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Attenuation of myocardial injury due to oxygen free radicals (OFR) by pretreatment with OFR or calcitonin gene-related peptide[1]

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KEY WORDS electrolysis; reactive oxygen species; calcitonin gene-related peptide; protein kinase C; heart function tests; hemodynamics

AIM: To study the cardioprotective effects of oxygen free radicals (OFR) and calcitonin gene-related peptide (CGRP) pretreatment on myocardial damages due to OFR in isolated perfused rat heart. **METHODS:** The hearts were perfused in a Langendorff mode. OFR were generated by electrolysis of Krebs-Henseleit (K-H) solution. **RESULTS:** OFR pretreatment reduced the impairment of cardiac contractile function, the decrease of coronary flow and the increase of creatinine kinase (CK) release due to OFR, and the effect exhibited period dependence and cycle-dependence. 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), an inhibitor of protein kinase C, abolished the protection of OFR pretreatment (CK release = 110 ± 7, 215 ± 23, 169 ± 14, 240 ± 30, and 113 ± 19 $kU \cdot L^{-1}$ for control, OFR, OFR pretreatment, OFR pretreatment plus H-7, and H-7, respectively). CGRP pretreatment also protected the myocardium damages elicited by OFR in isolated perfused rat heart. **CONCLUSIONS:** OFR or CGRP pretreatment protected myocardium against injury elicited by OFR, and the effect of OFR pretreatment was related to the activation of PKC.

Ischemic preconditioning was defined as a resistance of myocardium to a subsequent more prolonged period of ischemic damage after the heart was insulted one or more brief periods of ischemic stress[1]. The cardioprotection of ischemic preconditioning may be due to the releases of endogenous myocardial protective substances[2].

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Ischemia-reperfusion injury is related to the excessive production and accumulation of oxygen free radicals (OFR). The cardioprotective effect of ischemic preconditioning was abrogated by superoxide dismutase (SOD), a scavenger of superoxide anion, and a brief exposure to low concentrations of reactive oxygen metabolisms such as superoxide anion and H_2O_2 reduced infarct size in the rabbit heart[3]. Thus, OFR may play an important role in the mediation of ischemic preconditioning.

Calcitonin gene-related peptide (CGRP), a 37-amino acid neuropeptide, was a principal transmitter in sensory nerves and widely distributed in the cardiovascular system. Besides vasodilation, CGRP protected the blood vessel endothelial cell and ischemic myocardium[4,5]. CGRP pretreatment prevented ischemia-reperfusion damage in isolated rat heart[6]. The present study was to examine whether OFR and CGRP pretreatment protects myocardium injury elicited by OFR in isolated perfused rat heart.

MATERIALS AND METHODS

Drugs and chemicals SOD was purchased from Changsha Biochemical Factory (Changsha, China); 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) and CGRP were purchased from Sigma Chemical Co.

Isolated heart preparation Wistar rats (n = 70, 170 ± s 10 g, either sex), obtained from the Laboratory Animal Center of Hunan Medical University (Changsha, China), were decapitated. The hearts were excised and immersed in ice-cold K-H solution (4 ℃). The aorta was mounted onto a cannula, and retrogradely perfused with K-H solution (37 ℃, pH 7.4) at 10 kPa aerated with 95 % O_2 + 5 % CO_2, according to the modified Langendorff procedure[7].

Two platinum electrodes were placed on the right atrium. A square wave electronic stimulator (SEN-3201, Nihon Kohden, Japan) was used to pace the hearts at 250 beats $\cdot$ min^{-1} with pulse of 4 ms and 3 V. A small latex balloon on the tip of a polyethylene tube (PE 50) was inserted across the mitral valve into the left ventricle, and connected to

pressure transducer and a polygraph recorder (LMS-2B, Chengdu Instrument Factory, China) for measurement of left ventricular pressure (LVP) and its first derivative (LV dp/dt_{max}). The left ventricular diastolic end pressure was adjusted to 0.66 kPa and this balloon volume was maintained. Coronary flow (CF) was measured by timed collection of coronary effluent for measurement of creatinine kinase (CK).

