Effect of Acetylsalicylic Acid on Metabolism and Contractility in the Ischemic Reperfused Heart

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The effect of acetylsalicylic acid (ASA) on high-energy phosphates (adenosine triphosphate: ATP, creatine phosphate: CrP, inorganic phosphate: Pi) and intracellular pH during myocardial ischemia and reperfusion was studied using phosphorus 31-nuclear magnetic resonance ($^{31}$P-NMR) in the isolated rabbit hearts. Coronary flow, left ventricular systolic developed pressure (LV Dev.P) and left ventricular end-diastolic pressure (LVEDP) were also measured. Langendorff hearts perfused at 37°C with the perfluorochemical emulsion Fluosol-43 were subjected to 15 min and 30 min of zero-flow ischemia and to 15 min of low-flow ischemia (coronary perfusion pressure = 20 mmHg) followed by 65 min of reperfusion (control, Group I). ASA (0.28 mmol/L) was infused either for the entire experimental period from beginning 45 min prior to ischemia (Group II) and infused immediately after reperfusion (Group III). During ischemia, Group II showed a significant suppression of the decrease in the ATP level and pH with both zero-flow and low-flow ischemia compared to those in the other groups, and moreover the increase in Pi and the decrease in CrP in low-flow ischemia were also suppressed. In Group III, the ATP level during reperfusion was significantly higher than that in Group I, but was not significantly different from that in 30 min zero-flow ischemia. In 30 min zero-flow ischemia, Pi, CrP and coronary flow after reperfusion in Group II tended to recover to preischemic values. There were no differences in LV Dev. P among the 3 groups. In conclusion, ASA has a protective effect on myocardial high-energy phosphates during ischemia and reperfusion in rabbit hearts.

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Aspirin (acetylsalicylic acid) was first made available in 1899 and is the most commonly used anti-inflammatory analgesic agent. This drug has been known to have an antiplatelet effect since 1970, when Weiss et al. first reported that it had an inhibitory effect on thrombosis in the carotid and femoral arteries. A number of several studies have demonstrated the preventive effect of aspirin on cerebral and myocardial infarction since 1971, when Vane first reported that it had an inhibitory effect on prostaglandin biosynthesis. This action is believed to be based on the antiplatelet effect of aspirin, i.e., it inhibits TxA2 production by inhibiting platelet cyclooxygenase and thus blocking the arachidonate cascade. In addition, as recently reported by Karmazyn et al., this drug aspirin also has a direct protective effect on myocardial metabolism in isolated rat ischemic myocardium that is independent of its antiplatelet effect. However, the mechanism of this effect has not been elucidated in detail, and to our

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knowledge there have been no reports on the influence of this drug on myocardial energy metabolism. In this study, we assessed the effects of aspirin on ischemia and reperfusion injury by using phosphorus-31 nuclear magnetic resonance (\(^{31}\)P-NMR) to measure myocardial energy metabolism and cardiac function in isolated rabbits perfused with platelet-free artificial blood.

In the present study, different types of ischemia (zero-flow and low-flow ischemia for 15 min and zero-flow ischemia for 30 min) were experimentally induced and the effect of aspirin on each of them was determined. Treatment with this drug was found to reduce tissue damage due to abnormal myocardial energy metabolism during ischemia and reperfusion.

MATERIALS AND METHODS

Experimental Design

The perfusate was an artificial blood substitute, FC-43 (Green Cross Cor, Osaka, Japan) with the following composition (in mmol/L): NaCl 103, KCl 4.6, CaCl\(_2\) 2.5, NaHCO\(_3\) 25, MgCl\(_2\) 2.1, glucose 10.0, hydroxyethyl starch 3.0 W/V%, Pluronic F-68 2.56 W/V% and FC-43 20.0 W/V%. This was oxygenated with a gas mixture of 95% O\(_2\) + 5% CO\(_2\) using a bubble-type artificial lung (Japan Medical Supply Co, Ltd, Hiroshima, Japan), adjusted to pH 7.40, at a constant perfusion pressure of 80 mmHg. Temperature of the perfusate was maintained at 37°C with a heater coil (Hakko Shoji Co., Ltd, Tokyo, Japan) and double-coated tubes. The perfusate was recycled to the artificial lung after passing through the heart. A 40 \(\mu\)m Swank filter (Nipro Corp, Tokyo, Japan) was placed within this route to remove any contaminants. Male Japanese white rabbits weighing about 1.5 kg were anesthetized by an intravenous injection of sodium pentobarbital (30 mg/kg). The chest was opened and heparin sodium (1,000 IU/kg) was injected through the right atrial appendage. The heart was removed quickly and after aortic cannulation without delay the retrograde Langendorff perfusion was initiated.

