J. Pharm. Pharmacol. 1999, 51: 1049–1058
Received November 13, 1998
Accepted March 10, 1999

Inhibition of Na^+ Channel or Na^+/H^+ Exchanger Attenuates the Hydrogen Peroxide-induced Derangements in Isolated Perfused Rat Heart

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Abstract

The effect of tetrodotoxin, a specific inhibitor of the Na^+ channel, and 5-(*N*,*N*-dimethyl)-amiloride, a specific inhibitor of the Na^+/H^+ exchanger, on the mechanical and metabolic derangements induced by hydrogen peroxide (H_2O_2) was studied in the isolated perfused rat heart. The isolated rat heart was perfused aerobically at a constant flow rate and driven electrically.

H_2O_2 (600 μM) decreased the left ventricular developed pressure and increased the left ventricular end-diastolic pressure (i.e. mechanical dysfunction), decreased the tissue levels of adenosine triphosphate and adenosine diphosphate (i.e. metabolic derangement), and increased the tissue level of malondialdehyde (i.e. lipid peroxidation). These mechanical and metabolic derangements induced by H_2O_2 were significantly attenuated by tetrodotoxin (3 μM) or 5-(*N*,*N*-dimethyl)-amiloride (15 μM). Neither tetrodotoxin nor 5-(*N*,*N*-dimethyl)-amiloride modified the tissue malondialdehyde level, which was increased by H_2O_2. In the normal (H_2O_2-untreated) heart, neither tetrodotoxin nor 5-(*N*,*N*-dimethyl)-amiloride affected the mechanical function and energy metabolism.

These results suggested that inhibition of the Na^+ channel or Na^+/H^+ exchanger was effective in attenuating the H_2O_2-induced mechanical dysfunction and metabolic derangements in the isolated perfused rat heart.

It has been generally accepted that the primary mechanism of action of anti-anginal drugs is improvement of the myocardial oxygen balance between supply and demand by either an increase in coronary flow or a decrease in cardiac mechanical function, or both. Therefore, nitrates, β-adrenoceptor antagonists and Ca^{2+}-channel inhibitors have been used widely for treatment of patients with ischaemic heart disease. The beneficial action of inhibitors of the Na^+ channel, such as tetrodotoxin (Duff et al 1988) and lidocaine (Okamura et al 1982; Takeo et al 1988; Nakamura et al 1989), and that of inhibitors of the Na^+/H^+ exchanger, such as amiloride (Nasa et al 1997) and its derivative (Dennis et al 1990; Meng & Pierce 1990; Maddaford & Pierce 1997), on the ischaemia–reperfused heart have been demonstrated in animal models. Interestingly, inhibitors of the Na^+ channel (Okamura et al 1982; Duff et al 1988; Takeo et al 1988) and those of the Na^+/H^+ exchanger (Dennis et al 1990; Meng & Pierce 1990; Nasa et al 1997) are effective in attenuating ischaemia–reperfusion damage, even when they do not exert a significant effect on haemodynamics. Therefore, the protective action of these inhibitors may not be due to improvement of the myocardial oxygen balance induced by haemodynamic effects, but possibly due to inhibition of intracellular Na^+ accumulation during ischaemia (Pike et al 1993; Van Emous et al 1997). Nevertheless, the detailed mechanism of the protective action of inhibitors of the Na^+ channel and Na^+/H^+ exchanger on the ischaemia–reperfusion damage remains unclear.

During ischaemia and reperfusion, reactive oxygen species, such as superoxide anion, hydroxyl radical, hydrogen peroxide (H_2O_2) and singlet oxygen, are generated intra- and extracellularly in

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the myocardium and endothelium (Hess & Manson 1984; Lucchesi 1990; Loesser et al 1991). These reactive oxygen species cause lipid peroxidation of the cell membrane and myocardial derangements including mechanical dysfunction and energy deficiency (Hess & Manson 1984; Nakaya et al 1987; Loesser et al 1991). Therefore, reactive oxygen species are an important factor inducing myocardial damage during ischaemia and reperfusion. We (Hara et al 1993; Hara & Abiko 1996) have demonstrated that treatment of the isolated perfused rat heart with H_2O_2 inflicts severe damage on the mechanical function and energy metabolism, including lipid peroxidation. According to the results of a study using nuclear magnetic resonance (Yanagida et al 1995), H_2O_2 increases intracellular Na^+ concentration in the myocardium. Kim & Akera (1987) reported that the H_2O_2-induced increase in intracellular Na^+ concentration may be due, in part, to the inhibition of the sarcolemmal Na^+,K^+ATPase. An increase in intracellular Na^+ concentration could cause intracellular Ca^{2+} overload through the Na^+/Ca^{2+} exchanger, and hence myocardial derangements (Goldhaber 1996). Therefore, inhibitors of the Na^+ channel and Na^+/H^+ exchanger may be effective in attenuating H_2O_2-induced myocardial derangements via inhibition of Na^+ entry into the myocardial cell.

There is no report, however, on the action of Na^+-channel inhibitors Na^+/H^+-exchange inhibitors against the H_2O_2-induced myocardial damage. In addition, there is no report on whether the cardioprotective effects of these drugs are based on inhibition of lipid peroxidation in the myocardium. This study examined whether a Na^+-channel inhibitor or a Na^+/H^+-exchange inhibitor protects the H_2O_2-induced derangements in both mechanical function and energy metabolism and attenuates the H_2O_2-induced lipid peroxidation. We chose tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride as a specific Na^+-channel inhibitor and a specific Na^+/H^+-exchange inhibitor, respectively, because tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride may attenuate the H_2O_2-induced increase in intracellular concentration of Na^+ by different mechanisms.

