Oxidant stress with hydrogen peroxide attenuates calcium paradox injury: role of protein kinase C and ATP-sensitive potassium channel

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Received 24 March 1997; accepted 30 September 1997

Abstract

Objective: We tested the hypotheses that low concentration of \( \text{H}_2\text{O}_2 \) attenuates the Ca\(^{2+} \) paradox (Ca\(^{2+} \) PD) injury, and that activation of protein kinase C (PKC) and/or ATP-sensitive potassium channel (K\(_{\text{ATP}} \)) are involved in the protective effects of \( \text{H}_2\text{O}_2 \).

Methods: Langendorff-perfused rat hearts were subjected to the Ca\(^{2+} \) PD (10 min of Ca\(^{2+} \) depletion followed by 10 min of Ca\(^{2+} \) repletion). Functional and biochemical effects of \( \text{H}_2\text{O}_2 \) and other interventions on the cell injury induced by the Ca\(^{2+} \) PD were assessed.

Results: In the Ca\(^{2+} \) PD hearts pretreated with 20 \( \mu \text{mol/l} \) \( \text{H}_2\text{O}_2 \), left ventricular end-diastolic pressure and coronary flow were significantly preserved. Furthermore, peak lactate dehydrogenase release was significantly decreased and ATP contents were more preserved, compared with non-treated Ca\(^{2+} \) PD hearts. \( \text{H}_2\text{O}_2 \)-treated hearts also showed remarkable preservation of cell structure. Addition of a specific PKC inhibitor, chelerythrine during \( \text{H}_2\text{O}_2 \) treatment completely abolished the beneficial effects of \( \text{H}_2\text{O}_2 \) on the Ca\(^{2+} \) PD. Similarly, an activator of PKC, Phorbol 12-myristate 13 acetate mimicked the protection by \( \text{H}_2\text{O}_2 \). Furthermore, pretreatment with a K\(_{\text{ATP}} \) opener, cromakalim also provided protection similar to \( \text{H}_2\text{O}_2 \) against the Ca\(^{2+} \) PD injury. However, a specific K\(_{\text{ATP}} \) inhibitor, glibenclamide was not able to completely block the effects of \( \text{H}_2\text{O}_2 \). Conclusions: These findings suggest that pretreatment with low concentration of \( \text{H}_2\text{O}_2 \) provides significant protection against the lethal injury of Ca\(^{2+} \) PD in rat hearts. PKC-mediated signaling pathways appear to play a crucial role in the protection against the Ca\(^{2+} \) PD injury. © 1998 Elsevier Science B.V.

Keywords: Calcium; K\(_{\text{ATP}} \) channel; Myocytes; Protein kinase C; Preconditioning

1. Introduction

It is believed that reactive oxygen species (i.e., \( \text{H}_2\text{O}_2 \), \( \cdot \text{O}_2 \), \( \cdot \text{OH} \), and \( \cdot \text{O}_3 \)) are implicated in the pathogenesis of ischemia/reperfusion injury [1,2]. These reactive species are generated during ischemia/reperfusion and cause injury to myocardial cells [3–9]. However, a brief exposure of myocytes to oxygen radicals generated by reaction of xanthine oxidase with xanthine elicits both early (60 min later) and late (24 h later) protection against anoxia/re-oxygenation injury [10]. On the other hand, extended perfusion with exogenous \( \text{H}_2\text{O}_2 \) [11] generated through xanthine and xanthine oxidase reaction [12] causes cardiac dysfunction and irreversible pathological changes. It is based on our previous study [10] that a brief exposure of cardiac cells to \( \cdot \text{O}_2 \) / \( \text{H}_2\text{O}_2 \) provides protection against subsequent prolonged ischemia. Furthermore, \( \text{H}_2\text{O}_2 \) which is produced as a result of \( \text{O}_2 \) dismutation, has been reported to cause the activation of ATP-sensitive potassium channels (K\(_{\text{ATP}} \)) [13–15] and protein kinase C (PKC) [16–18]. The accumulated experimental findings suggest that K\(_{\text{ATP}} \) and PKC are actively involved in preconditioning phenomenon [19–23].

