Inhibitory Effect of KW-3049, A New 1, 4-Dihydropyridine Calcium Antagonist, on the Reduction of Myocardial Creatine Kinase Activity and High-Energy Phosphate Content in Rats Subjected to Coronary Artery Ligation

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The effect of KW-3049, (±)-(R* )-2,6-dimethyl-4-(m-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid (R*)-1-benzyl-3-piperidinyl ester, methyl ester hydrochloride on myocardial infarction in rats was examined, in comparison with some other drugs. Extension of myocardial infarction was assessed by separately determining the tissue creatine kinase (CK) activity of left ventricular free wall (LVFW) and that of interventricular septum. Loss of CK activity was limited to LVFW until 6 h after the ligation of the left main coronary artery, while 24 h after the ligation, it extended to the septum. Therefore, the effects of drugs were examined mainly in rats following 6 h of coronary artery ligation.

Pretreatments with KW-3049 at 1 and 3 mg/kg (p.o.), given 1 h before coronary artery ligation, significantly reduced the loss of CK activity of LVFW by 45.3 and 39.7%, respectively. Also, post-treatment with KW-3049 at 30 μg/kg (i.p.), given 10 min after the ligation, significantly reduced the loss of CK activity by 30.6%. On the other hand, nifedipine (3, 10 mg/kg, p.o.), propranolol (100 mg/kg, p.o.), OKY-1581 (100 mg/kg, p.o.) and BM 13.177 (100 mg/kg, p.o.), each of which was given 1 h before coronary ligation, did not significantly reduce the loss of CK activity.

Coronary artery ligation for 6 h significantly decreased the myocardial contents of adenosine triphosphate (ATP) and creatine phosphate (CP). Pretreatments with KW-3049 at 1 and 3 mg/kg (p.o.) reduced the decrease in ATP and CP.

These results suggest that KW-3049 possesses a superior cardioprotective effect in comparison with the other drugs examined. The possible implications for the protection by KW-3049 are discussed.

Keywords — KW-3049; nifedipine; propranolol; OKY-1581; BM13.177; myocardial infarction; conscious rat; creatine kinase activity; adenosine triphosphate; creatine phosphate

Introduction

Acute myocardial infarction is one of the major causes of death, and there has been a great deal of interest in the possibility of reducing the size of infarction by pharmacological interventions. Many experimental studies have been carried out to determine the feasibility of limiting the size of infarction in many animal species. Several studies have shown that calcium antagonists have beneficial effects on the ischemic myocardium, although the protective effect was not observed in some studies.

KW-3049 is a newly developed 1,4-dihydropyridine calcium antagonist (Fig. 1), which is characterized by long-lasting antihypertensive and antianginal activities. In the present study, using a rat model of myocardial infarction, we have examined the feasibility of KW-3049 and some other agents to salvage ischemic myocardium by estimating the tissue creatine kinase (CK) activity. Furthermore,

![Fig. 1. Chemical Structure of KW-3049, (±)-(R* )-2, 6-Dimethyl-4-(m-nitrophenyl)-1,4-dihydropyridine-3, 5-dicarboxylic Acid (R*)-1-benzyl-3-piperidinyl Ester, Methyl Ester Hydrochloride](image-url)
the effects of KW-3049 on the contents of adenosine triphosphate (ATP) and creatine phosphate (CP) in the ischemic myocardium were determined.

Materials and Methods

Operative Procedure — Male Wistar rats weighing 250—350 g were used for the experiments. Myocardial infarction was produced by occlusion of the left coronary artery according to the method of Selye et al. 123

Under ether anesthesia, an about 2 cm incision was made at the left fourth intercostal space. The heart was exteriorized through a thoracotomy and the left coronary artery was ligated at 2 to 3 mm from its origin, using a 3-0 silk suture. After the ligation, the heart was positioned back in the thoracic cavity and the chest was closed, evacuating the air from the thorax. The postoperative mortality was 20 to 40%.

The heart of surviving animals was excised at fixed times after coronary artery ligation and the extension of ischemic damage in the myocardium was examined by analyzing the activity of CK in the tissue. The effects of drugs were examined by comparing the CK activity of myocardium in drug-treated rats with that in vehicle-treated rats. In some experiments, the contents of ATP and CP in the heart were analyzed.

Tissue Analysis — CK activity in the myocardium was measured as follows. The rats were anesthetized with pentobarbital sodium (60 mg/kg, i.p.), and the hearts were excised and placed in ice-cold 50 mM Tris HCl (pH 7.5). The interventricular septum (non-ischemic region) and left ventricular free wall (LVFW) (ischemic region) were isolated from the surrounding tissues such as atria and right ventricular free wall, and then the septum and LVFW were separately analyzed. Both tissue samples (wet weight: 250—400 mg) were homogenized in 3 ml of 50 mM Tris HCl (pH 7.5) containing 1 mM EDTA at 0°C using a glass-glass homogenizer. Homogenates were centrifuged at 18000 × g and 0°C for 20 min. The supernatants were assayed for CK activity according to the method of Rosalki, 13 and for protein concentration according to the method of Lowry et al. 14 The tissue CK activity was expressed as IU/mg protein.

