDP in the ratio 1:10 is similar to the aggregation response on ADP alone, only in the case of ITP a reduction is observed.

However, when ADP in combination with UDP, GDP, or ITP is used in the ratio 1:100 a significant reduction in the maximal aggregation response is observed in vitro in all cases.

**Experiment C**

Cytochemical and biochemical studies using the ADP analogue ADP-β-S as the substrate for glomerular phosphatase activity demonstrate that this substrate is not hydrolyzed by the glomerular enzyme (Fig 1) and inhibits glomerular ADPase activity (Fig 2). However, when this...
ADP-β-S is applied to stimulate platelet aggregation in vitro, a significant aggregation response is observed (Fig 6).

In addition, platelet aggregation ex vivo is significantly increased in normal kidneys perfused with ADP-β-S as compared to aggregation in kidneys perfused with native ADP. As can be seen in Fig 7, the average percentage of fibrinogen-positive glomeruli after ADP-β-S perfusion is 61.6% ± 13.8%, whereas after perfusion with native ADP this percentage is 27.2% ± 9.7% (P < .005).

### DISCUSSION

In this study, experiments were performed to elucidate the role of glomerular ADPase activity as a platelet inhibitory mechanism without influencing other antithrombotic mechanisms or prothrombotic modalities. Therefore, glomerular ADPase activity was selectively inhibited by using competitive substrates on the one hand and an ADP analogue on the other hand in an ex vivo kidney perfusion model.

Biochemical and cytochemical data confirm the presence of both ADPase and UDPase activity in the glomeruli of rats. As reported previously, both substrates give rise to a similar localization of reaction product throughout the filtration barrier at the electron-microscopical level (Fig 1).

Biochemical studies with isolated glomeruli are in agree-
ment with these results (Fig 2); glomerular UDPase activity is of the same magnitude as glomerular ADPase activity. Moreover, ADPase and UDPase activities are strongly inhibited by ADP-β-S, indicating that ADP-β-S is a steric inhibitor of the active site of both ADPase and UDPase. From these results it can be deduced that UDP binds to the same binding site as ADP and, therefore, UDP can be used as a competitive substrate to inhibit glomerular ADPase activity. ATP, a known competitive substrate for ADP binding sites, cannot be used here because the enzymatic degradation products (ADP, adenosine monophosphate, and adenosine) influence platelet aggregation directly. ADP-β-S was applied in this biochemical experiment as an inhibitor of the receptor site because this ADP analogue is nondegradable by glomerular phosphatase activity (Fig 2); therefore, phosphate release during the incubation period can exclusively be attributed to ADPase or UDPase activity. From these results it can be concluded that UDP can be used as a competitive substrate to inhibit glomerular ADPase degradation and, secondly, that ADP-β-S is an inhibitor of glomerular phosphatase activity.

The first principle of enzyme inhibition was tested using the alternate kidney perfusion system described previously. Using this model, intraglomerular platelet aggregation can be studied without involvement of the coagulation cascade. Human platelets are used in this bioassay to evaluate thrombotic tendency because they can be readily detected in rat tissue using immunologic techniques and because they can be obtained in sufficient amounts for perfusion studies. Human platelets, like rat platelets, aggregate in response to ADP. As illustrated in Fig 3, abundant intraglomerular platelet aggregation occurs in normal kidneys (ie, kidneys with normal ADPase activity) exclusively when UDP is supplemented in excess to the platelet stimulating agonist (ie, ADP). Also, quantitative evaluation of the data shows a significant increase in platelet aggregation in normal kidneys exclusively when UDP is combined with ADP in the perfusion system (Fig 4), whereas after perfusion with UDP alone this increase is not observed. Moreover, this proaggregatory effect of UDP in combination with ADP is only observed ex vivo; in vitro UDP does not induce platelet aggregation even at high concentrations (50 μmol/L) nor does it interfere with ADP-induced platelet aggregation (Fig 5). Therefore, the aggregation-increasing effect of UDP ex vivo cannot be attributed to direct effects on platelets. Therefore, it can be concluded that specific inhibition of ADP degradation occurs by excess of UDP, and this subsequently leads to a significant increase in intraglomerular platelet aggregation in intact kidneys.

This principle is also clear in kidneys with reduced ADPase activity after ADR injection (Fig 4, right set of columns). These rats show a significant increase in platelet aggregation after perfusion with the substrate ADP as compared to untreated rats, which is in agreement with previous studies, but UDP alone has no effect. This demonstrates that an ADP-dependent antithrombotic mechanism is impaired in ADR-treated rats and other mechanisms, ie, glomerular TXA₂ production, are not involved in the present study 48 hours after the injection. The increased urinary thromboxane excretion of ADR-treated rats is observed in the full-blown nephrotic condition.

