Significance of Splanchnic Proteases in the Production of a Toxic Factor in Hemorrhagic Shock

By Thomas M. Glenn and Allan M. Lefer

ABSTRACT

The pathogenesis of circulatory shock has been previously associated with the plasma accumulation of a myocardial depressant factor (MDF). The plasma accumulation of MDF in cats subjected to hemorrhagic shock was associated with marked increases (P<0.01) in activities of plasma cathepsins A–E. The pH optima for three of the five cathepsins were in the range of 4.8–5.2, a pH range close to that found intracellularly during shock. Moreover, pancreatic and hepatic tissue from these cats exhibited significant decreases in total specific activities for these enzymes, compared with minimal changes in heart, duodenum and spleen. Decreases in total pancreatic cathepsin activities were associated with marked ultrastructural changes in pancreatic acinar cells primarily characterized by vacuolization of the lysosomes. Incubation of homogenates of splanchnic viscera of unshocked dogs revealed that significant concentrations of MDF were produced only in incubated pancreatic homogenates. Infusion of MDF in amounts half that produced by a cat pancreas into intact anesthetized cats yielded a significant circulatory depression indicated by a progressive decline in cardiac output and mean arterial blood pressure and a 54% decrease in cardiac work performance within 60 minutes. These data indicate that substantial amounts of lysosomal proteases are released during shock and that they operate in a suitable pH range to account for the production of quantities of MDF that are sufficient to induce a marked degree of cardiac depression.

KEY WORDS

myocardial depressant factor (MDF) myocardial performance cat

cathepsins pancreas

methylprednisolone dog

The impairment of myocardial performance in circulatory shock has been shown to be associated with the plasma accumulation of a myocardial depressant factor (MDF) in hemorrhagic (1), endotoxic (2), cardiogenic (3), pancreatitis (4), and splanchnic ischemia (5) shock, in experimental animals and in man (6). MDF is a small peptide which exerts a pronounced negative inotropic action on the isolated papillary muscle (7, 8), isolated perfused heart (9), and heart of the shocked animal (10).

Splanchnic hypoperfusion appears to be the common denominator in all of the forms of circulatory shock in which MDF has been found (11). It has been suggested that hypoperfusion of the splanchnic bed produces a critical degree of ischemia or hypoxia or both which alters cellular integrity and the physiologic status of subcellular organelles such as lysosomes (12). Indeed, a direct positive relationship has been found between the plasma MDF accumulation in shocked animals and plasma activities of lysosomal hydrolases (11). Although a number of other investigators (13–16) have noted increases in circulating activities of lysosomal hydrolases in circulatory shock, none has been able to...
attribute any direct or indirect deleterious actions of these enzymes on the pathogenesis of circulatory shock.

The primary aims of the present investigation were: (1) to relate the alterations in pancreatic ultrastructure with plasma and tissue lysosomal enzyme activities during the course of hemorrhagic shock; (2) to characterize the lysosomal response of each splanchnic tissue to hemorrhage and to determine the pH optima of splanchnic lysosomal proteases; (3) to localize the tissues involved in the production of MDF, and (4) to study the circulatory effects of MDF in the anesthetized intact cat, in order to evaluate the role of splanchnic proteases and MDF in the pathogenesis of circulatory shock.

**Methods**

**SHOCK PROTOCOL**

Healthy adult cats of either sex weighing between 3.0 and 3.7 kg were anesthetized with pentobarbital sodium (30 mg/kg iv). The left femoral artery, right carotid artery, and right femoral vein were cannulated, and heart rate, mean arterial blood pressure (MABP), and central venous pressure were recorded continuously on a Beckman type RB dynograph using Statham P23 pressure transducers. Heparin sodium (1500 U/kg) was administered intravenously 30 minutes before inducing hemorrhagic shock by bleeding the animals from the femoral artery into a siliconized Mariotte bottle into which a mixture of 95% O2 and 5% CO2 was bubbled at a preset hydrostatic pressure. The hydrostatic pressure in the bottle was set so that the MABP of the cat was maintained at 45 mm Hg until 40% of the shed blood was spontaneously taken up by the animal. At this time, the remaining 60% was infused under positive pressure with care taken so that the central venous pressure did not increase more than 2 to 3 cm H2O during infusion. Survival time is defined as the time from completion of reinfusion of all blood to the time when the MABP spontaneously declined to 60 mm Hg. At this time, blood samples were collected and the experiment terminated. This standardized hemorrhagic shock protocol resulted in a mortality rate of 98%.

