Blood Coagulation and Fibrinolysis in SART-Stressed (Repeated Cold-Stressed) Rats and Drug Effects on the Altered Hemostatic Parameters

Taeko Hata, Atsufumi Kawabata and Eiji Itoh
Department of Pharmacology, Faculty of Pharmacy, Kinki University, Kowakae, Higashi-Osaka 577, Japan

ABSTRACT — Blood coagulation and fibrinolytic activity was studied in SART (specific alternation of rhythm in temperature)-stressed animals found to exhibit thrombocytopenia and prolonged bleeding time, and drug effects on the abnormalities were evaluated. 1) SART-stressed rats revealed prolongation of activated partial thromboplastin and thrombin time, no change in prothrombin time, decreased plasma fibrinogen levels, and shortened euglobulin clot lysis time (ELT). Antithrombin III and α2-plasmin inhibitor activity remained constant following stress exposure. 2) During stress, fibrinogen levels declined from day 5 and remained depressed up to day 14. Reduction in ELT developed in a similar manner to fibrinogen. 3) Decreased fibrinogen levels were prevented by consecutive doses of tranexamic acid, an antifibrinolytic, and Neurotropin, a sedative analgesic. Shortened ELT was counteracted by chronic treatment with Neurotropin and alprazolam, an anxiolytic. Single administrations of the above agents failed to affect either change. These results indicate that SART-stressed animals exhibit suppressed intrinsic coagulability and enhanced fibrinolytic activity, but normal extrinsic coagulability. Considering the previous report together with the above results, the hemostatic system under SART stress tends uniformly toward hemorrhage. Moreover, Neurotropin appears to improve and normalize hemostatic imbalance due to SART stress, a chronic form of stress.

Many studies point out that stress is involved in the development and/or process of diseases related to abnormal hemostasis, such as myocardial infarction, cerebrovascular ischemia and DIC (disseminated intravascular coagulation) (1-4). Some types of acute stress, including emotional stress and exercise, can affect the number and/or function of blood platelets, coagulation and fibrinolysis in humans (2, 4-7). However, hemostatic alterations under chronic stress have rarely been investigated, and there are few studies on the relationship between stress and hemostasis in laboratory animals.

When experimental animals such as rats and mice are chronically stressed by repeated sudden alteration in their ambient temperature according to a certain specific schedule, they exhibit various physiological disorders such as hypotension (8), altered local blood flow (8, 9), electrocardiographic abnormality (10), hyperalgesia (11), and behavioral change (12). These animals are called SART (specific alternation of rhythm in temperature)-stressed ones (13, 14), and regarded as an animal model for clinical autonomic nervous dysfunction (15). Hematological studies show that SART stress results in thrombocytopenia in rodents.
accompanied by prolongation of bleeding time (16) in addition to increases in erythrocyte and neutrophil counts and decreases in lymphocyte and eosinophil counts (17). Bleeding time doubles following SART stress in spite of the fact that there is only a 25% change in platelet count (16). Therefore, a decrease in platelet count cannot completely account for the prolonged bleeding time, suggesting the participation of other mechanisms. In an attempt to clarify their hemostatic profiles in more detail, the present study investigated coagulative and fibrinolytic activity in SART-stressed rats.

Additional experiments were conducted to evaluate the effects of Neurtropin, a sedative analgesic, and alprazolam, an anxiolytic, on altered hemostasis in SART-stressed animals. Both drugs have been reported to prevent thrombocytopenia and prolonged bleeding time caused by SART stress (16). Neurtropin also improves other abnormal symptoms seen in stressed animals (8-10, 12, 15, 17).

MATERIALS AND METHODS

Experimental animals and the stress procedure
Male Wistar rats (Japan SLC., Inc.) weighing 250–280 g were used. Exposure to SART stress was carried out as follows according to the previously described methods (14): The rats were alternately transferred between two cages placed in rooms maintained at 24°C and −3°C, respectively, at 1-hr intervals from 09:00 to 16:00. They were housed in cages in the −3°C room between 16:00 and 09:00 overnight. These procedures were continued for 7 days on ordinary occasions, and the stressed rats were subjected to blood analysis 1 hr after getting out of the cold room on the final morning.

Blood collection and preparation of platelet-poor plasma
Blood (5 ml) was obtained from the abdominal aorta under pentobarbital (Abott, Nembutal®) anesthesia using a plastic syringe containing 3.13% sodium citrate. Each rat was subjected once only to blood collection. Platelet-poor plasma was prepared by centrifuging the blood sample at 2,000 × g (3,000 rpm) and 4°C for 10 min.

