Nitric oxide and oxygen radicals induced apoptosis via bcl-2 and p53 pathway in hypoxia-reoxygenated cardiomyocytes

SHEN Jiangang (沈剑刚)[1,2], QIU Xingshen (丘幸生)[2], JIANG Bo (姜 泊)[2], ZHANG Deliang (张德良)[1], XIN Wenjuan (忻文娟)[1], Peter C.W. Fung[3] & ZHAO Baolu (赵保路)[1]

1. Laboratory of Visual Information Processing, Center for Brain and Cognitive Sciences, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China;
2. Institute of Chinese Medicine, First Military Medical University, Guangzhou 510515, China;
3. Division of Medical Physics, Department of Medicine, The University of Hong Kong, Pokfulam Road, Hong Kong, China

Correspondence should be addressed to Zhao Baolu (email: zhaobl@sun5.ibp.ac.cn)

Received January 4, 2002

Abstract Neonatal rat cardiomyocytes were subjected to 24 h of hypoxia 95%N_2/5%CO_2 and 24 h of hypoxia plus 4 h of reoxygenation 95%O_2/5%CO_2. 24 h of hypoxia increased the levels of NO, NO_2^-/NO_3^-, TBARS and LDH. 24 h of hypoxia plus 4 h of reoxygenation decreased the levels of NO, NO_2^-/NO_3^-, but further increased TBARS and LDH. The hypoxia up-regulated the expression of bcl-2, p53 and p21/waf1/cip1 but the reoxygenation down-regulated the expression of bcl-2, and further up-regulated p53 and p21/waf1/cip1. The hypoxia increased cell apoptosis and reoxygenation further increased both apoptotic and necrotic cell death. NO, NO_2^-/NO_3^-, TBARS, DNA fragmentation and cell apoptosis were enhanced by SNP and inhibited by L-NAME respectively. In addition, SOD/catalase down-regulated the expression of p53, p21/wafl/cipl and TBARS but up-regulated bcl-2 and increased indirectly the level of NO, NO_2^-/NO_3^-, and inhibited DNA fragmentation. The results suggest that hypoxia-induced cell death is associated with the activation of NO, bcl-2 and p53 pathway, while hypoxia-reoxygenation induced cell death via the generation of reactive oxygen species and activation of p53 pathway. The present study clarified that NO may be an initiative signal to apoptotic cell death and the activation of bcl-2, p53 and p21/waf1/cip1 pathway in hypoxic and hypoxia-reoxygenated cardiomyocytes.

Keywords: apoptosis, hypoxia-reoxygenation, nitric oxide, oxygen radical, bcl-2, p53, p21/waf1/cip1.

Two types of cellular demise can occur simultaneously in tissues or cultured cell by necrosis and apoptosis. Loss of membrane integrity, cell edema and break, and the cell components released out are the characteristics of necrosis. While the cell apoptosis is a program cell death coded by gene and activated serious endogenous enzymes[1]. Recent studies have demonstrated that myocardial ischemia-reperfusion injury resulted in apoptotic cell death in addition to tissue necrosis[2—4].

Oxygen stress is one of the reasons that caused cell apoptosis and the oxygen radicals in the stress cause the DNA fragmentation, activate poly-ADP-ribose transferase, and increase p53 and

cell apoptosis[5,6]. Increasing evidence demonstrates that NO is involved in the apoptotic process[7]. Superoxide anions react with NO to form peroxynitrite ($ONOO^-$) and damage cell[8]. NO donor causes accumulation of p53 and cell apoptosis[5,9]. Hypoxia myocardium generates NO by activating inducible NO synthase (iNOS) through cGMP pathway and induces cell apoptosis[10,11].

Oncogene bcl-2 and p53 are important factors that regulated cell apoptosis[12,13]. Recent studies suggested that apoptosis was associated with the accumulation of p53 and its target gene p21/waf1/cip1 in hypoxia[14]. However, arguments arose from the recent studies that suggested apoptotic cell death to be independent of p53 gene expression in hypoxia-reoxygenated or ischemia-reperfused cardiomyocytes[15]. At the same time, decrease of bcl-2 expression in cardiomyocytes was shown to be an important reason for cell apoptosis. Bcl-2 could inhibit the NO-induced cell apoptosis and break of poly-ADP-ribose-polymerase. Expression of bcl-2 could inhibit p53-induced cell apoptosis[16,17]. The involvement of bcl-2 in apoptotic cell death during hypoxia and reoxygenation injury was still a controversy[18]. Some reported bcl-2 expression increased and other reported it decreased in ischemia-reperfusion myocardium[19,20].

