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Increased expression of Gαq protein in the heart of streptozotocin-induced diabetic rats

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Abbreviations: cAMP, cyclic adenosine monophosphate; PKA, cAMP dependent protein kinase; G proteins, signal transducing GTP-binding proteins; PKC, protein kinase C; CaM kinase II, calcium/calmodulin dependent protein kinase II

Abstract

Heart disease is one of the major cause of death in diabetic patients, but the pathogenesis of diabetic cardio-myopathy remains unclear. In this experiment, to assess the significance of G protein signaling pathways in the pathogenesis of diabetic cardiomyopathy, we analyzed the expression of G proteins and the activities of second messenger dependent protein kinases: cAMP-dependent protein kinase (PKA), DAG-mediated protein kinase C (PKC), and calmodulin dependent protein kinase II (CaM kinase II) in the streptozotocin induced diabetic rat heart. The expression of Gαq was increased by slightly over 10% (P<0.05) in diabetic rat heart, while Gαs, Gαi, and Gβ remained unchanged. The PKA activity in the heart did not change significantly but increased by 27% (P<0.01) in the liver. Insulin treatment did not restore the increased activity in the liver. Total PKC activity in the heart was increased by 56% (P<0.01), and insulin treatment did not restore such increase. The CaM kinase II activity in the heart remained at the same level but was slightly increased in the liver (14% increase, P<0.05). These findings of increased expression of Gαq in the streptozotocin-diabetic rat heart that are reflected by the increased level of PKC activity and insensitivity to insulin demonstrate that alteration of Gαq may underlie, at least partly, the cardiac dysfunction that is associated with diabetes.

Keywords: G proteins, protein kinase, cAMP dependent protein kinase, protein kinase C, calcium/calmodulin dependent protein kinase II, streptozotocin-induced diabetes

Introduction

Diabetes mellitus is a complex metabolic disease caused by impairment of insulin signaling pathways, and the defect usually results from pancreatic β-cell deficiency and/or a deficiency of insulin (Kahn, 1994). This disease causes many chronic complications such as vascular disease, retinopathy, neuropathy, kidney disease, and heart disease, and the diabetic heart disease is one of the major causes of death in the patients. Although it has been recognized for many years that diabetes mellitus is associated with cardiac dysfunction caused by cardiomyopathy without significant coronary artery disease, the pathogenesis of diabetic cardiomyopathy remains unclear yet.

The Diabetes Control and Complications Trial Research Group reported that a strict glycemic control could prevent the onset and progression of diabetic complications (1993). Several hypotheses have been proposed to explain the various pathologic changes induced by hyperglycemia such as hyperosmolarity, glycation end products, oxidant formation, abnormality of sorbitol and myoinositol metabolism, and diacylglycerol (DAG) protein kinase C (PKC) activation (Greene *et al.*, 1987; Brownlee *et al.*, 1988; Williamson *et al.*, 1993). It is likely that glucose and its metabolites mediate their adverse effects by altering the various signal transduction pathways, which are used by various cells to perform their functions and to maintain cellular integrity.

Though the dysfunction of insulin signaling pathway plays a major role in the pathogenesis of diabetic mellitus and it's complication, many other signaling pathways are also involved in these processes. One of such pathways is heterotrimeric GTP-binding protein (G protein) which transduces a variety of signals across the plasma membrane by sequential interactions with receptor and effector proteins, e.g. second messenger-generating enzymes and ion channels. These interactions result from guanidine nucleotide-driven conformational changes in G-protein α subunits. Agonist-bound receptors catalyze the exchange of GDP for GTP on the α subunits of their cognate G proteins to promote dissociation of α from a high affinity complex of βγ subunits. Dissociated subunits are competent to modulate the activity of effectors. GTP hydrolysis ultimately returns Gα to GDP-bound state, thus allowing reformation of an inactive heterotrimer.

