Intravenous Bovine Testicular Hyaluronidase Depolymerizes Myocardial Hyaluronic Acid in Dogs with Coronary Artery Occlusion

ROBERT A. WOLF, LEE-YUH CHAUNG, JAMES E. MULLER, ROBERT A. KLONER, AND EUGENE BRAUNWALD

SUMMARY It has been shown that intravenously injected bovine testicular hyaluronidase reduces ischemic myocardial necrosis in animal models of myocardial infarction. The mechanism for the protective effect of hyaluronidase is unknown, but it has been suggested that this enzyme depolymerizes myocardial hyaluronic acid and thus improves interstitial transport. However, biochemical data showing that intravenous hyaluronidase depolymerizes myocardial substrates are lacking. The purpose of the present study was to test the ability of hyaluronidase to depolymerize myocardial hyaluronic acid. Thirty minutes after occlusion of the left anterior descending coronary artery, nine dogs were randomized to untreated (four dogs) or hyaluronidase-treated (five dogs; 5000 National Formulary Units/kg, iv, and 0.5 and 2.5 hours post occlusion) groups. The hearts were excised 4.5 hours postocclusion and the myocardium was homogenized. Myocardial bound hyaluronidase was heat inactivated, and hyaluronic acid was extracted following protease digestion. Myocardial hyaluronic acid content was determined colorimetrically and its molecular weight was studied by Sepharose 2B gel exclusion chromatography. There was no difference in hyaluronic acid recovery or molecular weight between the ischemic anterior and non-ischemic posterior halves of the hearts of both treated and untreated dogs. Hearts of the untreated dogs contained 3.44 ± 0.32 μg hyaluronic acid/mg protein, whereas hyaluronidase-treated hearts contained only 2.06 ± 0.23 μg hyaluronic acid/mg protein (P < 0.01). The fraction of hyaluronic acid that chromatographed with molecular weight greater than 4 × 10^6 daltons was 73 ± 2% in untreated and only 45 ± 5% in hyaluronidase-treated animals (P < 0.001). Therefore, treatment with hyaluronidase reduces both the amount and the molecular weight of myocardial hyaluronic acid in dogs with coronary artery occlusion. Circ Res 47: 88-95, 1980

BOVINE testicular hyaluronidase (BTH) has been shown to reduce ischemic myocardial necrosis in several animal models of myocardial infarction (Maroko et al., 1972; Braunwald and Maroko, 1976; Maclean et al., 1976, 1978; Kluger et al., 1977; Hillis et al., 1977; Kluger et al., 1978; Hofmann et al., 1979). In addition, preliminary data suggest that BTH limits the extent of necrosis in human myocardial infarction as assessed by electrocardiographic techniques (de Oliveira et al., 1959; Maroko et al., 1975; Maroko et al., 1977). BTH currently is employed in a multicenter clinical investigation designed to assess its value in reducing myocardial infarct size. It has been suggested that the myocardial protective effect of BTH results from depolymerization of hyaluronic acid (HA) in the heart. However, biochemical data demonstrating depolymerization of myocardial HA have not been obtained previously. Such data are needed to substantiate this suggested mechanism of action since mammalian serum is known to contain substantial amounts of naturally occurring hyaluronidase activity (Bollet et al., 1963; DeSaeguir and Pigman, 1967) as well as a circulating inhibitor of BTH (Mathews and Dorfman, 1955). In the present study we have isolated a BTH substrate, hyaluronic acid, from canine myocardium and studied quantitative and structural changes in myocardial HA catalyzed by BTH injected intravenously after coronary artery occlusion.

