European Heart Journal (2010) **31**, 1780–1791
doi:10.1093/eurheartj/ehp598

Amphetamines induce tissue factor and impair tissue factor pathway inhibitor: role of dopamine receptor type 4

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Received 29 March 2009; revised 1 December 2009; accepted 17 December 2009; online publish-ahead-of-print 29 January 2010

This paper was guest edited by Filippo Crea, Institute of Cardiology, Catholic University of the Sacred Heart, Rome, Italy

Aims Amphetamine intake is associated with acute vascular syndromes. Since these events are caused by arterial thrombosis and this in turn is triggered by tissue factor (TF), this study examines whether amphetamines regulate TF in human endothelial cells.

Methods and results Amphetamine (10^{-7}–10^{-4} mol/L) enhanced thrombin- and tumour necrosis factor (TNF)-α-induced as well as basal TF expression ($P = 0.029$, 0.0003, and 0.003 at maximal concentration), and TNF-α-induced plasminogen activator inhibitor (PAI)-1 expression ($P = 0.003$), whereas tissue factor pathway inhibitor expression was impaired ($P = 0.008$). Similarly, 3,4-methylenedioxymethamphetamine (10^{-7}–10^{-4} mol/L) enhanced TF expression ($P = 0.046$). These effects were paralleled by an increased TF activity ($P = 0.002$); moreover, clotting time of human plasma was accelerated by supernatant from amphetamine-treated cells ($P = 0.03$). Amphetamine enhanced TF mRNA expression via phosphorylation of the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK) and p38 ($P = 0.03$ and 0.033), but not c-Jun NH_2-terminal kinase (JNK; $P = 0.81$). The effect of amphetamine on TF expression was abrogated by the dopamine D4 receptor antagonists L-745,870 and L-750,667, but not D2 or D3 receptor antagonists; furthermore, L-745,870 blunted the amphetamine-induced activation of ERK and p38, but not JNK.

Conclusion Amphetamines induce endothelial TF expression via stimulation of dopamine D4 receptor and activation of the MAPKs p38 and ERK. These effects occur at clinically relevant amphetamine concentrations and may account for the increased incidence of acute vascular syndromes after amphetamine consumption.

Keywords Acute vascular syndromes • Tissue factor • Amphetamine • Dopamine receptor

Introduction

In the Western civilization, intake of psychostimulants such as amphetamine and 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy') has risen dramatically over the past decade; as a consequence, this phenomenon has now turned into a major public burden.[1] The problem is aggravated by the fact that the majority of consumers are young (≤45 years) and thus often faced with unexpected acute cardiovascular events.[2] Indeed, numerous publications report amphetamine-associated acute vascular syndromes including myocardial infarction and ischaemic stroke.[3–8]

The pathogenesis of amphetamine-related acute cardiovascular events is still uncertain. Vasospasm and prothrombotic state have been postulated as aetiological factors, but no solid evidence is available to support these assumptions.[3,4,9] The combination of vasospasm and thrombosis does indeed seem to play a role in coronary artery occlusion, indicating a dynamic interaction of vasoconstriction and thrombus formation in acute vascular syndromes.[10] Further,

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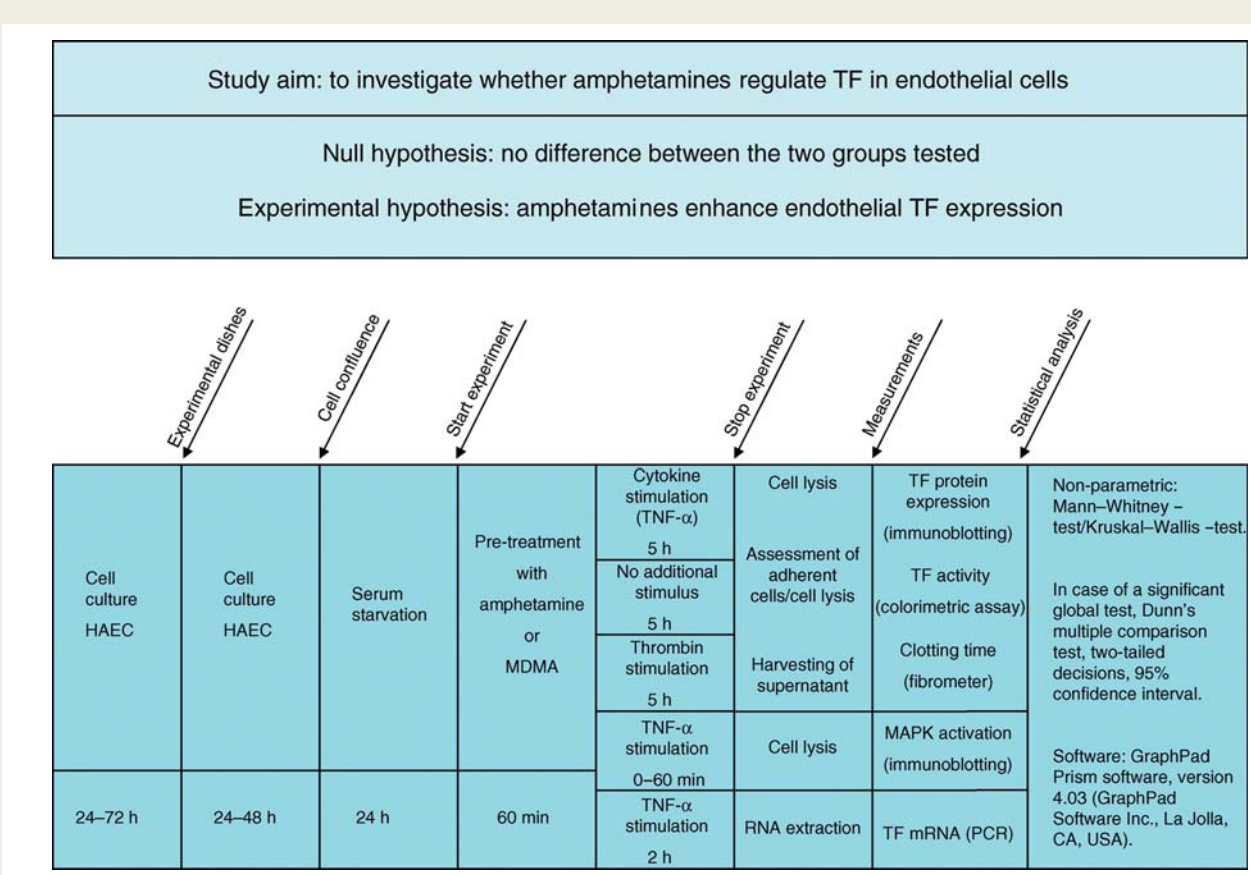

Figure 1 Study conduct of the *in vitro* experiments.

substances suspected to promote vasospasm, such as histamine and cocaine, are known to induce the endothelial expression of tissue factor (TF).[11,12] Tissue factor is the main trigger of coagulation and crucially involved in arterial thrombus formation, the central event in acute vascular syndromes.[13] In this context, it is important to note that amphetamines are known to activate mitogen-activated protein kinases (MAPKs), the major regulators of endothelial TF expression, in neuronal cells.[14] On the basis of these facts, we hypothesized that amphetamine may induce TF expression. Hence, this study was designed to examine the effect of amphetamines on TF expression in human vascular endothelial cells.

