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Regulation of protein kinase C by short term hyperglycaemia in human platelets in vivo and in vitro

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Abstract

Aims/hypothesis. Postprandial hyperglycaemia carries an increased risk of macrovascular disease even without Type II (non-insulin-dependent) diabetes mellitus. Chronic hyperglycaemia activates protein kinase C (PKC) in vitro and in vivo but it is not known whether PKC is regulated by short-term postprandial hyperglycaemia in vivo in humans. We investigated whether PKC is regulated in vivo in hyperglycaemic and hyperinsulinaemic infusion tests and correlated the results to stimulations in vitro.
Methods. Protein kinase C regulation was measured in platelets obtained from 8 healthy subjects who were infused with glucose and insulin for 2 h attaining peak concentrations of 16 mmol/l glucose and in platelets from 8 healthy young subjects, 8 older subjects without diabetes, and 10 older subjects with Type II diabetes after incubation in vitro with 16 mmol/l glucose or glucose and insulin. For precise quantification, a shortened PKC β1 standard protein was generated by bacterial expression and PKC α, β1, β2 and δ isoenzyme values were measured by immunoblot analyses.
Results. Hyperglycaemic and hyperinsulinaemic in vivo tests increased the amounts of PKC α, β1 and β2 in the membrane fraction of platelets to $225 \pm 87\,\%$, $164 \pm 22\,\%$ and $302 \pm 135\,\%$, respectively, when compared with the baseline values in young healthy volunteers ($n = 8$, $p < 0.05$). The expression of PKC δ did not change. In comparison to the recombinant PKC β1 standard protein, 5 ng PKC β1/μg protein was measured before the test and 2 ng/μg were translocated to the membrane fraction after the infusion. No change in the absolute amount of PKC β1 was detected. In contrast, after incubation in vitro PKC was not regulated by glucose or glucose and insulin in 8 young healthy subjects (age 26 ± 0.7 years) and in 8 older, healthy subjects (age $64{,}8 \pm 4$ years) although 100 nmol/l 12-O-tetradecanoylphorbol 13-acetate caused maximal activation. In marked contrast, PKC β1 and PKC β2, but not PKC α or PKC δ, were increased in vitro in the membrane fraction by $292 \pm 61\,\%$ and $432 \pm 88\,\%$ ($p < 0.05$) in 10 subjects with Type II diabetes mellitus matched for age, sex and BMI.
Conclusion/interpretation. We found that short-term hyperglycaemia activates PKC α, β1 and β2 in platelets of healthy persons making them potential candidates for mediating the increased cardiovascular risk of postprandial hyperglycaemia. Hyperglycaemia and hyperinsulinaemia did not cause short-term activation of PKC in platelets in vitro suggesting the existence of additional stimuli. Subjects with Type II diabetes showed a markedly altered reactivity of platelet PKC β in vitro indicating some diabetes-related regulation. [Diabetologia (2001) 44: 188–195]

Keywords Protein kinase C, diabetes mellitus, hyperglycaemic clamp hyperinsulinaemic clamp, platelets, PKC beta.

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Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; TPA, 12-O-tetradecanoylphorbol 13-acetate; HHIT, hyperglycaemic and hyperinsulinaemic infusion test.

Chronic diabetic complications depend on increased glucose concentrations as has been shown by the DCCT and UKPDS studies [1, 2]. The risk of macrovascular diseases, however, has been shown to increase even in the prediabetic state. Several studies have observed that increased postprandial rather than increased fasting plasma glucose is associated with the development of cardiovascular disease [3, 4]. Short-term but constant increases of plasma glucose therefore seem to be particularly important. The pathogenic principles mediating the effects of transient hyperglycaemia have not been established.

In the last two decades, evidence has accumulated indicating that the development of diabetic complications is multifaceted. Three major lines of changes in cellular physiology have been described. Firstly, increased aldose reductase activity leading to sorbitol accumulation and altered Na^+/K^+-ATPase activity [5]. Secondly, non-enzymatic glycation leading to the modification of proteins and lipoproteins and to the enhanced activation of endothelial cells [6, 7] and, thirdly activation of PKC in diabetes by de novo synthesis of diacylglycerol (DAG) which has been associated with altered cellular responses in vascular cells and other tissues [8]. Among these phenomena, non-enzymatic glycation and sorbitol accumulation require extended periods of hyperglycaemia whereas protein kinase C (PKC) could be activated by transient hyperglycaemia.

In diabetes mellitus, atherosclerosis develops earlier and with a higher frequency than in the population without diabetes. Platelets can contribute to this process because their reactivity determines the formation of thrombus. Increased patelet interaction in diabetes might result from platelet hypersensitivity to agonists or from changes of adhesion molecules and tissue mediators in the vessel wall [9, 10]. The concept of the platelet hypersensitivity has been derived mainly from animal studies or experiments in vitro but evidence from humans or ex vivo is limited.

