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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 affects 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 6 h. Insulin signalling pathway components and 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 IRS-1 and Akt phosphorylation in response to thrombin in cultured myotubes. Diminished Akt phosphorylation was alleviated by treatment with a PKC inhibitor. Thrombin directly increased basal glucose uptake ($p < 0.05$) that involved AMPK phosphorylation ($p < 0.01$) and this 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 in skeletal muscle cells through a PKC mediated mechanism, but did not affect the net action of insulin on glucose uptake. The direct stimulatory effect of thrombin on glucose uptake was mediated, at least in part, via an AMPK dependent mechanism. We conclude that thrombin activation results in multiple metabolic effects beyond increased thrombogenicity but does not include a decrease in insulin sensitivity (glucose uptake) in cultured human skeletal muscle cells.

1. Introduction

Thrombin is a multifunctional serine protease enzyme, involved in several biological processes with a primary function in the coagulation cascade (Bailey, Bettelheim, Lorand, & Middlebrook, 1951). 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 (Fan, Zhang, & Mulholland, 2005).

Distinct PARs are expressed in skeletal muscle. Three different PARs (PARs 1, PARs 3 and PARs 4) are shown to be activated by thrombin (Mackie et al., 2008). The activation of PARs by thrombin triggers different molecular pathways and includes: mobilisation of intracellular Ca^{+2}(Chevessier, Hantaï, & Verdière-Sahuqué, 2001), 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 (Gordon, Zhang, Stevenson, & Cosford, 2000). Protein kinase C, mitogen-activated protein (MAP) kinases (Arora, Ricks, & Trejo, 2007; Mackie et al., 2002; Ossovskaya & Bunnett, 2004) and AMPK are also involved in mediating the actions of thrombin (Thors, Halldórsson, & Thorgeirsson, 2004; Tokuda et al., 2012).

Hypercoagulation is a feature of patients with type 2 diabetes who are at high risk of thrombotic and vascular events (Carr, 2001). In type 2 diabetes, a strong association between metabolic abnormalities such as hyperglycaemia and the increased coagulation (elevated thrombin level) has been identified (Rao, Chouhan, Chen, Sun, & Boden, 1999), and decreased insulin sensitivity is associated with enhanced thrombin production (Romano et al., 2003).

Furthermore, there is an emerging evidence of correlation of the genetic basis of hypercoagulation and type 2 diabetes. High heritability indices were reported in a cohort of Mexican Americans for a number of coagulation cascade's factors, including prothrombin which was strongly associated with diabetes (Almasy et al., 2005).

Skeletal muscle is the primary peripheral tissue site of insulin stimulated glucose uptake (DeFronzo et al., 1981) and the key peripheral site of insulin resistance in type 2 diabetes (Cline et al., 1999).

Conflicts of interest: There is no conflict of interest for this work.

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

2. Material and methods

2.1. 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-IRS-1Tyr^{612} (ab66153) and Phospho-AS160 Thr^{642} [EPR2733] were purchased from Abcam. Phospho-Akt Ser^{473} (D9E), total Akt rabbit antibodies, Phospho-PKCζ/λThr$^{410/403}$ antibody (9378), phospho-AMPK Thr^{172} (40H9) rabbit antibodies and AMPKα (F6) mouse antibodies, total AS160 rabbit (C96A7)and total IRS-1 (L3D12) Mouse were supplied by New England Biolabs (Herts, UK. 2-Deoxy-D-[2, 6-3H] glucose was purchased from Hartmann Analytic (Germany). IL-6 ELISA kits were obtained from Qiagen (Sussex, UK).

2.2. 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 (Blau & Webster, 1981; Jackson et al., 2000). 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 5 ml 0.05% trypsin-EDTA for spin-digestion at 37 °C. After 15 min, the trypsin was removed, 5 ml media added and centrifuged at 1700 rpm for 5 min. 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 24 h to remove unattached cells and cell debris. Prior to stimulation, cells were seeded at a density of approximately 200,000 per 35 mm 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.

2.3. Immunofluorescent staining

Myoblasts were plated in cover slips in 6-well plates and, when confluent, allowed to differentiate for 7 days. 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 20 min. Cells were washed with PBS then permeabilized with 0.2% t-octylphenoxy-polyethoxyethanol (Triton X-100, Sigma-Aldrich) for 45 min. After washing with PBS, cells were incubated in blocking buffer (20% FBS in PBS) for 1 h 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 1 h. After washing with PBS, cells were incubated in Cy3-conjugated anti-rabbit IgG, diluted 1:500 in 0.05% FBS in PBS for 1 h 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.

2.4. Western blot

Cells were scraped into protein extraction buffer (100 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM EDTA, 25 mM KF, 1 mM benzamidine, 0.5 mM Na_3VO_4, 0.1% (v/v) Triton X-100, 1× protease inhibitor cocktail (Pierce), sonicated briefly and centrifuged at 13,400 rpm for 5 min at 4 °C before measuring protein concentration using the modified Bradford dye binding colorimetric method (Bradford, 1976) at 595 nm. 10 μg samples were loaded on 10% SDS-PAGE gels in loading buffer (0.125 M 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 1 h 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 and native-IRS 1 were used at 1:1000 dilutions, Phospho-Akt antibody was used at a 1:10000 dilution while native Akt was used at a 1:2000 dilution, phospho and native aPKC antibodies were used at a 1:1000 dilution, phospho and native AMPK antibodies were used at a 1:1000 and phospho and native AS160 were 1:500 dilutions.

2.5. Glucose uptake assay

Cells were cultured on 6 well plates and incubated with or without thrombin at 37 °C in serum free media for 6 h prior to use. After treatment, cells were washed twice with Kreb's buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.2 mM $CaCl_2$, 20 mM HEPES, pH 7.4), then treated with or without 100 nM insulin, 40 μM compound C or 10 μM cytochalasin B for 30 min. Hot glucose solution (0.1 mM 2-deoxy-glucose and 0.5μCi (2, 6-^{3}H) 2-deoxyglucose) was added for the last 10 min 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.

2.6. 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 μl. Total RNA was treated with DNase I and 200 ng was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a final volume of 20 μl.

2.7. 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. β2-microglobulin (β2M) was used as a reference gene with sequences: For; GCCTGCCGTGTGAACCAT, Rev; TTACATGTCT CGATCCCACTTACCTATC, Probe; FAM-TGACTTTGTCACAGCCCA-TAMRA. The concentration of both primers was 300 nM per reaction and 250 nM