To measure left ventricular (LV) pressure, a latex balloon was inserted into the left ventricle via a left atrial incision. The balloon was large enough so that no pressure was generated by itself over the range of left ventricular volume used in the experiment. The balloon was filled with bubble-free saline and attached to a pressure transducer (Millar Instruments Inc, Houston, USA) connected to a Polygraph system RM-6000 (Nihon Kohden Co, Tokyo, Japan). The balloon volume was set to produce a left ventricular end-diastolic pressure (LVEDP) of 10 mmHg. The isovolumic measurement of left ventricular developed pressure (LV Dev.P) was used as the an index of LV contractility.

To drain effluent from the left ventricular Thebesius vein, a polyethylene tube was inserted into the left ventricle via a left atrial incision. The heart was paced through the right ventricle with an agar wick soaked in saturated potassium chloride and encased in polyethylene tubing at 180 beats/min throughout all of the experiments. The pacing was performed by using an electronic stimulator SEN-3301 and an isolator SS-502 J (Nihon Kohden Co, Tokyo Japan). Coronary flow was measured by an electromagnetic flow probe (Nihon Kohden Co, Tokyo, Japan) attached around the ascending aorta. These data were recorded using a thermal-array recorder WS-681G (Nihon Kohden Co, Tokyo, Japan).

Measurement of High-Energy Phosphates and Intracellular pH by \(^{31}\)P-NMR

The heart was placed in an NMR sample tube of 25 mm in diameter, as shown in Fig 1. The temperature of the \(^{31}\)P-NMR device was maintained at 37°C. The \(^{31}\)P-NMR spectra were recorded with a JNM-GX 400 FT NMR spectrometer (JEOL, Tokyo, Japan) operating at 161.7 MHZ. Radiofrequency pulses of 45° were repeatedly applied at an interval of 1.0 sec. Spectra were obtained by accumulating 300 free induction decays. A quantitative analysis was performed by examining the relative intensities of the \(\beta\)-ATP (adenosine triphosphate), CrP (creatine phosphate), and Pi (inorganic phosphate) peaks. The area under each peak was integrated 5 times with a planimeter. The mean of five such readings was normalized as a percentage of the value given during the initial basal period. The distance of the intracellular Pi peak relative to the
intracellular CrP peak (pH-independent) was measured and the intracellular pH was determined using the following equation:

\[ \text{pH} = \text{pk} - \log(10 \times (\delta_A - \delta_B)/(\delta_A - \delta_O)) \]

pk = 6.79, \( \delta_A = 3.25 \), \( \delta_B = 5.75 \)
\( \delta_O \) (the chemical shift of Pi with respect to CrP)

**Measurement of 6-ketoPGF₁α**

In 15 min zero-flow ischemia, for the measurement of 6-ketoPGF₁α, solution from perfusate overflow was drawn 5 min before global ischemia and immediately after reperfusion. 6-ketoPGF₁α was measured by the radioimmunoassay method.

**Experimental Protocol (Fig 2)**

**Three models of global ischemia**

The hearts were subjected on 3 types of global ischemia models; (A) 15 min zero-flow ischemia model, (B) 30 min zero-flow ischemia model, and (C) 15 min low-flow ischemia model (coronary perfusion pressure = 20 mmHg). After 30 min of an initial 30 min warm-up period, 10 min of basal perfusion, 15 min or 30 min of global ischemia, and 65 min of posts ischemic reperfusion were successively performed. LV pressure and coronary flow were measured at 5 min intervals, and NMR spectra were recorded continuously.

