Protective Effects of Thromboxane Synthetase Inhibitors in Rats in Endotoxic Shock

W. Curtise Wise, James A. Cook, Perry V. Halushka, and Daniel R. Knapp

SUMMARY To evaluate the pathogenic role of thromboxane (Txs) in endotoxic shock, the potential protective effects of the Tx synthetase inhibitor, 7-(1-imidazolyl)-heptanoic acid (7-IHA) was assessed and compared to that of imidazole in the rat. 7-IHA (30 mg/kg, iv) administered iv 30 minutes prior to Salmonella enteritidis endotoxin (20 mg/kg, iv) improved the survival rate from 42% (n = 24) at 5 hours in the vehicle-treated rats to 100% (n = 11). By 24 hours, only 8% of the vehicle-treated rats survived, whereas 80% of the treated group survived. Venous plasma thromboxane B₂ elevation did not occur if 7-IHA (30 mg/kg) was administered iv 30 minutes prior to endotoxin (20 mg/kg, iv). However, 7-IHA did not inhibit endotoxin-induced elevations in plasma prostaglandin E₂. Endotoxin induced a significant reduction in the platelet counts in vehicle-treated rats from 780 ± 64 x 10⁴/mm³ to 179 ± 18 x 10⁴/mm³ (n = 6, P < 0.01) by 15 minutes, whereas, in imidazole-pretreated rats, the platelet count fell significantly less to 402 ± 55 x 10⁴/mm³ (n = 6, P < 0.05), and in the 7-IHA-pretreated rats, the platelet count fell to 541 ± 91 x 10⁴/mm³ (n = 5, P < 0.05). Fibrinogen/fibrin degradation products were significantly increased (P < 0.01) in response to endotoxic shock, but both imidazole and 7-IHA significantly decreased (P < 0.05) this elevation. Imidazole and 7-IHA pretreatment prevented lysosomal labilization in endotoxic shock as denoted by significantly decreased elevations in serum acid phosphatase and β-glucuronidase. Similarly, reduction in hepatic cellular damage, as assessed by decreased elevations of serum glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase, was apparent. These results are consistent with the hypothesis that TxA₂ plays a significant role in the pathogenesis of endotoxic shock. Circ Res 46: 854-859, 1980

ENDOTOXIC shock is associated with thrombocytopenia, disseminated intravascular coagulation (Müller-Berghaus, 1978), lysosomal labilization (Janoff et al., 1962), and hepatocellular dysfunction (Coalson et al., 1978). Additionally, pulmonary and mesenteric vasoconstriction have been reported in endotoxemia (Pennington et al., 1973; Brobmann et al., 1970). Recently, it has been shown that plasma thromboxane (Tx) levels rise in rats within 30 minutes after endotoxin administration (Cook et al., 1980). Imidazole, a Tx synthetase inhibitor (Cook et al., 1980), was demonstrated to improve survival to endotoxic shock and reduce the endotoxin-induced elevation of TxB₂ (Cook et al., 1980). Furthermore, rats deficient in essential fatty acids, which are devoid of the thromboxane precursor arachidonic acid, are resistant to the effects of endotoxemia (Cook et al., 1979). Since TxA₂ is a potent vasoconstrictor and platelet-aggregating substance (Samuelsson et al., 1978), we tested the effects of the TxA₂ synthetase inhibitors imidazole (Needelman et al., 1977; Moncada et al., 1977) and 7-(1-imidazolyl)-heptanoic acid (7-IHA) (Yoshimoto et al., 1978) on the pathophysiological sequelae of endotoxemia. Specifically we evaluated the effects of these agents to modify endotoxin-induced mortality, thrombocytopenia and coagulopathies, plasma thromboxane B₂ and prostaglandin E₂ elevations, as well as lysosomal integrity and hepatic function.

Methods

Male Long-Evans rats, 175–200 g (7–9 weeks old), used for these experiments came from a Long-Evans breeding colony maintained by the investigators. The rats were housed in plastic cages under conditions of constant temperature and controlled illumination and were allowed ad libitum access to food and water. Rat food was obtained from Wayne feeds (Allied Mills, Inc.) and contained a minimum of 24% protein, 4.5% fiber, and 4% fat.