Hypersensitivity of platelets in diabetes has been associated with increased sensitivity to pro-aggregatory agonists like ADP [11], thrombin [11, 12] or collagen [13] in diabetic rats and to platelet activating factor [14], ADP [11] or thrombin [15] in humans. Many of the pro-aggregatory agonists stimulate signal transduction pathways involving PKC as was shown in studies in vitro for purins [16], arachidonic acid [17] or thrombin [18]. The regulation of PKC therefore seems to play an important part in platelet activity.

Protein kinase C isoenzymes consist of a family of at least ten members which are divided into three groups. All PKC isoenzymes depend on fatty acids for their activation [19]. Platelets express predominantly the isoenzymes α, $\beta1$, $\beta2$, δ and, to a lesser extent, the atypical isoenzyme ζ [18]. The classical isoenzymes of PKC in human platelets comprise the α, $\beta1$, $\beta2$ type which are activated additionally by Ca^{2+} and DAG. The isoenzyme δ in platelets belongs to the novel isoenzymes which are activated without Ca^{2+} and the platelet isoenzyme ζ is an atypical one which is activated independently of Ca^{2+} or DAG.

These changes in diabetes were found to activate PKC. In cultured cells, increased PKC was observed in retinal endothelial cells, in aortic smooth muscle cells or renal mesangial cells after prolonged hyperglycaemia [20]. The classical and novel isoenzymes are activated in particular by diabetic changes as has been shown in the retina of diabetic rats for PKC α, $\beta1$, $\beta2$ and the novel isoenzyme ε [21], or in aorta and heart of diabetic rats for PKC $\beta2$ [22]. There are no data regarding human platelets.

This study aimed to answer the following questions. Firstly, are PKC isoenzyme contents altered in platelets of healthy humans by short-term hyperglycaemia in vivo suggesting an altered platelet function associated with postprandial hyperglycaemia? Secondly, which types of PKC are involved in this process? Thirdly, are alterations of PKC reproduced by glucose or glucose and insulin in vitro indicating the activation of the DAG pathway, and finally, does chronic hyperglycaemia in Type II diabetes mellitus alter platelet contents of PKC isoforms as a consequence of up-regulation or down-regulation of PKC? To answer these questions, a shortened PKC $\beta1$ protein was constructed and used as a quantitative standard in Western blots.

Subjects and methods

Subcloning and affinity purification of a recombinant PKC standard. The pUC12 containing PKC $\beta1$ cDNA (a generous gift of A. Ullrich, Martinsried, Germany) was restricted with NotI and HindIII. The NotI site was filled by the Klenow fragment. This cDNA-fragment was subcloned into a pQE32 expression plasmid (Qiagen, Hilden, Germany) which was restricted by SmaI and HindIII. After induction of the protein expression with IPTG in Top 10F' bacteria (Invitrogen) the tag of six histidines 3' terminal to the PKC-cDNA of the pQE32 vector was used to purify the recombinant PKC protein by a Ni-NTA agarose slurry (Qiagen, Hilden, Germany). Purified shortened PKC protein was quantitated by a modified Bradford assay and was used as an internal standard in immuno blotting analyses [23].

Platelet preparation. Blood was centrifuged with citric acid (1 : 9) and 5 μg PGE_1 for 10 min at 350 · g. Platelets in the plasma were sedimented for 10 min at 800 · g, washed with PBS containing 0.125 μmol/l prostacyclin (PGI_2) and were sedimented again. The purity of platelets was higher than 88 % as determined by flow cytometry.

Incubation of platelets in vitro. Approximately $5 \cdot 10^8$ platelets were incubated in Hepes buffer (10 mmol/l Hepes, pH 7.4, 134 mmol/l NaCl, 12 mmol/l $NaHCO_3$, 3 mmol/l KCl, 0.35 mmol/l NaH_2PO_4, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$) with 100 nmol/l 12-O-tetradecanoylphorbol 13-acetate (TPA) or

0.1 % (v/v) DMSO for 15 min or glucose, mannitol or insulin for 60 min. Platelets were then immediately sedimented for 2 min at 1000 · g, washed with PBS, sedimented again and resuspended in homogenisation buffer (see PKC preparation).

Hyperglycaemic and hyperinsulinaemic infusion tests. Healthy subjects were infused with glucose, insulin and sandostatin (0.5 μg/ml). At the beginning of infusion 25 μg sandostatin were injected. Glucose was infused at a rate of 480 mg/m^2 body surface · min, that of insulin was 25 mU/m^2 body surface · min. During the infusion, blood glucose was determined in 30 min intervals by the glucose oxidase method. A total of 20 ml blood was collected before the infusion and after 120 min in order to prepare the platelets.