There are more publications about the mechanism of hypoxia and ischemia induced cell apoptosis, but less about reperfusion and reoxygenation induced apoptosis. In order to identify the hypothesis that NO generated from hypoxia-reoxygenated cardiomyocytes may trigger cell death, we performed *in vitro* studies using cultured neonatal rat cardiomyocytes exposed to hypoxia and hypoxia followed by reoxygenation respectively. The expression of bcl-2, p53 in cardiomyocytes, NO concentration of culture supernatant, and DNA fragmentation were investigated to further address the role of bcl-2, p53 signal pathway in cell death of hypoxia-reoxygenated cardiomyocytes.

1 Materials and methods

1.1 Agents

DMEM (GIBCO), fetal calf serum (FCS, Kibbutz Beit Haemek, Israel), HEPES (Farco), bcl-2 antibody (N-19, Santa cruz), P53 antibody (CM-1, Novo), p21/waf1 antibody (C-19, Santa cruz), sheep-anti-rat FITC (Doko), Annexin V-Flous (Boechringer Mannheim), Propidium iodide (PI), NADPH, FAD, sodium nitroprusside (SNP), thiobarbituric acid (TBA), SOD, catalase, **Nù-nitro**-L-arginine methyl ester (L-NAME), cytosine arabinoside (Sigma). Other chemicals made in China were of analytical grade.

1.2 Cell model for hypoxia-reoxygenation injury

Neonatal rat cardiomyocytes were cultured according to the Tanaka method with minor modification[2]. In brief, the hearts from 2—3 days Wistar rats (Medical Zoology Center, the First Military Medical University) were minced and dissociated with 0.06% trypsin. The cells were incubated on 100-mm culture dish for 15 min at 37℃ with 100% relative humidity in a CO_2 incubator. Non-attached viable cells were collected and incubated in DMEM supplemented with

10% fetal calf serum penicillin 50 U/mL and streptomycin (50 μg/mL) for 6 h, followed by incubation in the same media supplemented with 10^{-6} mol/L cytosine arabinoside for 48 h to reduce the rate of non-cardiomyocytes. The cultured media were replaced by DMEM supplemented with 1% FCS and the cells were incubated for 24 h. Cardiomyocytes were incubated at 37℃ in an airtight incubator where normal air was pumped out with a vacuum pump and replaced by 95% N_2/5% CO_2 for 24 h to induce hypoxia injury. After 24 h of hypoxia, the gas was replaced by 95% O_2/5% CO_2 for another 4 h to produce hypoxia-reoxygenation injury. Before hypoxia, SNP (5 μmol/L), L-NAME (100 μmol/L), D-NAME (100 μmol/L), and SOD/catalase (100 U/mL each) were separately added to the cultured media and incubated with cardiomyocytes for 24 h of hypoxia or 24 h of hypoxia followed by 4 h of reoxygenation. The normal control cardiomyocytes were incubated under 95% air/5% CO_2.

1.3 Electrophoretic analysis of DNA fragmentation

DNA was extracted from cultured cardiomyocytes and the occurrence of DNA fragmentation was determined by agarose gel electrophoresis as in the previous method[21].

1.4 Annexin-V-FLUOS/propidium iodide (PI) double staining cytometry

Double staining for Annexin-V-FLOUS/PI was performed according to Vermes method[22,23]. After being washed twice with PBS, 10^6/mL cardiomyocyte cells were suspended in binding buffer (10 mmol/L HEPES/NaOH, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$). Annexin V-FLOUS and PI were separately added to a final concentration of 1 μg/mL in cell suspension. The mixture was incubated for 20 min in the dark at room temperature and then measured at 488 nm and 560 nm by FACScan (EPICS Elite). The cell populations of Annexin-V-FLOUS$^+$/PI$^-$ and Annexin-V-FLOUS$^+$/PI$^+$ were calculated respectively to represent apoptotic and necrotic cells and the cell without Annexin-V-FLOUS and PI as control.

