Effect of Hydrogen Peroxide on Intracellular pH in the Human Atrial Myocardium

Chao-Ming Chao[1], Jong-Shiaw Jin[2], Chien-Sung Tsai[3], Yin Tsai[4], Wei-Hwa Chen[5] Chao-Chin Chung[4] and Shih-Hurng Loh[4,*]

Departments of Dermatology[1], Pathology[2], Cardiovascular Surgery[3], Pharmacology[4] and Gynecology[5]
National Defense Medical Center
Taipei, Taiwan, Republic of China

Abstract

The cardiac injury observed during myocardial ischemia and reperfusion has been shown to be a consequence of a complex mechanism in which the accumulation of hydrogen peroxide (H_2O_2) and other oxygen free radicals (OFRs), and intracellular pH (pH_i) are believed to play a major role. However, the effect of H_2O_2 on pH_i has not been well characterized in the human atrial myocardium. In the present study, we superfused hydrogen peroxide into the human atrial tissue in order to assess the effects of oxygen free radicals on the pHi, and, furthermore, to test the ability of certain potential cardioprotective agents, including scavengers of the • OH free radical (N-(mercaptopropionyl)-glycine; N-MPG) and the HOCl free radical (L-methionine), to protect against oxidative-induced pH_i challenge. The human atrial tissues were obtained from patients undergoing corrective open-heart surgery. The ratiometric recordings of pH_i were measured using the pH-sensitive, dual-excitation and dual-emission fluorescent dye BCECF (2', 7'-bis(carboxyethyl)-5, 6-carboxyfluorescein acetoxymethyl ester). By continuously monitoring pHi changes in human atrial myocardium, we have found, for the first time, that (a) H_2O_2 (30 μM-3 mM) induced a significant dose-dependent intracellular acidosis, (b) N-MPG caused a significant block on the intracellular acidosis induced by 3 mM H_2O_2, whereas L-methionine did not, and (c) Hoe 694, a specific Na^+/H^+ exchanger (NHE) inhibitor, caused a similar extents like that induced by 3 mM H_2O_2. Our data suggest that the effects of H_2O_2 are caused mainly through the generation of • OH, which is attributed to the intracellular acidosis seen in the human atrial trabecular muscle. The possible underlying mechanism for H_2O_2-induced acidosis is likely due to its inhibition on the activity of NHE and other acid extruders, as the pH_i changes after H_2O_2 exposure could be detected even though the activity of NHE was completely blocked by 30 mM Hoe 694.

Key Words: human atrium; intracellular pH; fluorescent; oxygen free radicals; antioxidants; Na^+-H^+ exchanger

INTRODUCTION

Intracellular pH (pH_i) regulation is vital to maintain cellular homeostasis and hence the performance of the cell. Many cellular mechanisms are sensitive to changes in pH_i. These include enzyme activity (30), control of the cell volume (14), the regulation of cellular growth and differentiation (13), the kinetic properties of K^+ and Ca^{2+} channels (7), and cell contractility (6). Moreover, disturbances in the amount of acid or alkali within the cells of the heart can trigger major changes in the strength and rhythm of the heart beat. For example, during myocardial ischemia, the pH_i is substantially lowered (1). Moreover, during post-ischemia reperfusion, rapid recovery of the pH_i is observed (5). These pH_i disturbances have been claimed to be responsible for the reversible contractile dysfunction and malignant ventricular arrhythmias seen in cardiac myocytes and in other cell types (5, 23).

Corresponding author: Shih-Hurng Loh, Ph.D. Department of Pharmacology, National Defense Medical Center, Neihu P.O. box 90048-504, Taipei, Taiwan 840, R.O.C. Tel: 886-2-87924861, E-mail: shloh@ndmctsgh.edu.tw
Received: June 20, 2002; Revised: August 2, 2002; Accepted: August 30, 2002.

The episodes of ischemia and post-ischemic reperfusion occur not only in the condition of pathological disorders, but also in the procedure of certain kind of surgery. For example, coronary thrombosis, balloon angioplasty of blood vessels and open-heart surgery with cardioplegic arrest (2, 25). Furthermore, reperfusion of cardiac tissue after a reversible ischemic episode results in further, and usually more serious, cardiac dysfunction, such as stunning and arrhythmias, and other clinical complications (4, 12). Therefore, over the last few years, extensive progress has been made in exploring the underlying mechanisms of ischaemia/reperfusion-induced cardiac dysfunction. It has been found that oxidant injury plays a critical role in the development of ischemia/reperfusion-induced cardiac injury (27). For example, it has been demonstrated that the concentration of oxygen-derived free radicals, such as superoxide ($\bullet O_2^-$), hydroxyl ($\bullet OH$), and H_2O_2, can be dramatically increased following ischemia and reperfusion (34). Moreover, scavengers of oxygen free radicals, such as catalase and superoxide dismutase, can reduce ischemia/reperfusion-induced myocardial stunning and arrhythmias (2). As H_2O_2 is an important mediator of the production of other oxygen-derived free radicals, such as $\bullet OH$ and HOCl (3, 15, 31) , in the present study, various concentrations of H_2O_2 (30 μM-3 mM) were applied to the human atrial myocardium to mimic the effects of oxygen free radicals during ischemia and post-reperfusion.