Preparation of PKC and quantification of the protein concentration. Platelets were suspended in homogenisation buffer containing 20 mmol/l Tris, pH 7.5, 0.25 mmol/l sucrose, 10 mmol/l EGTA and 2 mmol/l EDTA with freshly added 1 mmol/l PMSF, 0.1 % (w/v) leupeptin, 0.1 % (w/v) aprotinin, 0.1 % (w/v) 2-mercaptoethanol, 20 mmol/l ALLNal, 3 μmol/l microcystin, 200 μmol/l sodium-ortho-vanadate. Platelets were sonified at 25 W for 10 s on ice three times. The homogenate was centrifuged at 100 000 · g for 30 min. The supernatant was collected as cytosol. The sediment was re-extracted with homogenisation buffer containing additionally 1 % (v/v) nonidet P-40 by sonification for 15 s at 25 W on ice. After incubation for 15 min on ice insoluble particles were sedimented at 14000 · g for 15 min. The supernatant was collected as membrane fraction. The concentration of protein was determined by a Bradford assay as modified previously [23].

Immuno blotting and laser densitometry. Protein extracts were separated on 8 % denaturing SDS-PAGE and transferred by semidry blotting to a PVDF membrane. There PKC-isoenzymes were detected by specific polyclonal antibodies against PKC α, β 1, β 2 or δ. Antibodies against PKC α were from Calbiochem (Homburg, Germany) and against β1, β2 and δ from Santa Cruz (Heidelberg, Germany). The PVDF membrane was blocked in TBS-T containing 10 mmol/l Tris, pH8, 150 mmol/l NaCl and 0.05 % (v/v) Tween-20 with 2 % (w/v) non-fat milk powder overnight at 4 °C. All antibodies were incubated in TBS-T with 0.5 % (w/v) non-fat milk powder for 1 h at room temperature. Signals were visualized by the ECL system of Amersham (Freiburg, Germany). Specific signals were quantified by laser densitometry (Molecular Dynamics, Sunnyvale, Calif., USA). Data gained by laser densitometry are partly given in arbitrary units reflecting the raw data of integrated signal areas of the respective analyses.

Construction and test of a shortened PKC β1 standard protein. In order to measure accurately the PKC protein signals in cytosolic and membrane protein fractions of platelets and to allow quantitative intraassay comparison we constructed a shortened PKC β1 to be used as a reference. This standard migrates at about 65000 M_r SDS gels and was expressed in bacteria. A tag of six histidines was added C-terminally to allow affinity purification with a Ni-NTA matrix of completely expressed PKC. Using this standard, a linear increase of the signal intensity of the PKC β1 standard protein between 1 and 15 · 10^{-9} g was determined by laser densitometry (Fig. 1). The highest signal intensity of the linear curve was used as cut-off value for the quantification of protein fractions. The purified PKC β1 standard protein was added to protein homogenates to measure the absolute amounts of PKC β1. Denaturating SDS-PAGEs were therefore carried out and polyclonal antibodies to PKC β1 were used. Because of the amino acid sequences, recognized by the polyclonal antibodies, of the shortened standard protein of PKC β1 and the cellular protein are identical, the antibodies recognize both types of denatured PKC β1.

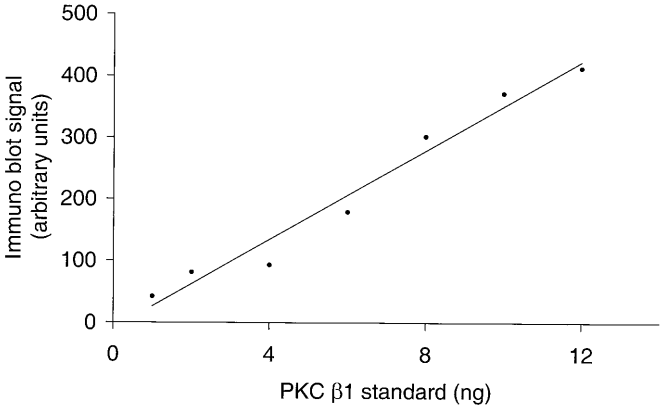

Fig. 1. Linearity of immuno blot signals of the affinity purified PKC β1 standard protein added to human platelet homogenates. Shortened PKC β1 standard protein was expressed in bacteria, purified with Ni-NTA and the concentration of the protein was determined by a Bradford assay. The purified protein was added to human platelet homogenates in various amounts and separated by 8 % SDS-PAGE. The PKC signals were developed by the ECL system. The signal intensities were determined by laser densitometry

