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Effects of thrombin on insulin signalling and glucose uptake in cultured human myotubes.

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ABSTRACT

Background; Hyper-coagulability (elevated thrombin) is a feature of type 2 diabetes and contributes to an increased risk of thrombotic and vascular events. Skeletal muscle is the key peripheral tissue site of insulin resistance in type 2 diabetes. Cultured human skeletal muscle cells were used to explore the effects of thrombin on insulin signalling and glucose uptake. We hypothesized that thrombin impairs insulin activity in human skeletal muscle cells which could link the hypercoagulability and insulin resistance in type 2 diabetes.

Methods; Human skeletal muscle cell cultures (myotubes) were treated with +/- 5 units/mL thrombin for 6hrs. Thrombin receptors were detected by immunofluorescence, insulin signalling pathway components (P-Akt / Akt) and P-AMPK /AMPK were examined by Western blotting. Real time PCR and glucose uptake assays were performed.

Results; There was a significant decrease ($p < 0.01$) in insulin mediated Akt phosphorylation in response to thrombin in cultured myotubes, which was alleviated by treatment with a PKC inhibitor. Thrombin did not inhibit the insulin activation of aPKC that is also involved in the pathway of insulin stimulated glucose uptake. Thrombin directly increased basal glucose uptake ($p < 0.05$) that involved AMPK phosphorylation ($p < 0.01$), and was partly repressed by compound C (AMPK inhibitor). Thrombin also significantly increased the gene expression level of both GLUT1 and GLUT4 in cultured human skeletal muscle cells.

Conclusion; Thrombin decreased insulin signalling via Akt in skeletal muscle cells through a PKC mediated mechanism, but did not affect the action of insulin mediated through the aPKC pathway of glucose uptake. Thrombin exerted a direct stimulatory effect on glucose uptake mediated via an AMPK dependent mechanism. We conclude that increased coagulability in type 2 diabetes results in multiple metabolic effects beyond increased thrombogenicity, and includes direct effects on insulin signalling and glucose metabolism.

Keywords: Thrombin, signal transduction, protein kinase C, AMPK, skeletal muscle metabolism.

Introduction:

Thrombin is a multifunctional serine protease enzyme, involved in several biological processes with a primary function in the coagulation cascade (1). It is generated by the cleavage of the precursor molecule prothrombin. Thrombin acts on the cell surface receptors known as protease activated receptors (PARs). The binding of thrombin to the PARs leads to proteolytic degradation of the N-terminal of the receptor domain leading to formation of new N-terminus that activates the receptor and initiates the intracellular signalling processes (2).

Distinct PARs are expressed in skeletal muscle. Three different PARs (PARs 1, PARs 3 and PARs 4) are shown to be activated by thrombin (3). The activation of PARs by thrombin triggers different molecular pathways and includes: mobilisation of intracellular Ca^{+2} (4), changes in the metabolism of membrane phospholipids and inhibition of adenylate cyclase. PI-3 kinase is also involved in PARs activation pathways as observed in mouse mast cells (5). Protein kinase C, mitogen-activated protein (MAP) kinases (6-8) and AMPK are also involved in mediating the actions of thrombin (9, 10).

Hypercoagulation is a feature of patients with type 2 diabetes who are at high risk of thrombotic and vascular events(11). In type 2 diabetes, a strong association between metabolic abnormalities such as hyperglycaemia and the increased coagulation (elevated thrombin level) has been identified (12), and decreased insulin sensitivity is associated with enhanced thrombin production (13).

Furthermore, there is emerging evidence of a genetic basis to hypercoagulation and type 2 diabetes. High heritability indices were reported in a cohort of Mexican Americans (14) for a number of factors in the coagulation cascade, including prothrombin that was also strongly associated with diabetes.

Skeletal muscle is the primary peripheral tissue site of insulin stimulated glucose uptake (15) and the key peripheral site of insulin resistance in type 2 diabetes (16).

We therefore set out to explore whether thrombin contributes directly to the insulin resistant state and investigated the effects of thrombin on insulin signalling and glucose uptake in cultured human skeletal muscle cells.

