HUMAN PLATELET PROTEIN KINASES IN DIABETIC RETINOPATHY

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1. Introduction

The pathogenesis of diabetic retinopathy is still unknown. Altered basement membrane, blood flow properties, oxygen transport, hormone production, cell metabolism, and disturbed hemostasis have been implicated as possible mechanisms [1]. Some of these presumed mechanisms may be mediated via protein kinase and phosphorylation of cellular proteins.

We have previously observed that platelet aggregation was increased in patients with diabetic retinopathy [2]. Platelet aggregation may be due to altered phosphorylation of the platelet membrane proteins mediated by a change of the activity or types of intracellular protein kinases. Our results, presented in this paper, show that platelets of patients with diabetic retinopathy exhibited increased activity levels of cAMP-independent protein kinase. Concomitantly, endogenous platelet protein phosphorylation was higher in diabetic patients than in control patients.

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2. Materials and methods

All biochemical reagents were obtained from Sigma Chemical Company. [γ-^{32}P]ATP, ammonium salt (2–10 Ci/mmol) was from New England Nuclear. Blood was obtained from patients with proliferative diabetic retinopathy in siliconized tubes containing 3.8% sodium citrate. Platelets were collected and lysed according to Zeller et al. [3]. A platelet count was obtained using the Coulter counter. Protein kinase activity was assayed in a total reaction volume of 0.2 ml for 10 min at 30°C as previously described by us [4]. Each assay was performed in triplicate. Heat-stable protein kinase inhibitor protein was prepared and partially purified from rabbit skeletal muscle [5]. Platelet lysates were subjected to chromatography on Whatman DEAE-cellulose (DE-52) pre-equilibrated in 20 mM Tris/5 mM dithiothreitol (pH 7.4). Elution was carried out with a linear KCl gradient (0.0–0.5 M KCl in 20 mM Tris/5 mM dithiothreitol (pH 7.4). Polyacrylamide gel electrophoresis for identification of endogenously phosphorylated proteins in platelet lysates was performed according to Ehrlich et al. [6].

Analysis of the data was based on methods of multivariate analysis of variance (and covariance) using least

squares estimation techniques [7]. However, the significance levels reported are based on the more familiar two sample univariate Student's t-statistic. Significance values of $P < 0.05$ were considered to be non-significant.

3. Results

3.1. *Effect of age on protein kinase activity*

Figures 1 and 3 show that total protein kinase and cAMP-independent protein kinase activities tended to decrease with age in both normal and diabetic patients. However, the negative correlation was not statistically significant for either group. Cyclic AMP-dependent protein kinase activity increased slightly with age in both normal and diabetic patients (fig.2). The positive correlation for patients in either group was marginally

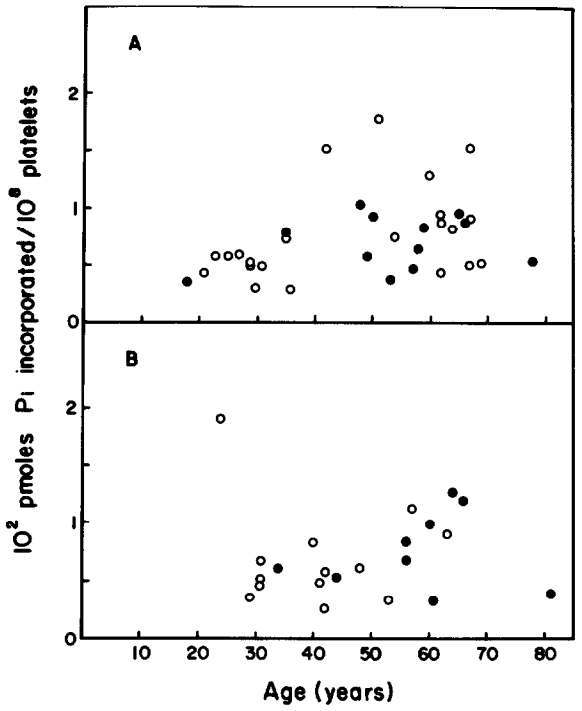

Fig.1. Total protein kinase activity in platelet lysates of normal and diabetic males (panel A) and females (panel B) as a function of age. Total protein kinase activity was measured as described under section 2 in the presence of 10^{-6} M cyclic AMP with protamine as substrate. •, normal; ○, diabetic patients.

