Paclitaxel Enhances Thrombin-Induced Endothelial Tissue Factor Expression via c-Jun Terminal NH₂ Kinase Activation

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Abstract—Paclitaxel is used on drug-eluting stents because it inhibits proliferation of vascular cells. Stent thrombosis remains a concern with this compound, particularly with higher dosages. This study investigates the effect of paclitaxel on tissue factor (TF) expression in human endothelial cells. Paclitaxel enhanced thrombin-induced endothelial TF protein expression in a concentration- and time-dependent manner. A concentration of 10⁻⁵ mol/L elicited a 2.1-fold increase in TF protein and a 1.6-fold increase in TF surface activity. The effect was similar after a 1 hour as compared with a 25-hour pretreatment period. Real-time polymerase chain reaction revealed that paclitaxel increased thrombin-induced TF mRNA expression. Paclitaxel potently activated c-Jun terminal NH₂ kinase (JNK) as compared with thrombin alone, whereas the thrombin-mediated phosphorylation of p38 and extracellular signal-regulated kinase remained unaffected. Similar to paclitaxel, docetaxel enhanced both TF expression and JNK activation as compared with thrombin alone. The JNK inhibitor SP600125 reduced thrombin-induced TF expression by 35%. Moreover, SP600125 blunted the effect of paclitaxel and docetaxel on thrombin-induced TF expression. Paclitaxel increases endothelial TF expression via its stabilizing effect on microtubules and selective activation of JNK. This observation provides novel insights into the pathogenesis of thrombus formation after paclitaxel-eluting stent deployment and may have an impact on drug-eluting stent design. (Circ Res. 2006;99:149-155.)

Key Words: acute coronary syndrome • thrombosis • stents • MAP kinase • signal transduction

Percutaneous intervention is common practice for treating acute coronary syndromes. Drug-eluting stents (DES), which are coated with antiproliferative agents, improve the outcome after coronary artery stenting. Paclitaxel, a microtubule-stabilizing drug eliciting cell cycle arrest in G₂/M phase, is used on DES because it reduces vascular smooth muscle cell proliferation and migration. Several randomized clinical trials have demonstrated that paclitaxel-eluting stents decrease intimal hyperplasia and restenosis, leading to reduced rates of major adverse cardiac events as compared with bare-metal stents (BMS). In contrast, the use of DES has not reduced the occurrence of stent thrombosis as compared with BMS. Acute, subacute, and late in-stent thromboses have been observed in patients treated with paclitaxel-eluting stents, particularly following cessation of clopidogrel therapy. Moreover, the SCORE trial, which analyzed the effect of a stent releasing higher paclitaxel concentrations than the TAXUS stent, had to be terminated because of increased rates of in-stent thrombosis. Although in-stent thrombosis after TAXUS stent implantation is less frequent, its rates may still be higher in “real world” patients than those reported in clinical trials. In addition, if in-stent thrombosis occurs, it is associated with high morbidity and mortality.

Tissue factor (TF) is a 47-kDa transmembrane glycoprotein which binds factor VIIa (FVIIa) and, in turn, activates FIX and FX, thereby, the TF/FVIIa complex is the principal initiator of coagulation. It is well documented that TF is highly expressed in atherosclerotic plaques and that initiation of coagulation is a key event in the pathogenesis of acute coronary syndromes. TF antigen and activity are indeed higher in plaques from patients with unstable angina or myocardial infarction than in those from patients with stable angina, and increased TF plasma levels are found in unstable angina and acute myocardial infarction. Given the important role of TF in acute coronary syndromes, it may be involved in the pathogenesis of in-stent thrombosis as well.

Rapamycin, another drug used with DES, does indeed enhance endothelial TF expression. The effect of paclitaxel...
on TF expression, however, is not known. This study was therefore designed to examine the effect of paclitaxel on TF expression in human endothelial cells.

Materials and Methods

Cell Culture and Morphology

Human aortic endothelial cells (HAECs) were purchased from Clonetics and cultured as described. Cells were grown to confluence in 3-cm culture dishes, rendered quiescent for 24 hours in medium containing 0.5% FCS, and then stimulated with 1 U/mL thrombin (Sigma). Paclitaxel (Sigma and Alexis) was added to the dishes 1 hour before stimulation. To assess cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase (LDH) was used according to the recommendations of the manufacturer (Roche). Cell morphology was evaluated by phase–contrast microscopy (Leitz DM IRB) at ×50 magnification and photographed (Olympus DP 50) without fixation.

