Modulation of Tissue Factor–Factor VIIa Signaling by Lipid Rafts and Caveolae

Vineet Awasthi, Samir K. Mandal, Veena Papanna, L. Vijaya Mohan Rao, Usha R. Pendurthi

Objective—Coagulation factor VIIa (VIIa) binding to its cellular receptor, tissue factor (TF), not only initiates the coagulation cascade but also induces cell signaling by activating G-protein coupled protease-activated receptors. The objective of the present study is to investigate the role of lipid rafts and caveolae in modulating TF-VIIa signaling and coagulant functions.

Methods and Results—TF-VIIa coagulant function was measured in factor X activation assay and the signaling function was evaluated in phosphoinositide hydrolysis and IL-8 gene induction. Buoyant density gradient centrifugation and immunofluorescence confocal microscopy were used to determine cellular localization of TF and protease-activated receptor 2. The data show that a substantial fraction of TF and protease-activated receptor 2 resides in lipid rafts/caveolae, and disruption of lipid rafts by cholesterol depletion or modification reduced TF-VIIa–induced cell signaling. Disruption of caveolae with caveolin-1 silencing had no effect on the TF-VIIa coagulant activity but inhibited the TF-VIIa-induced cell signaling.

Conclusion—Overall our data show that lipid raft/caveolae play a selective role in modulating the TF-VIIa signaling function without affecting the TF-VIIa coagulant activity. (Arterioscler Thromb Vasc Biol. 2007;27:1-2.)

Key Words: caveolae | factor VIIa | lipid rafts | signaling | tissue factor protease-activated receptors

Tissue factor (TF) is a cellular receptor for clotting factor VIIa, and the formation of TF-VIIa complexes on cell surfaces not only triggers the coagulation cascade but also transduces cell signaling via activation of protease-activated receptors (PARs), particularly PAR2.1,2 Although a number of recent studies provide valuable information on intracellular signaling pathways that are activated by TF-VIIa,3–5 the role of various cell surface components in mediating the interaction of TF-VIIa with PARs, and the subsequent signal transmittance are unknown. Unlike thrombin and trypsin, VIIa has to bind to TF to activate PARs. Although there is no quantitative data available on kinetics of PAR2 cleavage by TF-VIIa versus trypsin, a potent activator of PAR2, inability of TF-VIIa to trigger Ca2+ signaling and failure to desensitize signaling of subsequently added trypsin suggest that the TF-VIIa is a poor activator of PAR2.4–6 Despite this, a number of studies have shown that VIIa is as effective as trypsin or PAR2 agonist peptide in activating intracellular signaling pathways and gene expression.4,7 Although the potential mechanism for this phenomenon is unknown, compartmentalization of TF, PAR2, and G-proteins in the plasma membrane microdomains could facilitate a robust TF-VIIa–induced PAR2-mediated cell signaling.

Cholesterol plays a critical role in differentiating and maintaining cell surface microdomains of differing lipid composition. Cholesterol association with glycosphingolipids and other saturated long-chained lipids in biomembranes causes a phase transition in the membrane leading to segregated specialized microdomains, lipid rafts.8,9 Cholesterol- and sphingolipid-rich rafts in association with structural protein caveolin-1 form caveolae, flask-shaped invaginations in the plasma membrane.10,11 Various receptors and signaling molecules are shown to be concentrated in lipid rafts and caveolae. For example, when compared with the rest of plasma membrane, lipid rafts/caveolae are enriched in Src-family kinases, EGF-R, PDGF-R, various G-protein-coupled receptors and their down-stream signaling molecules, and a number of other signaling molecules.12–15 Further, cholesterol in plasma membrane can interact directly with membrane receptors and thus can have a strong influence on the affinity state, binding capacity, and signal transduction properties of receptors.14 Our recent studies with fibroblasts show that TF is localized in both lipid rafts and caveolae and disruption of these microdomains alters the coagulant activity of TF.15 Although certain G-protein-coupled receptors and G-proteins are known to be segregated into lipid rafts and caveolae,16–18 little is known whether G-protein-coupled PARs are segregated into lipid rafts and caveolae, and how such segregation might influence their activation by TF-VIIa and the subsequent cell signaling by coupling to G-proteins. To obtain answers to some of these questions, in the present study, we investigated the role of membrane cholesterol and...
lipid raft/caveolae in modulating the TF-VIIa signaling and coagulant functions in tumor cells.

Materials and Methods

Human breast carcinoma cell line MDA-MB-231 and COS-7 cells were obtained from ATCC (Rockville, Md). Quiescent monolayers were treated with methyl-β-cyclodextrin (mβCD) or filipin to disrupt lipid rafts/caveolae. Cell surface TF-VIIa activity was determined in factor X activation assay. TF antigen levels at the cell surface were determined by 125I-FVIIa or 125I-TF mAb binding assays. Phosphoinositide hydrolysis was determined by loading the cells with myo-[2-3H]inositol and monitoring the release of total 3H-inositol phosphates. Fluorescence microscopy was used for the measurement of calcium influxes in cells loaded with Fluo-4. Lipid rafts were separated by buoyant density gradient centrifugation. Total RNA was subjected to size fractionation on 1.0% agarose gel in formamide-degrading buffer system. Northern blot analysis was performed using 32P-labeled IL-8 cDNA. The fixed cells, either nonpermeabilized or formaldehyde fixed, were either washed in PBS, either washed in PBS, or permeabilized with 0.1% Triton X-100, were stained with antibodies against TF, PAR2, or caveolin-1, followed by Oregon Green or Rhodamine Red-conjugated secondary antibodies. Cleavage of PAR2 was evaluated in cells transfected transiently with alkaline phosphatase (AP)-PAR2 expression vector and measuring the activity of AP that was released into conditioned media following experimental treatments. Cells were transfected with control siRNA or caveolin specific siRNAs using SiPort amine.

