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A Cell Model System to Study Regulation of Phosphatidylinositol 3-Kinase and Protein Kinase B Activity by Cytokines/Growth Factors Produced By Type I Collagen Stimulated Immune Cells From Patients With Systemic Sclerosis

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Abstract

We have reported that posttranslational modification of systemic sclerosis patients' platelet phosphoinositide 1,3,4,5 kinase (PI 3-K) and protein kinase B (Akt) altered their enzymatic activities. In the present investigation, we have established a cell line model to study further the effects of posttranslational modification and modification by cytokines or growth factors of these two enzymes. Results from these studies suggest that posttranslational modification by phosphorylation of Akt and nitrotyrosylation of PI 3-K increases enzymatic activities, as was observed from SSc patients' platelets. These two signaling components are controlled by a different mechanism, which alters platelet reactivity towards the matrix components of vascular walls. We have used a megakaryotic cell line to study these two enzymes in the presence of cultured supernatants from peripheral blood mononuclear cells (PBMC), which were isolated from blood of SSc patients compared to controls including culture medium, rheumatoid arthritis, systemic lupus erythematosus, and osteoarthritis. The effect of the supernatants from SSc CI-stimulated PBMC cultures on both PI 3-K and Akt is specific.

Keywords

Systemic sclerosis; platelet; nitrotyrosylation of protein; nitric oxide synthase; collagen; protein kinase B

Introduction

Platelets from patients with SSc aggregate at an accelerated rate when incubated with type I collagen (CI) (1) and have increased expression of a 65 kDa non-integrin type I collagen receptor (2). We have previously shown that nitrotyrosylation of phosphatidylinositol 3-kinase

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(PI 3-K) is increased in platelets from patients with SSc and that PI 3-K enzymatic activity is also increased by posttranslational modification (3). We further identified nitrotyrosylation as one of the possible mechanisms which causes the increased PI 3-K activity (3). Both PI 3-K and protein kinase B (Akt) play a significant role in the platelet signaling transduction pathway, proposed as PI 3-K/Akt→eNOS→guanyl cyclase→cGMP-dependent protein kinase (4). PI 3-K is present in megakaryocytic cell lines and in human platelets. The generation of PI 3-K metabolites leads to the recruitment of the Akt to the plasma membrane through the PH domain of Akt. At the level of the plasma membrane, Akt is thought to undergo a conformational change and becomes activated by phosphorylation transduction signals from PI 3-K.

The metabolism of phosphoinositides plays a critical role in signal transduction and is triggered by a variety of stimuli. PI 3-K catalyzes the phosphorylation of phosphoinositol 1, 4, 5 to phosphoinositol 1, 3, 4, 5. Phosphoinositides have been shown to control the reorganization of the actin cytoskeleton (5,6) and are required for store-mediated Ca^{2+} entry in platelets (7). It has also been reported that lipid products of PI 3-K and PI 4-K are both required for ADP-dependent platelet spreading (8).

Rheological disturbances have an important role in promoting arterial thrombosis by enhancing the adhesive and signaling function of platelet integrin $\alpha\text{IIb}/\beta 3$. Jackson et al. (9) have reported that PI 3-K has a defined role in regulating the formation and stability of the integrin adhesion bonds necessary for activation of platelets. They developed an inhibitor of PI 3-K that results in unstable platelet thrombus formation. A recent report demonstrated that continued signaling via PI 3-K isoforms β and γ is required for platelet ADP receptor function in dynamic thrombus stabilization (10). It is important to study the mechanisms that regulate PI 3-K to gain a better understanding of its function.

Immune abnormalities are a cardinal feature of SSc. SSc T-cells are stimulated to produce a variety of cytokines when cultured with CI or constituent CI polypeptide chains [$\alpha 1(\text{I})$ or $\alpha 2(\text{I})$] and there are elevated levels of circulating cytokines in patients with SSc (11-16). To assess whether alteration in PI 3-K and Akt in platelets from patients with SSc might be a result of exposure to cytokines produced by CI stimulation of lymphocytes/monocytes, we cultured the human megakaryocytic line, MEG-01, in supernatants from cultures of CI stimulated peripheral blood mononuclear cells (PBMC) from patients with SSc or to cytokines/growth factors known to be present in such culture supernatants. In the present study, we present data showing these culture supernatants and certain cytokines/growth factors present. In the culture supernatants increase nitrotyrosylation of both PI 3-K and Akt, however, only the activity of PI 3-K is increased. This potentially links immune activation in patients with SSc to platelet abnormalities, characteristics of the disease, implications of which are discussed.

