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European Journal of Pharmacology 443 (2002) 169–177

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Hydrogen peroxide-induced intracellular acidosis and electromechanical inhibition in the diseased human ventricular myocardium

Shih-Hurng Loh [a,*], Chien-Sung Tsai [b], Yin Tsai [a], Wei-Hwa Chen [c], Guo-Jiueng Hong [b], Jeng Wei [d], Tzi-Hurng Cheng [a], Cheng-I Lin [a]

[a] *Department of Pharmacology, National Defense Medical Center, Neifu P.O. Box 90048-504, Taipei, Taiwan, Republic of China*
[b] *Department of Surgery, National Defense Medical Center, Cheng-Hsin General Hospital, Taipei, Taiwan, Republic of China*
[c] *Department of Gynecology, National Defense Medical Center, Cheng-Hsin General Hospital, Taipei, Taiwan, Republic of China*
[d] *Heart Center, Cheng-Hsin General Hospital, Taipei, Taiwan, Republic of China*

Received 10 January 2002; received in revised form 3 April 2002; accepted 9 April 2002

Abstract

Accumulation of oxygen free radicals is an important mediator of post-ischemia/reperfusion cardiac dysfunction. However, oxidative injury has not been well characterized in human cardiac tissues. In the present study, we superfused hydrogen peroxide (H_2O_2) into the diseased human ventricle in order to assess the effects of oxygen free radicals on the electromechanical parameters and the intracellular pH (pH_i), and to test the ability of certain potential cardioprotective agents, including scavengers of hydrogen peroxide (dibenzamidostilbene disulfonic acid; DBDS), the ·OH free radical (*N*-(mercaptopropionyl)-glycine; *N*-MPG), and the HOCl free radical (L-methionine), to protect against oxidative injury. Disease human ventricular tissues were obtained from patients undergoing heart transplantation. Electrophysiological experiments were performed using a traditional micropipette, while the pH_i was measured by microspectrofluorimetry. We found that (a) H_2O_2 (30 μM–3 mM) induced a significant dose-dependent intracellular acidosis, (b) H_2O_2 (30 μM–3 mM) had a notable dose-dependent biphasic effect on the contractile force (an increase, followed by a decrease), while moderate concentrations of H_2O_2 also inhibited the generation of action potential and increased the diastolic resting force significantly, and (c) *N*-MPG caused significant block of both the intracellular acidosis and the electromechanical inhibition induced by 3 mM H_2O_2, whereas L-methionine and DBDS did not. Our data suggest that the toxic effects of H_2O_2 are caused mainly through the generation of ·OH, which is attributed to the intracellular acidosis seen in the diseased human ventricle.

Keywords: Ventricle, human; Electrophysiology; Contractile force; Acidosis, intracellular; Fluorescent; Oxygen free radical; Antioxidant; Cardioprotective agent

1. Introduction

In the clinical situation, ischemia and post-ischemic reperfusion occur not only as a result of pathological disorders, such as coronary thrombosis, but also following certain surgical procedures, such as balloon angioplasty of blood vessels and open-heart surgery with cardioplegic arrest (Bolli, 1990; Pierce and Czubryt, 1995). Unfortunately, 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 (Bolli and Marban,1999; Goldhaber and Weiss, 1992). Ischaemia/reperfusion-induced cardiac dysfunction has become one of the most common causes of serious illness an early death in developed societies. There is increasing evidence that oxidant injury plays a critical role in the development of ischemia/reperfusion-induced cardiac injury (Salvatore et al., 1995). For example, it has been demonstrated that the concentration of oxygen-derived free radicals, such as superoxide ($\cdot O_2^-$), hydroxyl (·OH), and H_2O_2, can be dramatically increased following ischemia and reperfusion (Zwier et al., 1987). 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 ·OH radicals (Bolli, 1990; Farber et al., 1988; Goldhaber et al., 1989, Kloner et al., 1989). Moreover, scavengers of oxygen free radicals, such as catalase and superoxide dismutase, can reduce ischemia/

* Corresponding author. Tel./fax: +886-2-87924861.
E-mail address: shloh@ndmctsgh.edu.tw (S.-H. Loh).

0014-2999/02/$ - see front matter
PII: S0014-2999(02)01595-9

reperfusion-induced myocardial stunning and arrhythmias (Bolli, 1990).

In isolated guinea-pig ventricular myocytes, free radical-generating systems have been demonstrated to activate ATP-sensitive K^+ channels, decrease the calcium current, and induce cell shortening by irreversibly inhibiting glycolytic and oxidative metabolism (Goldhaber et al., 1989). Furthermore, Satoh and Matsui (1997) suggested that low concentrations of H_2O_2 (30–100 μM) alter the action potential duration (APD) and contractile force, but not the other electrical parameters in guinea pig cardiac muscles. In addition, a dose- and time-dependent action on the contractile force has been observed (Satoh and Matsui, 1997). In contrast, in rat ventricular myocytes, Ward and Giles (1997) showed that 50–200 μM H_2O_2 had no significant effect on the action potential, cell shortening, the transient outward K^+ current, and inwardly rectifying K^+ currents. 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 myocardium. As H_2O_2 is an important mediator of the production of other oxygen-derived free radicals, such as ·OH and HOCl (Bolli et al., 1989; Tsai et al., 1997; Ide et al., 2000), in the present study, various concentrations of H_2O_2 (30 μM–3 mM) were applied to the diseased human ventricular myocardium to mimic the effects of oxygen free radicals during ischemia and post-reperfusion.

