Protective Effect of FR183998, a Na⁺/H⁺ Exchange Inhibitor, Against Postischemic Injury After Normothermic and Prolonged Hypothermic Ischemia in Isolated Perfused Rat Hearts

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ABSTRACT—Inhibition of Na⁺/H⁺ exchanger has been reported to protect hearts from ischemia and reperfusion injury. However, the effect of Na⁺/H⁺ exchange inhibition on hypothermic ischemic injury has not been extensively studied and the results are inconsistent. The purpose of this study was to investigate whether inhibition of Na⁺/H⁺ exchange with FR183998 (5-(2,5-dichlorothiphen-3-yl)-3-[(2-dimethylaminoethyl)carbamoyl]benzoylguanidine dihydrochloride), a potent Na⁺/H⁺ exchange inhibitor, would show protective effects against postischemic cardiac dysfunction after hypothermic as well as normothermic ischemia and furthermore, after hypothermic cardioplegic arrest in isolated rat hearts. FR183998 (3.2 x 10⁻⁸ – 3.2 x 10⁻⁷ M) improved post-ischemic recovery of left ventricular developed pressure and suppressed the increase of left ventricular end diastolic pressure in a dose-dependent manner, after not only 45 min of normothermic ischemia but also 6 h of hypothermic ischemia. Furthermore, FR183998 (10⁻⁷ – 10⁻⁶ M) significantly reduced creatine kinase release during reperfusion after 3 h of hypothermic ischemia with cardioplegia. These results indicate that FR183998 has a potent protective effect on postischemic cardiac dysfunction after normothermic and hypothermic ischemia, and also on reperfusion injury after hypothermic cardioplegic arrest, suggesting that its effect would be additive to cardioplegia.

Keywords: Cardioplegia, FR183998, Na⁺/H⁺ exchange, Ischemia and reperfusion, Hypothermia

It has been shown that myocardial Na⁺/H⁺ exchange is a major mechanism for the regulation of intracellular pH (1, 2). H⁺ accumulation in ischemic heart stimulates Na⁺/H⁺ exchange, which leads to an increase of [Na⁺], followed by a secondary increase of [Ca²⁺] through Na⁺/Ca²⁺ exchange (3, 4). This Ca²⁺-overload is well recognized as a responsible mechanism for the detrimental myocardial tissue injury that becomes apparent after reperfusion such as arrhythmias, cellular necrosis and contractile dysfunction in hearts (5, 6). Furthermore, it has been demonstrated that inhibition of Na⁺/H⁺ exchange with amiloride and its derivatives (7 – 9) or structurally different compounds such as HOE694 (10, 11) and HOE642 (12, 13) results in marked protection against myocardial ischemia-reperfusion injury.

Although many studies have reported that inhibition of Na⁺/H⁺ exchange protects hearts from ischemic injuries, its effect on post-ischemic injury after hypothermic ischemia is not well recognized. In the clinical situation, the hypothermic condition is normally used during cardiac surgical procedures or heart preservation. The main principles of the hypothermic condition are energy preservation and decreasing the metabolic rate in myocardium. On the other hand, Askenasy et al. reported that Na⁺ influx was enhanced during ischemia at lower temperatures compared with normothermic ischemia (14). Under hypothermic ischemia, the process of ischemic damage such as energy consumption or ion balances is thought to be different from that of normothermic ischemia. Thus, it is very important to determine whether inhibitor of Na⁺/H⁺ exchange could protect hearts from ischemia-reperfusion injury after hypothermic ischemia as well as normothermic ischemia.

In the present study, we investigated the effect of FR183998 (5-(2,5-dichlorothiphen-3-yl)-3-[(2-dimethylaminoethyl)carbamoyl]benzoylguanidine dihydrochloride) on the functional recovery of isolated rat hearts after both normothermic and hypothermic ischemia. FR183998 has been characterized as a potent Na⁺/H⁺ exchange inhibitor
and showed marked antiarrhythmic and infarct size-limiting effects in anesthetized rats in our previous study (15). Therefore, the purpose of this study was to determine whether the structurally distinct Na\(^{+}/H\)\(^{+}\) exchange inhibitor FR183998 can protect hearts from contractile dysfunction after global ischemia at both normal and low temperature in isolated rat hearts. Furthermore, we also investigated whether FR183998 could show protective effects in isolated rat hearts subjected to hypothermic cardioplegic arrest to determine its additional protective effect compared with cardioplegia.

