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RESEARCH PAPER

Reversal of Increased Lymphocyte PC-1 Activity in Patients with Type 2 Diabetes Treated with Metformin

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Abstract

Background The plasma cell differentiation antigen (PC-1) is an inhibitor of insulin receptor tyrosine kinase activity, and has been implicated in the pathogenesis of insulin resistance in Type 2 diabetes. Metformin increases peripheral insulin sensitivity and, therefore, we have studied the effect of metformin treatment on lymphocyte PC-1 (ecto-alkaline phosphodiesterase I, APD) in patients with Type 2 diabetes.

Methods Basal, concanavalin A (Con A)-, and phorbol-12-myristate-13-acetate (PMA)-stimulated lymphocyte PC-1, aminopeptidase N (APN), and dipeptidylpeptidase IV (DPP IV) activities were determined in 16 patients with Type 2 diabetes before and after 3 months of metformin treatment.

Results Lymphocyte PC-1 in patients with Type 2 diabetes was increased significantly ($p < 0.001$) over control; however, metformin treatment brought its activity in unstimulated and Con A-stimulated lymphocytes to the control level. PMA-stimulated PC-1 in patients with Type 2 diabetes was 17-times higher than in controls, and was reduced to near the control level by 3-month metformin treatment. In Type 2 diabetes, PMA-stimulated ecto-DPP IV was significantly ($p < 0.005$) increased over control, but was reduced after metformin treatment.

Conclusion This study has shown an increased activity of lymphocyte PC-1 in Type 2 diabetes and its reversal by 3-month metformin treatment, corresponding to the improvement of insulin sensitivity. Data obtained are consistent with a role of PC-1 in insulin resistance and suggest a new mechanism of action for metformin via PC-1 inhibition. Copyright © 1999 John Wiley & Sons, Ltd.

Keywords Type 2 diabetes; metformin; insulin resistance; PC-1; aminopeptidase N; dipeptidylpeptidase IV

Introduction

Type 2 diabetes is a common disorder of middle-aged individuals characterized by high glucose levels which, if untreated, can cause serious complications and lead to early death. Hyperglycemia in Type 2 diabetes is caused by peripheral insulin resistance, resulting in decreased insulin-mediated glucose disposal, increased endogenous glucose production, mainly from liver, and inadequate pancreatic insulin secretion [1]. Insulin resistance is a major component of Type 2 diabetes. While a genetic contribution is likely, as yet none of several proposed candidate genes have been found to be

involved in the typically obese patients with Type 2 diabetes in explaining their insulin resistance. Defects in insulin receptor tyrosine kinase activity have been demonstrated in several tissues from insulin resistant subjects, but mutation in the insulin receptor gene occurs in only a small fraction of cases [2,3]. Therefore, other molecules that are capable of modulating the function of the insulin receptor are likely candidates in the search for the cellular mechanisms of insulin resistance.

The membrane glycoprotein plasma cell differentiation antigen (PC-1) has been purified as an inhibitor of insulin receptor tyrosine kinase activity and has been implicated in the pathogenesis of insulin resistance. PC-1 is an ectoenzyme possessing both alkaline phosphodiesterase I and nucleotide pyrophosphatase activities. Studies in skeletal muscle and adipose tissue of non-obese non-diabetic subjects have indicated that PC-1, by regulating insulin receptor function, may play a role in the degree of insulin sensitivity [4,5]. Increased expression of PC-1 and a decreased insulin receptor content in skeletal muscle was found to be involved in the insulin resistance of obesity in both patients with Type 2 diabetes and non-diabetic patients [6]. In subsequent studies, expression of PC-1 was found to be elevated in fibroblasts from other insulin resistant subjects [7]. PC-1 content in fibroblasts correlated closely with PC-1 content in muscle tissue. These studies indicated that the elevation of PC-1 content might be a primary factor in the cause of insulin resistance.