Assay of plasma coagulability
Activated partial thromboplastin time (APTT): A mixture of 0.1 ml of plasma with 0.1 ml of APTT reagent (Wako) containing phospholipids from rabbit brain and elaidic acid was incubated at 37°C for 5 min, followed by the addition of 0.1 ml of 0.02 M CaCl₂ solution. APTT was taken as the interval between the addition of CaCl₂ and the moment when the fibrin clot was visually detected.

Prothrombin time (PT): According to Quick’s one stage method, 0.1 ml of plasma was mixed with 0.2 ml of PT reagent (Wako) containing thromboplastin from rabbit brain and CaCl₂ in a water bath maintained at 37°C. Observation of the sample was then continued until formation of the fibrin clot.

Thrombin time (TT): Clotting time was measured immediately after the addition of 0.1 ml of 25 U/ml bovine thrombin (Sigma) solution to 0.1 ml of plasma at 37°C.

Determination of plasma fibrinogen concentration
The plasma fibrinogen level was determined according to a spectrophotometric technique reported by Tomikawa et al. (18). A mixture of plasma and 0.8% NaCl solution containing 6.8 mM CaCl₂ and 0.32 mM tranexamic acid (Aldrich) was incubated at 37°C in a water bath for 1 hr, resulting in fibrin clot formation. The protein content of this clot was determined by the method of Lowry et al. (19).

Estimation of fibrinolytic activity
As a fibrinolytic parameter, euglobulin clot lysis time (ELT) was measured by the method of Gallimore et al. (20) using a euglobulin fraction prepared according to Klufi et al. (21). Plasma was diluted with ice-cold water, adjusted to pH 5.9 by the addition of 2% acetic acid, and then allowed to stand in an ice bath for 1 hr. The precipitated euglobulin fraction was dissolved in 0.12 M acetate buffer
(pH 7.4). After adding bovine thrombin to the euglobulin solution thus obtained, the mixture was incubated in a water bath at 37°C. ELT was defined as the time interval between the addition of thrombin and complete disappearance of the formed clot.

**Determination of antithrombin III and α2-plasmin inhibitor activities**

Antithrombin III (AT III) and α2-plasmin inhibitor (α2-PI) activity was spectrophotometrically determined, using Testzym® AT III and APL kits (Kabi Vitrum AB), respectively. The results were expressed as a percentage of the activity of normal human plasma.

**Determination of blood cell counts, hemacrit and plasma protein**

The numbers of red blood cells (RBC) and white blood cells (WBC), and hemacrit were determined using a Coulter Counter (model SP, Coulter Electronics). Plasma protein concentration was measured according to Lowry et al. (19).

**Drug treatment**

Tranexamic acid (Aldrich) dissolved in physiological saline and an undiluted Neurotropin® (nonprotein component extracted from the skin of rabbits treated with vaccinia virus, Nippon Zoki) solution (20 mg/ml) were given intraperitoneally to rats. Alprazolam (a gift from Takeda Chemical Industries) was suspended in 0.5% CMC-Na solution and administered orally to rats. To evaluate acute effects, tranexamic acid and the other two drugs were administered once 30 and 60 min before blood collection, respectively. When chronic effects of the drugs were examined, the rats received each drug once daily, 7 times in all, until the day preceding blood analysis. Similarly, SART-stressed rats received daily administrations during the 7-day stress-exposure period.

**Statistical evaluation**

The results obtained were expressed as means with S.E. and statistically analyzed by the unpaired Student's t-test for two-group data or Newman-Keuls' test (22) after ANOVA for multiple-group data. Significance was set at P < 0.05.

**RESULTS**

**Coagulative and fibrinolytic profiles of SART-stressed rats**

The results obtained are shown in Fig. 1. In the plasma coagulability tests, SART-stressed rats exhibited slight but significant prolongation of APTT, reflecting the activity of the intrinsic blood coagulation system, whereas there was no change in PT, a parameter of activity in the extrinsic pathway. SART stress led to remarkable prolongation of TT in the rats. The plasma fibrinogen level in the SART-stressed rats was significantly less than that in unstressed ones, by as much as 27%. ELT, widely regarded as a parameter reflecting fibrinolytic activity, was shortened by 32% following stress.