**INFUSION OF MDF INTO INTACT CATS**

Ten cats of either sex weighing 2.8–3.5 kg were anesthetized with pentobarbital sodium (30 mg/kg iv). The right carotid artery and right femoral vein were cannulated; the femoral venous cannula was advanced into the inferior vena cava and served as the route of infusion of Krebs-Henseleit solution. A midline sternotomy was performed, and the chest was left open throughout the experiment. Following sternotomy, the animals were ventilated by intermittent positive pressure ventilation using a Harvard respirator, and a bilateral vagotomy was performed. The left atrium was then cannulated. Carotid arterial and atrial cannulas were connected to Statham P23 pressure transducers for monitoring of mean arterial and left atrial pressures. A nonoccluding, electromagnetic flow transducer was placed around the root of the aorta and mean and phasic flow were recorded with a Statham model M-4001 electromagnetic flowmeter. The diastolic plateau was taken as the zero-flow reference level, and the phase was zeroed periodically during the experiment using a Tektronix 502A oscilloscope. The transducer calibration factors were checked previously by placing the transducer around an excised aorta through which isotonic saline was driven by a variable height hydrostatic column of Krebs-Henseleit solution. All recordings were made on a six-channel Beckman type R dynograph recorder.

The basic technique employed in this study for the evaluation of myocardial performance by ventricular function curve analysis is the method of Lefer et al. (17), which is designed to test the maximal work performance or reserve capacity of the heart. Briefly, it involves the infusion of Krebs-Henseleit solution until aortic flow reaches a constant peak value, termed the plateau level, despite further increases in left atrial pressure. The increase in the rate of left ventricular work (left ventricular power) expressed in g-m/sec resulting from massive volume infusion was plotted against left ventricular filling pressure (left atrial pressure) to construct left ventricular function curves.

An initial left ventricular function curve was obtained for each cat after the completion of all surgery. Thirty minutes later, each of six cats was infused with 5 ml/kg of an MDF solution via the femoral vein. Five ml of this concentrated MDF solution contained approximately 60 MDF units/ml, which is half the amount of MDF produced by a cat pancreas in vitro. A second ventricular function curve was obtained 60 minutes after the infusion of MDF. Comparison of control and post-MDF infusion curves revealed the effect of MDF on cardiac reserve. In four control cats, ventricular function curves were evaluated initially and 2 hours after an infusion of an equivalent volume of Krebs-Henseleit solution (50–60 ml).
DETERMINATION OF TOTAL TISSUE LYSOSONAL ENZYME ACTIVITY

Cardiac, hepatic, splenic, pancreatic, and duodenal tissue samples were taken from shocked cats at the termination of the experiment and from unshocked, anesthetized cats. Tissue samples weighing 5 g were homogenized (1:5 w/v) in a 0.25M sucrose solution and centrifuged at 4°C and 1000 g for 20 minutes. The sedimentable fraction consisting primarily of cellular and nuclear debris was discarded and the supernatant fraction (S1) was centrifuged at 15,900 g for 30 minutes. This centrifugation yielded the S2 fraction and a pellet consisting of lysosomes and other subcellular particles of equivalent density. The S2 fraction was filtered and refrigerated. The lysosomal pellet was resuspended in 5 ml of Triton X-100 by 80 seconds of homogenization and centrifuged at 15,900 g for 30 minutes. The supernatant fraction (S3) resulting from this centrifugation was filtered and stored at 4°C until assay. The sedimentable fraction was discarded.

The lysosomal enzyme activity of the S3 fraction is termed the free lysosomal enzyme activity; the enzyme activity of the S3 fraction represents the intralysosomal or bound activity before release by the detergent Triton X-100. The sum of the enzyme activities of the S3 and S2 fractions equals the total lysosomal enzyme activity in the 5-g tissue sample. Alteration of total tissue enzyme activities was calculated by comparing the total enzyme activity of tissue from unshocked cats subjected to postoligemic shock.