In animal models, both *in vivo* and *in vitro*, the most damaging effects on the heart caused by oxygen free radicals seem to be due to $\bullet OH$ radicals (2, 9, 11, 20) . Furthermore, in cultured rat cardiac myoblasts, 100 μM H_2O_2 induces a marked decrease in the pH_i (33), and similar results are seen in cerebellar astrocytes and C6 glioma cells (31). These large pH_i reductions are suggested to result from the production of intracellular $\bullet OH$ as a result of H_2O_2 oxidation, i.e. via the Fenton reaction, since the acidosis is completely inhibited by two different potent membrane-crossing $\bullet OH$ scavengers (31). As yet, however, no pharmacological study has been performed on the effects of oxygen free radicals and the cardioprotective action of oxygen free radicals scavengers on the human atrial myocardium. In addition, it has been shown that the cardiac myocytes plasma membrane Na^+-H^+ exchanger (NHE) plays an important role in the maintenance of intracellular pH, sodium, and calcium ion homeostasis. From the results of various experimental studies, it is clear that NHE is an important mediator of ischemic-reperfusion injury of the heart (19, 21). Therefore, in the present study, Hoe 694, a specific and potent inhibitor of NHE, was applied in the human atrial myocardium to compare its action with that of H_2O_2.

Materials and Methods

Human Heart Tissue

Human atrial tissues were obtained from the hearts of 15 patients (age 62.4±3.3 years; 8 males and 7 females) undergoing corrective cardiac surgery for the open-heart surgery for the treatment of a variety of congenital (6 cases) and acquired (9 cases) heart diseases. Institutional rules for the protection of human subjects were observed. Prior to surgery, informed consent was obtained. Right atrial trabecular tissue, 0.5~1 mm in diameter and 3-5 mm long, was removed, as described previously (22), and immediately immersed in cold bicarbonate-containing Tyrode solution. The preparations were then perfused with oxygenated (95%O_2, 5% CO_2) Tyrode solution at 37°C. The pH of the solution was adjusted to 7.40±0.02 for experiments.

Chemicals and Solutions

All experiments were performed at 37°C in bicarbonate-buffered solutions (equilibrated with 95% O2, 5% CO_2) containing (in mM) NaCl 137, $MgCl_2$ 0.5, NaH_2PO_4 0.5, KCl 4, $CaCl_2$ 2.7, glucose 5.6, $NaHCO_3$ 22, and dextrose 5.6, the pH being adjusted to 7.4 using NaOH. BCECF-AM calibration solutions for pH_i recording contained (mM): KCl, 140; $MgCl_2$, 1; 10 μM nigericin; buffered with one of the following organic buffers: 20 mM 2-(N-morpholino) ethanesulphonic acid (MES, pH 5.5), 20 mM HEPES (pH 7.5) or 20 mM 3-(cyclohexylamino)-2-hydroxy-1-propane-sulphonic acid (CAPSO, pH 9.5), and were adjusted (37°C) to the correct pH with 4N NaOH. All chemicals were purchased from Sigma (USA) except that NaOH was purchased from Wako (Japan). When 20 mM NH_4Cl was used, it was added directly as solid to solution with osmotic compensation by replace the same concentration of NaCl, because NH_4Cl was easily dissolved in the solution. H_2O_2, N-(mercaptopropionyl)-glycine (N-MPG) and L-methionine were added, as stock solutions, to solutions shortly before use.

Measurement and Calibration of the Intracellular pH

Measurement of the pH_i has been described in detail elsewhere (33). In brief, the pH_i in the human atrial myocardium was measured using the pH-sensitive, dual excitation dual-emission fluorescent dye, BCECF-AM (2', 7'-bis(carboxyethyl)-5, 6-carboxyfluorescein acetoxymethyl ester, from Molecular Probes). The preparations were loaded with BCECF-AM (5 μM) by incubating them for 30 min at room temperature and were excited alternately