Unstressed and SART-stressed rats showed AT III activity of 121.8 ± 5.9% (n = 13) and 130.8 ± 7.0% (n = 13) and α2-PI activities of 120.7 ± 3.9% (n = 12) and 111.5 ± 4.0% (n = 13), respectively, when the activities were expressed as a percentage of the activity of normal human plasma. No significant differences could be found between the two groups in AT III and α2-PI activity.

Thus, SART-stressed rats appear to suffer extensive abnormalities in the coagulation-fibrinolysis system. In subsequent experiments, the relatively marked changes in plasma fibrinogen levels and ELT seen following stress according to the above measurements were examined for time-related fluctuations during exposure to stress; and also, drug effects on the alterations were evaluated.

**Evaluation of hemoconcentration or hemodilution in SART-stressed rats**

As seen in Table 1, rats exposed to SART stress for 7 days showed an increase in RBC and hemacrit, but no change in WBC and
plasma protein level. Relative fibrinogen level (fibrinogen/plasma protein) significantly decreased following stress exposure. As plasma protein level did not change, fibrinogen levels were given as mg/ml plasma in the following data.

**Time-related changes in plasma fibrinogen level and fibrinolytic activity during exposure to SART stress**

Figure 2 illustrates the time courses of fibrinogen levels during stress exposure. Fibrinogen concentration remained constant up to day 3 of SART stress, but fell suddenly on day 5, approximating a minimum. The decreased level was maintained with no further change up to at least day 14.

As shown in Fig. 3, ELT remained within normal limits up to day 3, although some shortening was temporally noted on day 1. Subsequently, it significantly decreased from day 5, particularly so on day 7, and the reduced time persisted up to day 14. Thus, changes in fibrinogen levels and ELT appear to develop in parallel.
Effect of tranexamic acid on decreased fibrinogen levels in SART-stressed rats

To examine the relationship between enhanced fibrinolytic activity and decreased fibrinogen levels in SART-stressed rats, the effect of tranexamic acid, an antifibrinolytic agent, on fibrinogen level was investigated in unstressed and stressed rats. The results are shown in Table 2. Single doses of tranexamic acid at 50–500 mg/kg failed to have any significant effect on the plasma levels of fibrinogen in either unstressed or SART-stressed rats. Consecutive administrations of tranexamic acid at a high dose, 500 mg/kg/day, significantly inhibited reduction in fibrinogen due to stress without affecting that in unstressed rats.

Effects of Neurotropin and alprazolam on alterations in fibrinogen levels and fibrinolytic activity caused by SART stress

Figure 4 shows the results for the effect of Neurotropin. Fibrinogen levels and ELT in unstressed and stressed rats were resistant to single administrations of Neurotropin at 50 and 100 mg/kg. Daily treatment with 100
mg/kg of Neurotropin blocked the decrease in fibrinogen levels and shortened ELT due to SART stress, without any significant effect on the unstressed rats.

As seen in Fig. 5, alprazolam at both single and daily doses of 1 and 2 mg/kg failed to affect fibrinogen levels in either unstressed or SART-stressed rats, whereas the chronic administration counteracted stress-induced ELT reduction without influencing ELT in the unstressed rats.

DISCUSSION

The present study indicates that SART-stressed rats exhibit suppressed intrinsic
Many investigators have demonstrated that acute stress, such as short-term exercise (5–7), emotional stress (5) and exposure to hypoxia (23) induces transient enhancement of the activity of both blood coagulation factor VIII and plasminogen activator (PA), leading to acceleration of blood coagulative and fibrinolytic activity. Few investigations have been conducted on the hemostatic response to chronic stress. Chohan et al. (24) reported that rats exposed to noise over a long period display complex hemostatic abnormalities such as a prolonged bleeding time, increased levels of fibrinogen and shortened APTT. In contrast, Palmblad et al. (25) found prolonged exposure of humans to a vigilance task to suppress the activities of intrinsic coagulation factors V, VIII and IX, but not to affect fibrinolytic potency.

In this study, rats subjected to SART stress, a chronic form of stress, exhibited prolongation of APTT, indicating suppressed activity of the intrinsic coagulation cascade, in contrast to the general response to acute forms of stress. Enhancement of fibrinolytic activity was continuously observed from day 5 during prolonged exposure to SART stress, differing from transient enhancement following acute stress (5). Considering the decreased platelet count and prolonged bleeding time (16), the hemostatic system appears to uniformly proceed toward hemorrhage during SART stress, which is also different in part from the response to chronic forms of stress as reported by Chohan et al. (24) and Palmblad et al. (25). Thus, hemostatic changes resulting from chronic stress present a complicated picture, and appear to vary according to type of chronic stress.

