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Effect of dipyridamole on glomerular mesangial cell ecto-5′-nucleotidase expression

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Abstract. Although dipyridamole has been extensively studied as an anti-aggregating agent, its mechanism of action has not been elucidated. Cultured mesangial cells were treated with dipyridamole 1–100 μM from 6–72 h. Ecto-5′-nucleotidase activity approximately doubled (from 115 ± 11 to 226 ± 14 nmol/min/mg) after treatment with 100 μM dipyridamole for 72 h. This effect was concentration- and time-dependent. Cycloheximide, an inhibitor of protein synthesis, did not alter basal 5′-nucleotidase activity. However, it prevented stimulation by 5 μM dipyridamole. Adenosine availability at the receptor sites was increased by dipyridamole and S-(p-nitrobenzyl)-6-thioinosine (NBTI), which inhibit adenosine uptake into the cell. Addition of dipyridamole or NBTI to the adenosine-treated mesagial cells produced an additive increase in ecto-5′-nucleotidase activity. Dipyridamole, through its effect on extracellular adenosine and ecto-5′-nucleotidase, may have an influence upon regulation of the glomerular microcirculation.
Key words. Mesangial cells; ecto-5′-nucleotidase; dipyridamole; adenosine.

Dipyridamole ('Persantin') has been extensively studied as an anti-aggregating agent; however, its mechanism of action has not been elucidated. Dipyridamole inhibits adenosine uptake and this effect prevents aggregation of platelets[1]. Dipyridamole was found to inhibit cAMP phosphodiesterase from platelets, and to inhibit aggregation by a common mechanism involving accumulation of intracellular cAMP[2,3]. Considerable evidence suggests that adenosine may play a major role in the regulation of coronary blood flow[4]. We have established that ecto-5′-nucleotidase of glomerular mesangial cells may be the main source of adenosine within the glomeruli, and that the activity of this enzyme is therefore likely to be essential in the regulation of glomerular microcirculation[5]. The aim of this work was to establish the effect of dipyridamole on ecto-5′-nucleotidase expression in cultured rat mesangial cells. Evidence is presented that dipyridamole up-regulates ecto-5′-nucleotidase expression in mesangial cells.

Materials and methods

Tissue culture flasks, dishes, and culture media were obtained from Flow Laboratories (Irvine, UK). Adenosine; adenosine-5′-monophosphate; adenosine deaminase, type VIII, from calf intestinal mucosa; dipyridamole; S-(p-nitrobenzyl)-6-thioinosine (NBTI); cycloheximide, and actinomycin D were purchased from Sigma (St. Louis, Missouri, USA).

Culture of mesangial cells. Primary cultures of mesangial cells were obtained from collagenase-treated glomeruli as previously described[6]. Kidneys were removed under pentobarbital anaesthesia from Sprague-Dawley rats and glomeruli were isolated by a sieving technique and centrifugation. Glomeruli were seeded in 25 cm^2 plastic flasks into 5 ml RPMI-1640 medium buffered with 20 mM HEPES and supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 ug/ml streptomycin sulfate, and 2 mM glutamine. Mesangial cells were subcultured on day 21, after treatment with 0.05% trypsin/0.02% EDTA, and transferred to Petri dishes. At confluency, mesangial cells were detached and seeded in 24-well plates for enzyme studies. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2-95% air. After two to three passages using 0.05% trypsin/0.02% EDTA, homogeneous cultures of mesangial cells were obtained. Mesangial cells were identified by light microscopy and indirect immunofluorescence. They had stellate appearance, overgrew each other, and showed a network of intracellular fibrils of myosin. They were not positive for anti-von Willebrand factor, antiurokinase or anticytokeratin.

Ecto-5′-nucleotidase assay. Enzyme activity was measured on intact cells in culture. The cells were washed first with a 30 mM Tris-HCl buffer pH 7.4 containing 130 mM NaCl, 0.25 mM EDTA, 0.125 mM EGTA, and 5.5 mM glucose. They were then washed with the incubation medium containing 30 mM Tris-HCl buffer pH 7.4, 130 mM NaCl, 5 mM $MgCl_2$, and 5.5 mM glucose. Incubation was started upon addition of 3 mM AMP to the medium and performed at 37 °C for 5–15 min; depending on the rate of enzyme activity, a time was chosen such that conditions of zero-order reaction were maintained. The amount of liberated inorganic phosphate was measured following the method of Gomori[7]. Cell protein was estimated according to the method at Lowry et al.[8], after appropriate digestion with 1N NaOH. 5′-Nucleotidase activity is expressed as nmol of

inorganic phosphate liberated per min and per mg of cell protein.

Means ± SD are given throughout. Statistical significance was estimated by using Student's *t*-test for unpaired values and by analysis of variance.

