EFFECTS OF PROPRANOLOL ON TISSUE NECROSIS IN EXPERIMENTAL MYOCARDIAL INFARCTION IN DOGS

KATSUHARU TSUCHIDA, RYUZABURO YAMAZAKI, KATSUYOSHI KANEKO AND HIRONAKA AIHARA

Research Center, Taisho Pharmaceutical Co., Ltd., 1-403, Yoshino-cho, Ohmiya, Saitama, 330, Japan
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We studied the effects of propranolol on degradation of cardiac structural proteins resulting from ischemia induced by 24 h ligation of the coronary artery in dogs. Degradation of myocardial myosin heavy chain, α-actin, and tropinin-I was used as an indicator of degradation of cardiac structural proteins. In dogs with left circumflex coronary artery ligation, propranolol, given orally in the dose of 10 or 30 mg/kg, significantly reduced degradation of cardiac structural proteins. This can be supported by the facts that treatment with propranolol, 10 or 30 mg/kg, reduced release of cathepsin B, L and D from lysosome to cytosol in the ischemic tissue and that the reduced acidity of the ischemic tissue was improved by treatment with propranolol, 30 mg/kg. In conclusion, propranolol delays the necrotic development of the severely ischemic myocardial tissue as shown by reduced protein degradation.

Keywords — myocardial infarction; structural protein; lysosomal enzyme; propranolol

INTRODUCTION

Propranolol, a β-adrenergic blocker, has been shown in experimental studies to limit infarct size following coronary occlusion.1−4 Numerous experimental animal studies have dealt with this agent’s beneficial effects within extremely short periods. Many patients with myocardial infarction cannot be treated within several hours with surgery of reperfusion.5 Accordingly, the effects of propranolol on experimentally-induced myocardial infarction must be considered in terms of reducing the length of process from initiation to recovery of an infarction. Thus, it is important to understand the necrotic process from tissue injury and necrosis to fibrosis of the ruined tissue. Completion of fibrosis can be considered as one of the most important phenomena indicating recovery from myocardial infarction.6 Protein degradation is assumed to progress mainly after the myocardial cells degenerate to an irreversible situation.7,8 The objective of this study is to examine the effects of propranolol on myocardial structural proteins degraded by ischemia in order to understand the necrotic process in the infarcted myocardium. The studies dealing with cell breakdown from the viewpoint of protein degradation in the course of myocardial necrosis have never been carried out with propranolol.

MATERIALS AND METHODS

Surgical Procedure and Experimental Protocol in 24 h-Coronary Ligated Myocardial Infarction — Adult mongrel dogs of either sex (weighing 8.5−11.5 kg) were anesthetized with sodium pentobarbital (25 mg/kg, i.v.). Ventilation was maintained at a rate of 40 cycles/min and a stroke volume of approximately 25 ml/kg was obtained by means of auffed endotracheal tube and a Shinano-respirator (SN-480-3). Standard limb leads were attached for recording lead II of the electrocardiogram by means of an electrocardiograph (Nihon Kohden MC-12) to confirm successful accomplishment of myocardial infarction. A left thoracotomy was performed in the fifth intercostal space and the pericardium was opened. The left circumflex coronary artery was isolated approximately 1 cm from its origin and ligated, and then incisions were closed. Hyperventilation was kept for 40−50 min after coronary ligation in an attempt to reduce mortality and then the ventilation rate was gradually decreased to 25 cycles/min at 80−90 min after ligation. Ventilation was removed 3 h after coronary ligation and all animals were left to recover for 24 h from anesthesia.

Propranolol hydrochloride (Sumitomo) was suspended in 5% gum arabic solution at a concentration of 30 mg/ml and the drug was administered intragastrically immediately after coro-
nary ligation.

Twenty-four hours after coronary ligation, the dogs were reanesthetized with sodium pentobarbital (10–30 mg/kg, i.v.) exsanguinated and the heart was removed.

**Myocardial Specimens and Preparation of Crude Structural Proteins** — Cross-section pieces of myocardium located at the central portion of the infarcted tissue and the normal tissue were taken from the left ventricle. The central portion of the infarcted tissue was defined near the top of the posterior papillary muscle. Crude structural proteins of heart tissue were extracted according to the methods of Katagiri. Approximately 100 mg of the cardiac tissue was homogenized in 9 volumes of 0.1 M NaCl solution containing 5 mM NaHCO₃ with an Ultra-Turrax (Janke & Kunkel IW) for 30 s. The homogenate was then centrifuged for 15 min at 1000 g and the precipitate was resuspended in 0.3 mM NaHCO₃ solution. This procedure was repeated several times to wash our soluble proteins to less than 0.5 μg/ml of protein in the supernatant fluid. The resulting residue was suspended in the supernatant fluid. The resulting residue was suspended in the same solution and used as a preparation of crude structural proteins. The method of Lowry et al. was used for protein determination.