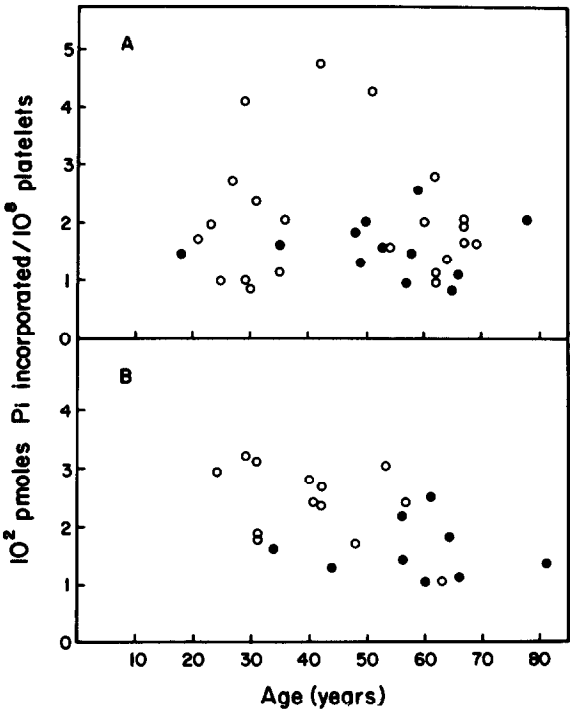

Fig.2. Cyclic AMP-dependent protein kinase activity in platelet lysates of normal and diabetic males (panel A) and females (panel B) as a function of age. Protein kinase activity was measured as described under section 2 in the presence of 10^{-6} M cyclic AMP, without and with saturating concentrations of heat-stable protein kinase inhibitor. The cyclic AMP-dependent protein kinase activity was derived by subtracting the protein kinase activity measured in the presence of the inhibitor (values of fig.3) from the total protein kinase activity measured in the absence of the inhibitor but in the presence of cyclic AMP (values of fig.1). •, normal; ○, diabetic patients.

significant ($P > 0.08$) and was due primarily to the age contribution by the diabetic males.

3.2. *Protein kinase activity in normal and diabetic patients*

Total protein kinase activity was significantly higher ($P < 0.029$) in diabetic than normal patients (fig.1 and table 1). Cyclic AMP-dependent protein kinase activity did not differ significantly in diabetic and normal patients, but cyclic AMP-independent protein kinase activity was significantly ($P < 0.009$) higher in diabetic than in normal patients (fig.3 and table 1). Both diabetic males and females had higher

Table 1
Comparison of protein kinase activity in normal and diabetic patients

Sex	Patients	Number of patients	Protein kinase activity (pmol P_i incorporated/min/10^8 platelets)		
			cAMP-dependent	cAMP-independent	Total
Male	Normal	12	684 ± 65	1581 ± 143	2265 ± 165
	Diabetic	22	760 ± 88	2066 ± 235	2793 ± 292
Female	Normal	9	764 ± 112	1612 ± 162	2376 ± 164
	Diabetic	13	712 ± 121	2434 ± 178	3146 ± 213
Significance level		*P*	$P < 0.819$	$P < 0.009$	$P < 0.029$

Total, cyclic AMP-dependent, and cyclic AMP-independent protein kinase activities were measured in individual platelet samples as described in legends of figs 1–3. In this table the protein kinase levels illustrated in figs 1–3 were averaged as listed in this table. The significance level is given for the two-tailed *t*-test statistic (54 degrees of freedom) for comparing means of normals (males and females) with diabetics (males and females). Each value is the mean ± S.D.

cyclic AMP-independent protein kinase activity than normal males and females but only the difference in the activity of the females was statistically significant ($P < 0.005$).