Methods

Surgical techniques have been described previously (Maroko et al., 1972). Briefly, mongrel dogs of both sexes weighing 20.4-36.3 kg were anesthetized with 25 mg/kg intravenous sodium thiopental and ventilated with a respirator (Harvard Apparatus Co., Inc.). The heart was exposed by thoracotomy in the left 5th intercostal space and suspended in a pericardial cradle. Ischemia of the anterior myocardial wall was produced by occlusion of the left anterior descending coronary artery (Maroko et al., 1972; Braunwald and Maroko, 1976) just proximal to the origin of the first diagonal branch. Myocardial ischemia was confirmed by the presence of cyanosis of the anterior wall, electrocardiographic
ST segment elevation of at least 1 mm and T wave changes in the limb leads. Thirty minutes after occlusion, each dog was randomized to untreated (four dogs) or BTH-treated (five dogs) groups. BTH-treated dogs received a rapid intravenous bolus of 5000 National Formulary Units (NFU) BTH/kg (hyaluronidase, type IV, Sigma Chemical Co.) injected in 10 ml 0.15 M sodium chloride at 0.5 and 2.5 hours post coronary occlusion. Each dog was killed 4.5 hours after occlusion by potassium chloride cardiac arrest.

**Extraction of Myocardial Hyaluronic Acid**

The method described by Orkin and Toole (1978) for isolation of chick embryonic heart HA was adapted to the canine myocardium. Immediately after potassium chloride arrest, the heart was excised, plunged into chopped ice, and bathed in ice cold 0.15 M sodium chloride. The myocardium was dissected free of atria, major vessels, and valvular apparatus and bisected for separate analysis into anterior and posterior halves along a plane perpendicular to the interventricular septum. The myocardium was homogenized for 60 seconds (VirTis Homogenizer, Virtis Co.) in 2 ml/g tissue of ice cold 0.15 M sodium chloride buffered by 0.1 M Tris (pH 7.2) with 0.01% sodium azide. The resulting homogenate was heated to 65°C for 35 minutes for thermal inactivation of BTH. A small aliquot of homogenate was saved for protein determination (Lowry et al., 1951), and the rest underwent three sequential 8-hour digestions with protease, 1 mg/ml (0.7 U protease/mg, type IV, Sigma Chemical Co.) at 56°C. Protease-digested homogenate was clarified at 20,000 rpm for 2 hours, boiled for 5 minutes to inactivate residual protease activity, and reclarified at 20,000 rpm for 30 minutes. Then 75 ml of myocardial extract was dialyzed for 24 hours against distilled water at 4°C, lyophilized, resuspended in 4 ml of 0.15 M sodium chloride, 0.1 M sodium acetate (pH 5.0) by constant stirring at 4°C for 7 hours, and clarified at 20,000 rpm for 30 minutes in preparation for gel exclusion chromatography. The modifications of the method of Orkin and Toole (1978) consisted of adding sodium azide to the extraction buffer for bacteriostasis, heating to inactivate tissue bound BTH after myocardial homogenization, and adding a third protease digestion of myocardial homogenate for HA extraction.

**Analysis of Myocardial Hyaluronic Acid**

Concentrated myocardial extract was loaded onto a 1.6 x 60 cm column of Sepharose 2B gel (Pharmacia) and chromatographed at 5 ml/hour in 0.15 M sodium chloride, 0.10 M sodium acetate (pH 5.0) at 4°C and collected as 0.7-ml fractions. Control and treated heart extracts were prepared and chromatographed under identical conditions. The anterior half of the myocardium which included the ischemic area was analyzed separately from the posterior non-ischemic half of each heart.

The Sepharose 2B gel column (carbohydrate exclusion limit 2 x 10^5 daltons) was standardized with linear dextrans of 2 x 10^3, 5 x 10^3, and 7 x 10^4 daltons (Pharmacia). Column void volume was determined with blue dextran. More than 95% of hyaluronic acid applied to the column was recovered in chromatographed fractions.

HA concentration in collected fractions was determined by a colorimetric method (Polansky et al., 1974; Hatae and Makita, 1974) which is based on the observation that an endosaccharidase prepared from *Streptomyces* species depolymerizes HA but does not cleave other glycosaminoglycans such as chondroitin or chondroitin sulfate (Ohya and Kaneko, 1970). HA concentration can be determined by exhaustive digestion of substrate with excess *Streptomyces* hyaluronidase (Fujisawa Pharmaceutical) followed by formation of the Reissig chromogen (Reissig et al., 1955). Reaction tubes lacking enzyme were incubated in parallel to serve as controls and parallel standards of known amounts of human umbilical HA (hyaluronic acid, Grade 1, Sigma Chemical Co.) were incubated with each assay. This assay is linear for HA concentrations up to 400 µg/ml (y = 0.002x-0.0007; r = 0.99; y = absorbance at 585 nm; x = µg HA/ml) with a limit of detection of 10 µg HA/ml.