Methods

Cell culture

Human aortic endothelial cells (HAECs; Clonetics, Allschwil, Switzerland) were cultured as described.[15] Cells were grown to confluence and were then serum withdrawn for 24 h by using EBM medium (Cambrex) supplemented with 0.5% FCS, before stimulation with 5 ng/mL tumour necrosis factor (TNF)-α (R&D, Minneapolis, MN, USA) or 1 U/mL thrombin (Sigma-Aldrich, Buchs, Switzerland) (*Figure 1*). Cells were pre-treated with amphetamine (Lipomed, Arlesheim, Switzerland) or MDMA (Sigma-Aldrich) for 60 min before stimulation with TNF-α. Dopamine, L-750,667, L-745,870, raclopride (all from Sigma-Aldrich), and NGB 2904 (Tocris Bioscience, Ellisville, MO, USA) were added to the dishes 60 min before stimulation (*Figure 1*). Cytotoxicity was assessed with a colorimetric assay to detect lactate dehydrogenase (LDH) release (Roche, Basel, Switzerland). The use of MDMA in this study was approved by the Swiss Health Authority (reference: AB-8/5-BetmG-07.001315).

Western blot

Protein expression was determined by western blot analysis as described.[16] Antibodies against human TF and tissue factor pathway inhibitor (TFPI; both from American Diagnostica, Stamford, CT, USA) were used at 1:2000 dilution. Antibody against plasminogen activator inhibitor (PAI-1; Santa Cruz) was applied at 1:4000 dilution. Antibodies against phosphorylated p38 MAPK (p38), p44/42 MAPK [extracellular signal-regulated kinase (ERK)], and c-Jun NH_2-terminal kinase (JNK; all from Cell Signaling, Danvers, MA, USA) were used at 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total p38, ERK, and JNK (all from Cell Signaling) were used at 1:3000, 1:2000, and 1:1000 dilution, respectively. Antibodies against Thr-34 and Thr-75 phosphorylated as well as against total dopamine-and-cAMP-regulated phosphoprotein 32 (DARPP-32) (Cell Signaling) were used at 1:1000 dilution. Anti-goat glyceraldehyde-3-phosphate dehydrogenase antibody (Chemicon, Temecula, CA, USA) was applied to ensure equal protein loading (1:20 000 dilution). Proteins were detected with a horseradish peroxidase-linked secondary antibody (Amersham, Munich, Germany).

Real-time polymerase chain reaction

Total RNA was extracted from HAEC using TRIzol Reagent (Invitrogen). Conversion of total cellular RNA to cDNA was carried out

with Moloney murine leukaemia virus reverse transcriptase and random hexamers (Amersham Bioscience) in a final volume of 33 μL using 4 μg of cDNA. All real-time polymerase chain reaction (PCR) experiments were performed in triplicate using the SYBR Green JumpStart kit (Sigma, Saint Louis, MO, USA) in an MX3000P PCR cycler (Stratagene, Amsterdam, The Netherlands). Each reaction (25 μL) contained 2 μL cDNA, 10 pmol of each primer, 0.25 μL of internal reference dye, and 12.5 μL of JumpStart *Taq* ReadyMix (containing buffer, dNTPs, stabilizers, SYBR Green, *Taq* polymerase, and JumpStart *Taq* antibody). The following primers were used: for TF: sense: 5′-TCCCCAGAGTTCACACCTTACC-3′ (bases 508–529 of F3 cDNA; NCBI no. NM 001993), antisense: 5′-CCTTTCTCC TGGCCCATACAC-3′ (bases 843–863 of TF cDNA; NCBI no. NM 001993); for human D4 dopamine receptor: sense: 5′-CCCACCCC AGACTCCACC-3′ (bases 963–980 of DRD4 cDNA; NCBI no. NM 000797), antisense: 5′-GAACTCGGCGTTGAAGACAG-3′ (bases 1202–1221 of DRD4 cDNA; NCBI no. 000797); and for human L28: sense: 5′-GCATCTGCAATGGATGGT-3′, antisense: 5′-CCTT TCTCCTGGCCCATACAC-3′. The amplification programme consisted of 1 cycle at 95°C for 10 min, followed by 35 cycles with a denaturing phase at 95°C for 30 s, an annealing phase at 60°C for 1 min, and an elongation phase at 72°C for 1 min. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. Polymerase chain reaction products were also analysed on an ethidium bromide-stained 1.5% agarose gel. In each real-time PCR run, a calibration curve was included that was generated from serial dilutions of the respective purified amplicons. Tissue factor mRNA stability was assessed in the presence of the transcriptional inhibitor actinomycin D (10 μg/mL, Sigma-Aldrich), which was added 2 h after TNF-α stimulation. Cells were harvested for RNA extraction at various time points (0–3 h) following actinomycin D treatment. The copy numbers for TF obtained in the real-time PCR analysis were normalized to those of L28, and the values are presented as per cent of the TNF-α alone group.

Gene silencing through siRNA transfection

Three siRNA sequences specific for the human dopamine receptor 4 (*D4*) were applied for knocking down *D4* expression using the N-TER™ Nanoparticle siRNA Transfection System (Sigma). These siRNA sequences were as follows: siRNA 1 (sense: 5′-CCGCCUCC AUCUUCAACCUdTdT-3′; antisense: 5′-AGGUUGAAGAUGGAGG CGGdTdT-3′); siRNA 2 (sense: 5′-CCCUCAUGGCCAUGGACGU dTdT-3′; antisense: 5′-ACGUCCAUGGCCAUGAGGGdTdT-3′); and siRNA 3 (sense: 5′-GCCUCCAUCUUCAACCUGUdTdT-3′; antisense: 5′-ACAGGUUGAAGAUGGAGGCdTdT-3′). Briefly, cells were transfected with a 15 nM siRNA Nanoparticle Forming Solution and incubated with transfection serum-free medium for the following 4 h at 37°C. Subsequently, cells were cultured in the serum-containing growth medium for 24–48 h at 37°C.

Tissue factor activity

Tissue factor activity was analysed at the luminal surface of adherent endothelial cells and in whole-cell lysates of HAEC using a colorimetric assay (American Diagnostica). TF/FVIIa converted factor X to factor Xa, which was measured by its ability to metabolize a chromogenic substrate. A standard curve with lipidated human TF was performed to assure that measurements were taken in the linear range of detection.

Clotting time

Clotting time was assessed using a Start fibrometer. Fifty microlitres of supernatant from HAECs were harvested and incubated with 50 μL of citrated human plasma for 1 min. Coagulation was initiated by the addition of 25 mM calcium chloride followed by analysis of clotting time.