Material and methods:**Chemicals and reagents:**

Thrombin was supplied by Sigma, both the PKC general inhibitor GF109203X and compound C were from Tocris Bioscience and the PKC specific inhibitor GÖ 6976 was from Calbiochem. Cell culture media was obtained from Lonza. FBS and trypsin-EDTA were obtained from Life Technologies (Paisley, UK). Chick embryo extract was purchased from Sera Labs International (Sussex, UK). Phospho-Akt^{Ser473} (D9E), total Akt rabbit antibodies, Phospho-PKC ζ/λ ^{Thr410/403} antibody (9378), phospho-AMPK^{Thr 172} (40H9) rabbit antibodies and AMPK α (F6) mouse antibodies were supplied by New England Biolabs (Herts, UK). β -actin (clone AC-15) antibody was purchased from Sigma. 2-Deoxy-D-[2, 6-³H] glucose was purchased from Hartmann Analytic (Germany).

Cell culture

Vastus lateralis muscle biopsies of healthy human subjects with no family history of type 2 diabetes were taken and satellite cells prepared as described previously (17, 18). Briefly, needle biopsies were collected in myoblast growth media (Ham's F10 media supplemented with 20% (v/v) FBS, 2% chick embryo extract, 1% penicillin-streptomycin), fibrous and fat tissues were dissected from muscle tissue, then the muscle tissue was cut into small pieces, washed with PBS 4 times to remove the adherent blood cells and transferred to a universal containing 5ml 0.05% trypsin-EDTA for spin-digestion at 37°C. After 15min, the trypsin was removed, 5ml media added and centrifuged at 1700rpm for 5mins. The pellet containing the satellite cells was resuspended in proliferation medium. The spin dissociation protocol was repeated a further 3 times, the pelleted satellite cells were pooled and plated in a T25 flask. Media was changed after 24h to remove unattached cells and cell debris. Prior to stimulation, cells were seeded at a density of approximately 200,000 per 35mm dish and grown to confluence before inducing differentiation. Differentiation was induced by changing the media to minimal essential media supplemented with 2% (v/v) FBS and 1% penicillin-streptomycin. All experiments were performed on day 7-8 after differentiation of myotubes; passages were between 5 and 8. Cell treatments were as described in the Figure Legends.

Immunofluorescent Staining:

Fully differentiated cells were plated in cover slips in 6-well plates. All steps were then carried out at room temperature, on a shaker set to a low-speed. Cells were washed with PBS and fixed with 10% formalin for 20mins. Cells were washed with PBS then permeabilized with 0.2% t-octylphenoxy-polyethoxyethanol (Triton X-100, Sigma-Aldrich) for 45-minutes. After washing with PBS, cells were incubated in blocking buffer (20% FBS in PBS) for 1h to block non-specific binding sites. Cells were then incubated with rabbit anti- human thrombin receptor IgG (Abcam, UK), diluted 1:500 in 0.05%

FBS in PBS for 1h. After washing with PBS, cells were incubated in Cy3-conjugated anti-rabbit IgG, diluted 1:500 in 0.05% FBS in PBS for 1h in the dark. After washing in PBS again cover slips were mounted onto slides using a drop of Vectasheild with DAPI. Fluorescent microscopy was performed on an Olympus CKX41 to visualise staining and images taken using QCapturePro60 software.

Western Blot:

Cells were scraped into protein extraction buffer (100mM Tris-HCl, pH 7.4, 100mM KCl, 1mM EDTA, 25mM KF, 1mM benzamidine, 0.5mM Na₃VO₄, 0.1% (v/v) Triton X-100, 1x protease inhibitor cocktail (Pierce), sonicated briefly and centrifuged at 13,400rpm for 5min at 4°C before measuring protein concentration using the modified Bradford dye binding colorimetric method (19) at 595nm. 10µg samples were loaded on 10% SDS-PAGE gels in loading buffer (0.125M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 0.004% (w/v) bromophenol blue). After separation, proteins were transferred onto a nitrocellulose membrane (0.45 µm pore size), using a mini-Hoeffer wet transfer system. Non-specific sites on the nitrocellulose membranes were blocked by incubation in Tris buffered saline tween (TBS-T) containing 5% (w/v) milk as a blocking buffer for 1h at room temperature. Membranes were incubated with monoclonal primary antibody (1°Ab) diluted in 5% blocking buffer TBS-T / 5% (w/v) milk, overnight at 4°C. After washing, membranes were incubated with a horseradish peroxidase (HRP) conjugated secondary antibody IgG, diluted in 5% blocking buffer. Detection was done using enhanced chemiluminescence and densitometry measurements performed using the Bio-RAD Molecular Imager GS-800 calibrated densitometer and Quantity One software. Phospho-Akt antibody was used at a 1:10000 dilution while native Akt was used at a 1:2000 dilution, β-actin was used at 1:10000, phospho and native aPKC antibodies were used at a 1:1000 dilution, phospho and native AMPK antibodies were used at a 1:1000 dilution.