1.5 ELISA detection of cell apoptosis

Cellular DNA fragmentation was determined using the cell death detection ELISA reagents (Boehringer Mannheim, Mannheim) following the manufacturer's instructions. The DNA fragmentation was expressed with the enrichment of histone-associated mono- and oligonucleosomes released into the cytoplasm. The enrichment factor (EF) was calculated according to absorption at 405 nm.

1.6 *bcl-2*, *p53* and *p21*/*waf1*/*cip1*/protein measurement

The expression of *bcl-2*, *p53* and *p21*/*waf1*/*cip1* proteins was detected as by the pervious method[24]. Fixed cells were washed twice in PBS containing 4% BSA (PBS/BSA). The 1∶100 dilution of the primary antibodies, *bcl-2*, *p53* and *p21*/*waf1*/*cip1* were added separately to the cell pellet and placed at 4℃ for 60 min. The cells were washed in PBS/BSA and incubated in a 1∶50 dilution of the secondary antibody, goat-anti-mouse FITC for 60 min. Excess antibody was rinsed

and the cells were suspended in PBS supplemented with PI (10 μg/mL) and RNase (1.0 mg/mL). Samples were analyzed using FACScan (EPICS Elite). At least 5000 cells were detected for each sample and the buffer without antibody as control and the percent of expressed proteins of *bcl-2*, *p53*, and *p21*/*waf1*/*cip1* were calculated.

1.7 LDH measurement

LDH was measured with LDH kit made by Zhongsheng Com. (Institute of Biophysics, Chinese Academy of Sciences) on UV-photospectrometer (Beckman DU-640).

1.8 NO measurement

The culture media were replaced by 3 mL DMEM buffer solution containing 1% FCS, 5 mmol/L DETC, 1 mmol/L $FeCl_2$. The cardiomyocytes were incubated at 37℃ for 30 min, then cooled down to stop the reaction, and 1∶5 volume ethyl acetate was added to the mixture[25]. After being shaken for 3 min, the solution was centrifuged at 10000 *g* for 6 min, the organic solvent layer was separated from the water and kept in the dark at 0—4℃ for ESR detection. ESR conditions: X-band, 100 kHz modulation with 3.2 G amplitude, microwave power 20 mW, central magnetic field 3380 G with sweeping 400 G, time constant 0.3 s.

1.9 Nitrite and nitrate determination

The concentrations of NO_2^-/NO_3^- in culture supernatants were measured by the previous method with minor modification[26]. In brief, cultured supernatant (200 μL) was incubated with 100 μL of nitrate reductase (90 mU/mL), 100 μL β-nicotinamideadenine dinucleotide phosphate (β-NADPH, 0.28 mmol/L), 100 μL of flavin adenine dinucleotide (FAD, 35 μmol/L) and 200 μL of potassium phosphate buffer (0.1 mol/L, pH 7.5) for 60 min at 25℃. After the solution was boiled for 3 min, an equal volume of Griess reagent (1∶1 mixture of 2% sulfanilamide in 5% H_3PO_4 and 0.2% N-(1-naphthyl) ethylenediamine dihydrochloride in water) was added to the reduced samples and incubated at 60℃ for 10 min. The absorption at 550 nm was measured using Bio-Rad 450 ELISA reader. A standard curve (0—20 nmol/mL) of sodium nitrate was indicated as NO_2^-/NO_3^- (nmol/mL).

1.10 TBARS measurement

The concentrations of TBARS in supernatants were measured by means of Ohkawa's method[24] at 532 nm using Beckman DU-640 spectrophotometer. Tetraethylox (10 nmol/L) as standard sample and the TBARS in the culture solution are expressed as nmol/mL.

1.11 Statistical analysis

All data were analyzed by Newman-Keult and *t*-test. When $P < 0.05$, statistical significance was accepted.

2 Results

2.1 Hypoxia-reoxygenation induced DNA fragmentation, apoptosis and necrosis

As shown in fig. 1, a ladder pattern of DNA fragmentation appeared in hypoxic (24 h) and hypoxia (24 h)-reoxygenated (4 h) cardiomyocytes. SNP increased but SOD/catalase and L-NAME inhibited the formation of DNA fragmentation, while D-NAME had no inhibitory effects on DNA fragmentation, suggesting that NO and superoxide anions may contribute to apoptotic cell death in hypoxic and hypoxic-reoxygenated cardiomyocytes.

