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Abstract

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)-alpha-induced as well as basal TF expression (P = 0.029, 0.0003, and 0.003 at maximal concentration), and TNF-alpha-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.
Amphetamines Induce Tissue Factor and Impair Tissue Factor Pathway Inhibitor

Role of Dopamine Receptor Type 4

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Short Title: Amphetamines Induce Tissue Factor

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Abstract

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} \text{ mol/L}) enhanced thrombin and TNF-\alpha induced as well as basal TF expression (p=0.029, p=0.0003, and p=0.003 at maximal concentration), and TNF-\alpha induced PAI-1 expression (p=0.003), while tissue factor pathway inhibitor (TFPI) expression was impaired (p=0.008). Similarly, methylenedioxy-methamphetamine (MDMA, 10^{-7}-10^{-4} \text{ mol/L}) enhanced TF expression (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 MAP kinases ERK and p38 (p=0.03 and p=0.033), but not 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.

Conclusions: Amphetamines induce endothelial TF expression via stimulation of dopamine D4 receptor and activation of the MAP kinases 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.
Key words: acute vascular syndromes, tissue factor, amphetamine, dopamine receptor
Introduction

In the Western civilization, intake of psychostimulants such as amphetamine and 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 ischemic stroke (3-8).

The pathogenesis of amphetamine-related acute cardiovascular events is still uncertain. Vasospasm and prothrombotic state have been postulated as etiologic 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, substances suspected to promote vasospasm, such as histamine and cocaine, are known to induce the endothelial expression of tissue factor (TF) (11, 12). TF 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 MAP kinases, the major regulators of endothelial TF expression, in neuronal cells (14). Based on these facts, we hypothetized 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.
Materials and 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 hrs by using EBM medium (Cambrex) supplemented with 0.5% FCS, before stimulation with 5 ng/mL TNF-α (R&D, Minneapolis, MN) or 1 U/mL thrombin (Sigma-Aldrich, Buchs, Switzerland) (Table 1). Cells were pretreated with amphetamine (Lipomed, Arlesheim, Switzerland) or 3,4-methylenedioxymethylamphetamine (MDMA; Sigma-Aldrich) for 60 min before stimulation with TNF-α. Dopamine, L-750,667, L-745,870, raclopride (all from Sigma-Aldrich), NGB 2904 (Tocris Bioscience, Ellisville, MOi) were added to the dishes 60 min before stimulation (Table 1). Cytotoxicity was assessed with a colorimetric assay to detect lactate dehydrogenase 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) 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 MAP kinase (p38), p44/42 MAP kinase (extracellular signal–regulated kinase [ERK]), and c-Jun NH2-terminal kinase (JNK; all from Cell Signaling, Danvers, MA) were used at 1:1000, 1:5000, and 1:1000 dilution.
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, Danvers, MA) were used at 1:1000 dilution. Anti-goat glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH; Chemicon, Temecula, CA) was applied to ensure equal protein loading (1:20000 dilution). Proteins were detected with a horseradish peroxidase–linked secondary antibody (Amersham, Munich, Germany).

Real-time PCR

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 PCR experiments were performed in triplicate using the SYBR Green JumpStart kit (Sigma) 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 tissue factor: sense 5'-TCCCCAGAGTTCACACCTTACC-3' (bases 508-529 of F3 cDNA; NCBI no. NM 001993), antisense: 5'-CCTTTCTCCTGGCCCATACAC-3' (bases 843-863 of TF cDNA; NCBI no. NM 001993); for human D4 dopamine receptor: sense: 5'-CCCACCCCAGACTCCACC-3' (bases 963-980 of DRD4 cDNA; NCBI no. NM 000797), antisense: 5'-GAACTCGGCGTTGAAGACAG-3' (bases 1202-1221 of DRD4 cDNA; NCBI no. 000797); for human L28: sense: 5'-
GCATCTGCAATGGATGGT-3’, antisense: 5’-CCTTTCTCCTGGCCCATACAC-3’.
The amplification program 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. PCR products were also analyzed 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. TF mRNA stability was assessed in the presence of the transcriptional inhibitor actinomycin D (10 ug/mL, Sigma-Aldrich, Buchs, Switzerland), which was added 2 hrs after TNF-α stimulation. Cells were harvested for RNA extraction at various time-points (0–3 hrs) 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 percent 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, Saint Louis, USA). These siRNA sequences were as follows: siRNA 1 (sense: 5’-CCGCCUCCAUUUCCAGGCdTdT-3’; antisense: 5’-AGGUUGAAGAGGAGGGCGdTdT-3’);  siRNA 2 (sense: 5’-CCCUCAUGGACGUGCdTdT-3’; antisense: 5’-ACGUUCCAGAGGAGGCGdTdT-3’);  siRNA 3 (sense: 5’-GCCUCAUCUACGCCUCCUdTdT-3’; antisense: 5’-ACAGGUUGAAGAGGAGGCGdTdT-3’). Briefly, cells were transfected with a 15 nM
siRNA Nanoparticle Forming Solution (NFS) 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.