**Sodium Dodecylsulfate (SDS)—Polyacrylamide Slab Gel Electrophoresis** — Gel electrophoresis in the presence of SDS was performed principally by the method of Laemmli. The protein samples were mixed with the same amount of a SDS sampling buffer containing 0.5 M Tris–HCl (pH 6.8), 50% glycine, 2% SDS, 10% beta-mercaptoethanol and 0.05% Bromphenol Blue, heated at 100 °C for 3 min and then kept at −20 °C. Forty μg of protein were applied to each lane. The slab gel was constructed in two parts; the upper part (5 cm in length) with 7.5% acrylamide and the lower part (6 cm in length) with 15% acrylamide. The upper part was used for analysis of high molecular weight proteins and the lower part for low molecular weight proteins. Electrophoresis was performed at a constant current of 20 mA for 4–5 h, after which the gel was immersed in 0.3% Coomassie Brilliant Blue, 10% acetic acid and 50% methanol for 30 min and destained in 10% acetic acid and 30% methanol. Proteins were identified by judging from SDS-polyacrylamide gels of the purified proteins obtained from canine heart muscle. Protein bands were traced by gel-scanner equipment, a Shimadzu TLC-scanner CS-910. The area under the peak of each band was determined by planimetry. This led to the percentage of each protein band versus the amount of whole protein applied. Then, the ratios of myosin heavy chain (MHC), α-actinin (α-Ac) and troponin-I (TN-I) versus actin (Ac) were calculated as the percentage of three proteins mentioned above over the percentage of actin (Ac). Relative amounts of three proteins in the central portion versus those in the normal portion were calculated by dividing the ratio in the central portion by that in the normal portion ×100. Student's t-test for paired data was used for determinations. (Before conducting the quantitative analysis of each protein content, SDS-polyacrylamide gel electrophoresis and planimetry were performed applying 5, 10, 20, 40 and 80 μg of the crude structural proteins to the same slab to examine whether the area of each protein depends on the concentration of the crude proteins. We determined 20 and 40 μg of the crude proteins appropriate for the quantitative analysis under this experimental condition because of the area being dependent on protein concentration.)

**Enzyme Assays** — The methods for production of experimental myocardial infarction was carried out according to the above mentioned methods without incisions being closed. Eighteen dogs (weighing 10.0–12.0 kg) were used. Twelve were given 10 or 30 mg/kg of propranolol and the remaining six were given vehicle only immediately after coronary ligation. Three hours after coronary ligation, the heart was removed.

Cross-sectional pieces of myocardium located in the central portion of the ischemic tissue and the normal portion were taken from each heart. Each piece of myocardium (100–200 mg) was homogenized with an Ultra-Turrax, in a solution containing 0.25 M sucrose, 10 mM Hepes buffer (pH 7.2), 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM KCl. The homogenate was centrifuged at 12000 g for 20 min and the precipitated fractions were resuspended in a solution containing 50 mM acetate buffer (pH 5.0), 0.15 M KCl and 1 mM EDTA. The supernatant and precipitated fractions were used for enzyme assays.

Total activity of cathepsins B and L of cardiac
muscle homogenate was determined by use of succinyl-L-tyrosyl-L-methionine-2-naphthylamide (Suc-Tyr-Met-Nap, synthetized in our laboratory) with some modification of the method of Barrett. The reaction mixture was 0.1 M acetate buffer (pH 5.0), containing 1 mM EDTA, 2 mM cysteine, 1 mg of Suc-Tyr-Met-Nap, and 10% dimethyl sulfoxide. The activity was calculated as μmol of substrate hydrolyzed for 1 min. Cathepsin D activity was determined using acid denatured 14C-methylated hemoglobin (bovine hemoglobin, obtained from Wako Chemicals) as a substrate in 0.1 M acetate buffer, pH 3.8, at 30°C. One unit (U) of enzyme activity is defined as that amount which hydrolyzed 1 μmol of substrate per min. Student's t-test for paired data was used for determinations.

