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ORIGINAL ARTICLE

Insulin inhibits tissue factor expression in monocytes

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Summary. *Objectives*: Platelets from healthy subjects are inhibited by insulin but type 2 diabetes mellitus (T2DM) platelets have become insulin-resistant, which might explain their hyperactivity. In the present study we investigated whether monocytes are responsive to insulin. *Methods and results*: LPS-induced tissue factor (TF) upregulation was measured in human monocytes and monocytic THP-1 cells in a factor Xa generation assay. Insulin (0.1–100 nmol L^{-1}) induced a dose-dependent inhibition in both cell types and in monocytes 100 nmol L^{-1} insulin inhibited cytosolic, membrane-bound and microparticle TF by 32 ± 2, 27 ± 3 and 52 ± 4% (n = 3). Insulin induced Tyr phosphorylation of the insulin receptor (INS-R) and formation of an INS-R – $G_i\alpha_2$ complex, suggesting interference with LPS-induced cAMP control. Indeed, insulin interfered with LPS-induced cAMP decrease and TF upregulation in a manner similar to an inhibitor of G_i (pertussis toxin) and agents that raise cAMP (iloprost, forskolin, IBMX) reduced TF upregulation. Although LPS failed to raise cytosolic Ca^{2+}, quenching of Ca^{2+} increases (BAPTA-AM) reduced and induction of Ca^{2+} entry (ionophore, P2X7 activation) enhanced upregulation of TF mRNA and procoagulant activity. Insulin interfered with MCP-1-induced Ca^{2+} mobilization but not with ATP-induced Ca^{2+} rises. *Conclusions*: Insulin inhibits TF expression in monocytes and monocyte-derived microparticles through interference with $G_i\alpha_2$-mediated cAMP suppression, which attenuates Ca^{2+}-mediated TF synthesis.

Keywords: insulin, monocytes, monocytes-derived microparticles, tissue factor, type 2 diabetes mellitus

Introduction

Patients with diabetes mellitus (DM) have a 2–8-fold higher risk of cardiovascular morbidity and mortality compared with matched controls [1]. Patients with type 2 DM (T2DM) show impaired insulin responsiveness or even complete insulin resistance and suffer from atherothrombotic complications and disturbed regulation of hemostasis with endothelial activation, hyperactive platelets, hypercoagulability and hypofibrinolysis [2,3]. The hypercoagulability in T2DM patients is accompanied by increased levels of plasma fibrinogen, factors VII, VIII, XI and XII, kallikrein and possibly von Willebrand factor [4]. Also levels of prothrombin fragment 1 + 2 and thrombin-anti-thrombin complexes are elevated, reflecting activation of the coagulation cascade. Of specific interest are the elevated levels of tissue factor (TF) [5,6], explaining the hypercoagulability and the cardiovascular complications observed in DM.

Under physiological conditions, monocytes express little TF. Upon contact with lipopolysaccharide (LPS), C-reactive protein, CD40 ligand, oxidized LDL and angiotensin II, TF expression increases in the cytosolic compartment and TF translocates to the plasma membrane, where it becomes a source for TF-rich microparticles. These particles are vehicles for so-called blood-born TF that in animal models for thrombosis initiates fibrin formation at the luminal site of the growing thrombus [7]. LPS binds and activates the Toll-like receptor 4 (TLR4). TLR4 signals to the IκB-kinase pathway and three mitogen-activated protein kinase (MAPK) pathways, leading to activation of transcription factors Egr-1, AP-1 and NF-κB [8]. In addition to pathways that promote TF synthesis, LPS initiates pathways that attenuate its production. Activation of the phosphatidyl inositol 3-kinase (PI3-K) – protein kinase B (PKB) pathway interferes with MAPK and transcription factors AP-1 and Egr-1 and the nuclear translocation of the NF-κB [9,10]. A second inhibitory route is formation of cAMP. The cAMP raising agents dibutyryl-cAMP, forskolin and 3-isobutyl-1-methylxanthine (IBMX) inhibit formation of TF mRNA, surface expression of TF protein and procoagulant activity due to interference with NF-κB and gene transcription [11,12].

Platelet functions are strongly enhanced by secreted ADP that binds to the P2Y12 receptor, which is coupled to the inhibitory G-protein of adenylyl cyclase, G_i. Activation of G_i initiates PI3-K-mediated activation of PKBα,β (Akt1,2), contributing to platelet activation, and inhibits adenylyl cyclase

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through $G_i\alpha$, which attenuates formation of cAMP, which is a potent platelet inhibitor [13–15]. In normal platelets insulin interferes with the P2Y12 pathway by signaling through its receptor (INS-R) and insulin receptor substrate-1 (IRS-1), inducing association of IRS-1 with the $G_i\alpha_2$ subunit. Tyr-phosphorylation of $G_i\alpha_2$ blocks G_i-mediated cAMP suppression and attenuates platelet functions. Platelets from T2DM patients have lost sensitivity to insulin and better respond to inducers of aggregation, release of secretion granules and generation of a procoagulant surface [16–18].

In the present study we investigated whether insulin interferes with TF expression in monocytes and monocytic THP-1 cells. We show that insulin concentrations in the physiological range interfere with cAMP suppression and TF formation in cells and monocytes-derived microparticles, making insulin resistance seen in diabetics a possible cause for the hyperactive monocytes seen in this disease.