**TF activity**

TF activity was analyzed 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. 50 μL 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 (> 2 groups), the 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 TF protein expression and activity

HAECs were stimulated with TNF-α (5 ng/ml) or thrombin (1 U/mL) for 5 hours in the presence or absence of amphetamines (Table 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 1A, Table 2). Similarly, amphetamine enhanced thrombin induced TF expression by 1.6-fold (n=4; p=0.029; Mann Whitney test; Figure 1C; Table 2) and basal TF expression by 2.2-fold (n=7; p=0.003; Mann Whitney test; Figure 1D; Table 2). Similar to amphetamine, MDMA (10⁻⁷-10⁻⁴ mol/L) enhanced TF expression by 1.7-fold at maximum concentration as compared to TNF-α alone (n=4; p=0.046; Mann Whitney test; Figure 1B; Table 2). 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 p=0.008, respectively; Mann Whitney test; Figure 2A and 2B; Table 2) and under basal conditions (n=6; p=0.002; Mann Whitney test; Figure 2D; Table 2). Amphetamine induced a similar relative increase in TF surface activity and TF whole cell activity (n=5; p=0.004; Mann Whitney test; Figure 2C; Table 2).

Amphetamines are not toxic

To control for toxic effects, HAECs were treated with increasing concentrations of amphetamine or MDMA for 6 hours. 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;
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=6; p=0.03; Mann Whitney test; Supplemental Figure 1; Table 2) and after stimulation with TNF-α (n=6; p=0.002; Mann Whitney test; Supplemental Figure 1; Table 2).

Amphetamine impairs TFPI and increases PAI-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 p=0.03, respectively; Mann Whitney test; Figure 3B and 3C; Table 2). A similar effect was observed under basal conditions (n=4; p=0.03; Mann Whitney test; Figure 3A; Table 2). 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 2).

Amphetamine enhances TF mRNA expression without affecting mRNA stability

Real-time PCR demonstrated that TF mRNA expression was induced after 2 hours of TNF-α stimulation (n=4; p=0.029; Mann Whitney test; Figure 4A). 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;
Amphetamine did not affect the stability of TF mRNA under these conditions (n=4; Figure 4B).

**Amphetamine enhances MAP kinase activation**

To assess whether amphetamine 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 (n=4; Figure 6A). Amphetamine enhanced phosphorylation of p38 and ERK (n=4-7; p=0.033 and p=0.03, respectively; Mann Whitney test; Figure 5A and B; Table 2), while that of JNK remained unaffected (n=4; p=0.81; Mann Whitney test; Figure 5A and B). No significant change in total expression of MAP kinases was observed at any time point with or without amphetamine. The MAP kinase 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 versus each inhibitor; Mann Whitney test; Figure 5C). Phosphorylation of dopamine-and-cAMP-regulated Phosphoprotein 32 (DARPP-32) was not altered in the presence or absence of amphetamine (data not shown). MAPK inhibitors were not toxic as assessed by LDH-release measured 5 hours after addition of TNF-α (n=3; data not shown).

