Recombinant Activated Protein C Attenuates Endothelial Injury and Inhibits Procoagulant Microparticles Release in Baboon Heatstroke

Abderrezak Bouchama, Corinne Kunzelmann, Mohammed Dehbi, Aaron Kwaasi, Abdelmoneim Eldali, Fatiha Zobairi, Jean-Marie Freyssinet, Dominique de Prost

Objectives—We tested the hypothesis that the antithrombotic and cytoprotective effects of recombinant human activated protein C (rhAPC) protect baboons against the lethal effects of heatstroke.

Methods and Results—Fourteen anesthetized baboons assigned randomly to rhAPC (n = 7) or control group (n = 7) were heat-stressed in a prewarmed incubator at 44 to 47°C until systolic blood pressure fell below 90 mm Hg, which signaled severe heatstroke. rhAPC was administered intravenously (24 μg/kg/h) for 12 hours at onset of heatstroke. Heat stress induced coagulation and fibrinolysis activation as evidenced by a significant increase from baseline levels in plasma levels of thrombin-antithrombin (TAT) complexes, tissue plasminogen activator, and D-dimer. Heat stress elicited cell activation/injury as assessed by the release of interleukin (IL)-6, soluble thrombomodulin, and procoagulant microparticles (MPs). rhAPC did not significantly reduce heatstroke-induced thrombin generation, and D-dimer and had no effect on fibrinolytic activity. In contrast, rhAPC infusion attenuated significantly the plasma rise of IL-6 and inhibited the release of soluble thrombomodulin and MPs as compared with control group. No difference in survival was observed between rhAPC-treated and control group.

Conclusions—rhAPC given to heatstroke baboons provided cytoprotection, but had no effect on heatstroke-induced coagulation activation and fibrin formation. Inhibition of MPs by rhAPC suggested a novel mechanism of action for this protein. (Arterioscler Thromb Vasc Biol. 2008;28:1318-1325)

Key Words: APC ■ heatstroke ■ hyperthermia ■ inflammation ■ procoagulant microparticles ■ primates

Heatstroke is a life-threatening condition characterized by a rapid rise in core temperature above 40°C and neurological disturbances, such as delirium, convulsions or coma, following exposure to a high ambient temperature. Despite cooling and optimal supportive care, heatstroke can progress to multiple organ injury/dysfunction (MOSD) and death.

The host inflammatory and hemostatic responses to severe heat stress are closely associated with MOSD and death. Endothelial injury and widespread microthrombi with hemorrhage and necrosis in most organs of the body are characteristic features in heatstroke victims at necropsy. Increased circulating proinflammatory cytokines was documented in human and experimental heatstroke, and their levels correlated with outcome. In laboratory rats, administration of IL-1 receptor antagonist at onset of heatstroke prevents severe arterial hypotension and improves survival. These observations suggest that endothelial cell injury, disseminated intravascular coagulation (DIC), and excessive inflammation are major pathological mechanisms in heatstroke.

Human activated protein C (APC) is an important natural anticoagulant protein with a number of beneficial cellular effects mediated either directly or through its interaction with endothelial protein C receptor and protease–activated receptor-1. These cytoprotective effects include antiinflammatory and antiapoptotic actions and endothelial barrier function protection. In a randomized clinical trial of patients with severe sepsis, rhAPC significantly decreased mortality. Recent observations suggest that therapeutic intervention with rhAPC can also alter the clinical course of heatstroke and improve survival. In humans, rhAPC given to sporadic cases of severe heatstroke resulted in resolution of MOSD and improved survival without bleeding complications. In a rat heatstroke model, administration of rhAPC attenuated the inflammatory response, normalized the coagulopathy, and thereby improved survival. However, this evidence remains insufficient to justify its use in human victims of heatstroke particularly as, because of interspecies
differences, extrapolation of data from small laboratory animals cannot predict reliably the human responses.