**Statistical Analysis**

Data are expressed as mean ± SEM. BTH-treated and untreated data groups are compared by Student's *t*-test for grouped data (Goldstein, 1964). Data from anterior and posterior halves of each heart were compared by the paired *t*-test. Data groups yielding *P* values less than 0.05 were accepted as significantly different.

**Results**

**Identification of Hyaluronic Acid in Canine Myocardium**

To confirm the identification of HA in myocardial extract, parallel samples of human umbilical HA (300 µg/ml) and Sepharose 2B chromatographed myocardial extract were digested with *Streptomyces* hyaluronidase. The absorption spectra of the Reissig chromogen (Reissig et al., 1955) formed from each of these digestion products are compared in Figure 1. These absorption spectra are very similar, with maximum absorption at 550 and 585 nm as originally described for the Reissig chromogen (Reissig et al., 1955). The similarity of these spectra and the specificity of *Streptomyces* hyaluronidase (Ohya and Kaneko, 1970) identify the product extracted from the myocardium as hyaluronic acid.

**Inactivation of Tissue-Bound BTH**

To prevent BTH-catalyzed postmortem changes in tissue HA, myocardium was maintained at 4°C during the homogenization procedure and subse-
It was shown that heating BTH to 65°C for 35 minutes produced irreversible thermal inactivation of BTH. A stock solution of 250 NFU BTH/ml in 0.1 M Tris (pH 7.2), 0.15 M sodium chloride was prepared. One aliquot of stock enzyme was heated to 65°C, a second aliquot heated to 60°C, and a control aliquot maintained at 37°C for 35 minutes. All three aliquots then were diluted into 0.1 M sodium formate (pH 3.7) and assayed at 37°C for 16 hours as previously described (Orkin and Toole, 1978). The aliquot heated to 60°C lost 78% of control activity, while BTH heated to 65°C was inactivated totally and did not recover activity during a 16-hour incubation with excess substrate (Fig. 2). Therefore, each myocardial homogenate was heated to 65°C for 35 minutes to inactivate tissue bound BTH.

Quantitative Analysis of Myocardial Hyaluronic Acid

The HA content of the anterior myocardial wall (including ischemic myocardium) was 3.44 ± 0.32 μg HA/mg protein in untreated dogs vs. only 2.06 ± 0.23 μg HA/mg protein in BTH-treated dogs (P < 0.01). The posterior myocardial wall contained 3.41 ± 0.24 μg HA/mg protein in untreated dogs, whereas BTH-treated hearts contained only 2.02 ± 0.16 μg HA/mg protein (P < 0.001). Therefore, there was significantly less HA detectable in the myocardium of BTH-treated dogs as compared to untreated animals, and this difference was significant both in the anterior and posterior myocardium (Fig. 3). There was no difference in anterior vs. posterior myocardial HA content in either the BTH-treated group or the untreated group in spite of the presence of ischemia in the anterior myocardial wall.