Sixteen distinct genes encode G protein α subunits in mammals. They are commonly divided into four subclasses based on amino acid sequence identity and function: Gs, Gi, Gq, and G_{12} (Berman and Gilman, 1998). One of more thoroughly characterized examples of G protein-mediated signal transduction is carried out by the hormone-sensitive adenyl cyclase system. Relevant receptors communicate with homologous G proteins, one of which (Gs) activates adenyl cyclase while the other (Gi) inhibits the enzyme. The second messenger (cAMP) mediates diverse cellular responses, primarily by activating cAMP-dependent protein kinase (PKA). In the case of Ca^{2+}-mobilizing agonist, G protein activation is followed by stimulation of phospholipase Cβ (PLCβ) to generate IP3 and DAG in the cytosol, which initiates the $[Ca^{2+}]i$ signal by release of Ca^{2+} from internal stores. PLCβ can be activated by each of the four Gq class α subunits or by Gβγ subunits released from G class proteins. IP3 and DAG in turn activates PKC (Hepler and Gilman, 1992).

Insulin mediated singal transduction which is dependent on the protein kinases and phosphatases have been reported to be altered in the diabetic animals (Ingebretsen *et al*., 1981; Parker *et al*., 1983; Smogorzewski *et al*., 1998), and these kinases are likely to be involved in the pathogenesis of diabetic heart complications. In this experiment, to assess the significance of G protein signaling pathways in the pathogenesis of diabetic myocardiopathy, we analyzed the expression of G proteins and the activities of second messenger dependent protein kinases, PKA, PKC, and calmodulin dependent protein kinase II (CaM kinase II) in the streptozotocin induced diabetic rat heart.

Materials and Methods

Animal treatment

All animal use followed the National Institutes of Health guidelines for the care and use of experimental animals. Male Sprague-Dawley rats (8 wk) weighing about 250 g were randomly divided into two groups, and one group was made diabetic by a single intraperitoneal injection of streptozotocin (65 mg/kg) in 50 mM citrate buffer, pH 4.5, whereas the control animal group was injected with the buffer only. In the diabetic group, the development of diabetes mellitus was confirmed by an urine test after one week of streptozotocin injection. After confirmation, diabetic rats were randomly divided into two groups. One diabetic group was injected with minimum amount of insulin for their survival throughout the experimental period, and the other group was treated with insulin (5 units per day) by subcutaneous injection. The rats were fasted for 5 h before the blood sampling, and the samples were assayed for glycohemoglobin by affinity chromatography (Glyc-Affin GHb, Iso Lab Inc., Akron, OH, USA) and for glucose using a glucose analyzer (Beckman Instruments Inc., Fullerton, CA, USA). Routine urine analysis was done by Diastix and Ketostix (Miles Sankyo Co., Tokyo, Japan).

All rats were maintained on normal animal chow and water ad libitum for 8-12 weeks before sacrifice. Rats were anesthetized with ether, and their hearts and livers were excised and rinsed with ice-cold saline. The tissues were immediately frozen in liquid nitrogen, and were stored at -80°C for assays of the G protein expression and protein kinase activity.

Preparation of tissue homogenate

Frozen tissues were thawed on ice, and then were homogenized with Polytron homogenizer in a homogenization buffer composed of 50 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM dithiothreitol. The protein content of the preparation was determined by the bicinchoninic acid method with bovine serum albumin as the reference standard (Smith *et al*., 1985).

The expression of G proteins and the activity of each protein kinase were assayed using assay kits of homogenate cAMP dependent protein kinase, protein kinase C and calmodulin-dependent protein kinase II bought from Upstate Biotechnology (Lake Placid, NY, USA).

Analysis of G protein expression by Western blot

Sixteen to 200 μg of the homogenate protein was separated on a 12.5% SDS polyacrylamide gel and transferred to nitrocellulose. The blot was blocked with 5% non-fat milk in phosphate buffered saline (PBS) for 1 h, and then incubated at room temperature with an antibody against each G protein (1 to 5 μg/ml) overnight. The antibodies were generated against carboxyl terminal peptides of each G protein and purified as described previously (Shin *et al*., 1995). The nitrocellulose paper was then washed with 0.1% Tween 20 in PBS and incubated with peroxidase-labeled goat anti-rabbit IgG antibody preparation (1 : 5000 dilution, Pierce, IL, USA) for 2 h at room temperature. The blot was washed with PBS and then incubated with enhanced chemiluminescence (ECL) substrate mixture (Amersham Inter-national plc, England) for 1 min, and then exposed to an X-ray film (AGFA Curix RPI) for 0.5-3 min to obtain an image.