We have recently developed a nonhuman primate model of heatstroke that mimics human heatstroke with the intent to assess novel therapy. The findings from nonhuman primates, more closely related to humans than small laboratory animals, may have direct applicability in humans and therefore form the basis for clinical trial. Using this model, we tested the hypothesis that administration of rhAPC may attenuate the DIC and excessive inflammation, to minimize MOSD and improve survival. Microparticles (MPs), soluble thrombomodulin (sTM), and interleukin-6 (IL-6) were used to assess cellular activation/injury, including endothelial cells and inflammation, respectively. MPs are plasma membrane-derived vesicles shed by most of the cell types of the vascular compartment, including endothelial cells on procoagulant, inflammatory, and apoptotic cellular activation. Hence, MPs reflect cell injury/death in vivo as demonstrated in many conditions, particularly those associated with vascular endothelium injury such as acute myocardial infarction, eclampsia, and diabetes. Thrombomodulin is a membrane-bound glycoprotein expressed at the surface of endothelial cells and also exists in soluble form in the plasma. Increased sTM may reflect increased membrane thrombomodulin expression or increased proteolytic cleavage and subsequent release into the circulation. Accordingly, an increased sTM level is considered as a marker of endothelial cell activation/injury in various disorders associated with inflammation and vascular damage in human and experimental primate models.

Methods

Baboons

The study protocol was approved by the KFSHRC Basic Research Committee and the Animal Care and Use Committee. The animals were handled in accordance with the American Physiological Society Guiding Principles in the Care and Use of Animals. Moreover, the number of animals was kept to the strict minimum necessary to test our hypothesis while addressing animal welfare.

On the day of the experiment, juvenile baboons (Papio hamadryas) weighing 4 to 6 kg were anesthetized, and arterial and venous catheters were inserted as described previously. Rectal temperature was measured with a rectal thermometer probe calibrated from 0 to 70°C. Vital signs were monitored continuously and recorded every 15 minutes for a period of 24 hour using a bedside monitor (Hewlett Packard).

Experimental Protocol

Induction of Severe Heatstroke

The animals were assigned randomly to treatment with rhAPC (n=7) or control (n=7) group and subjected to environmental heat stress in a prewarmed neonatal incubator maintained at 44 to 47°C until systolic blood pressure fell to <90 mm Hg, which was taken as the onset of systemic signs of severe heatstroke.

Cooling and Resuscitation

The animals were then removed from the incubator and allowed to cool passively at an ambient temperature of 26 to 29°C. A 10-mL bolus of normal saline was given as needed to maintain a mean arterial pressure >60 mm Hg. Baboons surviving for 7 days were considered permanent survivors.

rhAPC Treatment and Investigations

The treatment group received rhAPC (Drotrecogin alfa activated, Eli-Lilly) at a dose of 24 μg/kg/h intravenously at a constant rate for 12 hour, starting at onset of heatstroke. The dose of rhAPC was based on a dose-response relationship established in humans. The control group received a vehicle consisting of normal saline and 0.1% human albumin for 12 hour.

Collection of Blood Samples

Blood samples were collected in EDTA and citrate-treated tubes at baseline (B), and at the onset of heatstroke (T0), T1, T2, T3, T18, and T45 hours.

Arterial blood gases (corrected for core temperature), complete blood count, liver, renal, cardiac, and coagulation profiles were determined immediately using automated devices. Plasma was obtained by centrifugation at 3500 g (20 minutes at 15°C) as well as by a 2-step centrifugation procedure (1500 g for 10 minutes, then 12,000 g for 2 minutes) to make platelet-poor plasma. Aliquots were stored at −80°C until assayed.

Coagulation and Fibrinogen Assays

Prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen assays were performed by standard methods using automated coagulation analyzer (BCS, Dade-Behring). D-dimer was quantitated using a latex-enhanced, immunoturbidimetric test (D-dimer PLUS, Dade-Behring). Functional activity of α2 antiplasmin (α2 AP), plasminogen activator inhibitor (PAI), and antithrombin (AT) were measured in plasma using Berichrom reagents (Dade-Behring) on an automated BCS analyzer (BCS, Dade-Behring). Plasma antigen levels of protein C, free protein S, and tissue plasminogen activator (tPA) were measured using Asse-rachrom ELISA kits (Diagnostica Stago), and thrombin-antithrombin complexes were assayed using Enzygnost TAT micro (Behring).

Inflammation and Endothelial Cell Activation Markers

Plasma IL-6 and sTM were assayed using ELISA kits (Quantikine, R&D Systems and Diagnostica Stago, respectively) according to the manufacturers’ instructions.