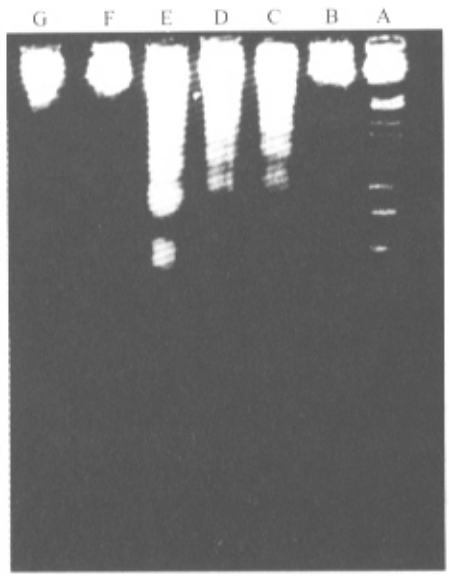

Fig. 1. Agarose gel electrophoresis analysis on DNA fragmentation in cultured cardiomyocytes. A, λDNA/*Eco*R Ⅰ+Hind marker; B, control; C, hypoxia; D, hypoxia-reoxygenation; E, hypoxia + SNP; F, hypoxia+L-NAME; G, HR+SOD/catalase.

To evaluate quantitatively DNA fragmentation, cell death detection ELISA method was employed in the study (fig. 2). The enrichment factor (EF) represents the enrichment of histone-associated DNA fragmentation and accounts for apoptosis of cardiomyocytes. EF was significantly increased by administration of SNP and inhibited by L-NAME in both hypoxic and hypoxia-reoxygenated cardiomyocytes, but D-NAME had no significantly inhibitory effects on it. In addition, SOD/catalase also decreased EF in hypoxia-reoxygen- ated cardiomyocytes. The results further support the hypothesis that NO and superoxide radicals may contribute to apoptotic cell death during hypoxia-reoxygenation injury.

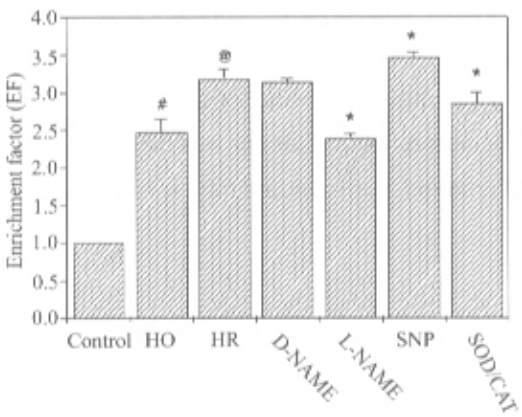

Fig. 2. Enrichment factor (EF) of DNA fragmentation in cultured cardiomyocytes detected by cell death ELISA. HO, Hypoxia; HR, hypoxia plus reoxygenation; L-NAME (100 μmol/L); D-NAME (100 μmol/L); SNP (5 μmol/L); SOD/CAT (100 U/ml, each); @, Vs. control, $p < 0.05$; *, Vs. HO, $p < 0.05$; #, Vs. HR, $p < 0.0$.

In order to differentiate necrotic cells from apoptotic cells, Annexin V-FLOUS was simultaneously applied with PI in hypoxia-reoxygenated cardiomyocytes. As shown in fig. 3, the vital cells are negative for both fluoresceinated annexin V binding and PI uptake (Annexin V^-/PI^-). The apoptotic cells are positive for fluoresceinated annexin V binding but negative for PI uptake (Annexin V^+/PI^-), while the necrotic cells are positive for both fluoresceinated Annexin V binding and PI uptake (Annexin V^+/PI^+). The statistic data are showed in fig. 4. 24 h of hypoxia alone induced mainly apoptotic cell death while 24 h of hypoxia plus 4 hours of reoxygenation

caused mainly necrotic cell death. The results indicate that hypoxia-reoxygenation not only mediates cardiomyocytes apoptosis but also induces cardiomyocytes necrosis. The rates of apoptosis and necrosis were increased by SNP and inhibited by L-NAME respectively in both hypoxic and hypoxia-reoxygenated cardiomyocytes, suggesting that NO contributes to apoptotic and necrotic cell death. It is interesting that SOD/catalase inhibited significantly both apoptotic and necrotic cell death in hypoxia-reoxygenated cardiomyocytes but without protective effects in hypoxic cardiomyocytes. The results suggest that superoxide anions contribute to cell death in hypoxia-reoxygenation injury, but not in hypoxia injury.