Structural Analysis of Myocardial Hyaluronic Acid

The molecular weight distribution of myocardial HA molecules was assessed by their elution pattern on a Sepharose 2B gel column. Anterior and posterior myocardial samples from each heart were chromatographed separately. The elution patterns for all 18 samples are summarized in Figure 4. There was a striking decrease in the high molecular weight fraction of HA isolated from BTH-treated hearts. In untreated hearts, anterior wall samples contained 2.54 ± 0.28 μg HA/mg protein which eluted between fractions 60 and 124, indicating a molecular weight greater than 4 × 10^6 daltons. In contrast, anterior myocardium from BTH-treated dogs contained only 0.90 ± 0.09 μg HA/mg protein with molecular weight greater than 4 × 10^6 daltons (P < 0.001). Similar changes in the molecular weight of HA were observed in the non-ischemic posterior myocardial samples. For untreated samples, 2.37 ± 0.16 μg HA/mg protein vs. 0.91 ± 0.14 μg HA/mg protein eluted with molecular weight greater than 4 × 10^6 daltons (P < 0.001).
Therefore, the high molecular weight component comprised 73 ± 2% of HA content from untreated anterior myocardium but only 45 ± 5% of HA from BTH-treated anterior myocardium (P < 0.001). In the posterior myocardium, 70 ± 1% untreated HA and 44 ± 5% BTH-treated myocardial HA chromatographed with molecular weight greater than 4 × 10^6 daltons (P < 0.005). A small but statistically insignificant increase in low molecular weight (less than 4 × 10^6 daltons) myocardial HA was seen in BTH-treated dogs (Table 1). There was no detectable difference in the high molecular weight content of anterior vs. posterior wall myocardial HA in either BTH-treated or control animals.

A measure of the average molecular weight of myocardial HA was calculated by determining the arithmetic mean fraction number of HA eluted from the column for each sample. As can be seen in Table 2 the average HA molecular weight for BTH-treated samples was lower than for untreated samples as evidenced by higher average fraction numbers for BTH-treated samples. This difference was statistically significant (P < 0.005) for both anterior and posterior myocardium and corresponds to an approximate average molecular weight of 5 × 10^6 daltons for untreated and 3 × 10^6 daltons for HA isolated from BTH-treated myocardium. However, a significant portion of the myocardial HA from untreated samples chromatographed in the void volume of the Sepharose 2B column (Fig. 4). For this reason, 5 × 10^6 daltons is necessarily an underestimation for the molecular weight of untreated myocardial HA. Therefore, the difference between the molecular weight of HA extracted from treated and untreated myocardium may actually be larger than revealed by the chromatographic patterns in Figure 4. The chromatographic discrimination of BTH-treated samples was very reproducible in the sense that the average fraction number for every BTH-treated sample was higher than for any of the untreated samples.

**Discussion**

BTH previously has been shown to cause certain biochemical changes in ischemic myocardium, including preservation of myocardial creatine kinase (Marcko et al., 1979), increased high energy phosphate stores (Rovetto, 1977), and preservation of myocardial glycogen deposits (Kloner et al., 1977). BTH has also been shown to reduce myocardial carbon dioxide tension in the ischemic heart (Hillis et al., 1978). Recent fluorescence data indicate that BTH decreases NADH accumulated in ischemic myocardium (Harden et al., 1979). In spite of these data, the molecular mechanism of the myocardial protective effect of BTH has been obscure. It has been suggested that intravenously injected BTH is delivered to the myocardium and digests myocardial substrates (Maroko et al., 1972). However, this assumption never was proven biochemically. No previous studies actually have shown that BTH depolymerizes myocardial hyaluronic acid or was capable of doing this in ischemic myocardium. This hypothesis is subject to several criticisms. Mammalian serum is known to contain a potent inhibitor of BTH enzymatic activity (Mathews and Dorfman, 1955). Serum BTH activity has an unusually short serum half life of approximately 2 minutes after intravenous injection in dogs, and sequential intravenous boluses fail to achieve sustained serum levels of enzyme (Muller et al., 1980). Finally, serum (Bollet et al., 1963; DeSalesgut and Pigman, 1987) and myocardial tissue (Orkin and Toole, 1978) have been shown to contain endogenous hyaluronidase activity which is thought to regulate in vivo hyaluronic acid metabolism. Therefore, intravenous BTH must overcome an endogenous serum inhibitor, a
FIGURE 4 Sepharose 2B gel exclusion chromatography showing reduction in molecular weight of HA in BTH-treated myocardium. Anterior myocardial samples (includes ischemic myocardium) shown in panel A, and posterior (non-ischemic) myocardial samples in panel B. Myocardial extracts from each dog were chromatographed separately. Bars indicate mean ± SEM. BTH-treated groups had much less HA eluting in early fractions (60 to 124) than untreated groups, indicating a marked reduction in high molecular weight HA. A significant portion of HA from untreated myocardial extract eluted in the column void volume. Therefore, the molecular weight difference between BTH-treated and untreated myocardial HA may be greater than revealed by Sepharose 2B chromatography. There are no significant differences between anterior and posterior myocardial extracts. Column void volume (V₀, carbohydrate exclusion limit) approximately 2 \times 10^6 daltons), and elution position of dextran standards of 2 \times 10^6, 5 \times 10^6, and 7 \times 10^6 daltons are shown at the top of each graph.