The density of visualized bands corresponding the control, diabetic, and insulin-treated diabetic rat G proteins was quantitated with an image analyzer (BioRad, Model GS-700, USA), and the density was compared with those of standard samples applied on the same gel.

Assay of PKA activity

In a microfuge tube, 10 μl of substrate cocktail containing

500 μM Kemptide and 10 μM cAMP, 10 μl of inhibitor cocktail containing 20 μM PKC inhibitor peptide and 20 μM Compound R24571, and 10 μl of tissue homogenate were added in order. Then 10 μl of the mixture containing 0.5 mM ATP, 75 mM $MgCl_2$ and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) was added, and incubated for 10 min at 30°C. After incubation, 25 μl of the mixture was blotted on the P81 paper square, and the paper was washed once with 0.75% phosphoric acid. The assay square was transferred to a scintillation vial containing 5 ml scintillation cocktail, and its radio-activity was counted in a scintillation counter (Model Tri-Carb 1600 CA, Packard Instrument Company, Meriden, CT, USA)

Assay of PKC activity

In a microfuge tube, 10 μl of substrate cocktail containing 500 μM PKC substrate peptide, 10 μl of inhibitor cocktail containing 2 μM PKA inhibitor peptide and 20 μM compound R24571, 10 μl of the lipid activator containing 0.5 mg/ml phosphatidylserine and 0.05 mg/ml diglyceride, and 10 μl of tissue homogenate were added in order. Then 10 μl of the mixture containing 0.5 mM ATP, 75 mM $MgCl_2$ and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) were added, and incubated for 10 min at 30°C. The procedures following the incubation were the same as those for PKA assay.

Assay of CaM kinase II activity

In a microfuge tube, 10 μl of substrate cocktail containing 500 μM Auto Camtide and 40 μg/ml calmodulin, 10 μl of inhibitor cocktail containing 2 μM PKA inhibitor peptide and 2 μM PKC inhibitor peptide, and 10 μl of tissue homogenate were added in order. Then 10 μl of the mixture containing 0.5 mM ATP, 75 mM $MgCl_2$ and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) were added, and incubated for 10 min at 30°C. The procedures following incubation were the same as those for PKA assay.

Statistical analysis

All data were analyzed on a personal computer using StatView SE+Graphics (Abacus Concepts Inc., Berkeley, CA, USA) software. Protein kinase activities were analyzed by non-parametric test using the Kruskal-Wallis test and the Mann-Whitney U test, and the difference with $P < 0.05$ among or between groups was considered a significant change.

Results and Discussion

The expression of signal transducing G proteins and the activity of second messenger dependent protein kinases were analyzed in the heart and liver homogenate of control, streptozotocin-induced diabetic, and insulin-treated diabetic rats respectively.

Expression of G proteins

Stimulatory G protein alpha subunit (Gαs) was detected as two bands each of which corresponded to 42 kD and 47 kD respectively, and alpha subunits of inhibitory G protein (Gαi) and Gq protein (Gαq) were visualized as one band (Figure 1a). The alpha subunit of Go was not detected enough to allow quantitative analysis by Western blot. The beta subunit of G proteins (Gβ) was also detected as a broad band indicating that it is composed of several isoforms. The expression of Gαq was increased by slightly over 10% ($P < 0.05$) in the

