Evaluation of Shock-related Cardiotoxic Peptide

Hiroaki KAWABATA, Mayumi YAHAGI, Katsuko MIZUMACHI and Shinkichi TEZUKA

The aim of the present work is to confirm the presence of MDF (myocardial depressant factor), which has long been postulated to be one of the cardiotoxic substances in the shock state. Twenty male mongrel dogs were divided into two groups, a hemorrhagic shock group (n = 10) and an endotoxic shock group (n = 10). Blood samples were obtained from each animal at 1, 2, 4, and 7 h after hypotensive events occurred. Inotropic properties of the plasma samples were evaluated by the isometric contraction of a cat papillary muscle preparation, and chromatographic analysis was performed on the peptides in the plasma. Developed tension of the muscle was increased significantly by changing the bathing medium from Krebs-Henseleit solution to plasma obtained 1 to 4 h after the onset of hemorrhagic and endotoxin induced hypotension. The positive inotropic change was associated with a significant increase in plasma epinephrine concentration. None of these plasma samples possessed a negative inotropic effect (i.e., the property of MDF activity). The elution profile by gel column chromatography of samples obtained from schocked animals was almost identical to that recognized as MDF. However, the presence of MDF was not confirmed by column parameters and color development by the ninhidrin reaction. In conclusion, we found no evidence to support the presence of cardiotoxic peptide in plasma of shocked animals. (Key words: shock, cardiotoxic factor, myocardial function)

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The mechanisms of cardiac failure during prolonged shock have been a subject of intensive investigation for many years. In order to understand these mechanisms, more than 10 kinds of humoral factors produced in the shock state have been postulated[1–4]. These factors are believed to have chemically different characteristics and to be produced in different types of shock. Among them, MDF, originally reported by Lefer et al.[5], is one of the most widely investigated substances and is reported to be a oligo-peptide (MW 800–1000) produced in ischemic pancreas[6–8]. However, MDF has not yet been purified as a single molecule.

On the other hand, several authors have failed to observe a cardiotoxic effect in plasma or blood from shocked animals. Hinshaw et al. showed that the cardiac performance of isolated dog hearts was not affected by perfusion with blood from host dogs in endotoxic [9,10] and splanchnic arterial occlusion shock[11,12]. Urschel et al.[13] also failed to observe a negative inotropic effect in plasma of shocked animals, using isometric contraction of papillary muscle preparations. Therefore, the question of whether a specific humoral factor(s) participates in the dete-

Department of Anesthesiology Teikyo University School of Medicine, Tokyo, Japan

Address reprint requests to Dr. Kawabata: Department of Anesthesiology, Teikyo University School of Medicine, 2-11-1, Kaga Itabashi-ku, Tokyo, 173 Japan

rioration of cardiac performance in shock remains controversial. The purpose of this study is to reevaluate cardiotoxic peptides, if any, in shock, and the biological activity and chemical nature of these substance.

Materials and Methods

Animal Preparation

Twenty male mongrel dogs, weighing from 10 to 15 kg, were used in this study. The dogs were allowed to take water, but not food, 12 h prior anesthesia. After intravenous injection of sodium pentobarbital (25 mg/kg body weight), the dogs were intubated and ventilated spontaneously. Catheters were, then, placed in the carotid artery, femoral artery and vein. A carotid arterial line was attached to a pressure transducer (P23ID; Gould-Statham Instruments, USA) by which blood pressure, heart rate, and pulse pressure were monitored continuously (361 Polygraph; NEC San-ei Co., Ltd., Japan). Femoral arterial and venous lines were used for bleeding and injection of endotoxin, respectively.

Shock Protocol

Ten dogs were subjected to hemorrhagic shock by bleeding into a reservoir until mean arterial blood pressure reached 40–50 mmHg, being maintained for 7 h. During the decompensatory phase of shock, the shed blood was reinfused to maintain blood pressure. Two- and 15-ml samples of arterial blood were collected separately before and at 1, 2, 4, and 7 h after the appearance of hypotension. The 2-ml sample of arterial blood was used for blood gas analysis and the 15-ml sample was for measurement of inotropic effects on heart muscle contraction. For this purpose, the 15-ml blood sample was immediately cooled on ice and centrifuged at 3000 rpm for 15 min at 1°C. Then the plasma was stored at −80°C until analysis. The arterial blood gas was analyzed by PHM72, Mk2 and BMS, Mk2 (Radiometer; Copenhagen). The other 10 dogs were subjected to endotoxic shock by a single injection of E. coli lipopolysaccharide (2 mg/kg: 0127, B8; Difco Lab., Detroit, MI) dissolved in 0.9% NaCl. Blood samples were collected in the same manner as those for hemorrhagic shock.