Many cellular mechanisms are sensitive to changes in the intracellular pH (pH_i). These include enzyme activity (Trivedi and Danforth, 1966), control of the cell volume (Grinstein et al., 1992), the regulation of cellular growth and differentiation (Grinstein et al., 1989), the kinetic properties of K^+ and Ca^{2+} channels (Carbone et al., 1981), and cell contractility (Bountra and Vaughan-Jones, 1989). During myocardial ischemia, the pH_i is substantially lowered (Allen and Orchard, 1987). Moreover, during post-ischemia reperfusion, rapid recovery of the pH_i is observed (Bond et al., 1991). 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 (Bond et al., 1991; Loh et al., 1996). In cultured rat cardiac myoblasts, 100 μM H_2O_2 induces a marked decrease in the pH_i (Wu et al., 1996), and similar results are seen in cerebellar astrocytes and C6 glioma cells (Tsai et al., 1997). These large pH_i reductions are suggested to result from the production of intracellular ·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 ·OH scavengers (Tsai et al., 1997); however, whether this occurs in the human myocardium is not yet known.

In order to investigate the relationship between the pH_i and electrophysiological parameters during exposure to oxygen free radicals, in the present study, we have used two different approaches, microspectrofluorimetry and conventional microelectrode techniques, in parallel experiments under the same conditions.

2. Materials and methods

2.1. Human heart tissue

With the approval of the institutional review committee and with prior informed consent, right diseased human ventricular myocardial tissue was obtained from 12 dilated cardiomyopathy patients (age 58.4 ± 3.6 years; 7 males and 5 females) undergoing heart transplantation. Diseased right ventricular trabecular tissue, 1 mm in diameter and 3–6 mm long, was removed, as described previously (Lin et al., 1985), and immediately immersed in cold bicarbonate-containing Tyrode solution. The preparations were then perfused at 37 °C with oxygenated (97%O_2/3%CO_2) bicarbonate-containing Tyrode solution, pH 7.40 ± 0.02 for experiments.

2.2. Electrophysiological and mechanical experiments

The diseased right human ventricular myocardia were driven by electrical stimuli at a frequency of 1 Hz. Transmembrane potentials were recorded using traditional glass microelectrodes filled with 3 M KCl and the contractile force was measured using a Gould UC2 force transducer, as described previously (Lin et al., 1985). Preparations were preloaded by equilibrating for 2 h under an optimal resting tension of 150 mg. The electrical and mechanical events were displayed simultaneously on a Gould 4072 oscilloscope and a Gould ES 1000 recorder. The action potential amplitude (APA), action potential duration at 50% and 90% repolarization (APD_{50} and APD_{90}, respectively), maximum diastolic potential (MDP), and twitch force were measured as described previously (Lin et al., 1994). To prevent possible unstable implement affecting the measured parameters of action potential during the experiments, we only used those preparations that have stable control (i.e. without losing implement for, at least, half an hour) to continue the subsequent protocols. Also, some of the phenomenon of totally inhibition on action potential will be double-checked by a few tries on re-implementing to ensure whether it's the true drug effect or simply the losing implement.

2.3. Measurement of the intracellular pH

Measurement of the pH_i has been described in detail elsewhere (Wu et al., 1996). In brief, the pH_i in the diseased human myocardium was measured using the pH-sensitive, dual excitation dual-emission fluorescent dye, BCECF-AM (Molecular Probes). The preparations were loaded with BCECF-AM (5 μM) by incubating them for 30 min at room temperature and were excited alternately with 490 and 440 nm wavelength light. The ratio of the 510 nm emission at

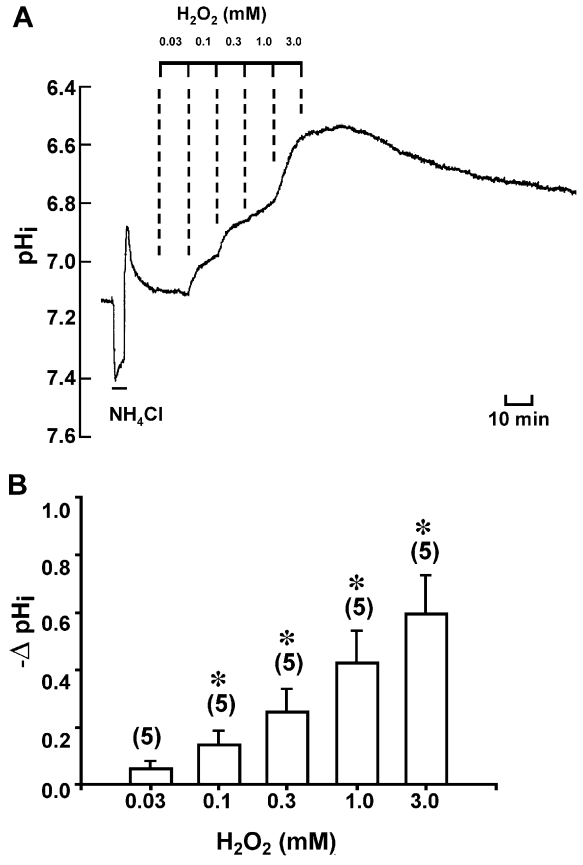

Fig. 1. Intracellular acidosis induced by H_2O_2 (H) in the diseased human ventricular myocardium. (A) The application of NH_4Cl (20 mM) and various concentrations of H_2O_2 (30 μM–3 mM) is shown by the bars below and above the pH_i trace. (B) Histogram showing the mean pH_i change induced by different concentrations of H_2O_2 in five 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 treating the drug.