**Dopamine D4 receptor mediates the effect of amphetamine on TF**

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
Dopamine indeed enhanced TNF-α induced TF expression in HAECs (n=6; p=0.002; Mann Whitney test; Figure 6A; Table 2). To determine whether dopamine receptors are involved in amphetamine induced TF expression, cells were pretreated 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. Pretreatment with L-745,870 abrogated amphetamine induced TF expression (n=5; p=0.02; Jonckheere-Terpstra test; Figure 6B; Table 2), 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 pretreatment with raclopride or NGB 2904 (n=4; p=1.0 and 0.46, respectively; Jonckheere-Terpstra test; Figure 6C and D; Table 2). 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=6; p=0.03 for amphetamine in non targeting siRNA transfected cells versus 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 versus amphetamine alone; Mann Whitney test; Figure 7A) and ERK (n=4; p=0.03 for amphetamine and L-745,870 versus amphetamine alone; Mann Whitney test; Figure 7B), while activation of JNK remained unaffected (n=4; p=0.34 for amphetamine and L-745,870 versus amphetamine alone; Mann Whitney test; Figure 7C). L-745,870 alone did not affect the TNF-α induced activation of MAP kinases (n=4; Figure 7). Total expression of MAP kinases 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 epidemiologic studies support this tendency. Amphetamine associated adverse cardiovascular events mostly occur in relatively young individuals with no previous history of cardiovascular disease. Consistent with this observation, myocardial ischemia 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 postmortem 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 MAP kinases 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 $5 \times 10^{-6}$ mol/L (1.1 μg/ml) was measured 6 hours 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 x 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 hemostatic 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 upregulate 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.

TF 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, both in quiescent and TNF-alpha 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-alpha induced
PAI-1 expression. PAI-1 is a serpin suppressing fibrinolysis by inhibiting the activity of tissue plasminogen activator (tPA). 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 behavioral 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). While 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). TF 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).

TF expression in response to a variety of stimuli is mediated by MAP kinase 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, MAP kinases, 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 MAP kinases p38 and ERK. These findings may offer a mechanistic insight regarding amphetamine-induced acute cardiovascular events.
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Conflict of Interest

None declared.
References


29. Feldpausch DL, Needham LM, Stone MP, Althaus JS, Yamamoto BK, Svensson KA, Merchant KM. The role of dopamine D4 receptor in the induction of behavioral sensitization to amphetamine and accompanying


Figure legends

Figure 1: Amphetamines enhance endothelial TF protein expression
   A. Amphetamine enhances TNF-α induced TF protein expression. *p<0.05 vs TNF-α alone. B. MDMA enhances TNF-α induced TF protein expression. *p<0.05 vs TNF-α alone C. Amphetamine enhances thrombin induced TF protein expression. *p<0.05 vs TNF-α alone D. Amphetamine induces basal TF protein expression. *p<0.05 vs unstimulated control. All blots are normalized to GAPDH expression.

Figure 2: Amphetamine induces TF activity
   A. Amphetamine enhances TNF-α induced TF surface activity. *p<0.05 vs TNF-α alone B. MDMA enhances TNF-α induced TF surface activity. *p<0.05 vs TNF-α alone C. Amphetamine induces a similar relative increase in luminal surface activity and whole cell activity of TF. p<0.05 vs control D. Amphetamine induces basal TF surface activity. *p<0.05 vs unstimulated control

Figure 3: Amphetamine inhibits endothelial TFPI protein expression
   A. Amphetamine inhibits basal TFPI protein expression. *p<0.05 vs unstimulated control B. Amphetamine inhibits TFPI protein expression in TNF-α stimulated cells. *p<0.05 vs TNF-α alone C. Amphetamine inhibits TFPI protein expression in thrombin stimulated cells. *p<0.05 vs thrombin alone

Figure 4: Amphetamine enhances endothelial TF mRNA expression without affecting mRNA stability
   A. Amphetamine enhances TNF-α induced TF mRNA expression. *p<0.05 vs
TNF-α alone. Values are determined by real-time PCR, indicated as percent of TNF-α alone, and normalized to L28 expression. B. Amphetamine (10^-4 M) does not affect TF mRNA stability. TF mRNA copies are normalized to L28 expression and expressed as percent of TNF-α.

Figure 5: Amphetamine enhances endothelial TF expression via MAP kinase activation

A. Phosphorylation pattern of MAP kinases p38, ERK, and JNK following amphetamine treatment in TNF-α stimulated cells. B. MAP kinase activation after 15 min of amphetamine treatment, expressed as increase over TNF-α alone. *p<0.05 vs TNF-α alone C. The MAP kinase inhibitors PD98059, SB203580, and SP600125, specifically inhibiting ERK, p38, and JNK, respectively, impair TF expression after TNF-α stimulation. *p<0.05 for TNF-α alone vs each inhibitor

Figure 6: Amphetamine enhances endothelial TF expression via dopamine D4 receptor

A. Dopamine enhances TNF-α induced endothelial TF expression. *p<0.05 vs. TNF-α alone B. The dopamine D4 receptor antagonist L-745,870 abrogates amphetamine-induced TF 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 TF expression is abrogated when D4 receptor gene is silenced through siRNA transfection. *p<0.05 vs versus amphetamine in D4R siRNA transfected cells.
**Figure 7: Amphetamine activates p38 and ERK via dopamine D4 receptor**

Blocking of the dopamine D4 receptor with L-745,870 abolishes amphetamine induced activation of p38 and ERK, while JNK phosphorylation remains unaffected. *p<0.05 vs amphetamine alone.