We examined the preconditioning effects of \( \text{H}_2\text{O}_2 \) on the Ca\(^{2+} \) paradox (Ca\(^{2+} \) PD) which is a far lethal experimental model than the sustained ischemia/reperfusion. A disruption of the electrical and mechanical properties of the cells, calcified mitochondria, a loss of the cellular contents, high-energy phosphate depletion and massive accumulation of calcium within the cells are common features of the Ca\(^{2+} \) PD [24–26]. Although Ca\(^{2+} \) PD
induces more severe cellular damage than ischemia/reperfusion injury (I/R). Ca\textsuperscript{2+} PD is a useful model for investigating the pathogenesis of Ca\textsuperscript{2+} overload-associated injury which also occurs during I/R. Nevertheless, a recent evidence suggests that a brief ‘Ca\textsuperscript{2+}’ stress attenuates the Ca\textsuperscript{2+} PD as well as ischemic damage [22,25].

Accordingly, the aim of the present study was to determine whether oxidative stress by H\textsubscript{2}O\textsubscript{2} reduces the lethal injury induced by Ca\textsuperscript{2+} PD and to examine the roles of K\textsubscript{ATP} and PKC in the H\textsubscript{2}O\textsubscript{2}-mediated effects on the Ca\textsuperscript{2+} PD injury.

2. Methods

2.1. Materials

H\textsubscript{2}O\textsubscript{2}, cromakalim, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical; glibenclamide and chelerythrine chloride were obtained from Research Biochemical.

2.2. Heart preparation

Male Sprague-Dawley rats weighing 250 to 300 g were anesthetized by intraperitoneal injection of 30 mg/kg pentobarbital. After intraperitoneal injection of 500 U/kg heparin sodium, hearts were removed and retrogradely perfused through the aorta in a noncirculating Langendorff apparatus with Krebs–Henseleit (KH) buffer consisted of (mmol/l) NaCl 118, KCl 4.7, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, CaCl\textsubscript{2} 1.8, NaHCO\textsubscript{3} 25, and glucose 11. The buffer was saturated with 95% O\textsubscript{2} – 5% CO\textsubscript{2} (pH 7.4, 37°C) for 50 min. Hearts were perfused at a constant pressure of 80 mmHg. A water-filled latex balloon-tipped catheter was inserted into the left ventricle through the left atrium and was adjusted to a left ventricular end-diastolic pressure of 5 mmHg during the initial equilibration. The distal end of the catheter was connected to a pressure transducer (Gould P23Db). Cardiac function was determined using the double product of heart rate multiplied by left ventricular developed pressure (LVDP) divided by 1000 [27]. LVDP was calculated from the difference between LV peak systolic pressure and end-diastolic pressure (EDP). Heart rate, left ventricular pressure, were monitored on a Grass 7D polygraph (model 7P20, Grass Instrument, Quincy, Mass.). After perfusion with the oxygenated KH buffer for an equilibration period of 20 min, a three-way stopcock above the aortic root was used to perfuse Ca\textsuperscript{2+}–free KH buffer for 10 min, followed by KH buffer containing Ca\textsuperscript{2+} for 10 min in order to induce a typical Ca\textsuperscript{2+} PD [26]. The coronary effluent was collected in a beaker, and coronary flow (CF) was determined volumetrically at the end of equilibration, during treatment period, the Ca\textsuperscript{2+} depletion period, and at 1.5, 3, 5, and 10 min during Ca\textsuperscript{2+} repletion. Glibenclamide and chelerythrine were directly adminis-

2.3. Experimental groups

After equilibration, hearts were randomly divided into following experimental groups:

Group 1: normal control. Hearts (n = 6) were perfused for 40 min with KH buffer as a normal control for different experimental groups.

Group 2: calcium paradox (Ca\textsuperscript{2+} PD). After 20 min perfusion with normal KH buffer, hearts (n = 6) were subjected to Ca\textsuperscript{2+}-free KH buffer for 10 min followed by Ca\textsuperscript{2+} containing KH buffer for 10 min.