CHEMICAL DETERMINATIONS

Plasma and tissue fractions were assayed for activities of cathepsins A–E. Cathepsin A activity was assayed with benzoylcarbonyl-L-glutamyl-L-tyrosine in a 0.1M acetate buffer at pH 5.0, according to the method of Iodice et al. (18). Cathepsin B activity was assayed with benzoyl-L-arginine amide in a 0.1M sodium citrate buffer at pH 5.0, according to the method of Greenbaum and Fruton (19). The concentration of the amino acids liberated in the assays of cathepsins A and B were calculated by the colorimetric ninhydrin method of Moore and Stein (20). Cathepsin C activity was assayed with glycylphenylalanine amide acetate as substrate in a 0.04M sodium veronal buffer at a pH of 7.2 according to the methods of De la Haba et al. (21). Cathepsin D activity was determined according to the method of Anson (22) using bovine hemoglobin substrate at a pH of 3.5. Cathepsin E activity was assayed using human serum albumin substrate at a pH of 2.5 by the method of LaPresle and Webb (23). The activities of all five cathepsins were expressed as specific activities (units of activity/mg tissue or plasma protein). Protein concentrations were determined using a microbiuret technique with the absorbance read at 300 nm and calibrated with micro-Kjeldahl determinations.

DETERMINATION OF CATHEPSIN pH OPTIMA

Lysosomal fractions of pancreas and liver were assayed at various hydrogen ion concentrations to determine the pH optima for cathepsins A–E. The same conditions were employed as in the original assays for these enzymes except that the buffers were adjusted to different pH values. Each fraction was assayed for the respective enzyme activity in triplicate. The mean activity at each pH was then determined and employed in the determination of the pH optima.

MDF PRODUCTION IN VITRO

Dogs weighing 18–25 kg were anesthetized with pentobarbital sodium (35 mg/kg iv), and the pancreas, spleen, duodenum, and liver were rapidly excised and placed in cold 0.25M sucrose solution. The tissues were then vigorously homogenized in Krebs-Henseleit solution (1:3 w/v) for 10 minutes in a Waring Blender. Aliquots of these homogenates were stored at 4°C for assay of MDF activity. The remainder of the tissue homogenates was divided into two fractions. The first fraction was diluted with two volumes of Krebs-Henseleit solution; the second fraction was diluted with two volumes of fresh plasma obtained from donor dogs. Both fractions were then subjected to 2.5 hours of incubation with agitation at 38°C. Following incubation, both the unincubated and incubated fractions were ultracentrifuged and processed for assay of MDF.

DETERMINATION OF RESIDUAL MDF ACTIVITY

Pancreases were removed from four cats in late postoligemic shock and from four time-matched sham-shocked cats and placed in cold Krebs-Henseleit solution. Each pancreas was weighed, and homogenized in five volumes of Krebs-Henseleit solution for 10 minutes in a Waring Blender. The homogenates were immediately ultracentrifuged and processed for assay of MDF activity.

PROCESSING TISSUES AND PLASMA FOR MDF ACTIVITY

Plasma and tissue samples were ultracentrifuged using dialysis tubing (Nojica tubing, viscose process, Union Carbide Corporation, 30-mm flat width) at 4°C under a pressure of 200–250 mm Hg for 24–36 hours. The ultrafiltrates were collected, and 10-ml aliquots were lyophilized. The lyophilized samples were then reconstituted to 20% of their original volume and applied to a column containing Bio-gel P-2 (200–400 mesh) suspended in Krebs-Henseleit solution according to the method of Lefer and Martin (24). Elution

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TABLE 1

<table>
<thead>
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<tbody>
<tr>
<td>Before oligemia</td>
<td>5.8 ± 1.2</td>
<td>7.3 ± 0.8</td>
<td>4.3 ± 0.2</td>
<td>2.7 ± 0.3</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>At reinfusion</td>
<td>23.4 ± 7.3*</td>
<td>13.0 ± 1.4*</td>
<td>9.6 ± 2.0*</td>
<td>7.8 ± 1.8*</td>
<td>16.2 ± 3.4*</td>
</tr>
<tr>
<td>Late postoligemia</td>
<td>28.1 ± 7.6*</td>
<td>13.8 ± 0.9*</td>
<td>9.0 ± 1.5*</td>
<td>10.2 ± 1.8*</td>
<td>14.9 ± 2.2*</td>
</tr>
</tbody>
</table>

All values are mean enzyme specific activities (units/mg protein) ± se. (See Methods for definition of each enzyme unit). Numbers in parentheses are number of samples assayed. *P < 0.01.

