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A Na^+–H^+ exchange inhibitor (SM-20550) protects from microvascular deterioration and myocardial injury after reperfusion

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Abstract

Na^+–H^+ exchange inhibitors may reduce myocardial damage after reperfusion. However, their effects on microvascular deterioration are not known. We examined the potency of a novel Na^+–H^+ exchange inhibitor, SM-20550 [*N*-(Aminoiminomethyl)-1,4-dimethyl-1*H*-indole-2-carboxamide methanesulfonate], and its effects on microvascular damage after reperfusion. In an in vitro study, the Na^+-H^+ exchange inhibiting activity of SM-20550 was about 10 times greater than that of ethylisopropyl amiloride. In in vivo experiments, we occluded the left circumflex coronary artery in 29 dogs for 2 h and then reperfused for 5 h. SM-20550 was administered either before ischemia ($n = 11$) or before reperfusion ($n = 7$). Another 11 dogs served as controls. We found that SM-20550 not only improved coronary vasodilator responses to acetylcholine and adenosine after reperfusion, but also reduced infarct size ($P < 0.01$). Intramyocardial bleeding, which should reflect microvascular damage, was not found in dogs with SM-20550 treatment. Infarct size was correlated inversely with collateral blood flow in control (both, $P < 0.01$) but not in SM-20550-treated animals. Furthermore, SM-20550 significantly suppressed ventricular fibrillation during both ischemia and reperfusion. These results suggest that protective effects of Na^+–H^+ exchange inhibitors on reperfused myocardium are due at least in part to microvascular protection.

Keywords: Na^+–H^+ exchange; 'No-reflow' phenomenon; Coronary flow reserve; Myocardial damage; Arrhythmia; Reperfusion

1. Introduction

Reperfusion of the ischemic myocardium during the early phase of myocardial infarction is a treatment of choice in the management of acute myocardial infarction (Hartzler et al., 1984; The ISAM Study Group, 1986). However, it is well known that reperfusion is often accompanied by ventricular arrhythmia, myocardial damage and microvascular deterioration (Braunwald and Kloner, 1985). To improve results and prognosis in the treatment of acute myocardial infarction, it is essential to understand the mechanisms underlying reperfusion injury.

Several investigators suggested the efficacy of pharmacological interventions to reduce reperfusion injury. In such reports, inhibition of Na^+–H^+ exchange proved to be a promising pharmacological intervention (Tani and Neely, 1990; Karmazyn and Moffat, 1993; Cross et al., 1996). However, its effects on microvascular deterioration have not yet been fully evaluated.

During myocardial ischemia, accumulation of intracellular H^+ results in an influx of Na^+ through the Na^+–H^+ exchanger (MacLeod, 1991). Then an increase in intracellular Ca^{2+} via the Na^+–Ca^{2+} exchange occurs (Lazdunski et al., 1985). The resultant intracellular calcium overload leads to cell death (Guarnieri, 1987; Wier, 1990). Therefore, reduction in $[Na^+]_i$ by inhibition of Na^+–H^+ exchange during ischemia may ultimately protect endothelial and myocardial cells against Ca^{2+} overload.

After reperfusion, microvascular deterioration develops. Histopathological studies suggest that reperfusion injury is accompanied by neutrophil plugging of capillary lumens in association with extensive disruption of endothelial cells

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(Engler et al., 1983; Forman et al., 1990). Such alterations result in a progressive decrease in blood flow ('no-reflow' phenomenon) (Kloner et al., 1974) and attenuation of coronary vasodilator reserve (Mehta et al., 1989a). Previous studies showed that interaction of endothelium with activated neutrophils and platelets during reperfusion plays an important role in the 'no-reflow' phenomenon (Mullane et al., 1988; Smith et al., 1988; Mehta et al., 1988; Westlin and Mullane, 1989). On the other hand, Na^+–H^+ exchange has been shown to be involved in the regulation of endothelial functions and activation of neutrophils and platelets in response to various stimuli (Sweatt et al., 1985, 1986; Simchowitz and Cragoe, 1986; Ghigo et al., 1988; Osaki et al., 1989; Stiffert et al., 1990).

Therefore, it is conceivable that a Na^+–H^+ exchange inhibitor would have protective effects on microvascular damage during ischemia and reperfusion. However, no previous studies have investigated the influence of Na^+–H^+ exchange inhibitors on microvascular damage induced by ischemia and reperfusion, including the 'no-reflow' phenomenon. Recently, SM-20550, which is a novel Na^+–H^+ exchange inhibitor, was developed (Matsui et al., 1998). The purpose of this study was to evaluate the activity of SM-20550 as a Na^+–H^+ exchange inhibitor and determine its effects on microvascular damage, arrhythmia, infarct size and cardiac function after reperfusion.

2. Materials and methods

2.1. Measurement of Na^+–H^+ exchange activities

To evaluate the activity of SM-20550 as a Na^+–H^+ exchange inhibitor, male Sprague–Dawley rats aged 6 weeks were purchased from Crea Japan (Tokyo, Japan). The experiment was approved by the Institution Animal Care and Use Committee at Sumitomo Pharmaceuticals Research Center (Osaka, Japan). Cardiac myocytes were prepared as follows: cardiac myocytes were isolated by an enzymatic digestion method described previously (Isenberg and Klockner, 1982; Yamamoto et al., 1990). Briefly, rats (6–7 weeks) were anesthetized with diethyl ether, and the hearts were removed and mounted on a modified Langendorff perfusion system for retrograde perfusion of the coronary circulation. The hearts were perfused first with normal Krebs solution (composition in millimolars: NaCl 112, KCl 4.7, $CaCl_2$ 2.2, $MgCl_2$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 14, bubbled with mixed gas, 95% O_2 and 5% CO_2) for 5 min, second with calcium-free Krebs solution (same as Krebs solution except calcium not added) for 10 min, third with calcium-free Krebs solution containing 0.015% collagenase (mixture of YK-102, Yakult and Type IA, Sigma) for 5–20 min, and finally with Kraftbruhe solution (composition in millimolars: KCl 70, KH_2PO_4 2, glutamic acid monopotassium 70, taurine 20, glucose 11, EGTA 0.5, HEPES 10, pH adjusted to 7.4 with Tris) for 5 min. The right ventricle wall was suspended in Kraftbruhe solution and the myocytes were dispersed by gentle stirring. After the cells sedimented, suspension buffer was replaced by calcium-free HEPES solution (composition in mM: NaCl 127, KCl 5.9, $MgCl_2$ 11, taurine 20, glucose 14, HEPES 10, pH adjusted to 7.4 with Tris), then normal HEPES solution (composition in mM: NaCl 137, KCl 5.9, $CaCl_2$ 2.2, $MgCl_2$ 1.2, glucose 14, HEPES 10, pH adjusted to 7.4 with Tris) was added stepwise to make the final Ca^{2+} concentration 2.2 mM.