Results

Mesangial cells were treated with dipyridamole 1–100 µmol for periods ranging from 6–72 h. Ecto-5'-nucleotidase activity after 6 h was not significantly changed. A small (not significant) increase in 5'-nucleotidase activity was observed at 24 h (data not presented). At 48 and 72 h an increase in ecto-5'-nucleotidase activity was observed, which was significant with 5 µmol dipyridamole ($p < 0.05$), and highly significant ($p < 0.01$) at higher concentrations (fig.).

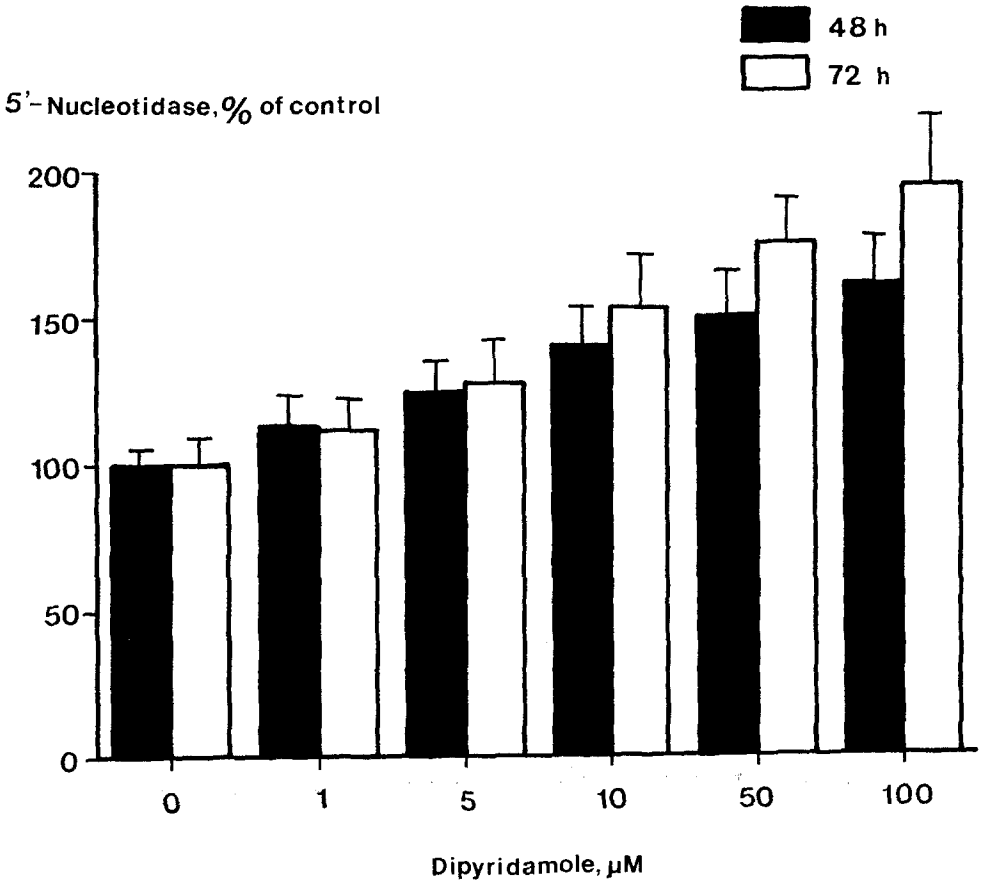
The effect of dipyridamole on ecto-5'-nucleotidase involves RNA and protein synthesis, since the dipyridamole-induced increase in 5-nucelotidase activity was inhibited by both actinomycin D, a blocker of RNA synthesis, and cycloheximide, a blocker of protein synthesis (table 1).

Table 1. Effect of cycloheximide and actinomycin D on dipyridamole-induced ecto-5'-nucleotidase activity of rat mesangial cells

	nmol/min/mg
Control	85.5 ± 8.2
Dipyridamole, 5 µM	127.4 ± 12.7
Cycloheximide, 0.2 µg/ml	84.0 ± 11.5
Dipyridamole 5 µM + Cycloheximide, 0.2 µg/ml	82.9 ± 14.3
Actinomycin D, 0.2 µg/ml	72.1 ± 9.0
Dipyridamole 5 µM + Actinomycin D, 0.2 µg/ml	79.4 ± 4.5

Cells were incubated for 72 h with the agents indicated. Values are means ± SD of 4 determinations. Results were analysed using two-way analysis of variance. The effects of dipyridamole, cycloheximide and actinomycin D are significant ($p < 0.05$).

Dipyridamole is known to inhibit cellular uptake of adenosine and to potentiate the action of exogenous adenosine. Comparative effects of dipyridamole and NBTI, another inhibitor of adenosine uptake, on adenosine- induced ecto-5'-nucleotidase of rat mesangial cells, are presented in table 2. Effects of adenosine and dipyridamole, as well as those of adenosine and NBTI, were additive. Adenosine-5'-monophosphate (5'-AMP)



Effect of dipyridamole on ecto-5'-nucleotidase activity of glomerular mesangial cells. Cells were cultured with dipyridamole in the concentrations indicated, for 48 and 72 h. Enzyme activity is expressed as % of control ± SD of 4 determinations. Results were analysed using one-way analysis of variance. Effect of dipyridamole was significant in the concentrations studied both at 48 and 72 h ($p < 0.05$ and $p < 0.01$, respectively).