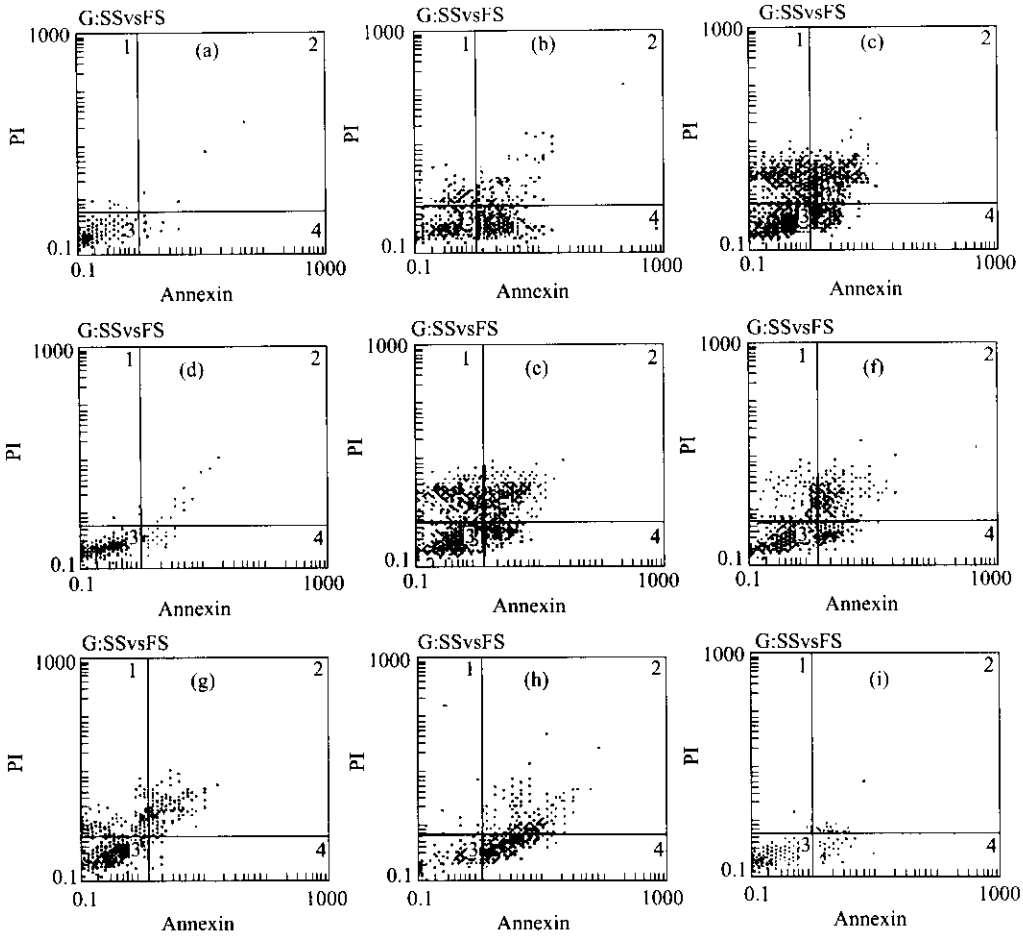

Fig. 3. Analysis of Annexin V-FLOUS / Propidium iodide (PI) double staining FACS measurement in cultured cardiomyocytes. (a) Normal control; (b) hypoxia (HO); (c) hypoxia-reoxygenation (HR); (d) HO+L-NAME (100 μmol/L); (e) HO+SNP (5 μmol/L); (f) HO+SOD/CAT (100 U/mL, each); (g) HR+L-NAME (100 μmol/L); (h) HR+SNP (5 μmol/L); (i) HR+SOD/CAT (100 U/mL, each). Region 3 represents the vital (Annexin V^-/PI^-) population; region 4 represents the apoptotic (Annexin V^+/PI^-) population, and region 2 represents the necrotic (Annexin V^+/PI^+) population.

