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Beneficial Action of a New Angiotensin-Converting Enzyme Inhibitor (SQ 14,225) in Hemorrhagic Shock in Cats

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SUMMARY A new angiotensin-converting enzyme inhibitor (CEI), SQ 14,225, was infused at 0.5 mg/kg per hr, iv, into cats to determine its effect in hemorrhagic shock. Cats were bled to a mean arterial blood pressure (MABP) of 40 mmHg for 160 minutes; this was followed by reinfusion and a 120-minute postoligemic observation period. Hemorrhagic shock and sham shock controls were given an infusion of the CEI or its vehicle (0.9% NaCl). The degree of converting enzyme inhibition was assessed by measuring pressor responses to angiotensin I and II and by radioimmunoassay determination of plasma angiotensin II concentrations. In vitro studies on cat papillary muscles and vascular smooth muscle strips revealed no direct inotropic or vasoactive effect of SQ 14,225. Nevertheless, hemorrhaged cats given the CEI demonstrated a significantly higher final arterial pressure than hemorrhaged cats given 0.9% NaCl (96 vs. 51 mm Hg) (P < 0.01), indicating a significant prolongation of circulatory stability which is closely related to survival. Circulating lysosomal hydrolase (i.e., cathepsin D) activity (3.5 vs. 11-fold increases) and total plasma proteolysis (25% vs. 100% increases) were significantly reduced in the shocked cats given the CEI compared to the untreated shocked animals. Formation of a myocardial depressant factor (MDF) also was significantly diminished by CEI treatment (26 vs. 62 U). These results indicate that CEI improved the hemodynamic and biochemical status of cats in hemorrhagic shock and suggest that blockade of angiotensin II formation may be beneficial in hemorrhagic shock. Abolition of other actions of converting enzymes (e.g., potentiation of bradykinin action or inhibition of proteolysis) may also be involved in the protective mechanisms.

RECENTLY an angiotensin-converting enzyme inhibitor (CEI), SQ 20,881, (bradykinin potentiating factor) has been shown to be beneficial in hemorrhaged animals. The actual mechanism of this protective action was not investigated, although the effectiveness of the blockade of angiotensin II formation was ascertained. Since angiotensin II is a potent splanchnic vasoconstrictor and also can damage the myocardium, angiotensin II may contribute to the pathophysiology of circulatory shock.

This is particularly relevant in hemorrhagic shock, since early hypoperfusion of the splanchnic organs and later myocardial impairment are processes in which angiotensin II may play an important contributory role.

The development of a more efficient CEI, SQ 14,225, provided a pharmacological method with which to attempt to characterize the role of angiotensin II in hemorrhagic shock, as well as to assess the effectiveness of converting enzyme inhibitors as therapeutic agents in shock. These possibilities were tested in cats subjected to a standardized hemorrhagic shock protocol with particular attention given to the consequences of lysosomal integrity and proteolysis as well as to hemodynamic status. Angiotensin II concentrations were monitored to test the completeness of the converting enzyme blockade and to evaluate the time course of changes in circulating angiotensin II concentra-
tions during hemorrhagic shock in nontreated animals.

The major purposes of this study were to: (1) examine the effect of SQ 14,225 in hemorrhagic shock, (2) elucidate possible mechanisms of action of the CEI, and (3) measure the magnitude of the responsiveness of the renin-angiotensin system to severe hemorrhage leading to a lethal form of circulatory shock.

Methods

Adult male cats (2.6 to 4.2 kg) were anesthetized with pentobarbital sodium (30 mg/kg, iv), and catheters were placed in the right carotid artery, and the left jugular vein to record mean arterial blood pressure (MABP) and central venous pressure (CVP) with Statham P-23Db pressure transducers. The trachea was intubated to ensure a patent airway and to allow for artificial ventilation if required during the oligemic period. Additionally, the right femoral artery and vein were cannulated with polyethylene catheters. The venous catheter served as a conduit for drug infusion, and the arterial catheter provided a route for bleeding and reinfusion of blood. A noncannulating electromagnetic flow probe of 1.5 mm internal diameter was fitted around the superior mesenteric artery and connected to a Statham model SP 2202 electromagnetic flowmeter to record superior mesenteric artery flow. Heparin was administered intravenously in a dose of 500 U/kg body weight. The blood reservoir, a Lucite chamber oxygenated with 95% O2 + 5% CO2, was employed to ensure stabilization of hemodynamic and metabolic parameters. The control reading was taken, and CEI (0.5 mg/kg per hr) or saline infusion commenced after a priming dose of 0.5 mg/kg or 1.5 ml of 0.9% NaCl. Four groups of cats were studied: (1) sham shock cats treated with the CEI, (2) sham shock cats given only the vehicle (0.9% NaCl), (3) hemorrhaged cats given the CEI, and (4) hemorrhaged cats receiving only the vehicle. The sham shock cats were subjected to all the surgical procedures experienced by hemorrhaged animals but were not bled. All hemorrhaged cats were bled to a mean arterial blood pressure (MABP) of 40 mm Hg, and this pressure was maintained for 150 minutes by the servo-pump. All shed blood then was reinfused slowly over 10 to 15 minutes, and the cats were observed for another 120 minutes. Five milliliter blood samples were collected at 0, 150, 170, 210, and 270 minutes for analysis of plasma angiotensin II, cathepsin D, amino nitrogen, and total plasma protein. An additional 30-ml blood sample was taken at 270 minutes for determination of myocardial depressant factor (MDF) activity. CEI or 0.9% NaCl was infused continuously for the 270-minute period.