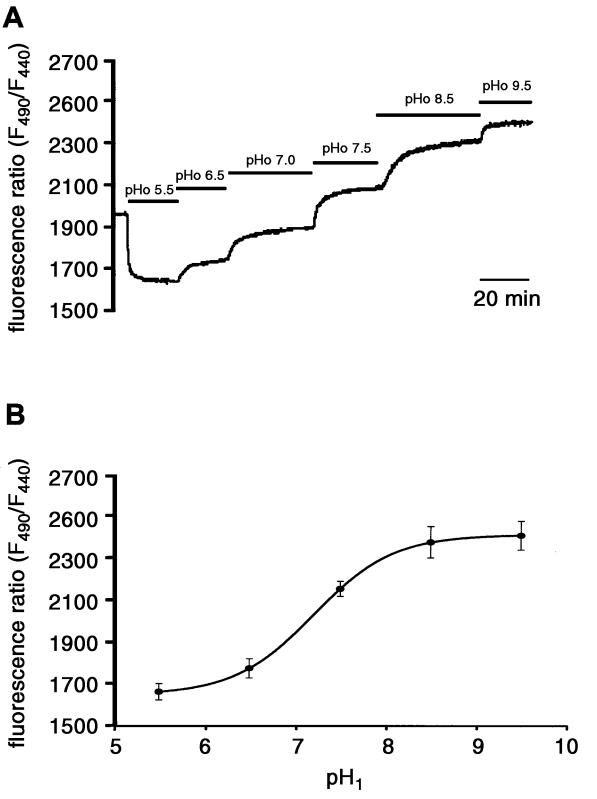

Fig. 1. In vivo calibration of intracellular pH. (A). The trace shows the BCECF fluorescence ratio (510 nm emission at 490 nm and 440 nm excitations) in human atrial myocardium loaded with BCECF-AM. The pH value in the calibration solution is shown above the record. Measurements were performed by microspectro-fluorimetry, after loading of the probe, with myocardium superfused in a buffer at various pH values (See Materials and Methods for details). Note that the addition of nigericin (10 μM) allowed rapid equilibration of external and internal pH. (B). Calibration curve of the ratio of the 510 nm emission at 490 nm and 440 nm excitation (F_{490}/F_{400}) of the fluorescent dye BCECF incorporated in cells, as function of pH.

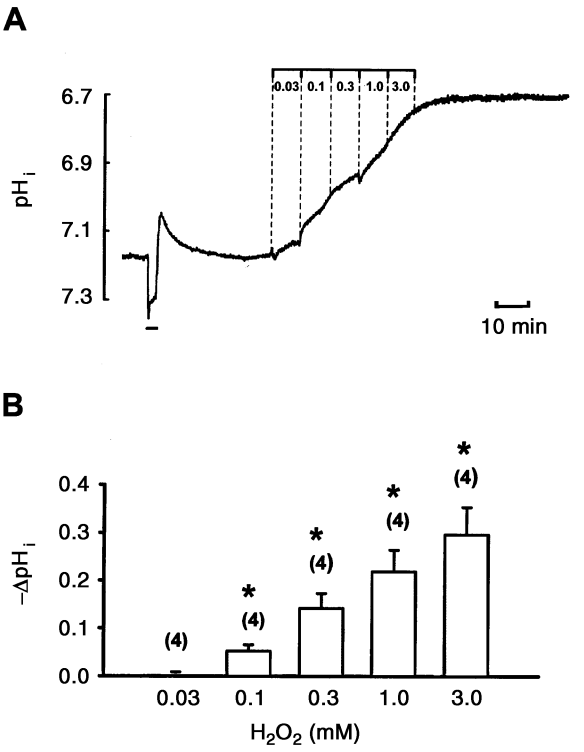

Fig. 2. Intracellular acidosis induced by H_2O_2 in the human atrial myocardium. A) The application of NH_4Cl (20 mM) and various concentrations of H_2O_2 (30 μM - 3 mM) is shown by the bars below and above the pH_i trace. B) Histogram showing the mean pH_i change induced by different concentrations of H_2O_2 in several experiments similar to that shown in A. The columns and bars represent the means ± S.E.M.; *P< 0.05 compared with in the absence of H_2O_2. Note that the change of pH_i induced by tested drug was measured at the 10th min after the treating the drug.

with 490 and 440 nm wavelength light. The BCECF fluorescence emission ratio of the 510 nm emission at 490 nm and 440 nm excitation (490/440) was calibrated using the K^+-nigericin method. Briefly, this consisted of exposing a BCECF-loaded cell to the three nigericin calibration solutions (listed above in *Solution* section). This clamps pH_i to the value of pH_o in the calibration solution (29). Figure 1A showed the emission ratio changes seen on perfusing atrial myocardium with calibration solutions with different pH values (5.5 ~ 9.5) in the presence of 10 μM nigericin. The emitted ratio 510 nm emission at 490 nm and 440 nm excitations (R; $R=F_{490}/F_{440}$) was increased as the pH value of superfusing solution was increased. R_{max} and R_{min} are, respectively, the maximum and minimum ratio values for the data curve. The overall sampling rate for the recorded fluorescent ratio (490 nm /440 nm) was 0.5 Hz in the experiment. Using the liner regression fit of the data (shown in the Figure 1B) obtained from 10 calibration experiments similar to that shown in Fig. 1A, the mean apparent dissociation constant (pKa) at 37 °C was found to be 7.14, very close to the value determined by other investigators (33). The following equation was used to convert the fluorescent ratio in to pH_i:

$$pH_i=pK_a+\log[(R_{max}-R)/(R-R_{min})] + \log(F_{440min}/F_{440max})$$

where R is the fluorescence ratio of the 510 nm emission at 490 nm and 440 nm excitation. R_{max} and R_{min} are, respectively, the maximum and minimum ratio values from the data curve and the pKa (-log of dissociation constant) is 7.16. F_{490min}/F_{440min} and F_{490max}/F_{440max} are the ratios of fluorescence measured at 440 nm of R_{min} and R_{max}, respectively.