Materials and Methods

Heart perfusion

The Asahikawa Medical College Committee on Animal Research approved the protocol of animal experiments in this study. Male Sprague–Dawley rats (9–10-weeks old; Sankyo Labo Service Corporation, Sapporo) were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.) 20 min after an injection with heparin (1000 units kg^{-1}, i.p.). After thoracotomy, the hearts were rapidly removed, and then retrograde perfusion was started using a cannula inserted into the aorta according to the Langendorff technique. The perfusion buffer was a Krebs–Henseleit bicarbonate (KHB) buffer containing (mM) NaCl 118, KCl 4·7, KH_2PO_4 1·2, $MgSO_4$ 1·2, $CaCl_2$ 2·5, $NaHCO_3$ 25 and glucose 11, equilibrated with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. The oxygen tension of the buffer measured by a blood gas analyser (Model 813, Instrumentation Laboratory, Lexington, MA) was about 550 mmHg. The hearts were initially perfused at a constant perfusion pressure of 80 cm H_2O. About 10 min after constant pressure perfusion, perfusion was switched to the constant flow perfusion (10 mL min^{-1}) using a microtube pump (Eyela MP-A, Tokyo-Rikakikai Instruments, Tokyo, Japan), and this flow rate was maintained throughout the experiment. The heart rate of spontaneously beating hearts was about 270 beats min^{-1}, but it was kept constant by pacing the heart at 300 beats min^{-1} with an electronic stimulator (3F46, San-Ei Instruments, Tokyo, Japan) during the course of the study. Rectangular pulses having 2-ms duration with a voltage of 6 V (about 3-times the threshold voltage) were applied to the left ventricle for this pacing.

As indices of mechanical function, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and left ventricular developed pressure (LVDP) were employed. The values of LVSP and LVEDP were determined from the left ventricular pressure (LVP) curves recorded continuously during the course of the study, and the LVDP value was calculated as LVSP minus LVEDP. For measurement of LVP, a saline-filled polyethylene cannula, connected to a pressure transducer, was inserted into the left ventricular cavity via the left atrium. Another pressure transducer was connected to the aortic cannula for the recording of coronary perfusion pressure. Before the start of each experiment, the heart was allowed to stabilize for 20 min under the conditions of constant flow perfusion.

Experimental protocol

The heart was perfused with KHB buffer at a constant flow throughout the whole course of the experiments (stabilization period for 20 min and observation period for 38 min). During the observation period, H_2O_2 and/or a drug (tetrodotoxin or 5-(*N*,*N*-dimethyl)-amiloride) were infused into the aortic cannula in which KHB buffer flowed. The

effect of tetrodotoxin or 5-(*N*,*N*-dimethyl)-amiloride on the mechanical function and energy metabolism in both H_2O_2-treated and H_2O_2-untreated (normal) hearts was examined. In the H_2O_2-treated heart experiments, the hearts were divided into five groups; vehicle, tetrodotoxin 1 μM, tetrodotoxin 3 μM, 5-(*N*,*N*-dimethyl)-amiloride 5 μM, and 5-(*N*,*N*-dimethyl)-amiloride 15 μM groups. In these groups, tetrodotoxin, 5-(*N*,*N*-dimethyl)-amiloride or vehicle (KHB buffer) was infused into the aortic cannula for 38 min at a constant flow rate of 0·1 mL min^{-1}. H_2O_2 was infused into the aortic cannula at the constant flow rate of 0·1 mL min^{-1} for 3 min from 5 min after the start of infusion of tetrodotoxin, 5-(*N*,*N*-dimethyl)-amiloride or vehicle. In the normal heart experiments, the hearts were divided into five groups; vehicle, tetrodotoxin 1 μM, tetrodotoxin 3 μM, 5-(*N*,*N*-dimethyl)-amiloride 5 μM, and 5-(*N*,*N*-dimethyl)-amiloride 15 μM groups. The experimental conditions and protocol in the normal heart experiments were essentially the same as those in the H_2O_2-treated heart experiments, except for an infusion of saline solution instead of H_2O_2 solution. In each group, LVSP, LVEDP and coronary perfusion pressure were continuously recorded over a 38-min observation period. To measure the tissue levels of high-energy phosphates, the heart was frozen at the end of the observation period (38 min after the start of infusion of tetrodotoxin, 5-(*N*,*N*-dimethyl)-amiloride or vehicle) with freezing clamps previously chilled in liquid nitrogen.

Some hearts in the vehicle group in the normal heart experiments and those in the vehicle, tetrodotoxin 3 μM and 5-(*N*,*N*-dimethyl)-amiloride 15 μM groups in the H_2O_2 heart experiments were frozen 10 min after the end of H_2O_2 infusion (18 min after the start of infusion of tetrodotoxin, 5-(*N*,*N*-dimethyl)-amiloride or vehicle) for measurement of the tissue level of malondialdehyde.

Biochemical analysis

The myocardial samples were stored in liquid nitrogen (−196°C) until the biochemical analysis was performed. Each frozen sample was pulverized in a mortar cooled with liquid nitrogen. Part of the pulverized tissue powder was weighed and put into an oven overnight to enable measurement of the tissue water content and dry weight of the tissue. The remainder of the tissue powder was used for determination of tissue levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), creatine phosphate and malondialdehyde. ATP, ADP, AMP and creatine phosphate were measured according to standard enzymatic procedures (Bergmeyer 1974). Malondialdehyde, a product of lipid peroxidation, was measured using HPLC instead of using thiobarbituric acid, because using the latter method may measure not only malondialdehyde but also other substances (Ceconi et al 1991). The HPLC procedure was performed according to the method of Koller & Bergmann (1989). Before measurement of the tissue malondialdehyde level, we confirmed that tetrodotoxin or 5-(*N*,*N*-dimethyl)-amiloride did not interfere with measurement of malondialdehyde. The procedure for measurements of energy metabolites and malondialdehyde was as described previously (Hara et al 1993; Hara & Abiko 1996).

