Differential effects of somatostatin on circulating tissue factor procoagulant activity and protein

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First published January 9, 2007; doi:10.1152/ajpendo.00483.2006.—The effects of somatostatin on circulating tissue factor procoagulant activity and protein. 

MATERIALS AND METHODS

Subjects

A total of 28 healthy volunteers participated in four different studies. Each volunteer was studied only once. The subjects’ ages, weights, heights, and body mass indexes are shown in Table 1. None of the subjects had a family history of diabetes or any other endocrine disorder, and none was taking any medication. Informed written consent was obtained from each participant after explanation of the nature, purpose, and potential risks of these studies. The study protocol was approved by the Institutional Review Board of Temple University Hospital, and all study subjects gave informed consent.

Study Design

The study volunteers were admitted to Temple University Hospital’s General Clinical Research Center on the evening before the studies. The studies began at ~8:00 AM after an overnight fast, with the subjects reclining in bed. A short polyethylene catheter was inserted into an antecubital vein for infusion of test substances; another catheter was placed into a contralateral forearm vein for blood sampling. That arm was wrapped with a heating blanket (~70°C) to arterialize venous blood.

The following four studies were performed (Table 1).

Hyperglycemic hyperinsulinemic clamp study (study 1), in which a 20% glucose solution was infused intravenously at variable rates that were adjusted to maintain plasma glucose at ~11 mmol/l (~200 mg/dl), and regular human insulin (Humulin; Eli Lilly, Indianapolis, IN) was infused at a rate of 12 pmol·kg⁻¹·min⁻¹ (2 mU·kg⁻¹·min⁻¹) to raise insulin concentrations to ~1.200 pmol/l (200 μU/ml). Stimu-
loration of endogenous insulin secretion was prevented by continuous infusion of SMS (305 nmol/h). Basal glucagon levels were maintained by continuous intravenous infusion of glucagon (0.25 ng·kg⁻¹·min⁻¹).

Basal glucose/basal insulin (BG/BI) clamps with infusion of SMS.

In the basal glucose/basal insulin (BG/BI) clamps with infusion of SMS (study 2), SMS was infused as described above, and insulin was infused at a rate of 0.33 pmol/kg·min⁻¹. In the basal glucose/basal insulin (BG/BI) clamps without SMS (study 3) but without infusion of insulin, SMS, and glucagon.

HG/HI clamps without SMS. The HG/HI clamps without SMS (study 4) were performed as described above (for study 1) but without infusion of insulin, SMS, and glucagon.

BG/BI clamps without SMS. In the BG/BI clamps without SMS study (study 4), only small amounts of glucose were infused when needed to maintain basal glucose concentrations. Studies 3 and 4 were part of a previously published study (23).

Blood Collections

Small blood samples (0.25 ml) were collected every 30–60 min initially and every 1–2 h later for measurement of blood glucose concentrations. In addition, blood samples were collected from antecubital veins without tourniquet-induced venostasis at 0, 6, 12, 18, and 24 h.

Assays

Plasma glucose was measured with a glucose analyzer using the glucose oxidase method and serum insulin by radioimmunoassay using an antisera with minimal (<0.2%) cross-reactivity with proinsulin (Linco Research, St. Charles, MO). Plasma SMS was measured by radioimmunoassay with a kit from ALPCO (Windham, NH).