Materials and methods

Materials

We obtained human recombinant insulin, Fura-2/AM, IBMX, ATP, wortmannin, protease inhibitor cocktail, sodium vanadate ($NaVO_3$) and LPS (*E. coli* 0111:B4) from Sigma (St Louis, MO, USA), prostacyclin from Schering AG (Berlin, Germany), H89 from Alexis Biochemicals (Lausen, Switzerland), protein G-Sepharose from Amersham (Uppsala, Sweden), forskolin, pertussis toxin from *Bordetella pertussis*, ionophore A23187, staurosporin and BAPTA(AM) from Calbiochem (La Jolla, CA, USA), LY294002 from Biomol (Plymouth Meeting, PA, USA) and MCP-1 from R&D Systems (Minneapolis, MN, USA). Factor X (FX) was purified from fresh-frozen plasma as described previously [19]. Recombinant Factor VIIa (rFVIIa) was from Novo Nordisk (Bagsværd, Denmark) and Innovin from Dade Behring (Liederbach, Germany). All other chemicals used were of analytical grade. Antibodies against the INS-R, INS-R phospho-$Tyr^{1150/1151}$, IRS-1 and horseradish peroxidase-labeled anti-rabbit antibody were from Cell Signaling Technology (Danvers, MA, USA), polyclonal antibodies against $G_i\alpha_2$, $G_s\alpha$, $G_q\alpha$, $G_z\alpha$ (T-19), PKBα,β total and PKBα phospho-Ser^{473} from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-phospho-Tyr monoclonal antibody 4G10 from Upstate Biotechnology (Bucks, UK), peroxidase-linked goat anti-mouse antibody from DAKO (Glostrup, Denmark), anti-annexin V-APC conjugated antibody from CALTAG laboratories (Burlingame, CA, USA) and anti-CD11b-PE conjugated antibody from Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

Monocytes, monocytic THP-1 cells and microparticles

Freshly drawn venous blood from healthy, medication-free volunteers was collected with informed consent into 0.1 volume of 130 mmol L^{-1} trisodium citrate according to procedures approved by the local Medical Ethical Review Board. Peripheral mononuclear cells were isolated by density-gradient centrifugation on Ficoll-Paque columns and monocytes were recovered by magnetic separation with anti-CD14 antibody coupled beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were resuspended in RPMI 1640 culture medium. For analyses that required large cell numbers, monocytic THP-1 (ATCC, Teddington, UK) were grown in RPMI 1640 culture medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% heat-inactivated fetal calf serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 50 μmol L^{-1} β-mercaptoethanol. For isolation of microparticles, monocytes were stimulated with 1 μg mL^{-1} LPS for 5 h (37 °C), centrifuged twice (350 ×*g*, 5 min, 22 °C), and the supernatant was collected. After a second centrifugation (17 000 ×*g*, 30 min, 4 °C), the pellet was collected and dissolved in 100 μL tris-buffered saline (TBS). For quantification of microparticle numbers, 5 μL freshly prepared microparticle suspension was labelled with annexin V-APC or with CD11b-PE (15 min, 22 °C, in darkness). Labelled particles were counted in a FACS Calibur (Becton, Dickinson and Company) qualibrated for particles < 1.5 μm. Supernatant of monocytes treated with 2 μmol L^{-1} staurosporin (5 h, 37 °C) served as positive control.

TF expression

For analysis of TF procoagulant activity, monocytes and THP-1 cells (1×10^6 cells) were stimulated for 5 h (37 °C) with 1 μg mL^{-1} LPS, a condition used in all experiments. Suspensions were centrifuged (350 ×*g*, 5 min, 22 °C) and the pellet dissolved in 100 μL TBS. Lysates were prepared by four times freeze/thawing and centrifuged (16 100 ×*g*, 5 min, 22 °C). Supernatant (cytosol fraction) and pellet (membrane fraction) were collected and incubated with 10 μg mL^{-1} FX, 5 U mL^{-1} rFVIIa and 5 mmol L^{-1} $CaCl_2$. FXa generation was measured in a fluorescence reader at 405 nm after addition of FXa substrate (Pentapharm, Basel, Switzerland). Procoagulant activity was expressed as ng per 10^7 cells based on a standard curve derived from serial dilutions of recombinant TF (Innovin). The cytosol fraction was adjusted for variations in protein content using the BCA assay.

For analysis of TF mRNA, 1 mL THP-1 suspension (5×10^6 cells mL^{-1}) was cultured in 6-well plates. Total RNA was isolated using the kit from Macherey-Nagel (Düren, Germany). cDNA was synthesized by reverse transcription on the PCR machine (PTC-200, MJ Research, Waltham, MA, USA). Quantitative real-time PCR was performed using TF primers 5′-CTC CCC AGA GTT CAC ACC TTA CC-3′ and 5′-CCG TTC ATC TTC TAC GGT CAC A-3′ and the fluorescent TF probe 5′-AGA CAA ACC TCG GAC AGC CAA CAA TTC A-3′. PBGD mRNA, which is constitutively expressed in cells, was measured for calibration.

IL-1β measurements

Monocytes (1×10^6 cells) were stimulated with LPS (5 h, 37 °C) and centrifuged (350 ×*g*, 5 min, 22 °C). Supernatant

and pellet were isolated, pellet was dissolved in 250 μL TBS, and Interleukin (IL)-1β was determined by ELISA (PeliKine Compact, Sanquin, Amsterdam, the Netherlands).