Measurement of Inotropic Effects

Inotropic effect of plasma from shocked animals was assayed by the isometric contraction of papillary muscle according to Lefer et al.[14] However, a slight modification of Justice et al.[15] was employed in our study to prevent formation and distortion of basal force development by gas bubbling, requiring a lesser sample volume. Twenty cats, weighing less than 2 kg, were sacrificed under ether anesthesia, and papillary muscles in the right ventricles were removed and submerged in an electric stimulation chamber containing 5 ml of modified Krebs-Henseleit solution (pH 7.40–7.45)[16]. The solution consisted of (mM/L) NaCl, 108; KCl, 4.75; $CaCl_2$, 2.54; KH_2PO_4, 1.19; $MgSO_4$, 1.19; $NaHCO_3$, 22.5; glucose, 10.0. The solution and the plasma samples were oxygenated by gas containing 95% O_2 and 5% CO_2. In this chamber, one side of the muscle was fixed to the bottom of the chamber and the other side was attached to a force transducer (UL-20; Minebea Co., Ltd., Japan). The papillary muscles were stimulated at a frequency of 1 Hz for 20 msec at 2 volts above threshold voltage[14]. Muscle length was determined according to a length-tension relationship to 80% of maximum tension. After obtaining a constant force development, the Krebs-Henseleit solution was quickly substituted by the plasma sample, which had been previously oxygenated and warmed in the other chamber. The observed changes in contractile force of the muscle were recorded on a thermal recorder (Rectigraph-8K; NEC San-ei, Co., Ltd., Japan). The contractile force of each papillary muscle was measured and analyzed in a series of five blood samples from a dog at 0, 1, 2, 4, and 7 h after hypotension was induced.

Catecholamine Determination

Plasma catecholamine (epinephrine and norepinephrine) concentrations were measured by high-performance liquid chromatography (655; Hitachi, Ltd., Japan) with a fluorometric detector (PD-540D; Japan Spectroscopic Co., Ltd.).

One ml of plasma sample with 5 ng of

carrier catecholamines were mixed with alumina equilibrated with 2 ml of Tris buffer (2 M Tris; EDTA-2 Na, 20 mg/ml; pH 8.6) and was vortexed for 30 min. After centrifugation at 3000 rpm for 5 min, the alumina was washed twice with deionized distilled water and transferred to a micro-V vial. Catecholamines were extracted from the alumina with 150 μl of 0.4 N perchloric acid after vortex mixing for 30 min. Then, 10 to 50 μl of extract were applied to a HPLC system. Exitation and emission wavelength were 280 and 315 nm, respectively. A silica-ODS column (4.6 × 250 mm) was used for separation of catecholamines. The composition of the eluate was as follows: 0.1 M phosphate buffer (pH 2.5); 50 mg/l of EDTA-2 Na; 1-octanesulfonic acid sodium salt 120 mg/l; acetonitrile 37 ml/l. These procedures were carried out as previously described[17,18].

Gel Chromatography and Chemical Analysis

Plasma samples (20 ml) taken before and 4 hours after the induction of hemorrhagic hypotension were subjected to selective membrane ultrafiltration using Diaflo Model 52 chamber with a YM-2 membrane (molecular weight cutoff < 1,000) at 4°C and 3 kg/cm^2 nitrogen gas transmembrane pressure. The ultrafiltrate was lyophilized and dissolved in 1 ml of glucose-free Krebs-Henseleit solution and then subjected to gel column chromatography (Bio-gel P-2, 200–400 mesh; Bio-Rad Lab., USA). The column, 85 × 1.5 cm (i.d.), was developed with glucose-free Krebs-Henseleit solution at a flow rate of 6.8 ml/h and the column effluent was monitored at 230 nm. Amino groups in each fraction were detected by the ninhydrin reaction. Bovine serum albumin (BSA), glycylglycine (Gly-gly), leucine (Leu), and phenylalanine (Phe) were used as molecular weight markers for assessing the separation capability of this system. These markers were mounted separately on the column and were detected by UV absorbance (230 nm) or by color development of the ninhydrin reaction (570 nm). All chemicals were obtained from Wako Pure Chemical Industries, Ltd.