Drugs

Tetrodotoxin (Sankyo Co., Ltd, Tokyo, Japan) and 5-(*N*,*N*-dimethyl)-amiloride (Research Biochemicals, Natick, MA) were dissolved in KHB buffer solution. H_2O_2 (Nacalai Tesque Ltd, Kyoto, Japan) was diluted with saline solution. Tetrodotoxin, 5-(*N*,*N*-dimethyl)-amiloride or vehicle (KHB buffer) solution was infused at a flow rate of 0·1 mL min^{-1} using an infusion pump into the inflow tube connected to the side arm of the aortic cannula. 5-(*N*,*N*-Dimethyl)-amiloride is unstable in white light so the drug solution was prepared under the light of a sodium lamp. In addition, the inflow tube and a syringe for drug infusion were shielded from light by an aluminum cover throughout the experiment. H_2O_2 or saline solution was also infused to the inflow tube at a flow rate of 0·1 mL min^{-1} using another infusion pump. The final concentration of tetrodotoxin in the perfusate (KHB buffer perfused at a flow rate of 10 mL min^{-1} plus tetrodotoxin solution infused at a flow rate of 0·1 mL min^{-1}) was set to 1 or 3 μM. Nevertheless, the concentration of tetrodotoxin changed from 1 to 0·99 μM or 3 to 2·97 μM when H_2O_2 was added to the perfusate, because total flow rate of perfusate increased from 10·1 to 10·2 mL min^{-1} during the H_2O_2 infusion. 5-(*N*,*N*-Dimethyl)-amiloride was also infused into the aortic cannula at a flow rate of 0·1 mL min^{-1} so that the final concentration of 5-(*N*,*N*-dimethyl)-amiloride in the perfusate was 5 or 15 μM, although the concentration changed from 5 to 4·95 μM or 15 to 14·85 μM during the infusion of H_2O_2. These small changes in the concentration of tetrodotoxin or 5-(*N*,*N*-dimethyl)-amiloride were about 1% and did not affect LVP. The final concentration of H_2O_2 in the perfusate including infusion solution was 600 μM. The reagents and enzymes used for biochemical analysis were purchased from Sigma Chemical Company (St Louis,

MO) or Aldrich Chemical Company (Milwaukee, WI).

Statistical analysis

All values are expressed as mean ± s.e.m. When data of mechanical function and coronary perfusion pressure were compared between vehicle-treated and drug-treated groups, statistical analysis was performed with a two-way repeated measures analysis of variance followed by Dunnett's test for multiple comparison (Figures 1, 2, 3 and 4). When metabolic data in the vehicle group were compared between H_2O_2-treated and normal (H_2O_2-untreated) groups, unpaired Student's *t*-test was employed (Figure 5). When other biochemical data were compared between vehicle-treated and drug-treated groups, statistical analysis was performed with a one-way analysis of variance followed by Dunnett's test for multiple comparison (Figure 5 and Table 1). A difference was considered statistically significant at $P < 0{\cdot}05$.

Results

*Effects of tetrodotoxin and 5-(*N,N *-dimethyl)-amiloride on H_2O_2-induced changes in mechanical function and coronary resistance*

In the normal (H_2O_2-untreated) heart, tetrodotoxin (1 and 3 μM) and 5-(*N*,*N*-dimethyl)-amiloride (5 and 15 μM) did not have any effect on the LVSP ($P > 0{\cdot}05$), LVEDP ($P > 0{\cdot}05$) or coronary perfusion pressure ($P > 0{\cdot}05$) during the whole course of the study (data not shown). Figures 1 and 2 show the effects of tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride, respectively, on the H_2O_2-induced changes in LVSP and LVEDP. Before starting the H_2O_2 infusion, there was no significant difference in the values of LVSP and LVEDP between the vehicle and tetrodotoxin groups and between the vehicle and 5-(*N*,*N*-dimethyl)-amiloride groups. In the vehicle group, H_2O_2 decreased LVSP temporarily but markedly ($P < 0{\cdot}001$). The LVSP, which had been decreased by H_2O_2, increased after the end of H_2O_2 infusion and returned to the initial level. H_2O_2 also increased LVEDP markedly ($P < 0{\cdot}001$), and the increase in LVEDP was accompanied by an increase in LVSP. The increase in LVEDP induced by H_2O_2 was prevented almost completely by tetrodotoxin (3 μM) or 5-(*N*,*N*-dimethyl)-amiloride (15 μM) ($P < 0{\cdot}05$ compared with the vehicle group). A low concentration of tetrodotoxin (1 μM) also attenuated the H_2O_2-induced increase in LVEDP ($P < 0{\cdot}05$ compared with the vehicle group). However, a low concentration of 5-(*N*,*N*-dimethyl)-amiloride (5 μM) was actually ineffective in attenuating the H_2O_2-induced increase in LVEDP.