For the measurement of pH_i, myocytes were loaded with the membrane permeable acetoxymethyl (AM) ester form of the pH-sensitive fluorescent indicator 2*,7*-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM, Wako, final concentration of 3 μM) for 30 min at room temperature. Myocytes loaded with BCECF-AM were then allowed to settle on a laminin-coated glass coverslip at the bottom of a small chamber mounted on the stage of a Nikon inverted microscope. After adherence to the coverslip, the myocytes were superfused (3 ml min^{-1}) with normal HEPES solution. Intracellular BCECF was illuminated at 450 and 490 nm and the BCECF-ratio (490 nm:450 nm) of the light signal emitted at 530 nm was measured by a fluorescence image analyzer (Argus-50, Hamamatsu Photonics). The emission intensity ratio (BCECF-ratio) was used as an index of pH_i (Scholz et al., 1995).

Na^+–H^+ exchange inhibition was determined by the inhibition of pH_i recovery from acidosis according to the methods of Nakanishi et al. (1991), Scholz et al. (1995) and Loh et al. (1996). Intracellular acidification was produced by an NH_4Cl prepulse technique: the cells were first perfused with normal HEPES buffer containing 20 mM NH_4Cl followed by perfusion with normal HEPES buffer. This measurement was performed under HCO_3^--free conditions, in which the cellular pH recovery from acidosis is restricted to Na^+–H^+ exchange. After the BCECF-ratio returned to its normal value, the acidification procedure was repeated again with drugs or vehicle. The percent recovery of the BCECF-ratio from the negative peak (delta-BCECF-ratio) at the 2 nd NH_4Cl prepulse against the first NH_4Cl prepulse was determined at each time course and plotted (BCECF-ratio recovery). As the BCECF-ratio recovery rate was linear until 2 min from the peak of acidosis (negative peak of BCECF-ratio), the percent of BCECF-ratio recovery at 2 min from the peak of acidosis with drugs against that with vehicle was determined as the Na^+/H^+ exchange inhibitory action of drugs.

2.2. Animal preparation

The in vivo study was conducted in accordance with the 'Ethical principles' set out by the Experimental Animal

Laboratory of Gunma University School of Medicine. Forty-two healthy, adult mongrel dogs of either sex ranging in weight from 15 to 25 kg (average weight, 22.4 ± 0.4 kg) were used for this study. The animals were randomly divided into three groups. They were anesthetized with pentobarbital sodium (30 mg kg^{-1}), intubated, and placed on assisted mechanical ventilation with a respirator (Harvard Apparatus, South Natick, MA). The rate (15–18 min^{-1}) and volume per stroke of the respirator (15–20 ml kg^{-1}) were adjusted to maintain arterial blood gases within the physiological range (pH 7.35–7.45, P_{CO_2} 35–45 mm Hg and $P_{O_2} > 80$ mm Hg). A left thoracotomy was performed in the fourth intercostal space, and the heart was suspended in a pericardial cradle. The left circumflex coronary artery was isolated proximal to the first marginal branch at a length of 2 cm. A small hydraulic cuff occluder was positioned around the left circumflex coronary artery just proximal to the first marginal branch to occlude the vessel. To exclude dogs with visible collateral blood flows, coronary angiograms were obtained in all dogs after occlusion of the vessel. In order to measure the left ventricular wall thickness, echocardiography (Toshiba SSH-65A, 3.5-MHz transducer) was performed in all dogs before insertion of a platinum tissue electrode (Unique Medical, Tokyo). The electrode was inserted into the endocardial myocardium in the center of ischemic and non-ischemic areas to measure regional myocardial blood flow (Aukland et al., 1964). The depth to which we inserted the electrode was more than two thirds of the left ventricular wall thickness. A small Teflon catheter (24 gauge) inserted into the left circumflex coronary artery distal to the cuff occluder was then connected to a pressure transducer and to an infusion pump for acetylcholine and adenosine. A pressure catheter was passed through the femoral artery and another one was inserted into the femoral vein to provide either SM-20550 or vehicle (ethylene glycol). For prevention of clotting, heparin was administered immediately after insertion of the catheter (3000 units i.v.) and continuously infused thereafter (500 units h^{-1}).

2.3. Experimental protocol

After completion of all surgical procedures, the dogs were allowed to stabilize for 20 min. A baseline ECG recording was done and heart rate, systemic arterial pressure and coronary blood flow were measured. Then, the left circumflex coronary artery was occluded in 29 dogs for 2 h and then reperfused for 5 h. In 11 dogs (SM-1 group), 0.17 mg kg^{-1} of SM-20550 (pH = 5.0; Sumitomo Pharmaceutical, Osaka, Japan) was administered intravenously 15 min before total occlusion. In seven other dogs (SM-2 group), SM-20550 (0.17 mg kg^{-1}) was administered 15 min before reperfusion. In both groups, SM-20550 (0.28 mg kg^{-1} h^{-1}) was continuously infused after the initial injection throughout the experiment during which acetylcholine and adenosine were administered. In another group of 11 animals (control group), vehicle was infused in the same manner. The electrocardiogram was monitored to evaluate heart rate, arrhythmias, and ST segment changes. Ventricular fibrillation was identified in accordance with the Lambeth Convention guidelines (Walker et al., 1988). Systolic, diastolic and mean artery pressure was also monitored. During ischemia, all dogs had a massive ST segment elevation in lead II. However, no dog showed marked alterations in hemodynamic conditions during ischemia. During ischemia, the mean artery pressure was > 90 mm Hg and heart rate was between 120 and 160 min^{-1} in all dogs. The occurrence of ventricular fibrillation was estimated during 2 h of ischemia and 15 min of reperfusion. During the experiment, all dogs that developed ventricular fibrillation accepted defibrillation (20 J) without antiarrhythmic drugs. With regard to the myocardial damage by defibrillation, Klein et al. (1991) reported that electric countershocks discharged outside the ischemic area have no effect on infarct size. Heart rate, systemic artery pressure, coronary artery pressure and left ventricular end-diastolic pressure were measured before ischemia, and at 15 min and at 5 h after reperfusion.