Insulin signaling

Aliquots of 1×10^6 THP-1 cells or monocytes were incubated with insulin (37 °C), collected in lysis buffer and analyzed for Tyr phosphorylation of INS-R, IRS-1, Gα-proteins, associations between these proteins and Ser phosphorylation of PKBα using appropriate antibodies, as previously described [16]. Band intensities were semi-quantified with ImageJ software.

cAMP and cytosolic Ca^{2+}

Monocytes (1×10^6 cells) were incubated with insulin as indicated and stimulated with LPS for 15 min (37 °C). Samples were acetylated according to manufacturer's instructions and cAMP levels were measured using the cAMP-EIA kit (Cayman Chemical, Ann Arbor, MI, USA). THP-1 cells (10×10^6 cells) were loaded with Fura-2/AM in the dark (1 h, 37 °C). Fluorescence was measured and changes in cytosolic Ca^{2+} content, $[Ca^{2+}]_i$, were analyzed as described [16].

Statistics

Data are expressed as mean ± SEM with number of observations n and were analyzed with the Student's test for unpaired observations. Differences were considered significant at $P < 0.05$.

Results

Insulin inhibits LPS-induced TF procoagulant activity in monocytes, THP-1 cells and monocyte-derived microparticles

In initial experiments, conditions were sought that optimally revealed interference with TF expression by insulin. Simultaneous addition of insulin and LPS showed no effect but a 15 min preincubation time induced maximal inhibition by insulin. In the absence of LPS, insulin failed to change the basal TF procoagulant activity (data not shown). LPS induced an increase of 84 ± 6% in cytosolic-TF and of 67 ± 2% in membrane-TF, which is equivalent to 42 ± 10 to 226 ± 39 and from 354 ± 30 to 1075 ± 139 ng 10^{-7} cells, respectively ($n = 3$). Insulin induced a dose-dependent inhibition, resulting in 32 ± 2% and 27 ± 3% ($n = 3$) fall in these fractions at 100 nM insulin (Fig. 1A). THP-1 cells showed a similar sensitivity to insulin with a 40 ± 9% fall in cytosolic TF at 100 nM insulin (Fig. 1B). LPS raised TF procoagulant activity in the microparticles from from 97 ± 26 to 311 ± 55 ng 10^{-7} monocytes, which was inhibited by 31 ± 8 and 52 ± 4% ($n = 3$) at 0.1 nmol L^{-1} and 100 nmol L^{-1} insulin, respectively (Fig. 1C). Control studies confirmed that insulin treatment left the number of annexin V-positive (Fig. 1D) and CD11b-positive (data not shown) particles unchanged, whereas staurosporin increased the number of microparticles with 40 ± 25% compared with untreated cells. Thus insulin decreased the TF procoagulant activity of microparticles derived from LPS-treated monocytes. To investigate whether inhibition by insulin was restricted to TF expression, the

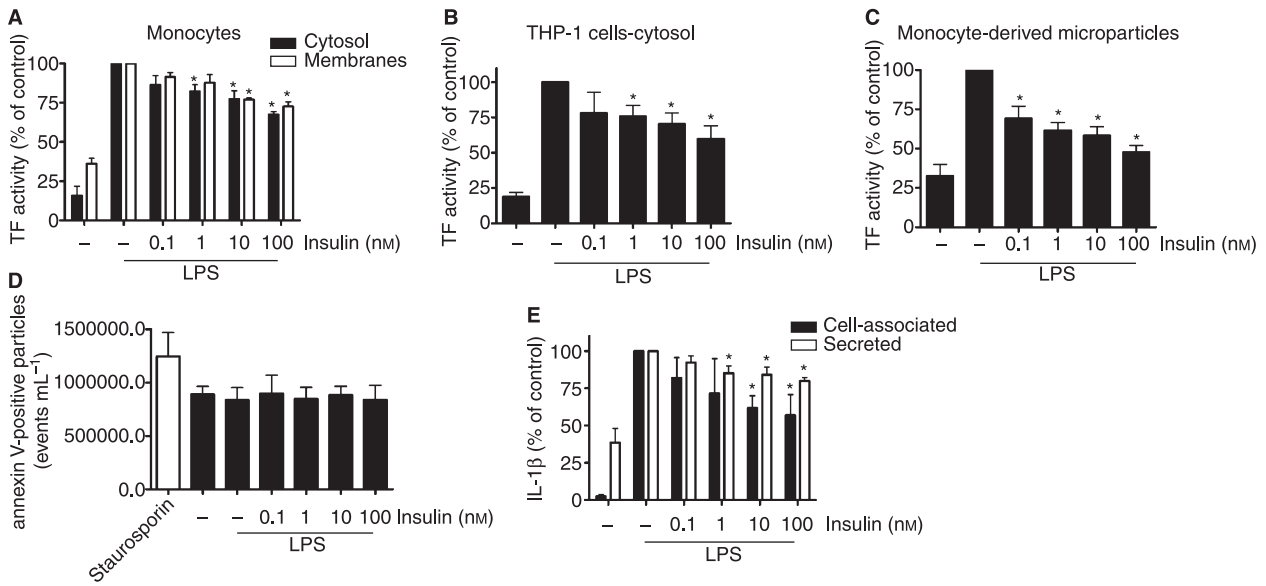

Fig. 1. Insulin inhibits LPS-induced upregulation of TF. Human monocytes and THP-1 were treated with 1 μg mL^{-1} LPS without and with preincubation with insulin (15 min, 37 °C). TF-procoagulant activity was measured in monocytes (A), THP-1 cells (B) and monocytes-derived microparticles (C). The number of microparticles was measured as annexin V-positive events using 2 μmol L^{-1} staurosporine (5 h, 37 °C) treatment as positive control (D). IL-1β surface expression and secretion were measured in monocytes (E). In the absence of LPS, insulin neither changed TF levels in monocytes (354 ± 30 ng 10^{-7} cells), THP-1 cells (82 ± 24 ng 10^{-7} cells) and monocytes-derived microparticles (97 ± 26 ng 10^{-7} monocytes) nor cell-associated and secreted IL-1β levels (583 ± 119 and 7626 ± 562 pg 10^{-7} cells). Data are percentages of controls (mean ± SEM, $n = 43$, * $P < 0.05$ vs. controls).