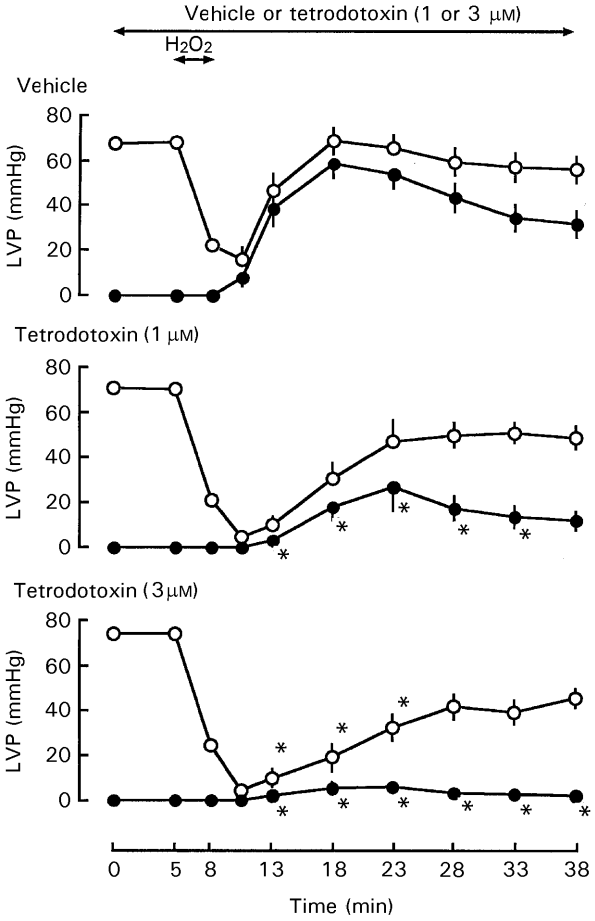

Figure 1. Effect of tetrodotoxin (1 and 3 μM) on the hydrogen peroxide (H_2O_2)-induced changes in left ventricular pressure (LVP). The changes in left ventricular systolic pressure (○) and left ventricular end-diastolic pressure (●) in the vehicle, tetrodotoxin 1 μM and 3 μM groups are recorded. Each value represents mean ± s.e.m. of 7–19 hearts. $^*P < 0{\cdot}05$ when compared with the vehicle group.

Figure 3 shows the effects of tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride on the H_2O_2-induced changes in LVDP. Before starting the H_2O_2 infusion, there was no significant difference in the LVDP value between the vehicle and tetrodotoxin or 5-(*N*,*N*-dimethyl)-amiloride groups. In the vehicle group, H_2O_2 decreased LVDP markedly ($P < 0{\cdot}001$). The decrease in LVDP induced by H_2O_2 was attenuated by tetrodotoxin (3 μM) or 5-(*N*,*N*-dimethyl)-amiloride (15 μM). Nevertheless, a low concentration of tetrodotoxin (1 μM) or 5-(*N*,*N*-dimethyl)-amiloride (5 μM) was actually ineffective in attenuating the H_2O_2-induced decrease in LVDP. These results indicated that tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride attenuated the mechanical dysfunction induced by H_2O_2.

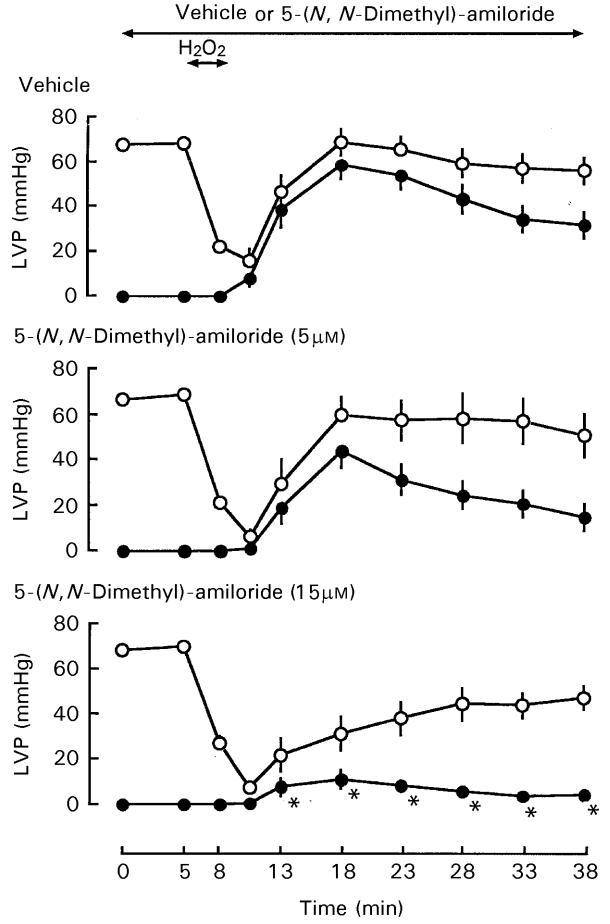

Figure 2. Effect of 5-(*N*,*N*-dimethyl)-amiloride (5 and 15 μM) on the H_2O_2-induced changes in left ventricular pressure (LVP). The changes of left ventricular systolic pressure (○) and left ventricular end-diastolic pressure (●) in the vehicle, 5-(*N*,*N*-dimethyl)-amiloride 5 μM and 15 μM groups are recorded. Hearts in the vehicle group are those in Figure 1. Each value represents mean ± s.e.m. of 9–19 hearts. $^*P < 0.05$ when compared with the vehicle group.