By means of a slight modification of the method described by Zamanis et al., 43 the contents of ATP and CP in the heart were measured in rats subjected to myocardial infarction for 6 h. The rats were anesthetized with pentobarbital sodium (60 mg/kg, i.p.). Immediately after thoracotomy, the hearts were frozen using an aluminum clamp precooled for several minutes in liquid nitrogen. The whole ventricle was homogenized in 4 ml of ice-cold, 6% perchloric acid and the homogenate was centrifuged at 18000 × g and 0°C for 15 min. The supernant was assayed for ATP and CP according to the previously described method, 153 and the precipitate was assayed for protein concentration according to the method of Lowry et al. 14 The contents of ATP and CP were expressed as nmol/mg protein. In the present study, we have examined the effects of KW-3049 (p.o.) on the contents of ATP and CP of hearts subjected to coronary artery ligation for 6 h, since KW-3049 (p.o.) was effective in reducing the myocardial infarction as estimated by the measurement of CK activity.

Drugs — KW-3049, OKY-1581 and BM13.177 were synthesized in our laboratories. The other drugs used were nifedipine (Bayer) and propranolol (HCl, Kyowa Hakko). For oral administrations, the drugs were dissolved or suspended in distilled water or 0.3% carboxymethylcellulose so as to make 1 ml of solution per 100 g of body weight. For intraperitoneal injection, KW-3049 was dissolved in saline containing 0.15% Tween 80 so as to make 0.1 ml of solution per 100 g of body weight.

Statistics — Values are expressed as the mean ± S.E. Comparison of the results between the treatment and vehicle groups was performed by using Student's t-test. For multiple comparison, analysis of variance and Duncan's test were used. p values of less then 0.05 were considered to be statistically significant.

Results

Time-Course of the Extension of Myocardial Infarction
Figure 2 shows the sequential change in CK activity of interventricular septum (non-ischemic area), LVFW (ischemic area) and the difference in the CK activity determined in the septum and LVFW. In non-operated control rats, there was no significant difference in CK activity in LVFW vs. septum (11.3 ± 0.5 vs. 11.5 ± 0.5 IU/mg protein). The CK activity of LVFW time-dependently decreased and the maximal loss of CK was attained 48 h after coronary artery ligation. Until 6 h after the ligation, the loss of CK was limited to LVFW whereas 24 h after the ligation, it extended to the septum. Therefore, up to 6 h after coronary artery ligation, the difference in CK activity between septum and LVFW can be used as a reliable index of ischemic damage to the myocardium. Thus, in the following experiments in which the effects of drugs were examined, only the difference in CK activity between septum and LVFW is illustrated except for the experiment involving 24 h coronary ligation.

**Effects of KW-3049 and Some Other Drugs on the Extension of Myocardial Infarction**

As shown in Fig. 3, pretreatments with KW-3049 at 1 and 3 mg/kg (p.o., 1 h) significantly attenuated the loss of CK from the ischemic myocardium following 6 h of coronary artery ligation by 45.3 and 39.7%, respectively. This result may represent a protective effect of prophylactically given KW-3049.

Pretreatments with nifedipine at 3 and 10 mg/kg (p.o., 1 h) did not significantly prevent

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**Fig. 2. Time-Course of the Extension of Myocardial Infarction after Left Main Coronary Artery Ligation in Conscious Rats**

A) Myocardial creatine kinase (CK) activities of septum, B) left ventricular free wall (LVFW) and C) the difference between non-ischemic (septum) and ischemic (LVFW) myocardium were expressed as IU/mg protein. Numbers in or immediately above the bar indicate the numbers of samples assayed. a) p < 0.05, b) p < 0.01, compared with the value of non-operated control (time 0 value).

**Fig. 3. Effects of KW-3049 on Myocardial Infarction (MI) in Conscious Rats**

KW-3049: A) 3, B) 1, C) 0.3 mg/kg, p.o. Bar heights indicate the difference in CK activity between non-ischemic (septum) and ischemic (LVFW) myocardium 6 h post ligation. Numbers in or immediately above the bar indicate the numbers of samples assayed. n.s.: not significant.
the loss of CK following 6 h of coronary artery ligation, though there was a tendency to alleviate it (Fig. 4).