A detailed Methods section is available in the online supplement at http://atvb.ahajournals.org.

Results

Localization of TF and PAR2 in Lipid Rafts and Caveolae

To investigate whether TF and PAR2 are colocated in cholesterol-rich sphingolipid rafts and/or caveolae in breast carcinoma cells, we isolated lipid rafts from MDA-MB-231 cell extracts based on their insolubility in Triton X-100, followed by their buoyant density in sucrose gradient. The gradient fractions were analyzed by immunoblotting for the presence of TF, caveolin-1, GM1, and PAR2. The lipid raft markers, GM1 and caveolin-1, were fractionated mostly into detergent-insoluble low-buoyant density fractions, fractions 3 to 7. A substantial amount of both TF and PAR2 are also distributed into these fractions (Figure 1A). When antigen levels were normalized to total cellular protein content, ≈32% of TF and PAR2 are localized in the low-density fractions (n=3). We further evaluated the localization of TF and PAR2 on the tumor cell surface by dual immunofluorescence confocal microscopy using specific antibodies against TF, PAR2, and caveolin-1. Caveolin-1 antibodies revealed punctate staining pattern marking the caveolae on the cell surface. Likewise, TF also displayed a significant punctate cell surface staining, indicating their elevated concentration within the discrete plasma membrane microdomains (Figure 1B). Overlap of TF and caveolin-1 fluorescence showed a high degree of colocalization of TF and caveolin-1 (0.68±0.05), suggesting TF localization in caveolae. Similar to TF, PAR2 also displayed significant punctuate staining and colocalization with caveolin-1 (0.49±0.01). A substantial amount of PAR2 at the cell surface is colocalized with TF (0.56±0.02; Figure 1B).

Effects of mβCD and Filipin Treatments on Membrane Cholesterol, Caveolae, and TF Activity

mβCD is a membrane-impermeable agent that binds to cholesterol with high specificity and depletes membrane cholesterol. Filipin does not deplete membrane cholesterol but alters the physical distribution of the cholesterol in the membrane by forming filipin–cholesterol complexes. Treatment of MDA-MB-231 cells with increasing concentrations of mβCD (1 to 10 mmol/L) reduced the cholesterol content in a dose-dependent manner (supplemental Figure IA, please see http://atvb.ahajournals.org); 10-mM mβCD treatment for 1 hour reduced the cholesterol content by ≈65%. In contrast, filipin treatment had no effect on the cholesterol content in the membrane. Examination of cells treated with mβCD and filipin under light microscopy revealed no gross differences in morphology between control and treated cells. Under our experimental conditions, these treatments had no significant effect on the cell viability or cell attachment to the culture dish (data not shown). Both mβCD and filipin treatment, as revealed by transmission electron microscopy, disrupted caveolar structures in MDA-MB-231 cells. While caveolae invaginations are clearly visible in control cells, there are very few morphologically recognizable caveolae in the cell membrane after cholesterol depletion/modification (supplemental Figure IB). Immunoblot analysis of detergent-resistant membrane in low-density fractions (Figure 2A). Determination of TF and PAR2 content by densitometry of immunoblots of sucrose gradient fractions revealed that TF and PAR2 content was decreased to 12% in mβCD-treated cells and to 17% in filipin-treated cells (32% of TF and PAR2 was localized in low-density fractions in control cells). Consistent with these data, immunofluorescence microscopy studies revealed that removal of plasma membrane cholesterol by mβCD treatment reduced TF and PAR2 association with the caveolae (Figure 2B). Colocalization analysis of TF or PAR2 with caveolin-1 at the plasma membrane from ≈80 to 100 randomly selected regions gave the following correlation coefficients: for TF and caveolin-1, control 0.68±0.05 and mβCD-treated 0.49±0.01 (P<0.001); and for PAR2 and caveolin-1, control 0.49±0.014 and mβCD-treated 0.41±0.012 (P<0.001). Filipin treatment reduced the TF colocalization with caveolin-1 18%.

Depletion of cholesterol from the plasma membrane by mβCD treatment reduced the cell surface TF-VIIa activity significantly, and the extent of decrease in the activity is correlated with the extent of cholesterol depletion from the plasma membrane (supplemental Figure IIA). In contrast, filipin treatment increased the cell surface TF-VIIa activity significantly. The opposing effects of mβCD and filipin on the TF-VIIa activity is consistent with our earlier observations made with fibroblasts. To address whether the cholesterol depletion/modification might have altered the availability of negatively charged phospholipids at the cell surface, which could influence the TF-VIIa activity, we examined the effect of mβCD (10 mmol/L) and filipin (5 μg/mL) on the ability of MDA-MB-231 cells to support factor Va/factor Va-catalyzed activation of prothrombin, which also depends
on negatively charged phospholipids. We found no differences in rates of thrombin generation in control cells and cells treated with either mβCD or filipin (supplemental Figure IIB). Analysis of annexin V binding to control, mβCD-treated, and filipin-treated cell by flow cytometry gave identical and overlapping fluorescence histograms (data not shown). These data rule out the possibility of potential alterations in negatively charged phospholipids as a reason for the altered TF-VIIa coagulant activity in mβCD-treated or filipin-treated cells.

