Supplemental Data

Distinct Functions of Activated Protein C Differentially Attenuate Acute Kidney Injury

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RESULTS

Effect of mutations on APC protease activity and plasma inhibition.

We compared the effect of these mutations on the catalytic efficiency to peptide substrate Glu-Pro-Arg-p-nitroanilide (S-2366) and as shown in Supplemental Table I, these mutations had no significant effect on the kcat/Km. We also assessed the effect of these mutations on interaction with serpin inhibitors as determined by their plasma inhibition and, as shown in Supplemental Table II, there was no significant difference in half-life of these molecules in rat or human plasma when compared to wild-type. Therefore, the mutations had no significant effect in altering the interaction with either synthetic substrates or plasma inhibitors at the active site.

Effect of variants K193E and L8W on PAR-1-dependent signaling and EPCR binding.
We have previously shown that APC induces Ca\(^{2+}\) flux in HUVECS \(^1\). K193E was also highly effective in inducing the PAR-1 dependent Ca\(^{2+}\) flux induced by APC in human endothelial cells (Supplemental Figure IA). In contrast, L8W was unable to induce a PAR-1 dependent response. In repeated experiments, K193E was significantly more effective than wtAPC in these assays of PAR-1 dependent signaling (Supplemental Figure IB). The PAR-1, as well as EPCR-dependency of the wtAPC and K193E effect in this assay was shown using blocking antibodies to both EPCR and PAR-1 as described in the Methods (Supplemental Figure IB). These antibodies have been previously shown to effectively block EPCR and PAR-1 in functional assays \(^2\).

As PAR-1 mediated signaling by APC has been shown to be dependent on EPCR, we determined the binding of L8W to EPCR as described in the Concise Methods. By flow cytometry to EPCR positive cells, L8W retained only an 8% percent of wtAPC binding at 80nM (Supplemental Figure IIA), consistent with a recent study suggesting that EPCR contacts the L8 position \(^3\). K193E and wtAPC bound to EPCR with equivalent affinity (Supplemental Figure IIB).

**Effect of APC on blood pressure following LPS challenge**

Shown in Supplemental Figure IIIA is the time course for the prevention of LPS-induced hypotension following a bolus dose of 100ug/kg APC with complete restoration of mean arterial pressure (MAP) by 3hrs. We assessed whether a lower concentration of APC might be effective and as shown in Figure IIIB, we observed a significant block in the reduction of MAP at a lower dose of 30ug/kg. We observed no alterations on heart rate with any of the three doses of APC (data not shown).
While we previously have shown that improving blood pressure alone in this models does not result in improvement in renal injury, control experiments were performed to determine if reduction in systemic blood pressure independent of LPS would result in a deficit in renal blood flow as determined by the CT method. Hypotension was induced by administration of sodium nitroprusside (SNP, 0.25 µg/kg), which resulted in a reduction in mean arterial pressure (MAP) similar to the reduction induced by LPS (control 120±2 vs. SNP 90.3±1.45 mmHg). Following renal blood flow measurements using CT, we did not observe any effect on renal blood flow (control, 37.78±3.96 vs. SNP, 37.24±2.4 (ml/min/100g tissue). These results are consistent with literature reports that renal blood flow can be maintained in the presence of hypotension, attributable to persistence of autoregulation in the kidney.

**Determination of APC-mediated PAR-1 agonism in the kidney**

Previous studies have demonstrated that APC induces the phosphorylation of extracellular signal-regulated protein kinase (ERK1/2) via PAR-1 activation. To determine whether K193E induced activation of phospho-ERK1/2 in the kidney post administration, kidney tissue was examined for the level of ERK1/2 from animals treated with both K193E and L8W. As shown in Supplemental Figure IV, the level of phospho-ERK1/2 was significantly increased in the K193E-treated animals but not in the L8W-treated animals. Along with the effect of K193E on other PAR-1-mediated markers, such as ADM (Figure 7), these data provide additional support that K193E activates PAR-1 in vivo.

