Acute changes in fibrinogen metabolism and coagulation after hemorrhage in pigs

Wenjun Z. Martini, David L. Chinkes, Anthony E. Pusateri, John B. Holcomb, Yong-Ming Yu, Xiao-Jun Zhang, and Robert R. Wolfe


EMORRHAGIC COAGULOPATHY (without neurological injury) constitutes 40% of injury-related death in civilian hospitals and on the battlefield (1, 3, 15, 18). Hemorrhagic coagulopathy includes an initial hypercoagulable state that may result in consumptive depletion of fibrinogen from progress to disseminated intravascular coagulation (DIC; see Refs. 2, 7, 8, 11, 12, 18, 22). DIC results in a paradoxical situation in which patients are hypocoagulable in terms of hemostasis and therefore cannot stop bleeding, and yet inappropriately deposit fibrin in the microvasculature, contributing to later organ failure in patients that survive the initial hemorrhagic insults. It appears that the availability and metabolism of fibrinogen may play a role in the development of the pathophysiological process. However, the relation between changes in fibrinogen metabolism to the development of clotting defects is poorly understood. The complexity of the clinical settings, such as tissue injury, blood loss, blood transfusion, and resuscitation, makes it difficult to clarify the mechanism contributing to the development of clotting disorders. Therefore, we have used an animal model that allowed us to define changes in fibrinogen metabolism under hemorrhage shock.

The essence of the coagulation process is the formation of fibrin from its precursor fibrinogen. Normal physiological coagulation requires a dynamic balance of complex systems of procoagulant, anticoagulant, and fibrinolytic processes. The pathological states involve dysfunction of these processes. Investigation of coagulation in trauma patients has been primarily based on hematological tests from clinical laboratories that assess levels of procoagulant and anticoagulant factors (1, 7, 12, 22). Among all the factors involved in the coagulation process, fibrinogen level has been reported to be the first to drop to pathophysiological levels in patients with coagulation defects (1, 6). The drop cannot be fully explained by blood loss and resuscitation (6). It is not clear whether the drop in fibrinogen level is the result of inhibited production, accelerated consumption, or both, since there has not been an in vivo technique available for simultaneous quantification of both production and consumption.

This study was designed to develop an in vivo technique to quantify fibrinogen synthesis and degradation simultaneously and independently. Using this technique, we investigated changes in fibrinogen metabolism in parallel to the changes in the coagulation process after a moderate hemorrhagic shock in a swine model. Changes in fibrinogen synthesis were assessed using a 6-h infusion of [1-13C]ketoisocaproate ([1-13C][KIC]) and [1-13C]phenylalanine ([1-13C][Phe]), and changes in fibrinogen breakdown were assessed by a 4-h infusion of d5-phenylalanine (d5-Phe). We tested the hypothesis that fibrinogen breakdown was accelerated and fibrinogen synthesis remained unchanged after hemorrhage. To correlate fibrinogen metabolic changes with functional changes in coagulation, thromboelastography (TEG) was used to measure changes in clot formation and fibrin lysis from fresh whole blood samples.

METHODS

Experimental design. This study was approved by the US Army Institute of Surgical Research Institutional Animal Care and Use Committee. Twelve pigs were randomly allocated to normal control...
The protocol of the primed constant infusion of stable isotopes is graphically depicted in Fig. 1. A sterile solution of stable isotope-labeled [1-13C]KIC, [1-13C]Phe, and d5-Phe was made in 0.45% saline and infused via the left femoral vein. On starting the infusion study, a priming dose of stable isotopes was given via the femoral vein containing [1-13C]KIC (30 μmol/kg), [1-13C]Phe (18 μmol/kg), and d5-Phe (18 μmol/kg). This was followed immediately by a constant infusion of tracer [1-13C]KIC (0.5 μmol·kg⁻¹·min⁻¹), [1-13C]Phe (0.3 μmol·kg⁻¹·min⁻¹), and d5-Phe (0.3 μmol·kg⁻¹·min⁻¹). The infusion of [1-13C]KIC and [1-13C]Phe was maintained for 6 h, and the d5-Phe infusion was maintained for 4 h (Fig. 1). The total tracer solution infused over 6 h was 125 ± 3 ml in C and 114 ± 6 ml in H. Blood samples (10 ml each) were collected hourly for 6 h with two additional samplings at 5.5 and 6.5 h. At 5 h, a bolus dose of sterile indocyanine green (ICG) dye solution (10 ml of 2.5 mg/ml) was given via the femoral vein, and additional blood samples (2 ml each) were collected upon injection and then at 5, 10, and 15 min for the assessment of plasma volume. Additional blood samples were drawn at baseline and 5 h (1 ml each) for blood clotting measurements.