Group 3: Ca\textsuperscript{2+} PD + H\textsubscript{2}O\textsubscript{2}. Hearts (n = 6) were perfused with KH buffer containing 20 μmol/l H\textsubscript{2}O\textsubscript{2} for 10 min. After 10 min wash-out period, hearts were subjected to Ca\textsuperscript{2+} PD as in group 2. Cytotoxicity of H\textsubscript{2}O\textsubscript{2} had been systematically investigated in our laboratory [11,12,28]. Onodera et al. [11] examined the dose- and time-dependent effects of exogenous H\textsubscript{2}O\textsubscript{2} and showed that perfusion of 100 μmol/l H\textsubscript{2}O\textsubscript{2} for 15 min caused no significant changes in cardiac function and cell morphology. We chose 20 μmol/l H\textsubscript{2}O\textsubscript{2} for this study as the appropriate concentration for causing minimal and reversible injury.

Group 4: H\textsubscript{2}O\textsubscript{2} and activation of PKC. To determine whether the beneficial effects of H\textsubscript{2}O\textsubscript{2} were mediated by PKC activation, we used a PKC activator, PMA, in order to mimic the beneficial effects by H\textsubscript{2}O\textsubscript{2} and a specific PKC inhibitor, chelerythrine to reverse effects of PMA during H\textsubscript{2}O\textsubscript{2} treatment. Hearts were perfused with KH buffer containing both PMA and chelerythrine.

Group 4A: Ca\textsuperscript{2+} PD + PMA. Hearts (n = 6) were perfused with KH buffer containing a PKC activator, PMA (1 nmol/l) for 10 min, prior to Ca\textsuperscript{2+} PD, and continued during Ca\textsuperscript{2+} PD.

Group 4B: Ca\textsuperscript{2+} PD + H\textsubscript{2}O\textsubscript{2} + chelerythrine. The protocol was similar to that of group 3, except chelerythrine chloride (0.02 μmol/min) was infused for 20 min prior to Ca\textsuperscript{2+} depletion and repletion (n = 7).

Group 4C: Ca\textsuperscript{2+} PD + PMA + chelerythrine. The protocol was similar to that of group 5A, except that chelerythrine chloride (0.02 μmol/min) was infusively infused during treatment with PMA (n = 6).

Group 5: H\textsubscript{2}O\textsubscript{2} and activation of K\textsubscript{ATP}. To elucidate the role of K\textsubscript{ATP} in H\textsubscript{2}O\textsubscript{2}-induced protective effects, we examined whether a K\textsubscript{ATP} opener, cromakalim, could mimic the effects of H\textsubscript{2}O\textsubscript{2} and a selective inhibitor of K\textsubscript{ATP}, glibenclamide, could abolish the salutary effects of H\textsubscript{2}O\textsubscript{2}. We also tested whether the use of an optimal dose of glibenclamide plus cromakalim could reverse its salutary effects.
Group 5A: Ca$^{2+}$ PD + cromakalim. Hearts ($n = 7$) were perfused with KH buffer containing $K_{ATP}$ opener, cromakalim (20 μmol/min) for 10 min, and then the hearts were subjected to Ca$^{2+}$ PD.

Group 5B: Ca$^{2+}$ PD + cromakalim + glibenclamide. The protocol was similar to group 4A, except glibenclamide (0.01 μmol/min), was intraaortically infused ($n = 6$). A preliminary study was done to determine the appropriate concentration of cromakalim (20 μmol/min) and glibenclamide (0.01 μmol/min) with reference to LDH release and ATP preservation.

Group 5C: Ca$^{2+}$ PD + H$_2$O$_2$ + glibenclamide. The protocol was similar to that for group 3, except glibenclamide (0.01 μmol/min), was intraaortically infused for 15 min followed 5 min wash out ($n = 7$).

2.4. Measurement of LDH

LDH, an indicator of myocardial tissue injury, was determined in coronary effluent [25]. This was assayed by a coupled-enzyme spectrometric technique using a Sigma assay kit. Measurement of enzyme activity was based on oxidation of lactate and the rate of increase in absorbance at 340 nm.