TABLE 1 Chromatography of Myocardial Hyaluronic Acid

<table>
<thead>
<tr>
<th></th>
<th>Anterior myocardium</th>
<th>Posterior myocardium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight greater than 4 \times 10^6 daltons*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount of HA in untreated group (µg HA/mg protein)</td>
<td>2.54 ± 0.28†</td>
<td>2.37 ± 0.16</td>
</tr>
<tr>
<td>Amount of HA in BTH-treated group (µg HA/mg protein)</td>
<td>0.90 ± 0.09‡</td>
<td>0.91 ± 0.14§</td>
</tr>
<tr>
<td>Molecular weight less than 4 \times 10^6 daltons‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount of HA in untreated group (µg HA/mg protein)</td>
<td>0.90 ± 0.06</td>
<td>1.03 ± 0.10</td>
</tr>
<tr>
<td>Amount of HA in BTH-treated group (µg HA/mg protein)</td>
<td>1.16 ± 0.21</td>
<td>1.11 ± 0.10</td>
</tr>
</tbody>
</table>

* Myocardial hyaluronic acid eluting between fractions 60 and 124 on Sepharose 2B column.
† P < 0.001 when compared to untreated anterior myocardial samples.
‡ P < 0.001 when compared to untreated posterior myocardial samples.
§ Myocardial hyaluronic eluting between fractions 125 to 200 on Sepharose 2B column.

The data in this study demonstrated a 40% reduction of HA detected in myocardial extract after two sequential intravenous boluses of 5000 NFU BTH/kg. Gel exclusion chromatography of myocardial HA revealed a selective decrease of high molecular weight HA (greater than 4 \times 10^6 daltons) after treatment with intravenous BTH. The low molecular weight myocardial HA remaining after BTH treatment may represent depolymerized high molecular weight HA, a BTH-inaccessible subclass of myocardial molecules, or newly synthesized myocardial HA.

The anterior and posterior halves of the myocardium were analyzed separately to determine if the presence of anterior wall ischemia altered BTH depolymerization of HA. There was no discernible difference in either the amount or structure of HA isolated from anterior vs. posterior myocardium in the control of BTH-treated groups.

A series of control experiments indicated that delays between animal sacrifice and HA extraction or postmortem manipulation of myocardium such as freeze-thaw reduced the amount of HA that was recovered from the myocardium. Furthermore, definition of the ischemic area using in vivo dye injection techniques (DeBoer et al., 1980) interferes with the colorimetric method used to detect hyaluronic acid.
HYALURONIDASE CLEAVES MYOCARDIAL HYALURONIC ACID/ Wolf et al.

Table 2 Average Fraction Number of Chromatographed Myocardial Hyaluronic Acid

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Anterior myocardium</th>
<th>Posterior myocardium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>113</td>
<td>111</td>
</tr>
<tr>
<td>2</td>
<td>109</td>
<td>113</td>
</tr>
<tr>
<td>3</td>
<td>103</td>
<td>111</td>
</tr>
<tr>
<td>4</td>
<td>106</td>
<td>109</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>107 ± 2</td>
<td>111 ± 1</td>
</tr>
<tr>
<td>BTH-treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>136</td>
<td>138</td>
</tr>
<tr>
<td>6</td>
<td>118</td>
<td>131</td>
</tr>
<tr>
<td>8</td>
<td>135</td>
<td>127</td>
</tr>
<tr>
<td>9</td>
<td>123</td>
<td>120</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>130 ± 4†</td>
<td>130 ± 3‡</td>
</tr>
</tbody>
</table>

* Myocardial hyaluronic acid was chromatographed on Sepharose 2B gel and the center of each peak of hyaluronic acid was estimated by calculating the arithmetic mean fraction number.
† P < 0.005 when compared to untreated anterior myocardial samples.
‡ P < 0.005 when compared to untreated posterior myocardial samples.
in this study is higher than the dose used in previous papers. At present there are no available data on the dose-response characteristics of HA depolymerization after BTH treatment. The time of onset and duration of the structural changes in myocardial HA also are unknown. Certain agents, notably heparin, are known to be inhibitors of in vitro BTH activity (Mathews and Dorfman, 1955), but their effects on in vivo activity have not been studied. This issue is of particular importance since many patients with acute myocardial infarction, who might benefit from BTH therapy, also receive heparin.