Western Blot Analysis

Protein expression was determined by Western blot analysis as described. Cells were lysed in 50 mmol/L Tris buffer, 25 μg was loaded per lane, and 10% SDS-PAGE was performed under reducing conditions. Resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) by semidry transfer. The antibody against human TF (American Diagnostica) was used at 1:2’000 dilution; antibodies against phosphorylated p38 mitogen-activated protein (MAP) kinase (p38), phosphorylated p44/42 MAP kinase (extracellular signal-regulated kinase [ERK]), and phosphorylated c-jun terminal NH2 kinase (JNK) (all from Cell Signaling) were used at 1:1’000, 1:5’000, and 1:1’000, respectively. Antibodies against total p38, total ERK, and total JNK (all from Cell Signaling) were used at 1:2’000, 1:5’000, and 1:1’000 dilution, respectively. The antibody against IκB-α (Santa Cruz) was applied at 1:1’000 dilution. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used to ensure equal protein loading at an antibody (Chemicon) dilution of 1:2’000. Proteins were detected with a horseradish peroxidase–linked secondary antibody (Amersham).

Real-Time PCR

Total RNA was extracted with 1 mL TRZol Reagent (Invitrogen) according to the recommendations of the manufacturer. Conversion of total cellular RNA to cDNA was performed with Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Amersham Bioscience) in a final volume of 33 μL using 4 μg of RNA. Real-time PCR amplification was performed in an MX3000P PCR cycler (Stratagene) using the SYBR Green JumpStart kit (Sigma) in 25 μL of reaction volume containing 2 μL of cDNA, 10 pmol of each primer, 0.25 μL of internal reference dye, and 12.5 μL of JumpStart Taq ReadyMix (buffer, dNTP, stabilizers, SYBR Green, Taq polymerase, and JumpStart Taq antibody). The total cDNA pool obtained served as template for subsequent PCR amplification with TF (F3)-specific primers (sense primer: 5′-TCCCCAGATTACACCTTAC-3′; bases 508 to 529 of F3 cDNA; National Center for Biotechnology Information [NCBI] no. NM 001993; antisense primer: 5′-TGACCAAGAACACAGCTCC-3′; bases 892 to 913 of F3 cDNA; NCBI no. NM 001993) using the following cycling parameters: 95°C for 10 minutes for 1 cycle; 95°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute, for a total of 40 cycles. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. L28 primers served as loading control. Products were separated by electrophoresis on a 1.6% agarose gel and visualized with ethidium bromide.

TF Surface Activity

TF surface activity was analyzed with a colorimetric assay (American Diagnostica) according to the recommendations of the manufacturer with some modifications as described. Cells were grown in 12-well plates, stimulated with thrombin, washed twice with PBS, and incubated with human FVIIa and FX at 37°C. This allowed the formation of a TF/FVIIa complex at the cell surface. This complex converted human FX to FXa, which was measured by its ability to cleave a chromogenic substrate. A standard curve was established with lipidated human TF to ensure that the results were in the linear range of detection (data not shown).

Figure 1. Paclitaxel enhances thrombin-induced TF protein expression. A, Paclitaxel enhances thrombin-induced TF protein expression in a concentration-dependent manner (n=6; *P<0.0005 vs thrombin alone). Values are given as percentage of TF expression in response to 5 hours of thrombin stimulation. Blots are normalized to GAPDH expression. B, Similar effect of paclitaxel on thrombin-induced TF protein expression after 1 hour and 25 hours of pretreatment (n=4; *P<0.005 vs thrombin alone, P=NS for 1 vs 25 hours). C, Paclitaxel enhances thrombin-induced TF surface activity (n=3; *P<0.005 vs thrombin alone). Values are given as percentage of TF surface activity in response to 5 hours of thrombin stimulation.
Statistical Analysis
Data are reported as mean±SEM. Unpaired Student’s t test was performed for statistical analysis. A probability value of <0.05 was considered to indicate a significant difference.