Statistics

Differences between mean data were compared by the non-parametric Mann–Whitney test and Jonckheere–Terpstra test, respectively, for non-Gaussian variables; however, due to the low number of experiments, those tests must not refer to the usual normal approximation. In case of a significant global test (more than two groups), Dunn's multiple comparison test was used for pairwise group comparison. All statistical decisions were made two-tailed with a 95% confidence interval. Data are reported as mean ± SEM. All tests used exact probability distributions. A two-tailed *P*-value of 0.05 was chosen as cut-off for statistical significance at hypothesis testing. Statistical analyses were performed using GraphPad Prism software, version 4.03 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Amphetamines enhance tissue factor protein expression and activity

Human aortic endothelial cells were stimulated with TNF-α (5 ng/mL) or thrombin (1 U/mL) for 5 h in the presence or absence of amphetamines (*Figure 1*). Amphetamine (10^{-7}–10^{-4} mol/L) enhanced TNF-α-induced TF expression in a concentration-dependent manner reaching a 1.8-fold induction ($n = 7$; $P = 0.0003$; Mann–Whitney test; *Figure 2A*; *Table 1*). Similarly, amphetamine enhanced thrombin-induced TF expression by 1.6-fold ($n = 4$; $P = 0.029$; Mann–Whitney test; *Figure 2C*; *Table 1*) and basal TF expression by 2.2-fold ($n = 7$; $P = 0.003$; Mann–Whitney test; *Figure 2D*; *Table 1*). Similar to amphetamine, MDMA (10^{-7}–10^{-4} mol/L) enhanced TF expression by 1.7-fold at maximum concentration when compared with TNF-α alone ($n = 4$; $P = 0.046$; Mann–Whitney test; *Figure 2B*; *Table 1*). The effect of amphetamine and MDMA on TF protein expression was paralleled by an increased TF surface activity both in TNF-α-stimulated cells ($n = 5$–6; $P = 0.002$ and 0.008, respectively; Mann–Whitney test; *Figure 3A* and *B*; *Table 1*) and under basal conditions ($n = 6$; $P = 0.002$; Mann–Whitney test; *Figure 3D*; *Table 1*). Amphetamine induced a similar relative increase in TF surface activity and TF whole-cell activity ($n = 5$; $P = 0.004$; Mann–Whitney test; *Figure 3C*; *Table 1*).

Amphetamines are not toxic

To control for toxic effects, HAECs were treated with increasing concentrations of amphetamine or MDMA for 6 h. Morphological examination did not reveal any changes ($n = 9$; data not shown); moreover, no signs of toxicity were detected by LDH release for any concentration of amphetamine or MDMA used ($n = 9$; data not shown).

Amphetamine accelerates clotting time of human plasma

To assess the functional relevance of amphetamine-induced TF expression, the effect of supernatant from amphetamine-treated HAECs on clotting time of human plasma was assessed. Clotting time was accelerated by supernatant from cells treated with amphetamine both under basal conditions ($n = 4$; $P = 0.03$; Mann–Whitney test; Supplementary material online, *Figure S1*; *Table 1*) and after

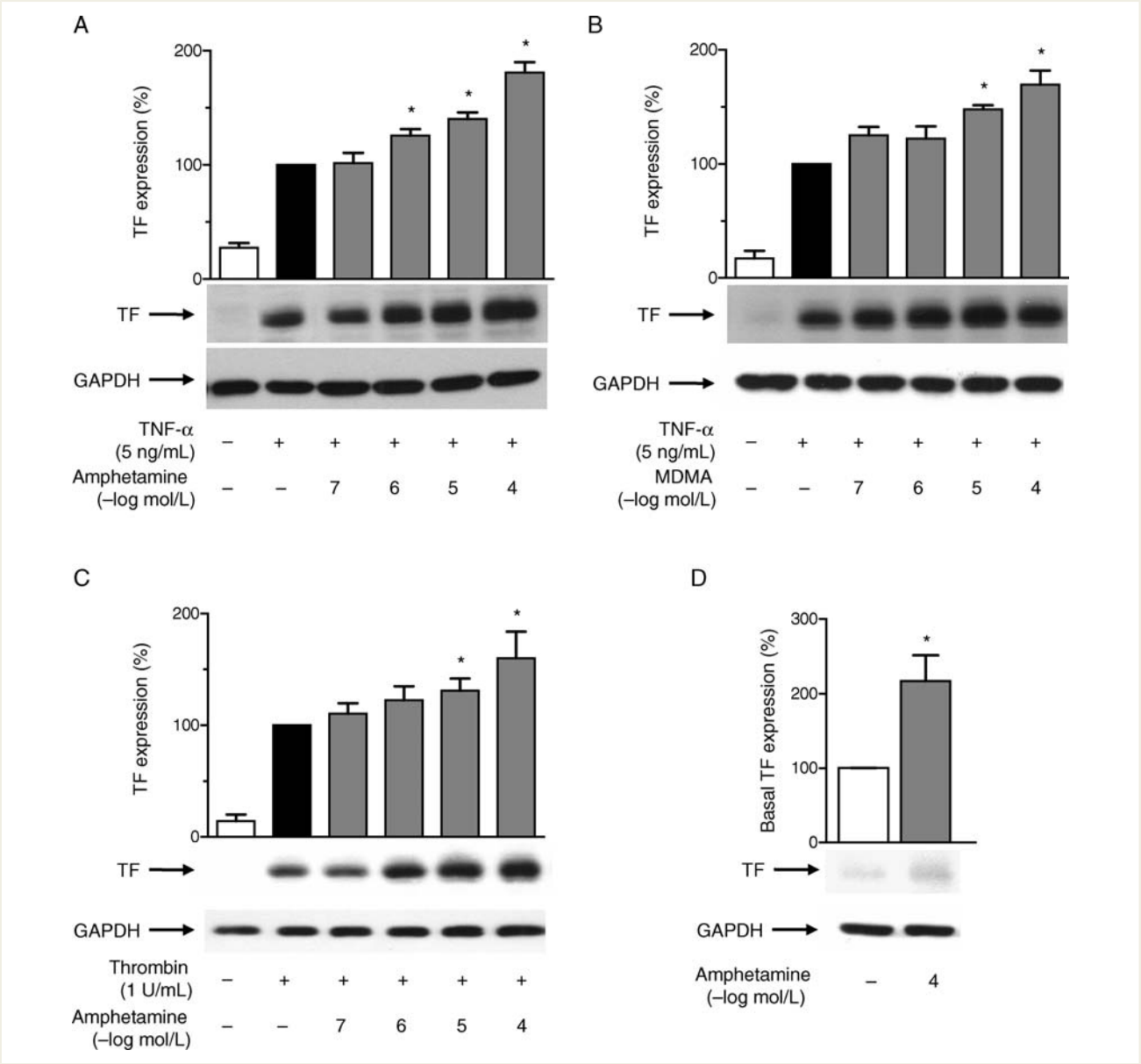

Figure 2 Amphetamines enhance endothelial tissue factor protein expression. (*A*) Amphetamine enhances tumour necrosis factor-α-induced tissue factor protein expression. *$P < 0.05$ vs. tumour necrosis factor-α alone. (*B*) 3,4-Methylenedioxymethamphetamine enhances tumour necrosis factor-α-induced tissue factor protein expression. *$P < 0.05$ vs. tumour necrosis factor-α alone. (*C*) Amphetamine enhances thrombin-induced tissue factor protein expression. *$P < 0.05$ vs. tumour necrosis factor-α alone. (*D*) Amphetamine induces basal tissue factor protein expression. *$P < 0.05$ vs. unstimulated control. All blots are normalized to glyceraldehyde-3-phosphate dehydrogenase expression.

stimulation with TNF-α ($n = 6$; $P = 0.002$; Mann–Whitney test; Supplementary material online, *Figure S1*; *Table 1*).

Amphetamine impairs tissue factor pathway inhibitor and increases plasminogen activator inhibitor-1 expression

Increasing concentrations of amphetamine (10^{-7}–10^{-4} mol/L) inhibited expression of TFPI in TNF-α- and thrombin-stimulated HAECs resulting in a 45 and 36% reduction, respectively ($n = 4$–5; $P = 0.008$ and 0.03, respectively; Mann–Whitney test; *Figure 4B* and *C*; *Table 1*). A similar effect was observed under basal conditions ($n = 4$; $P = 0.03$; Mann–Whitney test; *Figure 4A*; *Table 1*). In contrast, amphetamine (10^{-4} mol/L) increased endothelial PAI-1 expression following TNF-α stimulation ($n = 6$; $P = 0.003$; Mann–Whitney test; *Table 1*).