In summary, the data presented in this paper demonstrate a reduction in total HA content of myocardial extracts after intravenous injection of BTH. The reduction in HA content was due to a selective reduction in high molecular weight myocardial HA (greater than $4 \times 10^6$ daltons) and preservation of low molecular weight HA with an overall reduction in the average molecular weight of myocardial HA after BTH treatment. The magnitude of quantitative as well as structural changes in myocardial HA was similar in anterior myocardium containing ischemic tissue and posterior non-ischemic myocardium. These are the first biochemical data confirming the depolymerization of a myocardial substrate after intravenous BTH injection.

The biochemical techniques described in this paper have been shown to be effective in assessing in vivo activity of BTH. These methods can now be used to clarify further the important questions surrounding the use of BTH to preserve ischemic myocardium.

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References


Ohye J, Kaneko Y (1970) Novel hyaluronidase from Strepto-
Inhibition by Free Radical Scavengers and by Cyclooxygenase Inhibitors of Pial Arteriolar Abnormalities from Concussive Brain Injury in Cats

ENOCH P. WEI, HERMES A. KONTOS, W. DALTON DIETRICH, JOHN T. POVLISHOCK, AND EARL F. ELLIS

SUMMARY We studied the role of prostaglandins and free radicals in the induction of functional and morphological pial arteriolar abnormalities produced by concussive brain injury. Anesthetized cats equipped with a cranial window for the observation of the pial microcirculation were subjected to concussive brain injury using a fluid-percussion device following administration of cyclooxygenase inhibitors (indomethacin or AHR-5850) or the vehicle for the solution of these agents (NaCl or Na₂CO₃ solution). Pial arterioles from vehicle-treated animals displayed sustained dilation, reduced responsiveness to the vasoconstrictor effect of arterial hypocapnia, and a high density of endothelial lesions. Animals pretreated with cyclooxygenase inhibitors showed less pronounced vasodilation, normal responsiveness to hypocapnia, and a significantly reduced number of lesions. The vasodilation and reduced responsiveness to the vasoconstrictor effects of hypocapnia after brain injury also were inhibited by topical application of free radical scavengers (nitroblue tetrazolium, superoxide dismutase, or mannitol). The vessels from cats pretreated with free radical scavengers also had a lower density of endothelial lesions than controls. The results support the view that the immediate cause of cerebral arteriolar damage in concussive brain injury is the generation of free oxygen radicals associated with increased prostaglandin synthesis.


WE reported previously (Wei et al., 1980a) that concussive brain injury, induced by fluid percussion in cats, caused sustained relaxation of smooth muscle of cerebral arterioles, and discrete destructive lesions in the endothelium of these vessels. Despite the presence of endothelial injury, there was no evidence of platelet aggregation, either in vivo or in histological preparations. The combination of arteriolar dilation, endothelial injury, and absence of platelet aggregation suggested the possibility that the endothelial injury caused release of a vasodilator substance that inhibits platelet aggregation. Prostaglandin I₂ inhibits platelet aggregation (Moncada et al., 1977) and relaxes cerebral arterioles (Ellis et al., 1979). Furthermore, it is produced by the walls of blood vessels, including the large cerebral arteries (Hagen et al., 1979) and cerebral microvessels (unpublished observations), under normal circumstances, and its production is increased in the aortas of rats and rabbits after endothelial injury (Hornstra et al., 1978). It appeared desirable, therefore, to test the hypothesis that prostaglandins might be mediators of the arteriolar injury associated with experimental brain injury.
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