To make sure the preparations were in good condition, an intracellular acidosis was induced by an NH_4Cl prepulse (see left part of Fig. 2 for details) to

test the acid extruding activity of the cell, throughout the whole experiment. Briefly, the mechanism of the NH_4Cl prepulse technique relies upon the characteristic of incomplete dissociation. Although both the charged (NH_4^+) and uncharged (NH_3) species of a NH_4Cl exist at the same time in solution, the uncharged species of (NH_3) is lipid soluble and therefore able to permeate the lipid bi-layer of the cell membrane. In contrast, the charged species (NH_4^+) permeates relatively slowly, through various membrane protein routes. In other words, the whole process of the pre-pulse can be explained in terms of four phases as shown in the left part of Fig. 2: rapid entry (phase 1), slow recovery (phase 2), rapid exit (phase 3), and pH_i regulation (phase 4). Only those samples with good response went through the designed protocols. Moreover, to prevent the possible influence of fluorescent dye-leakage on the pH_i recording, the strength of both wavelength of 510 nm emissions at 490 nm and 440 nm excitation was continuously monitored in the oscilloscope and computer. The background fluorescence and auto-fluorescence were small (< 5%) and have been ignored.

Statistics

All data are expressed as the mean ± the standard error of the mean (S.E.M.) for n preparations. Statistical analysis was performed using one-way analysis of variance (one-way ANOVA), and Wilcoxon's signed rank test was used to test significance. A P value smaller than 0.05 was regarded as significant.

Results

H_2O_2 Induced Intracellular Acidosis

In pH_i measurement experiments, the steady-state pH_i value for the human atrial myocardium was found to be 7.24±0.02 (n=35). This value is similar to that reported previously in different animal models (8, 23, 32).

At the beginning of all pH_i measurement experiments, unless otherwise stated, a 20 mM NH4Cl pre-pulse was applied for about 10 min to induce an intracellular acidosis [(26); see Materials and Methods for details]. Only those tissues showing a rapid pH_i recovery immediately after the induced intracellular acidosis were used in experiments, thus guaranteeing that the tissue was in good condition. The left part of Figure 2A shows a typical NH_4Cl pre-pulse control, in which the induced acute intracellular acid load is followed by rapid recovery.

The effect of superfusion with H_2O_2 (30 μM-3 mM) on the pH_i of the human atrial myocardium is shown on the right part of Figure 2A. H_2O_2 treatment caused a concentration-dependent reduction of the pH_i. The highest concentration of H_2O_2 (3 mM) resulted in a large irreversible pH_i reduction. The histogram (Fig. 2B) shows the mean H_2O_2-induced pH_i reduction for 4 experiments similar to that shown in Figure 2A. The results clearly show that the H_2O_2-induced intracellular acidosis is concentration-dependent in the range of 0.03 mM to 3 mM.

This is the first demonstration of an H_2O_2-induced pH_i reduction in the human atrial myocardium, similar to that found in animal cells, such as cultured rat cardiac myoblasts (33), rat cerebellar astrocytes and C6 glioma cells (31).

Effects of Antioxidants on the Intracellular pH and H_2O_2-Induced Intracellular Acidosis

As H_2O_2 can readily cross the cell membrane and be converted, via the Fenton reaction, to • OH and, possibly, subsequently to HOCl, we wished to determine which of these chemical species were involved in the H_2O_2-induced intracellular acidification in the human atrial myocardium. The tissue was therefore pretreated for 30 min with N-(mercaptopropionyl)-glycine (N-MPG; 10 mM), a highly potent intracellular scavenger of the • OH free radical, and L-methionine (1 mM), a specific inhibitor of the HOCl free radical (15), both alone or in combination, before addition of 3 mM H_2O_2. Figure 3A shows a control experiment demonstrating that treatment with 3 mM H_2O_2 resulted in a time-dependent pH_i reduction (approximately 0.20 and 0.40 pH units after 5 and 10 min, respectively). As shown in Figure 3B, 1mM L-methionine alone had no significant effect on the intracellular acidosis induced by H_2O_2. Therefore, the result of Figure 3 suggested that the L-methionine, the scavenger of the HOCl free radical, could not prevent the H_2O_2-induced intracellular acidification. In the other word, the HOCl free radical is not attributed to the H_2O_2-induced intracellular acidification.

In contrast, as shown in the Figure 4A, pretreatment with 10 mM N-MPG significantly inhibited the H_2O_2-induced acidosis (reduction of only 0.16 pH units 30 min after H_2O_2 addition). Moreover, no dramatic pH_i changes was seen using the combination of 10 mM N-MPG and 1 mM L-methionine, as shown in Figure 4B. Note that the H_2O_2-induced intracellular acidosis was reversible in the group of N-MPG in combination with L-methionine (Fig. 4B). The summarized results for several experiments similar to that in Figure 3 and Figure 4 are shown in the histogram in Figure 5. The pH_i changes after 30 min treatment with the oxygen free radicals scavengers, N-MPG, L-methionine, and