Table 1. Clinical and biological characteristics of study subjects

	Healthy young subjects (n = 8)	Older non-diabetic subjects (n = 8)	Older diabetic subjects (n = 10)
Sex: men	5	4	5
women	3	4	5
Age (year)	26 ± 0.7	64.8 ± 4	67.5 ± 3
BMI (kg/m^2)	22.7 ± 1.4	25.9 ± 1.2	26.7 ± 1.2
Fasting blood sugar (mg/dl)	92 ± 7	89 ± 6	224 ± 29
HbA_{1c} (%)	5 ± 0.1	5.3 ± 0.2	9.2 ± 0.5
Total cholesterol (mg/dl)	191 ± 17	224 ± 20	218 ± 12
Triglycerides (mg/dl)	76 ± 10	126 ± 21	215 ± 60

Statistics. Data are presented as average ± SEM. Statistic evaluation was done by Mann-Whitney U test or Wilcoxon test as appropriate. A p value of less than 0.05 was considered statistically significant.

Results

Hyperglycaemic and hyperinsulinaemic infusion tests. In order to test whether PKC is regulated by the short-term effects of moderate hyperglycaemia and hyperinsulinaemia infusion tests (HHITs) were carried out on healthy subjects (Table 1). The subjects were infused with insulin and glucose for 2 h to achieve similar max-

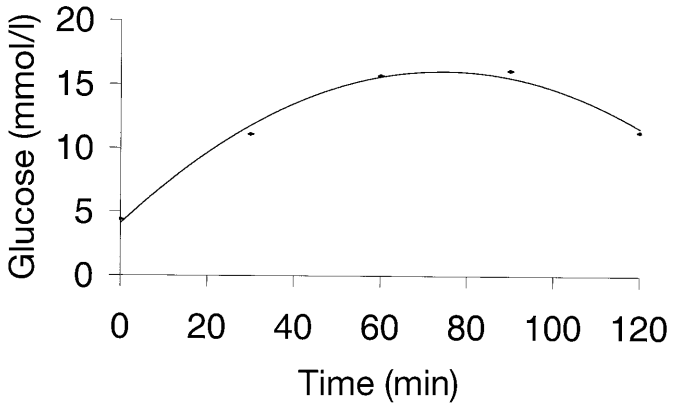

Fig. 2. Time course of plasma glucose concentrations during hyperglycaemic and hyperinsulinaemic infusion tests (HHITs, $n = 8$). Young healthy subjects were infused with glucose ($480\ mg \cdot m^{-2} \cdot min^{-1}$), insulin ($25\ mU \cdot m^{-2} \cdot min^{-1}$) and sandostatin ($0.5\ \mu g \cdot min^{-1}$). Samples were obtained as indicated

imum glucose concentrations and time profiles and to mimic post-prandial conditions. All infusions were controlled at 30 min intervals to ascertain the plasma glucose concentrations. The highest glucose concentration reached was 16 mmol/l (Fig. 2). Earlier results from our group showed that the amount of PKC in the cytosol and membrane fraction did not change in healthy persons when glucose increased from 2 mmol/l to 4 mmol/l in hyperglycaemic and hyperinsulinaemic conditions after 60 and 150 min [24]. In agreement with earlier studies, the response of PKC isoenzymes to the infusion in isolated platelets was measured using isoenzyme specific immuno blots. The translocation of PKC from the cytosolic to the membrane fraction, which is a prerequisite for activation, was determined for all patients in the study [25].

The response of the PKC $\beta 1$ relative to the added PKC $\beta 1$ standard protein was measured first. The standard protein showed homogenous staining over the whole blot membrane indicating accurate blot results. The PKC $\beta 1$ showed two specific signals at 76000 and 82000 M_r in the membrane and cytosol fraction. After 120 min of the infusion both signals of PKC $\beta 1$ in the membrane fraction increased to $153 \pm 14\,\%$ at 76000 M_r and to $164 \pm 22\,\%$ at 82000 M_r relative to the platelets isolated before the infusion (Fig. 3, $p = 0.04$). There was no change in the total amount of PKC $\beta 1$ protein before and after HHITs as the addition of the signal intensities of cytosolic and membrane signals showed relative to the added standard protein (Fig. 3). The total quantity of PKC before the HHIT was 5 ± 1.5 ng/µg for both signals of PKC $\beta 1$ in cytosol and membrane fraction and the translocated amount after the test was 2 ± 0.6 ng/µg.