Glucose uptake assay:

Cells were cultured on 6well plates and incubated with or without thrombin at 37°C in serum free media for 6 hours prior to use. After treatment, cells were washed twice with Kreb's buffer (136mM NaCl, 4.7mM KCl, 1.25mM MgSO₄, 1.2mM CaCl₂, 20mM HEPES, pH 7.4), then treated with or without 100nM insulin, 40µM compound C or 10µM Cytochalasin B for 30min. Hot glucose solution (0.1mM 2-deoxy-glucose and 0.5µCi (2, 6-³H) 2-deoxyglucose) was added for the last 10min before the reaction was stopped by washing with ice-cold phosphate buffered saline (PBS). Cells were lysed in 0.05% SDS before scintillation counting and protein determination.

RNA isolation and cDNA synthesis:

Total RNA was extracted from human skeletal muscle cells using the GenElute™ Mammalian Total RNA Miniprep kit (Sigma) following the manufacturer's instructions. Briefly, cells were lysed in lysis

buffer containing 1% β -mercaptoethanol and applied to a filtration column. An equal volume of 70% ethanol was added to the supernatant and passed through a nucleic-acid binding column. Bound RNA was washed sequentially in Wash buffers 1 and 2. Finally, the column was spin-dried and RNA eluted in a final volume of 50 μ l. Total RNA was treated with DNase I and 200ng was reverse-transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems) in a final volume of 20 μ l.

Quantitative real-time PCR:

Quantitative real-time PCR was performed on a Lightcycler 480 (Roche) using Taqman primers and probes. GLUT4 (Hs.380691) and GLUT1 (Hs.473721) were obtained from Applied Biosystems as predesigned Taqman primer-probe mixes and were used at the recommended 1:20 dilution. β 2-microglobulin (β 2M) was used as a reference gene with sequences: For; GCCTGCCGTGTGAACCAT, Rev; TTACATGTCTCGATCCCACTTACCTATC, Probe; FAM-TGACTTTGTACAGCCCA-TAMRA. The concentration of both primers was 300nM per reaction and 250nM for the probe. 10 μ l of Gene expression mastermix (Applied Biosystems) was added to each reaction with 20ng of template. Results were analysed using the standard curve method from a six-point serially diluted standard curve for GLUT1 and the $\Delta\Delta$ Ct method for GLUT4. Relative quantification was performed with data normalised to β 2-microglobulin.

Statistical analysis:

All results are expressed as mean $\pm$ standard error of the mean (SEM) unless where stated. Data were analysed using one-way ANOVA, and where significant, followed by t-test between groups. Statistical analyses were performed using GraphPad Prism (California) software.

Results:**Expression of thrombin receptor proteins:**

Immunofluorescent staining was carried out using a general anti-thrombin receptor antibody (rabbit anti-human thrombin receptor IgG), to visualise the distribution of thrombin receptor on the surface of human skeletal muscle cells (Supplementary Figure 1). From the images taken using fluorescent microscopy, it is clear that thrombin receptors (in red) are abundant and widely spread across the cell membrane.

Effects of thrombin on Akt (Ser473) phosphorylation:

The effects of thrombin on insulin signalling was investigated by treating human muscle cells in culture with thrombin then stimulating these cells with insulin and examining the degree of activation of the key downstream kinase PKB (Akt). As shown in Figure 1, exposure to thrombin resulted in a significant decrease ($p < 0.01$) in the phosphorylation of Akt^{Ser473} following insulin treatment.