**Administration of ASA (acetylsalicylic acid)**

The hearts were divided into the following 3 groups; (1) control group (CONT), which was not treated with ASA (acetylsalicylic acid, Green Cross Cor, Osaka, Japan) throughout all experiments the entire experiment (n=7), (2) Pre-ASA group, which was treated with ASA (0.28 mmol/L) for all periods from throughout the entire experiment beginning 45 min prior to global ischemia (n=7), and (3) Post-ASA group, which was also treated with ASA (0.28 mmol/L), but only immediately after posts ischemic reperfusion (n=7).

**Statistical Analysis**

Statistical analysis was performed by
ANOVA with Tukey's post hoc test. All values are expressed as the mean ± SEM, and P<0.05 was considered significant.

RESULTS
Fifteen-Minute Zero-Flow Ischemia

Left ventricular pressure and coronary flow during ischemia and reperfusion
There were no significant differences in LV Dev.P, coronary flow (Table I A) or LVEDP (Fig 3A) among the 3 groups during basal perfusion, global ischemia or reperfusion.

Myocardial metabolism during ischemia and reperfusion
Pi
There were no significant differences in the 3 groups during basal perfusion or global ischemia, as shown in Fig 4A. In the CONT group, Pi increased to 467% of the basal level in from 10—15 min period of global ischemia and recovered to 127% in 60—65 min after reperfusion. In the Pre-ASA group, after reperfusion, Pi was significantly lower than in the CONT group. This was 115% at end of experiment. In the Post-ASA group, after reperfusion Pi was not significantly different among the 3 groups.

CrP
CrP decreased to about 8% of the basal level during global ischemia, without any differences among the 3 groups, and showed a transient increase after reperfusion, an overshoot phenomenon, as shown in Fig 5A. The maximal value of the overshoot was greatest in the CONT group and the value was 126% of the basal value in 20—25 min period after reperfusion. The respective values were 118% in the Pre-ASA group and 117% in the Post-ASA group.

ATP
In the CONT group, ATP decreased to 55% of the basal level in 10—15 min period of global ischemia and recovered to 71% in 60—65 min after reperfusion as shown in Fig 6A. In the Pre-ASA group, ATP decreased to 79% of the basal level in 10—15 min period of global ischemia and recovered to 86% in 60—65 min period after reperfusion, both of these values were significantly higher than those in the CONT group. In the Post-ASA group, the respective values were 56% in 10—15 min period of global ischemia, and recovered to 82% in 60—65 min period after reperfusion. This recovery was better than that in the CONT group in 0—5 min after reperfusion.

pH
There were no significant differences in pH among the 3 groups during basal perfusion and reperfusion, as shown in Fig 7A.
TABLE I LEFT VENTRICULAR PRESSURE AND CORONARY FLOW OF GLOBAL
ISCHEMIA WITH AND WITHOUT ASA

A  15 min zero-flow ischemia

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>LVSP (mmHg)</th>
<th>LV Dev. P (mmHg)</th>
<th>Coronary Flow (ml/min per g wet heart-weight)</th>
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<td>Basal</td>
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<td>129±3</td>
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<tr>
<td>Reperfusion</td>
<td>25</td>
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<td>21±1</td>
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<tr>
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<td>30</td>
<td>87±5</td>
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B 30 min zero-flow ischemia

<table>
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<th>Time (min)</th>
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<th>LV Dev. P (mmHg)</th>
<th>Coronary Flow (ml/min per g wet heart-weight)</th>
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C 15 min low-flow ischemia

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<th>Time (min)</th>
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<tr>
<td></td>
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</table>

CONT: Control (N=7), Pre-ASA: ASA 45 min prior to ischemia (N=7), Post-ASA: ASA immediately after reperfusion (N=7). Left ventricular systolic pressure, and LV Dev. P: Left ventricular systolic developed pressure.

All values are mean±SE in each group.
*p<0.05 vs CONT.

During ischemia, pH sharply dropped but in the Pre-ASA group it remained significantly higher (6.45) than that in the CONT group (6.35). After reperfusion, pH showed a small rebound increase in all 3 groups.

6-ketoPGF₁α

6-ketoPGF₁α was significantly lower (693±12 pg/ml) in the Pre-ASA group than in the control group (1305±15 pg/ml) during basal perfusion. Immediately after reperfu-
Fig 3. Changes in LVEDP in the control (CONT), ASA 45 min prior to ischemia (Pre-ASA) and ASA immediately after reperfusion (Post-ASA) groups. (A) 15 min zero-flow ischemia group, (B) 30 min zero-flow ischemia group, (C) 15 min low-flow ischemia group (coronary perfusion pressure = 20 mmHg).