Based on our knowledge of the homogeneity of the blotting procedure with the PKC standard protein, the isoenzymes α, $\beta 2$ and δ were analysed. The

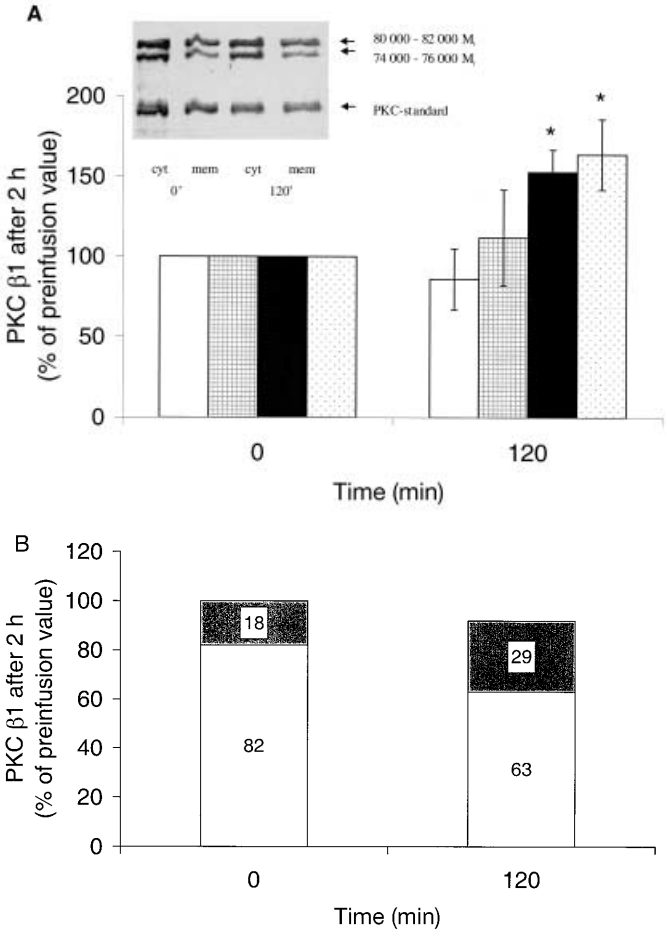

Fig. 3 A–B. (**A**) Comparison of cytosol (76000 M_r □, 82000 M_r ▦) and membrane (76000 M_r ■, 82000 M_r ▨) PKC $\beta 1$ signals before and after 120 min HHITs with co-detection of the PKC $\beta 1$ standard protein by immuno blots. Cell homogenates were prepared after HHIT and equal amounts of the respective protein fraction were subjected to SDS-PAGE. Signal intensities were determined by laser densitometry. Differences between the 76000 M_r signal and the 82000 M_r signal of the membrane fraction before and after the HHITs are indicated (Wilcoxon test; * $p < 0.05$). Cyt: cytosol fraction, mem: membrane fraction. (**B**) Diagram of determination of PKC $\beta 1$ content before and after 120 min HHITs in cytosol and membrane fractions. Cellular homogenates were prepared and analysed as in A). Signal intensities of cytosol (cyt, □) and membrane (mem, ■) fractions were added and expressed relative to the amount of preinfusion

isoenzyme α was translocated to the membrane fraction which increased to $225 \pm 87\,\%$ of the initial value (Fig. 4, $p = 0.04$). The PKC $\beta 2$ increased to $302 \pm 135\,\%$ of the initial value in the membrane fraction after the HHIT (Fig. 4, $p = 0.04$). No significant change was detected for the PKC δ isoenzyme (Fig. 4).

Incubation of platelets in vitro. To further analyse the results in vivo on the role of glucose or of insulin in the activation of PKC, platelets from three groups of subjects were incubated with glucose or in combina-

