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Effects of hydrogen peroxide on membrane fluidity and $Ca^{(2+)}$-transporting ATPase activity of rabbit myocardial sarcoplasmic reticulum[1]

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ABSTRACT This study was to investigate the effects of hydrogen peroxide on membrane fluidity and Ca^{2+}-ATPase activity of rabbit myocardial sarcoplasmic reticulum (SR). The membrane fluidity of SR was monitored by measuring the changes in the steady state fluorescence anisotropies (r_s) using diphenylhexatriene as a probe. The Ca^{2+}-ATPase activity was determined by assaying the amount of inorganic phosphate (P_i) released from ATP. It was found that the membrane fluidity (r_s; 0.154 ± 0.014 *vs* 0.113 ± 0.010, $P < 0.01$) and Ca^{2+}-ATPase activity (3.1 ± 1.3 *vs* 25.3 ± 2.4 μmol $P_i \cdot h^{-1}$/mg protein, $P < 0.01$) were reduced in SR exposed to H_2O_2 (2 mmol·L^{-1}) for 40 min. Catalase 20 μg·ml^{-1} completely prevented the SR damages caused by H_2O_2. H_2O_2 jeopardized the SR in a concentration- and time-dependent manner as measured by changes in r_s values and Ca^{2+}-ATPase activities, which were negatively correlated ($r = 0.981$, $P < 0.01$). These results suggest that H_2O_2 produces dysfunctions of the rabbit myocardial SR, and that the alteration of membrane fluidity may be one of the mechanisms responsible for the decrease of Ca^{2+}-ATPase activity.

KEY WORDS myocardium; sarcoplasmic reticulum; hydrogen peroxide; membrane fluidity; $Ca^{(2+)}$-transporting ATPase

Hydrogen peroxide is a well known oxidant and plays a key role in myocardial ischemia and reperfusion injury[1-3]. The processes that have been proposed as factors inperoxide-induced cell injury include decrease in ATP concentration, disturbances of intracellular Ca^{2+} homeostasis, and an increase in oxidized sulfhydrils. H_2O_2 could reduce the membrane fluidity of erythrocytes[4] and cardiomyocytes[5]. But the detailed mechanisms of cell damage by H_2O_2 are up to now not fully clarified. Nothing is known about the effects of H_2O_2 on SR membrane fluidity, and information about the effects of H_2O_2 on Ca^{2+}-ATPase activity remain limited. This study was undertaken to observe the alterations of membrane fluidity and Ca^{2+}-ATPase activity in SR when exposed to H_2O_2.

MATERIALS AND METHODS

Isolation of myocardial sarcoplasmic reticulum 11 rabbits (of either sex, weighing $2.6 \pm s$ 0.3 kg) were stunned and the hearts were rapidly excised and placed in ice-cold $NaHCO_3$ 10 mmol·L^{-1}. The left ventricles were trimmed of epicardium, endocardium, fat, and visible blood vessels. The myocardial tissue was minced and homogenized (1 g tissue in 5 vol $NaHCO_3$ 10 mmol·L^{-1}) thrice for 30 s with a ZS83-1 tissue homogenizer at a setting of half-maximal speed. The homogenate was centrifuged twice for 20 min at 14 000 $\times g$ at 4℃ in a RP-83T rotor of Hitachi ultracentrifuge to remove the nuclei, cell debris, and mitochondria. The supernatant from the second spin was centrifuged at 45 000 $\times g$ for 30 min. The resulting pellets were resuspended in 10 ml of KCl 0.6 mol·L^{-1}, histidine 30 mmol·L^{-1} at pH 7.0, and then sedimented again at 45 000 $\times g$ for 30 min. The final pellets were gently resuspended in 1 ml tris-chloride 10 mmol·L^{-1} (pH 7.0). The SR-enriched suspension was immediately stored at −40℃ for measurements of Ca^{2+}-ATPase activity and membrane fluidity within 24 h[6,7]. The protein content was determined colorimetrically[8], using bovine serum albumin as the reference standard.

Measurement of membrane fluidity Membrane fluidity of SR was monitored by the steady state fluorescence anisotropies (r_s). The fluorescence probe 1,

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6-diphenyl-1, 3, 5-hexatriene (DPH, 2 μmol·L^{-1}, in tetrahydrofuran) was added to 1 ml SR suspension (200 μg protein) to a final probe concentration of 1 μmol·L^{-1} and incubated at 37℃ for 3 h. The r_s value of DPH was measured using a M-850 spectrofluorometer (Hitachi, Japan) equipped with a temperature-controlled, thermally jacketed cell holder, λ_{ex} 360 nm and λ_{em} 430 nm. The r_s value was calculated by the formula: $r_s=[I_{VV}-G\,I_{VH}]/[I_{VV}+2G\,I_{VH}]$, where G= instrument correction factor, I_{VV} and I_{VH} = vertical and horizontal emission intensity respectively, when exciting light was vertically polarized[9].