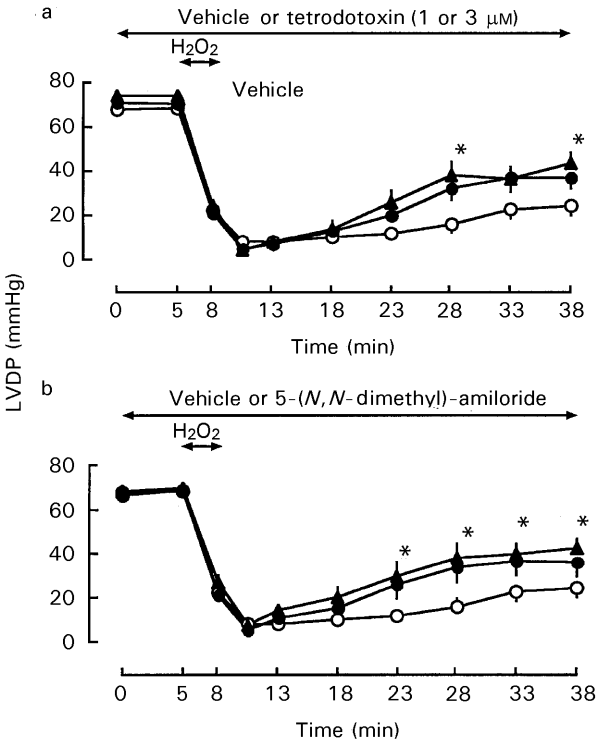

Figure 3. Effect of tetrodotoxin or 5-(*N*,*N*-dimethyl)-amiloride on the H_2O_2-induced changes in left ventricular developed pressure (LVDP). a. The changes in LVDP in the vehicle (○), tetrodotoxin 1 μM (●) and tetrodotoxin 3 μM (▲) groups. b. The changes of LVDP in the vehicle (○), 5-(*N*,*N*-dimethyl)-amiloride 5 μM (●) and 5-(*N*,*N*-dimethyl)-amiloride 15 μM (▲) groups. Values were calculated from the values of left ventricular systolic pressure and left ventricular end-diastolic pressure in Figures 1 and 2. Hearts in the vehicle group are the same hearts for a and b. Each value represents mean ± s.e.m. of 7–19 hearts. $^*P < 0.05$ when compared with the vehicle group.

Figure 4 shows the effects of tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride on the H_2O_2-induced changes in coronary perfusion pressure. Before starting the H_2O_2 infusion, there was no significant difference in the coronary perfusion pressure value between the vehicle and tetrodotoxin or 5-(*N*,*N*-dimethyl)-amiloride groups. In the vehicle group, H_2O_2 induced a transient and slight decrease in coronary perfusion pressure followed by a continuous and marked increase ($P < 0.001$). The increase in coronary perfusion pressure induced by H_2O_2 was attenuated by tetrodotoxin (3 μM) or 5-(*N*,*N*-dimethyl)-amiloride (15 μM) ($P < 0.05$ compared with the vehicle group). Nevertheless, a low concentration of tetrodotoxin (1 μM) or 5-(*N*,*N*-dimethyl)-amiloride (5 μM) did not attenuate the H_2O_2-induced increase in coronary perfusion pressure, except for 10·5 to 18 min after starting the tetrodotoxin (1 μM) infusion. These results indicated that tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride attenuated the increase in coronary perfusion pressure, and hence coronary resistance (because the perfusion flow was fixed) induced by H_2O_2.

*Effects of tetrodotoxin and 5-(*N ,N *-dimethyl)-amiloride on H_2O_2-induced changes in energy metabolism*

Figure 5 shows the tissue levels of ATP, ADP, AMP and creatine phosphate at the end of experiment in the normal (H_2O_2-untreated) and H_2O_2-treated hearts. In the normal (H_2O_2-untreated) heart, the tissue levels of ATP, ADP, AMP and creatine phosphate were similar between the vehicle and each of the tetrodotoxin or 5-(*N*,*N*-dimethyl)-amiloride groups. In the H_2O_2-treated heart, H_2O_2 decreased the tissue levels of ATP, ADP and creatine phosphate ($P < 0.05$ between the normal and H_2O_2-treated groups), and increased the tissue level of AMP ($P < 0.05$ between the normal and

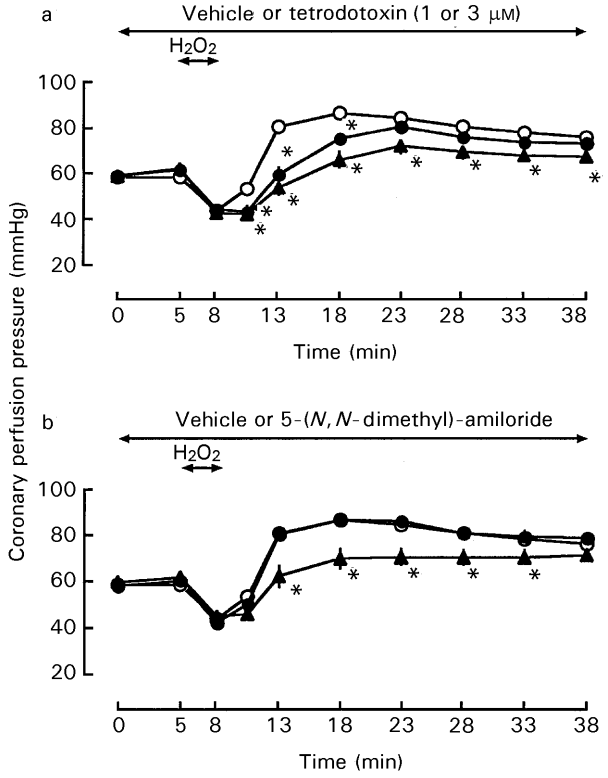

Figure 4. Effect of tetrodotoxin or 5-(*N*,*N*-dimethyl)-amiloride on the H_2O_2-induced changes in coronary perfusion pressure. a. The changes in coronary perfusion pressure in the vehicle (○), tetrodotoxin 1 μM (●) and tetrodotoxin 3 μM (▲) groups. b. The changes of coronary perfusion pressure in the vehicle (○), 5-(*N*,*N*-dimethyl)-amiloride 5 μM (●) and 5-(*N*,*N*-dimethyl)-amiloride 15 μM (▲) groups. Hearts were those in Figures 1 and 2. Hearts in the vehicle group are the same hearts for a and b. Each value represents mean ± s.e.m. of 7–19 hearts. $*P < 0.05$ when compared with the vehicle group.