Endotoxic shock was induced by administrating a single intravenous dose of Salmonella enteritidis endotoxin (20 mg/kg) to rats lightly anesthetized with ether. The endotoxin was a Boivin preparation from Difco, control no. 690467.

Platelet counts were determined using the method of Brecher and Cronkite (1950). Whole venous blood obtained from the tail vein was diluted...
in 1% ammonium oxalate, and platelets were counted in a hemocytometer.

Four hours after endotoxin administration, the rats were anesthetized with ether and exsanguinated from the inferior vena cava for determination of the following plasma constituents. Total serum acid phosphatase activity was assayed by a modification of the method of Andersch and Szczypinski (1947). Acid phosphatase activity was determined colorimetrically from the substrate of \( \text{p-nitrophenylphosphate}\), one unit of enzyme activity being defined as the amount of enzyme activity that will liberate 1 mol of \( \text{p-nitrophenol}\) per hour at 37°C. Serum \( \beta\)-glucuronidase activity was assayed by a modification of the procedure of Fishman et al. (1967). One unit of enzyme activity was defined as that amount of enzyme that will liberate 1 \( \mu\)g of phenolphthalein from phenolphthalein glucuronic acid per hour at 56°C. Plasma glutamic-oxaloacetic and glutamic-pyruvic transaminase were measured colorimetrically (Sigma Chemical Bulletin no. 505). Activity is expressed as Sigma Frankely (SF) units. One SF unit will form glutamate, \(4.80 \times 10^{-4}\) \(\mu\)mol/min, at pH 7.5 and 25°C.

The measurement of fibrinogen and fibrin degradation products in serum was based on the method proposed by Hawiger et al. (1970) using a commercially available kit (Sigma Chemical Co., no. 850). An estimate of the fibrinogen/fibrin degradation products (FDP) present is obtained by comparing the degree of cell staphylococcal clumping produced by the test serum with clumping observed using known amounts of fibrinogen.

Imidazole was obtained from Matheson Coleman and Bell, and 7-IHA was synthesized by reaction of ethyl \(7\)-bromohexanoate (Pfalz and Bauer) with the sodium salt of imidazole (prepared by treatment of imidazole with sodium hydride) in dimethylformamide. The resulting \(7\)-IHA ethyl ester was hydrolyzed with aqueous barium hydroxide. Barium ion was removed by precipitation with sulfate ion. The product was collected by freeze drying and purified by recrystallization from acetone. Both imidazole and \(7\)-IHA were diluted in phosphate-buffered saline (300 mOsmol/kg, pH 7.4) before administration. Both imidazole and \(7\)-IHA (30 mg/kg) were administered iv 30 minutes prior to endotoxin.

Radioimmunoassay of \(\text{TXB}_2\) and \(\text{PGE}\)

Blood, obtained from the inferior vena cava or abdominal aorta for \(\text{TXB}_2\) and \(\text{PGE}\) determinations, was collected in plastic syringes containing 0.1 ml indomethacin (4 mg/ml) dissolved in 0.1 m sodium phosphate buffer, pH 8.0, and 500 \(\mu\)l heparin. The blood was centrifuged (1500 g) for 20 minutes, and the plasma was collected and frozen at \(-20\)°C until extraction. To 1 ml of plasma, \([\text{H}]\text{TXB}_2\) or \([\text{H}]\text{PGE}\) (1500 counts/min) was added to correct for recovery losses. The plasma was acidified to pH 3.5 with formic acid and extracted twice with 3 volumes of ethyl acetate. The ethyl acetate layer was removed and dried under \(\text{N}_2\). The dried extract was reconstituted and applied to a silicic acid column (0.5 g) and the prostaglandin \(E\) and \(\text{TXB}_2\) fraction was collected and dried under nitrogen. The dried extract was reconstituted in 1 ml gelatin-phosphate-buffered saline. \(\text{TXB}_2\) was determined using a previously described radioimmunoassay (Burch et al., 1979). After incubation for 4 hours at 37°C, free \([\text{H}]\text{TXB}_2\) was separated from bound using charcoal dextran solution (Burch et al., 1979). The antibody was provided by Dr. J.B. Smith, Cardeza Foundation, Philadelphia, Pennsylvania. The minimum detectable amount in 1 ml of plasma was approximately 375 pg/ml. The antibody does not significantly cross-react (<0.04%) with other prostaglandins, and the interassay variability is 17% (\(n = 10\)). PGE was determined using a previously described radioimmunoassay after conversion to PGE (Webb et al., 1978).