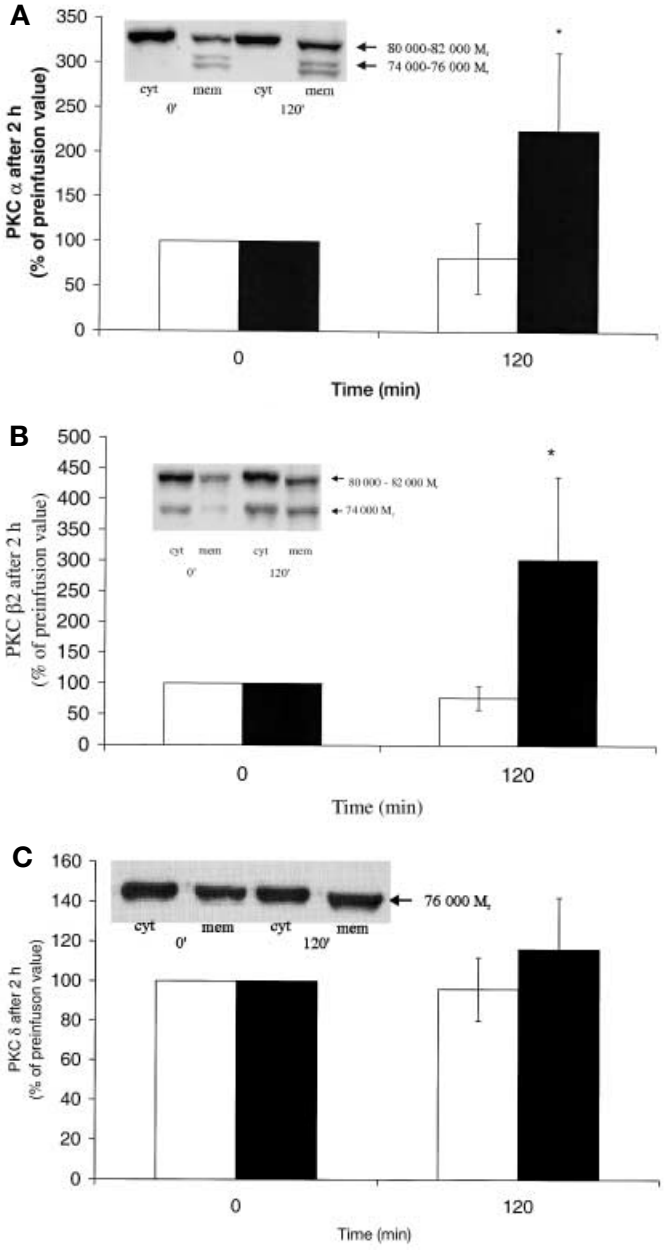

Fig. 4 A–C. Comparison of cytosol (cyt, □) and membrane (mem, ■) PKC signals before and after 120 min HHITs by immuno blots of (**A**) PKC α (**B**) PKC β2 and (**C**) PKC δ. Cellular homogenates were prepared and analyzed (see Fig. 3). PKC isoenzymes were detected with the respective isoenzyme specific polyclonal antibodies. Significant differences were found between the membrane contents of PKC α and PKC β2 before and after the HHITs by the Wilcoxon test, respectively (* $p < 0.05$)

tion with insulin. The first group comprised 8 healthy young subjects, the second 8 older subjects without diabetes and the third 10 older subjects with diabetes (Table 1). None of the isoenzymes in the platelets of young subjects or in the older subjects without diabetes changed after incubation for 1 h with glucose or after 15 min with insulin and glucose in cytosol or membrane (Table 2). As a control, an aliquot of

Table 2. Comparison of cytosol and membrane PKC signals before and after incubation of human platelets in vitro

Isoenzyme	PKC β1						PKC β2				PKC α				PKC δ			
	older non-diabetic $n = 8$				diabetic patients $n = 10$				older non-diabetic $n = 8$		diabetic patients $n = 10$		older non-diabetic $n = 8$		diabetic patients $n = 8$		older non-diabetic $n = 8$		diabetic patients $n = 10$	
Fraction	cyt 82000 M_r	cyt 76000 M_r	mem 82000 M_r	mem 76000 M_r	cyt 82000 M_r	cyt 76000 M_r	mem 82000 M_r	mem 76000 M_r	cyt	mem	cyt	mem	cyt	mem	cyt	mem	cyt	mem	cyt	mem
C	133 (19)	252 (48)	130 (46)	193 (28)[a, b]	120 (6)	172 (21)	147 (17)	56 (6)	133 (10)	91 (36)	109 (1)	97 (21)	126 (11)	97 (30)	108 (8)	61 (8)[a]	136 (6)	92 (16)	151 (37)	95 (20)
MAN	85 (9)	112 (21)	118 (57)	127 (37)	109 (13)	93 (22)	366 (42)[a, b]	162 (27)	122 (14)	123 (62)	89 (8)	436 (35)[a, b]	122 (13)	127 (71)	93 (6)	110 (16)	114 (11)	82 (37)	90 (16)	200 (41)[b]
LG	88 (19)	197 (61)	145 (43)	79 (9)	96 (14)	123 (22)	484 (168)[a, b]	145 (59)	108 (16)	126 (44)	65 (15)[a]	779 (385)[a, b]	95 (15)	116 (57)	102 (17)	212 (51)[a]	122 (6)	88 (13)	116 (18)	199 (49)
HG	96 (13)	161 (42)	127 (30)	73 (6)	83 (7)	92 (16)	292 (61)[a, b]	105 (21)	112 (9)	74 (12)	73 (10)[a, b]	432 (88)[a, b]	109 (7)	87 (23)	85 (10)	113 (22)	116 (11)	75 (8)	113 (11)	159 (37)[b]
C1	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)
TPA	48 (18)[a]	27 (11)[a]	613 (257)[a]	576 (297)[a]	57 (9)[a]	19 (2)[a]	698 (202)[a]	647 (166)[a]	28 (8)[a]	424 (226)[a]	56 (15)[a]	727 (259)[a]	16 (5)[a]	715 (224)[a]	100 (7)	152 (23)[a, b]	24 (7)[a]	190 (38)[a]	40 (15)[a]	219 (51)[a]
I + G	121 (22)	228 (66)	59 (11)	54 (8)	151 (33)	89 (68)	127 (27)	63 (12)	113 (6)	50 (13)	99 (5)	61 (25)	115 (16)	57 (12)	100 (7)	60 (33)	120 (4)	72 (7)	95 (11)	81 (19)