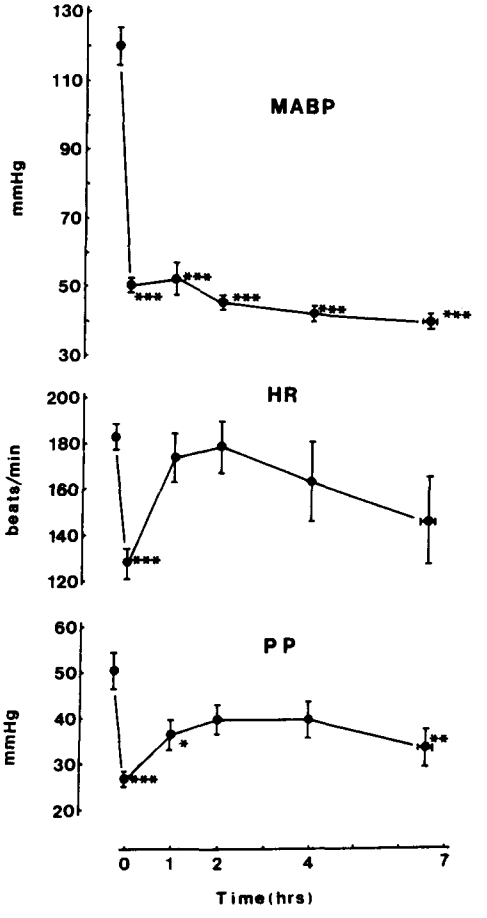

Fig. 1. Mean arterial blood pressure (MABP), heart rate (HR), and pulse pressure (PP) during hemorrhagic hypotension. Dogs were bled from a femoral artery and MABP held at 40–50 mmHg for 7 h. Values are expressed as means ± SEM for 10 dogs.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. pre-bleeding value.

Statistics

All data are expressed as mean ± SEM. Student's t-test was utilized for statistical significance, comparing the pre-shock values with those obtained after bleeding or endotoxin injection. Differences were considered significant when $P < 0.05$.

Results

In the hypotensive state, the mean survival time of the ten dogs was 6.6 ± 0.1 h, as shown in figure 1. Heart rate (HR) and

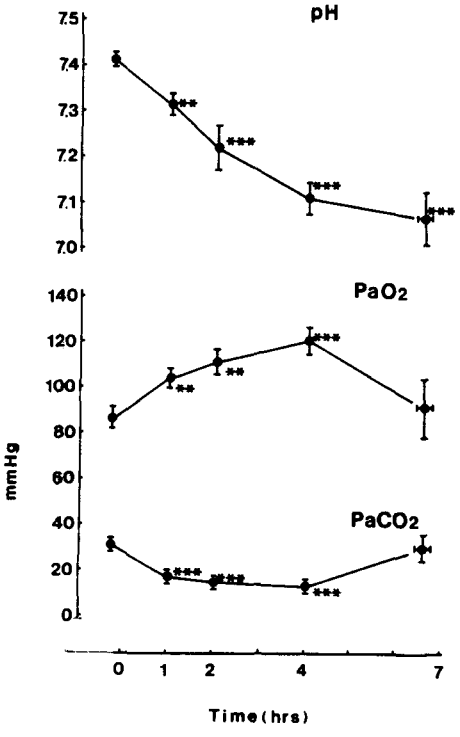

Fig. 2. Arterial pH, oxygen, and carbon dioxide tension (Pa_{O_2}, Pa_{CO_2}) during hemorrhagic hypotension. Statistical values as in figure 1.

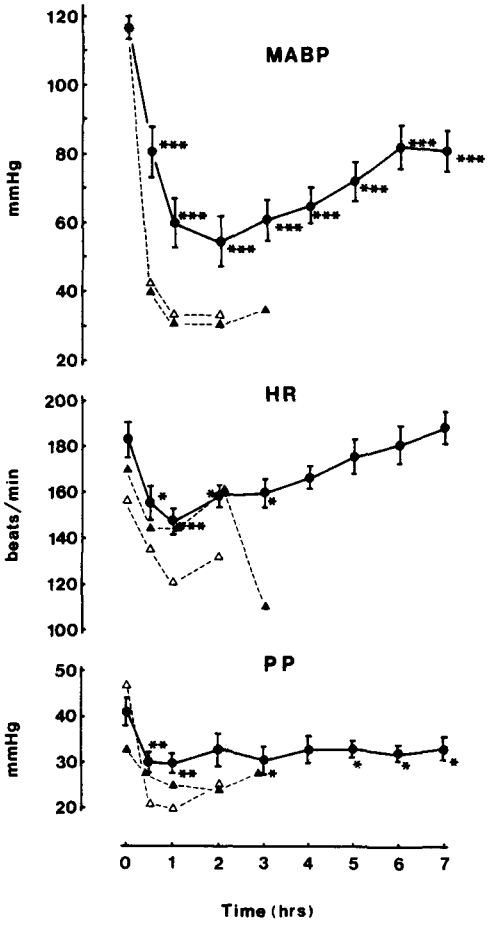

Fig. 3. Mean arterial blood pressure (MABP), heart rate (HR), and pulse pressure (PP) during endotoxin-induced hypotension. Two of ten dogs (△, ▲) died within 3 h after a single injection of endotoxin (2 mg/kg). Statistical values as in figure 1.