Generation of OFR OFR were generated by electrolysis of K-H solution[8]. Two platinum electrodes were placed into the flow tract above the heart, the anode was placed 4 cm and the cathode was 8 cm above the aorta. A stable direct current generated by a square wave stimulator was applied.

Creatinine kinase assay Myocardial injury was monitored by assaying CK level from hearts 40 min after OFR damages. The activity of CK was assayed by a spectrophotometer (751 G, Shanghai Analytic Instrument Factory, China).

Experimental protocols Hearts were equilibrated for 20 min before experiment. (1) Control group was perfused with K-H solution for 60 min. (2) OFR damage group was perfused with K-H solution subjected to electrolysis with 5 mA for 30 s, followed by K-H solution for 40 min. (3) OFR pretreatment group was perfused with K-H solution subjected to electrolysis with 1 mA for 10 - 30 s, followed by a 10-min fresh K-H solution before OFR damages. For the studies on the protective effects of pretreatment with OFR at various concentration, the OFR-treated hearts were further divided into (a) OFR pretreatment 1 (OFR-P 1) (10 s, one cycle); (b) OFR-P 2 (10 s, two cycles); (c) OFR-P 3 (20 s, one cycle); (d) OFR-P 4 (20 s, two cycles); (e) OFR-P 5 (30 s, one cycle); and (f) OFR-P 6 (30 s, two cycles). In the case of pretreatment with two cycles of electrolysis, about 10 min was allowed between perfusions with electrolyzed K-H solution for the contractile function to return to the stable level. (4) H-7 treatment group was perfused with H-7 5 $nmol \cdot L^{-1}$ for 10 min followed by fresh K-H solution for 50 min. (5) OFR damage plus SOD group was perfused with SOD 200 $kU \cdot L^{-1}$ for 5 min before OFR damages and for 5 min further. (6) OFR pretreatment plus SOD group was perfused with SOD 200 $kU \cdot L^{-1}$ for 5 min before OFR pretreatment (1 mA, 30 s) and for 5 min further. (7) OFR pretreatment plus H-7 group was perfused with H-7 5 $nmol \cdot L^{-1}$ for 5 min before OFR pretreatment and for 5 min further. (8) OFR damages plus CGRP pretreatment group was perfused with CGRP 5 $nmol \cdot L^{-1}$ for 5 min and perfused with fresh K-H solution for 10 min before OFR damages.

Statistics All the data were analyzed using ANOVA and the Newman-Keuls t test.

RESULTS

Effect of OFR pretreatment on myocardium damages due to OFR In the control group, continuously perfused rat hearts were observed for 60 min. There were no changes in contractile function (LVP, LV dp/dt_{max}) and CF. In the OFR damage group contractile function and CF were decreased, and CK release was increased ($P < 0.01$ *vs* control) (Tab 1).

OFR pretreatment group showed a slight decrease in the contractile function and CF compared with baseline value [LVP (kPa) = 15.7 ± 0.8 *vs* 14.4 ± 1.3 ($P > 0.05$), 16.1 ± 1.5 *vs* 13.5 ± 1.1 ($P < 0.05$) and 15.9 ± 1.1 *vs* 12.6 ± 1.6 ($P < 0.01$), LV dp/dt_{max} ($kPa \cdot s^{-1}$) = 749 ± 26 *vs* 689 ± 52 ($P < 0.05$), 756 ± 76 *vs* 605 ± 53 ($P < 0.01$) and 717 ± 66 *vs* 574 ± 36 ($P < 0.01$), CF ($mL \cdot min^{-1}$) = 11.4 ± 0.8 *vs* 10.6 ± 0.6 ($P > 0.05$), 10.8 ± 0.7 *vs* 9.4 ± 1.0 ($P < 0.05$) and 10.2 ± 0.9 *vs* 7.6 ± 1.2 ($P < 0.01$) for OFR-P 1, OFR-P 3 and OFR-P 5, respectively, $n = 5$]. The duration of the effects of OFR pretreatment was about 1 - 4 min.

OFR pretreatment (1 mA, 10 - 30 s) attenuated the reduction of the myocardial contractile function, CF, and the increase of CK release caused by OFR damages.