Modulation of TF-VIIa–Induced Signaling
To investigate the role of lipid rafts and caveolae in TF-VIIa–induced cell signaling, we studied the effects of cholesterol depletion and lipid raft/caveolae disruption on TF-VIIa–induced PI hydrolysis and IL-8 gene expression. VIIa treatment increased the rate of PI hydrolysis in MDA-MB-231 cells by >4-fold compared with untreated cells. Treatment of carcinoma cells with 10 mmol/L mβCD before their exposure to VIIa fully attenuated the TF-VIIa–induced increase in PI hydrolysis (Figure 3A). mβCD treatment had no significant effect on the basal PI hydrolysis measured in untreated cells. Restoration of membrane cholesterol to the cholesterol-depleted cells by cholesterol loading restored the TF-VIIa ability to induce PI hydrolysis (Figure 3B). This observation also rules out a potential nonspecific effect of mβCD because the cells were exposed to a same concentration of mβCD to load the cholesterol as that was used to deplete the cholesterol. Similar to mβCD, filipin treatment also markedly reduced the VIIa-induced PI hydrolysis in MDA-MB-231 cells. Consistent with these data, both mβCD and filipin treatments reduced the TF-VIIa–induced IL-8 gene expression (Figure 4). These data suggest that the cholesterol depletion or modification of membrane cholesterol, which...
disrupt lipid rafts and caveolae, impair the TF-VIIa–induced cell signaling.

In additional studies, we evaluated the effect of lipid raft/caveolae disruption by mβCD and filipin treatments on trypsin and PAR2 agonist peptide-induced PI hydrolysis and IL-8 gene expression. The data showed both mβCD and filipin treatments also impaired trypsin and PAR2 agonist peptide-induced PI hydrolysis and IL-8 gene expression (data not shown). These data are not completely unexpected because lipid raft/caveolae disruption is shown to impair coupling of some heterotrimeric G-proteins to their activated receptors,\textsuperscript{11,21} a step that follows after the activation of PAR2 at the cell surface. However, these data raise a question on whether lipid raft disruption impaired the TF-VIIa signaling because it disrupted TF-VIIa activation of PAR2 at the cell surface or impaired the interaction of G-proteins with their activated receptors.

To address this question, we investigated the effect of mβCD and filipin treatment on PAR2 agonist peptide-induced Ca\textsuperscript{2+} signaling. Exposure of MDA231 cells to PAR2 agonist peptide rapidly increased the concentration of intracellular Ca\textsuperscript{2+}, and pretreatment of MDA231 cells with mβCD (10 mmol/L) or filipin (5 μg/mL) had no effect on PAR2 AP-induced increase in intracellular Ca\textsuperscript{2+} (Figure 5A). Similar data were obtained with trypsin (because VIIa does not induce Ca\textsuperscript{2+} signaling, we cannot use this assay to test the effect of VIIa). Next, we investigated the effect of lipid raft disruption on the cleavage of PAR2 by TF-VIIa and trypsin. For these studies, COS-7 cells were transiently transfected with a PAR2 cleavage reporter construct (AP-PAR2). Extent of AP release into the conditioned medium correlates the extent of PAR2 cleavage. As shown in Figure 5B, both mβCD and filipin treatments markedly reduced TF-VIIa cleavage of PAR2. In contrast, they have no effect or slightly
increased trypsin cleavage of PAR2. These data suggest that lipid raft disruption specifically impaired TF-VIIa cleavage of PAR2.

Effect of Caveolin-1 Silencing on TF-VIIa Coagulant and Signaling Functions
Although both mβCD and filipin are widely used and became standard tools to disrupt caveolae, these reagents could potentially exert broad ranging effects unrelated to caveolae. To overcome this possibility, we selectively disrupted caveolae by inhibiting caveolin-1, an integral protein required for caveolae formation using siRNA technology. Transfection of MDA-MB-231 cells with caveolin siRNA markedly reduced the caveolin-1 protein expression and had no effect on either TF antigen or activity levels at the cell surface (supplemental Figure IIIA). Further, caveolin-1 silencing had no effect on the surface expression of TF as evidenced by similar TF-specific VIIa binding to control, negative siRNA, and caveolin-1 siRNA transfected cells (supplemental Figure IIIB). Consistent with these data, caveolin-1 silencing did not decrease the TF coagulant activity (supplemental Figure IIIC). Colocalization studies employing immunofluorescence confocal microscopy revealed a significant decrease in TF and PAR2 colocalization in caveolin-1 silenced cells compared with control cells. Caveolin-1 silencing, as expected, also had no effect on the expression of PAR2 as measured in Ca²⁺ signaling assay using PAR2 agonist peptide as the activator (data not shown). Caveolin-1 silencing reduced the TF-VIIa–mediated cleavage of PAR2 (Figure 6A) and IL-8 gene expression (Figure 6B). It is important to note that caveolin-1 silencing had no effect on trypsin cleavage of PAR2, which establishes that caveolin-1 silencing specifically affects TF-VIIa cleavage of PAR2. Caveolin-1 silencing significantly impaired TF-VIIa–induced expression of IL-8 (Figure 6B). In contrast to its marked effect on VIIa signaling, caveolin-1 silencing had a minimal effect on PAR2 agonist peptide-induced IL-8 expression (Figure 6B) and partial inhibitory effect on trypsin-induced IL-8 expression (data not shown).