Assay of Ca^{2+}-ATPase activity The ATPase activity was determined by colorimetric method measuring the inorganic phosphate (P_i) released from ATP[10]. For the assay of ATPase activity, SR vesicles (20 μg protein) were preincubated at 37℃ for 10 min in 0.4 ml of reaction medium containing histidine 50, (pH 7.4), $MgCl_2$ 3, KCl 111, EGTA 1 mmol·L^{-1}, and calcimycin 3 μg·ml^{-1} in the absence or presence of $CaCl_2$ 0.6 mmol·L^{-1}. The reaction was initiated by the addition of tris ATP 5 mmol·L^{-1} and terminated after 15 min by the addition of 2.5 ml stop solution which contained sodium bisulfite 4.3, *p*-methylaminophenol sulfate 1.4, ammonium molybdate 3.6 mg·ml^{-1}, and H_2SO_4 360 mmol·L^{-1}. The reagents in stop solution were also required for colorimetric determination of P_i. After 15 min at 25℃, the absorbance was read at 660 nm. The reaction rate in the absence of $CaCl_2$ was subtracted from that in the presence of $CaCl_2$ to obtain the Ca^{2+}-ATPase activity (μmol·P_i·h^{-1}/mg protein).

To observe the effects of catalase on H_2O_2-mediated alterations in membrane fluidity and ATPase activity, the SR was incubated with or without 2 mmol·L^{-1} H_2O_2 in the presence or absence of catalase (20 μg·ml^{-1}) for 25 min at 37℃.

Reagents ATP (Tris salt), calcimycin, and EGTA were obtained from Sigma Chemical Co, USA. DPH was purchased from Fluka AG, Switzerland. All other chemicals were of AR.

Statistics Results were presented as $\bar{x}\pm s$. Statistical differences between groups were determined by one-way ANOVA or *t* test.

RESULTS

Effects of H_2O_2 on membrane fluidity of SR The SR (200 μg protein·ml^{-1}) was incubated with various concentrations of H_2O_2 for a required period of time prior to measurement of fluorescence anisotropy. The value of r_s for DPH was elevated by H_2O_2 0.5 mmol·L^{-1}, followed by a further gradual increase in a concentration-dependent manner (Fig 1). The r_s value was increased after 15 min of incubation with H_2O_2, followed by a further slight increase in a time-dependent manner (Fig 2). Since the r_s value varies inversely with the membrane fluidity, the membrane fluidity of SR was shown to be reduced by H_2O_2 in a concentration- and time-dependent fashion.

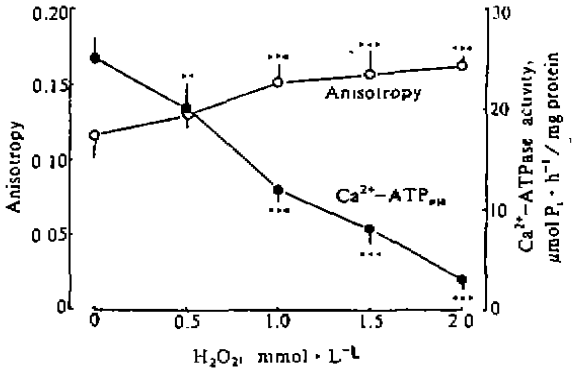

Fig 1. Effects of H_2O_2 on fluorescence anisotropy and Ca^{2+}-ATPase activity of rabbit myocardial SR. The SR was incubated with H_2O_2 for 40 min. $n=6$ or 11 rabbits, $\bar{x}\pm s$. $^{**}P<0.05$, $^{***}P<0.01$ vs control.

Effects of H_2O_2 on Ca^{2+}-ATPase activity of SR Before assaying the Ca^{2+}-ATPase activity, SR was incubated at 37℃ in reaction medium with or without H_2O_2. The Ca^{2+}-ATPase activity was depressed with H_2O_2 0.5 mmol·L^{-1} followed by a continuous decline in a concentration-dependent manner (Fig 1). H_2O_2 also produced a time-dependent inhibition on ATPase activity of SR (Fig 2). A negative correlationship was found between r_s values and ATPase activities of SR.