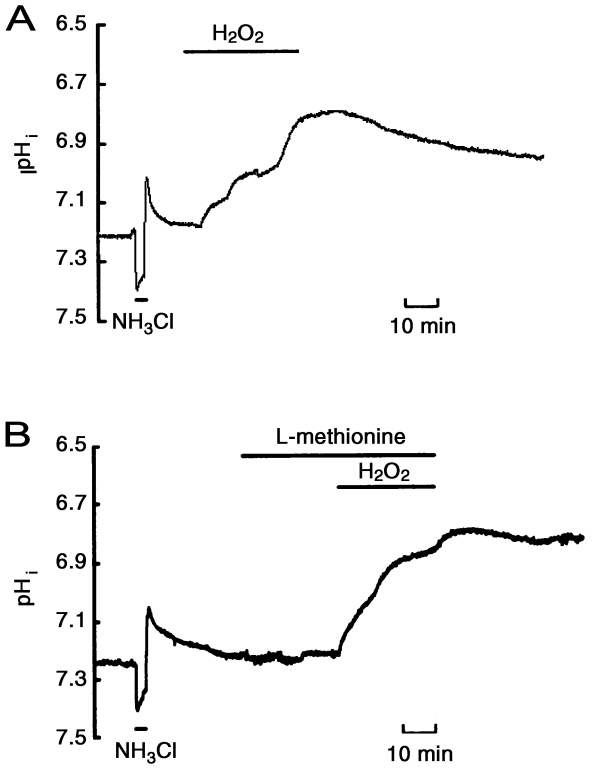

Fig. 3. Effect of the antioxidant, L-methionine on H_2O_2 -induced acidosis in the human atrial myocardium. In A, the recording shows that the H_2O_2 (3 mM)-induced acidosis is time-dependent. Recordings in part B shows that pretreatment of L-methionine (1 mM) alone for 30 min had no effect on the H_2O_2-induced acidosis. The application of NH_4Cl (20 mM), H_2O_2 (3 mM) and L-methionine (1 mM) is shown by the bars below and above the pH_i trace.

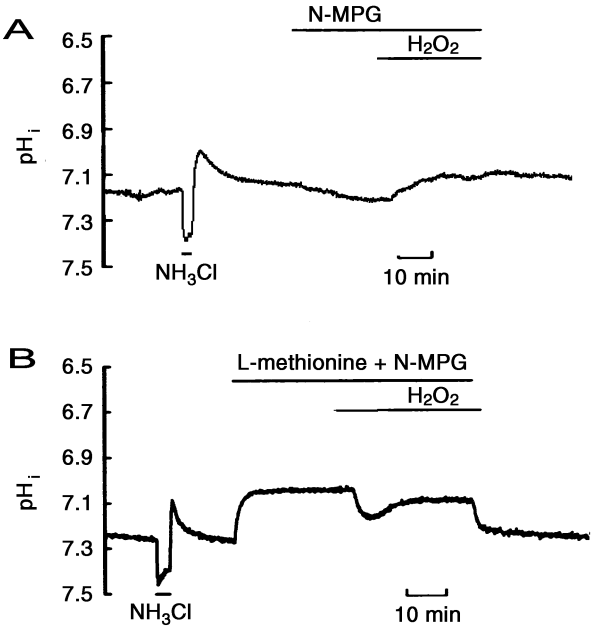

Fig. 4. Effect of the antioxidants, N-MPG alone or in combination of N-MPG and L-methionine, on H_2O_2-induced acidosis in the human atrial myocardium. In A, a typical recording shows that N-MPG (10 mM) alone significantly inhibited the acidosis, and B shows that this effect was not enhanced by addition of L-methionine (1 mM). The application of NH_4Cl (20 mM) and tested agents is shown by the bars below and above the pH_i trace.

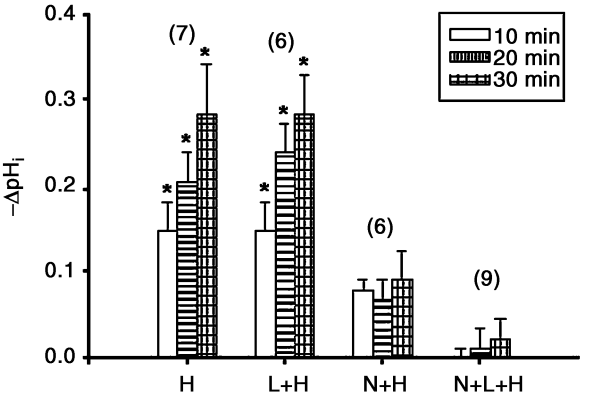

Fig. 5. A histogram showing the time-dependent (10, 20, or 30 min) pH_i change induced by H_2O_2 (H) and the effects of L-methionine (L) and N-MPG (N) averaged for several experiments similar to that shown in Fig. 3 and Fig. 4. The columns and bars represent the means ± S.E.M.; *P< 0.05 compared with in the absence of H_2O_2.

the combination of N-MPG and L-methionine (i.e. before exposure to H_2O_2) were -0.16±0.02 (P<0.05; n=6), 0±0.01 (P>0.05; n=6) ; -0.21±0.03 (P<0.05; n=9), respectively. In other words, the L-methionine did not change the basal pH_i value, while N-MPG did.