Amphetamine enhances tissue factor mRNA expression without affecting mRNA stability

Real-time PCR demonstrated that TF mRNA expression was induced after 2 h of TNF-α stimulation ($n = 4$; $P = 0.029$;

Table I Group comparison by the Mann–Whitney test

Parameter	Amphetamine (10^{-4} M), mean ± SEM	MDMA (10^{-4} M), mean ± SEM	Dopamine (10^{-4} M), DRA/siRNA, mean ± SEM	*n*	*P*-value of Mann–Whitney/ Jonckheere–Terpstra
TF protein expression					
TNF-α stimulation (amphetamine)	181.1 ± 9.0			7	0.0003
TNF-α stimulation (MDMA)		169.5 ± 12.5		4	0.046
Thrombin stimulation	160.1 ± 23 7			4	0.029
Basal	216.7 ± 34.6			7	0.003
TNF-α stimulation (dopamine)			215.5 ± 49.8	6	0.002
Dopamine 2R antagonist	174.0 ± 18.6		208.5 ± 42.1	4	1.0[a]
Dopamine 3R antagonist	174.0 ± 18.6		188.5 ± 42.7	4	0.46[a]
Dopamine 4R antagonist	174.0 ± 18.6		67.9 ± 20.0	5	0.002[a]
TFPI expression					
TNF-α stimulation	56.5 ± 9.3			5	0.008
Thrombin stimulation	74.6 ± 9.6			4	0.03
Basal	61.5 ± 4.7			4	0.03
PAI-1 expression (TNF-α)	207.2 ± 24.7			6	0.003
MAPK phosphorylation					
Pho-ERK	159.6 ± 9.1			4	0.03
Pho-p38	110.7 ± 16.4			7	0.033
Pho-JNK	102.1 ± 14.02			4	0.81
TF surface activity					
TNF-α stimulation (amphetamine)	159.3 ± 9.1			6	0.002
TNF-α stimulation (MDMA)		127.0 ± 5.3		5	0.008
Basal	168.0 ± 20.9			6	0.002
Whole-cell TF activity (TNF-α)	147.1 ± 10.3			5	0.004
Clotting time					
Basal	79.5 ± 5.3			4	0.03
TNF-α stimulation	85.6 ± 2.8			6	0.002
TF mRNA expression	203.6 ± 22.3			4	0.029
D4R siRNA transfection	180.4 ± 15.2		99.1 ± 19.8	5	0.03

Data are presented as mean ± SEM (per cent vs. control group). DRA, dopamine receptor antagonist.
[a]Jonckheere–Terpstra's test for multiple group comparison was applied. In case of a significant global test, Dunn's multiple comparison test was used for pairwise group comparison.

Mann–Whitney test; *Figure 5A*). Treatment with amphetamine enhanced TNF-α-induced TF mRNA expression in a concentration-dependent manner reaching a maximal induction of 2.1-fold ($n = 4$; $P = 0.032$; Mann–Whitney test; *Figure 5A*; *Table 1*). Amphetamine did not affect the stability of TF mRNA under these conditions ($n = 4$; *Figure 5B*).

Amphetamine enhances mitogen-activated protein kinase activation

To assess whether amphetamine alters MAPK activation, HAECs were examined at different time points after cytokine stimulation.

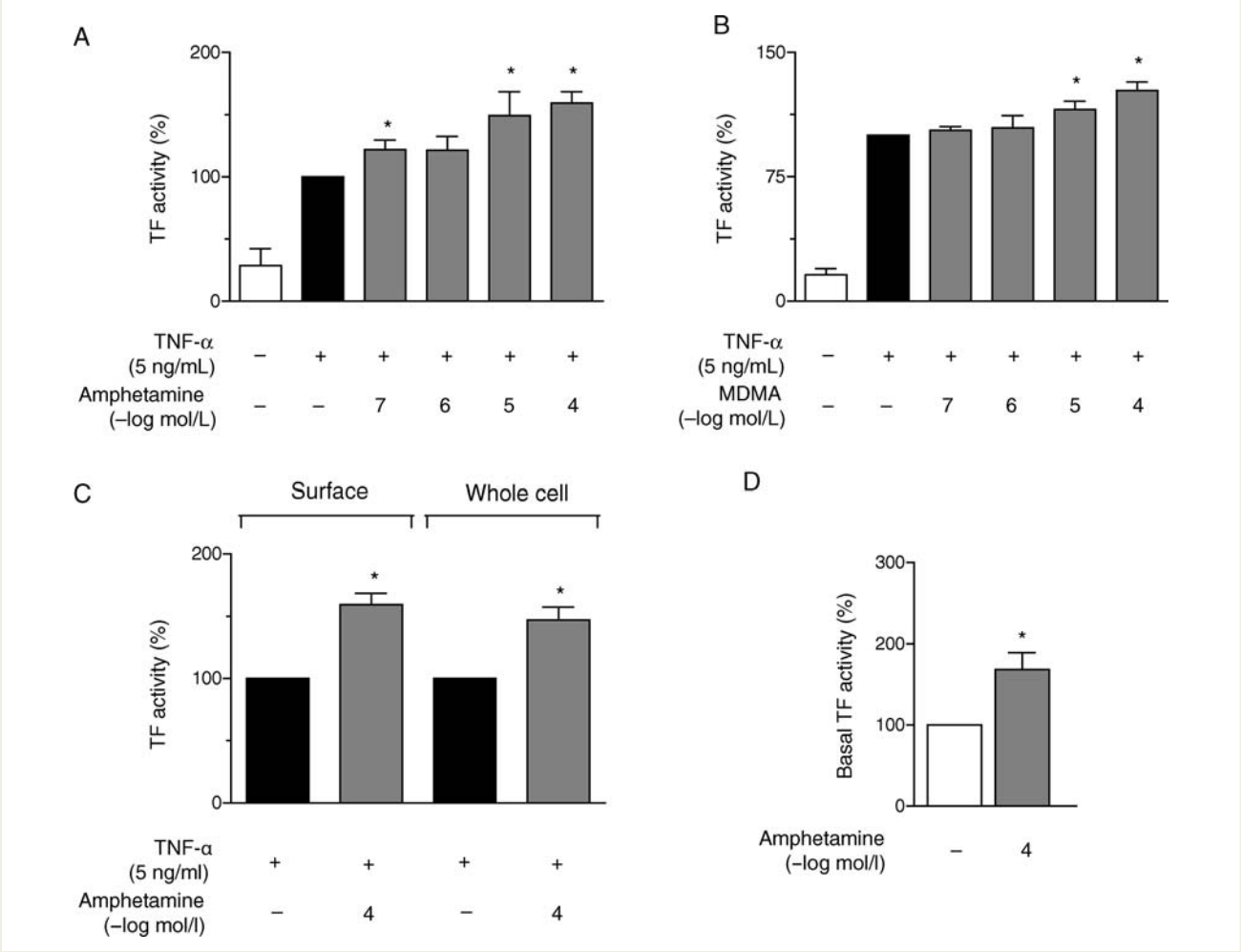

Figure 3 Amphetamine induces tissue factor activity. (*A*) Amphetamine enhances tumour necrosis factor-α-induced tissue factor surface activity. *$P < 0.05$ vs. tumour necrosis factor-α alone. (*B*) 3,4-Methylenedioxymethamphetamine enhances tumour necrosis factor-α-induced tissue factor surface activity. *$P < 0.05$ vs. tumour necrosis factor-α alone. (*C*) Amphetamine induces a similar relative increase in luminal surface activity and whole-cell activity of tissue factor. $P < 0.05$ vs. control. (*D*) Amphetamine induces basal tissue factor surface activity. *$P < 0.05$ vs. unstimulated control.