Role of PKC:

PKCs are involved in the insulin signalling pathways. To explore whether the inhibitory effect of thrombin on insulin-mediated Akt phosphorylation involves PKCs, we treated human skeletal muscle cells with thrombin and increasing concentrations of the PKC general PKC inhibitor (GF109203X). As shown in Figure 2A, exposure to GF109203X at 10 and 100nM reversed the inhibitory effect of thrombin on insulin-mediated Akt phosphorylation. PKC has many isoforms and several of them have been linked to the insulin signalling pathway. We therefore used the conventional PKC selective inhibitor Indolocarbazole (GÖ 6976) which is specific for the Ca⁺² dependent isoforms α and $\beta 1$ (20). As shown in Figure 2B, the inhibitory effect of thrombin on insulin-mediated Akt phosphorylation was reversed in a dose dependent manner by GÖ 6976 treatment.

Thrombin stimulates glucose uptake in cultured muscle cells:

To examine the effect of thrombin on glucose uptake in cultured human muscle cells, radiolabelled glucose uptake was measured in the presence and absence of thrombin. As shown in Figure 3, insulin increased glucose uptake vs basal in the absence of thrombin ($p < 0.05$). Insulin had the same effect in the presence of thrombin, but the increase did not reach statistical significance. Interestingly, basal glucose uptake was significantly increased in the presence vs absence of thrombin ($p < 0.05$).

Effects of thrombin on aPKC activation:

Insulin continued to increase glucose uptake in the presence of thrombin, which was unexpected in view of the inhibitory effect of thrombin on insulin mediated Akt activation. However, it is recognised that the insulin stimulation of glucose uptake is mediated through the parallel activation of Akt and aPKC. We therefore explored the influence of thrombin on the effect of insulin on aPKC activation by measuring the phosphorylation of PKC ζ/λ ^{Thr410/403}. Thrombin did not decrease the action of insulin on aPKC Figure 4 which could explain why insulin continued to promote glucose uptake in the presence of thrombin.

Thrombin increases AMPK activation:

To explore the possible mechanisms resulting in increased basal glucose uptake, the effect of thrombin on AMPK activation was assessed by the determination of AMPK^{Thr172} phosphorylation. As shown in Figure 5A, thrombin increased AMPK activation and this increase in AMPK activity was partially reversed by AMPK inhibitor 40 μ M compound C ($p < 0.01$).

We next explored the effect of 40 μ M compound C on glucose uptake. As shown in Figure 5B, compound C partially reversed the stimulatory effect of thrombin on glucose uptake. Taking these data together, thrombin increases glucose uptake that is in part mediated by the activation of AMPK.

Glucose transporter expression level:

GLUT1 is one of the major glucose transporters predominantly expressed in human muscle cells and represents the main way by which basal glucose uptake takes place (21), we showed that thrombin increases GLUT1 gene expression compared with non-thrombin treated cells as displayed in Supplementary Figure 2A.

Glut4 is the principle transporter involved in the insulin and muscle contraction responsive glucose uptake in skeletal muscle. GLUT4 was expressed in abundance in myotubes as a reflection of functional demands (22). Our results showed that thrombin causes an increase in GLUT4 gene expression in comparison with non-thrombin treated cells (Supplementary Figure 2B).

Discussion:

To our knowledge, this is the first work to investigate the metabolic effects of thrombin on skeletal muscle. In the present study, we documented that thrombin causes a decrease in insulin mediated Akt phosphorylation (a reduction in insulin signalling) in human myotubes which was mediated in part through classical PKC. In addition, we found that thrombin directly increased glucose uptake with evidence that this is facilitated through AMPK activation.

The activation of Akt by insulin is a crucial step in regulation of insulin signalling and insulin stimulated glucose uptake and metabolism (23, 24). In this study, thrombin decreased insulin stimulated Akt^{Ser473} phosphorylation. This result is consistent with the results obtained from other studies measuring the Akt activity in adipocytes (25) and endothelial cells (26), and this finding may help us to understand the link between hyper coagulation and decreased insulin sensitivity (insulin resistance). However, the results differ from some published studies on platelets which show that thrombin directly causes an increase in phosphorylation of Akt and activation of platelets (27, 28). This appears to be different to the situation in skeletal muscle, as we observed no activation of Akt by thrombin in myotubes in the absence of insulin.