Plasma Analysis

Plasma samples were analyzed for protein concentration by the biuret method of Gornall et al. The plasma activity of cathepsin D, a lysosomal enzyme marker, was assayed by the release of tyrosine from hemoglobin substrate according to the method of Anson. Proteolytic activity of plasma (i.e., plasma amino-nitrogen) was quantified by the ninhydrin method of Kabat. MDF activity was determined by a papillary muscle bioassay using the method of Lefer et al. Plasma was deproteinized and processed on Bio-Gel P-2 gel filtration columns, and the eluates were assayed on the isolated papillary muscles set up according to the method of Lefer, stimulated at 1 Hz at 37°C, and bathed in a modified Krebs-Henseleit solution. A depression in contractile force of 1% under these standardized conditions is equivalent to 1 MDF unit.

Angiotensin II concentrations were determined in plasma using 125I-labeled angiotensin and antiserum for 5-isoleucyl angiotensin II. Plasma samples were treated with 5 mM EDTA, an angiotensinase inhibitor that has been shown to prevent angiotensin degradation at this concentration. Aprotinin (5000 KIU/ml) also was added as an additional agent to prevent angiotensin II degradation. Aliquots of each sample were then quickly frozen in liquid nitrogen and stored at -20°C. The samples subsequently were assayed for angiotensin II within 1 to 2 weeks. Angiotensin II was isolated from plasma by fuller’s earth in excess (50 mg/ml plasma), and the angiotensin then was separated from the fuller’s earth with 0.88 M ammonia in methanol, as previously described. This solvent was evaporated and the angiotensin was reconstituted in a sodium acetate buffer. An extraction of at least 40% was obtained using this procedure.

Isolated Tissue Preparations

A variety of in vitro preparations were employed in an effort to characterize specific actions of the CEI. Papillary muscles from the right ventricles of cats were suspended in 10-ml chambers and electrically paced, according to the techniques described previously. Feline superior mesenteric arteries also were removed and cut into helical strips. These strips were placed in 20-ml chambers, bathed in Krebs-Henseleit solution, and subjected to a tension of 0.4 g. Pharmacological agents were added directly to the bath.

Isolated cat liver large granule fractions prepared from liver homogenates according to the method of Bridenbaugh et al. were incubated at 37°C with either the CEI or its vehicle (0.9% NaCl) to determine the direct lysosomal-stabilizing action of this agent. β-glucuronidase activity of liver large granule fractions was determined by the method of Talalay et al. In addition, cathepsin D activity also was determined in liver large granule fractions. Synthetic angiotensin I and angiotensin II were ob-
Statistics

All statistical analyses used Student's t-test, and a P value of less than 0.05 was accepted as significant.

Results

Figure 1 shows the time course of changes in MABP in the four experimental groups. Cats in all four groups exhibited comparable initial blood pressures. Sham shock cats experienced only small decreases in arterial pressure over the 4.5-hour experimental period. In contrast, hemorrhaged cats exhibited rapidly decreasing pressures after bleeding (B); the hypotension was maintained at 40 mm Hg for the entire 150-minute oligemic period, until terminated by reinfusion (R). Both hemorrhaged groups exhibited rapid increases in blood pressure after reinfusion, with the cats receiving the vehicle initially reaching higher pressures than those given the CEI. However, the MABP of the cats treated with vehicle then rapidly declined during the following 100 minutes to a final value of 51 ± 8 mm Hg (mean ± SEM). CEI treatment largely prevented this fall in pressure. In CEI-treated hemorrhaged cats, MABP declined to only 96 ± 9 mm Hg over the same time period. These results demonstrate a remarkable maintenance of systemic blood pressure by the CEI after hemorrhage, and can be regarded as showing a beneficial action on the circulatory system, since it enables the cats to sustain a normal tissue perfusion pressure.