The MAPKs p38, ERK, and JNK were transiently activated by TNF-α ($n = 4$; *Figure 7*). Amphetamine enhanced phosphorylation of p38 and ERK ($n = 4–7$; $P = 0.033$ and 0.03, respectively; Mann–Whitney test; *Figure 7A* and *B*; *Table 1*), whereas that of JNK remained unaffected ($n = 4$; $P = 0.81$; Mann–Whitney test; *Figure 7A* and *B*). No significant change in total expression of MAPKs was observed at any time point with or without amphetamine. The MAPK inhibitors SB203580, PD98059, and SP600125, which specifically act on p38, ERK, and JNK, respectively, impaired TF expression after TNF-α stimulation ($n = 4$; $P < 0.05$ for TNF-α alone vs. each inhibitor; Mann–Whitney test; *Figure 7C*). Phosphorylation of DARPP-32 was not altered in the presence or absence of amphetamine (data not shown). Mitogen-activated protein kinase inhibitors were not toxic as assessed by LDH release measured 5 h after addition of TNF-α ($n = 3$; data not shown).

Dopamine D4 receptor mediates the effect of amphetamine on tissue factor

Some of the psychotropic effects of amphetamine are known to be mediated by dopamine receptors. Endothelial cells express dopamine D2, D3, and D4 receptors, and these receptors are known to modulate endothelial activation. Expression of the dopamine D4 receptor in HAECs was confirmed by PCR ($n = 5$; data not shown). Dopamine indeed enhanced TNF-α-induced TF expression in HAECs ($n = 6$; $P = 0.002$; Mann–Whitney test; *Figure 6A*; *Table 1*). To determine whether dopamine receptors are involved in amphetamine-induced TF expression, cells were pre-treated with amphetamine, the D2 receptor antagonist raclopride, the D3 receptor antagonist NGB 2904, or the D4 receptor antagonists L-745,870 and L-750,667. Pre-treatment with L-745,870 abrogated amphetamine-induced TF expression ($n = 5$; $P = 0.002$;

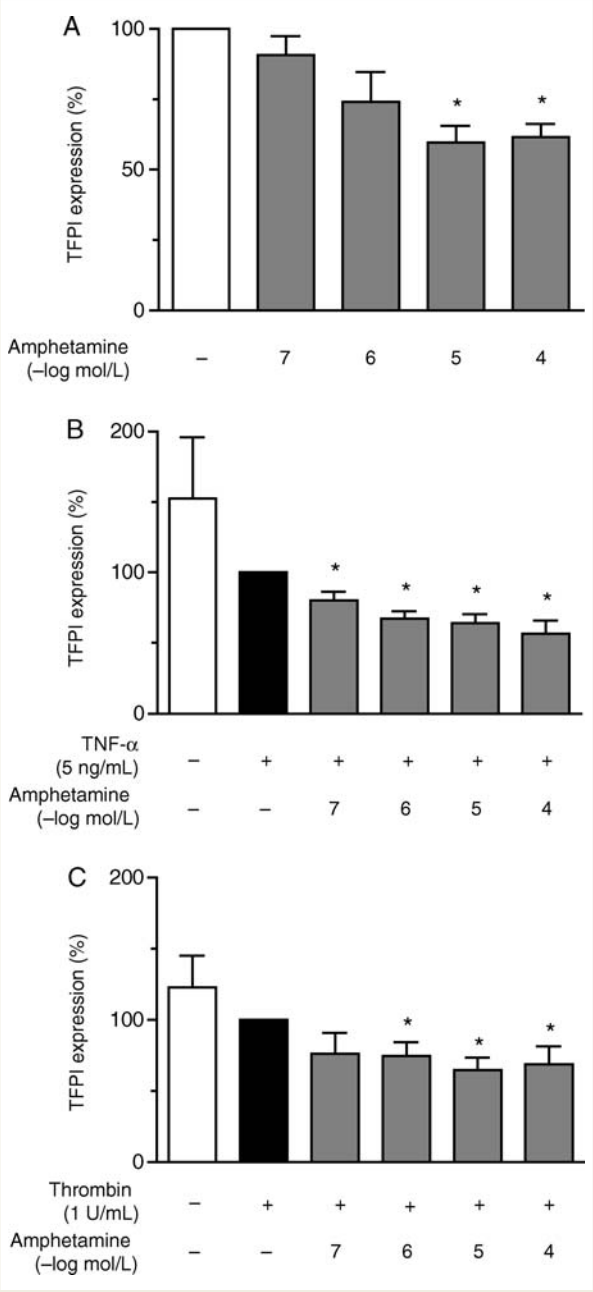

Figure 4 Amphetamine inhibits endothelial tissue factor pathway inhibitor protein expression. (*A*) Amphetamine inhibits basal tissue factor pathway inhibitor protein expression. *$P < 0.05$ vs. unstimulated control. (*B*) Amphetamine inhibits tissue factor pathway inhibitor protein expression in tumour necrosis factor-α-stimulated cells. *$P < 0.05$ vs. tumour necrosis factor-α alone. (*C*) Amphetamine inhibits tissue factor pathway inhibitor protein expression in thrombin-stimulated cells. *$P < 0.05$ vs. thrombin alone.

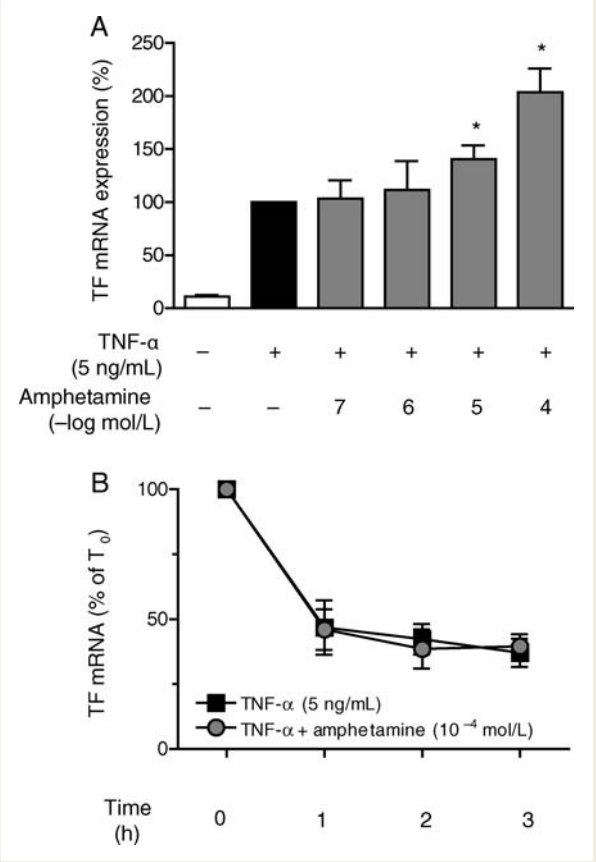

Figure 5 Amphetamine enhances endothelial tissue factor mRNA expression without affecting mRNA stability. (*A*) Amphetamine enhances tumour necrosis factor-α-induced tissue factor mRNA expression. *$P < 0.05$ vs. tumour necrosis factor-α alone. Values are determined by real-time polymerase chain reaction, indicated as per cent of tumour necrosis factor-α alone, and normalized to L28 expression. (*B*) Amphetamine (10^{-4} M) does not affect tissue factor mRNA stability. Tissue factor mRNA copies are normalized to L28 expression and expressed as per cent of tumour necrosis factor-α.