490 and 440 nm excitation (490/440) was calculated and converted to a linear pH using the following equation:

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

where R is the 510 nm emission at 490 nm excitation/510 emission at 440 nm excitation ratio, R_{max} and R_{min} are, respectively, the maximum and minimum ratio values from the calibration curve (data not shown), and pK is the dissociation constant for the dye, taken as 7.05. F_{440min}/F_{440max} is the ratio of the fluorescence measured at 440 nm of R_{min} and R_{max}. The overall sampling rate for the recorded fluorescent ratio (440/490 nm) was 0.5 Hz in the experiment. Throughout the whole experiment, the change of pH_i induced by tested drug was compared at the 10th min after the treating the drug, unless otherwise stated. To make sure the preparations were in good condition, an intracellular acidosis was induced by an NH_4Cl prepulse (see Figs. 1 and 2 for details) to test the acid extruding activity of the cell. 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 and 440 nm excitation was continuously monitored in the oscilloscope and computer. The background fluorescence and auto-fluorescence were small (< 5%) and has been ignored.

2.4. Chemicals and solutions

All experiments were performed at 37 °C in bicarbonate-buffered solutions (equilibrated with 97% O_2, 3% 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. Unless otherwise stated, all chemicals were purchased from Sigma

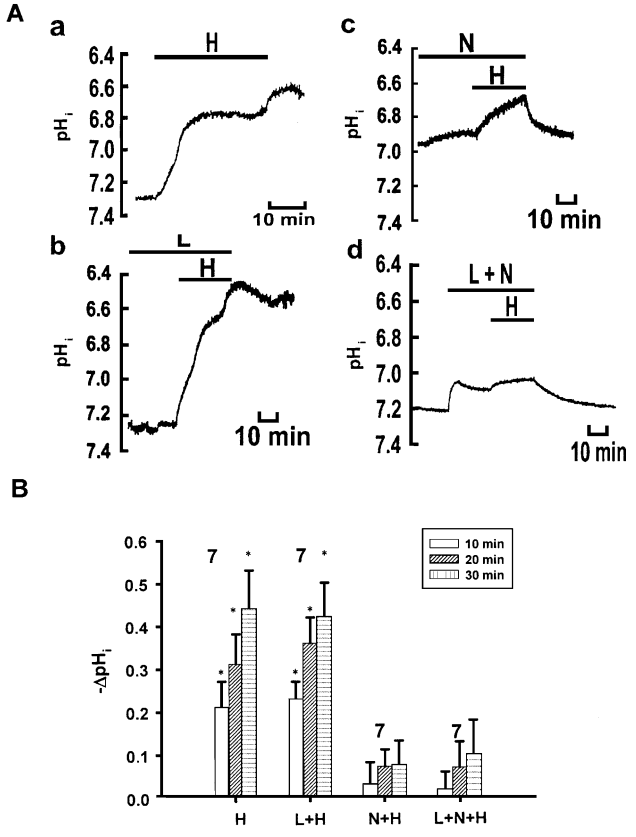

Fig. 2. Effect of the antioxidants, L-methionine (L) and *N*-MPG (N), alone and in combination (L + N), on H_2O_2 (H)-induced acidosis in the diseased human ventricular myocardium. In A, panel (a) shows that the H_2O_2 (3 mM)-induced acidosis is time-dependent, (b) shows that L-methionine (1 mM) alone had no effect on the H_2O_2-induce acidosis, (c) shows that *N*-MPG (10 mM) alone significantly inhibited the acidosis, and (d) shows that this effect was not enhanced by addition of L-methionine (1 mM). B is a histogram showing the time-dependent (10, 20, or 30 min) pH_i change induced by H_2O_2 and the effects of L-methionine and *N*-MPG averaged for seven 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.