Statistical Analysis

Student’s \(t\)-test was used to determine differences in enzyme levels between untreated and treated groups. The \(\chi^2\) test was employed for mortality studies. Confidence limits were placed at 95% for significance. All data are expressed as the mean ± SEM.

Results

To investigate the protective effects of blocking the synthesis of \(\text{TXA}_2\), we determined the effects of the \(\text{TX} \) synthetase inhibitor \(7\)-IHA. \(7\)-IHA (30 mg/kg) administered iv 30 minutes prior to endotoxin significantly improved the survival time (Fig. 1). Only 8% of the vehicle-treated rats were alive at 48 hours, whereas 80% of the \(7\)-IHA-pretreated rats were alive at 48 hours and a similar survival rate was observed at 72 hours.

Plasma \(\text{TXB}_2\) and Prostaglandin \(E\)

Plasma samples were obtained from the inferior vena cava and abdominal aorta 30 minutes after endotoxin administration. The \(\text{TXB}_2\) level in the aortic sample was 570 ± 29 pg/ml (\(n = 4\)) and the venous \(\text{TXB}_2\) sample was 1164 ± 106 pg/ml (\(n = 4\)). Both levels were significantly (\(P < 0.01\)) higher than in the abdominal aorta sample and the venous \(\text{TXB}_2\) level was significantly (\(P < 0.01\)) greater than in the venous sample. Thus, all subsequent samples were obtained from the vena cava.

Within 30 minutes after endotoxin injection, plasma venous \(\text{TXB}_2\) levels increased from undetectable levels (<375 pg/ml) in normal control rats (\(n = 10\)) to 2054 ± 524 pg/ml (\(P < 0.01\)). Plasma venous PGE levels also increased from 146 ± 33 pg/ml (\(n = 5\)) in non-shock controls to 2161 ± 606 pg/ml (\(n = 5\)) 30 minutes after endotoxin administra-
tion (Cook et al., 1980). In rats pretreated with 7-IHA (30 mg/kg, iv) 30 minutes before endotoxin, plasma venous TxB$_2$ levels failed to rise significantly above values for normal control rats. Plasma PGE levels, however, were elevated, (1456 ± 447 pg/ml) in 7-IHA-pretreated group ($n=5$) and did not vary significantly from shock controls.

**Effects of Imidazole and 7-IHA on Endotoxin-Induced Thrombocytopenia**

Within 15 minutes after the administration of endotoxin, platelet counts decreased from 780 ± 64 x 10$^3$/mm$^3$ to 179 ± 18 x 10$^3$/mm$^3$ ($P < 0.001$) in the vehicle-treated rats and returned to the control values within one hour (Table 1). However, at 4 hours, platelet counts in the endotoxin control group had decreased to 232 ± 56 x 10$^3$/mm$^3$ ($P < 0.05$). None of these control animals survived past 10 hours. In the imidazole-pretreated group, endotoxin decreased the platelet count from 872 ± 49 x 10$^3$ platelets/mm$^3$ to 402 ± 55 x 10$^3$ within 15 minutes, a value significantly greater ($P < 0.01$), than that observed in the control group. As was seen in the control group, platelet counts returned to control values within 1 hour. At 4 hours, however, the platelet count was significantly ($P < 0.01$) higher than in the control group. Similarly, 7-IHA decreased the severity of the thrombocytopenia. In rats pretreated with 7-IHA, endotoxin decreased platelet counts from 972 ± 92 x 10$^3$/mm$^3$ to 541 ± 91 x 10$^3$/mm$^3$ after 15 minutes. This fall in platelet count was significantly less ($P < 0.01$) compared to that in the control group. The 15-minute platelet count in the 7-IHA group was not significantly different from that in imidazole-pretreated group. In contrast to the above two groups, the platelet counts in the 7-IHA-pretreated rats returned to control levels within 30 minutes and at 4 hours, the thrombocytopenia was significantly ($P < 0.05$) less severe than in the vehicle-treated group.