Calculations. Plasma fibrinogen fractional synthesis rate (FSR) was calculated using the formula

\[
FSR = \frac{[EB]_0 - [EB]_t}{(EF \times t)}
\]

where [EB]₀ is the enrichment of fibrinogen-bound amino acids, EF is the precursor enrichment, t is the time point when d5-Phe infusion was stopped (5 h), and t is the time point when infusion of [1-13C]Phe and [1-13C]KIC was stopped (7 h), and t is time. In using tracer [1-13C]Phe to calculate FSR, [EB]₀ was fibrinogen-bound phenylalanine enrichment and EF was plasma free phenylalanine enrichment at the steady state. In using tracer [1-13C]KIC to calculate FSR, [EB]₀ was fibrinogen-bound leucine enrichment, and EF was the plasma free leucine enrichment at the steady state.

Plasma fibrinogen fractional breakdown rate (FBR) was determined by calculating the fractional rate of loss of labeled fibrinogen after the d5-Phe tracer was stopped. When the d5-Phe tracer was stopped, the plasma phenylalanine enrichment did not immediately go to zero because it took time for the labeled phenylalanine to be cleared from the blood, so labeled fibrinogen continued to be produced during this period. To account for this continued label incorporation, we determined the predicted fibrinogen-bound d5-Phe enrichment [as-
suming that there was no loss of labeled phenylalanine (denoted EBpred) using the formula

\[ EB_{\text{pred}} = EB_{(t_1)} + FSR \times EF \times t \quad (2) \]

where \( EB_{(t_1)} \) is the starting fibrinogen bound phenylalanine enrichment when \( d_5 \)-Phe infusion was stopped, \( EF \) is the enrichment of plasma phenylalanine, and \( t \) is the amount of time that elapsed from the starting fibrinogen-bound phenylalanine measurement. To account for the delay between the time when plasma phenylalanine is taken up to the time that it appears in bound fibrinogen, \( EF \) is the free phenylalanine enrichment \( 1 \) h before the time of the fibrinogen-bound enrichment. Note that Eq. 2 is just a rearrangement of FSR Eq. 1 where \( EB_{\text{pred}} \) is \( EB_{(t_2)}. \)

The actual measured fibrinogen-bound phenylalanine enrichment (denoted \( EB_{\text{act}} \)) will be lower than the above predicted enrichment to the extent that the label is irreversibly lost. Therefore, the FBR can be calculated using the formula

\[ FBR = \frac{(EB_{\text{act}} - EB_{\text{act}}) \times (EB_{(t_1)} \times t)}{EB_{(t_1)}} \quad (3) \]

The numerator of this equation represents the amount of tracer that is irreversibly lost (relative to the amount of tracer). Dividing this value by the initial enrichment \( |EB_{(t_1)}| \) gives the percentage of the initial enrichment that was irreversibly lost, and dividing also by the time gives the fractional rate that the tracer is lost. It is assumed that the fractional rate of loss of unlabeled fibrinogen is the same as the labeled fibrinogen.

The plasma fibrinogen absolute synthesis rate was calculated by multiplying FSR by plasma fibrinogen concentration and plasma pool size. Similarly, plasma fibrinogen absolute breakdown rate was calculated by multiplying FBR by plasma fibrinogen concentration and plasma pool size.

Statistical analysis. All results are expressed as means ± SE. Comparisons between the groups in fibrinogen synthesis, breakdown, liver enzyme activities, substrate concentrations, and changes in blood clotting measurements were made with Student’s \( t \)-test. Statistical significance was set at the 0.05 level.

RESULTS

Physiological data. All measurements in the control group remained unchanged during the study. Mean arterial pressure decreased immediately after hemorrhage from 89 ± 4 mmHg in C to 47 ± 4 mmHg in H (\( P < 0.05 \)), followed by a rebound to 68 ± 5 mmHg in H after 30 min. Similarly, cardiac output decreased immediately after hemorrhage from 3.4 ± 0.4 l/min in C to 1.8 ± 0.2 l/min in H (\( P < 0.05 \)), followed by a rebound to 2.9 ± 0.4 l/min in H after 30 min. Within 1 h after hemorrhage, there was a significant increase in blood lactate concentration and decreases in Hct, fibrinogen concentration, and plasma total protein content. Afterward, there were no changes within each group from 1 to 7 h in measurements of Hct (29.2 ± 0.7% in C and 25.4 ± 1.1% in H, \( P < 0.05 \)), platelet count (316 ± 33.3 × 10^9/ml in C and 268 ± 11.1 × 10^9/ml in H, \( P < 0.05 \)), fibrinogen concentration (185.8 ± 15.5 mg/dl in C and 120.6 ± 2.4 mg/dl in H, \( P < 0.05 \)), and total protein concentration (5.14 ± 0.21 g/l in C and 3.91 ± 0.15 g/l in H, \( P < 0.05 \)). Plasma volume, measured by ICG dye at 5 h, was 49.7 ± 0.5 ml/kg in C and 34.0 ± 0.4 ml/kg in H (\( P < 0.05 \)).