Results

Cats subjected to the hemorrhagic shock protocol exhibited mean bled volumes of 37.9 ± 1.0 ml/kg and were maintained in an oligemic state for 129 ± 11 minutes. Mean survival time after reinfusion in these cats was 86 ± 12 minutes. They had a mean plasma MDF activity of 58 ± 5 MDF units during postoligemia compared to 19 ± 3 MDF units in the plasma of unshocked control animals. A positive correlation was also found in these studies between the plasma activities of the five cathepsins and the plasma MDF activity. Table 1 summarizes the alterations in plasma activity for cathepsins A-E in cats subjected to hemorrhagic shock. Following the reinfusion of the shed blood, there was a significant elevation of enzyme activity of all five cathepsins (*P < 0.01). These increases in cathepsin activities were maintained for the duration of the experimental period.

TABLE 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>+27 ± 25</td>
<td>-3 ± 8</td>
<td>+19 ± 18</td>
<td>+9 ± 14</td>
<td>+16 ± 26</td>
</tr>
<tr>
<td>Duodenum</td>
<td>+14 ± 7</td>
<td>-10 ± 4</td>
<td>-18 ± 5*</td>
<td>+5 ± 3</td>
<td>-12 ± 8</td>
</tr>
<tr>
<td>Liver</td>
<td>-43 ± 10*</td>
<td>-42 ± 7*</td>
<td>-65 ± 18*</td>
<td>-44 ± 5*</td>
<td>-49 ± 7*</td>
</tr>
<tr>
<td>Pancreas</td>
<td>-58 ± 5*</td>
<td>-62 ± 6*</td>
<td>-51 ± 4*</td>
<td>-69 ± 2*</td>
<td>-39 ± 2*</td>
</tr>
<tr>
<td>Spleen</td>
<td>+7 ± 4</td>
<td>-40 ± 7*</td>
<td>-6 ± 3</td>
<td>-10 ± 7</td>
<td>-5 ± 6</td>
</tr>
</tbody>
</table>

Cathepsin activity expressed as mean percent change from control cats = se. Tissues from five shocked cats and five unshocked cats were studied. *P < 0.01; †P < 0.05.
To determine whether increased plasma lysosomal protease activity reflected changes in total tissue enzyme activities and ultrastructural changes in tissue lysosomes, determinations were made of total splanchnic tissue lysosomal hydrolase activities. Alterations in total cathepsin A-E enzyme activity in heart, liver, pancreas, spleen, and duodenum during hemorrhagic shock are summarized in Table 2. Each activity is expressed as the mean percent change from the activity of tissues in the unshocked control cats. In contrast to splanchnic tissues, the heart did not exhibit any significant alteration in the activity of any of the five cathepsins. Splenic tissue exhibited a significant decrease only in cathepsin B, and duodenum a significant decrease only in cathepsin C. However, the specific activity of all five cathepsins showed marked decreases in liver and pancreas. Furthermore, cathepsin A, B, and D activity declined to a greater extent in the pancreas than in the liver. The decrease in total tissue cathepsin activity can be accounted for by the increased plasma activities of these enzymes and demonstrates the marked fragility of the splanchnic lysosomes, in particular, those of the pancreas and liver, during hemorrhagic shock.

Marked ultrastructural changes also occurred concomitant with the alterations in total specific pancreatic cathepsin activities.
FIGURE 3

Electron micrograph of a portion of a cat pancreatic acinar cell (F) and selected lysosomes from adjacent cells (A–E) in a pancreas from a cat in postoligemic shock when the MABP had declined to 60 mm Hg. Note the large intracisternal spaces and the irregular appearance of the endoplasmic reticulum (ER). The lysosomes are characterized by large vacuoles and the absence of inclusion bodies.