Discussion
Unlike thrombin and trypsin, VIIa has to bind its cellular receptor, TF, to activate PARs. Therefore, TF-VIIa could only activate PARs that are in the close vicinity of TF. Unless TF and PAR2 are present in high density, it is unlikely that TF-VIIa encounters PAR2 on the cell surface. This raises an important question on how TF-VIIa could activate PAR2-mediated cell signaling. In this study, we show that a fraction of both TF and PAR2 are compartmentalized in cholesterol-rich specialized plasma membrane domains, lipid rafts/caveolae, in breast carcinoma cells (MDA-MB-231), a cell system in which TF-VIIa was shown to induce robust PAR2-mediated cell signaling. Depletion or sequestration of plasma membrane cholesterol impaired the TF-VIIa–induced cell signaling in these cells. Silencing of caveolin-1, an integral protein of caveolae, diminished the TF-VIIa–induced cell signaling without affecting the TF-VIIa coagulant activity. Overall, the data presented in this report suggests that sequestration of TF and PAR2 in lipid rafts/caveolae, probably coupled with differential segregation of heterotrimeric G-proteins into these microdomains, facilitates an effective environment for TF-VIIa–induced cell signaling.

Cholesterol plays a critical role in differentiating and maintaining cell surface microdomains of differing lipid composition, particularly sphingolipid rafts. Cholesterol- and sphingolipid-rich rafts in association with a structural protein, caveolin-1, form caveolae, flask-shaped invaginations in the plasma membrane. Because these microdomains have been shown to be enriched with a variety of signaling molecules, it is believed that they play a role in compartmentalizing signaling molecules at the cell surface and modulating signaling functions. Although certain G-protein-coupled receptors and G-proteins are known to be segregated into lipid rafts and caveolae, we are not aware of any report on whether PARs are segregated into lipid rafts. Consistent with our recent observations in fibroblasts, TF in MDA-MB-231 tumor cells is also localized in lipid rafts/caveolae. Further, our studies also show that a fraction of PAR2 is colocalized with TF presumably in lipid rafts/caveolae. Segregation of a fraction of PAR2 with TF into the lipid rafts may facilitate PAR2 access to VIIa bound to the
TF-VIIa cleavage of PAR2 in these microdomains could activate the signaling pathways as these microdomains were also shown to be enriched with certain heterotrimeric G-proteins and a number of other signaling molecules.

The integrity of cholesterol-rich membrane microdomains appears to be critical for the TF-VIIa–induced PAR2-mediated cell signaling. Depletion of membrane cholesterol, which leads to the loss of the caveolar structure as observed by transmission electron microscopy, reduced TF and PAR2 association with low-density membrane microdomains/caveolae, and impaired the TF-VIIa–induced PI hydrolysis and IL-8 gene expression. Although the depletion of membrane cholesterol also impairs the assembly of TF-VIIa complex at the cell surface, this alone cannot explain the complete loss of TF-VIIa–induced cell signaling in the cholesterol-depleted cells. In this context, it is pertinent to point out that cholesterol depletion by 10 mmol/L mβCD treatment reduced the TF-VIIa activation of factor X by ~50%, whereas it completely attenuated the TF-VIIa–induced cell signaling. The importance of the integrity of cholesterol-rich membrane microdomains in the TF-VIIa–induced cell signaling is better illustrated in cells where membrane cholesterol is sequestered rather than depleted.

Filipin, which sequesters the membrane cholesterol by forming cholesterol-filipin complexes, impaired the signaling function of TF-VIIa despite the increased TF-VIIa coagulant activity in these cells. The increased TF-VIIa coagulant activity observed in filipin-treated cells could have been the result of increased concentration of cholesterol in membranes patches because filipin treatment is shown to result in cholesterol aggregation in the membrane or movement of TF from inactive glycosphingolipid-rich microdomains to active anionic phospholipid region of the membrane.

Although exact underlying mechanisms are not fully known, it is well-established that most of the TF activity at the cell surface is encrypted. Ultrastructural localization of TF in smooth muscle cells, activated endothelial cells, and fibroblasts showed that a fraction of TF in these cells is associated with caveolae. Based on the increased TF activity and the enlargement of caveolar structures in smooth muscle cells after their detachment, Mulder et al speculated that caveolae-associated TF might function as a latent pool of procoagulant activity, which can rapidly be activated at sites in which vessel wall integrity is lost. Recently, Lupu et al reported that caveolae may regulate TF activity indirectly through regulation of tissue factor pathology inhibitor activity. These investigators showed caveolin-1 silencing decreased the tissue factor pathology inhibitor activity on activated endothelial cells and thereby increases TF activity by several fold. The data presented in this report show that caveolin-1 silencing had no significant effect on TF coagulant activity at the surface of tumor cells. Similar results were also obtained in lung fibroblasts (data not shown). These data strongly suggest that the caveolae are not negative regulators of TF procoagulant activity, as previously thought. In this context, it may be pertinent to note that, unlike endothelial cells, other cell types synthesize little or no tissue factor pathology inhibitor and most of it was secreted and not associated with the cell surface. Similarly, MDA-MB-231 tumor cells, the cell model system used in the present study, do not produce tissue factor pathology inhibitor (unpublished data of the authors). Thus, it is unlikely that tissue factor pathology inhibitor plays a role in localizing TF into caveolae or modulating TF-VIIa activity or signaling in our model system.

In contrast to its lack of effect on TF coagulant activity, caveolin-1 silencing significantly reduced the TF-VIIa–induced IL-8 gene expression in monolayers of MDA-MB-231 cells treated with varying concentrations of mβCD or filipin. Total RNA was extracted and subjected to Northern blot analysis to probe IL-8 gene expression. Data shown in (A) and (C) represent mean ± SEM from 4 to 6 experiments, and representative Northern blots were shown in (B) and (D). *Significantly (P < 0.05) differs form the value obtained with control (not treated with mβCD or filipin) cells exposed to VIIa.