With respect to the influence of air temperature on hemostasis, transient enhancement of fibrinolytic activity (26) and increase in blood viscosity and platelet count (27) have been noted in humans in response to acute cold exposure. Epidemiologic studies demonstrate mortality from cardiovascular ischemia and cerebrovascular disturbance to rise linearly as atmospheric temperature falls from autumn to winter (28); and air temperatures were found to be positively correlated with the activity of factor VII and AT III, but negatively correlated with fibrinolytic activity (29). SART stress-induced enhancement of fibrinolytic activity and enhanced fibrinolytic activity but show normal extrinsic coagulability.

### Table

| Treatment          | Fibrinogen level (mg/ml) | Euglobulin lysis time (min) |
|--------------------|--------------------------|----------------------------|
|                    | (mg/kg/day x times)      | 0  | 50 | 100 |
| Non-stress         |                          | 0  | 50 | 100 |
| Control            |                          | 0  | 50 | 100 |
| Alprazolam 2 x 1   |                          | 0  | 50 | 100 |
| SART stress        |                          | 0  | 50 | 100 |
| Control            |                          | 0  | 50 | 100 |
| Alprazolam 1 x 1   |                          | 27.1 | 22.1 |
| 2 x 1              |                          | 27.1 | 22.1 |

Fig. 5. Effect of alprazolam on the alterations in plasma fibrinogen level and fibrinolytic activity in SART-stressed rats. * * P < 0.01, compared to the SART-stressed control.
activity as seen here may offer some clues for explaining the above findings.

SART-stressed rats showed considerable prolongation of TT as well as a decrease in fibrinogen levels. TT is a parameter reflecting change in fibrinogen levels, although it is partly influenced by AT III activity and other factors. Considering that AT III activity remained constant even after exposure to SART stress, decreased fibrinogen levels appear to contribute mainly to a large extent to prolonged TT. Consequently, further investigation on TT was not done. Since the decrease in fibrinogen proceeded in parallel with the shortening of ELT during SART stress and was inhibited by chronic treatment with a high dose of tranexamic acid, the lower fibrinogen levels seen in stressed rats may reasonably be attributed to enhanced primary fibrinolysis. Similarly, the clinical report of Takada et al. showed tranexamic acid to improve the decrease in fibrinogen in a patient with DIC, resulting in the prevention of severe bleeding (30). Time-related changes in fibrinogen level and ELT during SART stress corresponded to those in platelet count reported previously (16), suggesting that most hemostatic alterations may develop simultaneously under SART stress. In conclusion, in SART-stressed animals, interactive correlations may exist among hemostatic alterations in their developmental stage, and prolonged bleeding time (16) should be discussed holistically from a "hemostatic balance" viewpoint. However, since enhanced fibrinolysis but not decreased fibrinogen was improved by chronic doses of alprazolam, these two changes may be independent events.

It is important to determine whether SART stress produces hemoconcentration or hemodilution. Generally, hemoconcentration is regarded as a stress symptom. Among parameters shown in Table 1, an increase in RBC and hematcrit is widely accepted as an important parameter reflecting hemoconcentration. Therefore, hemoconcentration may occur during SART stress. As relative fibrinogen level also decreased, however, the change in fibrinogen level under SART stress cannot be explained by hemoconcentration or hemodilution.

Some investigators support the idea that fibrinolysis may be controlled by the central nervous system, since plasma PA activity increases following electrical stimulation of the median eminence in rats (31), and enhancement of fibrinolysis induced by hypothermia in dogs is abolished by pretreatment with chlorpromazine (32). Considering that the SART stress-induced enhancement of fibrinolytic activity was improved by the anxiolytic alprazolam, fibrinolytic enhancement may be associated with central nervous system activity. That alprazolam may also generate antifibrinolytic effects in stressed rats by suppressing the action of platelet-activating factor (PAF) remains a possibility, since alprazolam has an antagonistic potency toward PAF (33), which releases PA from the vascular endothelium (34).

Chronic treatment with Neurotropin prevented both the alterations in fibrinogen level and ELT observed after SART stress. In addition, our previous paper showed Neurotropin to normalize prolonged bleeding time and decreased platelet count due to SART stress. Neurotropin may thus be concluded to possibly modulate and normalize hemostatic imbalance in SART-stressed animals.

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