Given the reported effects of NHE inhibitors in preventing arrhythmia during post-ischemic reperfusion (19, 21, 23, 28), we investigated whether Hoe 694, a specific and potent inhibitor of NHE, could protect against H_2O_2-induced pH_i acidosis in the human atrial myocardium. As shown in Figure 6, as expected, pretreatment with 30 μM Hoe 694 reduced the pH_i due to inhibition of NHE (-0.18±0.03 pH unit; P< 0.05, n=3). However, Hoe 694 pretreatment didn't prevent the 3 mM H_2O_2 induced-pH_i acidosis (-0.48±0.14 pH unit; P< 0.05, n=3; see Fig. 6). Moreover, the present results suggest that the underlying mechanism of H_2O_2-induced pH_i acidosis in the human atrial myocardium is due to the inhibition of NHE, as the rate and amplitude of pH_i decreasing in both groups are of similar extent.

Discussion

Using the technique of microspectrofluorimetry, we have, for the first time, demonstrated that H2O2 (0.1 mM-3 mM) caused marked intracellular acidosis in the human atrial myocardium (Fig. 2A). This effect was dose- and time-dependent (Fig. 2 and Fig. 3). The underlying mechanism for the H_2O_2-induced

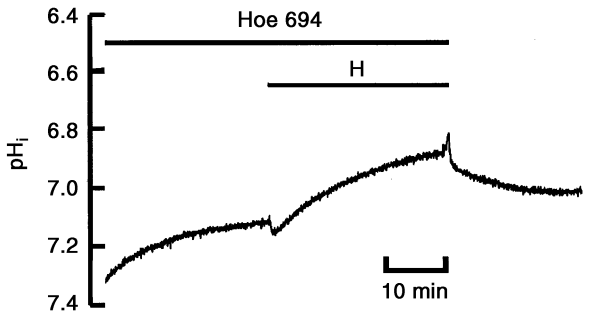

Fig. 6. Effects of Hoe 694 in the human atrial myocardium. The periods of application of Hoe 694 (30 μM) and H_2O_2 (H; 3 mM) are shown by the bars immediately above the pH_i trace.

acidosis is probably the intracellular production of • OH after H_2O_2 influx (i.e. via the Fenton reaction). This conclusion is strongly supported by the fact that the H_2O_2-induced intracellular acidosis was blocked by the specific potent • OH scavenger, N-MPG (Fig. 4A, B), but not blocked by the HOCl scavenger, L-methionine (Fig. 3B). Similar observations have been reported in animal models (33). These results indicated that the intracellular concentration of HOCl produced by H_2O_2 entry makes little or no contribution to the H_2O_2-induced pH_i reduction. In other words, our present study in the human atrial trabecular myocardium is in agreement with the hypothesis that • OH plays a critical role in the development of ischemia/reperfusion-induced cardiac injury, such as stunning (2, 9, 11, 20, 34).

NHE plays a pivotal role in the regulation of pH_i by removing protons that are continuously generated under normal cellular homeostatic processes, as well as during ischemia (19, 23). The NHE is relatively inactive at normal intracellular pH (7.0-7.3), but it becomes 10 times more active as intracellular pH decreases to 6.7 (5, 16). During ischaemia and reperfusion of the myocardium, cell injury, necrosis, stunning, and arrhythmias can occur. The mechanism by which this occurs may involve the NHE (18, 23, 24). As the NHE is a major uptake system for Na+ during acidosis (23), the resulting increase in the intracellular Na^+ concentration may trigger calcium influx via the Na^+/Ca^{2+} exchanger (10). This could result in intracellular calcium overload, leading to arrhythmias and even cell death. Hoe 694, a specific and potent NHE inhibitor, has recently been found to be remarkably effective in suppressing post-ischemic reperfusion arrhythmias and cellular reperfusion damage in animal models (17). However, no such studies have yet been carried out on the human atrial myocardium. Furthermore, in experiments on isolated mammalian papillary muscle, Bountra and Vaughan-Jones (6) found that intracellular acidosis can sometimes result in increased, rather than decreased, contraction, especially when the pH_o was maintained at 7.40. Similarly, in rat trabeculae, Orchard (24) found that acidosis can increase the cytosolic $[Ca^{2+}]_i$. Therefore, according to our present study, we propose that the • OH-induced changes of contractile force were strongly correlated with the induced intracellular acidosis and the subsequent changes of $[Na^+]_i$ and $[Ca^{2+}]_i$. Especially, it has been claimed that the OH-induced acidosis is due to inhibition of the glycolytic pathway, with hydrolysis of intracellular ATP and the resultant intracellular acidification (33). Hoe 694, an NHE inhibitor, has been shown to have mark protective and antiarrhythmic effects in different models of cardiac ischemia and reperfusion, both *in vitro* and *in vivo* (17, 18). However, in the present study, Hoe 694 failed to show protective effects against H_2O_2-induced pH_i acidosis (Fig. 6; n=3). The explanation for this is probably that the suggested underlying mechanism for the described protective effects of Hoe 694 is its inhibition of acid extrusion, whereas the H_2O_2-induced cardiac injury, seen in our present study, seems to be caused by direct damage or inhibition on the activity of NHE, rather than to activation of the NHE and subsequently increases of $[Na^+]_i$ and $[Ca^{2+}]_i$. Our results therefore suggest that the caridoprotective effects of Hoe 694 indicated in the other study (17, 23) are not mediated by the anti-oxidative properties in the human atrial myocardium. Moreover, given the fact that the smaller resting pH_i was affected by ~ 0.05 pH units during the pretreatment of N-MPG (Fig. 4A) and decreased by ~ 0.2 pH units during that of L-methionine + N-MPG (Fig. 4B), but not in that of L-methionine alone (Fig. 3B), this indicated that N-MPG *per se*, instead of L-methionine, could slightly acidify pH_i. Whether this slight intracellular acidification caused by N-MPG per se contributes to the protective effect on cardiac functions (i.e. pre-conditioning) requires further study. In addition, we speculate that the structure of carboxylic acid groups in N-MPG underlies the phenomenon of N-MPG-induced acidification and the transient reversal effect of H_2O_2 on pH_i acidification. However, the exact mechanisms for these waits further experiments.

