Catabolism of Streptokinase and Polyethylene Glycol-Streptokinase: Evidence for Transport of Intact Forms Through the Biliary System in the Mouse

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The catabolism of streptokinase (SK) and polyethylene glycol derivatives of SK (PEG-SK) were studied in mice. The clearance and catabolism of SK-plasmin (SK:Pm) and PEG-SK:Pm activator complexes were also investigated. Native 125I-SK cleared rapidly ($t_{1/2} = 15$ minutes) from the circulation, with the majority of the ligand accumulating in the liver and gastrointestinal (GI) tract and a substantial fraction also localizing in the kidneys. SK, which was removed from the plasma by the liver, was secreted into bile and then the GI tract. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that 125I-SK recovered from liver and bile was homogeneous and of the same molecular weight (mol wt $\approx 50,200$) as native SK. PEG-125I-SK cleared slowly ($t_{1/2}$ greater than 200 minutes), with more than 80% of the preparation localizing in liver and GI tract. The PEG-125I-SK secreted into the bile was also intact. The bile containing 125I-SK was incubated with stoichiometric amounts of plasminogen and electrophoresed under nondenaturing conditions. This study demonstrated that the secreted SK was able to form SK:Pm complexes. SDS-PAGE also showed activation of 125I-Pg that was incubated with recovered bile containing the SK. 125I-SK:Pm catabolism was also studied. In these experiments, the mol wt $\approx 42,000$ fragment obtained when SK is cleaved by plasmin was found in the bile. This fragment of 125I-SK was not recovered as part of a complex with plasmin, consistent with our previous observations that catabolism of SK:Pm involves transfer of the plasmin to plasma proteinase inhibitors while SK is catabolized independently. By contrast, when PEG-125I-SK:Pm was injected into mice, only intact PEG-125I-SK was found in the bile, consistent with our previous observations that the PEG derivatization blocks its degradation by plasmin.

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The STREPTOKINASE (SK), a widely used thrombolytic drug, is a well-characterized bacterial protein that forms high affinity stoichiometric complexes with human plasminogen (Pg). On complex formation with SK, a change in conformation within the molecule results in active site specificity distinct from that of plasmin. The Pg originally present in the complex is itself rapidly converted to plasmin. The SK-plasmin activator complex (SK:Pm) cleaves the Arg 560-Val 561 peptide bond of Pg generating plasmin. Pg activation by SK is species-specific; eg, murine Pg does not react with SK. SK has been used as a fibrinolytic agent for several decades in the treatment of acute myocardial infarction, deep-vein thrombosis, and other thrombosis-related pathogenic states.

Some limitations to SK therapy have been imposed by its bacterial origin. SK therapy is occasionally complicated by immune responses from patients possessing moderate to high titers of anti-SK antibodies. Polyethylene glycol (PEG) conjugation to proteins substantially alters properties such as antigenic recognition, and stability. Derivatization blocks its degradation by plasmin.

The clearance mechanisms of SK, PEG-SK, and their respective complexes with Pm are not well-understood, and the fate of SK cleared alone or in complex with Pm has not been investigated in detail. The objectives of this study were to explore the clearance pathway of SK and PEG-SK in detail, characterize their respective Pm complexes, and examine the effect of Pm on SK in vivo.

MATERIALS AND METHODS

Reagents. Bovine serum albumin, ammediol, and 3,4-dichloroisoumarin (DCI) were obtained from Sigma Chemical Co (St Louis, MO); 1,1-carbonyldimidazole and iodobeads were obtained from Pierce Co (Rockford, IL). Na125I was purchased from New England Nuclear (Boston, MA). Lactoperoxidase coupled to Sepharose was obtained from P-L Biochemicals, Inc (Milwaukee, WI). The Kabikinase (KabiCo, Stockholm, Sweden) product H-D-Val-L-Leu-L-Lys-p-nitroanilide-2HCl (S-2251) was used as the plasmin substrate and obtained from Helena Laboratories (Beaumont, TX). The Aldrich Co (Milwaukee, WI) supplied the PEG of molecular weight (mol wt) 1,900 (PEG-2). The Fisher Co supplied Spectrapor dialysis tubing (Spectrum Medical, Inc, Los Angeles, CA). All reagents were of the finest grade available.