Whole blood TF-PCA and TF antigen. TF was measured in whole blood cell lysates with a two-stage clotting assay using recombinant FVIIa (American Diagnostica, Factor X (Enzyme Research Laboratories, South Bend, IN), and normal human plasma containing phospholipid vesicles, as described previously (9, 23). This assay measures cell-bound and microparticle-associated TF in lysed whole blood. Blood samples (1 ml) were drawn into 0.1 vol of 3.8% sodium citrate as anticoagulant and frozen at −70°C until assayed. Blood lysates and cellular components were collected from aliquots of whole blood subjected to three cycles of freezing and thawing followed by centrifugation and washing and were finally suspended in HBSA buffer (10 mmol/l HEPES, 137 mmol/l NaCl, 5.38 mmol/l KCl, 5.55 mmol/l glucose, and 0.1% bovine serum albumin, pH 7.5) as described (12). The final membrane pellet, resuspended in HBSA buffer, was used as a source of TF. TF-PCA was measured using a two-stage clotting assay. Recombinant human TF Ortho Recombiplastin (Ortho Diagnostic Systems, Raritan, NJ) was used as a standard. A log plot of TF vs. clotting time was linear in the 1–1,000 U/ml range (r = 0.99). The interassay coefficient of variation (CV) was 8.6%; the intra-assay CV was 7.0%. The TF antigen (TF-Ag) was determined by using the IMUBIND TF-Ag ELISA (American Diagnostica).

**Table 1. Subjects’ characteristics**

<table>
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<tr>
<th>Sex (M/F)</th>
<th>Age, yr</th>
<th>Height, cm</th>
<th>Weight, kg</th>
<th>BMI, kg/m²</th>
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<tr>
<td>7 M/3 F</td>
<td>38.3±2.7</td>
<td>176.0±2.8</td>
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<tr>
<td>4 M/1 F</td>
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<td>184.9±2.9</td>
<td>103.9±6.0</td>
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<td>5 M/1 F</td>
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<td>173.0±4.8</td>
<td>86.4±6.0</td>
<td>28.9±1.8</td>
</tr>
<tr>
<td>7 M/0 F</td>
<td>28.7±2.6</td>
<td>184.9±2.9</td>
<td>91.0±5.6</td>
<td>27.6±1.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. SMS, somatostatin. *These subjects participated in a previously published study (23). P = not significant in all cases.

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**Fig. 1. Plasma glucose, serum insulin, and serum somatostatin (SMS) concentrations in normal volunteers during 24 h of high-glucose/high-insulin clamping with (n = 7) and without (n = 10) SMS (HG/HI ± SMS) and during 24 h of basal glucose/basal insulin clamping with (n = 5) and without (n = 5) SMS (BG/BI ± SMS). Shown are means ± SE.**
Thrombin-antithrombin complexes and prothrombin fragment 1.2.
Thrombin generation was assessed by determination of thrombin-antithrombin (TAT) and prothrombin fragment 1.2 (F1.2) in plasma both by ELISA (Enzygnost, Dade Behring, Marburg, Germany).

CD40 ligand expression on platelets.
Platelet-rich plasma was obtained from citrated whole blood by centrifugation at 200 g for 15 min. Platelet-rich plasma was diluted threefold with modified HEPES-Tyrodes buffer and incubated with phycoerythrin-labeled anti-human CD154 (CD40 ligand) or correspondingly matched isotype control phycoerythrin mouse IgG1K antibody for 20 min. Cells were fixed in 1% formaldehyde for 10 min at room temperature. The samples were diluted 10-fold with HEPES-Tyrodes buffer and analyzed (10,000 platelets) within 2 h on a BD FACScan flow cytometer. The CD40L-positive platelets were determined by flow cytometry with gating on 1% of isotype control-positive cells.

Monocyte-platelet aggregates.
These were measured as described previously (22). Citrated whole blood samples were diluted 1:4 with modified HEPES-Tyrodes buffer, fixed (1.1% formaldehyde), and incubated with FITC-labeled anti-human CD41a (GPIIb-IIIa) antibody for 20 min at room temperature. As control, corresponding matched-isotype FITC-labeled anti-mouse IgG1K antibody was used. Samples were analyzed using the BD FACScan flow cytometer. The percentages of positive monocyte-platelet aggregates were identified by gating CD41a-positive events based on monocyte size and light scattering properties; 4,000 monocytes were analyzed.