LPS-induced surface appearance and secretion of IL-1β were measured. Both responses were inhibited by insulin (Fig. 1E). Thus, a physiological relevant concentration of 1 nM insulin or more inhibits the generation of TF-procoagulant activity as well as IL-1β secretion, suggesting that insulin triggers a general reduction in the responsiveness of monocytes to stimulation by LPS.

Insulin signaling in THP-1 cells and monocytes

Having established that insulin reduced LPS-induced TF upregulation, we characterized the signaling pathways involved. In platelets, insulin affects INS-R, IRS-1 and a specific isoform of the G_i family, $G_i\alpha_2$, through a series of Tyr phosphorylations and transient formation of an IRS-1 - $G_i\alpha_2$ complex. Similar studies in monocytic THP-1 cells revealed that insulin induced a dose- and time-dependent phosphorylation of the INS-R (Fig. 2A) and IRS-1 (Fig. 2B). Phosphorylation of the receptor was detected after 5 min, persisted for 1 h and was maximal at 500 nmol L^{-1} insulin. In contrast, IRS-1 phosphorylation was maximal after 15 min and returned to prestimulation values after 30 min. Thus, activation of INS-R and signal generation occurred under conditions where insulin interfered with TF synthesis. In platelets, phosphorylation of IRS-1 induces association with $G_i\alpha_2$ but attempts to demonstrate a similar complex in monocytes had no success. In a search for alternative means for G_i inactivation we found that the INS-R associates directly with $G_i\alpha_2$ in THP-1 cells (Fig. 2C). These findings suggest that insulin activates pathways in monocytes through direct receptor - $G_i\alpha_2$ contact. Immunoprecipitates of the activated INS-R failed to show co-association of $G_s\alpha$, $G_q\alpha$ and $G_z\alpha$ (data not shown). These findings indicate that activation of INS-R triggers the exclusive association with $G_i\alpha_2$. Complex formation was maximal after 15 min stimulation and thereafter declined (Fig. 2C). Similar results for the transient binding between the INS-R and $G_i\alpha_2$