Proteins. Pg was purified from human plasma by affinity chromatography on lysine-Sepharose as described by Deutsch and Mertz and modified by the method of Brockway and Castellino. SK (Kabikinase) was obtained from the Kabik Co, and purified by the method of Brockway and Castellino. The homogeneity of the SK was verified by analysis using a mono Q ion exchange column on a Fast Protein Liquid Chromatography Pharmacia System (Pharmacia, LKB, Piscataway, NJ). The column was eluted with a buffer system consisting of NaP, 50 mmol/L; sodium borate, 10 mmol/L, pH 8.0 (Buffer A), and a limit buffer identical to Buffer A except for the inclusion of NaCl, 1.0 mol/L.

Protein concentrations. Concentrations of purified proteins were determined using the following extinction coefficients ($\lambda = 280$ nm) and mol wts: SK, A $1%/1$ cm $= 7.5$, mol wt $\approx 50,200$; Pg, A $1%/1$ cm $= 16.8$, mol wt $\approx 92,000$.

Protein radiolabeling. Proteins were radiolabeled with 1.0 mCi Na125I according to the method provided by the Pierce Co. All material used in experiments was freshly labeled, and no preparation demonstrated more than 5% nonprecipitable counts after precipitation with 10% trichloroacetic acid. Radioactivity was measured in an LKB Clnia Gamma Counter (Pharmacia).

PEG activation. PEG was activated according to the method of...
Beauchamp et al. PEG was dissolved in dioxane at 37°C at a final concentration of 50 mmol/L. 1,1'-Carbonyldimidazole was added to yield a final concentration of 0.5 mol/L and the solution was incubated at 37°C for 2 hours with stirring. PEG solutions were dialyzed extensively against water using Spectrapor mol wt ~ 1,000 inclusion limits. Once activated, PEG preparations were lyophilized and stored at −20°C. PEG was coupled to SK under alkaline conditions: 1 μmol/L SK in 10 mmol/L sodium borate, pH 8.5 for 48 hours at 4°C with 40 mmol/L activated PEG.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gradient ammiodial gel electrophoresis, 5% to 15%, was performed according to the method of Bury. Proteolytically active samples were incubated with 50 μL DCl and then reduced with 10 mg/mL dithiothreitol (DTT) in SDS sample buffer. Nondenaturating PAGE was also performed. Autoradiographs were made with Kodak XAR-Omatic film (Eastman Kodak, Rochester, NY).

Plasma elimination studies. Female CD-1 mice were injected intraperitoneally with 50 μL of 100 mg/mL ketamine, 10 mg/mL acepromazine mixed at a ratio of 2:1 for clearance experiments. Radiolabeled proteins were injected into the lateral tail vein of the mice and 25-μL blood samples were obtained from retroorbital venous plexus punctures 5 to 10 seconds after injection and at various intervals. Radiolabeled ligands were injected in the presence of various unlabeled ligands for some of the studies described. Only freshly labeled ligands were used, and the amounts injected ranged from 0.15 to 30 μg. Heparinized capillary tubes used for blood collection were analyzed in a gamma counter. Plasma elimination studies were always performed at least in duplicate (n = 2 to 24).

Organ distribution, tissue, and plasma studies. These studies were performed in duplicate. Blood samples for electrophoresis were collected from retroorbital bleeds in 100-μL heparinized capillary tubes, mixed with 20 μL 3.7% sodium citrate, and centrifuged in a Fisher microfuge at 4°C for 5 minutes. Plasma was reduced in SDS sample buffer and electrophoresed. Organs were recovered at approximately one half-life after cervical dislocation. Organs were placed on ice and each was washed at least three times before radioactivity was determined. Wash buffer consisted of cold 150 mmol/L NaCl, 20 mmol/L NaF, 10 mmol/L EDTA, pH 7.4. Livers were homogenized in a hand-held glass homogenizer with 10 mL of cold wash buffer. Homogenates were centrifuged at 3,500 rpm at 4°C for 10 minutes. The lipid layer was removed and homogenates were centrifuged at 3,500 rpm and then reduced in 100 μL of SDS sample buffer for SDS-PAGE. Gallbladders were trimmed free of any adhering liver tissue, washed, ruptured, and counted for bile in the gamma counter. The bile volume ranged from 10 to 40 μL. Bile was reduced in SDS-PAGE. The a,M was added to remove plasmin from the a,M-Pm and a free SK fragment of mol wt 42,000 so that its mobility can be compared with the 125I-SK fragment found in bile after injection of 125I-SK:Pm.11 Pg rather than the SK was labeled for some studies.