**Statistical Analysis**
A one-way ANOVA was used to test for significant differences between studies with Student-Newman-Keuls post hoc analysis. If data were not normally distributed, the Kruskal-Wallis one-way ANOVA with Dunn’s post hoc analysis was used. To test for differences across time, a one-way repeated-measures ANOVA with Student-Newman-Keuls post hoc analysis was used. Statistical differences between day 1 (baseline) and day 2 (24 h) were determined with a paired t-test and, if not normally distributed, with the Wilcoxon signed rank test. Statistical analyses were performed using SigmaStat for Windows (version 2.0; SPSS, Chicago, IL). Statistical significance was defined as P < 0.05. All results are presented as means ± SE.

**RESULTS**

**Glucose, Insulin, and SMS Levels**
Glucose concentrations were clamped at 5.9 ± 0.1 and 5.5 ± 0.1 mmol/l during the BG/BI studies with and without SMS, respectively, and at 11.6 ± 0.3 and 11.6 ± 0.1 mmol/l.
during the HG/HI studies with and without SMS, respectively (Fig. 1).

Insulin levels were clamped at 69 ± 16 and 63 ± 10 pmol/l during the BG/BI studies with and without SMS, respectively, and at 1,708 ± 240 and 863 ± 257 pmol/l (P < 0.02) during the HG/HI studies with and without SMS, respectively (Fig. 1).

SMS was clamped at 252 ± 3 and 224 ± 33 pmol/l, respectively, during the BG/BI + SMS studies and the HG/ HI + SMS studies (Fig. 1).

**Effect of SMS on Basal TF**

**TF-PCA and TF-Ag.** In the control study (BG/BI × 24 h), TF-PCA remained at basal levels of ~20 U/ml. Addition of SMS raised TF-PCA 1.8-fold (from 23 ± 4 to 42 ± 5 U/ml, P < 0.003; Fig. 2).

Similarly, basal TF-Ag levels remained unchanged in the control study, whereas addition of SMS raised TF-Ag 2.3-fold (from 149 ± 15 to 339 ± 13 pg/ml, P < 0.001; Fig. 2).

**TF on monocytes.** The percentage of monocytes expressing TF was unchanged after 24 h of BG/BI [15.6 ± 5.9 vs. 14.5 ± 5.5%, not significant (NS)]. Addition of SMS raised TF expression by 36% (from 16.6 ± 1.6 to 22.6 ± 1.7%, P < 0.001; Fig. 2).

**Plasma FVIIa, TAT, and F1.2**

The SMS-induced increase in circulating TF-PCA and Ag was associated with a 30% decrease in plasma FVIIa levels (from 69.4 ± 3.8 to 48.9 ± 3.7, P < 0.001), whereas FVIIa levels remained unchanged after 24 h of BG/BI without SMS (Fig. 3).

Basal TAT and F1.2 levels remained unchanged during BG/BI and tended to increase with the addition of SMS (Fig. 3).

**Effect of SMS on HG/HI-stimulated TF**

**TF-PCA and TF-Ag.** During the HG/HI clamps, TF-PCA rose 8.6-fold (from 20 ± 1.1 to 172 ± 5.9 U/ml, P < 0.001; Fig. 4). Coinfusion of SMS abolished the HG/HI-induced increase in TF-PCA; i.e., it reduced TF-PCA to the level seen with BG/BI + SMS (Fig. 4A). TF-PCA increased continuously during the entire 24-h HG/HI study (Fig. 4B).

During the HG/HI clamps, TF-Ag rose 8.6-fold (from 138 ± 13 to 1,195 ± 84 pg/ml, P < 0.001). Coinfusion of SMS suppressed the HG/HI-stimulated increase in TF-Ag by only 26% (from 1,195 ± 84 to 882 ± 80 pg/ml, P < 0.02; Fig. 4C).

**TF-PCA vs. TF-Ag.** Changes in TF-PCA correlated linearly and significantly with changes in TF-Ag for both HG/HI (r = 0.93).