The biguanide metformin (dimethylbiguanide) is an oral antihyperglycemic agent widely used in the management of Type 2 diabetes. In the United Kingdom Prospective Diabetes Study, patients receiving metformin had better blood glucose control than patients receiving conventional treatment [8]. Patients receiving metformin, compared with the conventional group, showed a risk reduction of 32% for any diabetes-related endpoint, and 36% for all cause-related mortality. Metformin is the initial drug of choice in the obese patient with Type 2 diabetes; however, its exact mechanism of action is still unknown. Metformin produces suppression of endogenous glucose output [9] and increased peripheral insulin sensitivity [10]. Metformin may stimulate insulin action by elevation of intracellular calcium in addition to activation of receptor tyrosine kinase [11].

The aim of this study was to evaluate activity of lymphocyte PC-1, ecto-alanine aminopeptidase N (APN, EC 3.4.11.2), and ecto-dipeptidylpeptidase IV (DPP IV, EC) activity in patients with Type 2 diabetes. The effect of 3-month metformin treatment on these lymphocyte ectoenzymes was also studied.

Materials and methods

Sixteen patients (9 females, 7 males) with Type 2 diabetes, as defined by the National Diabetes Data Group [12], who had a glycosylated hemoglobin value above the upper limit of normal and plasma C-peptide concentration of at least 1.5 ng/ml were studied. Patients with Type 2 diabetes receiving dietary therapy or treatment with a sulfonylurea were included. Patients were excluded if they had abnormal renal or hepatic function or had a recent atherosclerotic event. After a 2-week washout period, during which any previous drug therapy was discontinued, the patients received 1000 mg of metformin (ICN Pharmaceuticals, Belgrade) twice daily orally for 3 months.

A control group of nine non-diabetic subjects (5 females, 4 males) of similar age to the patients with Type 2 diabetes was studied. These individuals had no personal history of, or first degree relative with, diabetes (Type 1 or Type 2), hypertension or dyslipidemia. None of the control subjects were taking medication.

Isolation and culture of human PBMC

In order to assess cytokine production, peripheral blood mononuclear cells (PBMC) were isolated from 10 ml of freshly drawn heparinized (50 IU/ml) blood, layered over Ficoll-Hypaque (Lymphoprep, Nyegard, Oslo, Norway), washed twice in RPMI 1640 (Flow Laboratories, Irvine, UK) culture medium containing 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), and resuspended at a concentration of 2×10^6/ml in the same medium supplemented with 10% fetal calf serum (FCS). PBMC were incubated for 48 h at 37°C in an atmosphere of 95% air and 5% CO_2. Stimulation studies were performed for 48 h by adding Concanavalin A (Con A) or phorbol-12-myristate-13-acetate (PMA) (Sigma, St Louis, MO, USA), 25 μg/ml or 10 ng/ml, respectively, to the incubation medium.

Ectoenzymes of lymphocytes

Basal, Con A-, and PMA-stimulated lymphocyte PC-1, APN and DPP IV activities were determined. Non-adhering cells from the culture plates were transferred to centrifuge tubes after appropriate washing with saline, substrate was added, and enzyme activity determined as previously described [13–16]. The following substrates were used: *p*-nitrophenyl thymidine 5′-phosphate for PC-1, alanine *p*-nitroanilide for APN, and gly-pro *p*-nitroanilide for DPP IV (all from Sigma).

PC-1 (APD) was determined in 50 mM Tris–HCI buffer, pH 8.0, 130 mM NaCl, 1 mM $MgCl_2$, with 1.5 mM *p*-nitrophenyl thymidine 5′-phosphate as substrate [13,14]. Incubation was carried out at 37°C for 3–10 min with gentle agitation, under zero-order kinetic conditions. The enzyme reaction was stopped with 0.1 ml of 1 M sodium hydoxide. The *p*-nitrophenol formed was measured at 405 nm.