RESULTS

Plasma elimination of SK and PEG-SK. Clearance of 125I-SK at doses ranging from 0.15 to 30 μg resulted in identical plasma half-lives of approximately 15 minutes (n = 24) (Fig 1). This is in agreement with our previous studies, and clearance closely follows first order kinetics. Clearance of PEG-2-125I-SK (n = 2) demonstrated a half-life of greater than 200 minutes (Fig 1). SDS-PAGE showed that 125I-SK recovered from retroorbital venous plexus blood was not degraded (Fig 2). Additionally, 125I-SK that was cleared by the liver was also undegraded and demonstrated the same mol wt as native 125I-SK (Fig 3). 125I-SK that was present in bile was homogeneous and of the same mol wt as native SK (n = 4). Nondenaturing PAGE of 125I-SK from blood, liver, or bile, and control 125I-SK showed the same mobility (data not shown). The lack of degradation of PEG-125I-SK in bile was also shown by SDS-PAGE (Fig 3).

Organ distribution. Table 1 summarizes the major organ distribution and recovery of catabolized 125I-SK and PEG-2-125I-SK after injection of 30-μg samples in each case. Native SK was cleared primarily by the liver (44.1%), with a substantial portion present in the kidneys (30.9%). Significantly less PEG-125I-SK was removed by the renal clearance (10.9%). A large portion of 125I-SK and PEG-125I-SK was transferred to the gut during clearance, 20.7% and 50.9%, respectively.

Competition studies. Competition studies showed only moderate increases in plasma half-lives when 100- to 5,000-
fold excesses of unlabeled to labeled ligand (0.15 µg) were coinjected (Fig 4). Due to the significant renal component of SK clearance, nephrectomies were performed by ligating the kidneys in an attempt to define more clearly the competition effect (Fig 4). While competitions with a 5,000-fold excess of unlabeled SK in nephrectomized animals prolonged half-lives to some extent, 10,000-fold excess with nephrectomy showed no further competition (data not shown). This inability to saturate clearance pathways with plasminogen activators has been observed previously. In one experiment, 125I-SK was coinjected with a 10,000-fold molar excess of unlabeled SK and a 160,000-fold molar excess of albumin simultaneously with no effect on clearance (data not shown).

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**Fig 1.** Plasma elimination of 125I-SK and PEG-2-125I-SK. The percentage of radiolabeled ligand remaining in circulation was calculated relative to an initial time point drawn 5 seconds after injection. SK was unmodified (△) or coupled to PEG-2 (○).

**Fig 2.** SDS-PAGE of recovered 125I-SK. Lanes a through d represent blood samples taken at 5 seconds, 5 minutes, 10 minutes, and 15 minutes after injection. Lane e is an SK control, lane f is a liver homogenate, and lanes g and h are bile samples.

**Fig 3.** SDS-PAGE of recovered PEG-2-125I-SK. Lane a represents native 125I-SK, lane b PEG-2-125I-SK, lane c PEG-2-125I-SK from bile.
### Table 1. Organ Distribution of SK and PEG-SK Recovered Radioactivity (%)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Autopsy Time</th>
<th>Liver/GB</th>
<th>Kidneys*</th>
<th>Spleen</th>
<th>GI†</th>
<th>Heart</th>
<th>Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK‡</td>
<td>20</td>
<td>44.1</td>
<td>30.9</td>
<td>1.8</td>
<td>20.7</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>PEG-2-SK</td>
<td>170</td>
<td>29.3</td>
<td>10.9</td>
<td>2.1</td>
<td>50.9</td>
<td>3.3</td>
<td>3.5</td>
</tr>
<tr>
<td>SK:Pg§</td>
<td>50</td>
<td>11.0</td>
<td>6.0</td>
<td>2.0</td>
<td>78.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>PEG-2-SK:Pg</td>
<td>65</td>
<td>34.9</td>
<td>18.1</td>
<td>3.6</td>
<td>36.6</td>
<td>3.7</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Abbreviation: GB, gallbladder.
*Bladder included.
†GI, stomach, small and large intestine.
‡SK is the labeled ligand in all cases.
§Data taken from Rajagopalan et al. and included for comparison with the autopsy data for PEG-2-SK:Pm.

Similarly, a 1,000-fold molar excess of human lactoferrin failed to alter plasma elimination of $^{125}$I-SK (data not shown).

**Binding to and activation of plasminogen by catabolized $^{125}$I-SK.** Bile containing the $^{125}$I-SK was also incubated with Pg for 10 minutes at 22°C and then electrophoresed under nondenaturing conditions. Figure 5 illustrates the difference in migration of the $^{125}$I-SK:Pg complex formed with bile-derived $^{125}$I-SK and native $^{125}$I-SK. This study shows that the $^{125}$I-SK which has been recovered from the murine system retains the ability to complex with Pg.