MATERIALS AND METHODS

Animals

The experimental work was performed in accordance with the regulations of the Animal Ethical Committee of Fujisawa Pharmaceutical Co., Ltd. Male Wistar rats (8 – 10 weeks of age) and male Sprague-Dawley rats (9 – 10 weeks of age), purchased from Nihon SLC (Shizuoka), were used. All animals were group housed (5 – 6/cage) in an air-conditioned room under a 12-h light-dark cycle. They received ordinary laboratory food and water ad libitum. Each animal was used only once.

Functional recovery after normothermic and hypothermic ischemia in isolated rat hearts

In these experiments, a modified Langendorff isolated rat heart system was used (16). Male Sprague-Dawley rats were heparinized with 300 IU/kg and anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally. After 15 min, hearts were excised and placed in ice-cold saline, a cannula was inserted into the aorta, and the heart was rapidly mounted on to the Langendorff perfusion system. Isolated hearts were then perfused via the cannulated aorta at a constant perfusion pressure of 60 mmHg with a modified Krebs-Henseleit buffer (composition: 118.5 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 25.0 mM NaHCO\(_3\), 1.4 mM CaCl\(_2\), 11.1 mM glucose). The buffer was gassed with 95% O\(_2\) / 5% CO\(_2\), and the temperature was maintained at 37°C. Perfusion buffer was filtered through a 0.22-μm porosity membrane. Left ventricular developed pressure (LVDP) and heart rate (HR) were monitored through a water-filled latex balloon inserted into the left ventricle via the left atrium and connected to a pressure transducer with a polygraph (365-IB; NEC San-ei Instruments Ltd., Tokyo).

After introduction of the balloon into the left ventricle, left ventricular end diastolic pressure (LVEDP) was set at 10 mmHg. After 20 min of stabilization, FR183998 was infused for 10 min, and the perfusion was stopped to start the ischemic period.

The temperature of the hearts during ischemia was kept at 37°C for 45 min in normothermic global ischemia, while 17°C for 6 h in hypothermic global ischemia. After each ischemic period, all hearts were reperfused with the perfusion buffer at 37°C and hemodynamic parameters were continuously monitored for 30 min. The postischemic functional recoveries were expressed as a percentage of the pre-ischemic value at the end of the stabilizing period.

In these experimental protocols, hearts showing under 120 mmHg of LVDP during stabilization or under 200 beats/min HR were excluded. LVDP data were expressed as percentage recovery of LVDP during reperfusion compared with the value at the end of stabilization.

Measurement of CK leakage in isolated perfused rat hearts after hypothermic cardioplegic arrest

For this study, a modified protocol described by Galinanes et al. was used (17). Isolated rat hearts were prepared by the same method described above. Hearts of male Wistar rats were excised, cannulated via the aorta and immediately infused with St. Thomas’ Hospital cardioplegic solution (composition: 110.0 mM NaCl, 16.0 mM KCl, 16.0 mM MgCl\(_2\), 10.0 mM NaHCO\(_3\), 1.2 mM CaCl\(_2\), pH 7.8) with or without FR183998 on a Langendorff perfusion system, at a constant perfusion pressure of 45 mmHg at 25°C. During this infusion period cardioplegic arrest was obtained. Hearts were then immersed in the same cardioplegic solution and subjected to 3 h of hypothermic (25°C) global ischemia. After the ischemic period, hearts were reperfused with Krebs-Henseleit buffer at 37°C for 60 min, at a constant perfusion pressure of 65 mmHg. During the reperfusion period, coronary flow was measured by timed collection of effluents every 15 min and 1 mL of each effluent was stored at −80°C. In this study, we measured creatine kinase (CK) release as an index for irreversible cellular damage. The CK concentrations (U/mL) in each effluent were measured within a week by an auto-analyzer (80FR; Toshiba, Tokyo). Total CK leakage (U/60 min) was calculated by the obtained CK concentration (U/mL) and coronary flow (mL/15 min) during 60 min of reperfusion.

Materials

FR183998 (Fig. 1) was synthesized by Fujisawa Pharmaceutical Co., Ltd. (Osaka).