2.4. Analytical procedures

2.4.1. Coronary vascular resistance

Regional myocardial blood flows in ischemic and non-ischemic area were measured in all dogs. They were estimated by continuous recording of the tissue hydrogen concentration as previously reported (Aukland et al., 1964). In short, mixed gas containing 10% hydrogen was inhaled for 1 min to be saturated and washout of hydrogen from the myocardium was measured by diffuse electric currents in each area. The myocardial desaturation curve was then obtained. Analysis of this curve enabled us to estimate regional myocardial blood flow as follows: F (ml min^{-1} per 100 g) = $\lambda \times 69.3/(T/2)$, where F is the blood flow, λ is the tissue/blood partition coefficient for hydrogen (= 1) and $T/2$ is the time in minutes for the concentration in the tissue to be reduced to half of its value. To evaluate the accuracy of the polarographic hydrogen-clearance technique for measuring regional myocardial blood flow in the epicardial, midcardial, and subendocardial layers, we further compared the results with those of the colored-microsphere method (Hale et al., 1988) in another series of dogs with left circumflex coronary artery occlusion ($n = 18$). Colored polystyrene microspheres were obtained from Triton Technology (San Diego, CA). In short, yellow microspheres (15 μm in diameter) were agitated in an ultrasonic mixer and injected into the left atrium. A reference arterial blood sample was obtained from the femoral artery at a rate of 7 ml min^{-1} starting 10 s before the start of the injection and continued for 2 min. Then, tissue samples (2–3 g wet weight) from the three myocardial layers were

obtained for the measurement of myocardial blood flow and digested in an alkaline solution, and the microspheres were recovered and counted. Tissue blood flow was calculated from the equation as described (Hale et al., 1988). Previous investigators also confirmed the good agreement between myocardial blood flow obtained from tissue desaturation curves and that measured directly by coronary venous outflow (Aukland et al., 1964). In order to estimate the reproducibility of the polarographic hydrogen-clearance technique for measurement of regional myocardial blood flow, we repeated this measurement in another 18 dogs. Regional coronary vascular resistance was calculated from the mean coronary artery pressure/mean regional myocardial blood flow as previously described (Johnson et al., 1988).

2.4.2. Coronary arterial vasodilatory responses

Before ischemia, at 15 min and at 5 h after reperfusion, acetylcholine (0.1 μg kg^{-1} min^{-1}) and adenosine (40 μg kg^{-1} min^{-1}) were administered by infusion pump into the left circumflex coronary artery over 4 min. Then, coronary vascular resistances in response to acetylcholine and adenosine were assessed in controls and dogs with SM-20550 treatment (SM-1 and SM-2 groups) in pre-ischemia and at 15 min and at 5 h after reperfusion. Furthermore, to investigate whether the responses to acetylcholine and adenosine after reperfusion were dose-dependent in SM-1 and control groups, the various loading doses of acetylcholine (0.0001–0.1 μg kg^{-1} min^{-1}) or those of adenosine (0.04–40 μg kg^{-1} min^{-1}) were injected into the left circumflex coronary artery in the same manner in pre-ischemia and at 15 min and at 5 h after reperfusion.

2.4.3. Histopathological examination

A specimen of myocardium from the reperfused area was obtained and placed in 10% buffered formalin and processed for light microscopic examination. Sections of the myocardium stained by hematoxylin and eosin were evaluated for histopathological examination.

2.4.4. Myocardial myeloperoxidase activity

Myeloperoxidase activity in the myocardium, a specific enzymatic marker of neutrophil infiltration into the reperfused myocardium, was measured as previously reported (Mehta et al., 1989a,b). In short, the myocardium from the reperfused area was homogenized in 0.5% hexadecyltrimethylammonium bromide and dissolved in 50 mM potassium phosphate buffer at pH 6.0. After sonication, the homogenates were centrifuged at 30,000 rpm for 15 min. The supernatants were collected and reacted with 0.167 mg ml^{-1} of *O*-dianisidine dihydrochloride and 0.0005% H_2O_2 in 50 mM phosphate buffer at pH 6.0. The rate of alteration in absorbance was measured by spectrophotometer at 460 nm. One unit of myeloperoxidase activity was defined as that degrading 1 μmol H_2O_2 min^{-1} at 25°C.

2.4.5. Measurements of risk area and infarct size

After completion of the experiment, the left circumflex coronary artery was reoccluded and 20–30 ml of 0.5% methylene blue was administered through the left atrium to determine the anatomic area at risk. In all dogs, ventricular fibrillation was induced by electrical stimulation. After cardiac standstill, the whole heart was removed. Following removal of the right ventricle and atria, the left ventricle was sliced parallel to the atrioventricular groove in approximately 1 cm thick sections. The area at risk was defined as portions of the myocardium unstained by methylene blue. Sliced heart tissues were incubated in 1% triphenyl tetrazolium chloride in phosphate buffer at pH 7.8 and 37°C for 10 min to define the necrotic or irreversibly injured myocardium (Fishbein et al., 1981). A blinded investigator determined cumulative infarct size and area at risk with computer-assisted planimetry of each section. The risk area was indicated as a percentage of the left ventricular area. Infarct size was also expressed as a percentage of the area at risk.

2.5. Drugs

In the rat study, SM-20550 (Sumitomo Pharmaceutical (Osaka)) was dissolved in distilled water. Ethylisopropyl amiloride (Sigma; lot No. 36H4166) was dissolved in 10 (w/w)% dimethyl sulfoxide. In a dog model of reperfusion, SM-20550 [*N*-(Aminoiminomethyl)-1,4-dimethyl-1*H*-indole-2-carboxamide methanesulfonate] was dissolved in ethylene glycol. Other chemicals were purchased from Sigma (Poole, UK).

2.6. Statistical analysis

Regional myocardial blood flow calculated from polarographic hydrogen-clearance technique data was compared with that calculated with colored microspheres. To evaluate the agreement between the two techniques, we calculated linear regression and correlation coefficient. To examine the inter-technique bias and limits of agreement, Bland–Altman analysis (Bland and Altman, 1986) was used. In addition, in order to assess the reproducibility of the polarographic hydrogen-clearance technique of regional myocardial blood flow, pairs of consecutive inhalations of hydrogen were performed at various levels of ischemia. The results calculated from these two inhalations were compared by linear regression and correlation coefficient. The difference between flow values estimated from paired inhalations was calculated and normalized by the mean.

Each value was expressed as mean ± S.E.M. To compare hemodynamic data and coronary vascular resistance, the data were subjected to analysis of variance followed by the Bonferroni correction. Statistical significance was accepted at the 5% level ($P < 0.05$).