Figure 2 illustrates the plasma accumulation of cathepsin D activity in the four experimental groups. Both sham shock groups developed only slight increases in plasma cathepsin D activity during the course of the experimental period. However, hemorrhaged cats receiving 0.9% NaCl manifested high plasma cathepsin D activities for the duration of the experimental period, increasing from an initial value of 1.5 ± 0.3 to 16.4 ± 3.0 U at 270 minutes; this is an 11-fold elevation. Hemorrhaged cats receiving the converting enzyme inhibitor demonstrated increasing cathepsin D activities during oligemia, but the post-reinfusion increases were very slight. Plasma cathepsin D activity increased from 2.0 ± 0.8 U initially to 6.9 ± 1.4 U at 270 minutes, a 3.5-fold increase from control values in hemorrhaged cats given SQ 14,225. These two hemorrhaged groups differed significantly from each other at all three post-reinfusion times (P < 0.025), indicating a lessening of lysosomal enzyme release. SQ 14,225 and its vehicle were incubated in plasma samples containing high cathepsin D activity, without any effect of the CEI on cathepsin D. Thus, SQ 14,225 does not inhibit cathepsin D activity. Since the increase in circulating lysosomal hydrolase activity is usually associated with increasing severity of circulatory shock, this effect on circulating lysosomal enzyme activity appears to be beneficial.

Figure 3 illustrates the levels of proteolytic activity (i.e., free amino-nitrogen groups) present in cat plasma. All groups exhibited equivalent amino-nitrogen values at 0 time. Furthermore, both groups of sham shock cats demonstrated stable amino-nitrogen levels for the entire 270-minute experimental period. However, both hemorrhaged groups exhibited increasing amino-nitrogen values during
oligemia, with the cats receiving the CEI exhibiting a plateau in plasma proteolysis after reinfusion. The hemorrhaged group receiving the CEI actually showed a decline in proteolysis after reinfusion, with values decreasing from 9.3 ± 0.9 U (mean ± SEM) at reinfusion to 7.5 ± 0.4 U at 270 minutes. This equaled that of sham shock cats given the drug. As expected, cats given the vehicle showed a further increase in proteolysis after reinfusion, with final values reaching 11.7 ± 1.3 U. The final value in hemorrhaged cats given CEI was significantly lower than that in hemorrhaged cats given only the vehicle (P < 0.025), indicating a significant amelioration of the proteolysis associated with hemorrhagic shock in response to the converting enzyme inhibitor.

Figure 4 shows final plasma MDF activities of the four experimental groups. Sham shock cats exhibited low values of MDF (i.e., 17 ± 3 and 19 ± 3 U with SQ 14,225 or vehicle treatment, respectively). Hemorrhaged cats given only the vehicle developed high circulating levels of MDF (i.e., 63 ± 6 U), indicative of severe circulatory shock. In contrast, CEI treatment of shock animals significantly prevented the accumulation of MDF, final levels being only 27 ± 4 U, a value significantly different from the vehicle-treated shock cats (P < 0.001). These data also provide evidence for an anti-shock action of the CEI, since MDF formation is believed to be a critical factor in the circulatory decompensation occurring in shock.17

The maximum volumes of blood removed and body weights of the two groups of hemorrhaged cats are shown in Table 1. These two groups had almost identical average body weights, and maximum volumes of blood removed also were very similar (i.e., 31 ml/kg with the saline vehicle and 30 ml/kg with the addition of the CEI). These findings indicate that there was no significant difference between the two hemorrhaged groups with respect to the magnitude of hemorrhage to which both groups were subjected. Thus, the beneficial actions of SQ 14,225 cannot be attributed to a potential action of the drug in reducing the severity of the shock state itself.

Table 2 summarizes the plasma angiotensin II concentrations in the four groups. A large increase in the angiotensin II concentration was observed in hemorrhaged cats given only the vehicle; values rose from 108 to 1300 pg/ml at 150 minutes. CEI treatment of shock animals significantly blunted this increase; values increased from 109 to only 501 pg/ml. Final values in the bled groups were 792 and 456 pg/ml, respectively, in the vehicle- and CEI-

**Table 1: Body Weights and Bleeding Volumes in Hemorrhaged Cats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hemorrhagic shock + SQ 14,225</th>
<th>Hemorrhagic shock + vehicle</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>3.2 ± 0.2</td>
<td>3.4 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Maximum bleeding volume (ml/kg)</td>
<td>29.8 ± 2.6</td>
<td>31.0 ± 2.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