The inhibition of Akt activation by thrombin seems to be via a PKC related mechanism. Protein kinase C is a serine-threonine kinase that is expressed abundantly in skeletal muscles. Different PKC isoforms are expressed and classified according to their structure and regulatory cofactors of activation into three families as: conventional, novel and atypical (29). PKC isoforms play different roles in modulating biological activities such as growth, differentiation and signal transduction in different cell lines including human skeletal muscle (30, 31).

With regard to the insulin signalling pathway, different PKC isoforms exert different effects, that range from an activation of signal transduction in response to insulin such as PKC delta and zeta isoforms, to an inhibition of signal transduction such as PKC theta isoform (32). The diversity of functions of PKC isoforms in response to insulin in skeletal muscle are summarized in Supplementary Figure 3.

GF109203X is a PKC general inhibitor that has been shown to inhibit the activation of PKC in skeletal muscles (33, 34). The addition of the PKC general inhibitor improves insulin sensitivity in human skeletal muscles through an improvement of IRS1 tyrosine phosphorylation (35). IRS1 is an upstream stimulator of Akt through PI3K activation. We found that GF109203X reversed the inhibitory effects of thrombin on insulin signalling through an enhancement in Akt phosphorylation. Our findings are in line with those observed in endothelial cells in which GF109203X reversed the inhibitory effect of thrombin on growth factor stimulation of Akt (26).

Trying to define the specific PKC isoform involved in the effect of thrombin on insulin action, a specific PKC α isoform inhibitor has been used. PKC α has been shown to be constitutively associated with IRS-1 and this linkage causes attenuation in insulin activation, but upon insulin stimulation there is a dissociation from the IRS-1 and an increase in downstream activity towards increasing insulin signalling (36-38). Inflammatory cytokines, like TNF α , were shown to increase PKC α -IRS1 association, leading to insulin resistance. Sampson and Cooper (32), in their review, suggested that any stimuli that strengthen the PKC α -IRS1 association would inhibit insulin signalling that could contribute to insulin resistance. We explored whether thrombin might act through the same mechanism. We found that selective inhibition of the cPKC isoform using the GÖ 6976 (cPKC specific inhibitor) reversed the inhibitory effect of thrombin on insulin mediated Akt phosphorylation. This would suggest that PKC α is involved in the inhibitory effects of thrombin on the insulin signalling pathway.

While we found clear evidence that thrombin decreased insulin signalling in human skeletal muscle cells, we also found that thrombin directly increased glucose uptake in both the basal and to some extent in the insulin stimulated state. This is consistent with the findings of Kanda and Watanabe (39) who showed that thrombin causes an increase in glucose uptake in vascular smooth muscle cells in an Akt independent way.

In this study, we observed that thrombin causes an increase in AMPK activation. AMPK is a serine threonine kinase that acts as a metabolic sensor in skeletal muscle. AMPK is activated upon any condition that causes the cell to be in a state of decreased ATP either from decreased production or from an increased consumption. Activated AMPK causes switching off of the metabolic processes that consume energy (protein synthesis, lipid synthesis and glycogen synthesis) and simultaneously switching on processes that increase energy production including glucose uptake (40). Our observation in human muscle cells are consistent with results obtained from studying different cell lines like vascular endothelium, in which thrombin directly causes an increase in AMPK^{Thr172} phosphorylation and activation of AMPK downstream effects (10).

The increase of AMPK activity was attenuated with the use of a potent and selective inhibitor of AMPK (compound C). Our usage of compound C as an inhibitor to AMPK activation is consistent with data obtained in other studies (10, 41, 42). The observed decrease in AMPK activity by using compound C was associated with a decrease in thrombin mediated glucose uptake in the presence of compound C.

Having observed an inhibitory effect of thrombin on Akt phosphorylation and insulin signalling, we were surprised to observe that insulin stimulated glucose uptake tended to increase in the presence

of thrombin however, it was statistically not significant. It is recognised that insulin stimulation of glucose uptake is mediated through both Akt and aPKC activation distal to PDK1 (Supplementary Figure 3). We therefore, examined the effect of thrombin on aPKC phosphorylation and activation by insulin and found no inhibitory effect, like that observed in Akt phosphorylation. This would suggest that insulin action is maintained in the presence of thrombin through the activation of aPKC that effectively bypasses the inhibitory effect of thrombin on Akt activation. Glucose is transported into the cell via a family of specialized transporter proteins (GLUTs) (43, 44). Different isoforms of GLUT have been identified in human skeletal muscles; GLUT1 and GLUT4 are the major contributors for glucose transport in basal and stimulating states respectively (21, 45-47). We found that thrombin increased the gene expression of both GLUT1 and GLUT4, which would be predicted to contribute to the observed increase in both basal and insulin mediated glucose uptake.