Materials and Methods

Materials

All chemicals were purchased from Fisher Scientific (St Louis, MO), Sigma Chemical Co. (Denver, PA), and BioRad (Hercules, CA). MEG-01 cells were purchased from American Type Culture Collection (Manassas, VA). Bovine CI was prepared as previously described (13).

PBMC Cultures

After informed consent, 30 ml of blood from ten patients with diffuse SSc, rheumatoid arthritis, systemic lupus erythematosus, and osteoarthritis were drawn into tubes. The PBMC were isolated by isopycnic centrifugation on histopaque (Sigma Chemical Co., St. Louis, MO) cushions as previously described (13), washed $\times 2$ in phosphate buffered saline (PBS), resuspended at 4×10^6 ml of Dulbecco's modified eagle's medium containing 9% fetal calf

serum (FCS), high glucose, 100 U/ml penicillin, 100 µg/ml streptomycin and non-essential amino acids hereafter referred to as “complete medium”. To two wells of a 48 well tissue culture plate (NUNC, Roskilde, Denmark) 500 µl aliquots of the PBMC suspension were added. Culture of PBMC (from 10 patients) were stimulated with native bovine CI (10 µg/ml) (3). After 6 days, culture supernatants were harvested, pooled together, and stored at -70°C until used in experiments described below.

Culture conditions for MEG-01 cells

MEG-01 cells were plated at a density of 1×10^5 cells/ml and cultured in RPMI 1640 medium with 2 mM L-glutamine, 10 mM HEPES, 10 mM Sodium pyruvate, 4.5 g/L glucose, adjusted to contain 1.5 g/L sodium bicarbonate, 10 mM HEPES, and 10% fetal bovine serum at 37°C and 5% CO₂.

MEG-01 treated with PBMC cultured supernatant

To two MEG-01 cell cultures as above, supernatant (20%) from cultures of CI-stimulated PBMC from patients with SSc or 20% “complete medium” containing 10 µg/ml CI (control medium) were added. After three days in culture, half of the cells were removed for analysis. The remaining cells were placed in the same media and cultured for an additional 4 days. Seven and ten days from initial culture, two-thirds of the cells were removed for Western blot analysis and analysis of enzyme activity. The remaining cells were placed into culture with fresh medium along with a fresh dose of PBMC culture supernatant as described above. Following fifteen days from initial culture, all the cells were harvested for analysis.

MEG-01 cells treated with various cytokines

Using cytokine/growth factor arrays (Ray Biotech, Inc., Norcross, GA), we found interferon (IFN)γ, hepatocyte growth factor (hHGF), chemokine ligand (CCL-24), interleukin (IL) 1β, 10 and 13 were over expressed in supernatants from CI stimulated PBMC cultures from patients with SSc (unpublished data). MEG-01 cells were prepared for culture as stated above but with the addition of various cytokines to individual flasks. Briefly, three days following initiation of culture; one-half of the cells were removed for analysis, while the remaining cells were left in culture for an additional 4 days in the same medium. Following seven and ten days in culture, the cells were harvested, and two-thirds of the cells were removed for analysis while one-third remained in culture with fresh medium and cytokines. Fifteen days following initiation of the cultures, all the cells were harvested for analysis. The following cytokines were diluted with phosphate-buffered saline (PBS)/0.1% BSA and added to cultures at the concentration indicated: IFNγ 10 ng/ml, hHGF 5 ng/ml, CCL-24 (10 ng/ml), IL-1β (5 ng/ml), IL-10 (10 ng/ml), and IL-13 (10 ng/ml).