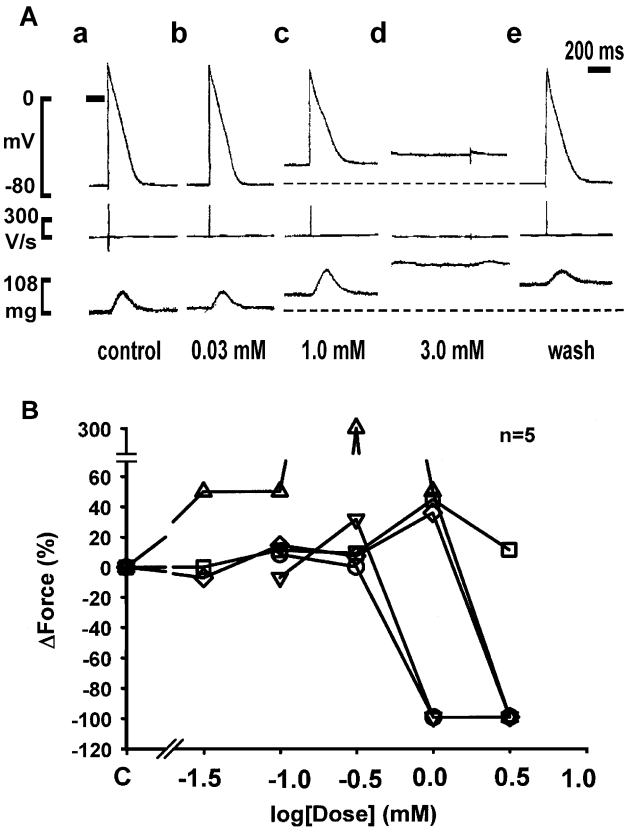

Fig. 3. Effects of various concentrations of H_2O_2 (0.03–3 mM) on the electrophysiological and mechanical parameters of the diseased human ventricular myocardium. In A, the action potential (upper trace) and its 0 phase depolarization (middle trace) and the contractile force (lower trace) were recorded (a) in Tyrode solution, (b–d) in the presence of 0.03 (b), 1 (c), or 3 (d) mM H_2O_2, and (e) after washout in drug-free superfusate. B shows the percentage change in contractile force versus the logarithm of the H_2O_2 concentration curve for five separate experiments similar to that shown in A; the different symbols indicate data from different human ventricular myocardial preparations. The change in the contractile force is expressed as a percentage change relative to the control value (C) before H_2O_2 exposure. Note that the resting diastolic force is increased dramatically by adding higher concentrations of H_2O_2 (A-c and A-d).

(USA) and Wako (Japan). When 20 mM NH_4Cl was used, it was added directly as solid to solution with osmotic compensation by replacing the same concentration of NaCl, because NH_4Cl was easily dissolved in the solution. H_2O_2, dibenzamidostilbene disulfonic acid (DBDS), *N*-(mercaptopropionyl)-glycine (*N*-MPG) and L-methionine were added, as stock solutions, to solutions shortly before use.

2.5. 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.

3. Results

3.1. Intracellular acidosis is induced by H2O2

In pH_i measurement experiments, the steady-state pH_i value for the diseased human ventricular myocardium was found to be 7.22 ± 0.03 ($n = 33$). This value is similar to that reported previously in animal models (Ellis and Thomas, 1976).

At the beginning of all pH_i measurement experiments, unless otherwise stated, a 20 mM NH_4Cl pre-pulse was applied for about 10 min to induce an intracellular acidosis (Roos and Boron, 1981) and 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 Fig. 1A shows a typical NH_4Cl pre-pulse control, in which

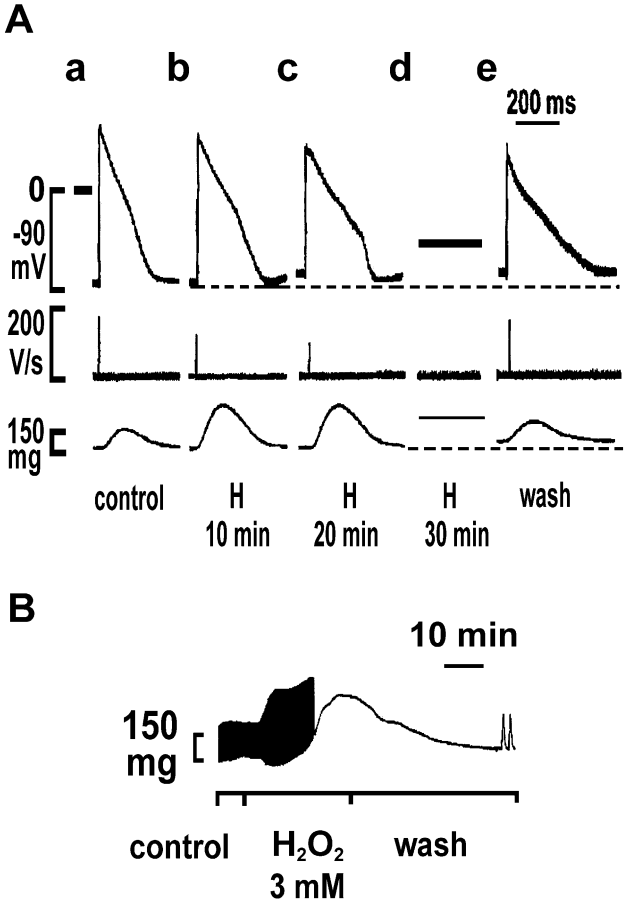

Fig. 4. Time-dependent effects of 3 mM H_2O_2 (H) on electrophysiological and mechanical parameters in the diseased human ventricular myocardium. In (A), the action potential (upper trace), maximal rate of rise of the 0 phase depolarization (middle trace), and contractile force (lower trace) were recorded at different times in the absence and presence of H_2O_2 in Tyrode solution (a: control; b, c, and d: after 10, 20, and 30 min exposure to H_2O_2, respectively; e: after washout 1 h). (B) Recordings of the contractile force at slow speed in the same experiment as that in A. Note that the diastolic resting force increases dramatically after 30 min in the presence of 3 mM H_2O_2 (A-d and B).

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 diseased human ventricular myocardium is shown on the right of Fig. 1A. H_2O_2 treatment caused a concentration-dependent reduction of the pH_i, and the highest concentration of H_2O_2 (3 mM) resulted in a large irreversible pH_i reduction. The histogram (Fig. 1B) shows the mean H_2O_2-induced pH_i reduction for five experiments similar to that shown in Fig. 1A. The results clearly show that the H_2O_2-induced intracellular acidosis is concentration-dependent in the range of 0.03 to 3 mM.