Figure 1 (E) is an electron micrograph of a section of a pancreatic acinar cell taken from an unshocked cat. A–D depict normal lysosomes from control cat pancreases. The lysosomes of the normal cat pancreas are characterized by the presence of numerous acid phosphatase-positive particles and inclusion bodies, and the relative absence of large vacuoles. Figure 2 (E) shows a section of a pancreatic acinar cell taken from a cat subjected to hemorrhagic shock just before reinfusion of the shed blood. A–D depict typical lysosomes in this pancreas. The lysosomes are larger and distended to a greater degree by large vacuoles than control lysosomes. A section of a pancreatic acinar cell obtained from a cat in late postoligemic shock is illustrated in Figure 3 (F). A–E show typical lysosomes from this cat pancreas. The lysosomes are devoid of inclusion bodies, and the vacuolization process has progressed beyond that observed during the oligemic period. The enlarged size of the lysosomes and the protruding vacuoles are indicative of increased lysosomal fragility and correlate well with the observed decreased tissue activities and increased plasma activities for the lysosomal enzymes.

Since MDF appears to be formed in the splanchnic tissues (particularly the pancreas) and since the intracellular pH of the splanchnic tissues is about 5.5 (26), data on the actual optimal pH range for the activities of cathepsins A–E would be of value in interpretation of the present results. The optimal pH for cathepsins A–E obtained in both liver and pancreas, the two organs which exhibited the greatest lysosomal fragility, are summarized in Table 3. The pH optima for the same enzyme obtained from liver and pancreas were within 0.1 pH units of each other and are thus listed together in Table 3 as a single value. The pH optima of cathepsins A–C are generally within the range found within splanchnic tissue cells, whereas those of cathepsins D and E are probably too low.

In additional experiments, the synthetic glucocorticoid, methylprednisolone \( (5 \times 10^{-4} \text{ M}) \) was added to the cathepsin enzyme incubation mixtures to determine whether this steroid altered cathepsin activities. These concentrations of methylprednisolone, which are comparable to those which have been shown to stabilize lysosomes in vitro, did not

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Cathepsin pH Optima in Pancreas and Liver</th>
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<tbody>
<tr>
<td>Cathepsin</td>
<td>Optimal pH range</td>
</tr>
<tr>
<td>A(^h)</td>
<td>4.8–5.2</td>
</tr>
<tr>
<td>B(^h)</td>
<td>4.6–5.0</td>
</tr>
<tr>
<td>C(^b)</td>
<td>6.8–7.2</td>
</tr>
<tr>
<td>D(^h)</td>
<td>3.2–3.6</td>
</tr>
<tr>
<td>E(^b)</td>
<td>2.3–2.7</td>
</tr>
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</table>

\( h \) = hydrolytic activity; \( t \) = transamidination reaction.
In Vitro Production of MDF by Homogenates of Splanchnic Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>N</th>
<th>Nonincubated</th>
<th>Incubated homogenate (2.5 hr)</th>
<th>K-H solution</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>3</td>
<td>20 ± 2.8</td>
<td>20 ± 3.3</td>
<td>16 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
<td>19 ± 2.7</td>
<td>25 ± 3.4</td>
<td>28 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>5</td>
<td>15 ± 4.1</td>
<td>59 ± 3.2*</td>
<td>61 ± 3.8*</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>3</td>
<td>15 ± 2.7</td>
<td>23 ± 9.8</td>
<td>5 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

All values are expressed as mean MDF units ± se.

*P < 0.001.

From anesthetized sham-shocked cats at a comparable interval of time. Pancreases from sham-shocked cats had very low residual MDF activity, comparable to that in the plasma of control animals not in shock. In contrast, the pancreases from shocked cats had MDF activities comparable to those found in the plasma of animals during postoligemic shock. The difference between the two groups was highly significant (P < 0.001).

MDF has been shown to exert a marked cardiodepressant action in the intact shocked animal; however, no significant hemodynamic effect of MDF has been demonstrated in the unshocked anesthetized animal. The hemodynamic alterations in cardiac output, MABP, and left atrial pressure following the infusion of MDF into intact cats are summarized in Table 5. Thirty minutes after the administration of MDF, there was a 28% decrease in cardiac output and a 27% decrease in MABP. Sixty minutes after the infusion of MDF, cardiac output and MABP were less than 45% of preinfusion values. Since there was a significant increase in left atrial pressure at this time, the data are indicative of cardiac exhibits any inhibitory or potentiating effect on the activities of cathepsins A-E in either pancreas or liver. All activities of the control solutions were within 2-5% of the solutions containing the methylprednisolone. Thus the beneficial effect of methylprednisolone in shock cannot be attributed to protease antagonism.