3. Results

3.1. Potency of SM-20550 as a Na^+–H^+ exchange inhibitor in rat cardiomyocytes

Na^+–H^+ exchange activities in isolated rat cardiomyocytes were measured by recovery of pH_i from acidosis induced by an NH_4Cl prepulse technique. This measurement was performed under HCO_3^- free conditions, in which the cellular pH recovery from acidosis is restricted to Na^+–H^+ exchange. After a first NH_4Cl prepulse, the pHi slowly recovered to the resting level. Then a second NH_4Cl prepulse was applied and test compounds were added to the perfusion solutions. Ethylisopropyl amiloride, a known Na^+–H^+ exchange inhibitor, suppressed the pH_i recovery from acidosis, and the approximate IC_{50} value was 10^{-7} M, which was similar to the result reported by Scholz et al. (1992, 1995). Fig. 1 indicates the inhibition of pH_i recovery from acidosis by SM-20550. SM-20550 (3×10^{-9}–10^{-7} M) concentration-dependently inhibited the recovery of pH_i, and the IC_{50} value was approximately 10^{-8} M. The Na^+–H^+ exchange inhibitory activity of SM-20550 was about 10 times more potent than that of ethylisopropyl amiloride.

3.2. Myocardial blood flow in the ischemic area

Fig. 2A shows the validity of the polarographic hydrogen-clearance technique to measure regional myocardial blood flow. Each regional myocardial blood flow value calculated with the polarographic hydrogen-clearance technique is presented against the corresponding colored microsphere reference value. There was a good correlation for the measurement of regional myocardial blood flow between colored microsphere and polarographic hydrogen clearance techniques ($r = 0.96$, $P < 0.01$) (Fig. 2A). Bland–Altman analysis (Bland and Altman, 1986), used to evaluate inter-technique agreement, proved a small bias of 7.6 ml min^{-1} per 100 g with the 95% limits of agreement

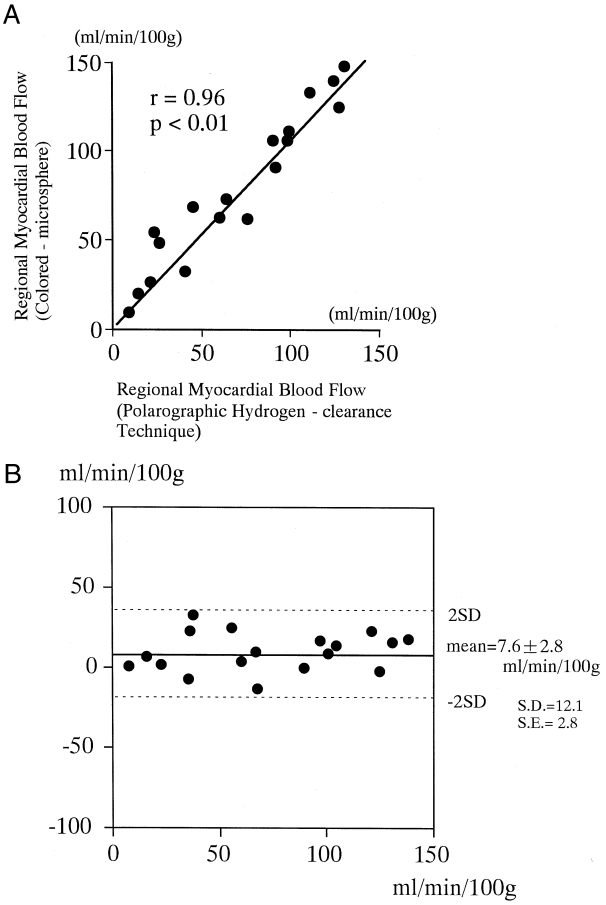

Fig. 2. (A) Regression plots of regional myocardial blood flow from the colored-microsphere and polarographic hydrogen-clearance techniques ($r = 0.96$, $P < 0.01$). RMBF (microsphere) = 1.044 × (polarographic RMBF) + 3.985; RMBF: regional myocardial blood flow. (B) Bland–Altman analysis revealed a small bias of 7.6 ml min^{-1} per 100 g with the 95% limits of agreement at 24.2 ml min^{-1} per 100 g.

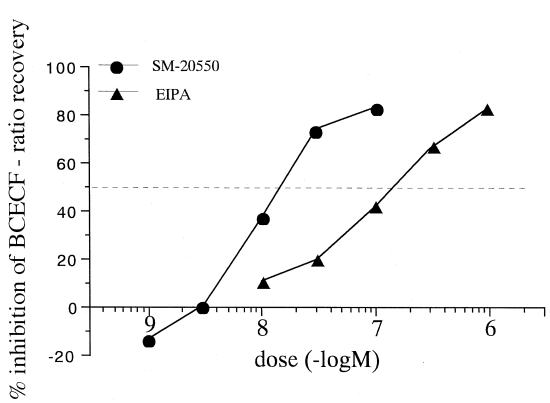

Fig. 1. Concentration-dependent inhibition by SM-20550 and ethylisopropyl amiloride (EIPA) of the BCECF-ratio recovery from acidification in isolated rat cardiomyocytes.

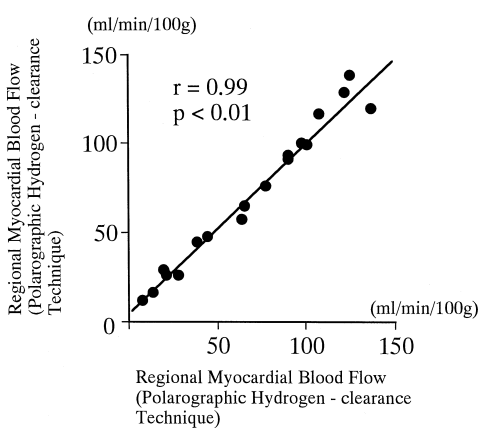

Fig. 3. Each value for regional myocardial blood flow during one inhalation of hydrogen is plotted against the value calculated during paired inhalation of hydrogen in our protocol. Correlation coefficient ($r = 0.99$) and linear regression ($y = 0.98\times + 2.4$) are shown.