Effect of SOD on OFR pretreatment and OFR damages SOD 200 $kU \cdot L^{-1}$ reduced OFR-induced myocardial injury, including an improvement of cardiac contractile function, an increase in CF and a decrease in CK release. SOD 200 $kU \cdot L^{-1}$ abolished the cardioprotective effect of OFR pretreatment, as reappearance of inhibitory effect of cardiac function and CF, and an increased CK release by OFR damages (Tab 2).

Effect of H-7 on OFR pretreatment H-7 5 $nmol \cdot L^{-1}$ abolished the protection of OFR pretreatment (Tab 2).

Effect of CGRP pretreatment on OFR damages Pretreatment with CGRP 5 $nmol \cdot L^{-1}$ for 5 min abrogated the impairment of heart function and the reduction of CF by OFR damages. The increased CK release by OFR damages was reduced by pretreatment with CGRP (Tab 2).

DISCUSSION

In the present study OFR pretreatment

Tab 1. Effects of OFR pretreatment on hemodynamic changes and creatinine kinase release elicited by OFR. $\bar{x} \pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* **control**; $^dP>0.05$, $^eP<0.05$, $^fP<0.01$ *vs* **OFR damage.**

	Hearts	Before damage	Time after oxygen free radicals pretreatment/min 10	20	30	40
			Left ventricular pressure (kPa)			
Control	6	16 0±0.2	16.0±0.2	16.0±0.2	15.9±0.3	15.9±0.3
OFR damage	6	16.1±0.9[a]	4.3±0.8[c]	5.8±0.3[c]	5.4±0.3[c]	4.3±0.5[c]
OFR-P 1	5	14.9±2.3[d]	4.6±0.9[d]	6.9±2.4[d]	7.2±2.6[d]	5.9±2.1[d]
OFR-P 2	5	14.2±1.3[d]	8.3±2.4[f]	8.5±1.1[e]	9.0±0.8[f]	8.0±0.8[f]
OFR-P 3	5	14.4±0.9[d]	9.6±1.2[f]	9.9±0.8[f]	10.1±1.4[f]	9.4±1.3[f]
OFR-P 4	5	14.9±1.7[d]	9.1±2.1[f]	10.7±1.1[f]	10.2±1.1[f]	8.8±1.3[f]
OFR-P 5	5	13.8±1.4[d]	10.6±1.4[f]	10.7±2.6[f]	10.9±2.1[f]	9.6±1.8[f]
OFR-P 6	6	12.8±1.9[e]	10.7±1.6[f]	13.1±1.4[f]	11.5±1.9[f]	9.8±1.4[f]
			Left ventricle dp/dt_{max} (kPa·s^{-1})			
Control	6	740±7	740±7	740±7	740±15	740±15
OFR damage	6	730±44[a]	180±59[c]	320±44[c]	140±37[c]	215±15[c]
OFR-P 1	5	690±44[d]	200±52[d]	290±104[d]	300±126[d]	250±96[d]
OFR-P 2	5	650±44[d]	350±104[f]	450±74[e]	410±37[e]	410±59[f]
OFR-P 3	5	610±81[d]	410±67[f]	430±44[e]	430±52[e]	410±67[f]
OFR-P 4	5	630±89[d]	450±59[f]	450±52[e]	420±67[e]	410±67[f]
OFR-P 5	5	670±59[d]	470±44[f]	510±96[f]	480±74[f]	440±74[f]
OFR-P 6	6	550±118[e]	430±81[f]	540±67[f]	480±81[f]	370±59[f]
			Coronary flow (mL·min^{-1})			
Control	6	10.6±0.6	10.6±0.6	10.5±0.7	10.5±0.7	10.5±0.7
OFR damage	6	10.5±1.0[a]	3.0±0.9[c]	4.9±1.1[c]	4.2±1.1[c]	3.3±1.0[c]
OFR-P 1	5	12.5±1.4[d]	4.3±1.2[d]	5.7±1.3[d]	5.1±1.3[d]	4.2±1.4[d]
OFR-P 2	5	10.3±1.2[d]	6.5±2.1[f]	5.7±1.4[d]	4.8±0.9[d]	4.6±0.6[d]
OFR-P 3	5	11.1±1.2[d]	5.5±1.1[f]	5.2±0.9[d]	4.8±0.7[d]	4.6±0.8[d]
OFR-P 4	5	9.8±1.0[d]	5.2±0.6[f]	4.9±0.4[d]	5.2±0.5[d]	5.1±0.5[e]
OFR-P 5	5	8.8±0.9[d]	5.9±0.6[f]	5.8±1.1[d]	5.3±0.6[d]	4.7±0.4[e]
OFR-P 6	6	7.3±0.9[f]	6.0±0.4[f]	6.1±0.9[d]	5.6±0.6[d]	4.8±0.4[e]
			Creatinine kinase release (mU·min^{-1})			
Control	6					110±7
OFR damage	6					215±23[c]
OFR-P 1	5					211±25[d]
OFR-P 2	5					187±19[d]
OFR-P 3	5					180±25[d]
OFR-P 4	5					176±15[e]
OFR-P 5	5					169±14[e]
OFR-P 6	6					160±20[f]