Jonckheere–Terpstra test; *Figure 6B*; *Table 1*), and a similar effect was observed with L-750,667 ($n = 3$; $P = 0.03$; Jonckheere–Terpstra test; data not shown). In contrast, the effect of amphetamine remained unaltered by pre-treatment with raclopride or NGB 2904 ($n = 4$; $P = 1.0$ and 0.46, respectively; Jonckheere–Terpstra test; *Figure 6C* and *D*; *Table 1*). When the D4 receptor gene was silenced through siRNA transfection, the effect of amphetamine on TF expression was abrogated, whereas non-targeting siRNA had no effect on amphetamine-induced TF expression ($n = 5$; $P = 0.03$ for amphetamine in non-targeting siRNA-transfected cells vs. amphetamine in D4R siRNA-transfected cells; Mann–Whitney test; *Figure 6E*). Blocking the dopamine D4 receptor with L-745,870 (10^{-5} mol/L) abrogated the amphetamine-induced phosphorylation of p38 ($n = 4$; $P = 0.03$ for amphetamine and L-745,870 vs. amphetamine alone; Mann–Whitney test; *Figure 8A*) and ERK ($n = 4$; $P = 0.03$ for amphetamine and L-745,870 vs. amphetamine alone; Mann–Whitney test; *Figure 8B*), whereas activation of JNK remained unaffected ($n = 4$; $P = 0.34$ for amphetamine and L-745,870 vs. amphetamine alone; Mann–Whitney test;

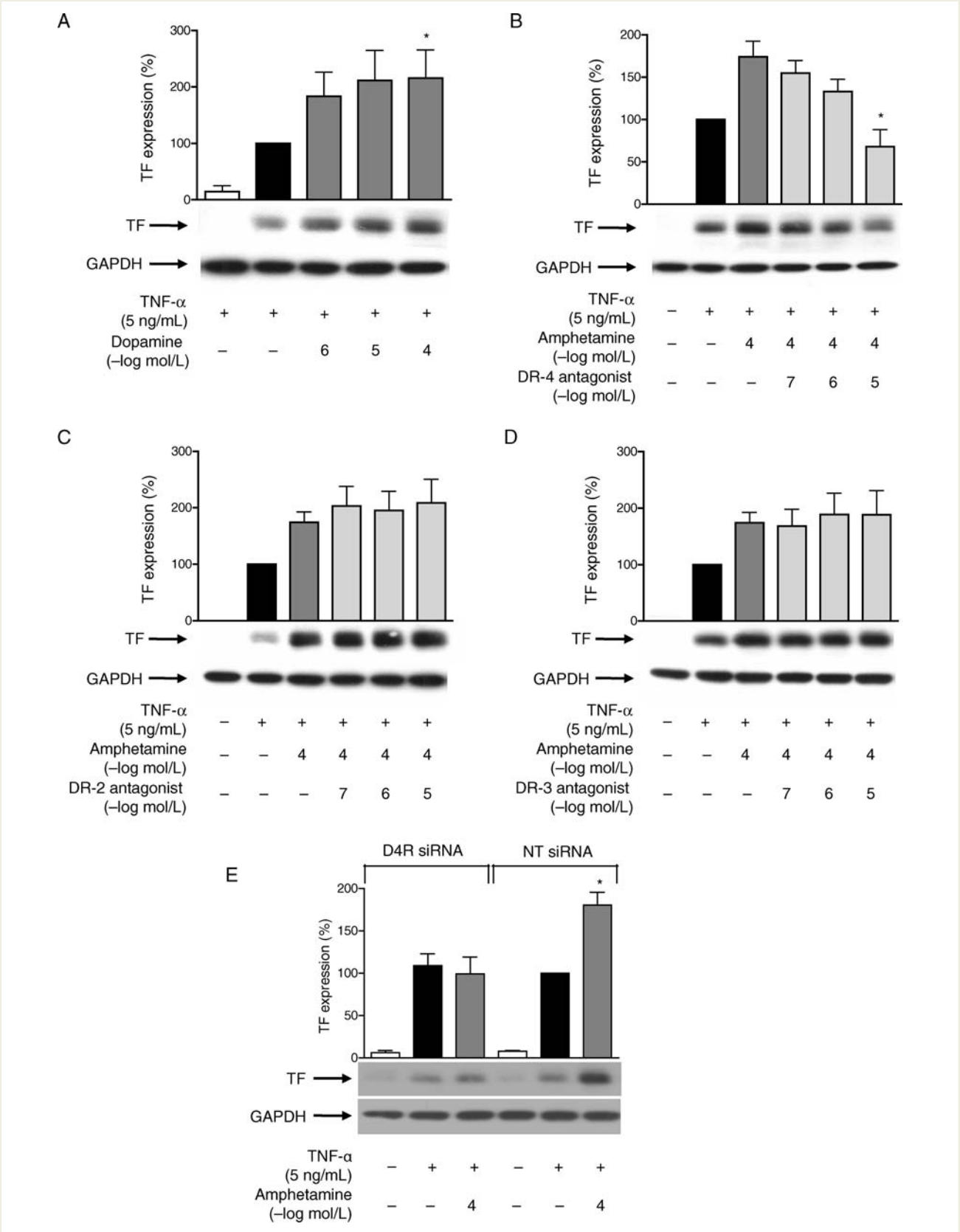

Figure 6 Amphetamine enhances endothelial tissue factor expression via dopamine D4 receptor. (*A*) Dopamine enhances tumour necrosis factor-α-induced endothelial tissue factor expression. *$P < 0.05$ vs. tumour necrosis factor-α alone. (*B*) The dopamine D4 receptor antagonist L-745,870 abrogates amphetamine-induced tissue factor expression. *$P < 0.05$ vs. amphetamine alone. (*C*) The effect of amphetamine remains unaltered by the D2 receptor antagonist raclopride. (*D*) The effect of amphetamine remains unaltered by the D3 receptor antagonist NGB 2904. (*E*) Amphetamine-enhanced tissue factor expression is abrogated when D4 receptor gene is silenced through siRNA transfection. *$P < 0.05$ vs. vs. amphetamine in D4R siRNA-transfected cells.