MPs Measurement and Quantification of TF Activity on TF-Bound MPs

MPs were isolated from platelet-poor plasma and captured by immobilized annexin V as previously described. The procoagulant phospholipid content (phosphatidylinerine [PS]) was determined by a prothrombinase assay using factor Xa (50 pm), factor Va (360 pm), prothrombin (1.3 μm/L), and 2.3 μm/L CaCl2 for 15 minutes at 37°C. The reaction was stopped by an excess of EDTA, and linear absorbance changes were recorded at 405 nm after addition of Chromozym TH (Roche Diagnostics) and converted to thrombin concentration by using a reference curve constructed with liposome of known PS concentrations. The amount of MPs was expressed as nanomolar phosphatidylycerine equivalent (nM PS eq).

Tissue Factor (TF) activit on MPs was measured after capture of the latter onto immobilized HTF1 monoclonal antibody against TF (a kind gift from Dr S.D. Carson, Omaha, Nebr) using the TF activity assay. TF was quantified through its ability to promote the activation of factor X (150 nmol/L) by FVIIa (5 nmol/L) in the presence of 5 mmol/L CaCl2 using the chromogenic substrate of FXa. The linear absorbance changes were converted to concentrations of FXa generated in this assay by reference to a standard curve.

Thermal Calculations and Statistical Analysis

Heat stress was quantified as described previously (please see supplemental materials, available online at http://atvb.ahajournals. org). All quantitative data are presented as median and interquartile ranges (IQR 25 to 75th percentile) unless stated otherwise, and their comparisons were performed using Kruskal-Wallis test. Comparisons between groups during the course of the observation period were performed using repeated measurements analysis of variance. Differences were considered significant at P<0.05. Pearson correlation coefficients were used to determine the degree of the linear
Effects of rhAPC on Coagulation and Fibrinolysis

As compared with baseline, heatstroke elicited DIC assessed by plasma markers namely significantly prolonged PT, aPTT, elevated D-dimer, and decreased platelet count (Table). The infusion of rhAPC was associated with a slightly prolonged aPTT and lower circulating D-dimer levels as compared with control group; this was not statistically significant.

Coagulation

Heatstroke-enhanced thrombin generation as evidenced by 4- and 6-fold increase from baseline levels in plasma TAT levels in rhAPC-treated animals and control baboons, respectively ($P<0.01$ for both groups; Figure 2A). There were slightly less TAT complexes in rhAPC-treated animals than in the control group, but these differences were not statistically significant.

An early and sustained decrease from baseline levels of protein C, free protein S and AT was noted in both groups (Figure 2A). rhAPC-treatment attenuated the decrease from baseline levels of Protein C ($P<0.01$), whereas the decrease in control group was not significant ($P>0.05$).

Fibrinolysis

rhAPC infusion did not alter heatstroke-induced fibrinolysis assessed by tissue-type plasminogen activator (t-PA), PAI, and $\alpha_2$-antiplasmin (Figure 2B). Heatstroke enhanced t-PA concentration by 8- to 9-fold from baseline values ($P<0.01$ for both groups). The acceleration of t-PA generation was accompanied by decrease in circulating $\alpha_2$-antiplasmin, a physiological inhibitor of the active fibrinolytic enzyme plasmin. There was significant inverse correlation between the time course of $\alpha_2$-antiplasmin and t-PA ($r=-0.565, P=0.002$) suggesting that generated t-PA induced the formation of plasmin. A rise in PAI activity was noted in both groups at T +3 hours and was sustained until T +18 hours with identical kinetics but lower magnitude in control group; this was not statistically significant (Figure 2B).

Effects of rhAPC on Cell Activation/Injury

rhAPC infusion attenuated significantly heatstroke-induced inflammation, cell activation/injury including endothelial cells as assessed by IL-6, MPs, and sTM release, respectively (Figure 3). Heatstroke elicited systemic inflammation as evidenced by an early and sustained increase of circulating IL-6 in both groups (Figure 3). The magnitude of IL-6 increase was lower in rhAPC-treated animals as compared with control animals, reaching statistical difference at T +18 hours: 45 (44 to 174) and 277 (234 to 440) pg/mL, respectively; ($P=0.04$).

rhAPC infusion significantly attenuated heatstroke-induced endothelial cell activation/injury as assessed by sTM compared with control group ($P=0.02$, ANOVA-repeated measures).