2.5. Measurement of tissue ATP

The heart was immediately frozen in liquid nitrogen after Ca$^{2+}$ depletion and repletion or as specified, and was freeze-dried for 24 h. 50 to 100 mg of freeze-dried tissue was crushed in precooled glass tube, and ATP was extracted with 5 ml of cold 6% trichloroacetic acid. The extract was analyzed for ATP by spectrophotometric method [25].

2.6. Morphological examination

Heart tissue from the mid ventricular wall was taken at the end of Ca$^{2+}$ repletion, and was cut into 1.0 mm pieces which were fixed with 2.5% buffered glutaraldehyde. A semi-quantitative estimate of cell damage was carried out on 1 μm-thick sections. Three randomly chosen blocks from each heart were examined for quantification of cell damage without prior knowledge of the treatment. Approximately 500 cells were analyzed in each heart, and one of three degrees of cell damage was assigned to each cell. Cell morphology was assessed according to the following classification [22,23]: (1) normal (compact myofibers with uniform staining of nucleoplasm, well-defined rows of mitochondria between the myofibrils, and no separation of opposing intercalated discs); (2) mild damage (same as above, except some vacuoles were present adjacent to the mitochondria); (3) severe damage (reduced staining of cytoplasmic organelles, clumped chromatin material, wavy myofibers, and granularity of cytoplasm; the cells with contraction band necrosis were added in this category).

2.7. Statistical analysis

All values are expressed as means ± SEM. Group comparisons were done by ANOVA with multiple comparisons or $t$-test when appropriate. A difference of $P < 0.05$ was considered significant.

3. Results

3.1. Ca$^{2+}$ paradox

During Ca$^{2+}$ repletion of Ca$^{2+}$-depleted hearts, the reddish color of the heart was lost, indicating a release of intracellular contents; LVEDP was significantly increased and coronary flow was significantly reduced, and no beating of hearts was observed (Table 1). At the end of the Ca$^{2+}$-free period, LDH release was not different from the control values. Immediately upon Ca$^{2+}$ repletion, LDH release increased multifold and peaked at 3 min (Fig. 1).

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Equilibration</th>
<th>Post-Ca$^{2+}$ + PD</th>
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<tbody>
<tr>
<td></td>
<td>HR-P product (mmHg/min$^{-1}$)</td>
<td>LVEDP (mmHg)</td>
</tr>
<tr>
<td>Ca$^{2+}$ PD ($n = 6$)</td>
<td>26.9 ± 1.9</td>
<td>4.8 ± 2.1</td>
</tr>
<tr>
<td>Ca$^{2+}$ PD + H$_2$O$_2$ ($n = 6$)</td>
<td>25.7 ± 0.8</td>
<td>5.3 ± 0.9</td>
</tr>
<tr>
<td>Ca$^{2+}$ PD + H$_2$O$_2$ + CH ($n = 7$)</td>
<td>25.8 ± 1.5</td>
<td>5.4 ± 1.1</td>
</tr>
<tr>
<td>Ca$^{2+}$ PD + H$_2$O$_2$ + CH + GB ($n = 7$)</td>
<td>26.6 ± 0.7</td>
<td>5.8 ± 0.9</td>
</tr>
<tr>
<td>Ca$^{2+}$ PD + CM ($n = 6$)</td>
<td>27.2 ± 0.3</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td>Ca$^{2+}$ PD + CM + GB ($n = 6$)</td>
<td>28.2 ± 0.8</td>
<td>4.1 ± 2.3</td>
</tr>
<tr>
<td>Ca$^{2+}$ PD + PMA ($n = 6$)</td>
<td>27.9 ± 0.4</td>
<td>5.1 ± 4.8</td>
</tr>
<tr>
<td>Ca$^{2+}$ PD + PMA + CH ($n = 6$)</td>
<td>26.6 ± 0.5</td>
<td>5.1 ± 0.2</td>
</tr>
</tbody>
</table>