2.2 Generation of NO, NO_2^-/NO_3^- in hypoxia-reoxygenated cardiomyocytes

The ESR spectra in fig. 5 came from incubation of $(DETC)_2Fe^{2+}$ with cardiomyocytes of hy-

poxia-reoxygenation and control. The signal at $g = 2.034$ with $a_N = 12.5$ G comes from complex $(DETC)_2Fe^{2+}$-NO. The signal increased with addition of L-arginine and decreased with addition of L-NAME, indicating that the NO came from NOS.

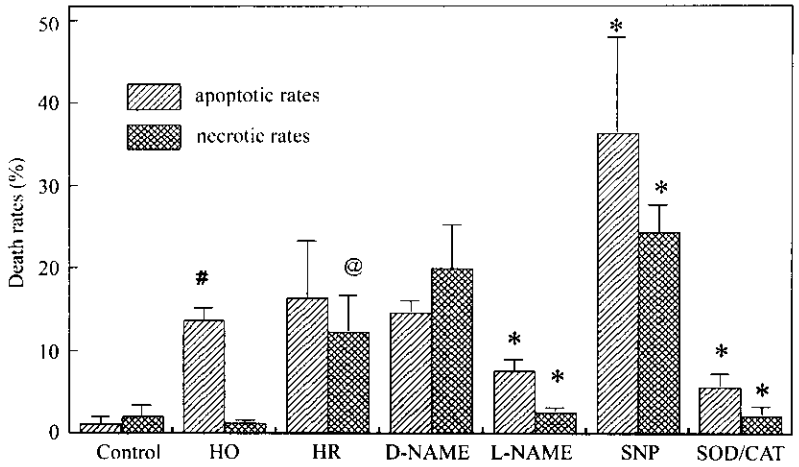

Fig. 4. Apoptotic and necrotic cell death rates (%) in cultured cardiomyocytes based on Annexin V/ PI double staining FACS measurement. HO, Hypoxia; HR, hypoxia plus reoxygenation; L-NAME (100 μmol/L); D-NAME (100 μmol/L); SNP (5 μmol/L); SOD/CAT (100 U/mL, each). @, Vs. control, $p < 0.05$; *, Vs. HO, $p < 0.05$; #, Vs. HR, $p < 0.05$, $n = 3$—5.

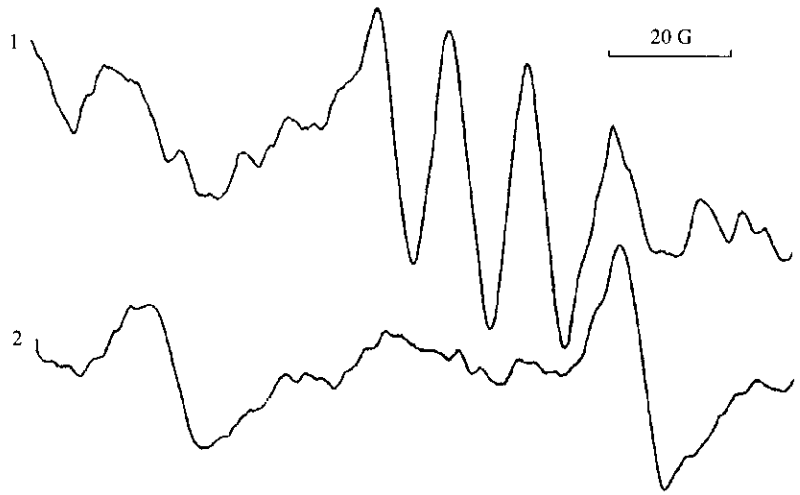

Fig. 5. ESR spectra of NO generated from 1, hypoxia-reoxygenated cardiomyocytes and trapped by $(DETC)_2Fe^{2+}$; 2, control without cardiomyocytes. Other conditions are described in sec. 1.

As shown in table 1, the level of NO_2^-/NO_3^- was significantly increased by 24 h of hypoxia, while reduced after reoxygenation. The level of NO_2^-/NO_3^- was decreased by administration of NOS inhibitor L-NAME and increased by NO donor SNP. Administration of SOD/catalase increased significantly the level of NO_2^-/NO_3^-.

2.3 TBARS and LDH leakage

As shown in table 1, the levels of TBARS and LDH in the media were slightly increased by 24 h of hypoxia and significantly augmented by 4 h of reoxygenation as NO_2^-/NO_3^- decreased.