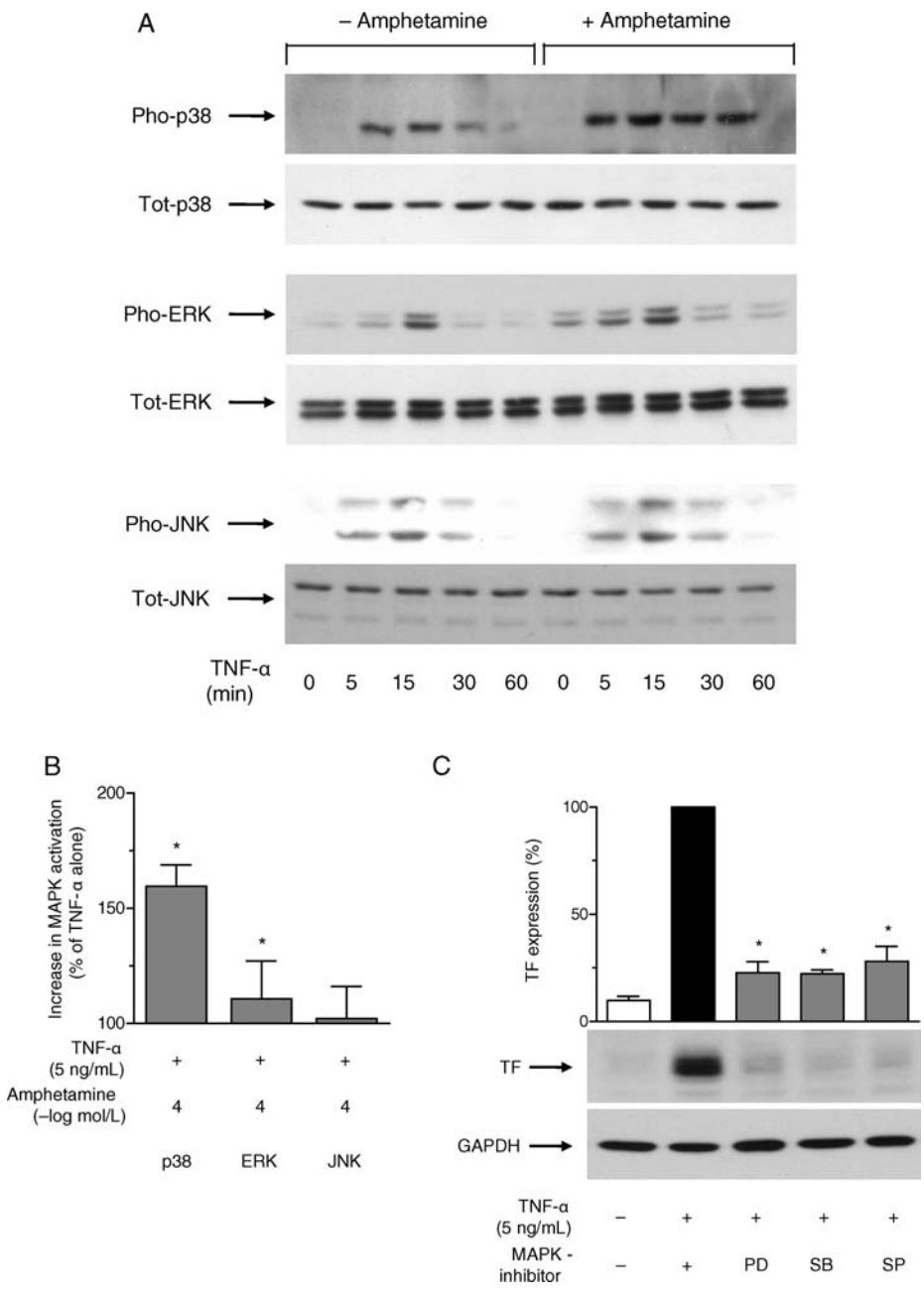

Figure 7 Amphetamine enhances endothelial tissue factor expression via mitogen-activated protein kinase activation. (*A*) Phosphorylation pattern of mitogen-activated protein kinases p38, extracellular signal-regulated kinase, and c-Jun NH_2-terminal kinase following amphetamine treatment in tumour necrosis factor-α-stimulated cells. (*B*) Mitogen-activated protein kinase activation after 15 min of amphetamine treatment, expressed as an increase over tumour necrosis factor-α alone. *$P < 0.05$ vs. tumour necrosis factor-α alone. (*C*) The mitogen-activated protein kinase inhibitors PD98059, SB203580, and SP600125, specifically inhibiting extracellular signal-regulated kinase, p38, and c-Jun NH_2-terminal kinase, respectively, impair tissue factor expression after tumour necrosis factor-α stimulation. *$P < 0.05$ for tumour necrosis factor-α alone vs. each inhibitor.

Figure 8C). L-745,870 alone did not affect the TNF-α-induced activation of MAPKs ($n = 4$; *Figure 8*). Total expression of MAPKs remained unchanged at all time under all conditions ($n = 4$; data not shown). Lactate dehydrogenase release did not reveal any cytotoxic effect of dopamine, raclopride, NGB 2904, L-745,870, or L-750,667 ($n = 3$; data not shown).

Discussion

Amphetamine consumption can lead to death due to cardiovascular events and is becoming an increasingly serious worldwide concern. Indeed, data from more than 30 case reports and 8 epidemiological studies support this tendency. Amphetamine-associated adverse

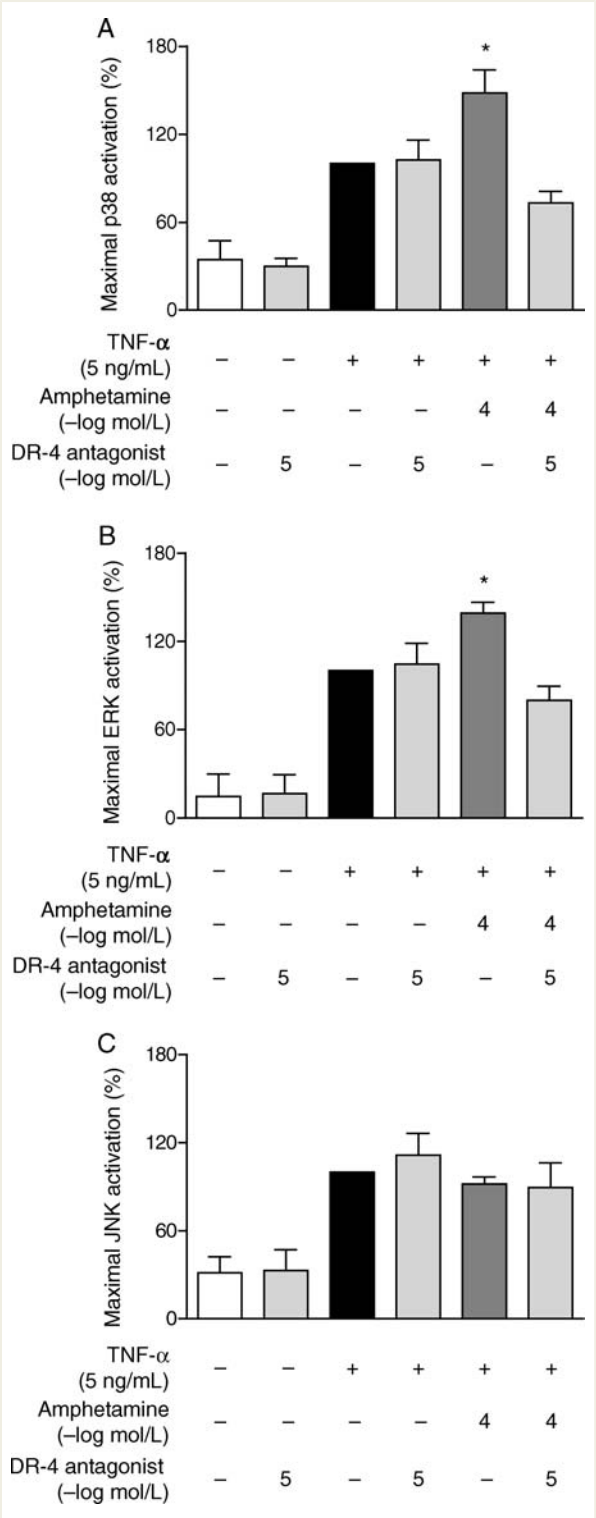

Figure 8 Amphetamine activates p38 and extracellular signal-regulated kinase via dopamine D4 receptor. Blocking of the dopamine D4 receptor with L-745,870 abolishes amphetamine-induced activation of p38 and extracellular signal-regulated kinase, whereas c-Jun NH2-terminal kinase phosphorylation remains unaffected. *$P < 0.05$ vs. amphetamine alone.

cardiovascular events mostly occur in relatively young individuals with no previous history of cardiovascular disease. Consistent with this observation, myocardial ischaemia with angiographically normal coronary arteries is documented in amphetamine consumers.[2,17] A recent report described severe anterior myocardial infarction with pump failure due to proximal thrombotic occlusion of the LAD in a 28-year-old amphetamine consumer.[18] Similar reports have demonstrated intravascular thrombosis, either angiographically or in post-mortem examination, in such patients.[18,19] In the present study, amphetamines are shown to induce TF expression and surface activity in human endothelial cells; this effect occurred at the transcriptional level and was mediated through the dopamine D4 receptor leading to activation of the MAPKs p38 and ERK. Since TF is the key trigger of thrombosis, our observations offer a potential mechanism for the occurrence of acute vascular syndromes in amphetamine consumers.