Additional experiments with other nucleoside polyphosphates, ie, GDP and ITP, demonstrated that the effect of competitive substrates on ex vivo platelet aggregation was not restricted to UDP, although GDP and ITP are less effective as compared with UDP. The reason for this is not clear, but a low glomerular GDPase activity has also been observed histochemically (personal observations, October 1988). The reduced effect of ITP ex vivo as compared with UDP may be related to a competitive effect of ITP on the platelet ADP receptors as has also been reported for ATP, thus impairing ADP-induced aggregation. ITP impairs ADP-induced aggregation in vitro at lower concentrations (ratio 1:10) as compared with UDP and GDP (Table 1).

Furthermore, it can be seen in Table 1 that all competitive substrates applied in this study impair ADP-induced aggregation at high concentrations in vitro (ratio 1:100), probably by a competitive effect on the platelet ADP receptor. Moreover, it is clear that none of the substrates applied in this study as a competitive substrate is able to induce aggregation directly, nor do they increase ADP-induced aggregation in vitro as compared with ADP alone. Therefore, results of ex vivo perfusion studies cannot be explained by direct effects of these nucleotides on platelets.

The second approach to demonstrate the importance of intraglomerular ADP-degrading activity in the prevention of intraglomerular platelet aggregation is the use of a nondegradable ADP analogue. ADP-β-S is not hydrolyzed by the glomerular enzyme, as is also demonstrated in Fig 2, but it is still capable of inducing platelet aggregation in vitro (Fig 6). While native ADP does not induce a significant intraglomerular platelet aggregation in the intact kidney ex vivo, it can be seen in Fig 7 that the nondegrad-
able ADP-β-S is able to induce a significantly increased platelet aggregation. These observations confirm that glomerular degradation of ADP is important for the prevention of intraglomerular platelet aggregation in the present model.

Taken together, these results demonstrate that selective reduction of glomerular ADPase activity, either by competitive substrates or by ADP-β-S, strongly enhances intraglomerular thrombotic tendency, as reflected by increased platelet aggregation in the rat kidney. Apparently glomerular ADPase exerts potent antithrombotic activity within intact rat kidneys by inhibiting platelet aggregation and subsequent thrombus formation.

A major advantage of this ex vivo model, as compared with the in vivo situation, is the possibility of impairing glomerular ADPase without influencing other antithrombotic mechanisms such as the eicosanoid synthesis. In vivo, reduction of glomerular ADPase activity, for instance by ADR injection, local X-irradiation, or induction of acute glomerulonephritis, can hardly be achieved without influencing these mechanisms or induction of aspecific damage to the vascular wall, which may facilitate platelet aggregation. Nevertheless, the question may emerge as to whether intraglomerular ADPase is also important in vivo as an antithrombotic mechanism. However, the role of membrane-associated ADPase in vivo is directly related to the importance of ADP as a stimulator of platelet aggregation in vivo. Although platelet aggregation in vivo can be initiated by a variety of stimuli, including TXA₂, platelet activating factor, collagen, and thrombin, ADP plays a major role in amplifying the hemostatic response. Moreover, at least the rat hemostatic response has been shown to depend more on ADP than on TXA₂. Also, in experimental glomerulonephritis in vivo, a strong association between intraglomerular platelet aggregation and decreased ADPase activity has been shown, supporting the notion that glomerular ADPase is a prominent antiaggregatory principle within glomeruli of the rat kidney in vivo.

In particular, because glomerular fenestrated endothelium enables a close contact between the bloodstream and the collagen of the GBM, platelet activation may readily occur after a slight perturbation of the capillary wall. Therefore, antithrombotic mechanisms within the glomerular capillary wall may be very important. Moreover, platelet aggregation can induce damage within glomeruli, and stimulate the coagulation cascade, or potentiate the inflammatory response. These observations stress the importance of a powerful antithrombotic mechanism within the intact glomerular capillary wall. In addition to other vessel wall-associated anticoagulatory mechanisms (ie, the fibrinolytic system and heparan sulphate proteoglycans) or endothelial PGI₂ and NO production, glomerular ADPase activity probably fulfills a major antithrombotic function in the rat kidney.

ACKNOWLEDGMENT

We thank H. Wierenga and H.R.A. Meiborg for performing the microphotography, and J. Donga for his excellent technical assistance. The collaboration of Dr C.Th. Smits-Sibinga, head of the bloodbank “Groningen, Drenthe,” is greatly appreciated.

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