Ca$^{2+}$ PD indicates calcium paradox; H$_2$O$_2$, hydrogen peroxide; GB, glibenclamide; CH, chelerythrine; PMA, phorbol 12-myristate 13-acetate; CM, cromakalim; Post-Ca$^{2+}$ PD, 10 min after Ca$^{2+}$ repletion; LVEDP, left ventricular end-diastolic pressure; HR-P product, heart rate-pressure product, CF, coronary flow, values are mean ± SEM. * $p < 0.05$ vs. the Ca$^{2+}$ PD group; $^*p < 0.05$ vs. equilibration.
Fig. 1. Effect of various interventions on LDH release in hearts subjected to Ca\(^{2+}\) PD. \(\text{H}_2\text{O}_2\) (20 \(\mu\text{mol}/\text{l}\)), PMA (1 nmol/\text{l}), and cromakalim (20 \(\mu\text{mol}/\text{l}\)) reduced LDH release. Beneficial effect of \(\text{H}_2\text{O}_2\) was completely abolished by PKC inhibition with chelerythrine \(\text{CH}\), 0.02 \(\mu\text{mol}/\text{min}\) and not completely by the \(\text{K}_\text{ATP}\) channel blocker, glibenclamide \(\text{GB}\), 0.01 \(\mu\text{mol}/\text{min}\).

Tissue ATP contents in Ca\(^{2+}\) PD hearts were obviously depleted as compared with the control hearts (Fig. 2).

In Ca\(^{2+}\) PD hearts, most of the cells were hypercontracted, and the cell membranes were ruptured, resulting in the extrusion of intracellular contents (Fig. 3, Table 2).

3.2. Effects of \(\text{H}_2\text{O}_2\) on Ca\(^{2+}\) paradox

At the end of Ca\(^{2+}\) repletion, a significant improvement in CF and LVEDP was observed in \(\text{H}_2\text{O}_2\)-treated hearts compared to hearts subjected to Ca\(^{2+}\) PD alone. Six in 10 hearts treated with \(\text{H}_2\text{O}_2\) resumed contraction. There were no significant differences in LDH release and tissue ATP levels between beating hearts and non-beating hearts after Ca\(^{2+}\) repletion (data not shown). In \(\text{H}_2\text{O}_2\)-treated hearts, maximum LDH release was reduced significantly and ATP contents were also preserved better than control Ca\(^{2+}\) PD hearts without \(\text{H}_2\text{O}_2\) treatment (Fig. 1, Fig. 2). The cellular structure was also markedly preserved in the \(\text{H}_2\text{O}_2\)-treated hearts: 45.2 \(\pm\) 7.5% of cells were normal; 14.2 \(\pm\) 2.9% and 40.6 \(\pm\) 4.9% were mildly and severely damaged respectively; and the damage was significantly lesser than that in the Ca\(^{2+}\) PD hearts without pretreatment (Table 2, Fig. 3).

Fig. 3. Effect of different interventions on the cell morphology. A: Normal heart. Nuclei showed uniform distribution of chromatin material (arrow). Compact and well-preserved myofibers were observed. (\(\times\) 320) B: Calcium paradox heart. All cells were so hyper-contracted that the membranes were ruptured. As a result, mitochondria were extruded from the cells (arrow). (\(\times\) 320) C: \(\text{H}_2\text{O}_2\)-treated heart. Myocytes were significantly preserved compared with nontreated calcium paradox heart (B). A few severely damaged cells were also observed (arrow). (\(\times\) 320) D: Heart treated with \(\text{H}_2\text{O}_2\) and glibenclamide. Most of myocytes were severely damaged. However, the degree of hypercontraction was less than in nontreated calcium paradox hearts. (\(\times\) 320) E: Heart treated with \(\text{H}_2\text{O}_2\) and chelerythrine. All cells were damaged similar to Ca\(^{2+}\) paradox. (\(\times\) 320) F: Cromakalim-treated heart. Approximately 20% cells was normal (arrowhead) and others were severely damaged (arrow). (\(\times\) 320) G: PMA-treated heart. Most of myocytes were well preserved with the exception of some damaged cells (arrow).
repletion. Chelerythrine exacerbated the maximum LDH release and caused depletion of tissue ATP contents in 
H₂O₂-treated hearts (Fig. 1, Fig. 2). Furthermore, the
degree of cellular damage in chelerythrine-treated hearts
was almost the same as in non-treated Ca²⁺ PD hearts
(Table 2, Fig. 3). Thus, PKC inhibitor abolished the bene-
ficial effects of H₂O₂ on the Ca²⁺ PD injury.
In a separate group of experiments, we determined
whether activation of PKC mimics the beneficial effects of
H₂O₂ pretreatment on the Ca²⁺ PD injury. With PKC
Table 2