APN activity was measured in Ca^{2+}-free phosphate buffered saline (PBS) containing 130 mM NaCl, 1 mM $MgCl_2$, 9 mM Na_2HPO_4, 9 mM NaH_2PO_4, 1.5 mM KH_2PO_4 3 mM KCl, and 3 mM alanine *p*-nitroanilide, at pH 7.4 [13,15]. Incubation was carried out at 37°C for 5–20 min with gentle agitation, under zero-order kinetic condi-

Table 1. Baseline characteristics of patients with Type 2 diabetes treated with metformin

Characteristic	Before metformin	After metformin
Age (years)	49.3 ± 2.8	
Duration of diabetes (years)	3.2 ± 0.4	
Weight (kg)	98.2 ± 4.2	
Body mass index (kg/m^2)	34.7 ± 4.2	
Fasting plasma glucose (mmol/l)	11.1 ± 0.9	9.1 ± 0.7
Glycosylated hemoglobin (%)	7.8 ± 0.7	7.1 ± 0.4
Fasting plasma insulin (μU/ml)	32.8 ± 9.3	19.8 ± 2.9
Fasting plasma C-peptide (ng/ml)	1.85 ± 0.26	2.27 ± 0.33

Values are means ± S.E.M.

tions. The enzyme reaction was stopped with 0.1 ml 10% (w/v) trichloroacetic acid, and the amount of *p*-nitroanilide formed was measured at 405 nm.

DPP IV activity was measured in 50 mM Tris–HCI buffer, pH 7.8, containing 130 mM NaCl and 1 mM $MgCl_2$, with 1.5 mM gly-pro-*p*-nitroanilide as substrate [13,16]. Incubation was carried out at 37°C for 3–10 min with gentle agitation, under zero-order kinetic conditions. The enzyme reaction was stopped with 0.1 ml 10% (w/v) trichloroacetic acid. The amount of *p*-nitroanilide formed was measured at 405 nm.

Results

Baseline characteristics of patients with Type 2 diabetes are presented in Table 1. These were obese diabetics with a body mass index of 34.7 ± 4.2 kg/m^2, compared to 23.1 ± 2.4 kg/m^2 in non-diabetic control subjects. Fasting plasma glucose was over the normal level, as was glycosylated hemoglobin. At 3 months, metformin lowered the mean fasting plasma glucose concentration by 2.0 mmol/l. The glycosylated hemoglobin values did not change significantly after metformin treatment.

Lymphocyte PC-1 activity

Unstimulated PC-1 activity in patients with Type 2 diabetes was significantly ($p < 0.001$) increased over the activity in healthy controls. However, after 3 months of metformin treatment a reversal occurred and no further difference was observed (Table 2).

Con A-stimulated lymphocyte PC-1 in patients with Type 2 diabetes was also significantly ($p < 0.001$) increased over control level, and metformin treatment reduced it to the control values. Expressed as a percentage of unstimulated activity, Con A-stimulated activity in Type 2 diabetes was about half of the control level, and the same percentage remained after metformin treatment.

PMA-stimulated lymphocyte PC-1 in patients with Type 2 diabetes was markedly ($p < 0.001$) increased being 79.6 vs 4.7 nmol/min per 10^6 lymphocytes in controls. Metformin treatment reduced this activity to the near normal level. Expressed as a percentage of unstimulated activity, PMA-stimulated activity in patients with Type 2 diabetes was increased by about 4-times over control ($p < 0.001$). Metformin treatment reduced this activity to the level of control.

Lymphocyte ecto-APN activity

Unstimulated, Con A-, and PMA-stimulated ecto-APN activity in patients with Type 2 diabetes was not different from healthy controls (Table 3). In patients with Type 2 diabetes, PMA-stimulated ecto-APN activity, expressed as percentage of unstimulated, was significantly higher than in controls (49% vs 18%); however, it was reduced to the control level by metformin treatment.