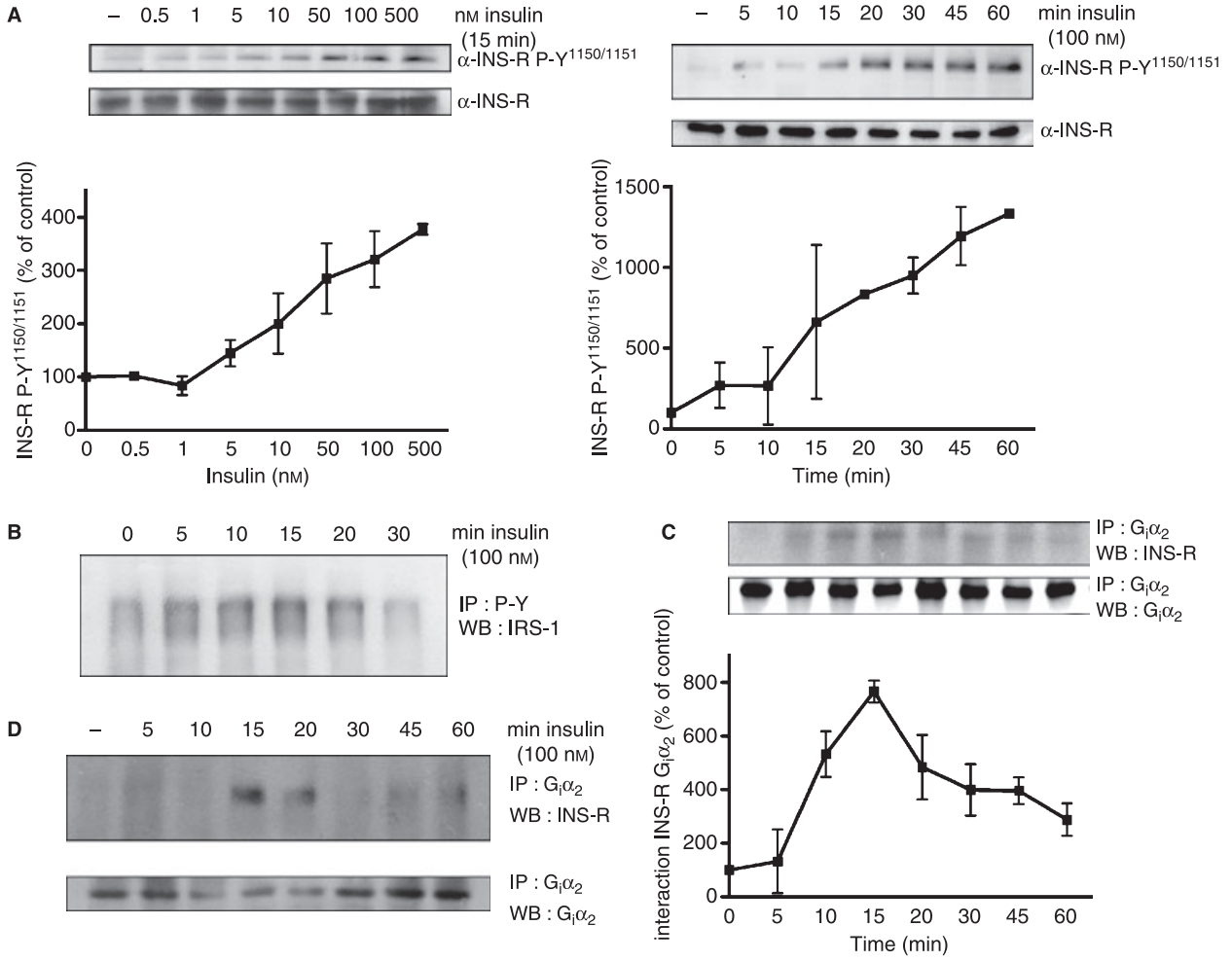

Fig. 2. Insulin signaling to $G_i\alpha_2$. THP-1 cells and monocytes were incubated with insulin at the indicated conditions (37 °C). For THP-1 cells phosphorylated and total INS-R was measured on Western blots (A) and immunoprecipitates of Tyr-phosphorylated proteins analyzed on Western blots show Tyr-phosphorylation of IRS-1 (B, representative example of $n = 3$). The insulin-induced association between INS-R and $G_i\alpha_2$ in THP-1 cells (C) and monocytes (D) in immunoprecipitates of $G_i\alpha_2$ are shown on Western blots. Band intensities were semi-quantified with ImageJ software and expressed as percentages of untreated cells (mean ± SEM, $n = 3$).

after insulin stimulation were seen in monocytes (Fig. 2D). This explains the 15 min preincubation time required for optimal inhibition of TF expression by insulin.

Control of cAMP by insulin

To confirm that interaction between INS-R and $G_i\alpha_2$ leads to inactivation of the G-protein, studies were repeated with pertussis toxin, a known inhibitor of the G_i family [20]. As observed with insulin, pertussis toxin inhibited LPS-induced upregulation of TF procoagulant activity, leading to a 49 ± 6% decrease (Fig. 3A). The cAMP level in monocytes is 14 ± 0.7 pmol 10^{-7} cells and decreased further to 4 ± 0.6 pmol 10^{-7} cells upon stimulation with LPS ($P < 0.01, n = 10$). Thus, LPS activates G_i, thereby inhibiting adenylyl cyclase and formation of cAMP. Pertussis toxin (Fig. 3A) and insulin (Fig. 3B) both interfered with the fall in cAMP induced by LPS, suggesting that insulin signaling to G_i inactivates the G-protein. To confirm that changes in cAMP directly interfered with TF synthesis, monocytes were treated with a stimulator of G_s (the prostacyclin analog iloprost), a stimulator of adenylyl cyclase (forskolin) and an inhibitor of phosphodiesterases (IBMX). All treatments inhibited LPS-induced TF synthesis and induced the expected increases in cAMP, confirming the role of cAMP as an inhibitor of TF upregulation (Fig. 3C). In platelets, cAMP exerts its inhibition through cAMP dependent protein kinase A. As expected, in the presence of the proten kinase A blocker H89, the effect of insulin had disappeared (Fig. 3D).

Earlier studies showed that TF expression is under negative control by the PI3-K-PKB pathway [9,10]. Because also in monocytes insulin is an activator of PKB (insert Fig. 3D), it is feasible that inhibition of TF upregulation by insulin is caused by stimulation of the PKB pathway. To evaluate this possibility, monocytes were incubated with wortmannin and LY294002, both potent inhibitors of PI3-K. The inhibitors induced a 1.5- to 2.0-fold increase in TF upregulation, illustrating the strong suppression of TF upregulation through this pathway in untreated monocytes. In the presence of wortmannin and LY294002, insulin reduced TF expression by 27 ± 3% and 37 ± 7% ($n = 3$), respectively, confirming an inhibitory role independent of the PI3-K–PKB pathway (Fig. 4D).