Experiments were then performed to determine if homogenates of normal splanchnic tissues could serve as a source of MDF, in vitro. Table 4 summarizes the MDF activities observed in unincubated and incubated homogenates of splanchnic tissues obtained from unshocked control dogs. Only incubated pancreatic homogenates produced significant quantities of MDF activity (P < 0.001). Unincubated pancreatic homogenates did not produce MDF, nor did hepatic, splenic, or duodenal homogenates regardless of whether they were incubated. Furthermore, pancreatic homogenates incubated with Krebs-Henseleit solution produced amounts of MDF comparable to those obtained upon incubation with plasma. Similar results were obtained in a smaller number of cat tissues. These data indicate that the enzymes, substrates, and cofactors required for MDF formation reside within the pancreas.

To extend the concept of the local pancreatic formation of MDF to the intact animal, pancreases from cats in postoligemic shock were removed, homogenized at 4°C, and immediately processed for residual MDF activity. Figure 4 compares the residual pancreatic MDF activity in cats in postoligemic shock to the MDF activity of pancreases taken from anesthetized sham-shocked cats at a comparable interval of time. Pancreases from sham-shocked cats had very low residual MDF activity, comparable to that in the plasma of control animals not in shock. In contrast, the pancreases from shocked cats had MDF activities comparable to those found in the plasma of animals during postoligemic shock. The difference between the two groups was highly significant (P < 0.001).

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TABLE 5

Hemodynamic Alterations in Cats after MDF Injection

<table>
<thead>
<tr>
<th>Time after addition of 5 ml/kg of MDF* (min)</th>
<th>Cardiac output (ml/min kg⁻¹)</th>
<th>Mean arterial blood pressure (mm Hg)</th>
<th>Left atrial pressure (cm H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>137 ± 14</td>
<td>134 ± 6</td>
<td>2.7 ± 0.37</td>
</tr>
<tr>
<td>15</td>
<td>129 ± 12</td>
<td>127 ± 5</td>
<td>3.1 ± 0.38</td>
</tr>
<tr>
<td>30</td>
<td>99 ± 13</td>
<td>98 ± 4</td>
<td>3.3 ± 0.41</td>
</tr>
<tr>
<td>45</td>
<td>77 ± 11</td>
<td>77 ± 3</td>
<td>4.0 ± 0.36</td>
</tr>
<tr>
<td>60</td>
<td>58 ± 15</td>
<td>59 ± 2</td>
<td>5.8 ± 0.39</td>
</tr>
</tbody>
</table>

All values are mean ± as for four cats.

*This constitutes an MDF activity equivalent to that produced in vitro from 3 g of cat pancreas.

depression. Anesthetized untreated controls did not exhibit significant alterations in MABP, central venous pressure, cardiac output, or heart rate over a 120-minute period.

When cardiac performance was assessed 60 minutes after MDF infusion by volume infusion, cardiac work performance (left ventricular power) was markedly depressed. Figure 5 shows typical left ventricular function curves for a cat infused with MDF (A) and for a control cat (B). There was no significant degree of cardiac depression in the untreated cat after 120 minutes, a finding which was typical of the results in the four control cats. However, in the cat receiving MDF, there was a marked decrease in peak left ventricular power 60 minutes after MDF infusion. In four cats tested before MDF infusion and 60 minutes after, a 54 ± 4% depression in cardiac power was observed. In two additional cats, left ventricular performance was assessed 15, 30, 45, and 60 minutes after MDF infusion. There was a progressive depression of left ventricular power over the 60-minute period, achieving a mean decrease of 59 ± 7% 60 minutes after MDF infusion. These data show that MDF can depress the completely normal intact heart to a degree comparable to that found in the late stages of circulatory shock.

Discussion

In 1962, Weissmann and Thomas (13) demonstrated the enhanced fragility of splanchnic lysosomes in endotoxic and traumatic shock and found this associated with an

![FIGURE 5](image_url)

Typical left ventricular function curves obtained from a cat 30 minutes before and 60 minutes after MDF infusion (A). B shows typical left ventricular function curves in unshocked cat at zero time and 120 minutes later. MDF infusion produced a 69% decrease in peak left ventricular power 60 minutes after MDF infusion. The increase in left atrial pressure at this time is consistent with findings of cardiac depression. The values for initial peak cardiac power in these two cats are comparable to the mean of 24.4 ± 1.8 g/m/sec obtained for all cats.