All values are means ± SEM. Numbers in parentheses indicate the number of cats in each group. NS = not significant.
treated cats. Thus, converting enzyme blockade by SQ 14,225 prevented about 70% of the increase in angiotensin II concentration in response to severe hemorrhage. The effectiveness of the converting enzyme blockade also was tested by angiotensin I and angiotensin II injections before and after infusion of SQ 14,225 in normal control cats. After infusion of SQ 14,225, only small increases in blood pressure or decreases in superior mesenteric artery flow (SMAF) were noted (i.e., an increase of 6 ± 1% and a decrease of 12 ± 4.3%, respectively), whereas the same angiotensin I dose produced a large pressor and splanchnic vasoconstrictor response before the CEI (i.e., an increase of 33 ± 3% and a decrease of 52 ± 12%, respectively). Both of these findings indicate an efficient inhibition of the angiotensin-converting enzyme by our protocol for SQ 14,225 administration. This inhibition of angiotensin I was specific, since the pressor and splanchnic vasoconstrictor responses to angiotensin II were not altered by SQ 14,225 in the same cats.

No direct effect of the CEI on superior mesenteric artery flow was observed in intact cats. Both hemorrhaged groups experienced severe declines in mesenteric flow during oligemia and after reinfusion. Final values were 8.1 ± 1.7 ml/kg in the CEI bled group and 5.1 ± 1.1 ml/kg in hemorrhaged cats receiving saline. Although the overall response of hemorrhaged cats given SQ 14,225 was improved, splanchnic blood flow was not significantly increased by CEI treatment during shock. Furthermore, isolated papillary muscles and superior mesenteric artery strips failed to respond to the CEI in the absence of direct inotropic and vasoactive effects of SQ 14,225 in the cat.

However, SQ 14,225 did exert cellular effects. Figure 5 demonstrates a lysosomal-stabilizing action of SQ 14,225 on isolated liver large granule fractions. Two representative lysosomal enzymes, /-glucuronidase and cathepsin D, were employed to monitor lysosomal membrane stability. /-Glucuronidase showed a decreased degree of release (i.e., 8.8 ± 2.7%), and cathepsin D showed a 3.7 ± 1.1% average inhibition of release when SQ 14,225 (5 µg/ml) was added to the LGF (liver large-granule fraction) incubates just prior to the start of the 30-minute incubation period. These results provide evidence for a membrane-stabilizing action of the CEI on liver lysosomal membranes.

Table 1 illustrates the effect of the CEI on the proteolytic activity of pancreatic homogenates. SQ 14,225 (5 µg/ml) decreased the proteolytic activity of the homogenates at 60 and 120 minutes by about 1 µmol serine/mg protein, a substantial inhibition of proteolysis. This finding of a decrease in pancreatic proteolysis indicates that SQ 14,225 exerts a significant anti-protease effect in pancreatic tissue. This may be of significance in preventing the formation of MDF by pancreatic acinar cells.

Discussion

The evidence accumulating in favor of an anti-shock action of angiotensin-converting enzyme inhibitors suggests a possible role of angiotensin II in the development of circulatory shock. The difficulty in evaluating the role of angiotensin in shock stems from the multiplicity of actions of converting enzyme inhibitors. Since angiotensin-converting en-

| Table 2 Plasma Angiotensin II Concentrations (pg/ml) in Anesthetized Cats |
|----------------|----------------|----------------|----------------|----------------|
| Time (min) | Sham + 0.9% NaCl | Sham + SQ 14,225 | Hem + 0.9% NaCl | Hem + SQ 14,225 |
| 0 | 81 ± 28 | 151 ± 62 | 109 ± 30 | 108 ± 33 |
| 150 | 154 ± 46 | 279 ± 91 | 501 ± 180 | 1300 ± 174* |
| 210 | 265 ± 90 | 440 ± 184 | 359 ± 80 | 1087 ± 212* |
| 270 | 246 ± 32 | 304 ± 63 | 315 ± 103 | 646 ± 240 |
| 330 | 241 ± 45 | 406 ± 140 | 456 ± 160 | 792 ± 229 |

All values are means ± SEM. Hem = hemorrhaged. Numbers in parentheses indicate the number of cats in each group.* P < 0.02 from appropriate sham at same time.

(5 µg/ml) was added to the LGF (liver large-granule fraction) incubates just prior to the start of the 30-minute incubation period. These results provide evidence for a membrane-stabilizing action of the CEI on liver lysosomal membranes.