Results
Paclitaxel Enhances TF Protein Expression and Surface Activity
HAECs were stimulated with thrombin (1 U/mL) for 5 hours in the presence or absence of paclitaxel (10⁻⁸ to 10⁻⁵ mol/L). Thrombin induced a 20-fold increase in TF expression as compared with baseline (n=7; P < 0.0001; Figure 1A). Pretreatment with paclitaxel enhanced thrombin-induced TF expression in a concentration-dependent manner; a maximal effect occurred at 10⁻⁵ mol/L and resulted in a 2.1-fold induction as compared with thrombin alone, corresponding to a 43.3-fold induction as compared with baseline (n=6; P < 0.0005; Figure 1A). The effect of paclitaxel was similar after a 1 hour as compared with a 25 hour pretreatment period (n=4; P=NS for 1 versus 25 hours; Figure 1B). The effect of paclitaxel was first observed after 3 hours and elicited a significant increase in thrombin-induced TF expression after 5 and 7 hours (n=4; P < 0.05; Figure 3B). In another time-course analysis, thrombin-induced TF expression was maximal after 6 hours of stimulation and decreased to 50% after 12 hours, 32% after 18 hours, and 27% after 24 hours; paclitaxel significantly enhanced thrombin-induced TF expression by 2.2-fold after 6 hours (n=4; P < 0.05) and by 1.4-fold after 12 hours (n=4; P < 0.05), although its effect did not reach statistical significance after 18 and 24 hours (n=4; P=NS) (data not shown). Consistent with these observations, Paclitaxel enhanced thrombin-induced TF surface activity by 56% (n=3; P < 0.005; Figure 1C). Paclitaxel alone did not affect basal TF expression (n=7; P=NS; Figure 1A). Paclitaxel did not affect endothelial cell morphology (Figure 2A) nor LDH release (Figure 2B) at any concentration used (n=5; P=NS).

Paclitaxel Selectively Activates JNK
Thrombin induced a transient phosphorylation of the MAP kinases p38, ERK, and JNK. Maximal activation of JNK was observed after 60 minutes, whereas that of p38 and ERK occurred after 5 minutes.¹¹ Paclitaxel significantly increased JNK phosphorylation after 15, 30, and 60 minutes by 3.7-, 3.2-, and 2.0-fold, respectively, as compared with thrombin alone (n=4; P < 0.05 for each time point; Figure 4A). Phosphorylation of p38 was slightly prolonged by paclitaxel after 15 minutes of stimulation (n=4; P < 0.005; Figure 4B), whereas all of the other time points remained unaltered (n=4; P=NS; Figure 4B). Phosphorylation of ERK was not affected by paclitaxel, except for a slight decrease at the 5-minute time point (n=4; P < 0.01; Figure 4C). Neither thrombin nor paclitaxel altered total expression of MAP kinases. Thrombin-induced IkB-α degradation was not affected by pretreatment with paclitaxel (n=3; P=NS; data not shown).

Paclitaxel and Docetaxel Exert Similar Effects on Both TF and JNK
HAECs were stimulated with thrombin (1 U/mL) for 5 hours in the presence or absence of paclitaxel or docetaxel (both at 10⁻⁶ and 10⁻⁵ mol/L). Similar to paclitaxel, docetaxel enhanced thrombin-induced TF expression by 2.2-fold as compared with thrombin alone (n=4; P < 0.05 for thrombin plus paclitaxel versus thrombin alone; P < 0.005 for thrombin plus docetaxel versus thrombin alone; P=NS for thrombin plus paclitaxel versus thrombin plus docetaxel; Figure 5A). Docetaxel did not affect endothelial cell morphology nor LDH release at any concentration used (n=3; P=NS; data not shown).

Similar to paclitaxel, docetaxel enhanced JNK phosphorylation as compared with thrombin alone. The increase in JNK activation after 2 hours of thrombin stimulation was 3.2-fold for paclitaxel and 2.2-fold for docetaxel (n=4; P=0.0001 for thrombin plus paclitaxel versus thrombin alone; P < 0.05 for thrombin plus docetaxel versus thrombin alone; P=NS for thrombin plus paclitaxel versus thrombin plus docetaxel; Figure 5B).

JNK Mediates the Effect of Paclitaxel and Docetaxel on TF
HAECs were pretreated with SP600125, a specific inhibitor of JNK. 90 minutes before stimulation with thrombin (1 U/mL). SP600125 (10⁻⁶ mol/L) reduced thrombin-induced TF expression by 35% (n=3; P < 0.01; Figure 5C). Moreover, SP600125 reduced the effect of paclitaxel on thrombin-induced TF expression by 110% and that of docetaxel by 105%, respectively (n=4; P < 0.0001 for paclitaxel versus paclitaxel plus SP600125; P < 0.05 for docetaxel versus docetaxel plus SP600125; Figure 5D). Hence, inhibition of JNK by SP600125 blunted the effect of paclitaxel and docetaxel on thrombin-induced TF expression (n=4; P=NS).
Discussion

This study reveals that paclitaxel enhances thrombin-induced endothelial TF protein expression and surface activity in a concentration- as well as time-dependent manner via its stabilizing effect on microtubules and selective activation of JNK.