Figure 5 shows the effect of propranolol, OKY-1581 and BM13.177 (100 mg/kg, p.o. 1 h) on the CK loss from the ischemic myocardium following 6 h of coronary artery ligation. Though propranolol and BM13.177 tended to prevent the CK loss, OKY-1581 was ineffective against the ischemic damage to the myocardium.

Figure 6 illustrates the effect of KW-3049 (3 mg/kg, p.o., 1 h) on the loss of CK from the ischemic myocardium following 24 h of coronary artery ligation. KW-3049 tended to alleviate the loss of CK from ischemic myocardium, but the effect was not statistically significant.

The effect of KW-3049 (30 μg/kg, i.p.) administered 10 min after coronary artery ligation on the loss of CK following 6 h of coronary artery ligation is shown in Fig. 7. As was the case...
in the experiments on prophylactically given KW-3049, it significantly prevented the loss of CK from the ischemic myocardium.

**Effects of KW-3049 on the Contents of ATP and CP in Ischemic Myocardium**

Following coronary artery ligation for 6 h, the myocardial contents of ATP and CK were significantly decreased in comparison with those in non-operated normal rats (Table I). In the rats treated with KW-3049 at 1 and 3 mg/kg (p.o., 1 h), the myocardial contents of ATP and CP were maintained at higher levels than those in vehicle-treated rats.

**Discussion**

Maclean et al.\(^{16}\) reported that the loss of tissue CK activity correlated well with the infarct size determined by histological and morphological measurements, and the measurement of CK loss is an accurate method of evaluating myocardial ischemic damage. Recently, Hock et al.\(^{17,18}\) adopted the difference in CK activity between septum and LVFW as a reliable index of ischemic myocardium following ligation of the left coronary artery in rats. In the present experiments, in which the time-course of the changes in CK activity of myocardium was measured, the CK activity of septum (non-ischemic area) did not change significantly up to 6 h after coronary ligation. Therefore, it was assumed that the difference in CK activity between the septum and LVFW is a reliable index of the extension of myocardial infarction following coronary ligation for up to 6 h.

Calcium antagonists have shown protective effects in some studies\(^{2-6}\) but not in others.\(^{7,8}\) In the present study, we found that KW-3049,

**Table I. Effects of KW-3049 (p.o.) on the Reduction of Myocardial Contents of ATP and CP in Rats Subjected to Myocardial Infarction for 6 h**

<table>
<thead>
<tr>
<th>Group</th>
<th>ATP (nmol/mg protein)</th>
<th>CP (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Normal</td>
<td>(13)</td>
<td>13.47 ± 1.20</td>
</tr>
<tr>
<td>2. MI + vehicle</td>
<td>(13)</td>
<td>6.11 ± 0.46</td>
</tr>
<tr>
<td>3. MI + KW-3049 (3 mg/kg)</td>
<td>(9)</td>
<td>10.25 ± 0.92</td>
</tr>
<tr>
<td>4. MI + KW-3049 (1 mg/kg)</td>
<td>(9)</td>
<td>8.37 ± 0.80</td>
</tr>
<tr>
<td>5. MI + KW-3049 (0.3 mg/kg)</td>
<td>(9)</td>
<td>6.89 ± 0.64</td>
</tr>
</tbody>
</table>

Statistics: \(^{c)}

1 vs. 2: \(p < 0.01\)  
1 vs. 3: \(p < 0.01\)  
1 vs. 4: \(p < 0.01\)  
1 vs. 5: \(p < 0.05\)  
2 vs. 3: \(p < 0.01\)  
2 vs. 4: \(p < 0.05\)  
2 vs. 5: n.s.

\(^{a)}\) Number of hearts examined. \(^{b)}\) Values are presented as the mean ± S.E. \(^{c)}\) Statistical significance of the difference between each group is shown. n.s.: not significant.
administered either before or immediately after coronary ligation, is able to alleviate significantly the extension of myocardial infarction, as estimated 6 h after the ligation. On the other hand, the pretreatment with nifedipine at 3 and 10 mg/kg (p.o.) did not significantly prevent the extent of infarction. Himori et al. 6) reported that nifedipine, repeatedly administered, was effective against myocardial infarction in rats subjected to 4 h of coronary artery ligation. Therefore, one feasible explanation for the ineffectiveness of nifedipine in the present study might be that the potential protective effect of nifedipine is shorter in duration than that of KW-3049.