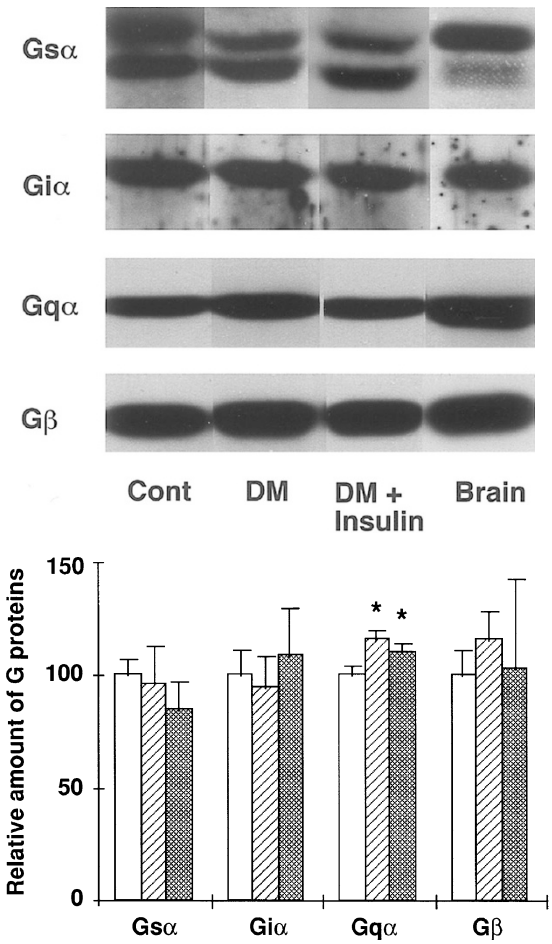

Figure 1. Expression of G proteins in the heart of streptozotocin-induced diabetic rats. a) Representative western blots of rat heart G proteins. Sixteen to 200 μg of the heart homogenate from control (Cont), diabetic (DM), and insulin-treated diabetic (DM + insulin) rats were analyzed for G protein expression by 12.5% SDS-PAGE and immunoblot. b) Histograms of G protein expression in rat hearts. Expression of each G proteins in the heart homogenate from the control (dotted bar), streptozotocin-induced diabetic (slant-lined bar), and insulin-treated diabetic (hatched bar) rats were analyzed by densitometry of the Western blots. Sixteen μg of the homogenate and RM antibody (1 μg/ml) was used for Western analysis of Gsα, 45 μg of the homogenate and AS antibody (1 μg/ml) for Giα, 200 μg of the homogenate and QL antibody (3 μg/ml) for Gqα, and 50 μg of the homogenate and SW antibody (1 μg/ml) for Gβ. Each group was composed of 4 or 5 rats. An asterisk represents the activity that differed statistically significantly from the control ($P < 0.05$).

heart of diabetic rat whereas Gαs, Gαi, and Gβ did not change significantly (Figure 1b). Treatment with insulin did not restore the elevated Gαq activity. Our result on the expression of Gαs, not affected by insulin deficiency, was agreeable with the findings of Wichelhaus *et al.* (1994) in the heart ventricles of streptozotocin-induced diabetic rat. The result of no change of Gαi expression in heart of diabetic rat was agreeable with the findings of Fu *et al.* (1994), but differed from the result of Wichelhaus *et al.* (1994). The G protein-coupled signaling pathways initiate a cascade of enzyme activation, such as PKA, PLC, PKC, CaM kinase etc. The increased expression of Gαq may reflect on the activation of protein kinases.

PKA activity

The cAMP-dependent PKA activity in the heart did not change significantly in either diabetic or insulin-treated diabetic group compared with the normal control group. In contrast, PKA activity in the liver of diabetic rats was increased by 27% ($p < 0.01$) and insulin treatment of diabetic rats reduced the increment of this kinase activity but not to the level of the normal control (12% increase, $P < 0.05$) (Figure 2).

The PKA is regulated hormonally by the pathway of membrane receptors, G proteins and adenylate cyclases, and plays a central role in the regulation of glycogen metabolism (Cohen, 1978). PKA was also involved in the regulation of heart contractile force (Keely and Eiring, 1979), and therefore, PKA activity was expected to change in diabetes heart. However, our results revealed no significant change in the enzyme activity of diabetic rat hearts. Similar results were observed in the heart of alloxan induced diabetic rats (Ingebretsen *et al.*, 1981). The unaltered activity of PKA may be harmonious with the unaltered expression of Gs and Gi proteins in that the activity of cAMP signaling pathway remains same in diabetic heart. Insulin stimulates glycogen synthetase by inhibiting cAMP-dependent protein kinase in rat liver (Mor *et al.*, 1981). Contrary to our results of PKA activity increase in the liver of diabetic rat, the decrease of the enzyme activity has also been reported (Weber *et al.*, 1977).