H_2O_2-treated groups). These changes of the tissue levels of energy metabolites induced by H_2O_2, however, were attenuated significantly by tetrodotoxin (3 μM) or 5-(*N*,*N*-dimethyl)-amiloride (15 μM) ($P < 0.05$ compared with the vehicle group). Nevertheless, a low concentration of tetrodotoxin (1 μM) or 5-(*N*,*N*-dimethyl)-amiloride (5 μM) failed to attenuate the H_2O_2-induced decrease in the tissue levels of ATP, ADP and creatine phosphate, although they attenuated the H_2O_2-induced increase in the tissue level of AMP. These results indicated that tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride attenuated the metabolic derangements induced by H_2O_2.

*Effects of tetrodotoxin and 5-(*N,N*-dimethyl)-amiloride on H_2O_2-induced lipid peroxidation*

There is a possibility that the beneficial effects of tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride on the H_2O_2-induced derangements relate to the reduction of lipid peroxidation that could be increased by H_2O_2. To examine this possibility, the tissue level of malondialdehyde was measured in the normal (H_2O_2-untreated), vehicle, tetrodotoxin 3 μM, and 5-(*N*,*N*-dimethyl)-amiloride 15 μM groups (Table 1). Previously, we reported (Hara et al 1993) that the tissue level of malondialdehyde, which had been increased by H_2O_2, decreased by prolongation of the period of heart perfusion after the end of H_2O_2 infusion, because malondialdehyde can be washed out into myocardial interstitial effluent (Koster et al 1985). Therefore, we measured the tissue malondialdehyde level shortly after the end of H_2O_2 infusion; 10 min was used as it corresponds to the time when H_2O_2 produced a marked increase in LVEDP and when the increase in LVEDP was inhibited by tetrodotoxin (3 μM) and 5-(*N*,*N*-dimethyl)-amiloride (15 μM). In the vehicle group, H_2O_2 increased the tissue malondialdehyde level by about three times the value in the normal group ($P < 0.05$). There was, however, no statistical difference in the tissue level of malondialdehyde between vehicle and tetrodotoxin 3 μM or between vehicle and 5-(*N*,*N*-dimethyl)-amiloride 15 μM groups. These results indicated that tetrodotoxin or 5-(*N*,*N*-dimethyl)-amiloride did not reduce the lipid peroxidation induced by H_2O_2.

Discussion

In this study, we used H_2O_2 as a reactive oxygen species for three reasons. Firstly, H_2O_2 and its metabolite, hydroxyl radical, are considered important in the pathogenesis of myocardial damage induced by ischaemia–reperfusion (Brown et al 1988; Loesser et al 1991). Secondly, H_2O_2 penetrates the cell membrane and reaches the intracellular site (Fisher 1988), and therefore it may cause severe damage to the cell. Thirdly, 600 μM H_2O_2 can inflict damage to the heart to a degree

Table 1. Effect of tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride on hydrogen peroxide (H_2O_2)-induced changes of the tissue level of malondialdehyde.

Group	n	Tissue malondialdehyde level (nmol g^{-1} dry weight)
Normal (H_2O_2-untreated)	8	9·9 ± 1·4*
Vehicle	12	29·7 ± 2·7
Tetrodotoxin (3 μM)	7	32·7 ± 3·2
5-(*N*,*N*-Dimethyl)-amiloride (15 μM)	8	36·5 ± 3·8

Each value represents the mean ± s.e.m. n represents the number of hearts. The tissue level of malondialdehyde was measured 10 min after the end of H_2O_2 infusion. The final concentrations of tetrodotoxin, 5-(*N*,*N*-dimethyl)-amiloride and H_2O_2 in the perfusate were 3, 15 and 600 μM, respectively. $*P < 0.05$ when compared with vehicle group.