In summary, the present study is the first to show that, in the human atrial myocardium, the • OH free radicals may be released in large amounts through the Fenton reaction from H_2O_2 and evoke marked intracellular acidosis. This acidosis is probably due to the inhibition in the activity of NHE. In terms of clinical implications, therefore, the present results suggest that the development of a more specific and potent • OH scavenger would be one potential approach to preventing certain pathological conditions, especially those involving oxidative challenge.

Acknowledgements

The authors would like to thank Dr. Cheng-I Lin, Dr. Mei-Ling Wu and Dr. Richard Vaughan-Jones for their encouragement and valuable opinions. Special thanks also would like express to Mrs. Shu-Fu Huang, and Ms Ho-Ling Peng for their technical assistance. This study was supported by grants from the National Science Council (NSC 89-2320-B-016-072; 89-2320-B16-018) and National Defense Medical Bureau, Taiwan, ROC.

References

1. Allen, D.G. and Orchard, C.H. Myocardial contractile function during ischemia and hypoxia. *Circ. Res*. 60: 153-168, 1987.
2. Bolli, R. Mechanism of Myocardial "Stunning". *Circulation* 82: 723-735. 1990.
3. Bolli, R., Jeroudi, M.O., Patel, B.S., Aruoma, O.I., Halliwell, B., Lai, E.K. and McCay, P.B. Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion: evidence that myocardial stunning is a manifestation of reperfusion injury. *Circ. Res*. 65: 607-622, 1989.
4. Bolli, R. and Marban, E. Molecular and cellular mechanisms of myocardial stunning. *Physiol. Rev*. 79: 609-341, 999.
5. Bond, J.M., Herman, B. and Lemasters, J.J. Protection by acidotic pH against anoxia/reoxygenation injury to rat neonatal cardiac myocytes. *Biochem. Biophys. Res. Comm* 179: 798-803, 1991.
6. Bountra, C. and Vaughan-Jones, R.D. Effect of intracellular and extracellular pH on contraction in isolated, mammalian cardiac muscle. *J. Physiol.* 418: 163-1871989.
7. Carbone, E., Testa, P.L. and Wanke, E. Intracellular pH and ionic channels in the Loligo Vulgaris giant axon. *Biophys. J*. 35: 393-413, 1981.
8. Ellis, D. and Thomas, R.C. Microelectrode measurement of the pH_i of mammalian heart cells. *Nature* 262: 224-2251976.
9. Farber, N.E., Vercellotti, G.M., Jacob, H.S., Pieper, G.M. and Gross, G.J. Evidence for a role of iron-catalyzed oxidants in functional and metabolic stunning in the canine heart. *Circ. Res*. 63: 351-360, 1988.
10. Frelin, C., Vigne, P. and Lazduski, M. The role of the Na^+/ H^+ exchange system in cardiac cells in relations to the control of the internal Na+ concentration. A molecular basis for the antagonistic effect of ouabin and amiloride on the heart. *J. Biol. Chem*. 259: 8880-8885, 1984.
11. Goldhaber, J.I., Ji, S., Lamp, S.T. and Weiss, J.N. Effects of exogenous free radicals on electromechanical function and metabolism in isolated rabbit and guinea pig ventricle: implications for ischemia and reperfusion injury. *J. Clin. Invest*. 8: 1800-1809, 1989.
12. Goldhaber, J.I. and Weiss, J.N. Oxygen free radicals and cardiac reperfusion abnormalities. *Hypertension* 20: 118-127, 1992.
13. Grinstein, S., Rotin, D. and Mason, M.J. Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. B*iochem. Biophy. Acta* 988: 73-97, 1989.
14. Grinstein, S., Woodside, M., Sardet, C., Pouyssegur, J. and Rotin, D. Activation of the Na^+-H^+ antiporter during cell volume regulation. Evidence for a phosphorylation-independent mechanism. *J. Biol. Chem*. 267: 23823-23828, 1992.
15. Ide, T., Tsutsui, H., Kinugawa, S., Suematsu, N., Hayashidani, S., Ichikawa, K., Utsumi, H., Machida, Y., Egashira, K. and Takeshita, A. Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. *Circ. Res*. 86: 152-157, 2000.