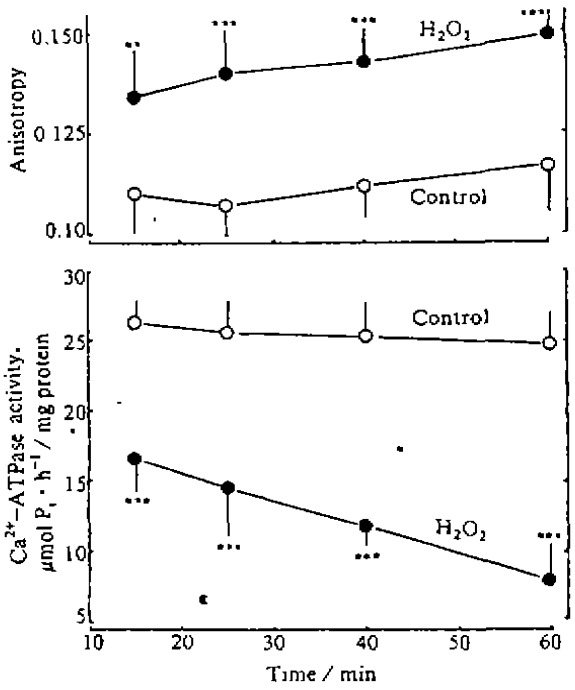

Fig 2. Effects of H_2O_2 (1 mmol·L^{-1}) on fluorescence anisotropy and Ca^{2+}-ATPase activity of rabbit myocardial SR. $n=5-6$, $\bar{x}\pm s$. $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

Effect of catalase on H_2O_2-mediated SR injury H_2O_2 increased the r_s values and decreased the Ca^{2+}-ATPase activity dramatically. These changes were abolished completely by catalase (Tab 1).

Tab 1. Effect of catalase (20 μg·L^{-1}) on H_2O_2 (2 mmol·L^{-1})-mediated sarcoplasmic reticulum injury. $n=5-6$ rabbits, $\bar{x}\pm s$. $^{*}P>0.05$, $^{*}P<0.01$ *vs* control. $^{\#}P<0.01$ *vs* H_2O_2.

Group	Fluorescence anisotropy	Ca^{2+}-ATPase activity, μmol Pi·h^{-1}/ mg protein
Control	0.113±0.010	25.3±2.4
H_2O_2	0.154±0.014^{c}	3.1±1.3^{c}
H_2O_2+catalase	0.118±0.011^{d}	23.2±3.0^{d}

DISCUSSION

The present work proved that the membrane fluidity, as reflected by decreases in the r_s values for DPH incorporated into the SR, was reduced by H_2O_2 attack in a concentration- and time-dependent fashion. These results were consistent with those observed in other biological membranes such as human erythrocyte membrane[4] and myocardial membranes[5,9].

There were contradictory experimental data regarding the changes in Ca^{2+}-ATPase activity after H_2O_2 exposure. Results in our study were consistent with those observed by Kukreja *et al*[11] and Scherer *et al*[12], but were in contrast with those obtained by Rowe *et al*[13] and Kaneko *et al*[14]. Rowe *et al*[13] found that exogenous H_2O_2 could uncouple Ca^{2+} transport from ATP hydrolysis leading to depression of Ca^{2+} uptake by SR. However, the Ca^{2+}-ATPase activity was not inhibited by H_2O_2. Kaneko *et al*[14] reported that Ca^{2+}-ATPase activity in sarcolemmal membranes was stimulated by H_2O_2. Lipid peroxidation and oxidation of SH groups were responsible for alterations in Ca^{2+}-ATPase activity[11-14]. Our results suggest another plausible explanation that the decrease of membrane fluidity may be related to the inhibition of SR Ca^{2+}-ATPase activity, since optimal membrane function requires the membrane to be in an adequately fluid state and alterations in fluidity interfere with the activity and kinetics of membrane-bound enzymes[15]. Furthermore, our results indicated that there was a good correlation between r_s values (representing the degree of membrane fluidity) and Ca^{2+}-ATPase activities in SR damaged by H_2O_2. It was, therefore, conceivable that the decrease of fluidity was another important mechanism contributing to the decrease of Ca^{2+}-ATPase activity of

SR attacked by H_2O_2.

In conclusion, these results demonstrate that H_2O_2 can directly cause dysfunction of rabbit myocardial SR, and that the decrease of membrane fluidity may be one of the mechanisms responsible for the decrease of Ca^{2+}-ATPase activity.

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393-396 (3)

过氧化氢对兔心肌浆网膜流动性及钙-转移腺苷三磷酸酶活性的影响

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摘要 用荧光偏振技术测定兔心肌浆网 (SR) 膜流动性，定磷法测定 Ca^{2+}-ATPase 活性，过氧化氢(H_2O_2)显著降低 SR 膜流动性和 Ca^{2+}-ATPase 活性；荧光各向异性值与 Ca^{2+}-ATPase 活性变化呈负相关；过氧化氢酶(20 $\mu g \cdot L^{-1}$)完全取消 H_2O_2 (2 $mmol \cdot L^{-1}$)对 SR 的损伤作用，表明 H_2O_2 能直接损伤 SR，膜流动性改变可能是 Ca^{2+}-ATPase 活性下降的原因之一.

关键词 心肌；肌浆网；过氧化氢；膜流动性；钙-转移腺苷三磷酸酶