pulse pressure (PP) were decreased significantly ($P < 0.001$) by bleeding. Both showed a maximum restoration at about 2 h after the beginning of hypotesion. On the other hand, arterial pH decreased gradually, and reached 7.07 ± 0.06 at the end of the hypotension period (fig. 2). Arterial P_{O_2} was increased and P_{CO_2} was decreased during 4 h period (fig. 2).

Endotoxin induced significant changes in mean arterial blood pressure (MABP), HR, and PP (fig. 3). Two of the ten dogs showed a rapid decrease in MABP and arterial pH, and died within 3 h (dotted line in fig. 3). The other 8 dogs survived for 7 h, showing a restoration of MABP, HR, and relatively constant values of arterial pH (fig. 4). Arterial P_{O_2} did not change significantly, but arterial P_{CO_2} decreased significantly over 7 h (fig. 4).

Figure 5 shows an example of the inotropic effects of plasma sampled at different stages of hemorrhagic hypotension. The arrows indicate timing of the change of medium from Krebs-Henseleit solution to plasma. The mean increment of the developed tension, expressed as percent increase against the tension in Krebs-Henseleit solution, was 132.5, 118.7, and 106.9% at 1, 2, and 4 h after induction of hemorrhagic hypotension, respectively (fig. 6A). These values were significantly different from the prebleeding value. However, the plasma samples from the animals at 7 h after the be-

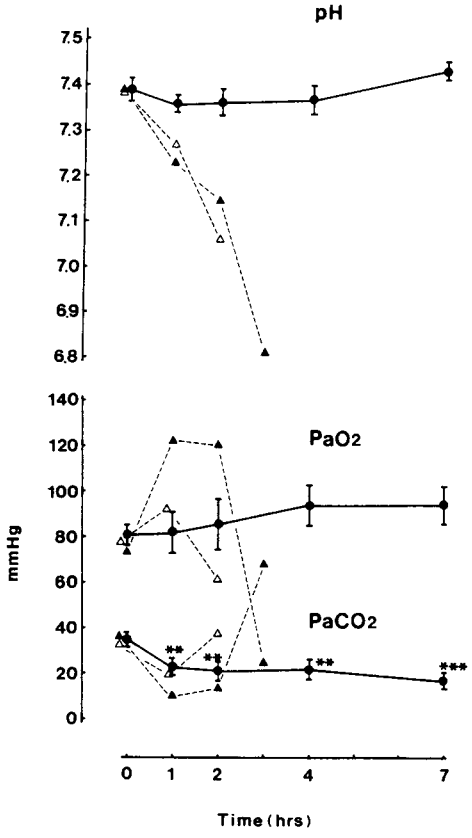

Fig. 4. Arterial pH, oxygen, and carbon dioxide tension (Pa_{O_2}, Pa_{CO_2}) during endotoxin-induced hypotension. Statistical values as in figure 1. Symbols (△, ▲) as in figure 3.

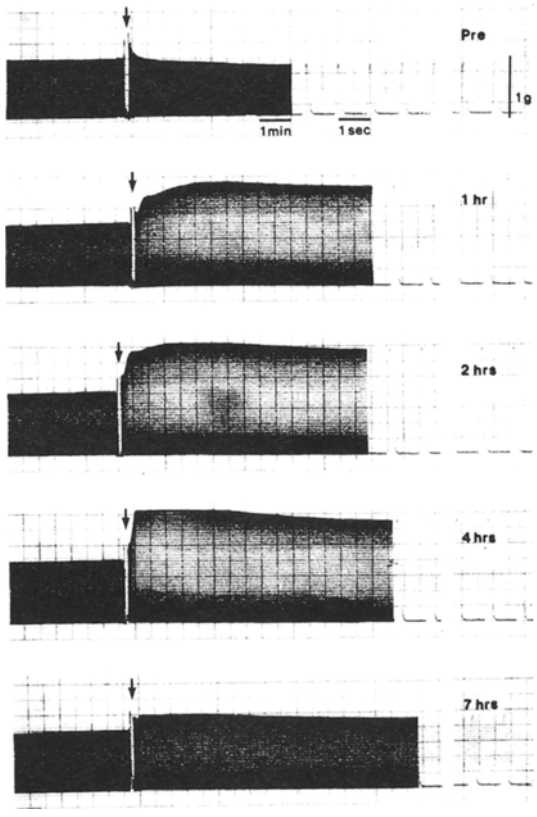

Fig. 5. An example of inotropic response of papillary muscle bathed in plasma obtained at different stages of shock. Inotropic effect of the plasma was tested by the isometric contraction of cat papillary muscle. Arrows indicate point at which medium was changed from Krebs-Henseleit solution to plasma. Positive inotropic effect is observed in plasma taken 1 to 4 h after induction of hemorrhagic hypotension.