Statistical analyses

The data are expressed as the mean ± S.E.M. for the number of experiments as indicated. Differences between groups in the recovery of LVDP, LVEDP and coronary flow rate were compared by one-way analysis of variance. Differences in CK release between groups were analyzed using the Kruskal-Wallis test. When this test indicated a significant difference, the data were further analyzed with
Dunnett’s multiple range test. Differences between groups were considered significant if the probability value was $P<0.05$.

RESULTS

Effects of FR183998 on functional recovery after normothermic and hypothermic ischemia

In both normothermic and hypothermic ischemia studies, treatment with FR183998 for 10 min did not show significant change from preischemic LVDP baseline values (Figs. 2A and 3A). Thus, there were no significant differences among the groups with respect to basal haemodynamic parameters. Also, heart rates were not significantly different among the control and FR183998-treated groups all through these protocols (242 ± 12, 243 ± 12 beats/min in the control and 3.2 ± 10$^{-7}$ M FR183998 group in the normothermic study and 261 ± 14, 246 ± 10 beats/min in the control and 3.2 ± 10$^{-7}$ M FR183998 group in the hypothermic study; each represents the value after 30 min of reperfusion).

The recovery of LVDP during 30 min of reperfusion after normothermic ischemia is shown in Fig. 2A. In the control group, reperfusion following 45 min of global ischemia resulted in poor recovery of LVDP. At 5 min of reperfusion, recovery of LVDP was 14 ± 6% of its preischemic value and gradually increased to 45 ± 7% by the end of reperfusion. Treatment with FR183998 before ischemia improved functional recovery in a concentration-dependent manner. Recoveries of LVDP after 30 min of reperfusion were significantly improved to 62 ± 2% and 76 ± 3% at concentrations of 10$^{-7}$ and 3.2 ± 10$^{-7}$ M, respectively.

LVEDP increased during normothermic ischemia in all groups, and there were no significant differences among these groups (Fig. 2B). In the control group, LVEDP markedly increased to 72 ± 13 mmHg after 5 min of reperfusion from 39 ± 1 mmHg at the end of ischemia, and it was slightly declined during the following reperfusion period. The increase of LVEDP after reperfusion was suppressed by FR183998 in a concentration-dependent manner. The reperfusion-induced increase of LVEDP was almost completely suppressed at a concentration of $3.2 \times 10^{-7}$ M (37 ± 9 mmHg after 5 min of reperfusion), and it was gradually decreased to the basal value. Statistically significant suppressions were obtained at $3.2 \times 10^{-7}$ M FR183998 after more than 10 min of reperfusion.
In the study of hypothermic ischemia, myocardial dysfunction was also observed after reperfusion in the control group. The recoveries of LVDP in the control group were $10 \pm 6\%$ and $51 \pm 3\%$, at 10 and 30 min of reperfusion, respectively. Treatment with FR183998 also improved recovery of LVDP in a concentration-dependent manner. The recoveries of LVDP at $3 \times 10^{-7} \text{M}$ FR183998 were $66 \pm 3\%$ and $74 \pm 3\%$, at 10 and 30 min of reperfusion, respectively, and these values were significantly higher than the control values (Fig. 3A).

During hypothermic global ischemia, ischemic contracture also occurred, and a further increase in LVEDP after reperfusion was observed in all groups. Treatment with FR183998 tended to reduce the rise in LVEDP during reperfusion, and the relevant figure (Fig. 3B) shows a significant reduction in LVEDP only at the 20-min reperfusion time point.

**Effects of FR183998 on CK leakage and coronary flow rate after hypothermic cardioplegic arrest**

Since the results shown above suggested that FR183998 could improve functional recovery of isolated rat hearts after not only normothermic but also hypothermic ischemia, we examined the effect of FR183998 in isolated rat hearts, with hypothermic cardioplegic arrest, to evaluate its effect under the condition as it is closer to that found in clinical cardiac surgery.