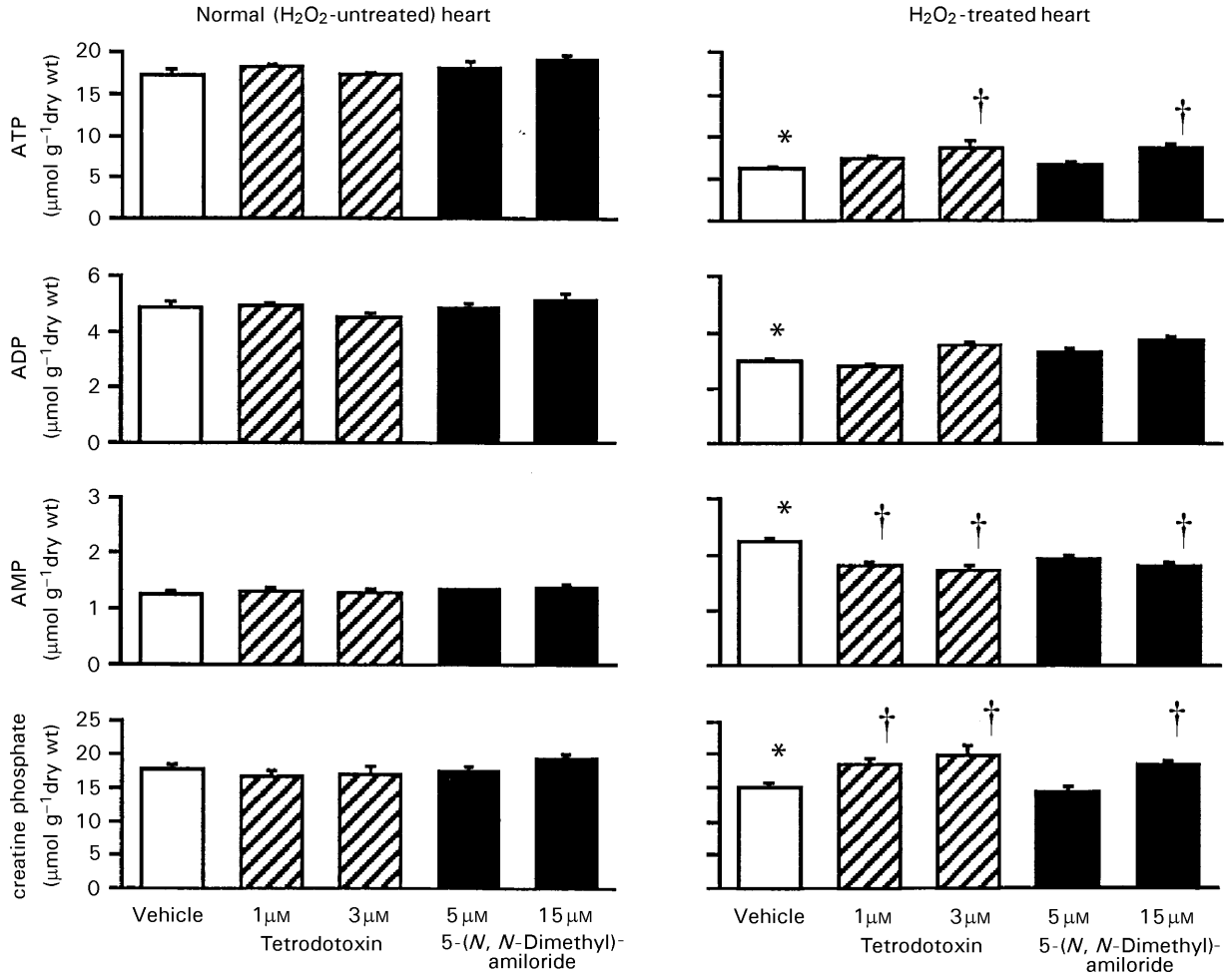

Figure 5. Effect of tetrodotoxin (1 and 3 μM) or 5-(*N*,*N*-dimethyl)-amiloride (5 and 15 μM) on the H_2O_2-induced changes in the tissue levels of high-energy phosphates. The tissue levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and creatine phosphate at the end of experiment (38 min after the start of tetrodotoxin, 5-(*N*,*N*-dimethyl)-amiloride or vehicle infusion) were measured in the normal (H_2O_2-untreated) and H_2O_2-treated hearts. Hearts in the H_2O_2-treated groups are those in Figures 1 and 2. Each value represents a mean ± s.e.m. of 5–7 hearts (the normal groups) and 7–19 hearts (the H_2O_2-treated groups). *$P < 0.05$ when compared with the corresponding normal group. †$P < 0.05$ when compared with the vehicle group in the H_2O_2-treated heart.

similar to that induced by ischaemia–reperfusion in terms of accumulation of lipid peroxides (Hara et al 1993). In this study, H_2O_2 produced mechanical dysfunction (as evidenced by an increase in LVEDP and a decrease in LVDP), an increase in coronary resistance (as evidenced by an increase in coronary perfusion pressure), and metabolic changes (as evidenced by a decrease in the tissue levels of ATP and ADP and an increase in the tissue level of AMP). These alterations induced by H_2O_2 were significantly attenuated by tetrodotoxin (3 μM) or 5-(*N*,*N*-dimethyl)-amiloride (15 μM), suggesting that both drugs have a beneficial effect against the H_2O_2-induced mechanical and metabolic derangements in the heart. The beneficial effect of tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride was observed on the H_2O_2-induced mechanical dysfunction in terms of recovery of LVEDP and LVDP but not LVSP, suggesting that both drugs improved the ability of the heart to relax. Cardiac contraction and relaxation are under control of the sarcoplasmic reticulum; contraction from release of Ca^{2+} from the sarcoplasmic reticulum and relaxation from its uptake of Ca^{2+}. H_2O_2 inhibits the Ca^{2+} uptake (Rowe et al 1983) and so it impairs sarcoplasmic reticulum function and hence causes mechanical dysfunction of the heart. The action of tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride on cardiac contraction and relaxation may be due to a reduction of Ca^{2+} entry into myocytes, which is a conceivable consequence of inhibition of Na^+ entry into myocytes.

The concentrations of tetrodotoxin (1 and 3 μM) and 5-(*N*,*N*-dimethyl)-amiloride (5 and 15 μM) used

did not modify mechanical function in the normal (H_2O_2-untreated) heart. These agents did not change coronary flow, because the hearts were perfused at a constant flow rate. It is unlikely, therefore, that the protective effects of tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride on the H_2O_2-induced myocardial derangements were due to preservation of energy caused by favourable action of these drugs on haemodynamics i.e. an energy-sparing effect. In fact, neither tetrodotoxin nor 5-(*N*,*N*-dimethyl)-amiloride had any effect on the tissue levels of high-energy phosphates in the normal, H_2O_2-untreated, heart.

Some radical scavengers or antioxidants have been demonstrated to protect the myocardium against oxidative stress (Nakaya et al 1987; Koller & Bergmann 1989). We have reported that in the isolated perfused rat heart, catalase, an H_2O_2 scavenger (Hara et al 1993), or propofol, an intravenous anaesthetic with an antioxidant action (Kokita & Hara 1996), attenuates H_2O_2-induced mechanical and metabolic derangements and lipid peroxidation. In this study, however, neither tetrodotoxin nor 5-(*N*,*N*-dimethyl)-amiloride affected the H_2O_2-induced lipid peroxidation, suggesting that the protective effect of tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride was not due to a radical scavenging or an antioxidant effect.