Circulating MPs were detected at baseline in both groups (Figure 3). The plasma concentration increased markedly by approximately 5- to 6-fold at T +1 hour, to reach a peak at T +3 hours before returning to baseline at T +45 hours in control animals. Infusion of rhAPC limited significantly the release of MPs into the circulation as compared with control group ($P=0.04$, ANOVA-repeated measures). There was a

Results

Development of Heatstroke

The animals in both groups were subjected to comparable heat stress indicated by maximal core temperature, time spent above 40.4°C and heat load (supplemental Table I). All animals responded with hyperthermia, tachycardia, and hypotension (Figure 1). There were no significant differences between rhAPC-treated and control animals over time in temperature, heart rate, and arterial blood pressure. There was also no significant difference between the 2 groups in amount of fluid given as a bolus to maintain MAP >60 mm Hg, urine output, and arterial oxygen concentration suggesting that rhAPC administration did not affect vascular permeability (Table). Infusion of rhAPC did not result in any bleeding complications, although intracerebral bleeding, a common site of rhAPC adverse effect, could not be ruled out in animals that succumbed while still under the influence of anesthesia.

Figure 1. Core temperature, systolic blood pressure, and heart rate in control and rhAPC-treated baboons at baseline (B) and after heatstroke. Values represent mean±SE in core temperature, systolic blood pressure (SBP) and heart rate at baseline (B) and after heat exposure. The difference between the 2 groups was tested by ANOVA-repeated measurements. NS indicates not significant. Arrow indicates both onset of heatstroke and rhAPC administration.

relationship between the continuous variables. Kaplan–Meier plot and log-rank-test were used for survival analysis.
significant correlation between circulating MPs and sTM levels in control and treated groups \((r=0.815, \ P<0.0001\) and \(r=0.646, \ P<0.0001\), respectively).

TF activity expressed on MPs was detectable at baseline in all baboons, and increased markedly in both groups after heat stress by approximately 3- to 4-fold (from baseline levels, \(\ P<0.05\)). rhAPC-treated baboons displayed high levels of TF-containing MPs up to T + 3 hours returning to baseline levels at T + 18 hours except in a single animal. This baboon exhibited a striking increase in TF-MP concentrations (6- to 15-fold) from the onset of heatstroke that was sustained up to T + 18 hours (Figure 3).

### Effects of rhAPC on Organ Injury/Dysfunction and Outcome

Heatstroke induced multiple organ system injury/dysfunction in kidneys, liver, heart, and skeletal muscles (Table). No significant difference was noted in the magnitude of increase in any of the biochemical markers of organ injury/dysfunction in rhAPC-treated or control animals. Three animals in the control and 2 in the treatment group survived (intent-to-treat analysis; \(\ P<0.05\); Figure 4). The median time of survival from onset of heatstroke to death was similar in both groups 2.95 (1.25 to 3.8) and 4.65 (2.92 to 14.9) h, respectively, \(\ P=0.33\).