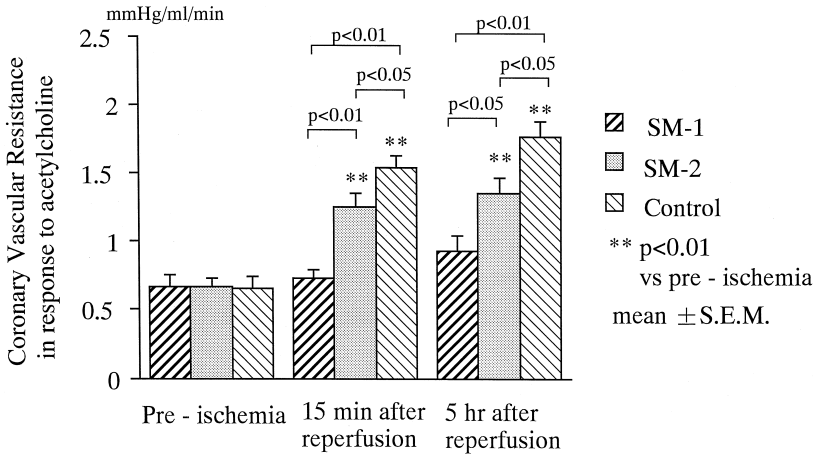

Fig. 4. Effects of intracoronary infusion of acetylcholine on coronary vascular resistance in ischemic area for the three reperfusion groups. Heights of bars are means; brackets represent ±S.E.M. values for each group.

at 24.2 ml min^{-1} per 100 g (Fig. 2B). The values for regional myocardial blood flow for paired inhalations of hydrogen to evaluate the reproducibility of the technique gave a linear regression of $y = 0.98 \times + 2.4$ ($r = 0.99$, $P < 0.01$) (Fig. 3). In repeated measurements, the intrapair difference normalized by the mean was 1.1 ± 9.5% (mean ± S.D.). At a given stenosis in the dogs without visible collateral blood flows, we also observed that subendocardial myocardial blood flow could reflect subepicardial flow because there was a good correlation for regional myocardial blood flow between the two layers ($RMBF_{subepi} = 1.23 \times RMBF_{subendo} + 8.82$, $n = 9$; $r = 0.96$, $P < 0.01$) (RMBF: regional myocardial blood flow).

Seven dogs with intractable ventricular fibrillation and six dogs in which we could not obtain adequate myocardial ischemia after total occlusion of the left circumflex coronary artery (< 20 ml min^{-1} per 100 g) were excluded from the present study, giving a total of 29 animals analyzed. Collateral blood flow to the ischemic area was comparable among the three groups (control group 7.7 ± 1.8; SM-1 group 7.5 ± 1.6; SM-2 group 7.4 ± 1.5 ml min^{-1} per 100 g).

3.3. Coronary vasodilator reserve

The effects of intracoronary administration of acetylcholine (0.1 μg kg^{-1} min^{-1}) and adenosine (40 μg kg^{-1} min^{-1}) on coronary vascular resistance in ischemic area after reperfusion for the three groups are shown in Figs. 4 and 5. In the case of SM-1, dogs in which SM-20550 treatment was started before ischemia, the coronary vascular resistances after acetylcholine and adenosine at both 15 min and 5 h after reperfusion were comparable to those in pre-ischemia (P: not significant). However, in control dogs,

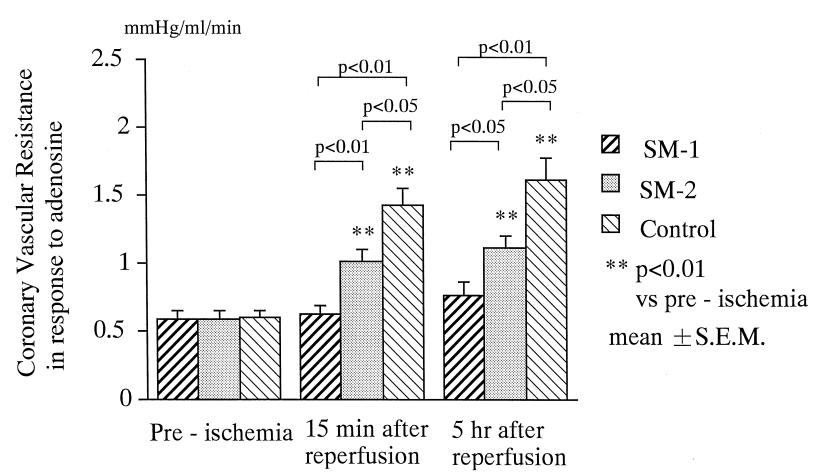

Fig. 5. Effects of intracoronary administration of adenosine on coronary vascular resistance in ischemic area for the three reperfusion groups. Heights of bars are means; brackets represent ±S.E.M. values for each group.

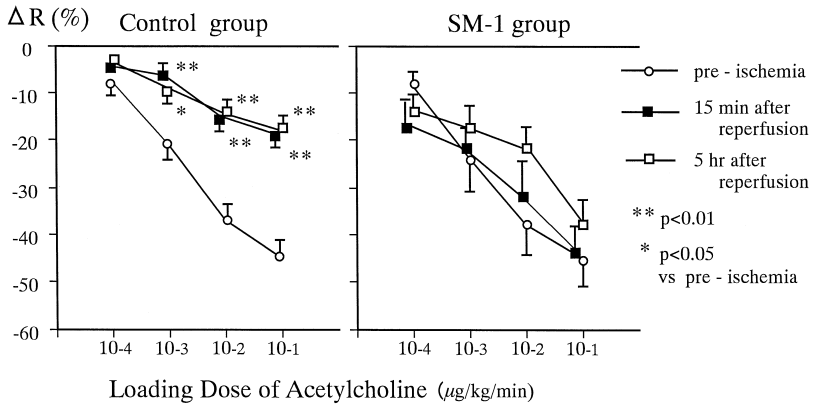

Fig. 6. Coronary vascular hemodynamic responses in ischemic area to an increase in loading dose of acetylcholine after reperfusion in the SM-1 and control groups. ΔR: % changes in coronary vascular resistance in response to acetylcholine. In the dogs with SM-20550 treatment before ischemia, the response to acetylcholine was preserved in a dose-dependent manner after reperfusion.

coronary vascular resistances in the presence of acetylcholine and adenosine after reperfusion were significantly greater than those in pre-ischemia ($P < 0.01$; both). After reperfusion, the coronary vascular resistances in the presence of acetylcholine and adenosine in the SM-2 group were significantly reduced as compared with those in the control ($P < 0.05$). Coronary vascular hemodynamic responses in ischemic area to an increase in the loading doses of acetylcholine and adenosine after reperfusion in SM-1 and control groups are shown in Figs. 6 and 7. The dose-dependent responses to these two drugs were preserved in the SM-1 but not in the control group.

3.4. Histopathology

In the controls, capillary plugging by neutrophils was the most characteristic finding in the subendocardial myocardium in the reperfused area (Fig. 8A). This contributes to the altered coronary flow reserve (Mehta et al., 1989a). The endothelial lining of the arterioles was often adhered to by neutrophils. We observed the complete occlusion of several vascular lumens by neutrophils in control dogs. Intramyocardial bleeding after reperfusion, which was observed in seven of the 11 control dogs, was not found in dogs with SM-20550 treatment ($P < 0.05$). In addition, the myocardial tissues obtained from the center of the reperfused area in controls showed several signs of early myocardial damage. These include wavy fibers and cell separation, cell contracture, and intense neutrophil infiltration of the intercellular spaces (Mehta et al., 1989b). In contrast, such findings that indicate microvascular deterioration and myocardial injury after reperfusion were less in the SM-1 group than in the control (Fig. 8B).