Fig 4. Changes in Pi in the control (CONT), ASA 45 min prior to ischemia (Pre-ASA) and ASA immediately after reperfusion (Post-ASA) groups. (A) 15 min zero-flow ischemia group, (B) 30 min zero-flow ischemia group, (C) 15 min low-flow ischemia group (coronary perfusion pressure = 20 mmHg).

Fig 5. Changes in CrP in the control (CONT), ASA 45 min prior to ischemia (Pre-ASA) and ASA immediately after reperfusion (Post-ASA) groups. (A) 15 min zero-flow ischemia group, (B) 30 min zero-flow ischemia group, (C) 15 min low-flow ischemia group (coronary perfusion pressure = 20 mmHg).
sion, 6-ketoPGF\(_{1\alpha}\) decreased slightly (526±12 pg/ml) in the Pre-ASA group, while it increased in the control group (1505±11 pg/ml).

**Thirty-Minute Zero-Flow Ischemia**

*Left ventricular pressure and coronary flow during ischemia and reperfusion*

There were no significant differences in LV Dev.P (Table IB) or LVEDP (Fig 3B) among the 3 groups during basal perfusion, global ischemia or reperfusion.

There were also no significant differences in coronary flow among the 3 groups during basal perfusion or global ischemia, as shown in Table IB. However, coronary flow was significantly higher in the Pre-ASA group than in the CONT group after reperfusion.

**Myocardial metabolism during ischemia and reperfusion**

*Pi*

There were no significant differences in Pi among the 3 groups during basal perfusion or global ischemia, as shown in Fig 4B.

*CrP*

There were no significant differences in CrP among the 3 groups during basal perfusion, global ischemia or reperfusion, as shown in Fig 5B.

**ATP**

ATP decreased to 25% of the basal level in 25–30 min period of global ischemia and recovered to 53% in 60–65 min after reperfusion in the CONT group, as shown in Fig 6B. In the Pre-ASA group, ATP
decreased to 34% of the basal level in 25-30 min period of global ischemia and recovered to 64% in 60-65 min period after reperfusion, both values were significantly higher than those in the CONT group. In the Post-ASA group, the recovery was similar to that in the CONT group.

pH
There were no significant differences in pH among the 3 groups during basal perfusion or reperfusion, as shown in Fig 7B. pH sharply dropped during ischemia, but it remained significantly higher in the Pre-ASA group (6.18) than that in the CONT group (6.06). After reperfusion, pH showed a rebound increase in all 3 groups.

Fifteen-Minute Low-Flow Ischemia

Left ventricular pressure and coronary flow during ischemia and reperfusion
There were no significant differences in LV Dev.P, coronary flow (Table 1C) or LVEDP (Fig 3C) among the 3 groups during basal perfusion, global ischemia or reperfusion. Coronary flow decreased to about 0.4 ml/min per gW during global ischemia.

Myocardial metabolism during ischemia and reperfusion
Pi
In the CONT group, Pi increased to 184% of the basal level in 10-15 min period of global ischemia, as shown in Fig 4C. In the Pre-ASA group, Pi was significantly lower (152%) in the same period of global ischemia than that in the CONT group. At 65 min after reperfusion, Pi had recovered to the control level in all 3 groups. It was 102% in the CONT group, 101% in the Pre-ASA group and 102% in the Post ASA group.

CrP
In the CONT group, CrP decreased to 70% of the basal level at in 10-15 min period of global ischemia, as shown in Fig 5C. In the Pre-ASA group, CrP was significantly higher (82%) than that in the CONT group both during this same period of global ischemia and in 0-5 min after reperfusion than in the CONT group. After reperfusion, an overshoot phenomenon was not observed.

ATP
ATP decreased to 72% in of the basal level in 10-15 min period of global ischemia and recovered to 81% in 60-65 min after reperfusion in the CONT group, as shown in Fig 6C. In the Pre-ASA group, ATP decreased to 80% in 10-15 min period of global ischemia and recovered to 89% in 60-65 min period after reperfusion, both values were significantly higher than those in the CONT group. In the Post-ASA group, ATP decreased to 72% in 10-15 min period of global ischemia, and recovered to 87% in 60-65 min period after reperfusion. This recovery was better than that in the CONT group in 0-5 min after reperfusion.

pH
There were no significant differences in pH among the 3 groups during basal perfusion or reperfusion, as shown in Fig 7C.