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**Fig. 4.** A: circulating TF-PCA in normal volunteers before (B) and after 24 h of HG/HI with and without SMS. B: serial TF-PCA values during HG/HI with and without SMS. C: circulating TF-antigen (TF-Ag) before (B) and after 24 h of HG/HI with and without SMS. D: correlations between TF-PCA and TF-Ag during 24 h of HG/HI. E: TF expression on monocytes obtained before (B) and after 24 h of HG/HI with and without SMS. Shown are means ± SE.
0.93, \( P < 0.001 \)) and the HG/HI + SMS studies (\( r = 0.93, \ P < 0.001 \)). However, SMS reduced the slope of the HG/HI correlation, indicating that SMS had drastically reduced the bioactivity of TF-Ag, i.e., its ability to activate Factor X in the presence of FVII (Fig. 4D).

TF on monocytes. TF expression on monocytes increased by 61% (from 19.8 ± 5.5 to 31.9 ± 5.0%, \( P < 0.002 \)) after 24 h of HG/HI. Coinfusion of SMS suppressed TF expression on monocytes to the level seen during BG/BI + SMS (\( +37\% \) vs. +36%; Figs. 2 and 4E).

Plasma FVIIa, TAT and F1.2, and CD40L

HG/HI reduced plasma FVIIa levels by 39% (from 83.6 ± 7.7 to 51.1 ± 5.1 mU/ml, \( P < 0.001 \)). SMS reduced this decrease to 26% (from 57.7 ± 8.8 to 42.5 ± 6.5 mU/ml, \( P < 0.003 \); Fig. 5).

SMS completely abolished the HG/HI-induced 6.5-fold increase of TAT, an indicator of thrombin formation. SMS completely abolished the 2.2-fold increase (from 1.0 ± 0.1 to 2.2 ± 0.3 nmol/l, \( P < 0.004 \)) of F1.2 seen with HG/HI alone (Fig. 5).

F1.2 is generated during the formation of thrombin from prothrombin and is therefore another sensitive indicator of thrombin generation.

Platelet CD40L Expression

To examine the effect of SMS on platelet activation, we determined platelet expression of CD40L. More than 95% of CD40L in the circulation is stored in platelets, is expressed rapidly on platelet surfaces after platelet activation, and is released into the circulation (2). HG/HI has been shown to activate platelets, as reflected by an increase in platelet CD40L expression (23).

During HG/HI, CD40L expression rose ~40%, from 32.9 ± 3.0 to 46.1 ± 3.7% (\( P < 0.001 \)). Coinfusion of SMS completely prevented this HG/HI-mediated increase in CD40L (29.2 ± 1.5 vs. 29.8 ± 1.7%). During BG/BI, coinfusion of SMS had no effect on CD40L expression (23.7 ± 3.5 vs. 25.2 ± 2.9%, NS).

Monocyte-Platelet Aggregates

Monocyte-platelet aggregates (%positive cells) did not change during 24 h of BG/BI (63.3 ± 8.9 vs. 63.4 ± 9.0%, NS) but increased during 24 h of HG/HI (from 72.3 ± 4.6 to 84.6 ± 2.5%, \( P < 0.05 \)). Coinfusion of SMS suppressed the HG/HI-mediated rise (74.2 ± 7.9 vs. 63.1 ± 7.8%, NS).

DISCUSSION

Effects of SMS on Basal TF

In this study, we found that SMS increased basal TF-PCA and TF-Ag about twofold in membranes and microparticles from circulating blood cells. Moreover, SMS produced an ~30% increase in TF expression on monocytes, the only hematopoietic cell with established ability to synthesize TF. These studies suggested that SMS stimulated TF synthesis.

SMS was also associated with a decrease in plasma concentration of the coagulation factor FVIIa. This, we believe, was due to enhanced cellular TF expression and increased binding of FVIIa to TF (23).