3.5. Myocardial myeloperoxidase activity

Neutrophil-specific myocardial myeloperoxidase activity in the reperfused region was significantly greater in the

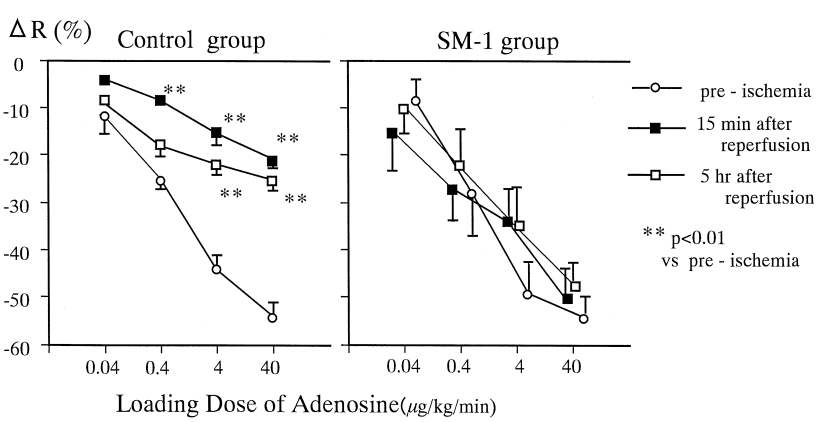

Fig. 7. Coronary vascular hemodynamic responses in ischemic area to an increase in loading dose of adenosine after reperfusion in the SM-1 and control groups. ΔR: % changes in coronary vascular resistance in response to adenosine. In the group SM-20550-treated before ischemia, the response to adenosine was preserved in a dose-dependent manner after reperfusion.

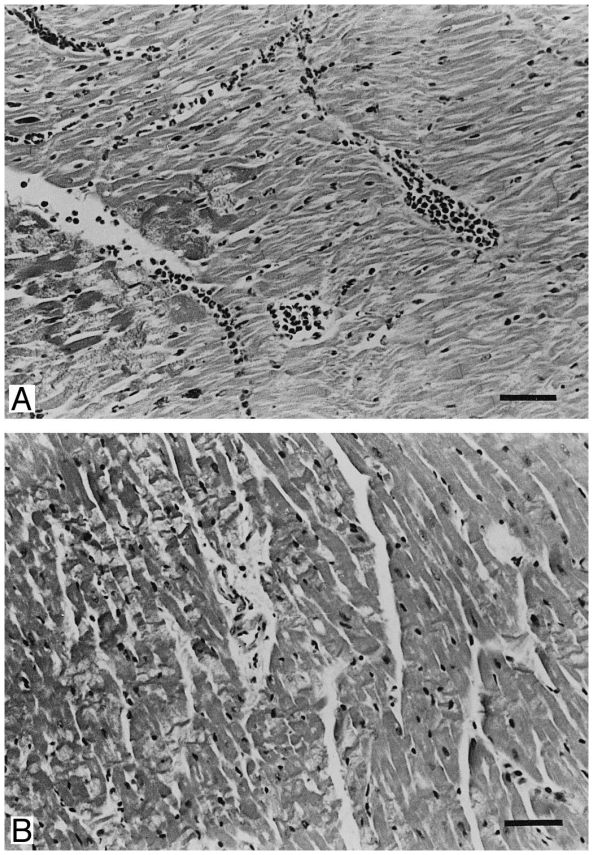

Fig. 8. Hematoxylin and eosin-stained section of myocardium examined under light microscopy from the reperfused area ((A) Control, (B) a dog with SM-20550 treatment before ischemia). SM-20550 reduced the capillary plugging by neutrophils and myocardial injury in reperfused area (B). Bar = 100 μm.

control than in the SM-1 group (control 0.84 ± 0.17; SM-1 group 0.36 ± 0.12 units mg^{-1} protein, $P < 0.01$).

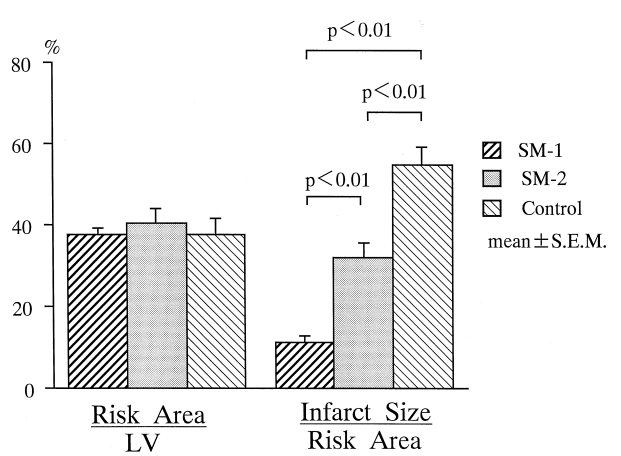

Fig. 9. Risk area was comparable among the three reperfusion groups. Infarct size was expressed as a percentage of the risk area after reperfusion in group SM-20550-treated before ischemia ($n = 11$), that treated just before reperfusion ($n = 7$) and control group ($n = 11$). Infarct size after reperfusion was significantly reduced in SM-20550-treated groups. LV: left ventricle.

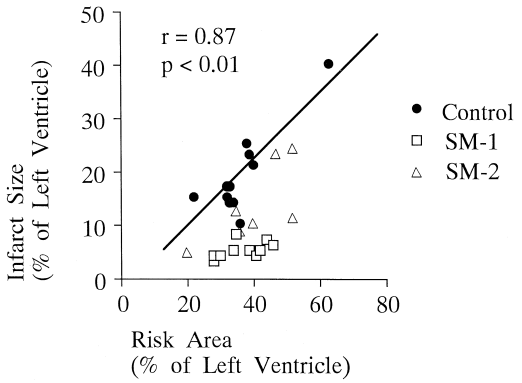

Fig. 10. The relationships between infarct size (% of LV) and the area at risk in the three reperfusion groups are illustrated. In the control group, there was a significant correlation between infarct size and the area at risk ($r = 0.87$, $P < 0.01$). However, in the both SM-20550-treated groups, infarct size is small and no significant relation exists between the two.

3.6. Risk area and infarct size

The risk area was comparable among the three groups (Fig. 9). When expressed as a percentage of the area at risk, infarct size in the SM-1 group was significantly reduced, but in the SM-2 group, it lay between the control and the SM-1 groups (control group 54 ± 5%, SM-1 group 11 ± 2%, SM-2 group 32 ± 4% :both $P < 0.01$ vs. control). It is noteworthy that SM-20550 more effectively reduced infarct size when administered pre-ischemia.

