Journal of Surgical Research **175,** 18–23 (2012)
doi:10.1016/j.jss.2011.06.055

ASSOCIATION FOR ACADEMIC SURGERY

Effect of Protein C Gene Mutation on Coagulation and Inflammation in Hemorrhagic Shock

Sigrid Burruss, M.D., Arthur Andakyan, B.S., Sergei Romanov, B.S., Natalya Semiletova, Ph.D., and Henry Cryer, M.D., Ph.D.[1]

Department of Surgery, University of California, Los Angeles, California

Originally submitted January 8, 2011; accepted for publication June 22, 2011

Introduction. **Trauma patients are at high risk of complications and death from coagulopathy and inflammatory organ failure. Recent evidence implicates protein C (PC) as a key mediator of this process. We hypothesized that a mutation in the PC gene would ameliorate the inflammatory and coagulopathic response to hemorrhagic shock (HS) and resuscitation.**

Methods. **FHH wild type and PC mutant rats underwent controlled hemorrhage for 120 min with 70% of blood volume removed. Rats were resuscitated with Ringers lactate (2x shed blood volume) and shed blood. Animals were sacrificed 4 h post-HS. Controls were untreated naïve rats.**

Results. **AST and NFkB lung protein levels were elevated similarly in both WT and mutants compared with naïve rats. Plasma fibrinogen levels decreased significantly with progression of HS compared with baseline (BL) levels and returned towards normal 4 h after resuscitation. PC activity was similar in both groups at BL (0.5 ± 0.08 *versus* 0.6 ± 0.14; $P = 0.14$) and decreased from BL by 53% ± 24% in WT ($P = 0.08$), by 67% ± 11% in mutants ($P = 0.03$) at sacrifice, and was not different between groups ($P = 0.29$).**

Conclusions. **Our model of HS and resuscitation produced a hypocoaguable, hyperinflammatory state with increased levels of NFkB and decreased levels of fibrinogen and PC levels. The mutated PC did not appear to alter these responses in our model of HS and resuscitation.**

Key Words: **trauma; hemorrhagic shock; coagulopathy; inflammation; protein C.**

INTRODUCTION

Massive hemorrhage remains a major cause of potentially preventable death following civilian and military trauma [1–5]. Multiple studies including data from our institution show that common consequences of hemorrhagic shock include persistence and exacerbation of uncontrollable bleeding from refractory coagulopathy leading to early death from exsanguination (Burruss S, Tillou A, Hiatt J, Cryer H. Factors associated with mortality in massively transfused trauma patients. AAST 2010. Boston MA. Poster Presentation). Even when coagulopathy is successfully controlled, multiple complications often occur in survivors of massive blood transfusion.

The mechanism of this coagulopathy remains poorly understood. Protein C (PC) activation is a promising mechanism that appears to be operative after hemorrhagic shock but before resuscitation. PC is activated when thrombin binds to thrombomodulin. Activated PC exerts its anticoagulant effects by binding to and inactivating Factors Va and VIIIa and by binding to the cell surface receptor, protease-activated receptor-1 (PAR-1), to provide both anticoagulant and several cytoprotective effects [6–8]. Recent evidence implicates PC as a key mediator in the process of coagulopathy and inflammatory organ failure [9–11].

This study was undertaken to further investigate the effects of PC in a model of rat hemorrhagic shock and resuscitation. We hypothesized that a mutation in the PC gene that is predicted to prevent the conformational change that exposes the active site of the enzyme upon activation would ameliorate the coagulopathic and inflammatory responses to hemorrhagic shock and resuscitation.

[1] To whom correspondence and reprint requests should be addressed at UCLA General Surgery, Box 957432, RRUMC #8501P, Los Angeles, CA 90095. E-mail: hcryer@mednet.ucla.edu.

0022-4804/$36.00

METHODS

Animal Preparation

FHH rat mutants were produced by the Medical College of Wisconsin using *N*-ethyl-*N*-nitrosourea (ENU), a potent mutagen. Using prediction models, a mutation in the PC gene changing arginine to glycine at the 312 amino acid position was predicted to create a nonfunctional activated PC protein (Table 1). Specifically, PolyPhen and Sorting Intolerant from Tolerant (SIFT) modeling programs were utilized. PolyPhen predicted this particular mutation to be "probably damaging," which indicates the best result from this program and has the lowest false positive rate. Changes in an amino acid sequence are entered into a probabilistic classifier to determine if the amino acid replacement occurs in a site that is critical for protein function [12]. This includes changes that are likely to destroy the hydrophobic core of a protein, electrostatic interactions, or other interactions with ligands. SIFT predicted this particular mutation to "affect protein function." This takes into consideration whether the substitution reduces protein stability or function by accounting for solvent accessibility and free energy among other things between the new and old amino acid [13].