DISCUSSION
In this experiment, an artificial blood substitute, FC-43, was used to mimic the physiological state as closely as possible. Since we could exclude effects of ASA on the platelet, we were able to examine its direct effect on the myocardium. Our previous study using FC-43 showed that methylprednisolone has a protective effect on myocardial high-energy phosphates during myocardial ischemia and reperfusion, but this effect is less than that of ASA in this study.

Zero-flow ischemia for 15 min was induced to produce a pathologic state similar to stunned myocardium. In addition, low-flow ischemia for 15 min was used as an angina and zero-flow ischemia for 30 min was used as a model of irreversible myocardial damage. These 3 kinds of global ischemia were induced and the effects of aspirin on ischemia and reperfusion injury were evaluated in each case.

In the control group, ischemia induced hemodynamic changes, such as a decrease in LV Dev.P and an increase in LVEDP. These parameters showed a transient increase depending on the intensity of ischemia immediately after the initiation of reperfusion, and thereafter returned to baseline with time. In addition, ischemia induced...
changes of affected myocardial metabolism, such as decreasing ATP, CrP, and pH and increasing Pi. Like the hemodynamic variables, these parameters showed changes that were dependent on the intensity of the ischemia and were recovered almost to the preischemic values after the initiation of reperfusion. However, the ATP level did not completely recover in any group. It is known that the extent of the ischemia-induced decrease in ATP is dependent on the intensity of ischemia, i.e., it is a function of the duration of ischemia and the decrease in regional tissue blood flow. In the present study, the myocardial ATP content also decreased in a manner that depended on the intensity of ischemia. It has been reported that irreversible injury does not occur if the myocardial ATP content during ischemia and reperfusion remains at 50% or more of its preischemic value. In the present study, the ATP level during ischemia was maintained at 50% or more of the preischemic value and no irreversible injury was considered to occur in the 15 min low- or zero-flow ischemia groups.

On the other hand, the ATP level decreased to 25% of the its preischemic value during ischemia and did not adequately recover after reperfusion in the 30 min zero-flow ischemia group. Considering the inadequate restoration recovery of Pi and LVEDP, 30 min of ischemia is considered to be the limit for preserving myocardial energy metabolism.

In the present study, the decrease in ATP and the fall in pH during ischemia were inhibited in all of the ASA pretreatment groups. In addition, the decrease in CrP during ischemia was inhibited in the low-flow ischemia groups as compared with the control and ASA post-treatment groups and the increase in Pi also tended to be inhibited. During reperfusion, the early restoration of ATP and better restoration of Pi were noted in the 15 and 30 min zero-flow ischemia groups, while inhibition of CrP overshoot was seen in the 15 min zero-flow ischemia group. Among the hemodynamic parameters, LVEDP and coronary flow showed a better improvement after reperfusion in the 30 min zero-flow ischemia group.

In the ASA post-treatment group, the ATP level tended to improve early after the initiation of reperfusion in the 15 min zero-flow and low-flow ischemia groups, and CrP overshoot was inhibited in the zero-flow group. However, no improvement of ATP, Pi, coronary flow, or LVEDP was noted in the 30 min zero-flow ischemia group. As in the ASA pretreatment groups, the LV Dev.P was not significantly different from that in the control group.

As described above, pretreatment with ASA had a myocardial protective effect against ischemia lasting for 30 min or less regardless of its severity, and ASA tended to maintain the myocardial ATP content throughout ischemia and reperfusion. In contrast, a beneficial effect of post-treatment with ASA was only obtained for 15 min of ischemia. In addition, acidosis during ischemia was only improved by pretreatment with ASA. Taken together, these findings show that ASA had a beneficial effect on myocardial energy metabolism during ischemia of short duration and in the subsequent reperfusion period.