Myocardial pH Measurement — This series of experiments were performed on eighteen dogs (weighing 9.0—12.0 kg). Animals were anesthetized with 30 mg/kg of sodium pentobarbital given intravenously. After performing a left thoracotomy under artificial respiration at a rate of 18 cycles/min and a stroke volume of approximately 30 ml/kg, the left circumflex coronary artery was isolated about 1 cm from its origin. A micro glass electrode (MI-410, Microelectrodes, Inc.) connected with a pH meter (Model 671, Shibata) was inserted into the region near the top of posterior papillary muscle. The pH sensitive tip (about 1 mm in diameter) of the electrode was placed about 5 mm deep from the surface of the left ventricular wall. The pH electrode was suspended by a spring so it would fluctuate with the beating of the heart. Changes in myocardial pH were recorded continuously with a pen recorder (Model 5184, Kanomax). The measurement of myocardial pH was carried out for 3 h after the coronary ligation. Experiments with electrode determined to be unappropriately positioned were excluded after anatomical analyses. The variance of analysis (one way) was used for determinations.

RESULTS
Cardiac Structural Proteins

Figure 1 shows the typical patterns of structural proteins obtained from the normal portion and the central portion of the infarcted tissue. The bands of MHC, α-Ac and TN-I decreased in width and intensity, whereas the component of Ac, tropomyosin (TM), myosin light chain 1 (MLC1) and myosin light chain 2 (MLC2) were preserved relatively well in the central portion of the infarcted tissue. Propranolol in doses of 10 and 30 mg/kg, but not 3 mg/kg, reduced the degradation of MHC, α-Ac and TN-I significantly. The compositions of each protein and the effect of propranolol on protein degradation are summarized in Table I.

Enzyme Activities

As shown in Figs. 2 and 3, both total activities of cathepsins B, L and cathepsin D were significantly decreased in the precipitated fraction taken from the central portion of the infarcted tissue 3 h after coronary ligation. In contrast, cathepsin activities were significantly increased in the supernatant fraction. The decrease in the activities of cathepsins in the precipitated fractions and the increase in the activities in the supernatant fractions were reduced significantly by treatment with propranolol, 10 and 30 mg/kg,

![FIG. 1. SDS-Polyacrylamide Gels of Cardiac Structural Proteins Obtained from the Normal Portion (N) and the Central Portion of the Infarcted Tissue (C) in the Control and the Propranolol (30 mg/kg)-Treated Dogs](image-url)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Ac</th>
<th>MHC</th>
<th>α-Ac</th>
<th>TN-I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>C</td>
<td>N</td>
<td>C</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>1.0</td>
<td>1.0</td>
<td>2.3</td>
<td>1.7 b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.06</td>
<td>±0.09</td>
<td>±0.01</td>
<td>(73.9%)</td>
</tr>
<tr>
<td>Propranolol</td>
<td>3 mg/kg</td>
<td>7</td>
<td>1.0</td>
<td>1.0</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.10</td>
<td>±0.07</td>
<td>±0.02</td>
<td>(87.0%)</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>8</td>
<td>1.0</td>
<td>1.0</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.09</td>
<td>±0.12</td>
<td>±0.01</td>
<td>(95.8%)</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>8</td>
<td>1.0</td>
<td>1.0</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.06</td>
<td>±0.13</td>
<td>±0.01</td>
<td>(100.0%)</td>
</tr>
</tbody>
</table>

N: normal portion; C, central portion. Figures in the brackets are expressed as percentages of normal portion values. Values are mean ± S.E.M., shown as the relative ratio compared with Ac. n represents number of experiments. a) p < 0.05, b) p < 0.01 versus normal portion. Ac, actin; MHC, myosin heavy chain; α-Ac, α-actinin; TN-I, troponin-I.

**FIG. 2.** Total Activity of Cathepsins B and L in the Normal Portion (N) and the Central Portion (C)

ppt; the precipitated fraction, sup; the supernatant fraction. Number of animals; control = 6, propranolol 10 mg/kg = 6, propranolol 30 mg/kg = 6. a) p < 0.05, b) p < 0.01 compared with the normal portion (N).

**FIG. 3.** Cathepsin D Activity in the Normal Portion (N) and the Central Portion (C)

ppt; the precipitated fraction, sup; the supernatant fraction. Number of animals; control = 6, propranolol 10 mg/kg = 6, propranolol 30 mg/kg = 6. a) p < 0.01 compared with the normal portion (N).
except for cathepsins B and L in the precipitate of propranolol treatment with 10 mg/kg.