Western blot

Cultured MEG-01 cells were harvested, washed with PBS by centrifugation and resuspended in PBS containing protease inhibitors (25 µM aprotinin, 25 µM leupeptin, and 1 µM PMSF). The suspension was sonicated for 20 sec at 4°C, and the protein concentration was determined. An aliquot of cell lysates (100 µg) plus an equal volume of 2X SDS-PAGE sample buffer were added, mixed, boiled for 3 min, and analyzed by 10% SDS-PAGE (17). Protein bands were electrophoretically transferred onto a nitrocellulose membrane, which was treated with 3% dried milk in TBST (20 mM Tris-500 mM NaCl-0.05% Tween 20, pH 7.4) to eliminate non-specific binding. The nitrocellulose sheet was then washed 3 times with TBS followed by incubation with anti-nitrotyrosine antibody (1/1000) overnight at 4°C. After washing 3 times with TBST, the nitrocellulose sheet was incubated with a second antibody (peroxidase conjugated goat anti-mouse IgG, 1/20000) for 2 h at room temperature. To facilitate visualization of bands, following three washes, the nitrocellulose sheets were treated with an

enhanced chemiluminescence (ECL) solution according to the manufacturer's description (Amersham Biosciences Corp., Piscataway, NJ) as routinely performed in our laboratory (9).

Assay for activity of PI 3-K

The activity of PI 3-K was measured, essentially as described by other investigators (8). Briefly, aliquots containing 50 µg of lysates of platelets from patients with SSc and from age, sex and race matched healthy donors were incubated in 50 µl of PI 3-K assay buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2 mg/ml sonicated phosphatidylinositol, 50 µM ATP, and 20 mM MgCl₂. The reactions were initiated by the addition of 30 µCi of [γ -³²P] ATP and incubated at 37°C for 10 min. The reactions were stopped by the addition of 20 µl of 6 N HCl and 160 µl of ice-cold chloroform: methanol (1:1). Following centrifugation, the lower organic phase was extracted for thin layer chromatography. The phospholipids were resolved on Silica Gel-60 plates (Merck) using chloroform/methanol/ammonium hydroxide/water (60:47:11.3:2) developing solvent for 210 minutes. Radioactive spots were visualized by autoradiography and exposure to iodine vapors. Identified spots were marked, scraped and placed in 10 ml ScintiVerse BD; and the radioactivity was measured in a Packard scintillation counter.

Immunoprecipitation of Akt

We obtained partially purified Akt from platelet lysates by immunoprecipitating an aliquot of platelet lysates (50 µg) with anti-Akt antibody (Cell Signaling Technology, Inc. 1/50) at 4°C overnight. A second antibody (1/50, goat anti-rabbit IgG was added to the mixture and incubated at 4°C for an additional 6 hours. The mixture was centrifuged (4°C) for 10 minutes and the pellet washed twice with 50 µl phosphate buffered saline. The pellet was resuspended in 40 µl of assay buffer and the activity was determined by the procedures described below.

Assay for activity of Akt

The activity of Akt was assayed by ELISA as described by the manufacturer (Stressgen, Victoria, Canada). Briefly, microtiter wells coated with a substrate peptide of Akt were primed with assay buffer for 10 minutes. At the end of priming, a 10 µl aliquot of containing 50 µg of SSc patient and control immunoprecipitates platelets lysates were added to triplicate wells in a final volume of 40 µl of a kinase assay buffer. The reaction was initiated by incubation at 30°C for 90 minutes with 10 µl of ATP. At the end of the incubation, the reaction was stopped by removing the incubated mixture by suction. An aliquot of anti-phosphate specific antibody was added and incubated at room temperature for 60 minutes. At the end of incubation, the microtiter wells were washed four times with wash solution (100 µl each). An aliquot of 40 µl of second antibody solution was added to each well and incubated at room temperature for 30 minutes. After washing four times with washing solution, an aliquot of 40 µl TMB was added to each wells and incubated at room temperature for 30 to 60 minutes. 20 µl of acid stopping solution was added to each well and read at 450 nm. A set of control samples was incubated with Akt inhibitor [SH-6 (Calbiochem, San Diego, CA) or staurosporine (Sigma, St. Louis, MO)] as background. All samples were assayed in triplicate.