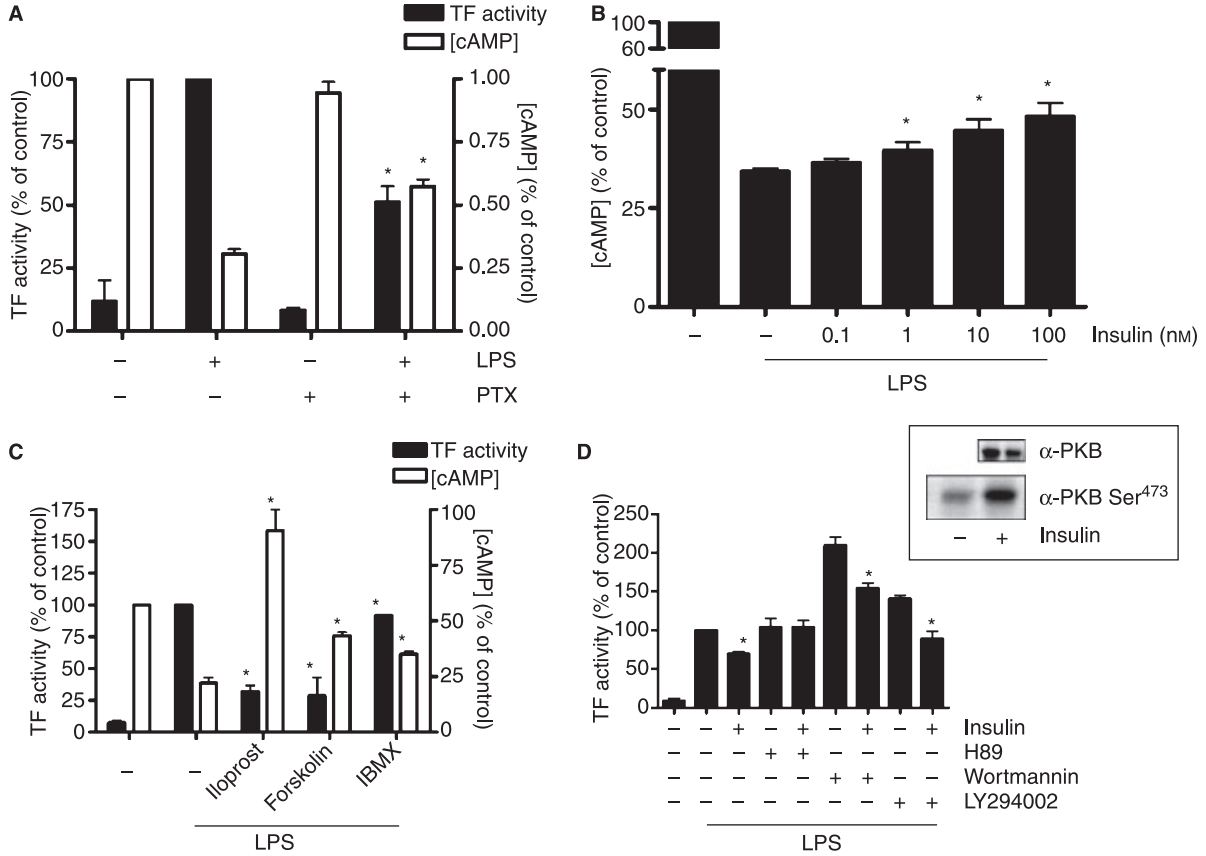

Fig. 3. Role of G_i/cAMP pathway in LPS-induced TF expression. Monocytes were preincubated at 37 °C with PTX (1 μg mL^{-1}, 2 h), insulin (0–100 nmol L^{-1}, 15 min), iloprost (1 μg mL^{-1}), forskolin (50 μmol L^{-1}) and IBMX (10 μmol L^{-1}, 15 min each), wortmannin (50 nmol L^{-1}, 30 min), LY294002 (10 μmol L^{-1}, 15 min), H89 (10 μmol L^{-1}, 15 min) before stimulation with LPS (1 μg mL^{-1}; A–D). cAMP levels in untreated cells and TF procoagulant activity induced by LPS were set at 100% (controls). Addition of inhibitors without LPS did not change basal TF levels, nor did cells treated with only vehicle change LPS-induced TF levels (data not shown). Lysates of THP-1 cells show increased Ser^{473} phosphorylation of PKB after stimulation with insulin (D, insert). Data are expressed as percentages of control (mean ± SEM, $n = 3$, for C $n = 5$; * $P < 0.05$ of controls).

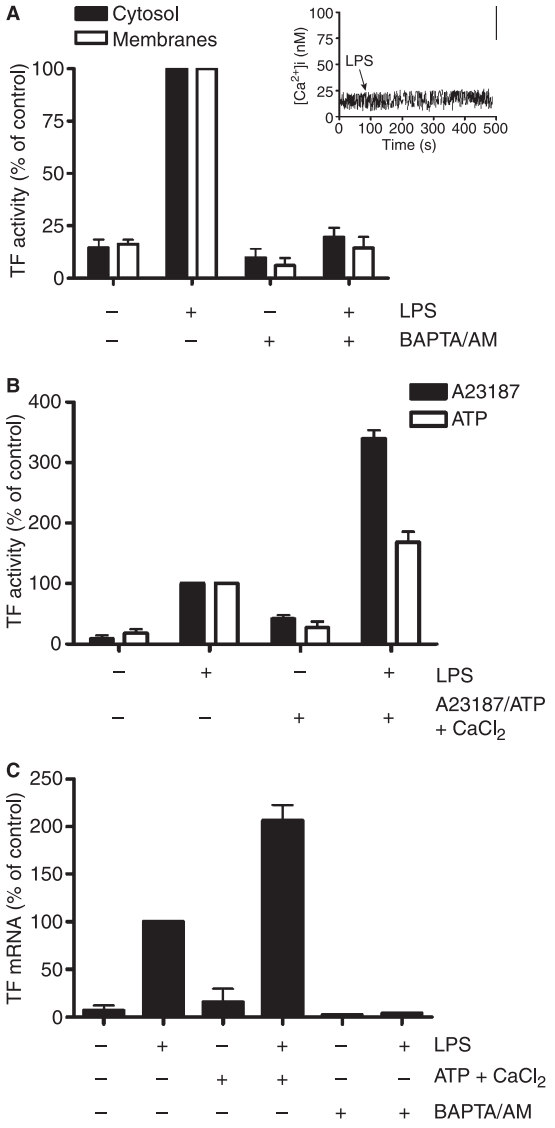

Fig. 4. LPS-induced TF upregulation depends on $[Ca^{2+}]_i$. THP-1 cells were incubated with Fura-2/AM at 37 °C and changes in $[Ca^{2+}]_i$ were measured after stimulation with LPS (1 μg mL^{-1}, A, insert). THP-1 cells were preincubated at 37 °C with BAPTA/AM (50 μmol L^{-1}, 15 min, A), Ca^{2+} ionophore A23187 (1 μmol L^{-1}, 15 min) with $CaCl_2$ (1 mmol L^{-1}, B) or ATP (10 μmol L^{-1}, 15 min) with $CaCl_2$ (1 mmol L^{-1}, B) and stimulated with LPS (1 μg mL^{-1} LPS). TF procoagulant activity was measured after 5 h incubation. TF mRNA was measured by quantitative real-time PCR analysis at the conditions indicated after 2 h stimulation with LPS (C).