16. Kaila, K., and Vaughan-Jones, R.D. Influence of sodium-hydrogen exchange on intracellular pH, sodium and tension in sheep cardiac Purkinje fibres. *J. Physiol*. 390: 93-118, 1987.
17. Kaplinsky, E., Ogawa, S., Michelson, E.L. and Dreifus, L.S. Instantaneous and delayed ventricular arrhythmias after reperfusion of acutely ischaemic myocardium: evidence for multiple mechanisms. *Circulation*. 63: 333-340, 1981.
18. Karmazyn, M. and Moffat, M. Role of Na^+/H^+ exchange in cardiac physiology and pathophysiology: mediation of myocardial reperfusion injury by the pH paradox. *Cardiovascular Research* 27: 915-924, 1993.
19. Karmazyn, M., Tracey, X., Humphreys, R.A., Yoshida, H. and Kusumoto, K. The myocardial Na^+-H^+ exchange, structure, regulation and its role in heart disease. *Circ. Res*. 85: 777-786, 1999.
20. Kloner, R.A., Przyklenk, K. and Whittaker, P. Deleterious effects of oxygen radicals in ischemia-reperfusion: resolved and unsolved issues. *Circulation* 80: 1115-1127, 1989.
21. Levitsky, J., Gurell, D. and Frishman, W. Sodium ion /hydrogen ion exchange inhibition: a new pharmacologic approach to myocardial ischemia and reperfusion injury. *J. Clin. Pharmacol*. 38: 887-897, 1989.
22. Lin, C.I., Chiu, T.H., Chiang, B.N. and Cheng, K.K. Electromechanical effects of caffeine in isolated human atrial fibres. *Cardiovas. Res.* 19: 727-733, 1985.
23. Loh, S.H., Sun, B. and Vaughan-Jones, R.D. Effect of HOE 694, a novel Na^+-H^+ exchange inhibitor, on intracellular pH regulation in the guinea-pig ventricular myocyte. *Br. J. Pharmacol*. 118: 1905-1912, 1996.
24. Orchard, C.H., Houser SR, Kort A.A., Bahinski A., Capogrossi M. C. and Lakatta E.G., Acidosis facilitates spontaneous sarcoplasmic reticulum Ca^{2+} release in rat myocardium. *J. Gen. Physiol*. 1987; 90: 145-165.
25. Pierce, G.N. and Czubryt, M.P. The contribution of ionic imbalance to ischaemia/reperfusion - induced injury. *J. Mol. Cell. Cardiol*. 27: 53-63, 1995.
26. Roos, A. and Boron, W.F. Intracellular pH. *Physiol. Rev*, 61: 296-434, 1981. 26. 26.
27. Salvatore, C., Cladio, C., Federica, D.E.G., Anna, F.P., Bruno, M., Marinella, C., Alessandro, P., Paolo, M., Ottavio, A., Frank, M. and Roberto, R. Oxidative stress during reperfusioon of human heats: potential sources of oxygen free radicals. *Cardiovasc. Res* 29: 118-125, 1995.
28. Scholz, W., Albus, U., Lang, H.J., Martorana, P.A., Englert, H.C. and Scholkens, B.A. Hoe 694, a new Na^+/H^+ exchanger inhibitor and its effects in cardiac ischemia. *British Journal of Pharmacology* 109: 562-568, 1993.
29. Thomas, J.A., Buchsbaum, R.N., Zimniak, A. and Racker, E. Intracellular pH measurements in Ehrlich ascites tumour cells utilizing spectroscopic probes generated in situ. *Biochemistry* 18: 2210-2218, 1979.
30. Trivedi, B., and Danforth, W.H. Effect of pH on the kinetics of frog muscle phosphofructokinase. *J. Biol. Chem*. 241: 4110-4112, 1966.
31. Tsai, K.L., Wang, S.M., Chen, C.C., Fong, T.H. and Wu, M.L. Mechanism of oxidation stress-induced intracellular acidosis in rat cerebellar astrocytes and C6 glioma cells. *J. Physiol*. 502: 161-174, 1997.
32. Vaughan-Jones, R.D., Eisner, D.A. and Lederer, W.J. Effects of changes of intracellular pH on contraction in sheep cardiac Purkinje fibres. *J. Gen. Physiol*. 89: 1015-1032, 1987. 33. 32. 32.
33. Wu, M.L., Tsai, K.L., Wang, S.M., Wu, J.C., Wang, B.S. and Lee, Y.T. Mechanism of hydrogen peroxide and hydroxyl free radical induced intracellular acidification in cultured rat cardiac myoblasts. *Circ. Res.* 78: 564-572, 1996.
34. Zwier, J., Rayburn, B., Flaherty, J. and Wiesfeldt, M. Recombinant superoxide dismutase reduces free radical concentrations in reperfused myocardium. *J. Clin. Invest.* 87: 1728-1734, 1987.