Lymphocyte ecto-DPP IV activity

Unstimulated and Con A-stimulated ecto-DPP IV activity was no different from controls. PMA-stimulated ecto-DPP IV activity in patients with Type 2 diabetes was significantly ($p < 0.005$) increased over control (Table 4). Metformin treatment reduced ecto-DPP IV activity to the control level.

Discussion

Insulin resistance of the skeletal muscle plays a key role in the development and progression of Type 2 diabetes. Available data suggest that insulin resistance is caused by an impaired signal from the insulin receptor to the glucose transport system and to glycogen synthase. The

Table 2. Lymphocyte PC-1 activity in Type 2 diabetes

Group	*N*	Unstimulated (nmol/min per 10^6 Ly)	Con A-stimulated (nmol/min per 10^6 Ly)	Con A-stimulated (% of unstimulated)	PMA-stimulated (nmol/min per 10^6 Ly)	PMA-stimulated (% of unstimulated)
1. Control	9	5.70 (3.6–22.1)	34.9 (9.7–112)	612 (170–1967)	4.70 (3.6–14.3)	82 (63–251)
2. Type 2 diabetes before metformin	16	23.4[a] (10.8–88.2)	73.8[a] (34.2–115)	315[b] (146–492)	79.6[a] (27.0–133)	340[b] (115–569)
3. Type 2 diabetes after metformin	16	9.40 (4.5–21.9)	33.5 (22.8–56.1)	356[b] (243–597)	8.35 (4.8–45.3)	89 (51–482)

Median values, and range in parentheses are given.
[a] $p < 0.001$ vs control and Type 2 diabetes after metformin.
[b] $p < 0.05$ vs control.

Table 3. Lymphocyte ecto-APN activity in Type 2 diabetes

Group	*N*	Unstimulated (nmol/min per 10^6 Ly)	Con A-stimulated		PMA-stimulated	
			(nmol/min per 10^6 Ly)	(% of unstimulated)	(nmol/min per 10^6 Ly)	(% of unstimulated)
1. Control	9	84.9 (35.1–135.6)	46.9 (8.8–75.9)	55 (10–89)	15.6 (7.4–32.5)	18 (9–38)
2. Type 2 diabetes before metformin	16	50.3 (35.1–82.5)	29.6 (16.6–56.7)	59 (33–113)	24.6 (6.9–51.4)	49[a] (14–102)
3. Type 2 diabetes after metformin	16	86.9 (26.5–130.9)	33.2 (15.0–73.7)	48 (17–85)	15.4 (4.5–32.2)	18 (5–37)

Median values, and range in parentheses are given.
[a] $p < 0.001$ vs control and Type 2 diabetes after metformin.

Table 4. Lymphocyte ecto-DPP IV activity in Type 2 diabetes

Group	*N*	Unstimulated (nmol/min per 10^6 Ly)	Con A-stimulated		PMA-stimulated	
			(nmol/min per 10^6 Ly)	(% of unstimulated)	(nmol/min per 10^6 Ly)	(% of unstimulated)
1. Control	9	23.2 (17.8–33.5)	19.0 (10.7–32.5)	82 (46–140)	16.9 (11.6–19.5)	73 (15–84)
2. Type 2 diabetes before metformin	16	26.6 (12.8–34.8)	18.8 (6.0–40.1)	68 (22–150)	23.5[a] (12.1–32.7)	88[b] (46–167)
3. Type 2 diabetes after metformin	16	23.1 (19.0–33.4)	20.5 (13.6–38.9)	89 (46–168)	18.5 (10.0–27.8)	80 (43–120)

Median values, and range in parentheses are given.
[a] $p < 0.005$ vs control.
[b] $p < 0.05$ vs control.