Changes in PKC activity

Total activity of PKC was increased by 56% ($P < 0.01$) in the heart of diabetic rats, and insulin treatment did not influence the increased activity of diabetic rat hearts (61% increase from the control, $P < 0.01$). The PKC activity also increased by 25% ($P < 0.05$) in the liver from diabetic group, and insulin treatment did not restore the increased enzyme activity in diabetic rats (20% increase from the control, $P < 0.05$) (Figure 3).

Our result is consistent with the report that cardiac PKC activity in diabetes increased in both cytosolic and membrane fractions in streptozotocin-induced diabetic rats (Tanaka *et al.*, 1991). The increase in PKC activity in diabetic heart might be in part resulted from the increased expression of Gqα observed in this experiment. It was recently reported that whole ventricle basal PKC activity was increased in BB/Wor diabetic rats (Giles *et al.*, 1998) and in streptozotocin-induced diabetic skeletal muscles (Given *et al.*, 1998). All these results suggest that increased PKC activity may play a pivotal role among insulin-dependent diabetes mellitus in the development of diabetic cardiomyopathy and skeletal muscle myopathy.

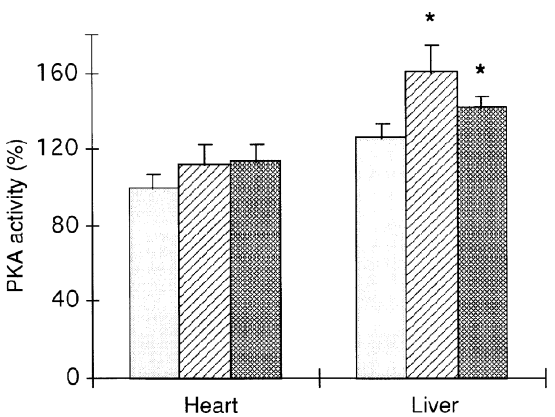

Figure 2. Activity of PKA in the heart and the liver of streptozotocin-induced diabetic rats. PKA activity was analyzed in the homogenate of the heart and the liver from the control (dotted bar), streptozotocin-induced diabetic (slant-lined bar), and insulin-treated diabetic (hatched bar) rats. The average enzyme activity and standard error were expressed as the percentage to heart PKA activity of the control group. Each group was composed of 10 rats, and two separate experiments were performed in duplicate. An asterisk represents the activity that differed statistically significantly from the control ($P < 0.05$).

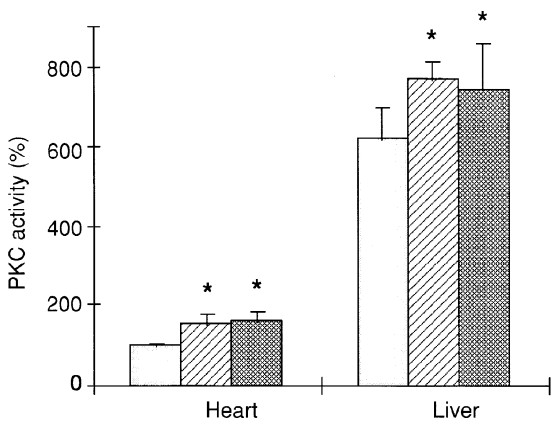

Figure 3. Activity of PKC in the heart and the liver of streptozotocin-induced diabetic rats. PKC activity was analyzed in the homogenate of the heart and the liver from the control (dotted bar), streptozotocin-induced diabetic (slant lined bar), and insulin-treated diabetic (hatched bar) rats. The average enzyme activity and standard error were expressed as the percentage to heart PKC activity of the control group. Each group was composed of 10 rats, and two separate experiments in duplicate were performed. An asterisk represents the activity that differed statistically significantly from the control ($P < 0.05$).