In conclusion, this is the first study to look at thrombin and insulin action/signalling in human skeletal muscle. We found that thrombin decreased insulin stimulated Akt activation which was mediated through PKC α , but thrombin had no effect on the parallel insulin stimulated aPKC pathway. Thrombin directly increased glucose uptake through an AMPK mediated mechanism.

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Figure legends:

Figure 1: Thrombin inhibits insulin stimulated Akt activation in cultured myotubes. Akt phosphorylation on Ser473 was measured by Western blot analysis. Cells plated onto dishes were treated with thrombin (5 U/ml) for 6h then stimulated with insulin (100 nM) for the last 10mins of thrombin treatment. (A) Shows representative blots of phosphorylated forms of Akt, blots of total Akt as well as a blot for β -actin respectively. (B) Shows the results of densitometry analysis of P-Akt/T-Akt in insulin stimulated cells both thrombin treated or non-treated cells. Values are expressed as the mean $\pm$ SEM (n = 8), **p<0.01.

Figure 2: PKC chemical inhibitor applied to the thrombin treated cells (A) PKC general inhibitor (GF109203X) in myotubes. Cells were treated with thrombin (5U/ml) for 6h then different doses of the inhibitors were added for the final 30min before cells were stimulated with 100 nM insulin for 10 mins. (A) Representative blots of phosphorylated forms of Akt, total Akt and the results of densitometry analysis of P-Akt/T-Akt in insulin stimulated cells with different doses of general inhibitor are shown. Values are expressed as the mean $\pm$ SEM (n = 6), *p< 0.05. (B) Conventional PKC selective inhibitor (GÖ 6976) in myotubes; the same conditions as described above for the general PKC inhibitor. (B) Shows representative blots of phosphorylated Akt, total Akt and the results of densitometry analysis of P-Akt/T-Akt in insulin stimulated cells with different doses of selective inhibitor. Values are expressed as the mean $\pm$ SEM (n = 4), *p<0.05, **p<0.01.

Figure 3: Glucose uptake in skeletal muscle myotubes, basal and insulin stimulated for both the thrombin (+) and non-thrombin (-) treated myotubes the values were represented as a mean $\pm$ SEM. Where n=5. *p<0.05.

Figure 4: Thrombin has no inhibitory effects on insulin stimulated aPKC activation in cultured myotubes. The phosphorylation of PKC ζ/λ ^{Thr410/403} was measured by Western blot analysis. Cells plated onto dishes were treated with thrombin (5 U/ml) for 6h then stimulated with insulin (100 nM) for the last 10mins of thrombin treatment. Representative blots of phosphorylation of PKC ζ/λ (Thr410/403), blots of total aPKC and the results of densitometry analysis of P- aPKC /T- aPKC in basal and insulin stimulated cells both thrombin treated or non- treated cells are shown. Values are expressed as the mean $\pm$ SEM (n = 4). *, p<0.05.

Figure 5: Thrombin induced basal glucose uptake by AMPK activation in cultured myotubes. (A) AMPK phosphorylation on Thr172 was measured by Western blot analysis. Cells plated onto dishes were treated with thrombin (5 U/ml) for 6h with or without 40 μ M compound C that was added for the last 30 mins. (A) shows representative blots of phosphorylated and total AMPK and the results of densitometry analysis of P-AMPK/ native AMPK for both thrombin and thrombin with compound C treated cells. Values are expressed as the mean $\pm$ SEM (n= 4). **p<0.01. (B) Basal glucose uptake in myotubes in response to AMPK inhibition. Basal glucose uptake in skeletal muscle myotubes for control, thrombin and thrombin with compound C, the values are expressed as mean $\pm$ SEM. Where n=4. *p<0.05.

Figure 1:

Figure 2:

(A): PKC general inhibitor (GF109203X).

(B): Conventional PKC selective inhibitor (Gö 6976).

Figure 3:

Figure 4:

Figure 5: