Caffeine induces endothelial tissue factor expression via phosphatidylinositol 3-kinase inhibition

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Summary

Tissue factor (TF) is the key activator of coagulation and is involved in acute coronary syndromes. Caffeine is often reported to increase cardiovascular risk; however, its effect on cardiovascular morbidity and mortality is controversial. Hence, this study was designed to investigate the impact of caffeine on endothelial TF expression *in vitro*. Caffeine concentration-dependently enhanced TF protein expression and surface activity in human endothelial cells stimulated by tumour necrosis factor (TNF)-α or thrombin. Caffeine inhibited phosphatidylinositol 3-kinase (PI3K) activity and this effect was comparable to that of the known PI3K inhibitor LY294002. Consistently, treatment of endothelial cells with LY294002 enhanced TNF-α induced TF expression to a similar extent as caffeine, and adenoviral expression of the active PI3K mutant (p110) reversed the effect of both caffeine and LY294002 on TF expression. Caffeine and LY294002 increased DNA binding capacity of the transcription factor nuclear factor κB, whereas the activation pattern of mitogen-activated protein kinases (MAPK) remained unaltered. Luciferase reporter assay revealed a caffeine dependent activation of the TF promoter, and RT-PCR revealed a dose dependent increase in TF mRNA levels when stimulated with caffeine in the presence of TNF-α. In conclusion, caffeine enhances TNF-α-induced endothelial TF protein expression as well as surface activity by inhibition of PI3K signalling. Since the caffeine concentrations applied in the present study are within the plasma range measured in humans, our findings indicate that caffeine enhances the prothrombotic potential of endothelial cells and underscore the importance of PI3K in mediating these effects.

Keywords

Tissue factor / factor VII, signal transduction, coronary syndrome, endothelial cells, nutrition

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Introduction

Arterial thrombosis is the critical event in acute vascular syndromes such as unstable angina, myocardial infarction, peripheral ischemia, and stroke (1–3). Thrombus formation is determined by activation of coagulation factors, in particular tissue factor (TF), a membrane-bound glycoprotein playing a central role in initiation and propagation of thrombosis. Its expression is upregulated by inflammatory mediators such as tumour necrosis factor (TNF)-α and can be detected in different cell types of the atherosclerotic plaque (4, 5).

Caffeine (1,3,7-trimethylxanthine) is the most widely used drug in the world; indeed, 80–90% of adults report regular consumption of caffeine-containing beverages such as coffee, tea, chocolate, and cola drinks. Caffeine is often regarded as a cardiovascular risk factor; however, there is conflicting epidemiological evidence on the relation of caffeine consumption to the occurrence of acute cardiovascular events (6–13). The conflicting evidence may in part be due to the various pleiotropic effects caffeine has on cells at physiologically achievable concentrations. A number of putative mechanisms for caffeine-related cardiovascular effects, such as its ability to inhibit phosphodiesterases (14), and to directly antagonise adenosine receptors (15), have been proposed, although the molecular basis of these effects has not been established.

Additionally, caffeine is a direct inhibitor of class I and II phosphatidylinositol 3 kinases (PI3K), with the most potent effects seen against the p110δ isoform, and this inhibitory effect may explain some of its physiological and pharmacological properties (14). PI3Ks are important negative regulators of TF expression in human endothelial cells (16, 17); and this pathway suppresses inflammation and coagulation in endotoxaemic mice (18). Targeting PI3K activity in various inflammatory conditions has become an active area of investigation (19, 20).

Based on the fact that a) the PI3k pathway negatively regulates TF expression and thrombus formation, and b) caffeine is a direct inhibitor of the PI3K pathway, we hypothesised that, under these conditions, caffeine may promote endothelial TF expression.

Materials and methods

Cell culture

Human aortic endothelial cells (HAEC) and vascular smooth muscle cells (both from Clonetics, Allschwil, Switzerland) were cultured as described (21). THP-1 cells (LGC Promochem, Molsheim, France) were cultured in RPMI containing 10% fetal calf serum. Adhering cells were grown to confluence in 3 cm dishes and rendered quiescent for 24 hours (h) before stimulation with 5 ng/ml TNF-α or 1 U/ml thrombin (both from Sigma, Buchs, Switzerland). Cells were pretreated with caffeine (Sigma) for 15 minutes (min) before stimulation, as preliminary experiments demonstrated that its effect was similar from 15 to 60 min of pretreatment (data not shown). LY294002 (Cell Signaling, Allschwil, Switzerland), 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX), 3,7-Dimethyl-1-(2-propynyl) xanthine (DMPX) (all from Sigma) were added to the dishes 60 min before stimulation. Cytotoxicity was assessed with a colourimetric assay to detect lactate dehydrogenase release (Roche, Basel, Switzerland).

Western blot

Protein expression was determined by Western blot analysis as described (22). Equal loading was confirmed by Ponceau S staining. Antibodies against human TF and tissue factor pathway inhibitor (TFPI) (both from American Diagnostica, Stamford, CT, USA) were used at 1:2,000 dilution. Antibodies against IκBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated p38 MAP kinase (p38), p44/42 MAP kinase (extracellular signal-regulated kinase [ERK]), and c-Jun NH2-terminal kinase (JNK; all from Cell Signaling) were used at 1:1,000, 1:1,000, 1:5,000, and 1:1,000 dilution, respectively. Antibodies against total p38, ERK, and JNK (all from Cell Signaling) were used at 1:2,000, 1:10,000, and 1:1,000 dilution, respectively. All blots were normalised to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Temecula, CA, USA) or α-Tubulin (aT) expression (1:20,000 dilution, Sigma).

TF activity

TF activity was analysed at the surface of HAEC using a colorimetric assay (American Diagnostica) according to the manufacturer's recommendations with some modifications as described (23). TF/FVIIa complex converted human FX to FXa, which was measured by its ability to metabolise a chromogenic substrate. A standard curve with lipidated human TF was performed to assure that measurements were taken in the linear range of detection.
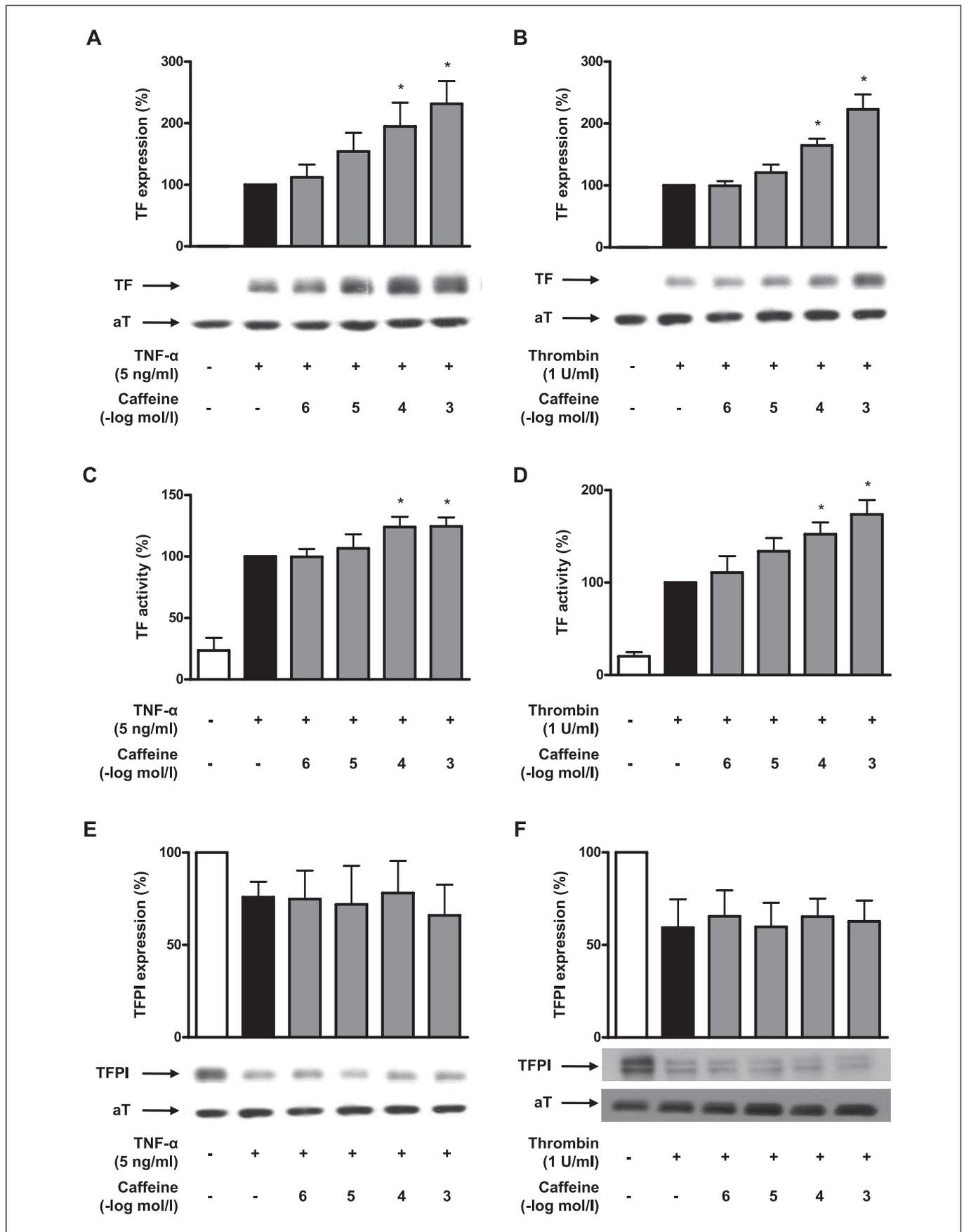
Real-time PCR

All real-time PCR experiments were performed in triplicate using the SYBR Green JumpStart kit (Sigma) in an MX3000P PCR cycler (Stratagene, Amsterdam, Netherlands). Each reaction (25 μl) contained 2 μl cDNA, 1 pmol of each primer, 0.25 μl of internal reference dye, and 12.5 μl of JumpStart Taq ReadyMix (containing buffer, dNTPs, stabilisers, SYBR Green, Taq polymerase, and JumpStart Taq antibody). The following primers were used: human tissue factor (*F3*): sense primer: 5'-TCCCCAGAGTTCACACCTTACC–3' (bases 508–529 of *F3* cDNA; NCBI no. NM 001993), antisense primer: 5'–TGACCACAAATACCACAGCTCC–3' (bases 892–913 of *F3* cDNA; NCBI no. NM 001993); human L28: sense primer: 5'–GCATCTGCAATGGATGGT-3', antisense primer: 5'–TGTTCTTGCGGATCATGTGT-3'. The amplification program consisted of one cycle at 95ºC for 10 min followed by 40 cycles with a denaturing phase at 95ºC for 30 seconds, an annealing phase at 60ºC for 1 min, and an elongation phase at 72ºC for 1 min. PCR products were analysed on an ethidium bromide stained 1% agarose gel, and a melting curve analysis was performed after amplification to verify the accuracy of the amplicon. In each real-time PCR run for F3 and L28, a calibration curve was included that was generated from serial dilutions of the respective purified amplicon.

In vitro PI3K assay

PI3K activity in endothelial cells was estimated using a commercially available PI3K ELISA kit (catalog number K-1000; Echelon, Salt Lake City, UT, USA) according to the manufacturer's instructions. Briefly, endothelial cells were lysed, supernatant was mixed with anti-PI3K p85 antibody (Millipore, Billerica, MA, USA) and incubated for 1 h. Then, protein A agarose beads were added to equal amounts of total protein and the samples rocked (4ºC) for 1 h at 4ºC. The immunoprecipitated PI3K was incubated with phosphatidylinositol-4,5-bisphosphate (PIP2) substrate and reaction buffer for 1 h. The amount of PIP3 formed from PIP2 by PI3-K activity was detected using a competitive ELISA.

NFκB DNA binding capacity

NFκB DNA binding activity in endothelial cells was estimated using a commercially available colorimetric NFκB assay (TransAM NFκB, cataolg number 40096; Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, for isolation of nuclear proteins a Nuclear Extract Kit (Active Motif) was ap-

plied. Nuclear extracts were then added to a 96-well plate on which oligonucleotide containing the NFκB consensus site (5′-GGG-ACTTTCC-3′) had been immobilised. Then, a primary antibody against an epitope on p65 that is accessible when NF?B is bound to its target DNA was added for 1 h. This was followed by addition of a secondary antibody conjugated to horseradish peroxidase. Colorimetric readout was achieved by spectrophotometry.

Adenoviral expression of PI3K active mutant rCD2-p110α

Generation of recombinant adenovirus expressing an active PI3K mutant (pEF-BOSrCD2-p110α) was carried out as described (24). For transduction, the vector was added to HAEC at 100 pfu/cell for 1 h and then removed. HAEC were kept in growth medium for 24 h and then serum-starved for 24 h prior to TNF-α stimulation in the presence or absence of caffeine or LY 294002 for 5 h.

TF promoter activity

The TF promoter (- 227 bp to +121 bp) was cloned upstream of the luciferase cDNA and the SV40 PolyA signal into the multiple cloning site of the helper vector VQAd5K-NpA (ViraQuest Inc., North Liberty, IA, USA). In a first step, HindIII and BamHI restriction sites of VQAd5K-NpA were used to insert a 2.7 kb HindIII/BamHI restriction fragment of pGL2-Basic vector (Promega, Madison, WI, USA) containing the luciferase cDNA and the SV40 PolyA signal. In a second step, a 0.3 kb KpnI restriction fragment from a human TF promoter plasmid including the TF minimal promoter kindly provided by Dr. Nigel Mackman (University of North Carolina, Chapell Hill; NC, USA) was ligated into the KpnI site of the resulting construct. The whole insert was sequenced to confirm its orientation and the absence of any nucleotide substitutions. This construct named VQAd5/hTF/Luc was used for production of an adenoviral vector (Ad5/hTF/Luc). For transduction, the vector was added to HAEC at 60 pfu/cell for 1 h and then removed. HAEC were kept in growth medium for 24 h and then serum-starved for 24 h prior to TNF-α stimulation for 5 h. Firefly luciferase activity was determined in cell lysates using a lumino-

Figure 1: Caffeine enhances TF expression in human aortic endothelial cells. Caffeine enhances TNF-α (A) and thrombin (B) -induced TF protein expression in a concentration-dependent manner. Values are indicated as percent of TNF-α or thrombin alone. *p<0.05 vs. TNF-α or thrombin alone. Caffeine enhances TNF-α (C) and thrombin (D) -induced TF surface activity in a concentration-dependent manner. Values are indicated as percent of TNF-α or thrombin alone. *p<0.05 vs. TNF-α or thrombin alone. Caffeine does not affect TNF-α (E) and thrombin (F) -induced TFPI protein expression. Values are indicated as percent of TNF-α or thrombin alone. p=NS vs. TNF-α or thrombin alone. Values are representative of at least four different experiments; all blots are normalised to α-Tubulin (aT) expression.
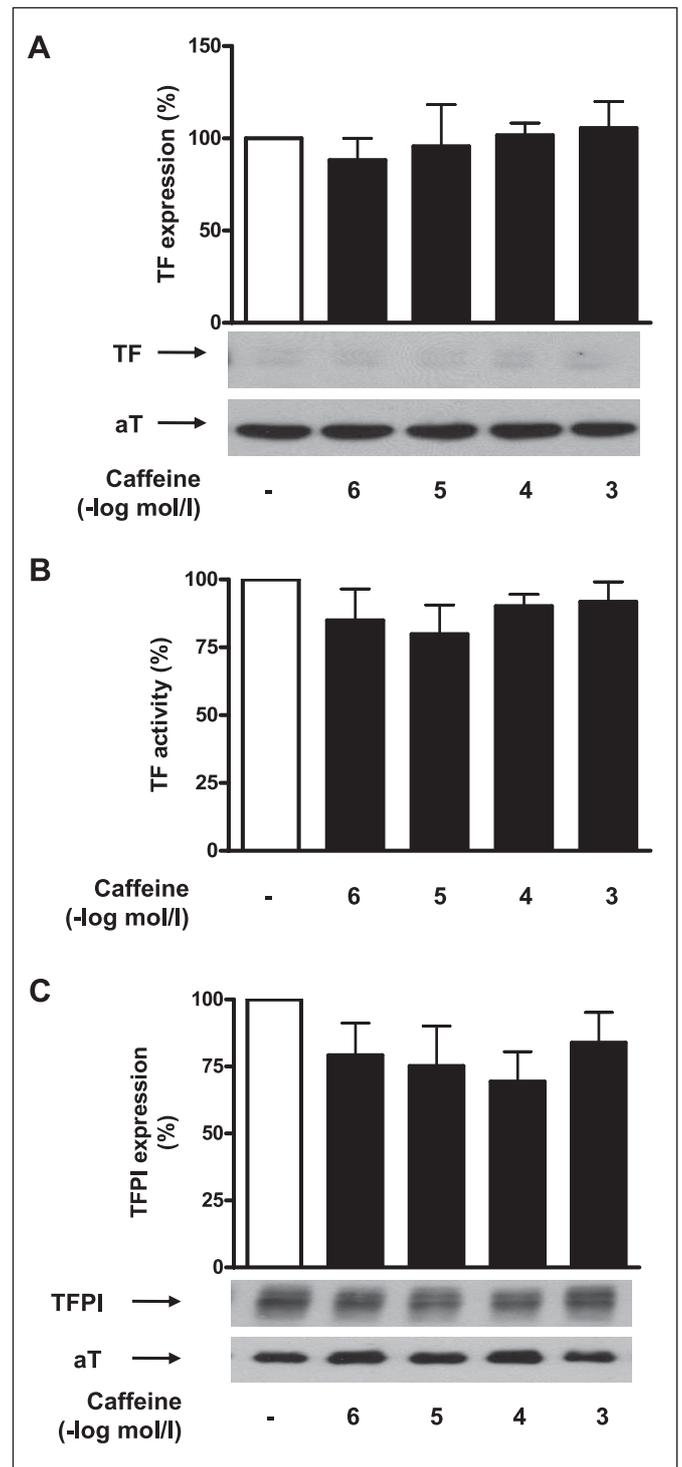
Figure 2: Caffeine does not affect basal TFPI and TF expression in human aortic endothelial cells. Caffeine does not alter TF protein expression under basal conditions (A). Values are indicated as percent of unstimulated control. p=NS vs. control. Caffeine does not alter TF activity under basal conditions (B). Values are indicated as percent of unstimulated control. p=NS vs. control. Caffeine did not alter endothelial TFPI expression under basal condition (C). Values are indicated as percent of unstimulated control. p=NS vs. control. Values are representative of at least four different experiments; all blots are normalised to α-Tubulin (aT) expression.
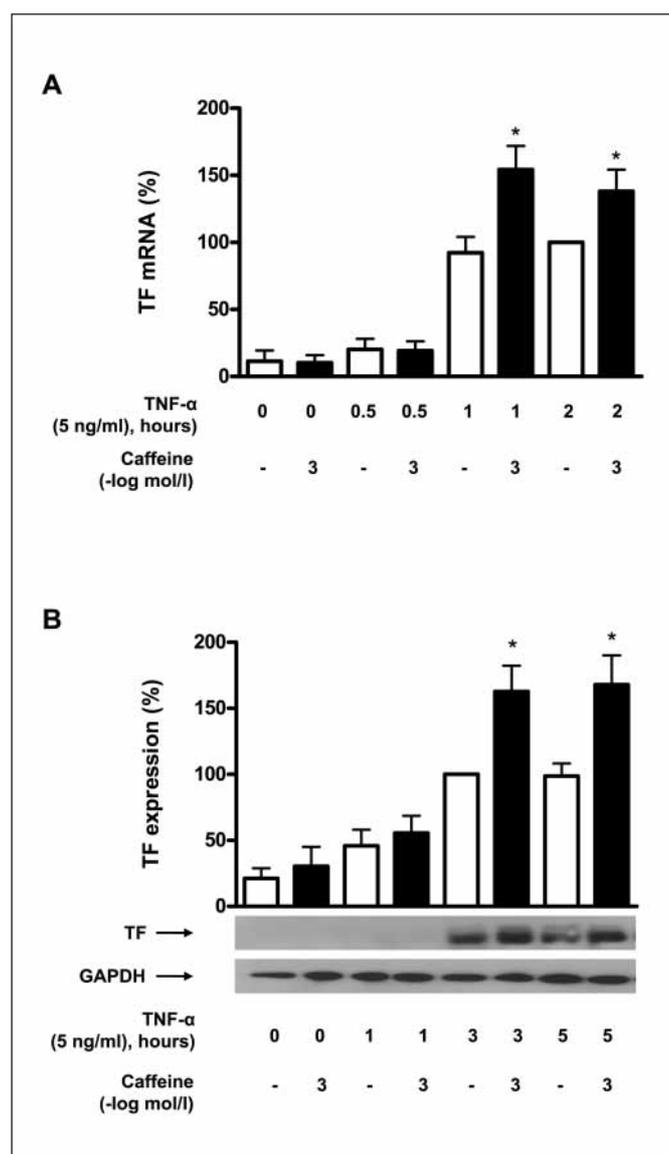
Figure 3: Caffeine increases TF mRNA and protein expression in a time-dependent manner. Caffeine enhances TNF-α induced TF mRNA expression after 1 h of stimulation (A). Values are determined by real-time PCR, indicated as percent of TNF-α alone, and normalised to L28 expression. *p<0.05 vs. TNF-α alone. Caffeine enhances TNF-α-induced TF protein expression after 3 h of stimulation (B). Values are indicated as percent of TNF-α alone. *p<0.05 vs. TNF-α alone.

meter (Berthold Technologies, Bad Wildbad, Germany). Protein concentration of the cell lysates was determined for normalisation of luciferase activity.

Statistics

Data are presented as mean ± SEM. Statistical analysis was performed by two-tailed unpaired Student's t-test or ANOVA with

post-hoc Tukey's test as appropriate. A probability value of <0.05 was considered significant.

Results

Caffeine enhances endothelial TF protein expression and activity

HAECs were stimulated with TNF-α (5 ng/ml) or thrombin (1 U/ml) for 5 h in the presence or absence of caffeine (10^{-6}–10^{-3} M). Caffeine at 10^{-4} M was calculated to correspond to 2–3 cups of coffee in a human weighing 70 kg(15, 25, 26). Caffeine enhanced TNF-α-induced TF expression in a concentration-dependent manner (n=9; p<0.05; ► Fig. 1A), with a maximal effect occurring at 10^{-3} M and resulting in a 2.3-fold induction as compared to TNF-α alone. Similarly, caffeine enhanced thrombin induced TF expression by 2.2-fold as compared to thrombin alone (n=7; p<0.05; ► Fig. 1B). The effect of caffeine on TF expression was paralleled by an increased TF surface activity which reached 1.3 times the level induced by TNF-α alone (n=6; p<0.05; ► Fig. 1C) and 1.7 times that by thrombin alone, respectively (n=5; p<0.05; ► Fig. 1D). Caffeine did not affect TF expression and TF activity under basal conditions (n=4; p=NS; ► Fig. 2A and B).

Similar to HAECs, TNF-α (5 ng/ml) and thrombin (1 U/ml) induced TF expression in THP-1 cells (n=11; p<0.05; data not shown) and vascular smooth muscle cells (VSMC) (n=4; p<0.05, data not shown). Caffeine, however, did not affect TF expression in either one of these cell types (n>4; p=NS; data not shown).

To exclude a cytotoxic effect, HAECs, VSMCs or THP-1 cells were incubated with caffeine for 5 h, and cell death was assessed by LDH release. No significant increase in LDH release of any cell type was observed for any concentration of caffeine used (n=4; p=NS; data not shown).

Caffeine does not affect TFPI expression

Treatment of TNF-α-stimulated HAECs with increasing concentrations of caffeine (10^{-6} to 10^{-3} M) did not affect TFPI expression (n=5; p=NS; ► Fig. 1E). Similarly, caffeine did not alter endothelial TFPI expression after thrombin stimulation (n=4; p=NS; ► Fig. 1F) or in unstimulated cells (n=8; p=NS; ► Fig. 2C).

Caffeine enhances TF mRNA and protein expression time-dependently

Real time-PCR revealed that treatment with caffeine (10^{-3} M) enhanced TNF-α induced TF mRNA expression in a time-dependent manner reaching a maximal induction of 1.6-fold after 1 h (n=5; p<0.05; ► Fig. 3A). Accordingly, stimulation with caffeine (10^{-3}
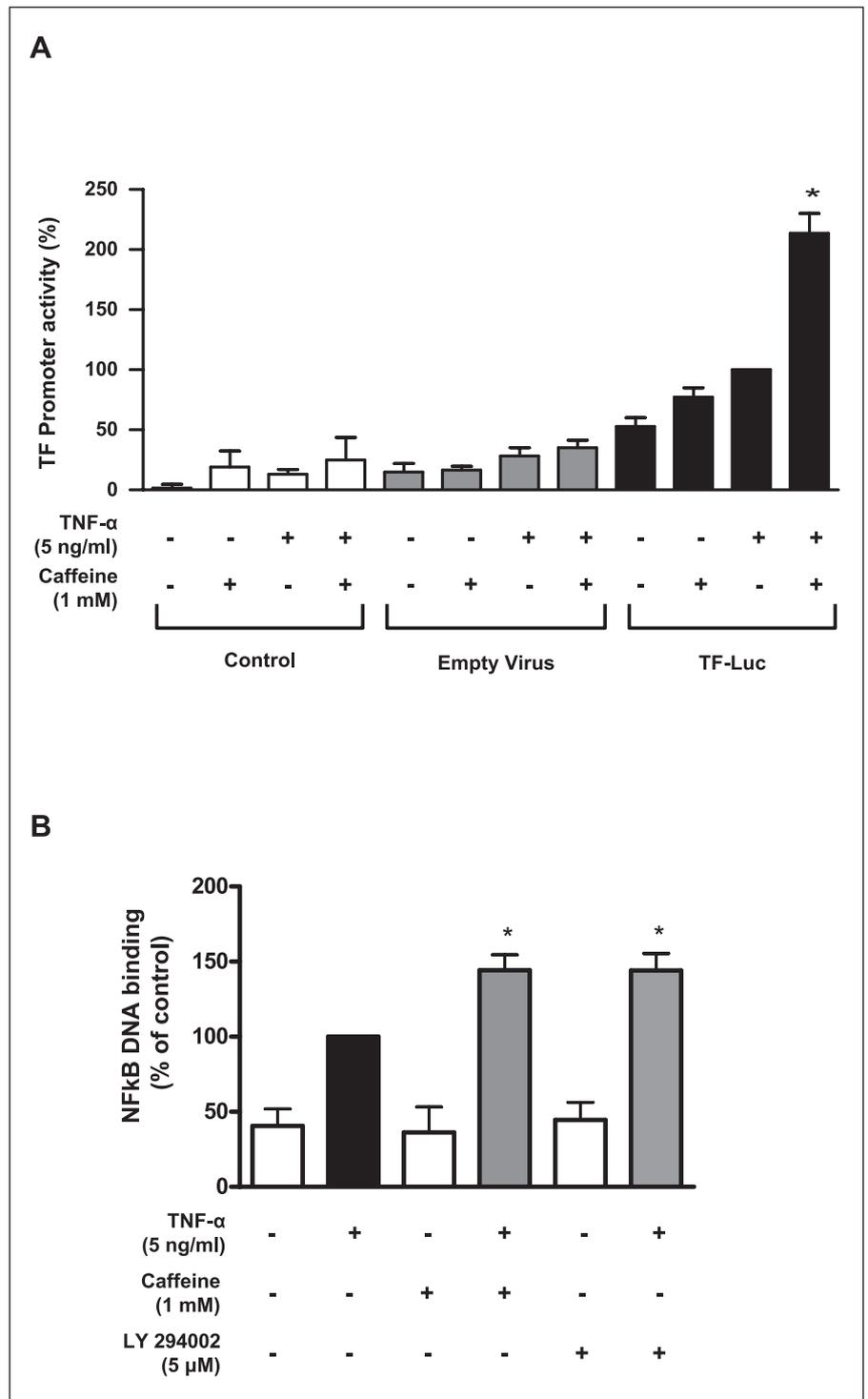
Figure 4: Caffeine enhances TF promoter activity and TNF-α-induced DNA binding of NFκB. Caffeine enhances TNF-α-induced TF promoter activation (A). Values are normalised to total protein concentration, are representative of at least four different experiments, and are indicated as percent of TNF-α alone *p<0.05 vs. TNF-α alone. Caffeine and LY294002 further enhances TNF-α-induced DNA binding of NFκB by 1.5-fold (B). Under basal condition NFκB DNA binding remains unaltered in the presence of caffeine or LY294002 (B). Values are indicated as percent of TNF-α alone. *p<0.05 vs. TNF-α alone.

M) led to a time-dependent induction of TF protein expression up to 1.7-fold the level induced by TNF-α alone; a maximal effect was observed after 5 h (n=5; p<0.05; ▶Fig. 3B). Moreover, treatment with caffeine after TNF-α enhanced TNF-α-induced TF protein expression in a time-dependent manner: the shorter the delay in caffeine administration after TNF-α stimulation, the more pronounced was its effect on TF expression; with 2 h delay TF induction was 1.8 fold the level induced by TNF-α alone after, with 4 h delay TF expression remained unaltered (n=5; p<0.05; data not shown).
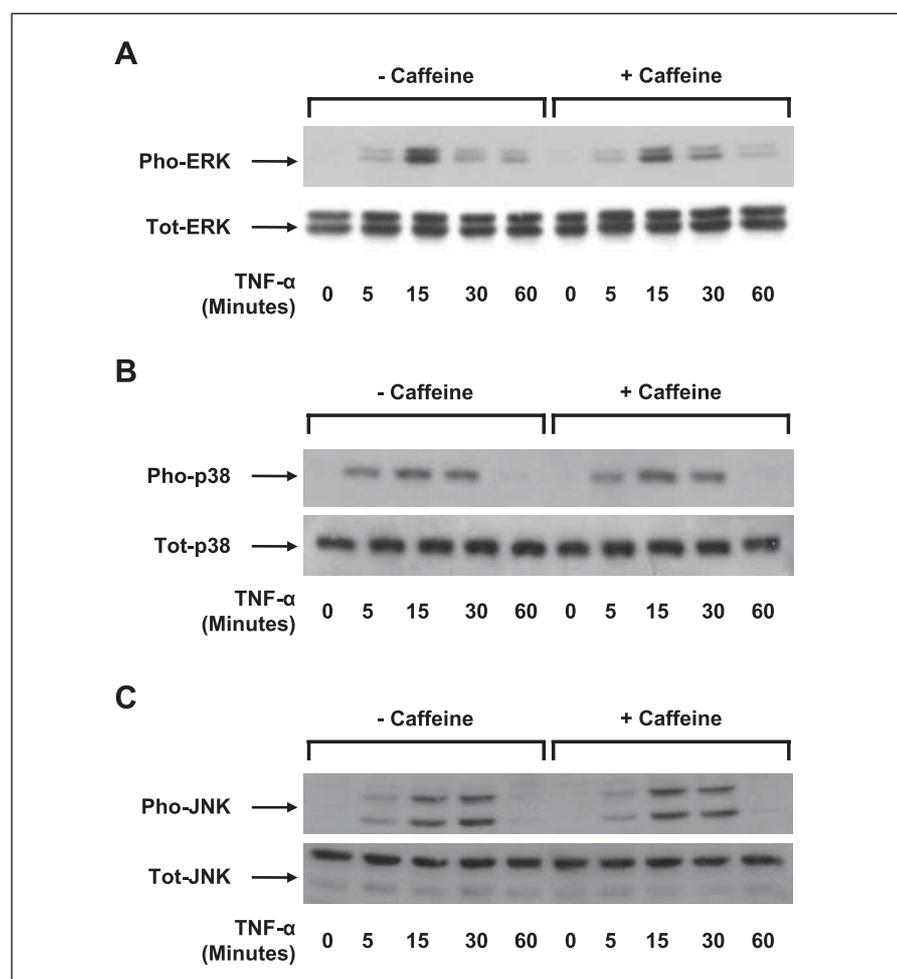
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Figure 5: Caffeine does not affect MAP kinase activation. Stimulation with TNF-α leads to transient phosphorylation (Pho) of the MAP kinases ERK (A), p38 (B), and JNK (C). Caffeine does not alter the pattern of MAP kinase activation. Total (Tot) levels of ERK, p38, and JNK remain unchanged. Values are representative of at least four different experiments.

Caffeine enhances TNF-α induced NFκB DNA binding and TF promoter activity

To assess whether the effect on TF mRNA was mediated by enhanced transcription, the impact of caffeine on TF promoter activity was analysed after transfection of HAEC with a plasmid expressing firefly luciferase under control of the human TF promoter (- 221 bp to +121 bp). Caffeine enhanced TF promoter activity by 2.1-fold as compared to TNF-α alone (n=4; p<0.05; ►Fig. 4A). Caffeine did not affect basal promoter activity (n=4; p=NS; ►Fig. 4A). Next, the effect of caffeine and LY294002 on NFκB activation in the presence and absence of TNF-α was examined. The capacity of NFκB to bind to its DNA consensus site (5′-GGGACTTTCC-3′) was determined in nuclear extracts by using a colourimetric assay. Caffeine further enhanced TNF-α-induced DNA binding of NFκB by 1.5-fold (n=4; p<0.05; ►Fig. 4B). This effect compared well to that of LY294002, which enhanced NFκB DNA binding by 1.5-fold in the presence of TNF-α (n=4; p<0.05; ►Fig. 4B). Under basal conditions, NFκB DNA binding remained unaltered in the presence of caffeine or LY294002 (n=4; p=NS; ►Fig. 4B). Nuclear translocation of NFκB, as determined by degradation of its inhibitor IκBα remained unaffected by both caffeine and LY294002 (n=3; p=NS; data not shown).

Caffeine does not alter MAPK activation

To assess whether caffeine alters MAP kinase activation, HAECs were examined at different time points after cytokine stimulation. The MAP kinases p38, ERK, and JNK were transiently activated by TNF-α (5 ng/ml; ►Fig. 5). Maximal activation of ERK, p38 and JNK was observed after 15 min. Quantitative analysis of four independent experiments revealed that this phosphorylation pattern remained unaffected by caffeine as compared to TNF-α alone (►Fig. 5, original records). Total expression of p38, ERK, and JNK remained unaltered at all time points examined (►Fig. 5).

Caffeine enhances TF expression by inhibiting PI3K

PI3K activation was determined by assaying PI3K lipid kinase activity in p85 immunoprecipitates. Caffeine (10^{-3} M) inhibited endothelial PI3K activity under both, basal and TNF-α stimulated conditions, by 72% and 68%, respectively (n=4; p<0.05; ►Fig. 6A). The effect of caffeine was less potent at lower concentrations (10^{-4} M; n=4; p<0.05 vs. control; data not shown). This effect of caffeine on
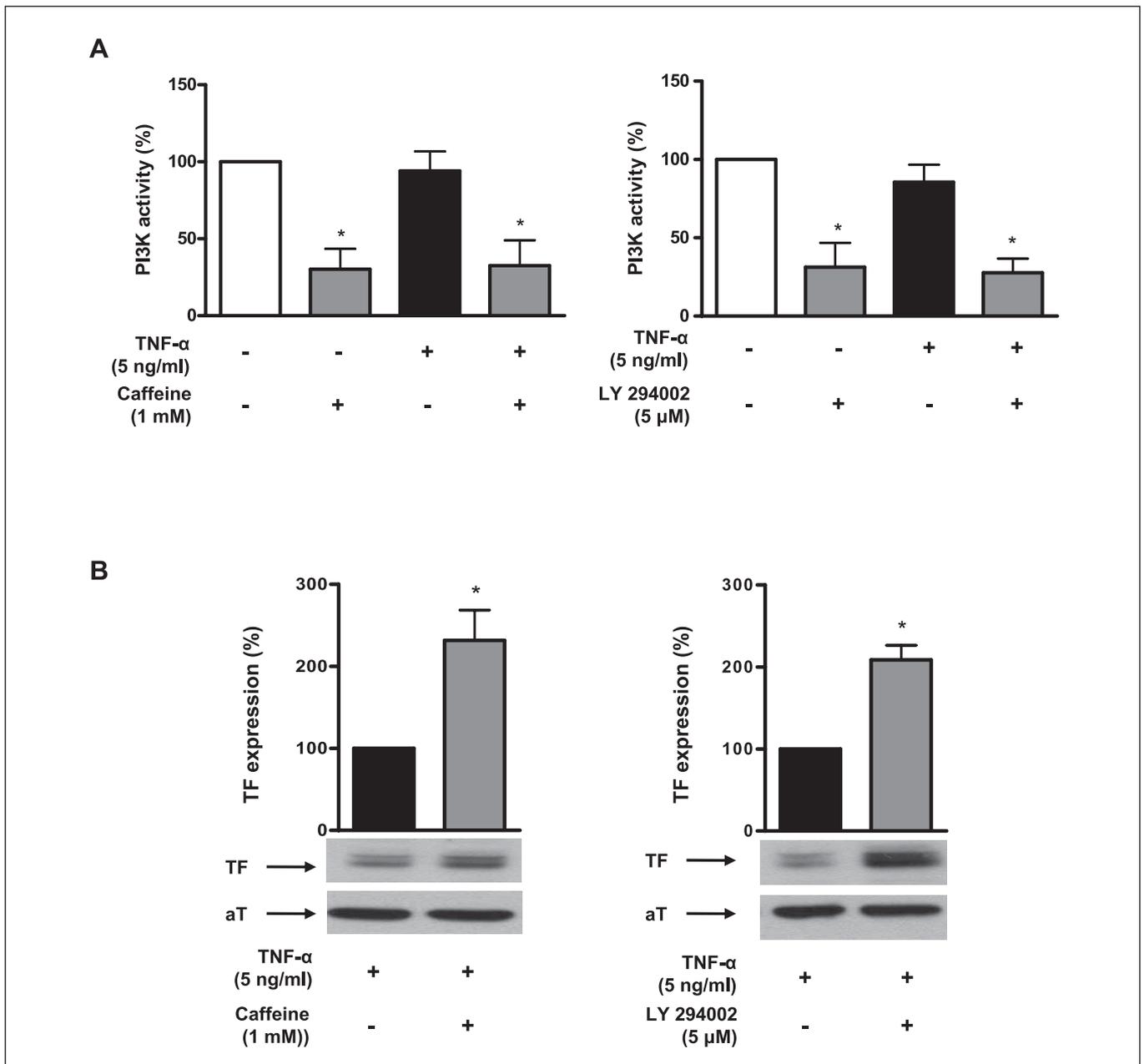
Figure 6: Caffeine inhibits PI3K activity. Caffeine (10^{-3} M) inhibits endothelial PI3K activity under basal conditions and in the presence of TNF-α (A, left panel). LY294002 (5×10^{-6} M) inhibits endothelial PI3K activity under basal conditions and in the presence of TNF-α (A, right panel). Values are determined by lipid kinase activity in p85 immunoprecipitates, are representative of at least four different experiments, and are indicated as percent of unstimulated controls. *$p < 0.05$ vs. unstimulated control. Caffeine enhances TNF-α-induced TF protein expression by 2.3-fold (B, left panel). This effect compares well to that of the PI3K inhibitor LY294002 which enhances TF expression by 2.1-fold (B, right panel). Values are indicated as percent of TNF-α alone and are representative of at least seven different experiments; all blots are normalised to α-Tubulin (aT) expression. *$p < 0.05$ vs. TNF-α alone.

PI3K was similar to that of the well known PI3K inhibitor LY294002 which inhibited PI3K activity by 69% under basal conditions and by 74% in TNF-α stimulated cells (n=4; p<0.05; ▶Fig. 6A right panel). When HAECs were treated with LY294002 before stimulation with TNF-α, TF expression was enhanced by 2.1-fold (n=7; p<0.05; ▶Fig. 6B right panel) as compared to TNF-α alone. These effects compared well to that of caffeine, which enhanced TF expression by 2.3-fold (n=9; p<0.05; ▶Fig. 6B left panel). Adenoviral overexpression of a constitutively active PI3K (rCD2-p110α) reversed the effect of caffeine (n=6; p<0.05 vs. control virus; ▶Fig. 7A) and LY294002 (n=6; p<0.05 vs. control virus; ▶Fig. 7B) on TF expression in TNF-α stimulated HAECs.

The effect of caffeine on TF expression is not mediated via adenosine receptors

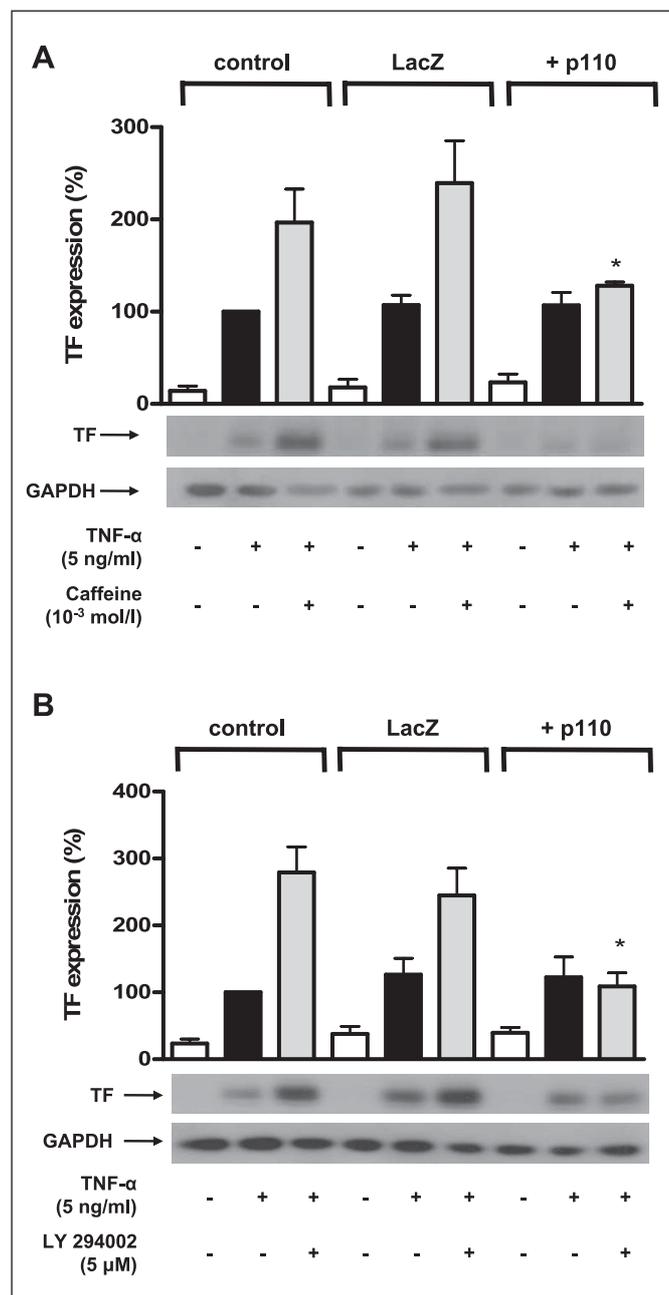Caffeine is a non-selective adenosine receptor blocker. Hence, the effect of the adenosine A1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and the A2a receptor antagonist 3,7-dimethyl-1-(2-propynyl) xanthine (DMPX) on TF expression was assessed. Pre-treatment with increasing concentrations of DPCPX or DMPX did not alter TNF-α-induced TF protein expression (n=4; p>0.2 for DPCPX or DMPX vs. % of the respective value for TNF-α alone, data not shown).

Discussion

This study demonstrates that caffeine increases endothelial TF expression and activity induced by inflammatory and procoagulant mediators and accelerates thrombus formation *in vivo*. This effect occurs at a transcriptional level and is mediated via PI3K inhibition leading to an increase of p65/NFκB DNA binding activity and induction of the TF promoter.

The estimated overall caffeine intake is 200–250 mg/person per day in the USA and Canada, and 300–400 mg/person per day in Northern Europe and Great Britain (26, 27). The caffeine concentration applied for *in vivo* experiments (10 mg/kg) and the dose used for *in vitro* experiments (10^{-4} M) correspond to intake of 250 mg caffeine or 2–3 cups of coffee by a human weighing 70 kg (15, 18–20, 25, 26); hence the caffeine concentrations applied in the presented experiments are physiologically relevant. This interpretation is in line with the observation that caffeine exerted its effect independent of whether it was added before or after TNF-α.

TNF-α and thrombin are both well-known inducers of endothelial TF expression. Caffeine enhanced TNF-α- and thrombin-mediated TF expression with similar potency and may thus increase TF expression under both inflammatory and prothrombotic conditions. Biologically active TF is located at the cell surface, and the effect of caffeine on TF expression was indeed paralleled by an increased TF surface activity. The latter, however, was less pronounced than that observed for protein expression, which may be related to the presence of encrypted TF or the distribution of TF in different cellular compartments. TF activity is inhibited by TFPI, and the balance between these factors is essential for vascular homeostasis (28). Caffeine did not affect endothelial TFPI expression under basal and stimulated conditions and in the presence of TNF-α or thrombin. Hence, our data indicate that the effect of caffeine on TF expression is not attenuated by a concomitant increase in TFPI expression and is specific for TF.

PI3Ks are key players in various inflammatory, autoimmune, and allergic processes. The inhibitory role of PI3K on TF expression is well documented, as inhibition of these kinases enhances TF protein expression in response to different mediators (17). PI3Ks are divided into three distinct classes based on their primary structure. The class I enzymes are further divided into α, β, γ, and δ isoforms based on their distinct p110 catalytic subunits and modes of regulation. Selective targeting of PI3K isoform p110β and p110γ has been proposed as an antithrombotic strategy and isoform-selective p110 inhibitors are currently developed by pharmaceutical companies (19, 20). Caffeine is known to inhibit the *in vitro* activity of PI3K with the most potent effect seen against

Figure 7: The effect of caffeine on TF expression is reversed by a constitutively active PI3K mutant. Adenoviral expression of the constitutively active PI3K mutant rCD2-p110 reverses the effect of caffeine on TF expression in TNF-α-stimulated HAECs (A). Adenoviral expression of the constitutively active PI3K mutant rCD2-p110 reverses the effect of LY294002 on TF expression in TNF-α-stimulated HAECs (B). Values are indicated as percent of TNF-α alone and are representative of at least four different experiments; all blots are normalised to GAPDH expression. *p<0.05 vs. TNF-α alone.

p110δ (14). The present study confirms that caffeine inhibited PI3K activity in endothelial cells and demonstrates that inhibition of PI3K by caffeine enhanced TF promoter activity leading to an increase in TF mRNA and protein expression. This effect was reversed by over-expression of a constitutively active PI3K p110α mutant in endothelial cells and compared well to that of the classic unspecific PI3K inhibitor LY294002. Hence, these observations suggest that caffeine may exert prothrombotic actions by inhibition of PI3K, particularly that of p110α isoform, at concentrations that are within the pharmacological and physiological range.

Extracellular adenosine levels vary within tissues and with different degrees of cellular stress; its plasma levels increase up to the micromolar range as a consequence of vascular inflammation and tissue ischaemia (29–32). Caffeine antagonises adenosine receptors in the cardiovascular system (33–35), and the effect of caffeine on TF protein is consistent with the observation that adenosine released from human umbilical vein endothelial cells inhibits TF expression in an autocrine or paracrine manner, and that adenosine receptor antagonists upregulate TF expression (29, 36). In our study, blocking either group of receptors did not alter endothelial TF expression, suggesting that the effect of caffeine does not depend on adenosine receptor inhibition under our experimental conditions. Thus, it is likely that the xanthin directly inhibits the PI3 kinase pathway.

Previous studies indicate that pharmacologic inhibition of PI3K enhances LPS-induced IκBα degradation and nuclear translocation of NFκB. However, PI3K has been shown to act both positively and negatively on NFκB-dependent gene expression, and exactly how the PI3K pathway regulates nuclear translocation of NFκB remains unclear (37, 38). We observed here that inhibition of PI3K did not affect degradation of IκBα in endothelial cells. However, we found a caffeine dependent increase in NFκB DNA binding capacity, and this effect was again congruent to that of LY294002.

Hence, these observations suggest that inhibition of PI3K by caffeine enhances nuclear activity of NFκB leading to an increase in TF promoter activity. Interestingly, the effect of caffeine on NFκB activation occurred only under inflammatory or prothrombotic conditions which supports the interpretation that caffeine requires additional stimuli to display its full effect on TF. Since caffeine consumption has been linked to an increased risk of cardiovascular events in some, but not all prospective studies (6, 8, 9), with some results supporting no association between coffee intake and cardiovascular disease (39) or even beneficial effects of caffeine on cardiovascular morbidity and mortality (7), this mechanism of action may explain, at least to some extent, the ongoing controversy.

MAP kinase activation mediates endothelial TF expression by promoting transcription in response to many stimuli (4, 22) and earlier studies have shown that MAPK activation is required for PI3K-dependent TF activation. Accordingly, p38, ERK, and JNK were transiently activated by TNF-α. Caffeine, however, did not af-

What is known about this topic?

- Tissue factor (TF) is the key activator of coagulation and plays a central role in initiation and propagation of thrombosis. Its expression is upregulated by inflammatory mediators such as tumour necrosis factor-α and can be detected in different cell types of the atherosclerotic plaque.
- Caffeine is often reported to increase cardiovascular risk; however, its effect on cardiovascular morbidity and mortality is controversial. Caffeine is a direct inhibitor of the phosphatidylinositol 3-kinase (PI3K) pathway
- PI3K negatively regulates TF expression and thrombus formation. Targeting PI3K activity in various inflammatory conditions has become an active area of investigation.

What does this paper add?

- Caffeine induces TF expression, thereby enhancing the prothrombotic potential of endothelial cells.
- Inhibition of PI3K signalling mediates caffeine induced TF expression leading to activation of nuclear factor κB and upregulation of TF.
- Caffeine concentrations applied in the present study are within the plasma range measured in humans. Caffeine intake may promote arterial thrombosis and its consumption should be controlled in patients at risk of cardiovascular complications.

fect the pattern of MAPK activation, indicating that the effect of caffeine on endothelial TF expression occurs independently of MAPK activation.

Caffeine did not enhance TNF-α-induced TF expression in THP-1 cells or VSMC, indicating that the effect of caffeine on TF is cell specific, and supporting the interpretation that caffeine specifically exerts its action on endothelial TF expression via the PI3K pathway; indeed, the latter does not exert any negative feedback on TF expression in VSMC or THP-1 cells (4).

Overall, our findings provide further evidence for a prothrombotic potential of caffeine, by its ability to directly inhibit phosphoinosite metabolism and thereby potentiate endothelial TF expression at sites of inflammation. Moreover, our study underlines the importance of the PI3K system in modulating cardiovascular disease. Since the effect of caffeine was reversed by over-expression of a constitutively active PI3K p110α mutant, however, the present data question the overall beneficial therapeutic effects of isoform selective p110 inhibitors, which have recently been proposed as a novel antithrombotic strategy. Further studies are needed to understand the mechanism by which PI3Ks are exerting their effects and to clarify the role of the selective PI3K inhibitors in specific inflammatory conditions.

Conflicts of interest
None declared.

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