The concentrations applied in the present study are within the plasma range observed *in vivo*; indeed, a plasma concentration of 5×10^{-6} mol/L (1.1 μg/mL) was measured 6 h following MDMA intake in a subject who developed massive thrombosis of the right coronary artery.[19] However, there is a large variability of amphetamine plasma levels in patients presenting to the emergency department, and up to 100-fold higher plasma concentrations in the range of 0.4–84.0 μg/mL for MDMA and 0.2–11.3 μg/mL (8×10^{-5} mol/L) for amphetamine were measured in amphetamine victims.[20]

In contrast to cocaine, amphetamine induced TF expression and activity in both quiescent and cytokine-stimulated endothelial cells.[12,21–23] Considering that the endothelium plays a pivotal role in modulating the haemostatic balance, this observation adds on to the evidence that amphetamine may trigger thrombus formation in the absence of inflammatory alterations, as it may occur in young amphetamine consumers. Furthermore, amphetamine enhanced TF expression in response to TNF-α, an inflammatory cytokine known to induce TF expression in endothelial cells.[13] Hence, amphetamines may up-regulate TF expression in an inflammatory environment as it is observed in patients exposed to cardiovascular risk factors, but without clinically manifest atherosclerosis. Inflammation is indeed an important trigger for the pathogenesis of arterial thrombosis,[24] and inflammatory markers such as C-reactive protein are significantly raised in patients suffering from myocardial infarction at a young age.[25] In addition, the enhancing effect of amphetamine on thrombin-induced TF expression suggests that amphetamine may not only induce, but also amplify, thrombus formation converting a small and clinically non-significant thrombus into an occlusive thrombosis leading to an acute vascular syndrome.

Tissue factor activity is counterbalanced by TFPI, and the equilibrium of these two factors is indeed essential in determining thrombus formation.[11,13] Besides its effects on TF, amphetamine reduced endothelial TFPI, in both quiescent and TNF-α- or thrombin-stimulated cells, indicating that the effect of amphetamine on TFPI does not depend on the activation status of the cells. Moreover, in parallel to enhancing TF and decreasing TFPI, amphetamine increased TNF-α-induced PAI-1 expression. Plasmi-

nogen activator inhibitor-1 is a serpin suppressing fibrinolysis by inhibiting the activity of tissue plasminogen activator. Therefore, amphetamine would indeed be expected to exert potent prothrombotic actions *in vivo* by modulating different mediators of haemostasis.

The functional relevance of the enhanced TF activity in amphetamine-treated HAECs was underlined by the observation that supernatant from amphetamine-treated cells accelerated clotting time of human plasma; this effect occurred with cells treated with amphetamine under both basal conditions and after cytokine stimulation. These data, although performed *in vitro*, add on to the evidence that amphetamine may trigger thrombus formation in amphetamine consumers by enhancing coagulation.

Dopamine receptors play a role in mediating the behavioural effects of amphetamine.[26–28] Earlier studies have convincingly shown that blockade of dopamine D4 receptor in rats prevents the acute stimulatory effects of amphetamine[29] and dopamine receptor blockade in mice attenuates amphetamine-induced hyperlocomotion.[28] Although many studies have investigated amphetamine-mediated signalling in the brain, no efforts have been made in identifying amphetamine-induced events in peripheral cells. Dopamine receptors are expressed in endothelial cells, can activate them, and mediate effects on coagulation factors.[30–32] Tissue factor induction by amphetamine was indeed abrogated by dopamine D4 receptor antagonists or by silencing the D4 receptor gene through siRNA transfection. In line with this observation, expression of this receptor in human endothelial cells was confirmed; furthermore, its activation by dopamine was able to induce endothelial TF expression. Considering the very similar chemical structure of amphetamine and dopamine, it is possible that amphetamine directly activates endothelial dopamine receptors; alternatively, amphetamine may increase locally synthesized extracellular dopamine by blocking dopamine transporters, as previously described in neuronal cells.[33,34] Most of the dopamine is indeed produced in non-neuronal locations such as renal tubules and endothelial cells of various organs;[35–37] the latter being underlined by the identification of L-DOPA decarboxylase by RT–PCR in cultured endothelial cells.[32]

Tissue factor expression in response to a variety of stimuli is mediated by MAPK activation leading to increased TF transcription.[13] Amphetamine indeed enhanced activation of p38 and ERK, but not JNK, resulting in enhanced TF mRNA expression; TF mRNA stability remained unaltered under these conditions. Inhibition of p38 and ERK impaired TF expression in response to TNF-α, confirming that the effect of amphetamine on p38 and ERK phosphorylation mediates the increase in TF expression. This signal transduction profile of amphetamine is consistent with recent observations demonstrating that p38 and ERK, but not JNK, are required for mediating amphetamine-induced forms of reward-related learning in the prefrontal cortex.[14] Blocking of the dopamine D4 receptor abolished activation of p38 and ERK, but not JNK, suggesting that amphetamine leads to activation of p38 and ERK via the dopamine D4 receptor. In line with this interpretation, stimulation of the dopamine D4 receptor produced a time- and dose-dependent increase in ERK activity in several brain regions.[38,39] Not surprisingly, MAPKs, in particular the ERK pathway, have been described as a key molecular process in selective brain regions in response to amphetamine, and pharmacological manipulation of the ERK pathway has been proposed as a potential treatment strategy for drug addiction.[40]

The present *in vitro* study has several limitations. We have not studied all the mechanism potentially influencing dopamine receptor signalling. Thus, further studies are needed to understand the molecular mechanism by which dopamine receptor signalling is involved in the regulation of haemostasis and vascular biology. Moreover, animal and clinical studies need to clarify whether an increased TF activity indeed accounts for the effects of amphetamines on thrombus formation *in vivo*.

In summary, this study demonstrates that amphetamine leads to an increase in endothelial TF expression and activity with a parallel decrease in TFPI expression. This effect is mediated by the dopamine D4 receptor and activation of the MAPKs p38 and ERK. These findings may offer a mechanistic insight regarding amphetamine-induced acute cardiovascular events.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Acknowledgement

The authors thank Helen Greutert for expert technical support.

Funding

This study was supported by the Swiss National Science Foundation (grant no. 3200B0-113328/1 to F.C.T. and grant no. 3100-068118.02/1 to T.F.L.), Bonizzi-Theler Foundation, Velux Foundation, Wolfermann Nägeli Foundation, MERCATOR Foundation, and the Swiss Heart Foundation.

Conflict of interest: none declared.

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