significantly attenuated the impairment of cardiac functions, the decrease of CF, and the increase of CK release due to OFR damages in the isolated perfused rat heart. These results, together with previous observations that exposure to reactive oxygen metabolisms reduced infarct size in the rabbit heart[3], suggest that OFR can mimic the beneficial effects of ischemic preconditioning, and that OFR pretreatment possesses a protection of the myocardium against different harmful factors.

Previous investigations have suggested that the regulation of ischemic preconditioning is involved in the PKC pathway[2,9,10]. Ischemic or adenosine-induced preconditioning increased PKC activity in myocardial tissues, and the protective effects of ischemic or pharmacological preconditioning were abolished by the PKC inhibitors[2,9,10]. In this study, the cardioprotection of OFR pretreatment

Tab 2. Effect of H-7, SOD, and CGRP on hemodynamic changes and creatinine kinase release elicited by OFR. $\bar{x} \pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control; $^dP>0.05$, $^eP<0.05$, $^fP<0.01$ *vs* OFR damage; $^gP>0.05$, $^hP<0.05$, $^iP<0.01$ *vs* + OFR-P 5.

	Hearts	Before damage	Time after oxygen free radicals pretreatment/min 10	20	30	40
			Left ventricuar pressure (kPa)			
Control	6	16.0±0.2	16.0±0.2	16.0±0.2	15.9±0.3	15.9±0.3
H−7 treatment	4	16.6±1.0[a]	16.6±1.0[a]	16.5±0.6[a]	15.7±0.3[a]	15.5±0.3[a]
OFR damage	6	16.0±1.0[a]	4.3±0.8[c]	5.8±0.3[c]	5.4±0.3[c]	4.3±0.5[c]
+SOD	5	17.8±1.8[d]	13.0±0.6[f]	11.8±2.4[f]	10.9±1.8[f]	10.1±2.1[f]
+OFR P−5	5	13.9±1.3[d]	10.6±1.4[f]	12.2±2.6[f]	10.9±2.1[f]	9.6±1.8[f]
+SOD&OFR P−5	5	15.7±1.0[g]	3.4±1.4[i]	3.5±2.1[i]	4.0±2.6[i]	4.0±2.6[i]
+H−7&OFR P−5	6	15.2±3.0[g]	4.3±1.0[i]	6.4±2.2[h]	4.2±1.3[i]	4.5±1.6[i]
+CGRP pretreatment	7	16.2±0.5[d]	8.8±2.1[f]	9.8±1.3[f]	8.0±1.1[f]	7.0±1.4[f]
			Left ventricle dp/dt_{max} (kPa·s^{-1})			
Control	6	740±7	740±7	740±7	740±15	740±15
H−7 treatment	4	790±74[a]	780±44[a]	770±22[a]	725±7[a]	725±7[a]
OFR damage	6	730±44[a]	180±59[c]	320±44[c]	280±37[c]	220±15[c]
+SOD	5	790±126[d]	580±30[f]	500±111[f]	470±81[f]	430±96[f]
+OFR P−5	5	670±52[d]	470±44[f]	510±96[f]	480±74[f]	440±67[f]
+SOD&OFR P−5	5	730±52[g]	140±67[i]	150±96[i]	170±118[i]	180±126[i]
+H−7&OFR P−5	6	670±155[g]	170±52[i]	280±44[i]	163±7[i]	180±59[i]
+CGRP pretreatment	7	760±30[d]	370±89[f]	430±74[e]	370±44[e]	310±52[e]