More males than females participated in these studies. We consider it unlikely, however, that this sex imbalance influenced the results, as neither sex nor age, nor weight, had any effect on TF-PCA in our previous studies (23).

Effects of SMS on HG/HI-Stimulated Circulating TF

An important observation was that SMS completely suppressed the large increase in TF-PCA caused by HG/HI. Specifically, the 8.6-fold increase in TF-PCA was reduced to the approximately twofold increase seen with SMS under conditions of BG/BI (Fig. 4A).

SMS also inhibited the increase in TF expression on monocytes exposed to HG/HI and completely suppressed, or at least
reduced, downstream events. For instance, the decrease in plasma concentrations of FVIIa was less with than without SMS, and the H/H-induced increases in circulating levels of FII.2, TAT, and CD40L expression on platelets and monocyte-platelet aggregates were completely suppressed. Surprisingly, however, the ~75% suppression of circulating TF-PCA was associated with only an ~25% suppression of TF-Ag. Hence, H/H plus SMS inhibited membrane-bound TF procoagulant activity more than TF-Ag. Thus, when SMS was infused, the circulating TF had less biological activity; i.e., it had reduced ability to bind FVIIa and to activate Factor X to Factor Xa. This was reflected in decreased thrombin generation (decreased FII.2 and TAT; Fig. 5).

Monocytes possess SMS receptors (5). Inhibitory effects of SMS similar to those on HG/H-induced increases in TF-PCA and TF-Ag have been observed by others. For instance, SMS has been shown to inhibit lipopolysaccharide-stimulated monocyte secretion of IL-6, TNF-α, and IL-1β, and surface expression of human leukocyte antigen-DR (a feature of activated monocytes) (14) and formyl-peptide stimulated superoxide release (12).

The reason for the marked disproportionate decrease in TF-PCA relative to that of TF-Ag is uncertain. It is known, however, that the functional ability of TF is dependent on the membrane microenvironment in which it exists. Under normal conditions, most cell surface TF is encrypted; i.e., the TF binds to FVIIa but is not capable of initiating coagulation or activating Factor X (24, 13). Monocyte activation with ionophore A23187 (which also enhances surface phosphatidylserine expression) markedly enhances the TF activity (4). Platelets play a major role in enhancing and decrypting TF activity (13). TF activity and decryption are regulated by interaction with microparticles (MP) derived from activated platelets and monocytes. Platelet MP can bind monocytes and neutrophils to produce highly procoagulant surfaces, interactions that require phosphatidylserine and p-selectin (6). A major functional effect of fusion of activated platelets with monocyte MP is increased proteolytic activity of the TF-VIIa complex (6). Thus, inhibition of platelet activation by SMS provides an explanation for the markedly decreased TF-PCA. Our studies show that both platelet CD40L expression and monocyte-platelet aggregates, two markers of platelet activation, are decreased by SMS (Fig. 5). Moreover, several previous studies have shown that SMS inhibits platelets (18, 15).

Clinical Relevance

TF is the primary physiological mechanism for initiation of blood coagulation (16, 10, 11). The earlier concept that blood coagulation is initiated only after vascular damage when blood is exposed to TF present in the adventitia of blood vessels or in atherosclerotic plaques has recently been broadened by the realization that there is a pool of blood cell-associated TF that is potentially thrombogenic (8, 16).

We (1) and others (17) have reported that patients with T2DM have increased blood levels of TF-PCA. These patients are usually hyperglycemic and hyperinsulinemic. They are known to be in a hypercoagulable state, which is likely to contribute to their greatly increased risk for arterial thrombotic events. It needs to be emphasized that blood coagulation is a complex process that is highly regulated by pro- and anticoagulant factors. Nevertheless, our demonstration that SMS prevented HG/H-induced activation of the TF pathway of blood coagulation may be useful therapeutically to reduce the procoagulant state and acute ASVD events in insulin-resistant, hyperinsulinemic diabetic patients.

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