3.7. Dependence of infarct size on area at risk and collateral flow

There was a close correlation between infarct size and the area at risk in control animals ($r = 0.87$, $P < 0.01$) (Fig. 10). In control dogs, infarct size was inversely correlated with collateral blood flow ($r = -0.95$, $P < 0.01$) (Fig. 11). However, in either the SM-1 or the SM-2 group,

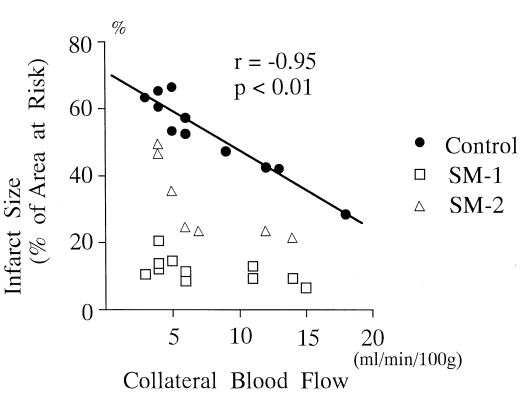

Fig. 11. The relationships between infarct size normalized to the area at risk and collateral blood flow in the three reperfusion groups are illustrated. In the control group, there is a significant negative correlation between infarct size and collateral blood flow ($r = -0.95$, $P < 0.01$). However, in both SM-20550-treated groups, infarct size is small and no significant relation exists between the two.

infarct size was independent of the area at risk or collateral blood flow (Figs. 10 and 11).

3.8. Cardiac function after reperfusion

During pre-ischemia and reperfusion, there was no significant difference in heart rate, mean artery pressure or mean coronary artery pressure among the three groups (Table 1). In the control and the SM-2 groups, left ventricular end-diastolic pressure at both 15 min and 5 h after reperfusion showed a significant elevation as compared with that for pre-ischemia (both, $P < 0.01$). However, in the SM-1 group, no significant increase in left ventricular end-diastolic pressure was observed after reperfusion.

Table 1
Comparison of hemodynamic status

	Pre-ischemia	15 min after reperfusion	5 hr after reperfusion
Heart rate (per min)			
Control group	141±2	134±3	137±5
Acetylcholine	141±1	139±2	137±4
Adenosine	141±4	139±4	135±5
SM-1 group	142±4	141±4	142±3
Acetylcholine	145±4	141±5	144±5
Adenosine	144±5	143±5	140±4
SM-2 group	143±3	142±5	140±6
Acetylcholine	142±5	139±5	138±5
Adenosine	143±6	141±4	139±6
Mean artery pressure (mm Hg)			
Control group	99±7	92±4	93±4
Acetylcholine	104±6	94±6	93±6
Adenosine	95±6	92±5	93±5
SM-1 group	100±6	104±5	103±3
Acetylcholine	100±6	101±4	100±4
Adenosine	99±5	101±7	104±5
SM-2 group	98±6	96±5	95±3
Acetylcholine	102±5	98±6	96±4
Adenosine	101±6	99±6	96±6
Mean coronary artery pressure (mm Hg)			
Control group	97±7	89±4	90±4
Acetylcholine	100±5	91±4	90±4
Adenosine	94±5	92±5	91±4
SM-1 group	98±6	101±6	101±4
Acetylcholine	98±5	99±4	100±4
Adenosine	96±5	97±6	97±4
SM-2 group	97±5	95±4	94±4
Acetylcholine	99±6	97±5	95±4
Adenosine	100±6	98±5	96±5
LVEDP (mm Hg)			
Control group	6±1	9±1[a]	10±1[a]
SM-1 group	6±1	7±1	7±1
SM-2 group	5±1	8±1[a]	9±1[a]

In the control and SM-2 groups, LVEDP significantly increased during reperfusion ($P < 0.01$).
Each value is expressed as means±S.E.M.
[a] $P < 0.01$ vs. pre-ischemia.
LVEDP: left ventricular end-diastolic pressure.

3.9. Incidence of ventricular fibrillation during ischemia and reperfusion

Of the 25 dogs without SM-20550 treatment during ischemia, 16 (64.0%) developed ventricular fibrillation. Seven of these 16 dogs were excluded from the study because they could not be resuscitated from ventricular fibrillation during ischemia by direct electric countershock. After reperfusion, six of the 11 dogs (54.5%) without administration of SM-20550 developed ventricular fibrillation. In contrast, no dog with SM-20550 treatment developed ventricular fibrillation during either ischemia or reperfusion. These results indicate that SM-20550 significantly suppressed ventricular fibrillation during both ischemia ($P < 0.01$) and reperfusion ($P < 0.05$).

4. Discussion

In the present study, we first demonstrated that inhibition of the Na^+–H^+ exchange protects coronary arteries from functional deterioration and histopathological damage after reperfusion. The protective effects of SM-20550 depended on the timing of treatment; its administration before ischemia was more effective than that before reperfusion. We further demonstrated that an improvement in the 'no-reflow' phenomenon by SM-20550 was accompanied by cardioprotective effects after reperfusion.

The results indicated that SM-20550 possesses a high potency as a Na^+–H^+ exchange inhibitor. That is, in isolated rat cardiac myocytes, the IC_{50} for SM-20550 is only 10 nM, whereas the IC_{50} of Hoe642 is reportedly between 1 μM and 0.1 μM (Scholz et al., 1995). Because Hoe642 was also reported to be about four times more potent than Hoe694, SM-20550 as a Na^+–H^+ exchange inhibitor seems to be at least forty times more potent than Hoe694. Furthermore, in an in vivo study using a rabbit model of ischemia and reperfusion, SM-20550 improved the reperfusion injury in a dose dependent manner without any side-effect (Matsui et al., 1998).

We observed that SM20550 inhibited the Na^+–H^+ exchange in rat cardiac myocytes. In cardiac myocytes, Na^+–H^+ exchange subtype 1 is mainly expressed (Piper et al., 1996). Accordingly, SM-20550 inhibits at least subtype 1 Na^+–H^+ exchange. On the other hand, Na^+–H^+ exchange subtypes 2 and 3 are selectively localized on the epithelial cells of the kidney (Tse et al., 1992, 1993). Administration of SM-20550 at a dose of 10 times greater than that used in the present study did not affect either the urine output or the excretion of Na^+, K^+, and Cl^- (unpublished data), indicating that SM-20550 has little effect on Na^+–H^+ exchange subtype 2 or 3.