**REFERENCES**


Supplemental Figure I. Determination of PAR-1 mediated properties of variants of APC by Ca flux. (A) Effect of anti-PAR-1 and anti-EPCR antibodies on the signaling of APC by calcium flux determination. Confluent HUVECs were incubated with the PAR-1 blocking antibody (ATAP2, 25 ug/ml) or the EPCR blocking antibody (RCR-252, 20 ug/ml) for 2 hours then cells were stimulated with 30 nM APC. The level of fluorescent counts were made relative to the time zero values for each experiment. (B) Effect of anti-PAR-1 and anti-EPCR antibodies on the signaling of APC and K193E quantified from calcium flux determination. The area under the curve (AUC) was determined using FLIPR software (v2.12, Molecular Devices, Sunnyvale). Results are mean ± sem, n=8.
Supplemental Figure II. Determination EPCR binding of variant APCs. (A) Determination of the amount of APC binding to EPCR-positive HUVEC cells by flow cytometry as described in the Concise Methods. The binding of APC in this assay is inhibited by the anti-EPCR antibody (data not shown). (B) Dose response for APC and K193E binding to EPCR. The Kd values were determined in a direct binding assay to EPCR as described in the Concise Methods.
Supplemental Figure III. Effect of APC on blood pressure in the LPS model. (A) Time course for the protection of APC at 100µg/kg bolus on mean arterial pressure (MAP). (B) Effect of different concentrations of APC (10, 30 and 100 µg/kg) on MAP at 3h post LPS administration. * P ≤ 0.05 as compared to sham, # P ≤ 0.05 as compared to LPS-treated group. Data are represented as mean ± sem, n= 6 per data group.
Supplemental Figure IV. Effect of APC variants on ERK1/2 in the kidney. Animals were treated with the APC variants and the kidney tissues examined for phosho-ERK1/2 as described previously. Data are represented as mean ± sem, n= 4 per data group.
Supplemental Figure V. Effect of APC and variants on renal pathology

Representative H & E staining of kidney sections from sham and APC treated animals. Lower magnification examples of treatments as described in Figure 6.
Supplemental Table I. Protease activity of wtAPC and variants

<table>
<thead>
<tr>
<th>Group</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$k_{cat} / K_m$ (mM$^{-1}$ • sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>0.98 ± 0.02</td>
<td>148 ± 2</td>
<td>151</td>
</tr>
<tr>
<td>APC-L8W</td>
<td>0.98 ± 0.03</td>
<td>144 ± 2</td>
<td>147</td>
</tr>
<tr>
<td>APC-K193E</td>
<td>0.83 ± 0.01</td>
<td>130 ± 1</td>
<td>157</td>
</tr>
</tbody>
</table>

*Activity was determined by measuring S-2366 amidolytic activity of APCs

Supplemental Table II. In vitro Plasma Half-life Determination

<table>
<thead>
<tr>
<th>APC</th>
<th>Plasma Half-life (min)</th>
<th>Human</th>
<th>Rat</th>
</tr>
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<tbody>
<tr>
<td>APC</td>
<td></td>
<td>23.2 ± 0.5</td>
<td>11.1 ± 0.1</td>
</tr>
<tr>
<td>APC-L8W</td>
<td></td>
<td>23.9 ± 0.3</td>
<td>10.0 ± 0.2</td>
</tr>
<tr>
<td>APC-K193E</td>
<td></td>
<td>20.2 ± 0.3</td>
<td>9.7 ± 0.1</td>
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</tbody>
</table>

$n = 4$

The half-life ($t_{1/2}$) was calculated by nonlinear regression analysis of the decay curves using the equation $t_{1/2} = \ln (2) / k_1 \text{ (app)}$ where $k_1 \text{ (app)}$ = the apparent first-order rate constant for inactivation.