The main clinical effect of ASA in patients with ischemic heart disease is its antiplatelet effect. It has become routine to administer a small dose of this drug to prevent acute myocardial infarction, but low doses must be used because of the aspirin dilemma. When administered at low doses, aspirin inhibits platelet TXA2 production but does not inhibit PGI2 production from the vascular wall. The mechanism of action of aspirin on myocardial ischemia has not yet been fully elucidated. To our knowledge, only Karmazyn et al. have studied the effect of aspirin on myocardial energy metabolism and its actions have never been elucidated in detail. The possible mechanisms involved in the myocardial protective effect of aspirin include (1) membrane stabilization, (2) inhibition of prostaglandin production, (3) inhibition of lactic acid production, (4) an antiarrhythmic effect, and (5) promotion of collateral blood flow. Its membrane-stabilizing effect may be explained as follows. Since aspirin is a weakly acidic nonsteroidal anti-inflammatory drug, it binds to the proteins found in biological membranes by hydrophobic bonds interaction, and consequently decreases membrane fluidity and permeability. In the case of the heart, this membrane-stabilizing effect
is considered to inhibit Ca\(^{2+}\) overload in myocardial cells during reperfusion. In addition, it may protect the mitochondria from Ca\(^{2+}\) overload and oxygen free radicals during ischemia and reperfusion and help to maintain the mitochondrial ATP content. Furthermore, it may prevent lysosomal disintegration and, which would in turn suppress the outflow of various enzymes phospholipase A\(_2\).

Endogenous prostaglandins are reported to be activated during ischemia and reperfusion, with the Ca\(^{2+}\) content in myocardial cells and mitochondria showing an increase, resulting in abnormal ATP production\(^{25}\). In addition, it has been reported that nonsteroidal anti-inflammatory drugs, which are cyclooxygenase inhibitors, reduce reperfusion injury by inhibiting the conversion of arachidonic acid to prostaglandins\(^{22}\).

Prostaglandins are produced by arachidonic acid released from membrane phospholipids by the action of phospholipase A\(_2\). The production and release of various prostaglandins increases during ischemia and reperfusion\(^{26-28}\). There are reports that prostaglandins increase the intracellular Na\(^+\) level by inhibiting Na\(^+\), K\(^+\) - ATPase, and also elevate the intracellular Ca\(^{2+}\) level via the Na\(^+\)-Ca\(^{2+}\) exchange system. In addition, prostaglandins may promote mitochondrial Ca\(^{2+}\) accumulation in the presence of intracellular Ca\(^{2+}\) overload and inhibit both oxidative phosphorylation and ATP production\(^{22,29}\). Accordingly, oxidative phosphorylation and ATP production may be increased by inhibiting prostaglandin production during ischemia and reperfusion. Thus, not only membrane stabilization but also the inhibition of prostaglandin production may be important in the protective effect of pretreatment with ASA.

The ATP level tended to improve even when ASA was administered at the initiation of postischemic reperfusion. However, ASA post-treatment was only effective for ischemia of short duration (15 min). The mechanism of action of ASA may be different between pretreatment and post-treatment, and it seems possible that prostaglandin receptors may be involved in its post-treatment effects. Post-treatment with ASA had no effect in the 30 min ischemia group, and therefore this duration of ische-

mia produced irreversible myocardial damage in isolated perfused rabbit heart.

The present study used an ASA concentration at which 85% to 95% of prostaglandin production was inhibited\(^{22}\). In general, anti-inflammatory drugs have a positive inotropic action. However, the ASA concentration used in the present study was low enough to have no influence on LV pressure parameters during basal perfusion\(^{30}\). Karmazyn et al\(^{8}\) have reported that aspirin decreased the accumulation of lactic acid. In the present study, the decrease in myocardial pH during ischemia was suppressed by pretreatment with ASA, and it seems possible that this effect may also be related to myocardial protection. However, the present study provided no data concerning the antiarrhythmic or collateral blood flow-promoting effects of this drug ASA. To our knowledge, there is no evidence that FC-43 alone has an effect on prostaglandins.

In conclusion, aspirin protected the myocardium from injury due to abnormalities in energy metabolism during short-term ischemia and subsequent reperfusion. This effect of ASA might be related to the membrane stabilization and the inhibition of prostaglandin production.

This drug seems to be effective for improving myocardial energy metabolism during ischemia, which suggests future clinical applications.

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