Results

SSc PBMC Culture Supernatants Increase MEG-01 PI 3-K and Akt Activity by different mechanism

To assess whether a possible link exists between T cell reactivity to CI (which is prevalent in SSc) and the altered PI 3-K and Akt activity, we chronically exposed the MEG-01 human megakaryocytic line in vitro to supernatants from culture of CI-stimulated SSc PBMC. MEG-01 cells chronically exposed to the culture supernatants exhibited increased

nitrotyrosylation of PI 3-K and Akt (Fig. 1, lane 2) compared to control supernatant-incubated cells (Fig. 1, lane 1). We then assayed both PI 3-K and Akt activities in the lysates from MEG-01 cells cultured with the supernatants. We found the activity of PI 3-K and Akt significantly increased in MEG-01 cells cultured with supernatants from cultures of type I collagen stimulated with PBMC from patients with SSc (Table I). We then used a Western blot by using anti-pAkt antibody to detect the content of pAkt in these samples. Fig. 2 shows the pAkt is increased in PBMC cultured supernatant (lane 2) compared to control (lane 1).

Specificity of the PBMC Culture Supernatant on MEG-01 cells

We have obtained supernatants from CI-stimulated PBMC cultures from three SSc patients (lanes 5 to 7) and 4 other disease controls [medium plus CI control (lane 1), one rheumatoid arthritis patient (lane 2), one systemic lupus erythematosus patient (lane 3), and one osteoarthritis patient (lane 4)]. We then cultured MEG-01 cells for 2 weeks with these supernatants and performed Western blots on the MEG-01 cell lysates. Fig. 3 shows that PI 3-K increase in nitrotyrosylation in SSc patients but not with medium plus CI control or other disease controls (panel B, lanes 5 to 7 compared to lanes 1 to 4). Panel A is the Western blot with probed anti-PI 3-K antibody to ensure equal amount of each sample.

We have analyzed the same set of samples by Western blot using anti-pAkt antibody. The SSc patients' PBMC supernatants enhanced expression of phosphorylated Akt in MEG-01 cells (Fig. 3, panel D lanes 5 to 7) compared to medium (lane 1) other disease controls [rheumatoid arthritis (lane 2), systemic lupus erythematosus (lane 3), and osteoarthritis (lane 4)]. Western blots of these same set of samples probed with anti-Akt showed about the same level of expression in all samples.

Cytokine/Growth Factor Effects on MEG-01 PI 3K and Akt

We next assessed effects of expression of PI 3-K and Akt by MEG-01 cells treated with six cytokines and growth factors which are present in supernatants from cultures of CI-stimulated PBMC from patients with SSc. Western analyses of lysates probed with anti-nitrotyrosine (Fig. 4) of supernatant treated MEG-01 cells showed that hHGF (lane 3) and IL-13 (lane 7) significantly decreased PI 3-K expression. CCL-24 (lane 4) did not change expression of Akt, but IL-13 treatment significantly decreased Akt expression. We assayed effects of cytokines/growth factors on MEG-01 PI 3-K activity (Table II). Among these cytokines and growth factors: IFN γ (185%) and IL-1 β (141%) increased PI 3-K activity. In contrast, an inhibitory effect was observed in IL-13 (56%) and hHGF (46%) treated cells.

Akt activity in these same lysates from cytokine/growth factor-treated MEG-01 cells was analyzed. IFN γ (158%) and IL-1 β (141%) significantly increased Akt activity. IL-13 (52%) significantly decreased Akt activity, while hHGF, IL-10, and CCL24 showed no significant effect (Table III).

Discussion

The increased activity and nitrotyrosylation of PI K3 and Akt (two enzymes important in regulating platelet function) that we previously described in platelets from patients with SSc (3) was reproduced in vitro in a human megakaryocytic cell line, MEG-01 cells, by prolonged exposure to cytokine/growth factor-rich supernatant from cultures of CI stimulated PBMC from patients with SSc. This raises the possibility that in SSc, cells of the immune system (e.g. T cells and monocytes) activated in vivo by exposure to autoantigens such as CI generate cytokines/growth factors that may be responsible for the increased activity and nitrotyrosylation of PI 3-K and Akt in megakaryocytes and platelets from patients with the disease.

We have previously reported that in patients with SSc there is an abnormality of the 65-kDa platelet type I collagen receptor (CIR) (2). This abnormal expression of the 65-kDa CIR is not found in platelets from patients with rheumatoid arthritis, fibromyalgia, osteoarthritis, gout, or systemic lupus erythematosus (2).