**Supplemental Figure 1: Amphetamine accelerates clotting time of human plasma**

Clotting time of human plasma in the presence of supernatant from amphetamine treated HAECs under basal conditions and in the presence of TNF-α. *p<0.05 vs unstimulated and TNF-α alone, respectively.

**Table 1:** Study conduct of the in vitro experiments.

**Table 2:** Group comparison by Mann Whitney test. Data are presented as mean±SEM (% versus control group). ** Jonckheere-Terpstra 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. DRA: dopamine receptor antagonist
Figure 1

A

B

C

D

TF expression (%)

TF

GAPDH

TNF-α (5 ng/ml)

Amphetamine (-log mol/l)

TF expression (%)

TF

GAPDH

TNF-α (5 ng/ml)

Amphetamine (-log mol/l)

TF expression (%)

TF

GAPDH

Thrombin (1 U/ml)

Amphetamine (-log mol/l)

basal TF expression (%)
Figure 2

A

TF activity (%)

TNF-α (5 ng/ml)  -  +  +  +  +  +  +
Amphetamine (-log mol/l)  -  7  6  5  4

B

TF activity (%)

TNF-α (5 ng/ml)  -  +  +  +  +  +  +
MDMA (-log mol/l)  -  7  6  5  4

C

TF activity (%)

surface  whole cell

TNF-α (5 ng/ml)  +  +  +  +  +
Amphetamine (-log mol/l)  -  4  -  4

D

basal TF activity (%)

Amphetamine (-log mol/l)  -  4
Figure 3

A

![Graph showing TFPI expression (%) in response to Amphetamine (log mol/l) at various concentrations.](image)

B

![Graph showing TFPI expression (%) in response to TNF-α (5 ng/ml) and Amphetamine (log mol/l) at various concentrations.](image)

C

![Graph showing TFPI expression (%) in response to Thrombin (1 U/ml) and Amphetamine (log mol/l) at various concentrations.](image)
Figure 4

A

TF mRNA expression (% of T0) vs. time (hours) for different treatments:
- TNF-α (5 ng/ml)
- Amphetamine (-log mol/l)

B

TF mRNA (% of T0) vs. time (hours) for different treatments:
- TNF-α (5 ng/ml)
- TNF-α + Amphetamine (10^(-4) mol/l)
Figure 5

A

- Amphetamine + Amphetamine

TNF-α (minutes) 0 5 15 30 60 0 5 15 30 60

Pho-p38  
Tot-p38  
Pho-ERK  
Tot-ERK  
Pho-JNK  
Tot-JNK

B

Increase in MAPK activation (% of TNF-α alone)

TNF-α (5 ng/ml) + + +  
Amphetamine (-log mol/l) 4 4 4  
p38 ERK JNK

C

TF expression (%)

TNF-α (5 ng/ml) - + + + +  
MAPK-Inhibitor - + PD SB SP
Figure 6

A

B

C

D

E
Figure 7

A

![Graph showing maximal p38 activation (%)]

- TNF-α (5 ng/ml)
- Amphetamine (-log mol/l)
- DR-4 Antagonist (-log mol/l)

B

![Graph showing maximal ERK activation (%)]

- TNF-α (5 ng/ml)
- Amphetamine (-log mol/l)
- DR-4 Antagonist (-log mol/l)

C

![Graph showing maximal JNK activation (%)]

- TNF-α (5 ng/ml)
- Amphetamine (-log mol/l)
- DR-4 Antagonist (-log mol/l)
### Table 1