Blood biochemical and clotting data. There were no differences between the two groups in circulating enzyme activities of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and \( \gamma \)-glutamyltranspeptidase during the study.

Changes of blood clotting time after hemorrhage are shown in Fig. 2. At 5 h, blood clotting time in H was shortened to 92.7 ± 1.6% of its baseline value compared with 104 ± 1.5% of its baseline value in C (\( P < 0.05 \)). There were no differences in fibrin clot lysis (LY60) between the two groups.

Isotope data and fibrinogen kinetics. The enrichments of plasma free leucine tracer-to-tracee ratio (TTR) \( (m + 1) \) in both groups reached plateau values (9.37 ± 0.35% in C and 9.07 ± 0.61% in H) after 1 h of [1-13C]KIC infusion (Fig. 3). Similarly, the enrichments of plasma free Phe TTR \( (m + 1) \) in both groups reached plateau values (21.23 ± 0.58% in C and 23.80 ± 1.30% in H) after 1 h of [1-13C]Phe infusion (Fig. 3). The calculated rate of appearance for phenylalanine, which is a reflection of whole body protein breakdown (21), was 1.42 ± 0.04 μmol·kg⁻¹·min⁻¹ in C and 1.27 ± 0.08 μmol·kg⁻¹·min⁻¹ in H (\( P = 0.16 \)).

The enrichments of fibrinogen-bound phenylalanine TTR \( (m + 1) \) and leucine TTR \( (m + 1) \) showed a linear increase during the infusion of [1-13C]Phe and [1-13C]KIC (Fig. 4). Plasma fibrinogen FSR was calculated from the increment of fibrinogen enrichments between 5 and 7 h of the infusion study. Fibrinogen FSR measured by [1-13C]KIC was 3.1 ± 0.4%/h in C and 4.4 ± 0.5%/h in H (\( P < 0.05 \)), and the FSR by [1-13C]Phe was 2.7 ± 0.2%/h in C and 4.2 ± 0.4%/h in H (\( P < 0.05 \)). It is worth mentioning that, within each group, the FSR values measured by these two tracers were close, and significant increases of FSR by hemorrhage were shown by both tracers. Fibrinogen absolute synthesis rate, calculated by multiplying FSR by fibrinogen concentration and plasma volume, was 2.5 ± 0.6 mg·kg⁻¹·h⁻¹ in C and 1.7 ± 0.2 mg·kg⁻¹·h⁻¹ in H by tracer [1-13C]Phe (\( P = 0.24 \), Fig. 5) and 3.0 ± 0.8 mg·kg⁻¹·h⁻¹ in C and 1.8 ± 0.2 mg·kg⁻¹·h⁻¹ in H by tracer [1-13C]KIC (\( P = 0.43 \)).

Fibrinogen FBR was calculated from the changes in fibrinogen-bound Phe enrichment TTR \( (m + 5) \) after the cessation of tracer \( d_5 \)-Phe infusion (Fig. 4). The FBR in H was 12.9 ± 1.8%/h, which was significantly accelerated from 3.6 ± 1.0%/h in C (\( P < 0.05 \)). The absolute breakdown rate, calculated by multiplying FBR by fibrinogen concentration and plasma volume, was also accelerated to 5.4 ± 0.6 mg·kg⁻¹·h⁻¹ in H compared with 3.0 ± 0.4 mg·kg⁻¹·h⁻¹ in C (\( P < 0.05 \), Fig. 5).
DISCUSSION

We have developed a new stable isotope technique that allows, for the first time, simultaneous and independent quantification of fibrinogen synthesis and breakdown in vivo. The simultaneous quantification of synthesis and breakdown provides comprehensive understanding of changes in fibrinogen metabolism under different physiological conditions. Using this new technique, we investigated changes in fibrinogen metabolism in pigs after a hemorrhagic shock. Our finding was that, acutely after a moderate hemorrhage shock, fibrinogen breakdown was accelerated, but synthesis remained unchanged. The acceleration in fibrinogen breakdown was associated with a shortening in blood clotting time. The deficit between fibrinogen production and consumption indicates a potential decrease in fibrinogen availability after hemorrhage.