ginning of hypotension did not induce significant change in the developed tension. Samples from dogs in endotoxic shock showed similar inotropic effects as observed in hemorrhagic shock (fig. 6B). None of the plasma samples obtained from either type of shocked animal showed negative inotropic effects in terms of papillary muscle contraction.

Figure 7 shows plasma epinephrine concentrations during hemorrhagic and endotoxin-induced hypotension. In each group, a significant increase was observed at 1 and 2 h after induction of hypotension, and thereafter both decreased. Changes in norepinephrine concentrations were similar to those observed in the epinephrine concentration in these two groups (fig. 8).

Chromatographic parameters such as void volume (V_o) and total bed volume (V_t) of the gel column were 60 ml and 152 ml, respectively. The elution patterns of the molecular weight markers, Gly-gly, Leu, and Phe, are shown in figure 9; their elution volumes were 96, 102, and 122 ml, respectively.

Figure 10 shows the elution profile of plasma ultrafiltrate from samples obtained from a dog in pre-hemorrhagic state (dotted line) and after 4 h of hemorrhagic shock (solid line). The post-shock plasma sample produced five prominent absorption peaks by chromatography which had not been observed in the pre-shock sample from the

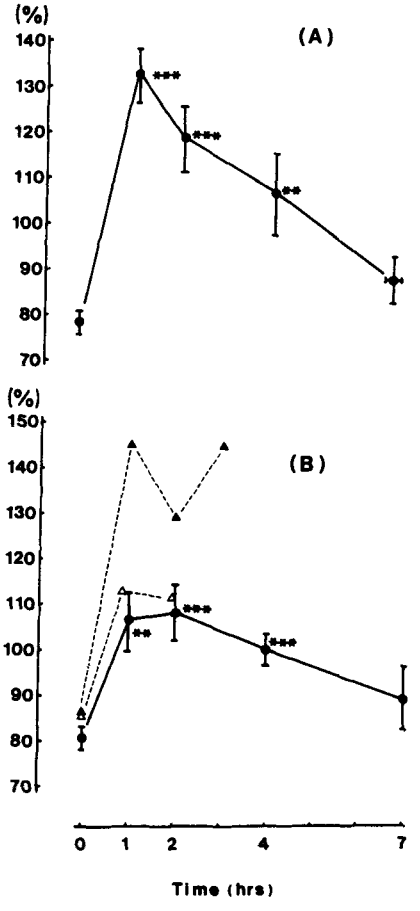

Fig. 6. Changes in inotropic effect of plasma from dogs in different stages of hemorrhagic (A) and endotoxic shock (B). Statistical values as in figure 1. Symbols (△, ▲) as in figure 3.

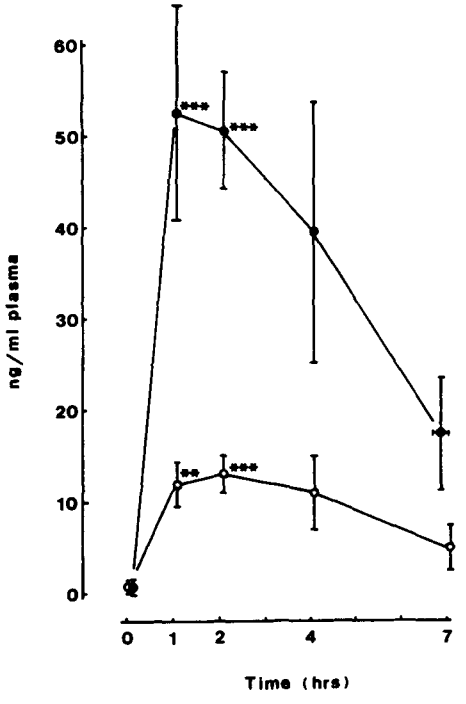

Fig. 7. Epinephrine concentrations in plasma from dogs in hemorrhagic (—●—) and endotoxic shock (—○—). Statistical values as in figure 1.

same animal. The fourth peak (corresponding to peak D in Lefer's study) in the shock sample closely corresponded to the parameter V_t. Figure 11 shows the color development (dotted line) after the ninhydrin reaction of the same fraction shown in figure 10. The elution volume of the main color peak corresponded to that of the molecular weight markers, Leu and Gly-gly. The fourth peak did not reveal color development.