Semiquantitative estimate of morphological damage in hearts subjected to Ca\(^{2+}\) paradox after various interventions

<table>
<thead>
<tr>
<th>Group</th>
<th>Degree of cell damage (% of cells)</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Normal control</td>
<td>98.7±0.4</td>
</tr>
<tr>
<td>Ca(^{2+}) PD</td>
<td>98.8±0.2</td>
</tr>
<tr>
<td>Ca(^{2+}) PD+H(_2)O(_2)</td>
<td>45.2±7.5</td>
</tr>
<tr>
<td>Ca(^{2+}) PD+H(_2)O(_2)+GB</td>
<td>40.6±4.9</td>
</tr>
<tr>
<td>Ca(^{2+}) PD+H(_2)O(_2)+CH</td>
<td>2.0±0.9</td>
</tr>
<tr>
<td>Ca(^{2+}) PD+CM</td>
<td>20.4±4.0</td>
</tr>
<tr>
<td>Ca(^{2+}) PD+CM+GB</td>
<td>0.9±0.8</td>
</tr>
<tr>
<td>Ca(^{2+}) PD+PMA</td>
<td>40.3±5.3</td>
</tr>
<tr>
<td>Ca(^{2+}) PD+PMA+CH</td>
<td>1.5±0.4</td>
</tr>
</tbody>
</table>

Values are mean SEM. *p < 0.05 vs. Ca\(^{2+}\) PD; \#p < 0.05 vs. the other groups except PMA. Ca\(^{2+}\) PD indicates Ca\(^{2+}\) paradox; GB, glibenclamide; CH, chelerythrine; PMA, phorbol 12-myristate 13-acetate; CM, cromakalim.

activation, the effects of LV functional recovery in PMA (1 nmol/l)-treated hearts were almost the same as H\(_2\)O\(_2\)-treated hearts. After Ca\(^{2+}\) repletion, 6 in 11 hearts treated with PMA resumed beating (Table 1). The maximum LDH release and tissue ATP levels in PMA-treated hearts were similar to H\(_2\)O\(_2\)-treated hearts (Fig. 1). LVEDP, coronary flow and tissue ATP level were also preserved in PMA-treated hearts as compared with Ca\(^{2+}\) PD hearts (Table 1, Fig. 2). Thus, PMA almost mimicked the salutary effects of H\(_2\)O\(_2\) on Ca\(^{2+}\) PD. Morphological examination of PMA-treated hearts also supported the biochemical findings (Table 2, Fig. 3). However, these beneficial effects by PMA were abolished by infusing chelerythrine. Chelerythrine reversed the salutary effects of H\(_2\)O\(_2\) on the Ca\(^{2+}\) PD injury which was almost similar to those in hearts treated with PMA plus chelerythrine (Table 2, Figs. 1 and 2).

3.4. Effects of H\(_2\)O\(_2\) and K\(_{ATP}\) activation on Ca\(^{2+}\) paradox injury

A lower concentration of glibenclamide (0.001 \(\mu\)mol/min) did not affect the H\(_2\)O\(_2\)-induced protection (Fig. 4). The maximum inhibitory effect of glibenclamide on H\(_2\)O\(_2\)-treatment was observed at 0.01 \(\mu\)mol/min, however, this concentration of glibenclamide could not completely reverse the H\(_2\)O\(_2\)-induced beneficial effects (Figs. 1, 2 and 4). A significant reduction in maximum LDH release and increased tissue ATP preservation were also observed in glibenclamide-treated hearts compared to control Ca\(^{2+}\) PD. However, ATP values were significantly less compared to H\(_2\)O\(_2\)-treated hearts (Fig. 2). The morphological findings are also in agreement with the above observations (Table 2, Fig. 3).
In separate series of experiments, we determined the effect of K\textsubscript{ATP} opener, cromakalim on the Ca\textsuperscript{2+} PD damage. The maximum effects of cromakalim were observed at 20 μmol/min (Fig. 5). This concentration of cromakalim significantly reduced LVEDP, maximum LDH release, preserved tissue ATP levels, and increased CF as compared with non-treated Ca\textsuperscript{2+} PD hearts. However, the degree of protection was less than H\textsubscript{2}O\textsubscript{2}-treated hearts (Figs. 1, 2 and 5). Morphological damage was also greater in hearts treated with cromakalim than H\textsubscript{2}O\textsubscript{2} (Table 2, Fig. 3). After glibenclamide (0.01 μmol/min) was infused in cromakalim-treated hearts, the salutary effects by cromakalim were completely reversed.