Paclitaxel is a lipophilic diterpenoid that binds to the β subunit of the tubulin heterodimer; this interaction promotes tubulin polymerization leading to the formation of stable nonfunctional microtubule bundles and promoting cell cycle arrest in G2/M phase.26,27 Via this mechanism, paclitaxel inhibits proliferation as well as migration of vascular smooth muscle cells and reduces restenosis rates in patients with...
coronary artery disease.\textsuperscript{3,4,7} Because of its lipophilic properties, paclitaxel accumulates in the vessel wall, reaching particularly high concentrations in the intima\textsuperscript{28,29}; local tissue concentrations are indeed 100-fold higher as compared with perfusate concentrations during ex vivo endovascular perfusion.\textsuperscript{28} In a porcine coronary artery stent model, tissue concentrations of paclitaxel reached 3.2 \(\mu\)g/g arterial tissue after 28 days and drop below detection limit within 3 months only.\textsuperscript{30} Similar tissue concentrations have been measured in a rabbit iliac artery stent model.\textsuperscript{31} Thus, the paclitaxel concentrations used in our study are comparable to local tissue concentrations after stent deployment.

In animal models, partial reendothelialization has been observed as early as 4 days after DES deployment, whereas complete reendothelialization occurs within 3 weeks.\textsuperscript{30,32} In humans, partial reendothelialization has been documented 2 weeks after stenting and is usually completed within 12 weeks.\textsuperscript{8,23} Paclitaxel-eluting stents have a biphasic drug-release profile in vitro, characterized by an initial burst during the first 48 hours after implantation, followed by a sustained low-level release for at least 2 weeks.\textsuperscript{6,34,35} Because of its lipophilic properties, however, very high paclitaxel concentrations have been measured up to 4 weeks after stent implantation in vivo, and the drug remains detectable for up to 12 weeks. Therefore, the time course of reendothelialization coincides with the presence of paclitaxel in the vessel wall after stent deployment. Thus, paclitaxel may indeed alter the biology of the endothelium within the stented area.

In-stent thrombosis has been described in patients treated with paclitaxel-eluting stents, particularly after cessation of antiplatelet therapy.\textsuperscript{8,10,12} Our data demonstrate that the effect of paclitaxel is maintained over prolonged time periods and that it becomes effective as soon as a stimulus like thrombin is present; hence, the data are consistent with the clinical observation that cessation of antiplatelet therapy is a risk factor for thrombosis of drug-eluting stents. Moreover, in view of the coronary paclitaxel concentrations after stent deployment, as well as the time course of reendothelialization, paclitaxel may indeed contribute to the development of subacute or late in-stent thrombosis by enhancing endothelial TF expression. This interpretation is supported by the results of the SCORE trial, which compared the QuaDDS stent (coated with the paclitaxel-derivative 7-hexanoyltaxol) to BMS; the trial had to be terminated prematurely because of very high rates of subacute and late in-stent thrombosis as

**Figure 5.** JNK mediates the effect of paclitaxel and docetaxel on TF expression. A, Similar to paclitaxel, docetaxel (10\textsuperscript{-6} and 10\textsuperscript{-5} mol/L) enhances thrombin-induced TF expression (n=4; \(P<0.005\) for thrombin+paclitaxel vs thrombin alone; \(P<0.005\) for thrombin+docetaxel vs thrombin alone; \(P=NS\) for thrombin+paclitaxel vs thrombin+docetaxel). Values are given as percentage of TF expression in response to 5 hours of thrombin stimulation. Blots are normalized to GAPDH expression. B, Paclitaxel and docetaxel enhance JNK phosphorylation as compared with thrombin alone (n=4; \(P=0.0001\) for thrombin+paclitaxel vs thrombin alone; \(P<0.05\) for thrombin+docetaxel vs thrombin alone; \(P=NS\) for thrombin+paclitaxel vs thrombin+docetaxel). Expression of total JNK is not affected. C, SP600125 (10\textsuperscript{-6} mol/L) reduces thrombin-induced TF expression to 65% of control (n=3; \(P<0.01\)). D, In the presence of SP600125 (10\textsuperscript{-6} mol/L), the effect of paclitaxel and docetaxel on thrombin-induced TF expression is blunted (n=4; \(P<0.0001\) for paclitaxel vs paclitaxel+SP600125; \(P<0.05\) for docetaxel vs docetaxel+SP600125; \(P=NS\) for paclitaxel+SP600125 vs thrombin alone; \(P=NS\) for docetaxel+SP600125 vs thrombin alone).
well as major adverse cardiac events. The increased rates of in-stent thrombosis have been primarily related to the high paclitaxel doses released by these stents, although an unfavorable effect of the stent design may have contributed. Interestingly, the paclitaxel derivative 7-hexanoyltaxol can be detected up to 10 mm proximal and distal to the stent margins, suggesting that paclitaxel may induce TF expression in the vessel segments proximal and distal to the stent as well.