**Fibrinogen/Fibrin Degradation Products (FDP)**

Having established that both imidazole and 7-IHA reduced the severity of the endotoxin-induced thrombocytopenia, we sought to determine the de-

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**Table 1 Platelet Counts ($\times 10^3$)**

<table>
<thead>
<tr>
<th>Group</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>780 ± 64*</td>
<td>179 ± 18†</td>
<td>556 ± 48†</td>
<td>643 ± 64 (6)</td>
<td>232 ± 56† (9)</td>
</tr>
<tr>
<td>Imidazole-</td>
<td>872 ± 49</td>
<td>402 ± 55‡</td>
<td>540 ± 51‡</td>
<td>640 ± 60 (6)</td>
<td>687 ± 95 (5)</td>
</tr>
<tr>
<td>treated</td>
<td>(7)</td>
<td>(6)</td>
<td>(11)</td>
<td>(10)</td>
<td>(5)</td>
</tr>
<tr>
<td>7-IHA-</td>
<td>972 ± 92</td>
<td>541 ± 91‡</td>
<td>977 ± 135</td>
<td>859 ± 39 (5)</td>
<td>422 ± 58‡ (12)</td>
</tr>
<tr>
<td>treated</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(12)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses = number of rats. Imidazole (30 mg/kg) and 7-(1-imidazolyl)-heptanoic acid (7-IHA) (30/kg) were administered iv 30 minutes prior to iv S. enteritidis endotoxin. The 15-minute control values were significantly lower ($P < 0.01$) than those of either imidazole or 7-IHA-pretreated groups.
† $P < 0.01$ compared to 0 time.
‡ $P < 0.05$ compared to 0 time.
TABLE 2  Plasma Fibrinogen/Fibrin Degradation Products (FDP) during Endotoxemia

<table>
<thead>
<tr>
<th></th>
<th>Values 4 hours post-endotoxin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
<td>58.5 ± 12*</td>
</tr>
<tr>
<td>Imidazole-treated (n = 5)</td>
<td>4.5 ± 0.8†</td>
</tr>
<tr>
<td>7-IHA-treated (n = 7)</td>
<td>10.5 ± 1.5†</td>
</tr>
</tbody>
</table>

Nonendotoxin-treated controls were <0.05 µg/ml. Numbers in parentheses = number of rats. Imidazole (30 mg/kg) and 7-(1-imidazole)-heptanoic acid (7-IHA) (30 mg/kg) were administered iv 30 minutes prior to iv S. enteritidis endotoxin.

* P < 0.05 compared to control.
† SEM.

Discussion

We have demonstrated previously (Cook et al., 1980) that plasma TxB2 increases within 30 minutes after iv administration of endotoxin in rats and that pretreatment with imidazole abolished the elevation and improved survival. Similarly, our results demonstrate that 7-IHA pretreatment also prevents elevation in plasma TxB2 levels during endotoxic shock and reduces mortality. The mechanism of the protective effect of these compounds is uncertain. Although both compounds inhibit thromboxane synthetase, imidazole has numerous additional actions which may be salutory, e.g., stabilization of lysosomal membranes and inhibition of fatty acid cyclooxygenase at higher doses (Moncada et al., 1977 and Puig-Muset et al., 1972). 7-IHA does not inhibit fatty acid cyclooxygenase (Yoshimoto et al., 1978), but its effect on lysosomal membranes is unknown. Additionally we have demonstrated that 7-IHA at 30 mg/kg did not inhibit PGE formation but did inhibit plasma elevation of TxB2 induced by endotoxin. Similar results were obtained in our previous studies with imidazole (Cook et al., 1980). These observations are consistent with the concept that 7-IHA and imidazole both exert their protective action via inhibition of thromboxane synthesis.

The reduction in thrombocytopenia induced by endotoxin following administration of either of these...
The decrease in lysosomal integrity and hepatic function seen in endotoxic shock was improved by pretreatment with imidazole or 7-IHA. This protective effect may be the result of preventing some of the earliest pathophysiological sequelae, e.g., disseminated intravascular coagulation, or may be due to a direct action of these compounds to stabilize lysosomal membranes.