Table 3 illustrates the effect of the CEI on the proteolytic activity of pancreatic homogenates. SQ 14,225 (5 µg/ml) decreased the proteolytic activity of the homogenates at 60 and 120 minutes by about 1 µmol serine/mg protein, a substantial inhibition of proteolysis. This finding of a decrease in pancreatic proteolysis indicates that SQ 14,225 exerts a significant anti-protease effect in pancreatic tissue. This may be of significance in preventing the formation of MDF by pancreatic acinar cells.

Table 3 Effect of SQ 14,225 on Proteolysis in Cat Pancreatic Homogenates

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Δ Proteolysis (µmol serine/mg protein)</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>60</td>
<td>-0.97 ± 0.21</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>120</td>
<td>-1.11 ± 0.40</td>
<td>&lt;0.05</td>
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</tbody>
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Values are paired means ± SEM of the difference between samples of the same homogenate incubated with 0.9% NaCl or SQ 14,225 for six experiments.
zyme also degrades bradykinin, CEIs potentiate the effects of bradykinin. Murthy et al. have shown that CEIs potentiate the vasodepressor effects of bradykinin. Other actions of CEI, such as a general peptidase inhibition, may occur as well. However, CEI does not interfere with prostaglandin or antidiuretic hormone release. A general antiprotease action of SQ 14,225 was observed on pancreatic homogenates in this study, as was a lysosomal-stabilizing action which may be related to inhibition of proteases that can damage lysosomal membranes. Both of these actions have been shown to be beneficial in treating shock. SQ 14,225, therefore, possesses at least two potentially beneficial mechanisms in circulatory shock on a subcellular level, presumably unrelated to any hemodynamic effect of the renin-angiotensin system.

Another protease inhibitor, aprotinin, also has been shown to be of value in hemorrhagic shock. This agent antagonizes a variety of proteases including trypsin, plasmin, and kallikrein. Since aprotinin reduces bradykinin concentration as it protects in shock, it thus renders less likely the bradykinin-potentiating effects of SQ 14,225 as being a significant component of its protective effect in hemorrhagic shock.

CEI infusion in hemorrhaged cats resulted in significantly higher postischemic MABP than in vehicle-treated bled cats. This higher pressure did not result from an elevation of blood pressure in the drug-treated cats but rather relates to a maintenance of pressure (i.e., a prevention of the reduction of MABP) after reinfusion. Maintenance, rather than elevation of pressure, is the probable explanation, because immediately after reinfusion, cats treated with the vehicle had higher MABP than did CEI-treated cats. Further evidence in favor of a lack of a direct action of CEI on MABP in the cat is the close similarity of the systemic pressures of the two sham shock groups. The conclusion drawn from these data is that SQ 14,225 lacks a direct hemodynamic action but confers a degree of protection in shock resulting in an improved post-reinfusion MABP.

SQ 14,225 diminished the accumulation of cathepsin D, the generation of peptide fragments, and the plasma MDF activity in vivo. The reductions in lysosomal and proteolytic activities probably result from direct biochemical actions of SQ 14,225, since in vitro studies demonstrated lysosomal stabilization and general antiprotease actions. MDF is thought to be formed from a combination of the actions of lysosomal and zymogenic proteases within pancreatic acinar cells; therefore, the lowered levels of this toxic peptide probably also are dependent on stabilization of lysosomal membranes and the antagonism of zymogenic proteases within pancreatic acinar cells.

The increase in plasma angiotensin II concentration in response to hemorrhage was effectively curtailed by the CEI. The role of this inhibition in the antishock actions of SQ 14,225 may be of great significance to survival in shock states. However, when SQ 14,225 was infused in two cats starting 2 hours after the onset of hemorrhage, no hemodynamic or biochemical protection was observed. These findings suggest that prevention of angiotensin II formation is a critical aspect of the beneficial effects of SQ 14,225 in hemorrhagic shock. The contributions of angiotensin II to the progression of the shock state must be elucidated by procedures designed specifically to eliminate it from the system. However, the splanchnic vasoconstrictor action of angiotensin II, as well as its potential damaging effect on the myocardium when high concentrations are circulating, suggests a possible shock-promoting role of angiotensin II.

In conclusion, results of the present investigation demonstrate significant beneficial effects of the converting enzyme inhibitor, SQ 14,225, in hemorrhagic shock. This effect probably is dependent on a combination of the following actions: (1) lysosomal stabilization, (2) general antiproteolytic actions, and (3) inhibition of angiotensin II formation. However, the actual contribution of the reduced angiotensin II levels or other antiprotease actions to the protection from effects of circulatory shock remains to be quantified.

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