Discussion

In this study, we aimed to evaluate the presence of cardiotoxic peptide(s) in plasma from shocked animals. We focused particularly on MDF. MDF is reported to be produced in an ischemic pancreas[6,7] and to be an oligopeptide[8] consisting of glycine, serine, glutamic acid, and an as yet unidentified amino acid[19]; however, the amino acid sequence of MDF has not been demonstrated. Therefore, the presence of this factor is still unclear.

Inotropic Effects of Plasma from Shocked Animal

As shown in figures 5 and 6, plasma from both dogs in hemorrhagic shock and those in endotoxic shock showed positive inotropic effects on papillary muscle contraction. These effects are apparently contradictory to that of MDF, whose activity was reported to increase progressively following injection of endotoxin[20] and onset of hemorrhagic shock[21]. Although the exact reasons for these contradictory observations remain to be explained, the positive effect we observed in this study appears to be

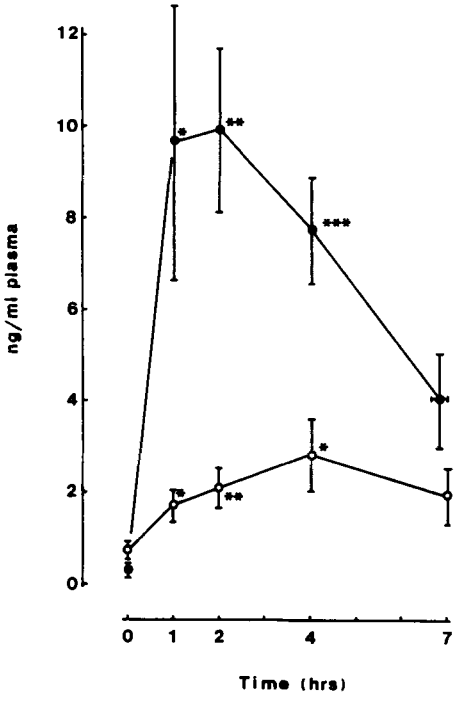

Fig. 8. Norepinephrine concentrations in plasma from dogs in hemorrhagic (—●—) and endotoxic shock (—○—). Statistical values as in figure 1.

due in part to increased level of plasma catecholamines, because the inotropic effect and the concentration of catecholamines changed in a similar direction over 7 h of hypotension (figs. 6, 7, 8). Since increased concentration of plasma catecholamines in the shock state have been observed by many authors[22–24], it seems reasonable to assume that our results reflect this common observation. It is not clear, on the basis of our results, why we did not observe a negative inotropic effect in our samples. However, Urschel et al.[13], using the isometric contraction of a papillary muscle preparation, reported an interesting phenomenon which may explain these controversial results. They demonstrated a positive inotropic effect in fresh plasma from a shocked animal that was immediately subjected to assay, but they also observed a negative inotropic effect in plasma that had been stored in a frozen state. In our study, blood samples were processed under precise cooling conditions and the plasma was stored at −80°C until analysis, while −10°C was emplyed in the literature[8]. Such difference in experimental condition may have caused a diverse results mentioned above. In addition, it is well known that the concentration of plasma catecholamines decreases at temperature below −20°C. Hinshaw et al.[9–11,25] conducted a cross-circulation technique (using an isolated heart supported by the circulating blood of an intact animal) to determine the cardiotoxic effects of blood from a shocked animal. This technique has the advantage of excluding the effects of processing procedures. However, these authors did not find any indication of cardiac deterioration in the isolated heart perfused by blood from a severely endotoxin-shocked dog[10], or by blood from the splanchnic region after 2 h occlusion of this area followed by declamping[11]. Moreover, they could not find MDF activity under conditions of beta-adrenergic blockade[25]. This suggests that our inability to detect MDF activity was not as a result of the high concentrations of catecholamines.

Another factor influencing the inotropic state of cardiac muscle is the age of the animals used as donors of papillary muscle. It is known that the cardiac response to beta-adrenergic stimulation decreases with age[26,27]. The ages of the donor cats, however, were not exactly known in our experiments and also in the literature[8,19]. We obtained papillary muscles from cats weighing less than 2 kg, and therefore those animals were believed to be relatively young. Considering all these factors, it is more likely that a clear positive inotropic effect on muscle contraction would therefore be observed.