### Table. Effects of rhAPC on Markers of Cell Injury and Organ System Dysfunction in Heatstroke Baboons

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>T + 3 h</th>
<th>T + 18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine, (\mu)mol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>54 (39 to 56)</td>
<td>120 (103 to 134)</td>
<td>105 (80 to 120)</td>
</tr>
<tr>
<td>rhAPC</td>
<td>55 (52 to 62)</td>
<td>192 (141 to 210)</td>
<td>107 (82 to 259)</td>
</tr>
<tr>
<td>CK, U/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>463 (259 to 571)</td>
<td>2077 (1107 to 2232)</td>
<td>6344 (3445 to 6658)</td>
</tr>
<tr>
<td>rhAPC</td>
<td>433 (362 to 656)</td>
<td>1504 (1355 to 1765)</td>
<td>2843 (2200 to 3325)</td>
</tr>
<tr>
<td>LDH, U/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>396 (384 to 481)</td>
<td>1746 (1459 to 2239)</td>
<td>2902 (948 to 3523)</td>
</tr>
<tr>
<td>rhAPC</td>
<td>456 (388 to 490)</td>
<td>1670 (1550 to 2463)</td>
<td>2010 (1879 to 3350)</td>
</tr>
<tr>
<td>AST, U/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>26 (18 to 32)</td>
<td>193 (115 to 242)</td>
<td>227 (123 to 325)</td>
</tr>
<tr>
<td>rhAPC</td>
<td>27 (24 to 35)</td>
<td>204 (121 to 250)</td>
<td>173 (119 to 362)</td>
</tr>
<tr>
<td>Troponin, (\mu)g/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.01 (0.01 to 0.01)</td>
<td>0.89 (0.43 to 5.45)</td>
<td>0.09 (0.03 to 0.28)</td>
</tr>
<tr>
<td>rhAPC</td>
<td>0.01 (0.01 to 0.06)</td>
<td>0.70 (0.38 to 3.69)</td>
<td>0.06 (0.02 to 0.21)</td>
</tr>
<tr>
<td>(\text{PaO}_2/\text{FiO}_2), mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>332 (323 to 341)</td>
<td>457 (427 to 457)</td>
<td>385 (334 to 436)</td>
</tr>
<tr>
<td>rhAPC</td>
<td>331 (317 to 344)</td>
<td>321 (309 to 333)</td>
<td>367 (324 to 410)</td>
</tr>
<tr>
<td>PT, sec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.8 (12.4 to 13.3)</td>
<td>18.8 (17 to 27)</td>
<td>15.8 (14.9 to 16)</td>
</tr>
<tr>
<td>rhAPC</td>
<td>11.8 (11.4 to 12.6)</td>
<td>19.0 (16.7 to 19.4)</td>
<td>16.1 (15.6 to 23.1)</td>
</tr>
<tr>
<td>aPTT, sec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>35 (33 to 39)</td>
<td>49 (45 to 62)</td>
<td>43 (41 to 44)</td>
</tr>
<tr>
<td>rhAPC</td>
<td>32 (30 to 33)</td>
<td>65 (54 to 66)</td>
<td>49 (42 to 71)</td>
</tr>
<tr>
<td>D dimer, ug/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>145 (63 to 224)</td>
<td>806 (441 to 2793)</td>
<td>502 (293 to 634)</td>
</tr>
<tr>
<td>rhAPC</td>
<td>111 (103 to 226)</td>
<td>605 (397 to 798)</td>
<td>521 (418 to 604)</td>
</tr>
<tr>
<td>Platelet, 10(^9)/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>528 (498 to 545)</td>
<td>254 (219 to 312)</td>
<td>173 (115 to 283)</td>
</tr>
<tr>
<td>rhAPC</td>
<td>453 (403 to 487)</td>
<td>252 (173 to 278)</td>
<td>83 (78 to 156)</td>
</tr>
<tr>
<td>Fibrinogen, g/L*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.2 (1.1 to 1.3)</td>
<td>0.95 (0.8 to 1.2)</td>
<td>1.6 (1.5 to 1.6)</td>
</tr>
<tr>
<td>rhAPC</td>
<td>1.2 (1.0 to 1.3)</td>
<td>1.0 (1.0 to 1.2)</td>
<td>1.2 (1.2 to 1.3)</td>
</tr>
</tbody>
</table>

Values represent median (IQR) in plasma creatinine, creatine kinase (CK), lactate dehydrogenase (LDH), aspartic aminotransferase (AST), troponin, partial pressure of oxygen and fraction of inspired oxygen (\(\text{PaO}_2/\text{FiO}_2\)), and prothrombin time (PT), activated partial thromboplastin time (aPTT), D-dimer, fibrinogen, and platelet count at baseline (B) and after heatstroke. No significant difference was observed in any of the variables (except fibrinogen indicated by the asterisk) between the 2 groups tested by ANOVA repeated measurements.
Figure 2. A, Time course of thrombin-antithrombin (TAT) complexes, protein C, free protein S, and antithrombin in control and rhAPC-treated baboons at baseline (B) and after heatstroke. Values represent mean±SE. The difference between the 2 groups was tested by ANOVA-repeated measurements. NS indicates not significant. B, Time course of tissue plasminogen activator (tPA) and α2-antiplasmin and plasminogen activator inhibitor (PAI) in control and rhAPC-treated baboons at baseline (B) and after heatstroke. Values represent mean±SE. The difference between the 2 groups was tested by ANOVA-repeated measurements. NS indicates not significant.
Early death, between 33 minutes and 4 hours attributable to refractory shock, was noted in 4 treated and 3 control animals, respectively. One baboon in each group died of multiple organ failure at 25 hours (control) and 90 hours (rhAPC-treated).