Figure 4. Membrane cholesterol depletion/modification impairs TF-VIIa–induced IL-8 gene expression. Monolayers of MDA-MB-231 cells were treated with varying concentrations of mβCD (A and B) or filipin (C and D), and then stimulated with VIIa (10 nM) for 90 minutes. Total RNA was extracted and subjected to Northern blot analysis to probe IL-8 gene expression. Data shown in (A) and (C) represent mean ± SEM from 4 to 6 experiments, and representative Northern blots were shown in (B) and (D). *Significantly (P < 0.05) differs from the value obtained with control (not treated with mβCD or filipin) cells exposed to VIIa.
duced cell signaling in tumor cells. It is likely that the loss of the structural integrity of caveolae might have caused the relocalization of TF and PAR2 at the cell surface without affecting their functions per se. Such reorganization could have placed the PAR2 beyond the physical reach of TF-VIIa, thus impairing the ability of TF-VIIa to activate PAR2. This hypothesis is supported by our observation that showed lipid raft/caveolar disruption by cholesterol binding drugs or caveolin-1 silencing impaired TF-VIIa cleavage of PAR2 but not trypsin cleavage of PAR2.

A number of G-protein-coupled receptors and their interacting proteins are known to be localized within lipid rafts and caveolin-enriched microdomains.16,21,33,34 Further, caveolins may act as scaffolding proteins to cluster and regulate signaling molecules targeted to the caveolae, such as Src-family tyrosine kinase, EGF receptor, and G-protein α sub-units.12 Therefore, disruption of lipid rafts or caveolae may also cause uncoupling of G-proteins and other signaling proteins with their membrane receptor. This could explain why lipid raft disruption by cholesterol binding drugs had no effect on trypsin activation of PAR2 at the cell surface but impaired trypsin-induced PAR2-mediated cell signaling.

In summary, the data presented herein demonstrate that TF localization at the cell membrane could influence different functions of TF differently. While caveolar localization of TF had no influence in propagating the procoagulant activity of TF, it is essential in supporting the TF-VIIa–induced cell signaling. The cholesterol content in the plasma membrane and not the structural integrity of the rafts/caveolae appear to influence the TF procoagulant activity, whereas the structural

**Figure 5.** Membrane cholesterol depletion/modification specifically impairs TF-VIIa activation of PAR2. A, MDA231 cells cultured in glass-chambered slides were loaded with Fluo-4 and treated with mβCD (10 mmol/L for 1 hour) or filipin (5 μg/mL for 15 minutes). After mounting the slide on a microscope stage, live fluorescence images were obtained for 30 seconds, and then control vehicle or PAR2 agonist peptide (SLIGKV, 25 μmol/L) was added to the cells, and the imaging was continued for 3 minutes. Increase in intracellular Ca²⁺ levels were shown as increase in fluorescence at 530 nm emission wavelength. B, COS-7 cells transiently transfected with TF plus AP-PAR2 expression vectors were treated with a control vehicle, mβCD (10 mmol/L for 1 hour), or filipin (5 μg/mL for 15 minutes), and thereafter exposed to VIIa or trypsin (10 nM) for 1 hour, then soluble AP activity in the conditioned medium was measured. Results are expressed as AP activity in cells treated with VIIa or trypsin minus that in cells not treated with proteases, but subjected to mβCD and filipin treatments (n=3 ±SEM). *Significantly (P<0.05) differs from the control cells exposed to the same protease.
major components of the rafts/caveolae, their structure is
peptide agonists) can access the PAR2 that is not readily
subsequent to FVIIa exposure because these proteases (or
facilitate the cells to respond to other proteases in tandem or
explain why some cells that express both TF and functional
PAR2 peptide-mediated cell signaling, whereas PAR2 localization is
membrane microdomains is critical for TF-VIIa to trigger
integrity of cholesterol-rich, caveolin-1–enriched microdo-
mains plays a greater role in regulating the TF-VIIa signaling.
Our data also suggest that localization of TF and PAR2 in
addition to reducing atherosclerosis.

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Disclosure

None.

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Figure 6. Effect of caveolin-1 silencing on TF-VIIa cleavage of PAR2 and PAR2-mediated IL-8 gene expression: A, MDA-MB-
231 cells were transfected with caveolin-1 siRNA or control siRNA. After 48 hours, the cells were infected with adenovirus
expressing AP-PAR2. Sixteen hours later, monolayers of MDA
231 were treated with control vehicle (NT), VIIa, or trypsin (10
mM) for 1 hour. The conditioned medium collected and AP activ-
ity was determined. Alkaline phosphatase activity released with
trypsin (10 mM) in control siRNA transfected cells was arbitrarily
as 100% of PAR2 cleavage (n=6, mean ±SD). B, MDA-
MB-231 cells were transfected with control siRNA or caveolin
siRNA were treated with control vehicle (NT), VIIa (10 nM), or
PAR2 peptide agonist (50

9262
mol/L) for 90 minutes. Total RNA
was extracted and subjected to Northern blot analysis for IL-8
mRNA expression, and hybridization signal intensities were
quantified using phosphor imaging (n=3). *Significantly (P<0.05)
differs from the corresponding control.

sensitive to the amount of cholesterol in the membrane, and
therefore the concentration of cholesterol in the membrane
not only regulates the TF-VIIa coagulant function but also the
TF-VIIa–induced cell signaling. Because recent studies sug-
gest that TF-VIIa, in addition to triggering blood coagulation,
plays a role in many pathophysiological processes,1,2 choles-
terol lowering could provide additional health benefits, in
addition to reducing atherosclerosis.