Ca^{2+} rises contribute to TF expression

In platelets, rises in cAMP levels attenuate aggregation and secretion through inhibition of Ca^{2+} mobilization and influx [16]. We investigated whether a similar mechanism controlled TF synthesis in THP-1 cells. Stimulation with LPS alone failed to change $[Ca^{2+}]_i$, in agreement with earlier observations (Fig. 4A, insert and [21]). The Ca^{2+} chelating agent BAPTA-AM abolished the rise in LPS-induced TF procoagulant activity almost completely, both in the cytosol and the membrane fraction (Fig. 4A). Incubation with BAPTA-AM alone did not change basal TF activity. AM-free BAPTA did not change TF expression, reflecting its incapability to cross membranes and demonstrating that these compounds left the Ca^{2+}-dependent factor Xa generating assay undisturbed (data not shown).

When $[Ca^{2+}]_i$ was raised by ionophore A23187 in the presence of extracellular Ca^{2+}, TF expression was 33.3 ± 0.03% of the rise induced by LPS, indicating that a rise in Ca^{2+} alone is a poor activator of TF expression. Together with LPS, this treatment raised TF expression by 240 ± 13%, illustrating strong stimulation by increases in $[Ca^{2+}]_i$ (Fig. 4B). ATP activates the P2X7 receptor and Ca^{2+} influx. In the absence of LPS, ATP had little effect, again illustrating the poor capacity of rises in $[Ca^{2+}]_i$ to induce TF expression. Together with LPS, ATP induced an increase of 68 ± 17% (Fig. 4B).

These results show that Ca^{2+} is required for LPS-induced upregulation of TF procoagulant activity and that increases in $[Ca^{2+}]_i$ strongly enhance this process. To investigate whether Ca^{2+} stimulated TF expression at the level of transcription or the post-transcriptional activation known as de-encryption [22], TF mRNA levels were measured. The slight rise in TF expression by ATP alone, the higher increase by LPS and especially the strong potentation by the combination of LPS and ATP seen at the level of TF procoagulant activity was paralleled by similar changes in TF mRNA expression. Also the inhibition TF procoagulant activity by BAPTA/AM was accompanied by inhibition of TF mRNA (Fig. 4C). Together, these data indicate that Ca^{2+} supports the signaling pathways that control transcription of the TF gene, independent from changes in the TF protein.

Insulin inhibits MCP-1–induced Ca^{2+} mobilization

To investigate whether the inhibition of TF upregulation by insulin was caused by attenuation of Ca^{2+} rises, THP-1 were stimulated with ATP after preincubation with increasing concentrations of insulin. Without insulin, this treatment raised $[Ca^{2+}]_i$ to 206 ± 12 nM (n = 4). The increase remained unchanged in the presence of insulin, which argues against inhibition by insulin through interference with the P2Y7 receptor (Fig. 5A,B). To assess an effect by insulin on Ca^{2+} mobilization, THP-1 cells were stimulated with 10 ng mL^{-1} MCP-1, which induced a rise to 36 ± 10 nmol L^{-1} (n = 3). Insulin induced a dose-dependent inhibition leading to a fall of 39 ± 7% at 100 nmol L^{-1} insulin (Fig. 5C,D). Apparently, pathways that control the mobilization of Ca^{2+} from internal stores are sensitive to inhibition by insulin.

Discussion

Insulin inhibits dose-dependently the upregulation of TF procoagulant activity in monocytes and THP-1 cells stimulated with LPS. The inhibition is caused by a mechanism that interferes with the regulation of cAMP and $[Ca^{2+}]_i$, and has a