Myocardial pH Measurement

As shown in Fig. 4, the mean pH value of the central portion of ischemic tissue decreased from 7.49 to 6.65, 1 h after the ligation in the control group, whereas the pH values in the propranolol (30 mg/kg, p.o.)-treated group were significantly higher than those in the control group during 3 h of myoccardial infarction.

DISCUSSION

The objective of our study was to determine whether propranolol would reduce degradation of cardiac structural proteins, because such biochemical events are considered to play an important role in the course of tissue necrosis from irreversible cell dysfunction to cell death and finally to fibrosis of necrotic tissue. Fibrosis means the completion of restoration of necrotic tissue. Some studies have shown that degradation of cardiac structural proteins is induced by experimental myocardial infarction\(^7,8,13\) and others have shown that lysosomal enzymes are released from lysosomes to cytosol during ischemia.\(^14-16\) However, there are no reports dealing with both protein degradation and enzyme release concomitantly. Our study indicated that propranolol reduced degradation of cardiac structural proteins, suggesting a delay of the development of necrosis and the following completion of restoration of necrotic tissue. Mildly ischemic myocardium is defined by a degree of limited coronary blood flow and the duration of the time of the limited coronary blood flow and it is certain that propranolol has beneficial effects in mildly ischemic myocardium. We demonstrated that ischemic myocardial cells contained smaller amounts of cathepsins B, L and D than normal cells in the precipitated fractions (lysosomal and/or mitochondrial fractions), and on the contrary, larger amounts in the supernatant fraction (cytosolic fraction). Propranolol treatment was shown to attenuate an alteration of cathepsin distribution. It can also be assumed that propranolol would prevent the change of distribution of various other lysosomal enzymes in addition to cathepsins, as shown by the inhibition of cathepsin release. The inhibitory effect of propranolol on the release of cathepsins B and L can be regarded as closely related to the reduced degradation of proteins.\(^17\) The reason that we chose 3 h as a time to measure enzyme activities is as follows: since creatine phosphokinase release from cell membrane started about 2–3 h after coronary ligation (data not shown), enzymes in cytosol (the supernatant fraction) well considered to decrease as a result of depletion through injured cell membrane more than 2–3 h after coronary ligation. In fact, cathepsin activities when measured 24 h after coronary ligation were decreased not only in the precipitated fraction but also in the supernatant fraction.\(^17\) This depletion may make the change of enzyme distribution (the release from lysosome to cytosol) obscure.

Moreover, we indicated that the pH of the ischemic tissue decreased from 7.49 to 6.65 1 h after the ligation and then began to return gradually towards the pre-ligation value during 3 h of coronary ligation. That is, the maximal decrease of pH value was 6.65. Since myocardial pH determined by a pH electrode is the pH of a mixture containing intra- and extracellular fluid, a change in myocardial pH thus determined does not indicate the exact change in the intracellular pH. However, it reflects alteration in the intracellular pH, which influences the extracellular pH. Propranolol treatment reduced the decrease of myocardial pH in the ischemic
tissue significantly in our experiment, as previously shown by Ichihara et al. in the infarcted tissue with a more temporary occlusion. This is thought to contribute in part to making acid proteinases, such as cathepsins, less active because the pH optima of cathepsins B and L are approximately 6.0 and cathepsin D, 4.0. From the viewpoint of ischemic tissue pH, cathepsins B and L are strong suspects, despite other proteinases considered to be involved in degrading myofibrillar proteins in the case of experimentally-induced myocardial infarction.

On the other hand, Wildenthal proposed the "Lysosomal Hypothesis" which proposes that early damage to lysosomes and release of lysosomal degradative enzymes in any way could contribute to the process by which potentially reversible ischemic damage progresses to irreversible infarction. In this respect, results are compatible with this hypothesis because propranolol prevented both enzyme release and protein degradation despite there being no direct evidence proving this hypothesis.

In conclusion, propranolol has protective effects on degradation of cardiac structural proteins such as MHC, α-Ac and TN-I in the experimentally-induced myocardial infarction. These effects are considered to be at least partly due to the reduction in release of degradative enzymes such as cathepsins B and L from lysosome to cytosol. These protective effects can be assumed to delay the pathological alterations in the irreversibly injured cells of the severely ischemic myocardium.

REFERENCES