The effect of paclitaxel was attributable to a specific action on endothelial cell function, as it neither affected cell morphology nor induced any toxicity. This is consistent with previous observations demonstrating that paclitaxel (10^-5 mol/L) does not induce any cell death in human pulmonary artery endothelial cells or aortic smooth muscle cells after 16 and 36 hours of incubation, respectively. The increase in TF surface activity was less pronounced than that in protein expression, which may be related to the presence of inactive encrypted TF on the cell surface or to the distribution of TF in several cellular compartments.

Thrombin induces TF expression at the transcriptional level via activation of the MAP kinases p38, ERK, and JNK. The increase in TF protein expression by paclitaxel was preceded by an enhanced TF mRNA expression. Consistent with this observation, paclitaxel augmented thrombin-induced JNK phosphorylation. Interestingly, the activation pattern of p38 and ERK was not affected, indicating that paclitaxel selectively activates JNK without affecting other signal-transduction molecules in endothelial cells. Consistent with this interpretation, IκB-α degradation was not altered by paclitaxel. Similar observations have been made in different cancer cell lines, demonstrating that the effect of paclitaxel on JNK activation is not restricted to the endothelium.

To assess whether JNK indeed mediates the induction of TF expression in response to thrombin and, in particular, to paclitaxel, endothelial cells were pretreated with SP600125, a selective inhibitor of JNK catalytic activity. SP600125 decreased thrombin-induced TF expression by approximately a third, indicating that JNK is not the only signal-transduction mediator regulating thrombin-induced TF expression. In contrast, the JNK inhibitor fully prevented the effect of paclitaxel, strongly suggesting that the effect of paclitaxel on TF expression is selectively mediated by the JNK pathway. However, it cannot be ruled out completely that other signal-transduction pathways may be involved as well.

The microtubule-stabilizing agent docetaxel was used to elucidate whether the increase in thrombin-induced JNK activation and TF expression by paclitaxel was related to perturbation of microtubule function. Both microtubule-stabilizing agents exerted a similar effect on JNK activation and TF expression, and SP600125 blunted the enhancing effect of both docetaxel and paclitaxel on thrombin-induced TF expression. Thus, the action of paclitaxel on JNK activation and TF expression seems to depend on stabilization of microtubule bundles rather than on a substance-specific effect. Consistent with this interpretation, JNK activation in response to changes in the microtubule cytoskeleton has been described in different cancer cell lines.

Paclitaxel enhanced thrombin-induced endothelial TF expression by 2.1-fold. Rapamycin augmented thrombin-induced TF expression to a similar extent, whereas the mechanisms of action of the 2 drugs differ completely: binding of rapamycin to its intracellular receptor FKBP-12 abrogates p70S6 kinase phosphorylation, leading to enhanced endothelial TF expression; whereas JNK activation remains unaffected. Large-scale clinical trials have demonstrated that patients receiving paclitaxel-eluting stents have similar rates of in-stent thrombosis as compared with rapamycin-eluting stents. This observation is consistent with the similar degree of TF induction in endothelial cells, suggesting that the latter may indeed be importantly involved in thrombosis of DES.

In conclusion, this study indicates that paclitaxel increases endothelial TF expression via JNK activation because of its microtubule stabilizing effect. The enhanced endothelial TF expression may favor thrombus formation after paclitaxel-eluting stent deployment, particularly when antithrombotic drugs are withdrawn or thrombin levels are elevated as it occurs in acute coronary syndromes. Therefore, these findings may have interesting implications for drug-eluting stent design.

Sources of Funding
This work was supported by the Swiss National Science Foundation (grant No. 3200B0-102232/1 to F.C.T., and grant No. 3100-068118.02/1 to T.F.L.), the Bonizzi-Theler Foundation, the Hartmann-Müller Foundation, the Olga Mayenfisch Foundation, the Herzog-Egli Foundation, and the Swiss Heart Foundation.

Disclosures
None.

References


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_Circ Res._ 2006;99:149-155; originally published online June 22, 2006; doi: 10.1161/01.RES.0000233379.92010.fd

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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