Tetrodotoxin selectively inhibits the Na^+ channel without affecting the Ca^{2+} channel (Tytgat et al 1990) and Na^+/Ca^{2+} exchanger (Requena et al 1985). According to binding studies (Catterall & Coppersmith 1981; Rogart 1987), the concentrations of tetrodotoxin used in this study, which attenuated the H_2O_2-induced derangements (1 and 3 μM), were capable of inhibiting the Na^+ channel in the rat heart (dissociation constant = 0·3 to 1 μM). In addition, we have found that lidocaine (Hara et al 1993) and dilazep (Hara & Abiko 1996), both of which have an inhibitory action on the Na^+ channel, attenuate H_2O_2-induced myocardial derangements. Those results suggested that inhibition of the Na^+ channel is effective in attenuating H_2O_2-induced myocardial derangements. Electrophysiological studies have demonstrated that H_2O_2 increases the Na^+ current in ventricular myocytes, but the increase is blocked by tetrodotoxin (Bhatnagar et al 1990; Ward & Giles 1997). Ver Donck & Borgers (1991) have shown that inhibition of the Na^+ channel with tetrodotoxin attenuates hypercontraction induced by singlet oxygen in cardiomyoctes, supporting our findings of the beneficial action of Na^+ channel inhibitors on reactive oxygen species. 5-(*N*,*N*-Dimethyl)-amiloride (15 μM), like tetrodotoxin, attenuated H_2O_2-induced mechanical and metabolic derangements in the heart, although the low concentration of 5-(*N*,*N*-dimethyl)-amiloride (5 μM) did not. 5-(*N*,*N*-Dimethyl)-amiloride (15 μM) is capable of inhibiting the Na^+/H^+ exchanger in the heart (half-maximal effective concentration = 7 μM) without significant action on the Na^+ channel, Ca^{2+} channel, Na^+/Ca^{2+} exchanger or Na^+,K^+ ATPase (Kleyman & Cragoe 1988; Dennis et al 1990). Hoque & Karmazyn (1997) have shown that HOE-642 (4-isopropyl-3-methylsulphonylbenzoyl-guanidine methanesulphonate), another specific inhibitor of the Na^+/H^+ exchanger, attenuated H_2O_2-induced derangements in the isolated perfused heart. These findings indicated that inhibition of the Na^+/H^+ exchanger was effective in attenuating H_2O_2-induced myocardial derangements. We did not measure the intracellular concentrations of H^+ and Na^+ in the H_2O_2-treated heart in the present study. It is possible that intracellular concentrations of both H^+ and Na^+ increase after H_2O_2 administration, because in the cultured cardiac myocytes H_2O_2 induces a decrease in intracellular pH (Wu et al 1996) and stimulates the Na^+/H^+ exchanger via activation of mitogen-activated protein kinase to increase the intracellular Na^+ concentration (Sabri et al 1998).

The results of this study demonstrated that either tetrodotoxin (3 μM) alone or 5-(*N*,*N*-dimethyl)-amiloride (15 μM) alone attenuated the H_2O_2-induced increase in LVEDP almost completely. A possible explanation for this finding is that there is a threshold concentration for intracellular Na^+ to stimulate Ca^{2+} influx via the Na^+/Ca^{2+} exchanger as demonstrated by Mullins et al (1983), and that either tetrodotoxin alone or 5-(*N*,*N*-dimethyl)-amiloride alone can attenuate the H_2O_2-induced increase in intracellular Na^+ concentration to the threshold level. Interestingly, Eng et al (1998) demonstrated that either tetrodotoxin alone or HOE-642 alone attenuated the ischaemia–reperfusion damage in rat hearts and that there was no additive effect when these drugs were given simultaneously. The concentrations of tetrodotoxin (3 μM) and 5-(*N*,*N*-dimethyl)-amiloride (15 μM) needed to protect the H_2O_2-treated heart are high enough to attenuate myocardial derangements induced by ischaemia–reperfusion in the isolated perfused heart (Duff et al 1988; Dennis et al 1990; Meng & Pierce 1990; Maddaford & Pierce 1997). Therefore, it is possible that the beneficial action of inhibitors of the Na^+ channel and the Na^+/H^+ exchanger against the H_2O_2-induced changes contributes to their protective effect on the myocardium against ischaemia–reperfusion damage.

In conclusion, inhibition of Na^+ channel or Na^+/H^+ exchanger is effective in attenuating

H_2O_2-induced mechanical dysfunction and metabolic derangements in the isolated perfused rat heart. This action of both inhibitors may contribute to their protective action on the myocardium against ischaemia–reperfusion damage. Nevertheless, we did not examine the effects of tetrodotoxin and 5-(*N*,*N*-dimethyl)-amiloride on intracellular concentrations of Na^+ and Ca^{2+} in the H_2O_2-treated heart. Therefore, further studies are needed to determine detailed mechanisms of the protective action of inhibitors of the Na^+ channel and the Na^+/H^+ exchanger on the H_2O_2-induced derangements.

Acknowledgements

The authors thank Mr Tadahiko Yokoyama for his technical assistance, and Ms Miwa Kashu and Ms Kaori Inaba for their secretarial assistance. We also wish to thank Mr Simon Bayley for his help in preparing the manuscript. This research was supported by the grant provided by the Hokkaido Heart Association of Japan and NISHINOMIYA Basic Research Fund of Japan.

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