Previous studies with β-adrenoceptor blocking agents on myocardial infarct in rats have produced varying results, 6,19-21) although the majority of experiments in dogs have shown beneficial effects of β-blockers on the ischemic myocardium. 22,23) In the present experiment using rats, propranolol tended to reduce the size of myocardial infarction, though the effect was not statistically significant. Our results are similar to those of Himori et al. 6)

Recently, evidence has accumulated that thromboxane A₂ (TXA₂) is involved in the pathophysiology of experimental myocardial ischemia. This has prompted us to examine the effects of OKY-1581, a thromboxane synthetase inhibitor, and BM13.177, a thromboxane A₂ receptor antagonist, on the myocardial infarction in rats. In contrast to the results of Burke et al. 24) who reported significant cardioprotection by OKY-1581 in cat models of myocardial ischemia, we could not demonstrate the ameliorating effect of OKY-1581 in rats. Schröer and Thiemermann 25) reported some beneficial effects of BM13.177 on acute myocardial ischemia in cats. On the other hand, in our rat model of myocardial infarction, BM13.177 slightly, but not significantly reduced the ischemic myocardial damage. These discrepancies of the results may be accounted for by the differences among the animal species examined, the dose regimens employed and the endpoints of infarction studied.

There are several reasons why a calcium antagonist might be beneficial to ischemic myocardium in general. First, calcium antagonists improve coronary collateral blood flow based on the well-known, potent coronary vasodilating properties of those agents. 26) A second reason is reduced myocardial oxygen demand due to decreases in afterload, preload and contractility. 27,28) Third, it has been hypothesized that calcium overload in the myocardium, which results from the depletion of ATP, is responsible for ischemic cell death, 29,30) and calcium antagonists might inhibit or delay cellular injury via the blockade of calcium entry into ischemic myocardium. It was reported that 31) rats have collateral blood flow of only 6% whereas dogs, cats and guinea-pigs have well-developed collateral circulation. Therefore, it seems unlikely that the improvement of collateral circulation plays a major role in the protection by KW-3049 of ischemic myocardium in rats. On the other hand, we have already observed 9) that, in conscious normotensive rats, KW-3049 at 1 mg/kg (p.o.) does not decrease systemic blood pressure or the double-product, an index of myocardial oxygen demand. In the present study, KW-3049 at 1 mg/kg (p.o.) reduced the extension of myocardial infarction in rats. These results suggest that the decrease in oxygen demand is not necessarily responsible for the appreciable protection of ischemic myocardium in rats. This is also in accordance with the present result that propranolol, which is known to have negative inotropic action, did not significantly reduce the extension of myocardial infarction in rats.

The decreased contents of high-energy phosphates in ischemic myocardium lead to elevation of myocardial cytosolic free Ca²⁺, resulting in cell death, because an uncontrolled rise in cytosolic Ca²⁺ will activate a number of energy-consuming reactions, notably those of the cardiac contractile proteins and of mitochondria. 29,30,32) The present results demonstrate that coronary artery ligation for 6 h in rats results in decreased myocardial contents of high-energy phosphates as well as the extension of myocardial infarction. Moreover, KW-3049 alleviated both the decrease in the contents of high-energy phosphates and the extension of infarction. Since KW-3049 possesses potent inhibitory effects on cardiac slow channels, 33) it seems reasonable to assume that
KW-3049 protected the ischemic myocardium by inhibiting the myocardial Ca²⁺-overload associated with the ischemia and the resultant decline of the contents of high-energy phosphates. The measurements of the cytosolic free Ca²⁺ in ischemic myocardium in the present model, however, require further investigation. The higher myocardial contents of ATP and CP in rats treated with KW-3049 could be attributed to the reduced extension of infarction, resulting in greater preservation of energy-producing activity.

Since acute myocardial infarction in humans is distinct from animal models in many respects, the extrapolation of the present results must be carried out with extreme caution. Although there has been little information regarding the effect of calcium antagonists on the extension of myocardial infarction in patients, most of the results are discouraging. Campbell et al. 1) ascribed the negative effects in most clinical trials to two reasons. First, therapy must be initiated much earlier than was carried out in these trials, where treatments were started 4 to 6 h after the onset of infarction. Second, the main clinical action of calcium antagonists is the alleviation of coronary vasospasm; however, thrombosis but not vasospasm is the final cause of coronary occlusion in the majority of patients. Recently, Reimer and Jennings 30) have suggested that the best chance for limitation for infarct size would occur in patients who had been treated on a long-term basis with calcium antagonists and in whom either spontaneously or therapeutically induced reperfusion occurred relatively early after the onset of acute coronary occlusion. Therefore, KW-3049, if administered in such situations, might salvage the ischemic myocardium in humans.

We have already reported 10) that KW-3049 has ameliorating effects on ischemic damage of myocardium of dogs subjected to a coronary occlusion and reperfusion, and that the effects of KW-3049 are more favorable than those of nifedipine in terms of the weaker deteriorating action of KW-3049 on cardiac performance. From these findings as well as the present results, it is suggested KW-3049 is a promising candidate for the treatment of ischemic myocardium, and deserves clinical investigation.

References

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