Discussion

The findings in the present study confirmed previous observations that heatstroke elicits systemic inflammation, activation, of coagulation and fibrinolysis, and endothelial cell injury.7–9,25 They also showed for the first time that heatstroke induced a concomitant and parallel increase of circulating MPs, unique markers of in vivo cell activation/injury on procoagulant, inflammatory or apoptotic stimulation.16,17 In addition, they provide evidence that a proportion of these MPs bear functionally active TF, suggesting that they may have contributed to the excessive coagulation activation of heatstroke.

Our results also demonstrated that administration of rhAPC intravenously for 12 hours to baboons with severe heatstroke, starting at the onset of heatstroke, was associated with marked reduction in circulating MPs together with sTM and attenuation in plasma rise of IL-6, during the infusion period compared with control animals. This suggested that rhAPC minimized cell activation/injury, particularly of the endothelium and lessened, albeit moderately, the host inflammatory response to heat stress. In contrast, rhAPC did not reduce heatstroke-induced tissue factor activity, thrombin generation, and fibrin formation. Finally, the present study revealed also that treatment of heatstroke with rhAPC did not improve survival.

Previous studies have reported that rhAPC reduces mortality in patients with high risk of death attributable to sepsis, although this benefit was subsequently challenged, particularly because the protective mechanisms were unclear.11,13,26,27 Consistent with its anticoagulant and anti thrombotic effects, rhAPC infusion in humans with sepsis led to a decrease in TAT complexes, concomitant with a more rapid normalization of protein C levels, resulting in a decline in fibrin formation as assessed by circulating D-dimer concentration.11,12 The results of the present investigation show that rhAPC exerted neither comparable inhibitory effect on coagulation activation and fibrin formation in baboons with heatstroke, nor improved survival.

One likely reason for the lack of demonstrable antithrombotic effect and clinical benefit may have been that the dose of rhAPC (24 μg/kg/h) in continuous intravenous infusion without loading dose, as derived from human sepsis studies, was not suitable in the setting of severe heatstroke.11,23 Human heatstroke is a medical emergency with rapid (hours) progression to MOSD and death.1,28,29 During heat waves, 40% to 60% of patients with heatstroke are hospitalized or found dead within 1 day of reported onset of illness.28,29 Moreover, when patients are hospitalized and receive optimal care, the mortality from heatstroke can exceed 60%.2,3 Although, in the present study, all the conditions for optimal treatment of heatstroke (immediate withdrawal from the environmental heat, cooling, and prompt fluid resuscitation) were met, the high mortality was not ameliorated. This reinforces the notion that the current therapeutic approach is inadequate for improved outcome in human heatstroke. Further, the rapid evolution to death (less than 4 hours in 4 rhAPC-treated baboons), suggested that a loading dose or higher infusion rate of rhAPC might have been more effective.23 This therapeutic approach is supported by studies both in baboons lethally challenged with Escherichia coli and rodent model of heatstroke, which showed that a large bolus or higher infusion rate (10- to 160-fold that used in this experiment), were required for demonstrable survival benefit.10 However, such an approach could be associated with an increase in hemorrhagic complications and would require careful evaluation.

Another possibility is that our sample size was too small to detect a survival benefit, as similar dosage used in human sepsis study required 1690 patients to detect a 10% difference in survival. However, this is unlikely as previous study using nonhuman primates demonstrated a striking improvement in...
outcome in a smaller number of animals than in the present investigation.10 Nonetheless, despite that no survival benefit was observed in our study, the fact that rhAPC treatment afforded cytoprotection supports our hypothesis and warrants further study using a higher rhAPC dosage.

Recent human and experimental studies provided evidence for additional beneficial cellular effects of rhAPC distinct from its antithrombotic activity, namely via modulation of gene expression for inflammation and apoptotic pathways and protection of microvasculature barrier function.13 In the present study, we demonstrated that administration of rhAPC unequivocally minimize cellular activation/injury in baboons with severe heatstroke as measured by 3 markers: MPs, sTM, and IL-6. Moreover, the strong correlation between circulating MPs and sTM suggests that this cytoprotective effect might have included the endothelial cells.