Catalytic amounts of $^{125}$I-SK present in bile were then incubated with $^{125}$I-Pg for 2 hours at 37°C in the presence of 10 mmol/L lysine. SDS-PAGE (Fig 6) showed activation of the $^{125}$I-Pg. While activation of $^{125}$I-Pg by bile-derived $^{125}$I-SK is less extensive than the control, these studies suggest that the recovered SK binds to Pg to produce an activator complex, which retains the ability to cleave Pg. Differences in activation between the control and bile-derived $^{125}$I-SK may be due to incomplete recovery of $^{125}$I-SK bound to gallbladder tissue. Attempts were also made to assay the plasmin generated by use of the substrate S-2251. However, the absorbance of the bile precluded obtaining an accurate end point. Attempts to purify bile-derived $^{125}$I-SK were not technically feasible because the volume of bile was so small.

**Clearance of $^{125}$I-SK-Pg activator complexes.** Activator complexes formed by 1:1 stoichiometric incubation of $^{125}$I-SK and unlabeled Pg were injected at a dose range of 10 to 30 µg per animal, recovered from bile, and electrophoresed. The plasma half-life of these complexes was approximately 3 minutes, with no evidence of dose-dependence consistent with previous reports. Electrophoresis of bile showed that the radioligand from the complex migrated at a position higher on the nondenaturing gradient gel than either native SK or SK:Pg (Fig 7). There were two possible explanations.

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![Fig 4. Plasma elimination of $^{125}$I-SK from circulation with 100- to 5,000-fold molar excesses of unlabeled ligand. The symbols are: control (○); 100-fold excess (▲); 1,000-fold excess (△); 5,000-fold excess (□); and 5,000-fold excess with nephrectomy (▲).](image-url)

![Fig 5. Nondenaturing PAGE of $^{125}$I-SK complexes with Pg. Lane a is native $^{125}$I-SK. Lane b is $^{125}$I-SK recovered from bile and complexed stoichiometrically with Pg.](image-url)
for this altered mobility: (1) the $^{125}$I-SK was still present in some form of complex with plasmin; and (2) the band represented a cleavage product of SK with altered charge. Previous studies showed that murine catabolism of SK:Pm involves transfer of the plasmin moiety to $\alpha_2$M with the SK undergoing catabolism by a distinct mechanism. No evidence exists for formation of a stable ternary intermediate with either murine or human $\alpha_2$M. While these studies were most consistent with the second hypothesis, it was decided to experimentally examine this question. Because incubation of $^{125}$I-SK:Pm complexes with $\alpha_2$M removes the plasmin, this treatment can be used to obtain the SK moiety of the complex. Incubation of the SK:Pm complex results in time-dependent cleavage of the SK to a fragment of mol wt $\sim$ 42,000.

Therefore, in control experiments, $\alpha_2$M was incubated with $^{125}$I-SK:Pm and the preparation electrophoresed in nondenaturing PAGE. The migration of the $^{125}$I-SK:Pm preparation obtained from bile was compared (Fig 8). As can be seen, the $^{125}$I-SK in all cases showed the same mobility. SDS-PAGE was used to confirm the fact that the $^{125}$I-SK originally present in the $^{125}$I-SK:Pm preparations consisted of the $^{125}$I-SK moiety of mol wt $\sim$ 42,000, as we have previously demonstrated (data not shown).

In another series of experiments, SK:$^{125}$I-Pm was injected into mice. These studies clearly indicated that plasmin and SK undergo separate catabolism, since only 4% as much $^{125}$I-plasmin was found in the gallbladder as $^{125}$I-SK when comparable amounts of SK:Pm were injected with SK or plasmin radiolabeled, respectively. Finally, clearance of PEG-$^{125}$I-SK:Pm was studied. PEG-$^{125}$I-SK:Pm activator complexes cleared slowly from circulation with approximately 50% of the material remaining in circulation at 60 minutes (data not shown). The majority of the radioactivity was recovered in the gastrointestinal (GI) tract and liver (Table 1). These studies are consistent with our prior observations, although autopsy data were not previously reported.