This is the first demonstration of an H_2O_2-induced pH_i reduction in diseased human ventricular myocardium, similar to that found in animal cells, such as cultured rat cardiac myoblasts (Wu et al., 1996) and rat cerebellar astrocytes and C6 glioma cells (Tsai et al., 1997).

3.2. The effects of antioxidants on the intracellular pH and H2O2-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 diseased human ventricular 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 (Ide et al., 2000), both alone or in combination, before addition of 3 mM H_2O_2. Fig. 2A-a shows a control experiment demonstrating that treatment with 3 mmol l^{-1} H_2O_2 resulted in a time-dependent pH_i reduction (approximately 0.20 and 0.52 pH units after 5 and 10 min, respectively). As shown in Fig. 2A-b, 1 mM L-methionine alone had no significant effect on the intracellular acidosis induced by H_2O_2. However, 10 mM *N*-MPG significantly inhibited the H_2O_2-induced acidosis (reduction of only 0.2 pH units 30 min after H_2O_2 addition (Fig. 2A-c) and no additive effect was seen using the combination of 10 mM *N*-MPG and 1 mM L-methionine (Fig. 2A-d). Note that the H_2O_2-induced intracellular acidosis was reversible only after *N*-MPG pretreatment. The summarized results for seven experiments similar to that in Fig. 2A are shown in the histogram in Fig. 2B. The pH_i changes after 30 min treatment with the oxygen free radicals scavengers, *N*-MPG, L-methionine, and the combination of *N*-MPG and L-methionine (no exposure to H_2O_2) were -0.16 ± 0.02 ($p < 0.05$), 0 ± 0.01; -0.21 ± 0.03 ($p < 0.05$), respectively ($n = 7$).

3.3. Electrophysiological and mechanical effects of antioxidants and H2O2

To examine the relationship between the H_2O_2-induced pH_i disturbances and cardiac dysfunction, parallel experi-

Table 1
Effects of H_2O_2 and antioxidants on action potential characteristics and force in the diseased human ventricular myocardia in Tyrode solution (3%CO_2/HCO_3^-; $pH_o = 7.4$)

	n	APA (mV)	MDP (mV)	APD_{50} (ms)	APD_{90} (ms)	V_{max} (V/s)	Force change (mg)	DRF change (%)
Control	5	107 ± 5	−86 ± 5	148 ± 35	260 ± 36	267 ± 89	125 ± 42	
H 10′	5	93 ± 6[a]	−81 ± 3	153 ± 27	295 ± 40	203 ± 98	271 ± 69[a]	67 ± 36[a]
H 20′	5	61 ± 31[a]	−52 ± 12[a]	210 ± 21[a]	335 ± 32[a]	40 ± 40[a]	269 ± 113[a]	128 ± 17[a]
H 30′	5	0 ± 0[a]	−36 ± 16[a]	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]	275 ± 68[a]
Control	5	101 ± 6	−85 ± 5	156 ± 19	296 ± 27	259 ± 68	118 ± 24	
L 30′	5	97 ± 4	−78 ± 7	178 ± 21	312 ± 20	213 ± 89	130 ± 2	6 ± 5
L+H 10′	4	90 ± 2[a]	−80 ± 11	197 ± 22	348 ± 52	109 ± 59[a]	204 ± 18[a]	49 ± 23[a]
L+ H 20′	4	0 ± 0[a]	−41 ± 9[a]	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]	111 ± 46[a]
L+ H 30′	4	0 ± 0[a]	−32 ± 14[a]	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]	249 ± 60[a]
Control	4	116 ± 4	−89 ± 5	150 ± 21	255 ± 18	233 ± 16	95 ± 22	
N 30′	4	117 ± 6	−84 ± 4	164 ± 16	248 ± 19	223 ± 16	104 ± 13	0 ± 0
N+H 10′	4	116 ± 5	−84 ± 4	170 ± 17	255 ± 21	229 ± 11	105 ± 14	3 ± 2
N+H 20′	4	115 ± 9	−87 ± 5	171 ± 16	273 ± 18	216 ± 21	110 ± 21	2 ± 2
N+H 30′	4	111 ± 10	−86 ± 4	176 ± 23	280 ± 15[a]	203 ± 20	112 ± 23	3 ± 2
Control	5	117 ± 8	−83 ± 7	175 ± 24	288 ± 32	237 ± 18	119 ± 34	
L+N 30′	5	121 ± 11	−82 ± 7	173 ± 23	283 ± 33	242 ± 22	124 ± 13	1 ± 0
L+N+H 10′	5	115 ± 10	−79 ± 5	207 ± 21	339 ± 28	257 ± 21	164 ± 42	2 ± 5
L+N+H 20′	5	113 ± 11	−79 ± 6	203 ± 25	324 ± 45	253 ± 26	107 ± 27	7 ± 6
L+N+H 30′	5	112 ± 14	−78 ± 10	210 ± 21	300 ± 35	200 ± 15	99 ± 39	5 ± 6

Values are the means ± S.E.M., *n* = number of preparations, APA = action potential amplitude, MDP = maximum diastolic potential, APD_{50} = action potential duration at 50% repolarization, APD_{90} = action potential duration at 90% repolarization, V_{max} = maximum upstroke velocity of phase 0 depolarization, DRF = diastolic resting force, H = H_2O_2, L-M = L-methionine, *N*-MPG, = *N*-(mercaptopropionyl)-glycine. Data were evaluated by one-way analysis of variance (one-way ANOVA) and Wilcoxon signed rank test was used to test significance.