The CK leakages after hypothermic cardioplegic arrest are shown in Table 1. The CK leakage in the control group (cardioplegia alone) during the first 15 min of reperfusion was $18 \pm 2 \text{IU/15 min}$, and it was slightly declined during the following reperfusion period. The addition of FR183998 to cardioplegic solution decreased CK leakage in a concentration-dependent manner, and significant reductions of CK release were observed at concentrations of $3 \times 10^{-7}$ and $10^{-6} \text{M}$. Total CK leakage during 60 min of reperfusion in the control group was $46 \pm 6 \text{IU/60 min}$. On the other hand, it was significantly reduced to $31 \pm 3$ and $28 \pm 3 \text{IU/60 min}$ at $3 \times 10^{-7}$ and $10^{-6} \text{M}$ FR183998, respectively (Fig. 4).

Figure 5 shows the coronary flow during each 15-min period of reperfusion. In the control group, the coronary flow rate during the first 15 min was $5.8 \pm 0.3 \text{ml/min}$, which declined slightly during the following reperfusion period. In FR183998-treated groups, the coronary flow rates tended to be higher than in the control group throughout the reperfusion period. A significant difference was obtained during the first 15 min of reperfusion at $10^{-6} \text{M}$ FR183998.

**Table 1.** Creatine kinase (CK) release from reperfused rat hearts after hypothermic cardioplegic arrest

| Time of reperfusion | Control (n = 10) | $10^{-7} \text{M}$ (n = 11) | $3 \times 10^{-7} \text{M}$ (n = 11) | $10^{-6} \text{M}$ (n = 11) |
|---------------------|-----------------|--------------------------|-------------------------------|--------------------------|
| 0 – 15 min          | 18.3 ± 1.6      | 17.6 ± 2.0               | 17.3 ± 1.9                    | 16.9 ± 1.5               |
| 15 – 30 min         | 11.0 ± 1.3      | 7.8 ± 1.3                | 8.1 ± 1.5                     | 6.8 ± 1.1                |
| 30 – 45 min         | 9.5 ± 1.9       | 5.9 ± 1.6                | 3.3 ± 0.8**                   | 3.3 ± 0.8**              |
| 45 – 60 min         | 7.4 ± 2.4       | 4.6 ± 1.7                | 1.9 ± 0.6**                   | 1.4 ± 0.3**              |

Values are expressed as the mean ± S.E.M. of 10 or 11 experiments. **$P<0.01$, compared with the control.**
DISCUSSION

In this study, we have investigated the effect of FR183998 on myocardial injury after various ischemic conditions in isolated rat hearts. FR183998 improved post-ischemic functional recovery after hypothermic as well as normothermic ischemia. Furthermore, the addition of FR183998 to hypothermic cardioplegia significantly reduced CK leakage from isolated hearts during the reperfusion period, suggesting that a protective effect of FR183998 against myocardial injury was also obtained, even after hypothermic cardioplegic arrest.

It is well known that Na\(^{+}\)/H\(^{+}\) exchange is an important regulator of intracellular pH during ischemia, causing reperfusion injury due to intracellular Na\(^{+}\) and Ca\(^{2+}\)-overload (3, 18). Although a number of studies have shown that Na\(^{+}\)/H\(^{+}\) exchange inhibition provides marked cardioprotective effects against cardiac arrhythmias, myocardial stunning and infarction (7–11, 19), few studies have reported its effect on functional recovery after both normothermic and hypothermic ischemia (16). FR183998 has been shown to have a more potent inhibitory effect on Na\(^{+}\)/H\(^{+}\) exchange than FR168888 and exerts markedly protective effects against the incidence of arrhythmias and myocardial infarction induced by ischemia and reperfusion in anesthetized rats (15). Thus in this study, we investigated the effect of FR183998 on cardiac dysfunction in isolated rat hearts after global ischemia at normal and low temperatures. To further characterize the cardioprotective effect of FR183998, a second study of hypothermic cardioplegic arrest was undertaken, as this condition is more likely to occur in clinical situations.