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Materials and Methods

Cell Culture
Human breast carcinoma cell line MDA-MB-231 and COS-7 cells were obtained from ATCC (Rockville, MD). The cells were cultured at 37°C in 5% CO₂ humidified incubator using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1.0 % penicillin/streptomycin/glutamine. Cells were cultured to 90% confluence, and then rendered quiescent by serum deprivation for overnight, and washed once with serum-free medium before they were used in experiments.

Plasma Membrane Cholesterol Depletion/Modification and Measurement of Membrane Cholesterol
Quiescent monolayers of MDA-MB-231 cells were washed once with serum-free medium and treated with varying concentrations of methyl-ß-cyclodextrin (mßCD) (0 to 10 mM) for 1 h or filipin III (0 to 5.0 µg/ml) for 15 min in the serum-free medium at 37°C. Following the treatment, cells were washed twice with buffer B (10 mM HEPES, 0.15 M NaCl, 4 mM KCl, 11 mM glucose, 5 mM Ca++, 1 mg/ml BSA, pH 7.4), removed from the dish by scraping, and pelleted by centrifugation at 3,000 rpm for 10 min using Eppendorf microcentrifuge. Cell pellets were suspended in 300 µl of TBS (20 mM Tris.HCl, 0.15 M NaCl, pH 7.0) containing 0.5% Tween-20. One hundred µl of sample was used for the determination of membrane cholesterol using Cholesterol CII kit (Wako Chemicals USA, Inc.).

Separation of Lipid Rafts by Buoyant Density Gradient Centrifugation
MDA-MB-231 cells cultured in T-75 flasks were treated with a control vehicle, mßCD (10 mM for 1 h) or filipin (5 µg/ml for 15 min) (3 flasks/treatment). At the end of the treatment, the cells were washed with serum-free DMEM and harvested by scraping the cells in 1.0 ml of ice-cold MES
buffer (25 mM MES, 150 mM NaCl, pH 6.5) containing 1.0% Triton X-100. Cells were homogenized with 10 strokes in a loose-fitting dounce homogenizer, and 1.0 ml of ice-cold 80% sucrose in MES buffer was added to homogenates. The homogenates were placed in the bottom of an ultracentrifuge tube (12 ml size), and layered with 30% and 5% sucrose (6:4 ratio) in MES buffer. The gradient was centrifuged at 150,000 X g for 18 h at 4°C. The gradient was fractionated manually by collecting 1 ml fractions from the top with minimal disturbance. The protein concentration of each fraction was determined using a BCA protein assay kit (Pierce Chemical Co, Rockford, IL). From each fraction, 30 µg protein was precipitated using 10% w/v trichloroacetic acid (TCA) and the pellets were suspended in 50 µl of SDS-PAGE loading buffer.

To determine the distribution of TF and PAR2 between low density (rafts) and soluble fractions, fractions 3 to 9 were taken as low-density fractions and 10 to 12 as soluble fractions. To estimate relative amounts of antigens present in various fractions, band intensities of immunoblots (exposed to X-ray film) were quantified by laser densitometry (Molecular Imager, Bio-Rad). Antigen levels were normalized to the total cellular protein content. In many experiments, a second set of film was exposed to immunoblots for a longer time to obtain images that are better suited for the presentation.

**Immunoblot Analysis**

Equal amount of protein (~10 µg) from the sucrose gradient fractions were subjected to SDS-PAGE (12% polyacrylamide gels) and transferred to polyvinylidene difluoride membrane (PVDF) using standard procedures. The membrane was blocked with blocking buffer (5% non-fat dry milk in TBS containing 0.1% Tween-20, pH 7.4). The membrane was incubated with primary antibodies for 3 h at room temperature (or overnight at 4°C), followed by three 15-min washings in TBS containing 0.1% Tween-20. Then, the membrane was incubated with appropriate HRP-conjugated secondary antibodies in the blocking buffer for 2 h. After washing
the membrane four times with TBS containing 0.1% Tween-20, the blot was developed using chemiluminescence detection kit (Perkin-Elmer).

**Determination of Cell Surface TF-VIIa Activity**

Monolayers of control, mßCD-, and filipin- treated cells were incubated with VIIa (10 nM) in buffer B for 5 min at 37°C, followed by the addition of substrate factor X (175 nM). At the end of 5 min reaction period, an aliquot was removed into stopping buffer (TBS containing 1 mg/ml BSA and 10 mM EDTA) and factor Xa in the sample was measured in a chromogenic assay as described earlier.\(^1\) Under the above experimental condition, factor X activation was linear for 15 min or more.