[a] $p < 0.05$, significantly different from the control value in each group.

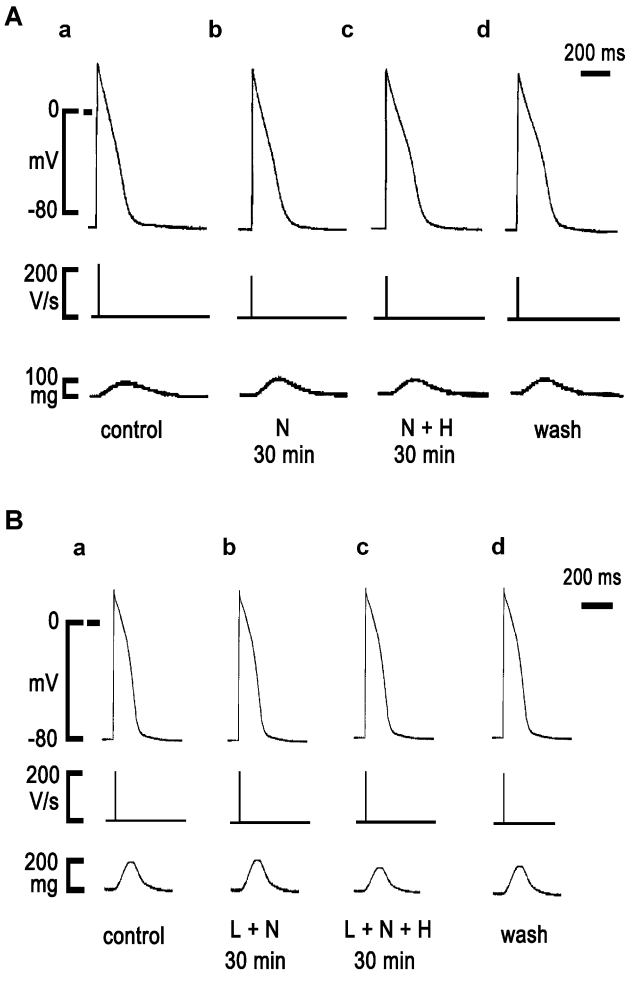

Fig. 5. Effect of adding *N*-MPG (N) alone or together with L-methionine (L) on the electrophysiological and mechanical parameters [action potential (upper trace) and its 0 phase depolarization (middle trace), and the contractile force (lower trace)] in the presence of a free radical generating system in the diseased human ventricular myocardium. (A) Recordings are (a) control, (b) in the presence of *N*-MPG (N), (c) in the presence *N*-MPG and 3 mM H_2O_2 (N + H), (d) after washout in drug-free superfusate. (B) Recorded (a) in control Tyrode solution, (b) in the presence of *N*-MPG plus L-methionine (L + N), (c) in the presence of *N*-MPG plus L-methionine (L + N) and 3 mM H_2O_2 (H), (d) after washout in drug-free superfusate.

ments on the electromechanical parameters of the human ventricular myocardium were performed. As shown in the original traces in Fig. 3A-b, 0.03 mM H_2O_2 had no obvious effect on the duration and amplitude of action potential (top trace) and contractile force (bottom trace), but reduced the depolarization of the 0 phase of the action potential (V_{max}; middle trace). In contrast, 1 mM H_2O_2 had a significant effect on the action potential characteristics, including prolongation of the APD_{50} and APD_{90} and inhibition of the V_{max} (Fig. 3A-c; top and middle traces); it also dramatically increased the contractile force and significantly elevated the diastolic resting force (bottom trace of Fig. 3A-c). Using 3 mM H_2O_2, the generation of action potential and contractile force were totally inhibited, while the diastolic resting tension showed an even more dramatic increase (Fig. 3A-d). These effects were slowly reversible after about 50 min washout in drug-free Tyrode solution (Fig. 3A-e). Similar results were obtained using another four ventricular preparations. In four out of five experiments similar to that shown in Fig. 3A, H_2O_2 had a dose-dependent biphasic effect on the contractile force, i.e. an increase at lower doses, followed by inhibition at higher doses, whereas, in the fifth preparation, only the initial increase was seen (Fig. 3B; note that each symbol represents an individual myocardium).