Platelets were isolated from whole blood and protein homogenates of cytosol and membrane fractions were analysed for PKCα, PKCβ1, β2 and δ, PKC levels were compared within a group of interest relative to C1 and separately for older non diabetic and diabetic patient. 4.4 mmol/l glucose 1 h (LG), 16 mmol/l glucose 1 h (HG), 16 mmol/l mannitol 1 h (MAN), 4.4 mmol/l glucose for 15 min (C1), 100 nmol/l TPA for 15 min (TPA), without incubation (C), 25 mU/ml insulin + 16 mmol/l glucose 15 min (I + G), cyt: cytosol, mem: membrane;
[a] $p < 0.05$ with Wilcoxon test within the group of interest compared to C1, [b] $p < 0.05$ with Mann-Whitney U test between older patients without diabetes and patients with diabetes; PKC levels are expressed as percentage of C1, values in parenthesis are SEM.

platelets was incubated with 12-O-tetradecanoylphorbol 13-acetate (TPA) as a potent PKC activator which very effectively translocated all isoenzymes to the membrane fraction. The increase of the PKC signal after TPA treatment was in the membrane fraction 715 ± 224 % for PKC α, 613 ± 257 % for 82000 M_r PKC β1 and 576 ± 297 % for 76000 M_r PKC β1, 424 ± 226 % for PKC β2 and 190 ± 39 % for PKC δ.

Platelets of patients with Type II diabetes displayed a large increase in membrane associated PKC β1 and PKC β2 after 1 h of incubation with 4.4 or 16 mmol/l glucose or 16 mmol/l mannitol compared with no incubation or 15 min incubation. Similarly, TPA caused a strong increase in membrane associated PKC β1 and PKC β2. The experiments were originally designed to compare high glucose (16 mmol/l) with low glucose concentrations (4.4 mmol/l) and with an osmotic control of the high glucose value with mannitol (16 mmol/l) after 1 h of incubation. All these incubations showed similar amounts of PKC without changes in isoenzymes level (Table 2). Thus, there was an extensive regulation of PKC β induced by stimuli associated with the incubation, independent of glucose concentrations. The upper 82000 M_r PKC β1 signal increased to 292 ± 61 % by stimulation with 16 mmol/l glucose ($p = 0.03$), to 484 ± 168 % for 4.4 mmol/l glucose ($p = 0.01$) and to 366 ± 42 % for 16 mmol/l mannitol ($p = 0.04$, Table 2). The amount of membrane associated PKC β2 increased by 432 ± 88 % with 16 mmol/l glucose ($p = 0.03$), by 779 ± 385 % with 4.4 mmol/l glucose ($p = 0.04$) and by 436 ± 35 % with 16 mmol/l mannitol ($p = 0.04$, Table 2). PKC δ and α levels did not change. Incubation with insulin and glucose did not change the amount of PKC isoenzymes. Therefore the amount of PKC β isoenzymes of subjects with diabetes specifically increased in vitro in response to a time dependent, submaximum stimulus.

It is not known whether PKC β is upregulated or downregulated in humans as a consequence of chronic activation in vivo due to hyperglycaemia and other metabolic abnormalities in Type II diabetes. The PKC β1 protein standard that we have developed helps to investigate this question in platelets which are fully exposed to metabolic fluctuations in the blood. The PKC β1 content in the cytosol of platelets of diabetic patients, older patients without diabetes and younger control subjects was 5.6 ± 0.4 ng/μg, 3.7 ± 1.6 ng /μg and 5.0 ± 1.5 ng/μg protein and was 0.7 ± 0.2 ng/μg protein, 0.7 ± 0.2 ng/μg and 0.9 ± 0.3 ng/μg protein in the membrane respectively. Therefore, no difference in the total amount of the protein content or in the relative distribution of the membrane fractions was observed in freshly isolated platelets of the three groups.

Discussion

Atherosclerosis involves a complex pathophysiology. This includes a proaggregatory and prothrombotic state which is caused by the increased expression of aggregatory mediators such as PAI-1 (plasminogen activator inhibitor-1) and by increased activation of platelets [26]. Hyperglycaemia markedly worsens the atherosclerotic predisposition. The mechanisms involved in humans are, however, only partially understood.