			Coronary flow (mL·min^{-1})			
Control	6	10.6±0.6	10.6±0.6	10.5±0.7	10.5±0.7	10.5±0.7
H−7 treatment	4	11.8±1.2[a]	12.7±1.6[a]	12.3±1.7[a]	12.8±1.5[a]	12.7±1.5[a]
OFR damage	6	10.5±1.0[a]	3.0±0.9[c]	4.9±1.1[c]	4.2±1.1[c]	3.3±1.1[c]
+SOD	5	11.9±1.4[d]	7.5±0.3[f]	6.1±0.4[d]	5.8±0.4[e]	5.5±0.6[e]
+OFR P−5	5	8.8±0.9[d]	5.9±0.6[f]	5.8±1.1[d]	5.3±0.6[d]	4.7±0.4[d]
+SOD&OFR P−5	5	10.7±1.0[g]	3.0±0.6[i]	3.2±0.3[i]	3.1±0.3[i]	3.1±0.3[h]
+H−7&OFR P−5	6	9.2±2.3[g]	3.0±0.8[i]	4.2±1.0[g]	3.5±1.0[b]	2.8±0.4[i]
+CGRP pretreatment	7	12.0±1.2[d]	6.4±1.2[f]	6.5±1.1[d]	5.4±0.7[d]	4.7±0.7[d]
			Creatinine kinase release (mU·min^{-1})			
Control	6					110±7
H−7 treatment	4					113±19[a]
OFR damage	6					215±23[c]
+SOD	5					115±17[f]
+OFR P−5	5					169±14[e]
+SOD&OFR P−5	5					221±27[h]
+H−7&OFR P−5	6					240±30[h]
+CGRP pretreatment	7					175±14[e]

was also abolished by the PKC inhibitor H-7. Similar effects have been seen in the studies on the hearts pretreated with purine/xanthine oxidase or H_2O_2[3]. These results indicate that OFR pretreatment protects the myocardium via the activation of PKC.

Recently, our works have shown that in the isolated perfused rat heart CGRP pretreatment reduces myocardial damages elicited by ischemia-reperfusion[6], adriamycin[11] or endothelin[12]. In this study, pretreatment with CGRP also protected against OFR-induced myocardium injury in the isolated perfused rat heart, in further support of the conclusion that CGRP pretreatment affords the cardioprotection.

In conclusion, the present results suggest: (1) OFR pretreatment protected against myocardial damages elicited by OFR, (2) the protection of

OFR pretreatment was related to the activation of PKC, and (3) CGRP pretreatment also protected the myocardium against OFR damages in the isolated perfused rat heart.

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氧自由基或降钙素基因相关肽预处置减轻氧自由基所致心肌损伤

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关键词 电解；活性氧族；降钙素基因相关肽；蛋白激酶 C；心功能测定；血流动力学

目的：研究氧自由基(OFR)及降钙素基因相关肽(CGRP)预处置对 OFR 所致离体大鼠心脏损伤的拮抗作用. **方法**：Langendorff 法灌流心脏，电解 K-H 液产生 OFR. **结果**：CGRP 或 OFR 预处置减轻 OFR 所致心脏收缩功能下降，冠脉流量减少和肌酸激酶(CK)释放增加. 蛋白激酶 C (PKC) 抑制剂 H-7 可取消 OFR 预处置的心脏保护作用(对照组，OFR 损伤组，OFR 预处置组，H-7 加 OFR 预处置组及 H-7 组的 CK 释放量分别是 110 ± 7，215 ± 23，169 ± 14，240 ± 30，113 ± 19 U·L^{-1}). **结论**：OFR 或 CGRP 预处置对 OFR 所致心肌损伤具有拮抗作用，该作用与 PKC 激活有关.