Chemical Characteristics of MDF

Plasma ultrafiltrates prepared from a dog before bleeding and 4 h after induction of hemorrhagic hypotension showed a similar chromatographic pattern to that reported previously (fig. 10). The fourth peak, which was claimed to include MDF by Lefer et al.[8], eluted at about the V_t position. However, the relationship between the column parameter (V_t) and the elution volume of the

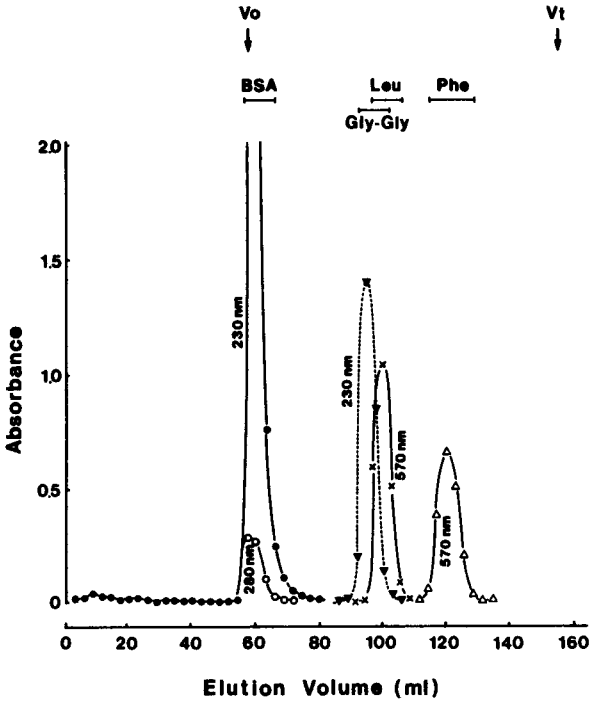

Fig. 9. Elution profile of molecular markers. Bovine serum albumin (BSA: —●—), Glycylglycine (Gly-gly: --▼--), Leucine (Leu: —×—), and Phenylalanine (Phe: —△—) are separately applied on the same column and detected by UV absorbance or ninhydrin reaction (λ = 570 nm). Molecular weights of these markers are as follows: BSA, 67,000; Gly-gly, 132.1; Leu, 131.2; Phe, 165.2.

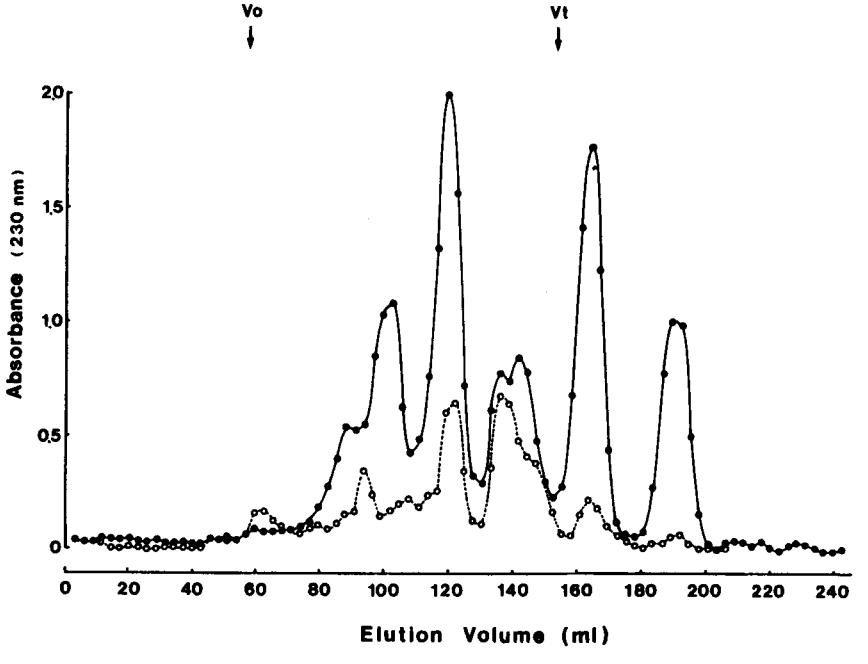

Fig. 10. Elution profile of plasma ultrafiltrate from a dog before (--○--) and during hemorrhagic shock (—●—). Samples were filtered through a YM-2 membrane and the filtrates were lyophylized. The lyophylized powder was dissolved in 1 ml of glucose-free Krebs-Henseleit solution and then applied to the column.