The Akt/PI 3-K pathway in different cell types can be activated by nitric oxide and other oxidants (18-20). The importance of Akt in regulating platelet function (secretion and aggregation) has been demonstrated by Stojanovic et al. (4). PI 3-K, which is upstream in the signaling pathway, promotes platelet function by activating Akt (21). We have found that the nitrotyrosylation of Akt did not change its enzymatic activity (22), suggesting that the activity of Akt can be controlled by other mechanism(s). We have shown that one of the mechanisms by which platelet Akt is activated is via phosphorylation (22) as has reported by others (23-25).

We further used individual cytokines or growth factors present in cultures of CI-stimulated SSc PBMC to evaluate individual cytokine/growth factor effects on PI 3-K and Akt. When we evaluated the effects of several cytokines and growth factors identified to be elevated in supernatants from cultures of CI-stimulated SSc PBMC, we found that both IFN- γ and IL-10 increased the activity of PI 3-K. In contrast, both hHGF and IL-13 significantly decreased PI 3-K activity. The activity of Akt in MEG-01 cells was increased by IFN- γ and IL-1 β but decreased by IL-13. The increased enzymatic activity observed is due to posttranslational modifications of PI 3-K by nitrotyrosylation or phosphorylation of Akt. Although the mechanism(s) involved in modulation of PI 3-K and Akt by cytokines/growth factors have not yet been determined, future investigation of these mechanisms could prove to be useful in understanding the processes that contribute to platelet dysfunction in SSc, and may provide information towards designing a therapeutic strategy against the disease.

The cytokines/growth factors studied were identified from SSc PBMC cultures and detected with Ray Biotech Human Cytokine/Growth Factor Antibody Arrays. Several growth factors and Th1 and Th2 cytokines were elevated in the culture supernatants. Although the unfractionated supernatant from CI-stimulated SSc PBMC cultures upregulated expression of PI 3-K and Akt, individual cytokines/growth factors found to be over expressed in the SSc PBMC supernatant by Ray Biotech arrays when added to the human megakaryotic cell line had no effect, stimulated or inhibited expression of PI 3-K and/or Akt, suggesting the immune system through specific cytokine/growth factors can modulate platelet PI 3-K and Akt expression. Both Th1 and Th2 cytokines were present in the supernatant from cultures of PBMC stimulated by CI (26). This has been observed by other investigators as well as by our own group (13,14). Sera from patients with SSc have been reported to contain predominately Th2 cytokines, although this may reflect an attempt by the immune system in patients with SSc to counter a Th1 response (27-29). Freshly isolated blood lymphocytes from patients with SSc produce both Th1 (IFN γ , IL-2) and Th2 (IL-4) cytokine (30). Culture CI-specific T cells form autoantigen CI from patients with SSc produce large amounts of IFN γ (31).

These data pose the question of whether the up regulation of PI K-3 and Akt in platelets from patients with SSc is acquired and induced by ongoing immune system activation in patients with the disease. If this is the case, then therapeutic strategies to correct the immune abnormalities in patients with SSc by immunosuppression agents or immunomodulation, may lead to normalization of platelet function by decreasing expression of SSc megakaryotic precursors of platelets to cytokines/growth factors.

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Figure 1.

Western blots analysis of PI 3-K and Akt in MEG-01 cell lines. MEG-01 cells were cultured for 15 days with supernatant from culture of CI stimulated PBMC from patients with SSc. Cells were harvested, washed, sonicated, and protein concentrations were determined. An aliquot of 100 μ g of each sample was analyzed by SDS-PAGE and Western blot with anti-nitrotyrosine antibody as described in the Materials and Methods Section. Lane 1: PBMC cultured supernatant of PBMC from SSc patients cultured with CI. Lane 2: fresh medium contained same amount of CI. The data shown are one of two experiments.

Figure 2.

Western blots analysis of pAkt in PBMC and control supernatant of MEG-01 cell lysates. We used Western blot of samples from Fig. 1 with anti-pAkt antibody (1/500) and developed with ECL solution. Lane 1: PBMC cultured supernatant of PBMC from SSc patients cultured with CI. Lane 2: fresh medium contained same amount of CI. The data shown are one of two experiments.

Figure 3. Specificity of the PBMC cultured supernatants on post-translation modifications. We have used three SSc patients PBMC culture supernatants (lanes 5 to 7) and compared to other controls [medium (lane 1), one rheumatoid arthritis (lane 2), one systemic lupus erythematosus (lane 3), and one osteoarthritis (lane 4)]. Panel A: anti-PI 3-K antibody; panel B: anti-nitrotyrosine antibody; Panel C anti-Akt antibody; and Panel D: anti-pAkt antibody.