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increase in plasma lysosomal hydrolase activity. These workers suggested that the enhanced release of the hydrolases during the course of circulatory shock may be involved in the pathogenesis of the lethality of the shock state. However, they were unable to directly demonstrate any deleterious action of the released lysosomal hydrolases or to provide evidence for an indirect action of lysosomal hydrolases during shock.

Recently, a close correlation has been found between plasma lysosomal enzyme activity and the plasma accumulation of a myocardial depressant factor (MDF) in a variety of forms of circulatory shock (11, 12). We (5) have previously demonstrated that the increase in circulating lysosomal hydrolase activity in splanchnic ischemia shock is associated with alterations in the functional integrity of pancreatic lysosomes and suggested that splanchnic lysosomal membranes become more permeable during shock, allowing a greater rate of release of hydrolase activity. These lysosomal alterations are a consequence of splanchnic hypoperfusion, a finding that appears to be a common denominator in many forms of circulatory shock (2, 3, 5, 27).

In the present investigation, increased plasma activities of cathepsins A–E were associated with marked changes in total cathepsin activities in the pancreas and liver but not in the heart. Splanchnic lysosomal enzymes contribute a large fraction of the total plasma lysosomal enzyme activity during shock. Thus diversion of thoracic duct lymph during the course of hemorrhagic shock prevented both the elevation of plasma lysosomal enzyme activities (16, 28) and the plasma accumulation of MDF (28). The major components of thoracic duct lymph in the anesthetized cat are derived from the splanchnic bed (29), further supporting the concept that splanchnic lysosomes are the major source of plasma lysosomal enzymes during hemorrhagic shock.

Significant decreases in total lysosomal enzyme activity occurred in the pancreas and liver during the present experiments, whereas the heart exhibited no significant enzyme changes. These findings suggest that either the heart is not subjected to as severe a degree of ischemia or hypoxia during hemorrhagic shock or that the cardiac lysosomes are less sensitive than splanchnic lysosomes to ischemia. Leighty (30) has demonstrated that selective clamping of the coronary vessels can lead to a decreased stability of cardiac lysosomes and a subsequent loss of intralysosomal enzyme content concomitant with a reduction in myocardial performance. Also, coronary vascular resistance decreases during hemorrhagic shock to maintain coronary blood flow (31). Thus the available evidence suggests that an insufficient degree of cardiac ischemia or hypoxia occurs during the course of hemorrhagic shock. However, no direct data are available concerning the sensitivity of cardiac lysosomes to the trauma of hemorrhagic shock. Nevertheless, one implication of these findings is that cardiac ischemia during oligemia does not contribute significantly to the impairment of cardiac performance which occurs in the late stages of circulatory shock (7, 10, 11).

The loss of intralysosomal cathepsin activity from the pancreas was associated with changes in the ultrastructure of pancreatic lysosomes. Electron micrographs of pancreatic tissue taken from animals at the end of the oligemic phase or in the late postoligemic phase showed an increase in pancreatic lysosomal size and an increased vacuolization of lysosomes. These changes are consistent with the concept of increased lysosomal fragility during shock. These ultrastructural changes are similar to the findings of Donath et al. (32), who demonstrated the presence of large vacuoles and cytoplasmic dissolution in the Golgi field of rat pancreases during hemorrhagic shock, although these workers did not specifically stain for lysosomes. Blair et al. (33) have reported comparable ultrastructural changes in hepatic lysosomes during hemorrhagic shock which were believed to indicate an increase in lysosomal fragility.

Several sequelae of splanchnic hypoperfusion act as a trigger mechanism for the release
of pancreatic lysosomal proteases in circulatory shock. Studies using an isolated perfused pancreas (12) have demonstrated that ischemia and hypoxia, but not acidosis, can provoke a marked release of lysosomal enzymes from pancreatic acinar cells. Thus the ischemia and hypoxia associated with the oligemic phase of hemorrhagic shock can directly alter the stability of pancreatic and hepatic lysosomes and result in the release of their acid proteases.