The HHITs were carried out in young healthy persons to measure whether PKC isoenzymes were rapidly regulated by high glucose and insulin concentrations independently of pre-existing diabetic conditions. The isoenzymes PKC α, β1 and β2 increased in the membrane fraction in vivo indicating their activation [25]. These data show that in addition to the long-term effects of glucose, the short-term induction of hyperglycaemic and hyperinsulinaemic conditions also effectively regulate PKC. The regulation of PKC shows primarily the response of platelets, which were enriched by 88 % from whole blood, rather than other blood cells. Nevertheless other blood cells could have contributed to the observed effects to some extent.

The same isoenzymes were regulated in this model as have been shown to be involved in the development of diabetic complications. This applies to PKC β which has been found to be altered in the vascular cells of diabetic mice [22] and which has been shown to be involved in nephropathy of rats [8] and to lead to heart failure in transgenic mice upon overexpression [27]. According to these models, an altered PKC β regulation is important particularly for the development of diabetic complications, at least after a long-term increase of glucose concentrations. Our study shows that short-term deviations in blood glucose in vivo affected the same PKC isoenzyme independently of pre-existing diabetic complications or long-term metabolic abnormalities. Our data therefore support the hypothesis with the concept that changes of PKC β regulation in diabetes precede the development of diabetic complications. Both PKC β1 and β2 might therefore be involved in the short-term and earlier changes of diabetes as well as in the later stages of diabetes.

Changes in the regulation of PKC could therefore appear at the beginning of the metabolic conversion in Type II diabetes. In agreement with this hypothesis, the normalization of altered PKC activity even in advanced stages of diabetes reduced diabetic nephropathy in rats [8] or macrovascular changes in mice [22] and a PKC β selective inhibitor ameliorated the blood pressure and filtration time in the retina of patients with diabetes [28]. The regulation of PKC β in response to short-term glucose stimuli was maintained in the advanced stages of diabetes under mild

hyperglycaemic conditions in Type II diabetes [24]. Short-term regulation of PKC in our model might thus mimic periods of changing blood glucose and insulin concentrations in diabetes leading to diabetic complications. In this context, it is noteworthy that periodic glucose fluctuations were proposed to be important for the development of diabetic complications and for the enhanced synthesis of collagen type III and IV [29].

Increased glucose and insulin concentrations, as mimicked in the HHITs, are characteristic of postprandial changes in obese subjects, even in the absence of diabetes. Because we could not distinguish these factors from the infusions of glucose and insulin, we attempted to identify their effects by incubating the platelets of the healthy, young subjects in vitro. The platelets were exposed to glucose concentrations of 4.4 or 16 mmol/l or to 16 mmol/l mannitol as an osmotic control in vitro. These incubations showed no response to any of the isoenzymes of PKC in vitro. We therefore concluded that glucose did not directly regulate platelet PKC in vitro. The response was not altered by the addition of insulin in vitro although the presence of an insulin receptor on platelets has been described even if the function has not been specified [30]. The regulation of PKC in vivo thus could be the result of postprandial alterations of GLP-1, GIP or glucagon, but there is no direct evidence for an effect of these hormones. Since PKC is sensitive to oxidation [31] it could, however, also be regulated by hyperglycaemia-enhanced oxidative processes [32] or by a postprandial increase in plasma free fatty acids and triglycerides or LDL receptors [33].

The increased content of PKC β1 and β2 in the platelet membrane in vitro was found to be specific for subjects with diabetes because the PKC isoenzymes of the control group without diabetes, and matched for age, sex and BMI showed no response. The differences of PKC regulation in subjects with Type II diabetes and control subjects could reflect long-term changes in diabetes such as enhanced oxidative processes or increased amounts of LDL, VLDL, free fatty acids or chylomicrons. These long-term effects of glucose could contribute to a hyperglycaemic memory of cells and to the alteration of signal transduction. The amplitude of the fluctuation of blood glucose concentration therefore seems to be relevant for the long-term activation of PKC [29]. Platelet hypersensitivity in subjects with Type II diabetes is a well known phenomenom [10]. Our results show that the facilitated activation of PKC β1 and PKC β2, but not of PKC α or PKC δ, could contribute to this hypersensitivity and represents a rather specific response with regard to PKC isoforms.

Our data provide further support for the concept that PKC β is the most sensitive isoenzyme when it comes to activation which might depend in particular on the mobilization of Ca^{2+} in the platelets of subjects with diabetes [34]. Accordingly, our investigations show that Ca^{2+}-sensitive isoenzymes were regulated in vivo or in vitro or both whereas PKC δ was not regulated. The regulation of PKC β in vivo and in vitro supports the idea that predominantly the isoenzymes of this group are potentially potent mediators of diabetic alterations to the cellular responses in humans.

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