<table>
<thead>
<tr>
<th>Cell culture HAEC</th>
<th>Cell culture HAEC</th>
<th>Serum starvation</th>
<th>Pretreatment with amphetamine or MDMA</th>
<th>Cytokine stimulation (TNF-α) 5 hrs</th>
<th>Cell lysis</th>
<th>Assessment of adherent cells/cell lysis</th>
<th>TF protein expression (Immunoblotting)</th>
<th>TF activity (colorimetric assay)</th>
<th>Clotting time (fibrometer)</th>
<th>MAPK activation (Immunoblotting)</th>
<th>RNA extraction</th>
<th>TF mRNA (PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-72 hrs</td>
<td>24-48 hrs</td>
<td>24 hrs</td>
<td>60 min</td>
<td>No additional stimulus 5 hrs</td>
<td></td>
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</tbody>
</table>

**Study aim:** to investigate whether amphetamine regulates TF in endothelial cells

**Null hypothesis:** no difference between the two groups tested

**Experimental hypothesis:** Amphetamines enhance endothelial TF expression

**Non-parametric:** Mann-Whitney-test/Kruskal-Wallis-test.

In case of significant global test Dunn's multiple comparison test, two-tailed decisions, 95% confidence interval.

**Software:** GraphPad Prism software, version 4.03 (GraphPad Software Inc., La Jolla, CA, USA).
### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Amphetamine 10-4M Mean ± SEM</th>
<th>MDMA 10-4M Mean ± SEM</th>
<th>Dopamine 10-4M DRA/siRNA Mean ± SEM</th>
<th>n</th>
<th>p value of Mann Whitney/Jonckheere-Terpstra</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TF protein expression</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- TNF-α stimulation (amphetamine)</td>
<td>181.1 ± 9.0</td>
<td></td>
<td>169.5 ± 12.5</td>
<td>7</td>
<td>0.0003</td>
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<tr>
<td>- TNF-α stimulation (MDMA)</td>
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<td>4</td>
<td>0.046</td>
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<tr>
<td>- Thrombin stimulation</td>
<td>160.1 ± 23.7</td>
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<td>4</td>
<td>0.029</td>
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<tr>
<td>- basal</td>
<td>216.7 ± 34.6</td>
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<td>7</td>
<td>0.003</td>
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<tr>
<td>- TNF-α stimulation (Dopamine)</td>
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<td>215.5 ± 49.8</td>
<td>6</td>
<td>0.002</td>
</tr>
<tr>
<td>- Dopamine 2R antagonist</td>
<td>174.0 ± 18.6</td>
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<td>208.5 ± 42.1</td>
<td>4</td>
<td>1.0**</td>
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<tr>
<td>- Dopamine 3R antagonist</td>
<td>174.0 ± 18.6</td>
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<td>188.5 ± 42.7</td>
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<td>0.46**</td>
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<tr>
<td>- Dopamine 4R antagonist</td>
<td>174.0 ± 18.6</td>
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<td>67.9 ± 20.0</td>
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<td>0.002**</td>
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<tr>
<td><strong>TFPI expression</strong></td>
<td>56.5 ± 9.3</td>
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<td>0.008</td>
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<tr>
<td>- Thrombin stimulation</td>
<td>74.6 ± 9.6</td>
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<tr>
<td>- basal</td>
<td>61.5 ± 4.7</td>
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<tr>
<td><strong>PAI-1 expression (TNF-α)</strong></td>
<td>207.2 ± 24.7</td>
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<tr>
<td><strong>MAPK phosphorylation</strong></td>
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<tr>
<td>- phoERK</td>
<td>159.6 ± 9.1</td>
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<td>4</td>
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<td>- phop38</td>
<td>110.7 ± 16.4</td>
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<td>- phoJNK</td>
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<td><strong>TF surface activity</strong></td>
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<tr>
<td>- TNF-α stimulation (amphetamine)</td>
<td>159.3 ± 9.1</td>
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<td>127.0 ± 5.3</td>
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<td>0.002</td>
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<tr>
<td>- TNF-α stimulation (MDMA)</td>
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<td>5</td>
<td>0.008</td>
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<tr>
<td>- basal</td>
<td>168.0 ± 20.9</td>
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<tr>
<td><strong>Whole cell TF activity (TNF-α)</strong></td>
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<td><strong>Clotting time</strong></td>
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<tr>
<td>- basal</td>
<td>79.5 ± 5.3</td>
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<tr>
<td>- TNF-α stimulation</td>
<td>85.6 ± 2.8</td>
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<td><strong>TF mRNA expression</strong></td>
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<td>99.1 ± 19.8</td>
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</table>