Table 2. Effects of dipyridamole and NBTI on adenosine-induced ecto-5′-nucleotidase of rat mesangial cells.

Treatment group	5′-Nucleotidase nmol/min/mg	%
Control	66.2 ± 2.5	100
Adenosine, 100 μM	79.8 ± 4.2*	120.5
Dipyridamole, 5 μM	103.5 ± 9.3**	156.3
Adenosine, 100 μM + Dipyridamole, 5 μM	137.3 ± 9.7**	207.4
NBTI, 5 μM	93.0 ± 3.6**	140.5
Adenosine, 100 μM + NBTI, 5 μM	103.8 ± 3.9**	156.8

Mesangial cells were cultured with adenosine and/or dipyridamole and NBTI for 48 h. Enzyme activity of control is 100%. The figures represent the means of 4 experiments ± SD. Results were analysed using Student's *t* test or two-way analysis of variance when combined effects were studied. The effects of dipyridamole and NBTI were additive to that of adenosine (no significant interaction).
* vs control: $p < 0.01$
** vs control: $p < 0.001$

and dipyridamole together strongly induced ecto-5′-nucleotidase of mesangial cells, being 66.7% more effective than adenosine and dipyridamole. Adenosine deaminase (ADA), which transforms adenosine into inosine, reduced this effect (table 3).

Discussion

Mesangial cells are both the source and target of adenosine. Their role as a source of adenosine is dependent on 5′-nucleotidase activity[5]. We have recently demonstrated that adenosine binding to its A_2 receptor sites on mesangial cells was associated with cAMP production and cAMP-induced expression of 5′-nucleotidase[9].

In the present study evidence was obtained that dipyridamole, a commonly used anti-aggregation agent, induces ecto-5′-nucleotidase in cultured rat mesangial cells. This effect was found to be time- and concentration-dependent. Actinomycin D and cycloheximide prevented the dipyridamole-induced increase in ecto-5′-nucleotidase activity, which suggested that transcription and enzyme synthesis are responsible for the increased activity.

Dipyridamole inhibits adenosine uptake into the mesangial cells, as does NBTI. Both agents produced an increase in 5′-nucleotidase activity, additive to that of adenosine. Dipyridamole was more potent than NBTI. This could be explained by differences in ability to prevent adenosine uptake, and by the inhibitory effect on cAMP phosphodiesterase that has been demonstrated for dipyridamole. The expression of ecto-5′-nucleotidase in rat mesangial cells has been shown to be induced by cAMP, whether the reason for cAMP accumulation is stimulation of adenylate cyclase activity or inhibition of cAMP phosphodiesterase[10].

Induction of mesangial cell ecto-5′-nucleotidase, which hydrolyzes extracellular adenine nucleotides to adenosine, could have an impact on the glomerular filtration rate. Adenosine is a local mediator, producing a decrease in the glomerular filtration rate via mesangial cell contraction[11], constriction of the afferent arteriole[12], and inhibition of renin release[13]. Dipyridamole induction of mesangial cell ecto-5′-nucleotidase may influence glomerular microcirculation in various kidney disease and other disease states. Coagulation has been considered as a mechanism of glomerular injury in many glomerular and vascular kidney diseases[14]. Several therapeutic protocols have included antiplatelet agents and anticoagulants in the treatment of various forms of glomerulonephritis. The success of such therapy has been variable, owing to the recruitment of coagulation mechanisms following endothelial injury only being secondary[15]. A better understanding of the pathological processes and the mechanisms of action of antiplatelet drugs may have a positive impact upon treatment.

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Table 3. Effect of dipyridamole on adenosine and adenosine-5′-monophosphate induced 5′-nucleotidase of rat mesangial cells.

Treatment group	Ecto-5′-nucleotidase	
	nmol/min/mg	%
Control	84.0 ± 7.1	100
Dipyridamole, 5 μM	108.1 ± 9.8*	128.6
Adenosine, 0.4 mM	98.9 ± 0.7*	117.7
Adenosine, 0.4 mM + dipyridamole, 5 μM	141.8 ± 9.9**	168.8
Adenosine, 0.4 mM + dipyridamole, 5 μM + ADA, 0.5 U/ml	103.1 ± 8.1*	122.7
5′-AMP, 0.4 mM	103.3 ± 10.8*	130
5′-AMP, 0.4 mM + dipyridamole, 5 μM	197.8 ± 15.2**	235.5
5′-AMP, 0.4 mM + dipyridamole, 5 μM + ADA, 0.5 U/ml	116.0 ± 12.0*	138.1

Mesangial cells were cultured with adenosine or 5′-AMP for 48 h. Additions as indicated. Enzyme activity of control is 100%. The figures represent the mean of 4 experiments ± SD.
* vs control $p < 0.05$
** vs control $p < 0.001$.

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