An impairment of coronary vasodilator reserve in the reperfused area suggests the occurrence of the 'no-reflow' phenomenon (Mehta et al., 1989a,b). In the control group,

we found that coronary vasodilator responses to both acetylcholine and adenosine after reperfusion were significantly attenuated, indicating the development of the 'no-reflow' phenomenon in the reperfused area. However, treatment with SM-20550 starting just before reperfusion protected the coronary artery from functional impairment after reperfusion. There are two possible mechanisms by which inhibition of the Na^+–H^+ exchange could improve microvascular damage after reperfusion. First, the improvement in microvascular damage after reperfusion may arise from a reduction in myocardial injury by SM-20550. Na^+–H^+ exchange inhibitors are known to protect myocardium from injury during reperfusion (Tani and Neely, 1990; Klein et al., 1995; Cross et al., 1996). Second, the reduction in microvascular damage by SM-20550 may result from its direct effects on endothelium, neutrophils and platelets after reperfusion. With regard to endothelium, we observed that addition of 10^{-6} M SM-20550 to the culture media significantly increased the viability of endothelial cells following anoxiareoxygenation (unpublished data), indicating that SM-20550 may protect endothelial cells from deterioration after reperfusion. In addition, many investigators have reported salutary effects of Na^+–H^+ exchange inhibitors on various cellular processes contributing to the occurrence of reperfusion injury (Ghigo et al., 1988; Osaki et al., 1989; Stiffert et al., 1990; Sweatt et al., 1985, 1986). For instance, treatment with Na^+–H^+ exchange inhibitors can attenuate the production of leukotriene B_4, a potent chemotactic and chemokinetic agent, in neutrophils via the blockade of cytoplasmic alkalinization. As to the activation of platelets, inhibition of the Na^+–H^+ exchange can also reduce the synthesis of thrombin-induced platelet activating factor by endothelial cells. Accordingly, it is likely that the suppressive effects of a Na^+–H^+ exchange inhibitor on the interaction between endothelium, activated neutrophils and platelets during reperfusion may result in an improvement in microvessel damage. However, further studies will be needed to explore the mechanism by which SM-20550 reduces microvascular deterioration after reperfusion.

Our histopathological study revealed that microvascular damage such as intramyocardial bleeding and the myocardial injury after reperfusion were less severe in SM-20550-treated dogs than in the controls. In fact, the intramyocardial bleeding after reperfusion observed in control dogs was not found in dogs with SM-20550 treatment. Previous investigators reported capillary plugging by neutrophils to be the most characteristic finding in reperfused myocardium (Mehta et al., 1989b). Indeed, in myocardial tissues obtained from the center of the reperfused area, we found capillary plugging by neutrophils in control dogs but not in dogs with SM-20550 treatment. Moreover, myocardial myeloperoxidase activity in the reperfused area was significantly reduced in dogs with SM-20550 treatment as compared with that in the controls. An increase in myocardial myeloperoxidase activity indicates the presence of large numbers of neutrophils in the myocardium (Mehta et al., 1989b). Therefore, both histopathological and biochemical analyses of the reperfused myocardium indicated that SM-20550 may reduce neutrophil infiltration, suggesting the contribution of a Na^+–H^+ exchange inhibitor to the protection against microvascular and myocardial damage after reperfusion.

In both the control and SM-2 groups, left ventricular end-diastolic pressure after reperfusion was significantly higher than that pre-ischemia. In contrast, when SM-20550 treatment was started before ischemia (SM-1 group), there was no significant difference in left ventricular end-diastolic pressure between pre-ischemia and after reperfusion. These results indicate that inhibition of the Na^+–H^+ exchange throughout the period of ischemia and reperfusion could improve cardiac dysfunction after reperfusion. However, a similar study using Hoe694 revealed no improvement in cardiac dysfunction after reperfusion (Klein et al., 1995). The discrepancy between results of the two studies might be due to the different potencies of SM-20550 and Hoe694.

Infarct size in dogs with SM-20550 treatment was significantly less than that in control dogs. Furthermore, in the SM-20550 treatment groups, infarct size in the dogs treated pre-ischemia was significantly less than that in dogs treated just before reperfusion. This indicates that the reduction in infarct size was an effect of SM-20550 throughout the period of ischemia and reperfusion. In the control group, infarct size was correlated positively with the area at risk and inversely with collateral blood flow, the area at risk and collateral blood flow could be the two major determinants of infarct size in the controls after reperfusion. In contrast, in dogs with SM-20550 treatment (SM-1 and SM-2 groups), infarct size was not dependent on either the area at risk or collateral blood flow. This finding is consistent with the fact that SM-20550 treatment yielded no improvement in the degree of ischemia in spite of a reduction in infarct size. Therefore, it is likely that the direct effects of SM-20550 on myocardial cells result in the reduction in myocardial damage after reperfusion. That is, accumulation of intracellular H^+ occurs during myocardial ischemia. Moreover, after the start of reperfusion, rapid washout of extracellular H^+ induces an intracellular to extracellular H^+ gradient. Under both conditions, an influx of Na^+ through the Na^+–H^+ exchanger occurs (Lazdunski et al., 1985; MacLeod, 1991). Following such an influx, an increase in intracellular Ca^{2+} via the Na^+–Ca^{2+} exchange (Lazdunski et al., 1985) would favor accumulation of intracellular Ca^{2+}. On the contrary, reduction in $[Na^+]_i$ by inhibition of Na^+–H^+ exchange during ischemia and reperfusion may ultimately attenuate the activation of the Na^+–Ca^{2+} exchange and then protect cells from Ca^{2+} overload. Indeed, amiloride, a Na^+–H^+ exchange inhibitor, can reduce the increase in intracellular Ca^{2+} during ischemia and reperfusion in the isolated rat heart (Tani and Neely, 1989).

Treatment with SM-20550 significantly reduced the occurrence of ventricular fibrillation during ischemia and reperfusion. Because an increase in intracellular Ca^{2+} has been proposed to play an important role in the development of arrhythmias during ischemia and early reperfusion (Opie et al., 1988), it is possible that a potent Na^+–H^+ exchange inhibitor, SM-20550, may prevent ventricular fibrillation by inhibition of an increase in intracellular Ca^{2+}. Indeed, during the coronary reperfusion of the isolated rat heart, a Na^+–H^+ exchange inhibitor afforded significant protection against reperfusion-inducedventricular fibrillation (Mochizuki et al., 1993; Yasutake et al., 1994).

However, antiarrhythmic effects of Hoc694 were not mentioned in a previous report about a work with ischemic and reperfused porcine heart (Klein et al., 1995).

Our present study demonstrated for the first time that Na^+–H^+ exchange plays an important role in the development of microvascular deterioration after reperfusion in dogs. The results of our investigation may help to design pharmacological interventions aimed at reducing microvascular reperfusion injury and infarct size.

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