Figure 4.

Effect of cytokine/growth factor on MEG-01 PI 3-K and Akt. MEG-01 cells were incubated with six cytokines and growth factors, which are present in supernatants from cultures of CI-stimulated PBMC from patients with SSc. Western blot analyses of lysates probed with anti-nitrotyrosine (Fig. 3). Panel A shows Western blot of PI 3-K and Panel B shows Western blot of Akt. Cells treated with different cytokines/growth factors: control (lane 1), IFN γ (lane 2), hHGF (lane 3), CCL 24 (lane 4), IL-1 β (lane 5), and IL-10 (lane 6), and IL-13 (lane 7).

TABLE I

Effect of Supernatant from Cultures of CI Stimulated PBMC on the Activity of PI 3-K and Akt

Treatment	PI 3-K activity (cpm/50 μ g)	Akt activity (450 nm/50 μ g)
Control	3757 $\pm$ 185	0.696 $\pm$ 0.041
SSc patients' PBMC cultured supernatant	5552 $\pm$ 75	0.841 $\pm$ 0.072

p = 0.001

p = 0.0384

MEG-01 cell lysates (50 μ g) were used to assay PI 3-K activity as described in the Materials and Methods Section. Data shown are the $\pm$ SEM Mean of duplicate measurements. An aliquot of 50 μ g was immunoprecipitated with anti-Akt then assayed its activity also described in the Material and Methods Section. Data shown are Mean $\pm$ S.D. of triplicate determinations. Data were analyzed by students 2 sample to test. The data shown are combined from two experiments.

TABLE II
Effect of Cytokines or Growth Factors on the Activity Of PI 3-K

Cytokines or growth factors	PI 3-K activity (CPM/25 µg)	% Control
Control	1937	100
IFN γ	3581	185
hHGF	892	46
CCL24	2361	122
IL-1 β	2733	141
IL-10	2316	119
IL-13	1271	56

MEG-01 cells were cultured with reagents listed for 15 days at 37°C in a humidified incubator containing 5% CO₂. Culture medium was changed every 7 days with the addition of fresh reagent. At the end of 15 days, cells were removed with 1 mM EDTA in PBS, washed with PBS, and resuspended in PI 3-K assay buffer. After a brief sonication, the content of protein was determined by BioRad DC Protein Assay kit. Each assay tube contained 50 µg of protein and other assay mixtures as described in the Materials and Methods Section. The activity of PI 3-K was assayed as described in the Materials and Methods Section. Data expressed are the mean of duplicate determination. The data shown are from one experiment representative of two that gave similar results.

TABLE III

Effect of Cytokines or Growth Factors on the Activity of Akt

Cytokines or Growth factors	Activity of Akt (450 nm)	Mean $\pm$ S.D.	% Control (p value)
Control	0.394	0.435	0.404
IFN γ	0.636	0.637	0.411 $\pm$ 0.02
hHGF	0.365	0.432	0.65 $\pm$ 0.02
CCL24	0.322	0.414	0.419 $\pm$ 0.05
IL-1 β	0.582	0.581	0.419 $\pm$ 0.05
IL-10	0.275	0.374	0.580 $\pm$ 0.002
IL-13	0.200	0.245	0.344 $\pm$ 0.06
			0.212 $\pm$ 0.03
			100
			158 (0.0002)
			102
			101
			141 (0.00016)
			84 (0.145)
			52 (0.0006)

MEG-01 cells were cultured with the reagents as listed for 15 days at 37°C in a humidified incubator containing 5% CO₂. Culture medium was changed every 7 days with the addition of each reagent. At the end of 15 days, cells were removed with 1 mM EDTA in PBS, washed with PBS, and resuspended in Akt assay buffer. After a brief sonication, the content of protein was determined by BioRad DC Protein Assay kit. Each assay tube contained 50 μ g of protein and other assay mixtures as described in the Materials and Methods Section. The activity of Akt was assayed according to the method described in the Materials and Methods Section. Data are expressed as Mean $\pm$ SD of triplicate determinations. The data shown are from one experiment representative of two that gave similar results.