**Binding Studies**

Factor VIIa and TF mAB were labeled by using Iodo-Gen (Pierce Biotechnology, Rockford, IL) coated tubes and Na\(^{125}\)I according to the manufacturer's technical bulletin and as described previously.\(^2\) Cell surface binding of \(^{125}\)I-VIIa or \(^{125}\)I-TF mAB (TF9H10) was performed essentially as described in our earlier publication.\(^3\)

**Phosphoinositide Hydrolysis**

Cells cultured in 12-well plate were washed once with serum-free M199 medium and loaded overnight with 2 μCi/ml myo-[2-\(^3\)H]inositol in the same medium. Following the loading, the cells were washed once with the serum-free M199 medium and treated with varying concentrations of either mßCD or filipin, for 1 h and 15 min, respectively, at 37°C. The cells were then washed and treated with 20 mM LiCl\(_2\) in 20 mM HEPES (pH 7.2) containing 1 mg/ml BSA with or without agonist for 2 h. At the end of the treatment, the cells were washed with HEPES buffer and extracted with 20 mM formic acid for 1 h at 4°C. Released total \(^3\)H-inositol phosphates were quantified as described earlier.\(^4\) Briefly, the formic acid extracts containing intracellular inositol
phosphates were loaded onto Dowex AG 1-X8 anion exchange columns (2.5 cm X 1 cm), and the columns were washed with 3.0 ml of 40 mM NH₄OH (pH 9.0), followed by 3.0 ml of 40 mM NH₄ formate before the bound inositol phosphates were eluted with 3.0 ml of 40 mM NH₄ formate containing 0.1 M formic acid. The total ³H-inositol phosphates released were quantified by measuring ³H-radiolabeled counts using a beta counter (Beckman LS6500).

**Measurement of Cytosolic Ca²⁺ Release**

Fluorescence microscopy was used for the measurement of calcium influxes. Fibroblasts were seeded in 8-well chambered cover slips (Nunc, Rochester, NY) at a density of 10,000 cells/well. After 24 h, cells were washed and incubated with 4 µM Fluo-4/AM Ester (Molecular Probes, Inc; Eugene, OR) for 45 min in buffer B in a humidified atmosphere of 37°C/5% CO₂. The cells were then washed with buffer B, and the chambered cover slip was placed on the stage of Nikon Eclipse TE2000-5 (Japan) microscope equipped with a CCD camera. Live images of fluorescence were recorded by exciting the Fluo-4 probe with 488 nm light and monitoring 530 nm emission using Perkin Elmer Ultraview LC1 confocal microscopy system. After 30 sec of image acquisition, the test compound was added to the cells and the recording was continued for a further 250 sec. Results are represented as the change in fluorescence intensity over time.

**Northern Blot Analysis**

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was dissolved in 1 x RNA secure (Ambion, Austin, TX). Ten microgram of RNA was precipitated and dissolved in RNA sample buffer (20 mM MOPS, pH 7.0 containing 1.0 mM EDTA, 5 mM sodium acetate and 4% formaldehyde) and subjected to size fractionation on 1.0 % agarose gel in formaldehyde buffer system. Northern blot analysis was performed essentially as described earlier using ³²P labeled IL-8 cDNA. The hybridized membrane was exposed to X-ray film, and the hybridization signal intensities were quantified by
exposing the membrane to phosphor screen, and analyzing it using a phosphor imager (Molecular Imager, Bio-Rad, Richmond, CA).

**Immunofluorescence Microscopy**

MDA-MB-231 cells were plated in 8-well chambered glass slides (Nunc Inc.). Twenty four h after the plating, they were treated with specific agents as indicated in Results or figure legends, and then fixed for 1 h in 4% paraformaldehyde + 0.1% glutaraldehyde at 4°C in phosphate-buffered saline (PBS). The fixed cells, either nonpermeabilized or permeabilized with 0.1% Triton X-100 in PBS for 10 min, were blocked with 3% goat serum in PBS for 1 h at room temperature. The cells were stained overnight at 4°C with specific antibodies as indicated in figure legends, followed by incubation with Oregon Green- (excitation/emission wavelengths, 490/510 nm) or Rhodamine Red- (excitation/emission wavelength, 590/620 nm) conjugated secondary antibodies for 60 min at room temperature. The immunostained cells were viewed under Nikon Eclipse TE2000-S microscope at 60 X optical lens (oil) at room temperature. Images were acquired by scanning the cells sequentially with 2-µm increments using UltraVIEW LCI scanning confocal system (Perkin Elmer, Boston, MA) with 488- and 568- nm excitation laser lines, and equipped with digital CCD camera (Ultra Pix, Hamamatsu Photonics, Japan) with a resolution of 1344 x 1024 x 12. Perkin Elmer’s ImagingSuite™ (version 5.2) Acquisition & Processing Software was used for the acquisition of images and measuring the co-localization by determining the correlation coefficient of overlap of the green and the red fluorescence. A total of seventy two to 144 cells from 3 or more experiments were selected for colocalization analysis. From each cell, about eight randomly selected rectangular regions (fixed size) of the plasma membrane, covering ~25% of the surface area of the cell, was used for the analysis. The scanned images were imported into Power Point (Office 2000 software) for compilation of figures.
Transmission Electron Microscopy

Following control and experimental treatments, monolayers of MDA-MB-231 cells were fixed for 1 h at 4°C in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Following the fixation, cells were washed thrice with the cacodylate buffer and rinsed once with Milli-Q water. The fixed cells were stained in 1% aqueous uranyl acetate for 30 min in dark at 4°C, washed in deionized water, subsequently dehydrated in graded ethanol and embedded in epoxy resin. Thin sections (0.5 µm) were cut perpendicular to dish. The sections were mounted on copper grids (300 mesh size) and stained in 0.5% aqueous uranyl acetate for 10 min, followed by 2% lead citrate for 5 to 10 min. The grids were washed thoroughly in deionized water, dried, viewed and photographed under an electron microscope (JOEL 3000).