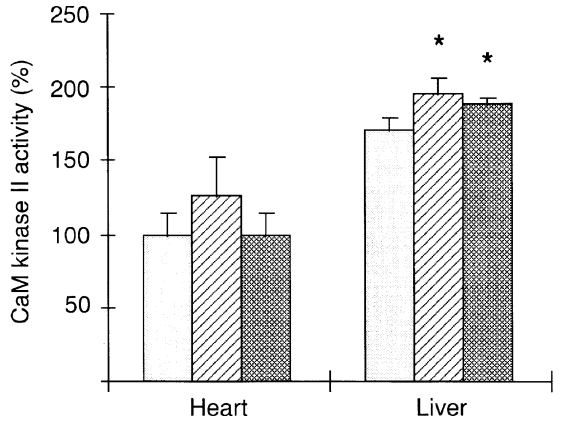

Figure 4. Activity of CaM kinase II in the heart and the liver of streptozotocin-induced diabetic rats. CaM kinase II activity was analyzed in the homogenate of the heart and the liver from the control (dotted bar), streptozotocin-induced diabetic (slant lined bar), and insulin-treated diabetic (hatched bar) rats. The average enzyme activity and standard error were expressed as the percentage to heart CaM kinase II activity of the control group. Each group was composed of 10 rats, and two separate experiments in duplicate were performed. An asterisk represents the activity that differed statistically significantly from the control ($P < 0.05$).

Immunoreactivity and activity of PKC increased significantly in the liver membrane of Zucker diabetic fatty rat (Considine *et al*., 1995). Hence it is possible that PKC-mediated phosphorylation of some component in the insulin signaling cascade contributes to the insulin resistance observed in non-insulin dependent diabetes mellitus. However, some decrease in hepatic PKC activity was also reported (Pugazhenthi *et al*., 1990), and thus it needs further study to explain the discrepancy in changes of the enzyme activity in diabetic liver.

Changes in CaM kinase II activity

CaM kinase II activity did not change significantly in the heart of diabetic and insulin treated diabetic rats. The enzyme activity increased slightly in the liver of the diabetic group (14% increase from the control, $P < 0.05$), and insulin injection did not restore the increase of the enzyme activity (10% increase from the control, $P < 0.05$) (Figure 4).

CaM kinase II is widespread in nature, and phosphorylates various proteins in response to Ca^{2+} signals. It regulates a broad array of cellular functions including metabolism of carbohydrate, lipid and amino acid, neurotransmitter synthesis and release, ion channels, calcium homeostasis and gene expression (Hanson and Schulman, 1992). Streptozotocin-induced diabetes was reported to cause a significant decrease in calmodulin level of smooth muscle suggesting that the altered smooth muscle calmodulin might contribute to the defective contractile response in diabetes and this change might be resistant to insulin therapy (Aydin *et al*., 1996). Calcium-dependent protein kinase activity was reported to decrease in sciatic nerve of streptozotocin-induced diabetic rats (Gabbay *et al*., 1990). In our study, CaM kinase II activity remained unchanged in diabetic heart in spite of increased Gαq expression, but increased slightly in the liver. Diabetes related alterations in the Ca^{2+}/calmodulin protein kinase II-dependent phosphorylation of Na^{+}-H^{+} exchange were reported in ventricular myocytes of rats (Le Prigent *et al*., 1997). Although our results showed no significant changes in the CaM kinase II activity in the heart of streptozotocin-diabetic rats, it may not sufficient evidence to exclude the role of the CaM kinase II in the pathogenesis of diabetic cardiomyopathy**.** It needs further studies to clarify the role of the enzyme(s) in the development of the heart disease.

In this experiment, we found the increased expression of Gαq and increased PKC activity in diabetic rat heart, in contrast to the unaltered expression of Gαs and Gαi as well as the activity of PKA and CaM kinase. These findings suggest that the Gqα protein and PKC may be involved in the pathogenesis of the heart diseases in diabetes mellitus.

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