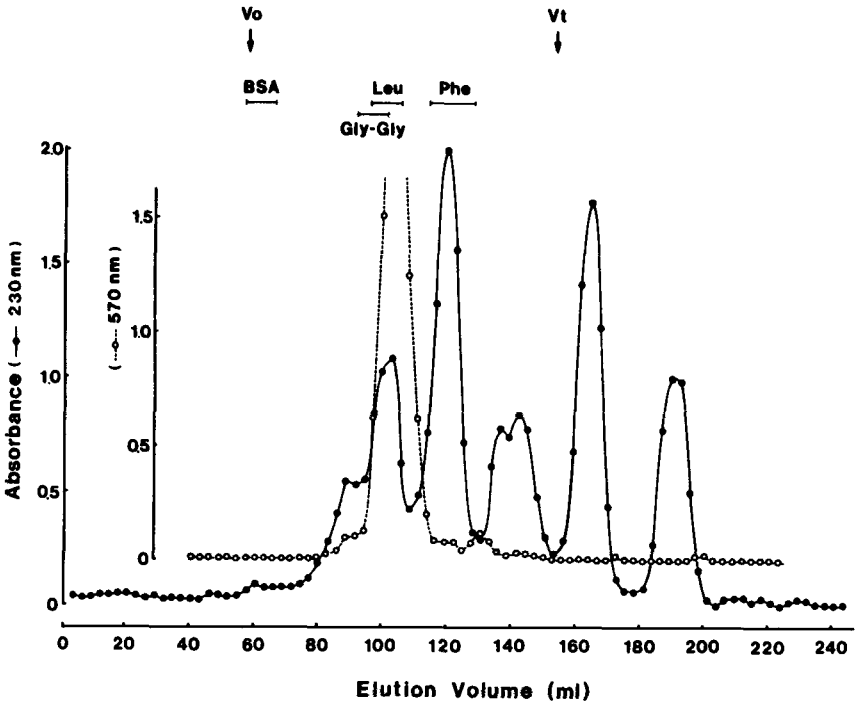

Fig. 11. Detection of amino group (--○--) in each fraction of the column eluate. An aliquot of each fraction from a shocked dog was subjected to the ninhydrin reaction. UV absorbance (—●—) is the same as figure 10. Elution volume of the main color peak, which consists mainly of free amino acids in plasma, corresponds to that of Gly-gly and Leu. The fourth peak does not reveal color development.

molecular marker (Gly-gly) in our study was very different from their results, that is, the elution volume of Gly-gly and the V_t in our study were 96 ml and 152 ml, respectively, whereas those values were 250 ml and 160 ml in Lefer's study (their V_t value of 160 ml is calculated from their methods[8]). Since the dipeptide does not consist of aromatic amino acids and has no affinity for acrylamide gel, there is no possibility that Gly-gly elutes at the volume of 1.5 times V_t on the gel chromatograph. Other evidence in support of the validity of our results is shown in figures. 9, 10, and 11. Leu and Gly-gly (which have similar molecular weights) eluted closely at about 100 ml, and the elution position corresponded to that of the main color peak representing free amino acids in the plasma ultrafiltrate (fig. 11). Furthermore, as shown in figure 11, the fourth peak did not reveal color development by the ninhydrin reaction (open circles). Considering these results, it is reasonable to conclude that the fourth peak consisted of molecules of smaller molecular weight than the peptide.

Wangensteen et al.[28] demonstrated the presence of cardio-depressant activity in a fraction of column eluate (corresponding to peak D in Lefer's study) whose peak was caused by a high concentration of salts. Although we did not investigate whether such high concentrations of salts were eluted at the fourth peak, it is likely that the peak included a large amount of salts, as judged by the column parameter mentioned above.

Other myocardial depressant factors[3,4] have been postulated in recent years. One of those factors is reported to be a peptide (MW 1000–2500) consisting of nine amino acids produced in pancreas of dog

after incubation at 37°C for 2 h in vitro[3]; this factor was assessed in isolated perfused hearts and by membrane movement of contracting myocytes in vitro. Another putative depressant factor was recognized in human sera from patients with a reversible left ventricular depression[4]; this factor was assessed in culture of newborn rat beating heart cells. These factors, including MDF, are considered to influence cardiac performance in shock only after they have induced cardiac depression in intact animal, or they have induced further cardiac depression in the shocked animal.

In conclusion, we found no evidence to support the presense of cardiotoxic peptide(s) in plasma of shocked animals in the present work.

To understand whether a specific humoral factor(s) participates in the heart function in shock, further precise studies on the cardiac performance and in vitro studies on cardiotoxicity of blood samples from shocked animals are needed.

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