Fig. 4 shows that the inhibition of the electrophysiological parameters induced by H_2O_2 (3 mM) was time-dependent and the ventricular myocardium became unexcitable at about 20–30 min, a similar time-course to that seen in the pH_i experiments. In particular, as illustrated in the bottom trace of Fig. 4A, the effects of 3 mM H_2O_2 on the contractile force were not only time-dependent, but also biphasic, i.e. an increase, followed by a decrease, similar to results seen in guinea pig cardiac muscles (Satoh and Matsui, 1997). This

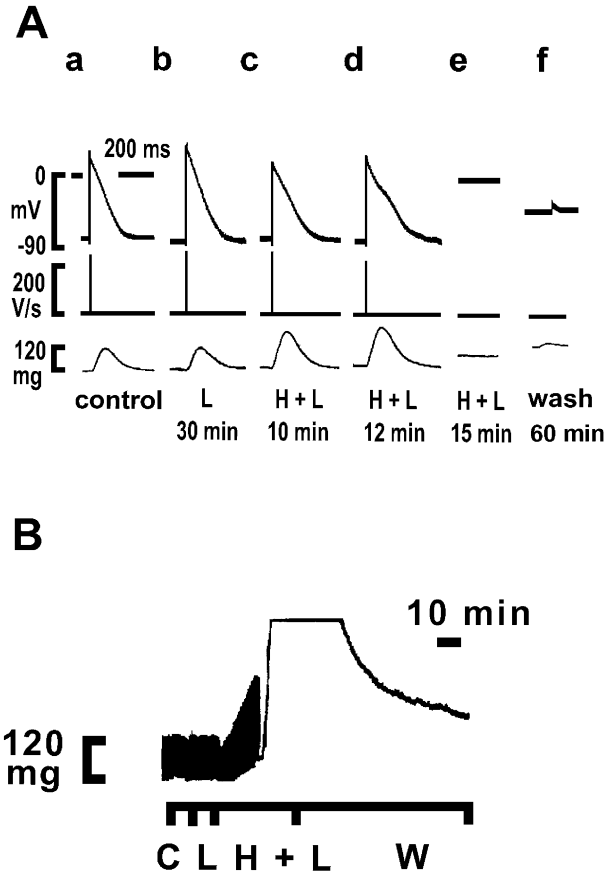

Fig. 6. Effects of L-methionine (L) on electrophysiological and mechanical parameters in the diseased human ventricular myocardium. Effect of L-methionine (1 mM) on the action potential (upper trace) and its 0 phase depolarization (middle trace), and the contractile force (lower trace). Recordings (a) in control Tyrode solution, (b) in the presence of 1 mM L-methionine, (c–e) in the presence of 1 mM L-methionine and 3 mM H_2O_2(H) for 10 (c), 12 (d) and 15 (e) min, and (f) after washout in drug-free superfusate. (B) Recordings of the contractile force at slow speed in the same experiment shown in (A).

effect can be seen more clearly in the continuous slow recording trace (Fig. 4B). The results of five similar experiments showing the time-dependent effects of 3 mM H_2O_2 on electrophysiological and mechanical parameters are summarized in Table 1.

3.4. Electromechanical effects of antioxidants on H2O2-induced injury

Since the changes induced in the electrophysiological and mechanic experiments by exposure to 3 mM H_2O_2 had a similar time-course to the pH_i changes, experiments were performed under the same conditions used in the pH_i experiments to see whether oxygen free radicals scavengers could protect human ventricular myocardium from the H_2O_2-induced-inhibitory effects on electromechanical characteristics. Pretreatment with *N*-MPG (10 mM, 30 min) itself had no significant effect on the electromechanical characteristics (Fig. 5A-b), but afforded significant protection against perfusion with H_2O_2 (3 mM for 30 min) (Fig. 5A-c; compare with Fig. 4A-d). A similar cardioprotective effect was seen using the combination of 1 mM L-methionine and 10 mM *N*-MPG (Fig. 5B). The results of several similar experiments are summarized in Table 1. In addition, as seen in the pH_i experiments, L-methionine (1 mM) had no protective effect against the H_2O_2-induced inhibition of electromechanical parameters (Fig. 6A). Fig. 6B shows that H_2O_2 in the presence of L-methionine still had a biphasic effect on the contractile force and increased the diastolic resting force in a time-dependent manner. Table 1 summarizes the data for several experiments similar to that shown in Fig. 6A and B. Moreover, as shown in Fig. 7, pretreatment with the intrinsic H_2O_2 scavenger, DBDA (1 mM), for 30 min did not prevent the H_2O_2-induced inhibitory effects on electromechanical parameters.

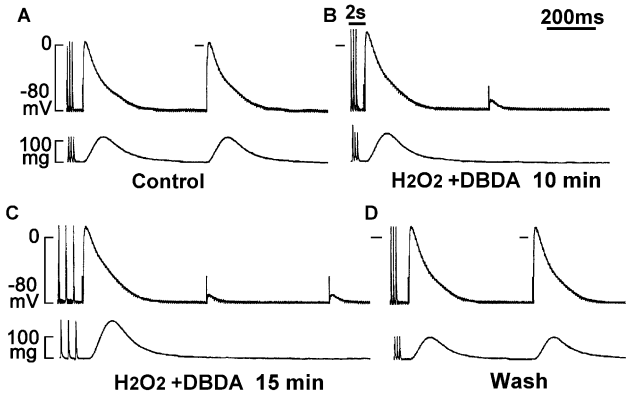

Fig. 7. Effects of DBDA on electrophysiological and mechanical parameters in the diseased human ventricular myocardium. Effect of DBDA (1mM) on the action potential (upper trace) and contractile force (lower trace). Recordings (A) in control Tyrode solution (in the presence of 1mM DBDA 30 min), (B) in the presence of 1 mM DBDA and 3 mM H_2O_2 for 10 min (C), 15 min, and (D) after washout in drug-free superfusate. Note that two recording speeds have been used, as shown above the scale bar.