Recent studies from our laboratory (Cook et al., 1979) have also demonstrated that rats deficient in essential fatty acids, depleted of arachidonic acid, are resistant to bacterial endotoxin and do not manifest elevated plasma TxB$_2$ levels following endotoxin administration (Cook et al., 1980). Likewise, 13-azaprostanoic acid (an antagonist of thromboxane A$_2$) (LeBreton et al., 1979) improved the survival from endotoxemia (Cook et al., 1980). The present study coupled with previous observations allows us to hypothesize that endotoxin causes a release of arachidonic acid from one or more tissues resulting in increased synthesis of TxA$_2$, followed by intravascular platelet aggregation and subsequent sequelae of endotoxemia. Thus, inhibition of TxA$_2$ synthesis can alter the pathogenic events of endotoxic shock. The further delineation of the sites of synthesis and the role of TxA$_2$ in the pathogenesis of endotoxic shock clearly merits further investigation.

References

Pennington DG, Hyman AL, Jaques WE (1973) Pulmonary
Effects of Atrial Pacing on Atrio-Sinus Conduction and Overdrive Suppression in the Isolated Rabbit Sinus Node

GERHARD STEINBECK, RALPH HABERL, AND BERNDT LÜDERITZ

SUMMARY We evaluated the effects of atrial pacing on atrio-sinus conduction and overdrive suppression in 15 isolated rabbit sinus node preparations. Decreasing the pacing cycle length from 400 to 200 msec increased atrio-sinus conduction time from 35 ± 10 msec (± SD) to 72 ± 25 msec, and various degrees of atrio-sinus block developed. Increase of conduction time was associated with a decrease of maximum diastolic potential and a marked decrease in action potential amplitude of the sinus node fibers, which gradually returned to control values after cessation of drive; both the changes in conduction times and transmembrane potentials were most pronounced in dominant pacemaker fibers of the sinus node and gradually diminished toward the atrium. An increase in pacing rate decreased the slope of phase 4 depolarization of dominant pacemaker cells after drive (overdrive suppression), until atrio-sinus block ensued; the latter accounted for both the shortening and increasing variation of sinus node recovery time following rapid drive. We conclude that neither pacing-induced release of acetylcholine nor stimulation of an electrogenic sodium pump provide a major contribution to overdrive suppression of sinus node pacemaker cells as the latter likely does in Purkinje fibers. Depression of phase 4 depolarization depended on the prior rate of beats propagated to the sinus node. Sinus node recovery time cannot be interpreted only in terms of overdrive suppression of the automatic process but is the result of a complex interaction between conduction and impulse formation in the sinus node.


RAPID atrial pacing to induce overdrive suppression (Rosen et al., 1971; Mandel et al., 1971; Narula et al., 1972) and premature atrial stimulation (Strauss et al., 1973) recently have been introduced into clinical practice to measure sinus node recovery time and sinoatrial conduction time. To test the validity of the latter diagnostic procedure, we previously investigated the response of the isolated rabbit sinus node to ectopic atrial premature beats using an extensive sinus node-mapping procedure (Steinbeck et al., 1978). We demonstrated that, following a premature beat (elicited late in atrial diastole, but early enough to capture the dominant pacemaker fibers of the node), retrograde impulse propagation from the atrium to the sinus node (atrio-sinus conduction) is more rapid than antegrade propagation from the sinus node to the atrium during spontaneous rhythm (sinoatrial conduction) (Steinbeck et al., 1978). The earlier the premature beat is elicited in the atrial cycle, the longer the time for conduction from the atrium to dominant sinus node fibers (Bonke et al., 1969; Miller and Strauss, 1974). In this study, we used the same mapping technique as previously to evaluate the atrio-sinus conduction pattern during rapid atrial pacing. Results provide evidence that the rate of atrial stimulation strikingly affects atrio-sinus conduction, which in turn was found to be a major determinant for overdrive suppression of the sinus node pacemaker and, hence, the length of the recovery interval after cessation of drive.

Methods

Young rabbits (body weight 2-3 kg) were killed by a blow to the neck. The thorax was opened, and the heart was removed rapidly. The right atrium,
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doi: 10.1161/01.RES.46.6.854

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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