impaired response of the insulin receptor tyrosine kinase, documented in Type 2 diabetes, appears to contribute to the pathogenesis of the signaling defect. Defects of insulin receptor tyrosine kinase activity have been demonstrated in several tissues of insulin resistant subjects, but mutations in the insulin receptor gene occur in only a small fraction of cases [17]. Therefore, the reduced kinase activation is not caused by mutations within the insulin receptor gene. An inhibitor of insulin receptor tyrosine kinase activity was isolated and identified as membrane glycoprotein PC-1. Preliminary data suggest a direct relationship between PC-1 and the insulin receptor. A study has suggested that the phosphodiesterase activity of PC-1 is not necessary for its inhibition of the insulin receptor [18]. However, the mechanisms whereby PC-1 inhibits insulin receptor signaling remain to be elucidated.

We have demonstrated that alkaline phosphodiesterase I, the enzyme activity of PC-1, was enhanced in both unstimulated and PMA-stimulated lymphocytes of patients with Type 2 diabetes. The median value of unstimulated PC-1 activity in patients with Type 2 diabetes was 5.1 times higher than in controls. However, PMA-stimulated activity in patients with Type 2 diabetes was 17-times higher than in controls. Metformin treatment for 3 months restored normal PC-1 activity in both unstimulated, Con A- and PMA-stimulated lymphocytes. Thus improvement of peripheral insulin sensitivity, observed during metformin treatment [10], could probably be explained by a reduced activity of PC-1, observed in our study.

Data obtained are consistent with a role of PC-1 in insulin resistance and suggest a new mechanism of action for metformin via PC-1 inhibition. The question of whether the metformin directly influences PC-1 or whether correction of the hyperglycemia improves this function could not be answered. However, a relatively small decrease of glucose from a mean of 11.1 to 9.1 mmol/l, and a decrease of PC-1 from a median of 23.4 to 9.4 in unstimulated lymphocytes, and from 79.6 to 8.35 nmol/min/106 Ly in PMA-stimulated cells after a 3-month treatment with metformin, is consistent with the view of a direct inhibition of PC-1 by metformin. Further study is needed to answer this question.

Alkaline phosphodiesterase I (EC 3.1.4.1)/nucleotide pyrophosphatase (EC 3.6.1.9) is a single membrane protein possessing both enzymatic activities. First, it hydrolyzes polyribonucleotides or polydeoxyribonucleotides, which have a free 3′-OH group, sequentially liberating 5′-nucleoside monophosphates by cleavage of the phosphodiester bonds. Secondly, it hydrolyzes the pyrophosphate bond present in nicotinamide adenine dinucleotide (NAD) releasing 5′-adenosine monophosphate (5′-AMP) and nicotinamide mononucleotide [19,20]. The nucleotide pyrophosphatase activity is involved in the regulation of glycoprotein synthesis by controlling the availability of the nucleotide sugars [21]. In addition, it represents the first step in the degradation of NAD, the following being the conversion of 5′-AMP into adenosine by 5′-nucleotidase. The phosphodiesterase activity results in the cleavage of nucleotides from nucleic acids and thus may be involved in the catabolism of

extracellular DNA. The cell surface antigen PC-1, which is characteristic of murine B cells at a late stage of their differentiation, has been identified as being the alkaline phosphodiesterase I protein [22] indicating that this enzyme, as others, corresponds to previously described blood cell surface antigens.

Lymphocyte-APN activity in diabetic patients was not found to be different from the control level. Thus the effect of diabetes on lymphocyte alkaline phosphodiesterase seems specific, since diabetes had no effect on APN activity.

DPP IV releases the N-terminal dipeptides from oligopeptides with proline or alanine as the penultimate amino acid. DPP IV occurs preferentially on the surface of CD4 cells and the enzyme activity increases with T cell activation [23]. DPP IV inhibition improves glucose homeostasis in rats [24,25]. In the present study, PMA-stimulated ecto-DPP IV of patients with Type 2 diabetes was found to be significantly increased over control, but was reduced after metformin treatment. This effect of metformin could also contribute to the better blood glucose control in humans.

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