4. Discussion

The present study demonstrates that low concentration of H\textsubscript{2}O\textsubscript{2} provides cardioprotection to rat hearts subjected to Ca\textsuperscript{2+} PD. The effects of H\textsubscript{2}O\textsubscript{2} are mimicked by PMA and are completely blocked by PKC inhibitor, chelerythrine thus indicating the potential role of PKC activation in the H\textsubscript{2}O\textsubscript{2}-induced protection. Glibenclamide, a specific inhibitor of K\textsubscript{ATP} reduced but did not abolish the H\textsubscript{2}O\textsubscript{2}-induced effects. However, cromakalim, a specific K\textsubscript{ATP} opener yielded less protection than H\textsubscript{2}O\textsubscript{2} or PMA. These findings suggest that K\textsubscript{ATP}, at least partly, acts as an end effect of H\textsubscript{2}O\textsubscript{2}-induced protection.

A number of studies have indicated that the lethal cellular damage induced by Ca\textsuperscript{2+} influx during Ca\textsuperscript{2+} repletion phase (see review [24]). Therefore, H\textsubscript{2}O\textsubscript{2}-induced protection appears to be due to attenuation of prominent increase in [Ca\textsuperscript{2+}], during Ca\textsuperscript{2+} PD. In the Langendorff-perfused rat hearts, Nayler et al. [29] studied the mechanism of the Ca\textsuperscript{2+} entry into the myocardium during Ca\textsuperscript{2+} PD and suggested that Ca\textsuperscript{2+} entry consisted of at least two components, the transient initial component through voltage-dependent Ca\textsuperscript{2+} channel and the second component through Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger. Furthermore, they suggested that the major increase of Ca\textsuperscript{2+} influx occurs via the Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger. Using isolated myocytes, Lambert et al. [30] also reached the same conclusions as Nayler and colleagues.

It has been proposed that the activation of K\textsubscript{ATP} leads to the shortening of action potential duration subsequent to increased K\textsuperscript{+} efflux and decreases Ca\textsuperscript{2+} influx via voltage-gated Ca\textsuperscript{2+} channels and consequently cellular damage is reduced [19]. Recently, Behring et al. [31] have demonstrated that K\textsubscript{ATP} openers depressed the increase in cytosolic Ca\textsuperscript{2+} during sustained ischemia and reperfusion as determined by NMR and this effect was correlated with attenuation of ischemic contracture. We showed that the effects of cromakalim on Ca\textsuperscript{2+} were less pronounced than H\textsubscript{2}O\textsubscript{2}. This result indicates that Ca\textsuperscript{2+} extrusion by mechanisms associated with K\textsubscript{ATP} during Ca\textsuperscript{2+} PD is not sufficient enough to yield the same protective effects as H\textsubscript{2}O\textsubscript{2} or PMA on the Ca\textsuperscript{2+} PD. In addition, the partial attenuation of H\textsubscript{2}O\textsubscript{2}-mediated effect by K\textsubscript{ATP} inhibitor further suggests that H\textsubscript{2}O\textsubscript{2} also activates K\textsubscript{ATP}.