FHH wildtype (WT) and mutant rats, 15-wk-old, (Medical College of Wisconsin) with weight ranging from 300 to 390 g, were anesthetized with 1 uL per 1 gm of pentobarbital (50 mg/mL); 3% isoflurane was used throughout the procedure as necessary to maintain appropriate anesthesia. The left femoral artery was cannulated with a polyethylene catheter (PE50; Solomon Scientific, San Antonio, TX, USA) and hemodynamic data was collected using a micro-medic blood pressure analyzer. The left femoral vein was cannulated with a PE50 catheter and was used for blood withdrawal. A body temperature probe (Oakton IR thermometer, Vernon Hills, IL, USA) was used to monitor body temperature. Temperature was maintained at 36.6°C using heating pad and heating lamp. Four animals were included in the wildtype group and eight animals were included in the mutant group. Naive animals ($n = 3$) underwent no interventions.

Experimental Design

Controlled hemorrhage was achieved by blood withdrawal through the femoral vein. One mL aliquots were removed at 5 min intervals until 70% of blood volume was removed. Rats were resuscitated with Ringers lactate (2x shed blood volume) and shed blood after 2 h of shock and sacrificed 4 h later.

Tissue Harvest

At the time of sacrifice, a midline incision was made and the lung and liver were removed. One portion was placed in formalin for histology and another was washed with PBS and stored at 80°C until time of tissue analysis.

Blood Sampling and Analyses

Venous blood samples (1 mL) were collected at baseline, at initiation of shock (SBP 35 mmHg), after 60 and 120 min of shock, after Ringers lactate resuscitation, after shed blood resuscitation, and at time of sacrifice. Blood samples were spun at −4°C at 3000 RPM and plasma was removed. Plasma was immediately analyzed for fibrinogen and factor V levels using StaCompact from Diagnostima Stago (Parsippany, NJ, USA). Not every plasma sample from each time point was analyzed due to limited amount of available volume. Samples were subsequently stored at −80°C. Plasma samples were analyzed for PC activity using the Biophen chromogenic assay (Aniara Corp., Mason, OH, USA). PC is measured following a specific activation with Protac, an enzyme extracted from snake venom. Activated PC then specifically cleaves the chromogenic substrate SaPC-21 (specific for activated PC) releasing paranitroanaline, which is measured by a color change at 405 nm. There is a direct relationship between the color development and PC activity in the tested sample [14].

Tissue Analysis

RNA from each sample was extracted using RNA-Bee and converted into cDNA using Superscript III one step reverse transcriptase-PCR with platinum Taq high fidelity, following the manufacture's protocol (Invitrogen, Carlsbad, CA, USA). Primers were designed for specific genes using primer software using the National Center for Biotechnology Information (NCBI) database and the size of their PCR products were less than 500 bp. Primers for EGR-1, thrombin, and Protein C were given as follows: EGR-1 (sense 5′-CTCCCAACACTG ACATTTTTCC-3′, antisense 5′-GAAATTACGCATGCAGATTCG-3′), thrombin (sense 5′-CTCCTCGAGCGTGGCCGGCTG-3′, antisense 5′-CTCCGCGTCGGTGGTGCGTC-3′), protein C (sense 5′-GAATT TTCCATGTGGGAAACTGG-3′, antisense 5′-CATCCTTGATTCTGT CGCTTTG-3′) all from Invitrogen. PCR was performed on a Chromo4 thermal cycler (MJ/BioRad, Hercules, CA, USA) with 40 cycles of 45 s at 94°C and 1 min at 55°C. Control RNA provided by Invitrogen was used as an internal control. Each sample was run and analyzed using the Fotodyne and Total lab TL100 program. Results were normalized against a housekeeping gene and presented in ng.

Western blots were performed for liver tissue. Primary antibodies against Bcl-xL (Stressgen, Kampenhout, Belgium) and NFkB (Cell Signaling, Danvers, MA, USA) were used. Whole cell lysates were prepared from harvested liver samples using a mammalian cell lysis kit (Sigma, St. Louis, MO, USA). Protein content was assessed by spectrophotometry and equalized among samples. Forty ug of protein sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% gels and transferred onto nitrocellulose membranes (BioRad). Membrane was blocked with 5% milk in TBS solution and incubated with Bcl-xL antibody (1:1000 in 5% milk) overnight. The primary antibody was detected by incubating membrane with antirabbit secondary antibody (1:20,000 with 5% milk) for 1 h at room temperature. For NFkB, membranes were blocked with 5% milk and incubated with primary antibody (1:1000, 2.5% milk) overnight. Primary antibodies were detected using antirabbit secondary antibody (1:20,000, 2.5% milk) for 1 h. Chemiluminescence detection was performed with HRP Chemiluminescent Substrate Reagent Kit Novex ECL (Invitrogen). Standard photographic procedure was used to develop films and the detected bands were analyzed with densitometer scanning from TotalLab.

TABLE 1

Protein C Mutant

Genome position	Mutation	AA position	AA change	Affected domain
Chr18:24590568	T/C:A/G	312	L312P	Trypsin
Wild type sequence: catcgccctgctccgcctgtcccagccagccacacTctctaaaaccatagtgcccatctgtctgccgaaca				
Mutant sequence: catcgccctgctccgcctgtcccagccagccacacCctctaaaaccatagtgcccatctgtctgccgaaca				