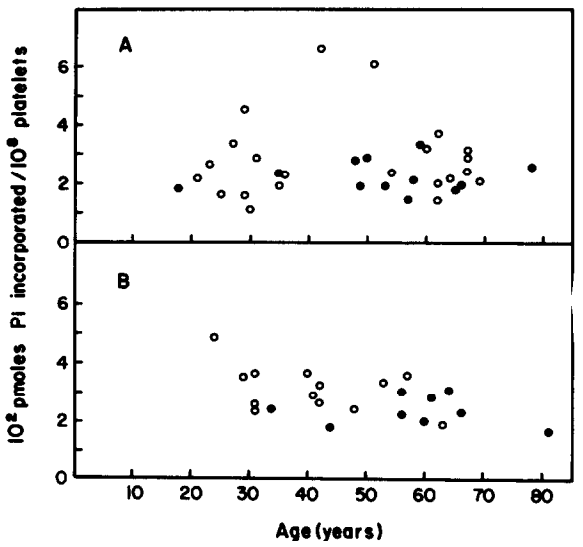

Fig.3. Cyclic AMP-independent protein kinase activity in platelet lysates of normal and diabetic males (panel A) and females (panel B) as a function of age. Protein kinase activity was measured as described under section 2 in the presence of 10^{-6} M cyclic AMP and saturating concentrations of heat-stable inhibitor with protamine as substrate. •, normal; ○, diabetic patients.

3.3. *Multiplicity of cyclic AMP-dependent protein kinase*

Since no quantitative differences of cyclic AMP-dependent protein kinase levels were identified in normal and diabetic patients, platelet soluble preparations were subjected to DEAE-cellulose chromatography to identify qualitative changes in type I versus type II cyclic AMP-dependent protein kinase isozyme patterns. DEAE-cellulose chromatography of platelet homogenates revealed the presence of the two cyclic AMP-dependent protein kinase isozymes eluting at 0.18 and at 0.25 M NaCl (data not shown). The chromatographic profiles of protein kinase activity did, however, not differ qualitatively or quantitatively in preparation from normal or diabetic patients.

3.4. *Effect of cyclic AMP on endogenous protein phosphorylation*

To study the effect of cyclic AMP on the phosphorylation of endogenous platelet proteins by their endogenous protein kinases, platelets were incubated in the presence of [γ-^{32}P]ATP without and with 10^{-6} M cyclic AMP. After phosphorylation the protein preparations were subjected to polyacrylamide slab gel electrophoresis [6]. After autoradiography of the slab gels it was found that cyclic AMP had no significant stimulatory effect on protein phosphorylation in either normal or diabetic patients. However,

under identical phosphorylation conditions we consistently found higher levels of ^{32}P incorporation into platelet proteins from diabetic patients than from normal patients (data not shown). These findings were expected in view of the identified higher activity of cyclic AMP-independent protein kinase in diabetic patients. Electrophoresis revealed no qualitative changes of composition of platelet proteins in normal and diabetic patients.

4. Discussion

Our results indicate that platelet protein kinase levels were significantly elevated in patients with proliferative diabetic retinopathy. The elevated activity was mainly contributed by the cAMP-independent protein kinase. The increase of cyclic AMP-independent protein kinase activity was reflected by an increased incorporation of ^{32}P into platelet proteins of diabetic patients.

There have been several reports suggesting that growth hormone may be implicated in the etiology of diabetic retinopathy [8–11]. However, Christensen [12] has also described the beneficial effects of hypophysectomy and hypothyroidism on capillary fragility in diabetics. Steroid and thyroid hormones are believed to casue protein phosphorylation primarily through stimulation of cAMP-independent protein kinases [13]. In this study we have reported an increase in cAMP-independent protein kinase levels in diabetics. We believe that a correlation between cAMP-independent protein kinase, thyroid and steroid hormones may exist in diabetics with proliferative retinopathy. This correlation may be involved in the observed regression of diabetic proliferative retinopathy after hypophysectomy [9,10,12].

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