AP-PAR2 Expression Vector

AP-PAR2 expression vector was constructed following a strategy similar to that of AP-PAR1. Briefly, secreted human placental alkaline phosphatase was fused to human PAR2 at Ser31 with an intervening linker region containing the FLAG epitope (DYKDDDD) using the following primers: 5´-GGGGTACCGCCACCATGCTGGGG CCCTGCA-3´ (SEAP forward primer); 5´-CCATCGATGTCATCGTCATCCT TGTA-3´ (SEAP reverse primer); 5´-CCATCGATGATTACAAGGATGACGATGACAGATCCTCTAAAGGAAG AAG-3´ (PAR2 forward primer); and 5´-CGGAATTCTCAATAGGAGG TCTTAACAG-3´ (PAR2 reverse primer). SEAP forward primer has KpnI restriction endonuclease linker on its 5´ end, and SEAP reverse primer and PAR2 forward primer contain the nucleotide sequence for FLAG epitope followed with ClaI site at the its 5´ end. The gel purified PCR products were first cloned into pGEMTeasy vector and then transferred into mammalian vector pcDNA 3 (Invitrogen). After the SEAP-FLAG was cloned into pcDNA3 using the KpnI and EcoRI restriction endonuclease, FLAG-PAR2 was inserted into the pcDNA3 using the ClaI and EcoRI. The amino acid sequence of the fusion region reads as ……GTTDAAHPGDYKDDDD IDDYKDDDRSSKGR↓SLIGKVD… where AP
sequence is underlined, FLAG sequence italicized, and native PAR2 sequence is shown in bold, and ↓ indicates activation cleavage site (amino acids ID are included to introduce Clal restriction site). The entire sequence of the construct was verified to make sure that the construct was devoid of errors.

**Quantification of Soluble Alkaline Phosphatase Released by PAR2 Cleavage**

COS-7 cells were transfected transiently with AP-PAR2 expression plasmid using FUGENE6. MDA-MB-231 cells were transduced with AP-PAR2 adenovirus (10 pfu/cell). Conditioned media collected from AP-PAR2 transfected cells following experimental treatments was centrifuged for 5 min at 14,000 rpm in Eppendorf’s tabletop centrifuge to remove any cell debris. Alkaline phosphatase activity in the supernatant medium was quantified using a chemiluminescence’s substrate from BD Bioscience Great EscAPE SEAP detection kit.

**Inhibition of Caveloin-1 by siRNA**

MDA-MB-231 cells were transfected with control siRNA or caveolin specific siRNAs (Ambion, sense sequences, GGAGUUAGUGGAUUACUGCtt and GGAGAUCGACCUGGUCAACtt, respectively) using SiPort amine. Briefly, when cells reached 70% confluency, cells were washed once with serum-free medium, and then incubated with 200 nM siRNA + 4% SiPort amine in the serum-free medium. After 3 h, an equal volume of the medium containing serum (10% v/v) was added to the cells. Thirty six to 48 h post transfection, the monolayers were serum-deprived overnight before they were subjected to experimental treatments.
References


**Figure I.** Effects of mßCD and filipin treatments on membrane cholesterol content and caveolar structures. Monolayers of MDA-MB-231 cells were treated with varying doses of mßCD or filipin for 1 h and 15 min, respectively. At the end of treatment, cells were washed and used for determining cholesterol content (A) or transmission electron microscopy analysis (B). Data shown in the panel A is mean ± SEM (n = 3).

**Figure II.** Effects of mßCD and filipin treatments on cell surface TF coagulant and prothrombinase activities. Panel A: Monolayers of MDA-MB-231 cells were treated with varying concentrations of mßCD or filipin, for 1 h and 15 min, respectively. After the treatment, the monolayers were washed and then incubated with VIIa (10 nM) and the substrate factor X (175 nM) for 5 min at 37°C to measure the rate of TF-VIIa activation of factor X. * denotes significantly (p <0.05) differs form the control (untreated) (n = 3 to 6, mean ±SEM). Panel B: Monolayers of MDA-MB-231 cells were treated with a control vehicle, mßCD (10 mM for 1 h) or filipin (5 µg/ml for 15 min). After washing the cells in buffer A, prothrombin (100 µg/ml, 1.4 µM), factor Xa (0.1 nM) and factor Va (10 nM) were added to the cells in buffer B, and the rate of thrombin generation was monitored in a chromogenic assay. In the absence of cells, thrombin generation was insignificant (less than 0.1 U/ml thrombin in 10 min). Symbols as follow, (○) control, (●) mßCD-treated, and (■) filipin-treated (n=4 to 6, mean ±SEM).

**Figure III.** Caveolin-1 silencing does not alter TF expression or the coagulant activity. MDA-MB-231 cells were transfected with caveolin-1 siRNA or control siRNA. Forty eight h after transfection, the monolayers were processed for immunoblotting to confirm caveolin-1 knock-down (panel A), and for measurements of 125I-VIIa binding to cell surface TF (panel B) and TF-VIIa activation of factor X (panel C) (n = 4, mean ± SEM).
Figure I

**A**

Bar graph showing the membrane cholesterol content (% control) at various concentrations of mβCD and Filipin. The x-axis represents the concentration levels of mβCD (1.0, 5.0, 10.0 mM) and Filipin (0.1, 1.0, 5.0 µg/ml), while the y-axis represents the membrane cholesterol content. There are bars for Control and treated conditions, with asterisks indicating significant differences.

**B**

Images comparing Control, mβCD-treated, and Filipin-treated conditions. The images show morphological changes before and after treatment.
Figure III

Panel B: 

- Cav-1 si RNA
- Neg. siRNA
- Non-transfected

TF

Cav-1

125I-FVIIa bound (f mole/well)

Control
Neg. siRNA
Cav-1 siRNA

Panel C:

F Xa generated (nM/ml/min)

Control
Neg. siRNA
Cav-1 siRNA

TF

Cav-1