Previous investigations of fibrinogen catabolism included 125I-labeled fibrinogen administration and subsequent daily blood samplings for 5–14 days (4, 5, 16, 19, 20). The radioactivity decay curve during the sampling period was used to calculate fibrinogen turnover. This approach was used as the only in vivo means in the past to monitor clotting defects in patients and animals under pathophysiological states (4, 5, 16, 19, 20). As a comparison, there are a number of advantages of our new technique over the 125I-fibrinogen method. First, we used stable isotopes rather than radioactive isotopes, which is a safer approach. Second, we measure FSR and FBR independently and simultaneously, whereas studies using 125I-fibrinogen only measure degradation. This is an important advantage in cases such as the present study in which synthesis and breakdown are not equal after hemorrhage. Third, our method provides fast and timely measurements to monitor the coagulation process, since it only takes 6 h as opposed to 5–14 days in the 125I-fibrinogen approach. Fourth, unlike the 125I-fibrinogen method, in which the exogenously given fibrinogen is not directly comparable to the endogenous fibrinogen, the labeled fibrinogen in this study is produced endogenously by incorporating naturally occurring stable isotopes.

Fig. 3. Changes in plasma free leucine (Leu) and Phe enrichments in control (○, n = 6) and hemorrhage (●, n = 6) groups during the infusion of [1-13C]KIC (6 h), [1-13C]phenylalanine ([1-13C]Phe, 6 h), and d5-phenylalanine (d5-Phe, 4 h). Data are expressed as means ± SE. TTR, tracer-to-tracee ratio.

Fig. 4. Changes in fibrinogen-bound Leu and Phe enrichments in control (○, n = 6) and hemorrhage (●, n = 6) groups during the infusion of [1-13C]KIC (6 h), [1-13C]Phe (6 h), and d5-Phe (4 h). Data are expressed as means ± SE.

Fig. 5. Fibrinogen synthesis and breakdown rates in control (n = 6) and hemorrhage (n = 6) groups. *P < 0.05, control vs. hemorrhage groups. Data are expressed as means ± SE.
previous $^{125}$I-fibrinogen method, problems such as uneven labeling of exogenous fibrinogen, modification of the half-life of fibrinogen from the in vitro procedure, possible tag shifts between different proteins, and dilution from endogenous fibrinogen synthesis remain unresolved (13–14). All of these unresolved problems are likely to compromise the validity of fibrinogen degradation quantification using the $^{125}$I-fibrinogen method.

Under the physiological situations in this study, the steady-state enrichment level of the infused tracer (Fig. 3) is mainly determined by the turnover rate of the trace pool rather than the absolute concentration of the trace in the metabolic pool (21); and the determination of FSR of fibrinogen is based on the slope of fibrinogen enrichment increment (Fig. 4) secondary to the steady-state plasma enrichment of the infused tracer (Fig. 3). It is also worth noting that none of the equations we used to calculate fibrinogen fractional synthesis or breakdown has a parameter of concentration. Therefore, we consider that, even if bleeding and dilution from the isotope infusion might alter precursor and product concentration to some degree, since the measurements were made under a quasi-steady state, our results of FSR and FBR should reasonably reflect the dynamic nature of fibrinogen metabolism. The absolute synthesis (or breakdown) rate was calculated as FSR multiplying plasma volume and fibrinogen concentration. Because plasma volumes and fibrinogen concentrations were directly measured in each animal group in this study, it is, therefore, not necessary to correct or adjust plasma volume for the assessments of absolute synthesis and breakdown rates.

In this study, [1-$^{13}$C]Phe, d-$^{5}$Phe, and [1-$^{13}$C]KIC were infused at the same rates ($\mu$mol·kg$^{-1}$·min$^{-1}$) in the control and hemorrhage groups. The total volume infused over 6 h was similar in control (125 ± 3 ml) and hemorrhage (114 ± 6 ml) groups. The similar volume of tracer might cause relatively more dilution in the hemorrhage group because of its blood loss before the isotope infusion. This additional dilution might possibly decrease clotting factor levels in hemorrhaged animals, resulting in possible prolonged clotting time compared with that in control animals. Thus our clotting time measurement, which was shortened ~10% after hemorrhagic shock, might possibly underestimate the hemorrhage effect on clotting time. However, because the infused volume during the 6 h was small compared with total blood volume, this possible underestimation was likely to be minimal. Therefore, we consider that our clotting time results are reasonable.

In summary, we developed a stable isotopic model to quantify fibrinogen synthesis and breakdown independently and simultaneously in pigs. We found that, acutely after a moderate hemorrhage, fibrinogen breakdown was accelerated, whereas fibrinogen synthesis remained unchanged, resulting in a net loss of fibrinogen availability. This study provides an in vivo tool to investigate long-term or multifactorial effects on fibrinogen metabolism after hemorrhagic shock.

ACKNOWLEDGMENTS

We thank Suzanne Christensen, John Uszolwicz, Michael Scherer, and Douglas Cortez for technical assistance in animal studies and Amy Newland for assistance in editing the manuscript. We appreciate support received from the Veterinary Service Support Branch and Laboratory Support Branch at the U.S. Army Institute of Surgical Research.

GRANTS

This study was supported by the U.S. Army Medical Research and Medical Command. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

REFERENCES