Further evidence supporting the importance of lysosomes and the subsequent formation of MDF in circulatory shock can be obtained from studies on the mechanism of the protective action of glucocorticoids in hemorrhagic shock (5, 34). Thus methylprednisolone, a synthetic glucocorticoid, prevents the plasma accumulation of lysosomal proteases and therefore MDF formation, via the stabilization of pancreatic lysosomal membranes (5, 34, 35). In this regard, it has been demonstrated that hypoxia-induced release of intralysosomal enzyme activity in the perfused pancreas can also be prevented by prior administration of methylprednisolone (12). Methylprednisolone, however, does not appear to antagonize lysosomal cathepsins once they are released from lysosomes. In the present study, methylprednisolone in concentrations equivalent to or higher than those used to stabilize lysosomal membranes exerted no significant inhibitory effect on free catheptic activity.

Determinations of the pH optima for pancreatic and hepatic cathepsins showed that at least three of the enzymes (cathepsins A–C) have pH optima approximating that of splanchnic tissues during circulatory shock. No previous data are available on the pH optima of pancreatic cathepsins. The pH of splanchnic tissues in shock has been estimated by the pH of splanchnic lymphatics to be about 5.5–6.5 (26), a value close to the optimal pH for the activity of cathepsins A–C. Cathepsins D and E would not be expected to be as important as the others in the production of MDF since their pH optima of 2.3–3.6 is well below that which would be expected in cytoplasm or extracellular fluid of the pancreas.

Previous studies (4, 5, 7) have suggested the pancreatic formation of MDF rather than a generalized formation (from plasma protein). Thus MDF has been shown to be produced by selective pancreatic ischemia (7) and in acute hemorrhagic pancreatitis (4). In this regard, total pancreatectomy before the induction of hemorrhagic shock not only prevented the plasma accumulation of MDF but also significantly increased survival after reinfusion. Seifert (36) has suggested that the pancreas produces humoral factors which may play an important role in the pathogenesis of shock by contributing to the circulatory collapse observed in shock states, but has not identified any specific toxic agent. In the present study, the in vitro formation of MDF from incubated pancreatic homogenates supports these findings. Furthermore, the concentrations of MDF produced from incubated pancreatic homogenates were comparable whether the incubation medium was Krebs-Henseleit solution or plasma. This indicates that plasma does not play an essential role as a substrate source in the formation of MDF, and that the substrate(s), cofactor(s) and enzyme(s) which react to form MDF are all normally present in pancreatic tissue. Moreover, the absence of significant amounts of MDF in unincubated pancreatic homogenates suggests that MDF is not a normal endogenous pancreatic constituent existing in storage form which can subsequently be released during circulatory shock.

The central role of the pancreas in the production of MDF during hemorrhagic shock was substantiated by the finding of high residual MDF activity in pancreases taken from cats in postoligemic shock. The MDF activity of these pancreases without prior incubation was comparable to that found in the plasma of cats in postoligemic shock. These findings indicate that the pancreas continues to produce MDF during shock. Moreover, an additional amount of MDF remains within the pancreas late in postoligemic shock, possibly because lymphatic flow is
very low at this time (28) or because the MDF concentration gradient across the pancreas is small. In either case, the intrapancreatic MDF would not be transported away from the pancreas.

After MDF is formed in the pancreas, it is transported to the systemic circulation, where it exerts marked cardiodepressant effects on the heart (10). However, the negative inotropic effect of MDF had not been previously demonstrated in the intact unshocked animal. Intravenous infusion of MDF into intact anesthetized cats in amounts equivalent to that present in the plasma of cats in postoligemic shock resulted in a profound shock state 30-60 minutes after MDF infusion. Sixty minutes after MDF infusion, these animals had a cardiac output and an MABP closely resembling that of the shocked cats 60 minutes after reinfusion of the shed blood. In both cases the decreases in both variables were comparable at this time, indicating that total peripheral resistance had not significantly changed. In addition, a marked impairment of cardiac performance was observed at this time. These findings indicate that MDF is capable of impairing the normal unsensitized heart of the intact animal to a degree comparable to that found in circulatory shock, thus reinforcing the significance of MDF as a potent toxic factor contributing to the lethality of circulatory shock.

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References


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