**DISCUSSION**

SK remains an important drug in fibrinolytic therapy despite the introduction of newer thrombolytic agents. Previous studies from this laboratory suggest that PEG-SK derivatives have substantially improved therapeutic properties such as reduced antigenicity and prolonged plasma half-lives. The catabolism of plasmin complexes of SK and PEG-SK have been studied in the murine clearance model.
the samples from lanes b through e demonstrated the expected mol wt 42,000 fragment of "I-SK recovered from bile (Fig 7. lane b). SDS-PAGE of the samples from lanes b through e demonstrated the expected mol wt ~ 42,000 carboxyl terminal fragment of SK (data not shown).

However, the fate of SK in these complexes has not been studied in detail.

When SK is infused into patients at therapeutic levels, α2-antiplasmin is the initial inhibitor of the resultant plasmin. However, this inhibitor is rapidly depleted because of its low concentration in plasma, and subsequently α2M is the major antiplasmin.25,26 Similar results are observed in the murine model.21 The catabolism of plasmin in complex with SK has been studied in vivo in the mouse model12,21 and in vitro in murine and human plasma.21,25 In human plasma, the plasmin is transferred preferentially to α2-antiplasmin,25 while in murine plasma the plasmin is preferentially transferred to α2M21,25 with release of free SK observed in both cases.21,25 However, human α2M in the absence of α2-antiplasmin is a potent inhibitor of SK:Pm, and detailed analysis of this reaction confirms the transfer of plasmin to α2M with release of SK in a degraded form of mol wt ~ 42,000.25 Thus, under therapeutic conditions in humans where α2-antiplasmin is depleted, SK:Pm catabolism should involve an interaction with α2M.

For these reasons, the murine model is an appropriate one for study of SK:Pm catabolism, despite the fact that murine α2-antiplasmin is not involved in catabolism of SK:Pm complexes in this species. Moreover, since murine Pg does not react with SK,3 the model allows for the comparison of the catabolism of native SK and the mol wt ~ 42,000 degradation product of SK obtained after SK reacts with Pg.12,20

Using the murine model, we now observe that both SK and its mol wt ~ 42,000 fragment are secreted intact from the liver into the bile. Moreover, native SK retains its ability to complex and activate Pg. PEG-SK catabolism was shown to involve a similar mechanism. This observation is important for two reasons. First, the derivatization of SK lysine residues by PEG prevents degradation of SK so that the mol wt ~ 42,000 fragment is not produced.15 Second, Beckman et al11 have shown that coupling of PEG to superoxide dismutase favors endothelial uptake of the conjugate. Hence, PEG-SK may have shown a significantly different catabolic pattern.

It is somewhat surprising that a protein of bacterial origin such as SK should be secreted by liver in an intact form. Hepatic uptake and transport pathways for a number of mammalian proteins such as albumin, immunoglobulin A (IgA), transferrin, lactoferrin, and asialoglycoproteins have been studied in detail.22-26 In the present studies, we examined the possibility that some of these pathways might be involved in SK catabolism. The competition studies performed do not suggest that bulk phase transport32 or the metalloprotein clearance pathways33,34,35 are involved in the catabolism. It is important to note that non-denaturing PAGE of 125I-SK cleared and recovered in bile showed no change in migration compared with native 125I-SK. Thus, an association with a carrier protein is not evident nor is the acquisition of a secretory component common to receptor-mediated hepatic transport of IgA suggested.39,40

Circulatory proteins found in bile have a variety of potential pathways for transport. They may take transcellular routes across the hepatocyte resulting in direct fusion with the bile canaliculus, or initially be subject to lysosomal degradation and then be deposited in bile via the bile canaliculus. They may follow a paracellular route with or without nonparachymal cell mediation. Additionally, biliary proteins may diffuse laterally across the hepatocyte plasma membrane after insertion into the sinusoid or may be transported from periductal epithelia.33 A range of proteins using these various pathways have been studied as noted above.32,40 Hydrolytic enzymes of the lysosome are likely to preclude it as an intermediate vesicle during biliary transport of SK considering the intact and functional state of SK in bile. Direct transcellular transport and exocytosis into the bile canaliculus, initiated by hepatocyte surface endocytosis, is one plausible route for SK transport. However, the possibility of insertion into the sinusoidal domain of the hepatocyte plasma membrane followed by lateral diffusion to the canaliculus cannot be excluded by our results.

In summary, we have shown that intact and functional SK is secreted by the liver into bile. Although clearing at a slower rate, PEG-SK appears to be removed by the same mechanism. The latter observation is important in view of the potential role of PEG-SK as a fibrinolytic agent.
REFERENCES


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Catabolism of streptokinase and polyethylene glycol-streptokinase: evidence for transport of intact forms through the biliary system in the mouse

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