FR183998 improved the recovery of LVDP after 45 min of normothermic ischemia in a concentration-dependent manner. Significant decreases in LVEDP were also observed in FR183998-treated groups. These results are in agreement with other studies, showing that amiloride analogues improved developed tension and decreased resting tension during reperfusion in isolated rat hearts (8), and that HOE694 attenuated the post-ischemic contractile dysfunction and increase of LVEDP during reperfusion in isolated rabbit hearts (20). Furthermore under hypothermic ischemic conditions, FR183998 produced significantly greater recoveries of LVDP during reperfusion and LVEDP tended to be lower in FR183998-treated groups than in the control group. In terms of hypothermic ischemia, most studies have used cardioplegic solution to obtain an improved recovery of post-ischemic function, and inhibition of Na\(^{+}\)/H\(^{+}\) exchange afforded more protective effects on functional recovery (16, 21). However Askenasy et al. have shown that administration of amiloride during hypothermic ischemia in isolated rat hearts perfused with Krebs-Henseleit solution resulted in a deterioration of post-

![Fig. 4. Effects of FR183998 on creatine kinase (CK) leakage during 60 min of reperfusion after 3 h of hypothermic global ischemia with cardioplegia in isolated rat hearts. FR183998 treatment was performed during the ischemic period. Values are the means ± S.E.M. (n = 10 or 11). *P<0.05, compared with the control group.](image1)

![Fig. 5. Coronary flow rates during 60 min of reperfusion after 3 h of hypothermic global ischemia with cardioplegia in isolated rat hearts. Coronary flow rates were calculated every 15 min of reperfusion. FR183998 treatment was performed during the ischemic period. Values are the means ± S.E.M. (n = 10 or 11). *P<0.05, compared with the control group.](image2)
Snabaitis and Chambers have demonstrated that amiloride significantly reduced intracellular Na⁺ thoroughly. Even under hypothermia, Na⁺ hypothermic cardioplegia have not been investigated as on not only cardiac function but also increase of coronary flow rate compared to the cardioplegia control group. These results agree with previous studies using cardioplegia at low temperature. Yamauchi et al. showed that pretreatment with FR168888 enhanced the recovery of ventricular function during reperfusion in isolated rat hearts subjected to 5 h of hypothermic ischemia following cardioplegic arrest (16). Kupriyanov et al. also reported that addition of amiloride caused a significant increase in pressure-rate product in isolated pig hearts arrested by hyperkalemic buffer followed by 15 h of cold preservation (10°C) (21). In the study by Kupriyanov et al., the perfusion pressure remained lower in the amiloride-treated group, and they speculated that the inhibition of Na⁺/H⁺ exchange by amiloride could reduce the increase of intracellular Na⁺ and subsequent Ca²⁺ entry by Na⁺/Ca²⁺ exchanger, which may be associated with the protective effects both in cardiomyocytes and vascular cells. In our study, an increase of coronary flow was also observed in the FR183998-treated groups. Although precise mechanisms were not elucidated in this study and further investigations are required, these results suggest that there is an additional protective effect of Na⁺/H⁺ exchange inhibition on not only cardiac function but also increase of coronary flow after hypothermic cardioplegic arrest.

The effects of inhibition of Na⁺/H⁺ exchange during hypothermic cardioplegia have not been investigated as thoroughly. Even under hypothermia, Na⁺/H⁺ exchange is still active, although its activity is attenuated (16). Inhibition of Na⁺/H⁺ exchange with amiloride or ethylisopropyl amiloride significantly reduced intracellular Na⁺ and water accumulation in rat hearts during storage at 4°C for 12 h (22). Snabaitis and Chambers have demonstrated that HOE694 showed additional protective effects compared with tetrodotoxin (TTX) alone in the study of TTX-induced cardioprotection against post-ischemic function and CK leakage in rat hearts subjected to 8 h of preservation at 7.5°C (23). Furthermore combination of TTX, HOE694, furosemide (an inhibitor of Na⁺/K⁺/2Cl⁻ cotransporter) and 2,3-butanedione monoxide (a calcium desensitizer) exerted further protective effects. They concluded that each component can act additively to have beneficial effects against myocardial injury related to Na⁺ and Ca²⁺ overload under these conditions. Based on these studies, it is suggested that activation of Na⁺/H⁺ exchange contributes, at least partially, to postischemic injury under hypothermic ischemia, and inhibition of this antipporter would show beneficial effects likewise after hypothermic cardioplegic arrest.

In conclusion, FR183998 has potent cardioprotective effects in isolated perfused rat hearts subjected to hypothermic as well as normothermic ischemia. Addition of FR183998 in cardioplegia exerted further protective effects likewise after hypothermic cardioplegic arrest. Although these models can not be directly extrapolated to the clinical situation, these results suggest that pharmacological inhibition with FR183998 may be practical for clinical use as a therapeutic agent in cardiac surgery and heart preservation.

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