PKC [32] is believed to be an important regulatory enzyme of the myocardium under both normal and abnormal conditions. The abolition of H\textsubscript{2}O\textsubscript{2}-mediated protection by PKC inhibition strongly points out the pivotal role of PKC in signal transduction pathways leading to protection against the Ca\textsuperscript{2+} PD. This is further strengthened by the fact that the protection can be duplicated by PMA before induction of the Ca\textsuperscript{2+} PD. The pathway by which H\textsubscript{2}O\textsubscript{2} activates PKC is not clear. It is known that H\textsubscript{2}O\textsubscript{2} activates PKC [16–18], although the mechanism by which H\textsubscript{2}O\textsubscript{2} activates PKC is not clear. Gopalakrishna et al. [16] demonstrated that treatment with low concentration of H\textsubscript{2}O\textsubscript{2} for a brief period increases PKC activity, which declined after longer periods of H\textsubscript{2}O\textsubscript{2}-exposure. H\textsubscript{2}O\textsubscript{2} is known to increase [Ca\textsuperscript{2+}]\textsubscript{i} through various mechanisms [33]. We have recently demonstrated that a transient rise in Ca\textsuperscript{2+} as a result of Ca\textsuperscript{2+} preconditioning [22,23] mediates the translocation of PKC-α and PKC-δ, which seem to participate in different signal transduction pathways leading to cardiac protection against lethal injury associated with the Ca\textsuperscript{2+} overload [25] and ischemia [22,23]. The role of PKC in preconditioning has been fairly established by several investigators [20–22,33,35]. There is likelihood that PKC stimulation influences various membrane ion channels which are important in regulating the ions movement.

Activation of K\textsubscript{ATP} by PKC has recently been reported by several investigators [33–35]. Light et al. [35] suggests that PKC can activate K\textsubscript{ATP} in physiological level of tissue ATP concentration as observed in brief (within 5 min) preconditioning ischemia, although K\textsubscript{ATP} usually opens subsequent to decrease in tissue ATP level [36]. Treatment with low concentration of H\textsubscript{2}O\textsubscript{2} as well as brief ischemia hardly influences tissue ATP contents [11]. Thus, the mechanism of protection by H\textsubscript{2}O\textsubscript{2} may be due to its direct activation of K\textsubscript{ATP} or indirectly mediated by PKC. However, H\textsubscript{2}O\textsubscript{2}-induced signaling pathway appears to be more complicated, since our results show that the protection was blocked totally by chelerythrine but only partial loss of protection was observed with glibenclamide. Thus it appears that PKC activation by H\textsubscript{2}O\textsubscript{2} contributes to the cardioprotection through the opening of K\textsubscript{ATP} as well as other mechanisms. For example, the activation of PKC results in decrease in [Ca\textsuperscript{2+}]\textsubscript{i} through the modification of L-type Ca\textsuperscript{2+} channels [37] and SR Ca\textsuperscript{2+}-ATPase [38]. Furthermore, activated PKC might indirectly reduce Ca\textsuperscript{2+} influx through Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger. PKC enhances Na\textsuperscript{+}–K\textsuperscript{+} ATPase activity [39], which is usually depressed by severe myocardial injury, leading to decrease in Na\textsuperscript{+} accumulation [40]. The latter mechanism may reduce the Ca\textsuperscript{2+} influx through Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger in myocardium subjected to the Ca\textsuperscript{2+} PD.
It is generally believed that the massive production of oxygen radicals after sustained ischemia and reperfusion is involved with the pathogenesis of ischemia/reperfusion injury. However, exposure of hearts to lower concentration of reactive oxygen species before prolonged ischemia may prime hearts with alterations of ion channels through PKC activation, resulting in protection. A recent report from Tritto et al. [41] in which ischemic preconditioning was induced by low concentration of oxygen radicals and was blocked with oxygen radical scavengers supports our hypothesis that low dose of oxygen radicals plays a crucial role in cardioprotection by preconditioning phenomenon.

In summary, we have demonstrated that H$_2$O$_2$ triggers the protective responses and that PKC plays a crucial role in the signal transduction pathways leading to protection against the lethal cellular injury induced by the Ca$^{2+}$ PD.

Acknowledgements

This study was supported in part by NIH research grants HL23597 and HL55678 from the National Heart, Lung and Blood Institute. The authors thank Atif Ashraf for technical assistance.

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