Emerging evidence suggests that circulating MPs are not only procoagulant but may act as true long-range signals for inflammation or apoptosis on cells different from their cell of origin.16,17 MPs can carry membrane antigens and cytoplasmic contents from their cell of origin including TF, adhesion molecules, and apoptotic ligands.16–19 Accordingly, MPs can initiate or perpetuate coagulation, inflammation, and apoptosis and thereby contribute to many disease processes. Therefore lowering the MP levels or modulating their characteristics has become an important therapeutic goal.16,17,20

The role of MPs in the pathophysiology of heatstroke and the clinical benefit of their modulation by rhAPC are yet to be elucidated. Nonetheless, in the present study, a large amount of procoagulant MPs, as measured by prothrombinase assay, is released in the circulation, which in addition express functionally active TF. Intravital microscopy in living mice demonstrated that TF associated MPs contributed to fibrin formation during thrombus propagation and growth.30 Also, MPs shed under pathological conditions were shown to induce endothelial injury/dysfunction.16–17 Consequently, one can postulate that MPs triggered or amplified the coagulation activation and contributed to the endothelial injury/dysfunction observed in heatstroke baboons. The present observation that rhAPC was associated with decreased total MPs and their procoagulant potential together with marked reduction of sTM raises the possibility of a novel mechanism of action of this protein through modulation of membrane-shed MPs that deserves further studies. These investigations should include the phenotyping of MPs aimed at identifying their main cellular origin(s), provided relevant cross-reactive antibodies are available. Recent in vitro data demonstrating that addition of rhAPC to monocyte or endothelial cells stimulated with lipopolysaccharide (LPS) and tumor necrosis factor (TNF)α enhances significantly the release of MPs bearing functional EPCR support this hypothesis.31

In conclusion, our findings show that rhAPC given to baboons suffering severe heatstroke provides cytoprotection, although without conferring survival benefit. Additional animal studies using an rhAPC dose regimen suitable for the explosive course of severe heatstroke, namely bolus or early higher dose, are warranted, and this may result in improved clinical outcome providing that such an approach would not result in higher rate of hemorrhagic complications.

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Disclosures
None.

References


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**Thermal calculations**

Heat stress was quantified by determining the heat load, a product of magnitude of \( T_C \) above 40.4°C and duration of hyperthermia as described previously\(^9\). Core body temperature was recorded at 15min intervals and heat load (°C-min.) was calculated as \( \Sigma \) time interval (min.) \( [T_C (°C) \text{ above } 40.4°C - 40.4°C] \).

**Table I. Heat stress responses in control and rhAPC -treated baboons subjected to heat (Ta=44-47°C, rh= 33-39%)**

<table>
<thead>
<tr>
<th>Heat Response</th>
<th>Control</th>
<th>rhAPC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>4.2 (4.1-4.4)</td>
<td>4.0 (3.9-4.2)</td>
<td>0.22</td>
</tr>
<tr>
<td>Weight loss %</td>
<td>4.8 (0.0-7.3)</td>
<td>2.5 (0.0-4.9)</td>
<td>0.88</td>
</tr>
<tr>
<td>Tc maximum (°C)</td>
<td>43.7 (43.5-43.8)</td>
<td>43.7 (43.6-44.1)</td>
<td>0.61</td>
</tr>
<tr>
<td>Time to &gt;40.4°C (min)</td>
<td>150 (150-195)</td>
<td>195 (140-225)</td>
<td>0.70</td>
</tr>
<tr>
<td>Heat load (°C .min)</td>
<td>317 (276-402)</td>
<td>357 (288-422)</td>
<td>0.65</td>
</tr>
<tr>
<td>Duration of heat exposure</td>
<td>255 (200-260)</td>
<td>240 (180-273)</td>
<td>0.95</td>
</tr>
</tbody>
</table>

\( T_c \) = core temperature, \( T_a \) = ambient temperature, \( r_h \) = relative humidity

Statistical comparisons were made by Kruskal Wallis test between control and rhAPC-treated baboons. Values are median and interquartile range (25-75\(^{th}\) percentile).