4. Discussion

Using the technique of microspectrofluorimetry, we have, for the first time, demonstrated that H_2O_2 (0.1–3 mM) caused marked intracellular acidosis in the diseased human ventricular myocardium (Fig. 1A). This effect was dose- and time-dependent (Figs. 1 and 2). The underlying mechanism for the H_2O_2-induced 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. 2A-c,d), but not blocked by the HOCl scavenger, L-methionine (Fig. 2A-b,d) or the intrinsic H_2O_2 scavenger, DBDA (Fig. 7). Similar observations have been reported in animal models (Wu et al., 1996). 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 addition, our data showed that H_2O_2 (>0.1 mM) caused significant effects on electromechanical parameters, such as the APD, 0 phase depolarization, and resting membrane potential (Fig. 3), and induced biphasic increase/decrease in twitch amplitude and monotonic increase in resting tension (Figs. 3 and 4). Indeed, similar results have been obtained in guinea-pig heart muscles (Satoh and Matsui, 1997), in which the induced biphasic response was claimed by the production of ·OH free radical. Their finding is in good agreement with our results that the specific ·OH free radical scavenger, *N*-MPG, provided significant protection against the H_2O_2-induced inhibition on the electromechanical parameters (Fig. 4), whereas HOCl and intrinsic H_2O_2 scavengers (L-methionine and DBDA, respectively) did not (Figs. 5–7).

The above results strongly suggest that the intracellular ·OH free radical is responsible not only for the H_2O_2-induced pH_i acidification, but also for the induced electromechanical effects. In other words, our present study in the diseased human ventricular tissue is in agreement with the hypothesis that ·OH injury plays a critical role in the development of ischemia/reperfusion-induce cardiac injury, such as stunning (Bolli, 1990; Farber et al., 1988; Goldhaber et al., 1989; Kloner et al., 1989). Moreover, the oxygen free radicals-induced time-dependent changes in our present study also support the report that the initial burst of oxygen free radicals generation peaks 2 min after reflow, and production continues for up to 3 h (Kaplinsky et al., 1981; Zwier et al., 1987; Bolli, 1990). However, whether the present result derived from diseased ventricular tissues (heart failure) is different from that of healthy ones require further study to clarify. Also, note that the large S.E.M. was commonly existed in different groups of our present experiments, as shown in the Table 1. Although it seems to us that the large variation was simply due to the nature biological difference among different samples, the other possibility, such as the influence of disease condition, require further

study. Moreover, the other possibilities that may contribute to cardiac stunning, such as NO (Hattler et al., 1994), cytokines (Finkel et al., 1992), and prostaglandins (Faber and Gross, 1989), require further investigation.

In skinned cardiac fibers, Fabiato and Fabiato (1978) found that a decrease of pH_i resulted in reduced contractile force, while an increase had the opposite effect. Experiments in mammalian cardiac Purkinje and ventricular tissue showed that the inotropic effect of changing the pH_o was mediated by a change in the pH_i (Bountra and Vaughan-Jones, 1989; Vaughan-Jones et al., 1987). Moreover, in experiments on isolated mammalian papillary muscle, Bountra and Vaughan-Jones (1989) 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 et al. (1987) found that acidosis can increase the systolic $[Ca^{2+}]_i$. This variability in the contractile response to intracellular acidosis is due to an effect of acidosis on the intracellular Na^+ concentration. Developed tension in cardiac tissue is markedly dependent on the $[Na^+]_i$ (Eisner et al., 1984). The inotropic response of cardiac tissue to acidosis is therefore modulated by the negative inotropic effect of the $[H^+]_i$ in combination with changes in the $[Na^+]_i$, leading to changes in the $[Ca^{2+}]_i$, i.e. Ca^{2+}-overloading. Therefore, according to our present study, we propose that the ·OH-induced changes of contractile force was 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 (Wu et al., 1996). In addition to the intracellular acidification contribution to ·OH-induced myocardium injury, we should also consider other possibilities, such as (1) the competition between Ca^{2+} and H^+ for common binding sites (e.g. the intracellular buffer); (2) increased sarcolemmal permeability to Ca^{2+} ions; (3) effect on transmembrane calcium current inactivation; (4) mitochondrial impairment; (5) damage to the Ca^{2+} release channel of sarcoplasmic reticulum; and (6) activation of ATP-sensitive K^+ channel; and inhibition of the glycolytic pathway (Goldhaber et al., 1989; Holmberg et al., 1991; Satoh and Matsui, 1997; Wu et al., 1996). Moreover, given the facts that the resting pH_i was decreased by ~ 0.2 pH units during the pretreatment of *N*-MPG (Fig. 2A-c) and during that of L-methionine + *N*-MPG (Fig. 2A-d), but not in that of L-methionine alone. This indicated that *N*-MPG per se, instead of L-methionine, could slightly acidify pH_i. However, whether *N*-MPG per se acidifies the resting pH_i through its chemical structure property (i.e. with carboxylic acid group) or other mechanisms require further study.

In summary, in terms of clinical implications, the results of our present study support the idea that the underlying mechanism by which oxygen free radicals induce cardiac dysfunction is most probably the intracellular production of·OH, which damages the tissue related with the induced intracellular acidosis in combination with changes in the changes of the $[Na^+]_i$ and $[Ca^{2+}]_i$. Thus, 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. Mei-Ling Wu and Dr. Richard Vaughan-Jones for their encouragement and valuable opinions. Special thanks are also expressed 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.

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