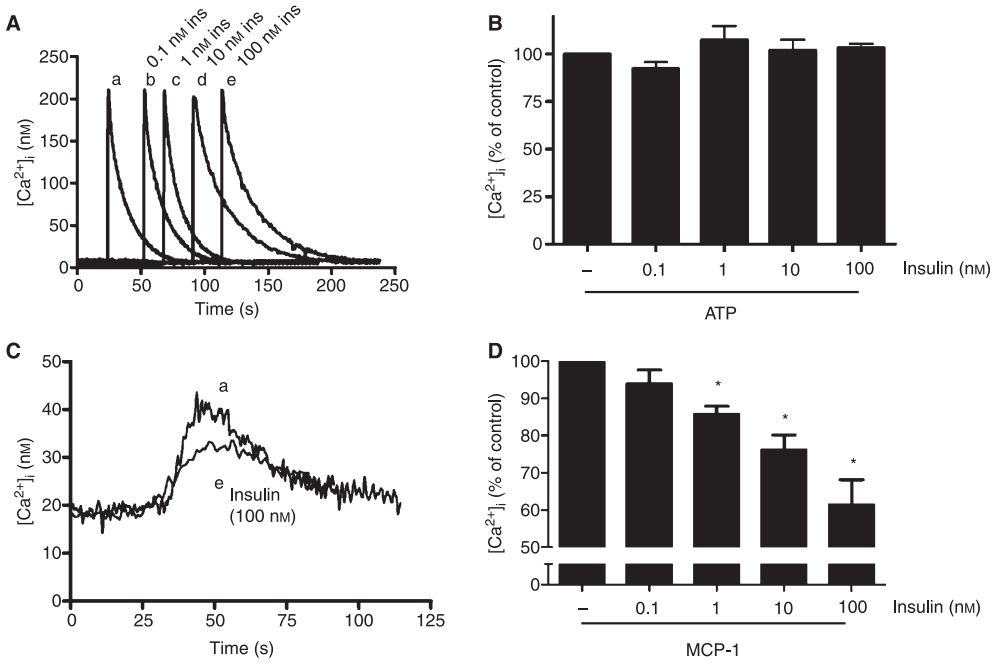

Fig. 5. Insulin inhibits MCP-1-induced Ca^{2+} mobilization. In THP-1 cells rises in $[Ca^{2+}]_i$ were measured at 37 °C upon stimulation with ATP (10 μmol L^{-1}, A,B) and MCP-1 (10 ng mL^{-1}, C,D). Curve (a) represents Ca^{2+} mobilization in the absence of insulin. Curves (b, c, d, e) represent the Ca^{2+} mobilization after preincubation with insulin (15 min, A,C). Agonist-induced increase in $[Ca^{2+}]_i$ was expressed at 100% (control) (B,D). Insulin alone did not change basal $[Ca^{2+}_i]$ (19 ± 2 nmol L^{-1}). (Futher details as in Fig. 3).

great impact on the expression of TF. Because this property also affects TF expression on monocyte-derived microparticles, it is likely to interfere with the contribution of blood-born TF to thrombus formation. Insulin also reduces surface expression and secretion of IL-1β consistent with earlier findings [23] and might therefore act as a general suppressor of monocyte activation.

Inhibition by insulin is the result of activation through Tyr phosphorylation of the INS-R (on residues 1150/1151) inducing a transient INS-R – $G_i\alpha_2$ complex. It is difficult to confirm that insulin inhibits the GTPase activity of a Gα protein [24], but the observation that it aborts the suppression of cAMP synthesis by LPS is proof for interference with a $G_i\alpha$ subunit. Because $G_i\alpha_2$, but not $G_s\alpha$, $G_q\alpha$, $G_z\alpha$, interacts with the INS-R after stimulation with insulin, blockade of this G-protein subunit is the cause for inhibition of TF expression.

The cause for the inhibition by insulin must be sought in the control of cAMP. Agents that stimulate cAMP production or inhibit cAMP degradation raise cAMP and induce a fall in TF procoagulant activity. The observation that LPS reduces cAMP thereby inducing optimal upregulation of TF expression implies that the LPS receptor signals to a $G_i\alpha$ subunit, possibly $G_i\alpha_2$. Previous studies [11,12] and our own data show that elevated cAMP levels inhibit TF expression in monocytes. cAMP induces the expression of several genes mediated by protein kinase A through the phosphorylation of the cAMP response element-binding protein (CREB) and the subsequent recruitment of the co-activator CREB-binding protein (CBP). Besides association with CREB, CBP binds to p65 *in vitro* and *in vivo*. Functional assays suggest that a competition occurs between CREB and p65 for limiting amounts of CBP, which can lead to a decrease in association of CBP with p65, thus inhibiting NF-κB-dependent transcription [25]. In addition to direct interference with TF transcription, cAMP might inhibit TF expression by suppressing rises in $[Ca^{2+}]_i$. In THP-1 cells LPS does not trigger a rise in $[Ca^{2+}]_i$, but the importance of basal $[Ca^{2+}]_i$ and rises in $[Ca^{2+}]_i$ induced by entry and mobilization is evident. Previous studies support a role for $[Ca^{2+}]_i$ in the activation of the transcription factor NF-κB in RAW 264.7 cells [26] and dendritic cells [27]. A similar mechanism might be operational in monocytes.

T2DM patients suffer from atherothrombotic complications. Their atherosclerotic plaques contain high levels of apoptotic microparticles [28]. They are mainly of monocytic and lymphocytic origin and retain almost all TF activity, suggesting a direct link between shed microparticles and plaque thrombogenicity [28]. Indeed, active TF has been identified within thrombi formed in coronaries [29,30]. The observation that insulin suppresses TF expression in monocytes from healthy individuals raises the possibility that insulin-resistance in T2DM patients leads to hyperactivity of monocytes, increased TF expression and microparticles enriched in TF. This would increase the risk for cardiovascular disease.

In healthy individuals, insulin suppresses the activity of platelets through interference with cAMP suppression by the P2Y12 receptor [16]. In T2DM patients, platelets have lost their responsiveness to insulin and show hyperactivity upon stimulation. The clopidgrel-like inhibitor AR-C69931 MX makes T2DM platelets equally responsive as normal platelets in the presence of insulin, suggesting that clopidogrel is a suitable

target to normalize the function of T2DM platelets [17]. Monocytes lack the P2Y12 receptor and alternative approaches must be sought to normalize the function of T2DM monocytes, if these cells have indeed become insulin-resistant. Preliminary experiments with periodate-oxidized ATP show that such a correction is feasible through interference with the P2X7 receptor, thereby suppressing the stimulation by Ca^{2+} without interfering with the basal TF expression.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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