Table 1 Changes of NO (μmol/L), TBARS (μmol/L) and LDH (U/L) in the culture solution of cardiomyocytes (mean ± S.E, $n = 3$)

Group	NO_2^-/NO_3^-	TBARS	LDH
Control	2.47 ± 0.09	4.10 ± 0.21	2.60 ± 0.31
HO	14.28 ± 0.78*	5.33 ± 0.43*	7.82 ± 0.47*
HR	12.32 ± 0.27$^{\#}$	7.41 ± 0.42$^{\#}$	12.77 ± 0.56$^{\#}$
HR+SNP	18.24 ± 2.07$^{\Delta}$	9.85 ± 0.28$^{\Delta}$	16.35 ± 0.28$^{\Delta}$
HR+L-NAME	6.35 ± 0.63$^{\Delta}$	5.52 ± 0.27$^{\Delta}$	7.50 ± 0.63$^{\Delta}$
HR+D-NAME	11.57 ± 1.42	7.49 ± 0.37	10.78 ± 0.55
HR+SOD/CAT	10.73 ± 0.69	5.07 ± 0.28$^{\Delta}$	8.06 ± 0.95$^{\Delta}$

HO, Hypoxia; HR, hypoxia-reoxygenation; *, Vs control $p < 0.05$; $^{\#}$, Vs. HO, $p < 0.05$; Δ, HR, $p < 0.05$.

TBARS and LDH were significantly decreased in the media of hypoxia and hypoxia-reoxygenated cardiomyocytes in the presence of SOD/catalase and L-NAME but no effect by the presence of D-NAME, indicating that NO and oxygen radicals played an important role in the hypoxia-reoxygenation cardiomyocytes injury.

2.4 Expression of bcl-2, p53 and p21/waf1/cip1 protein

24 h of hypoxia increased the expression of bcl-2, p53 as well as p21/waf1/cip1 protein in cardiomyocytes and bcl-2 decreased but p53 and p21/waf1/cip1 protein further increased by following 4 h of reoxygenation (figs. 6—8). Administration of L-NAME or SOD/catalase inhibited significantly the expression of p53 and p21/waf1/cip1 and up-regulated bcl-2, while SNP up-regulated p53 and down-regulated bcl-2 but no effect on p21/waf-1/cip-1. There was no effect of D-NAME on the above proteins. The results suggested that apoptotic cell death induced by NO or reactive oxygen species may be via the alterations of the expression of bcl-2, p53 and p21/waf1/cip1 in cardiomyocytes of hypoxia-reoxygenation injury.

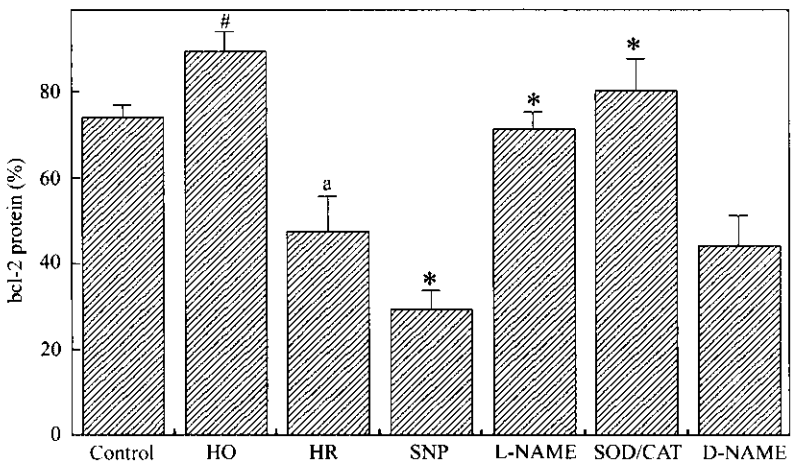

Fig. 6. Expression rates (%) of bcl-2 proteins in cultured cardiomyocytes. HO, Hypoxia; HR, hypoxia plus re-oxygenation; L-NAME (100 μmol/L); D-NAME (100 μmol/L); SNP (5 μmol/L); SOD/CAT (100 U/mL, each). @, Vs. control, $p < 0.05$; *, Vs. HO, $p < 0.05$; #, Vs. HR, $p < 0.05$, $n = 3$—5.

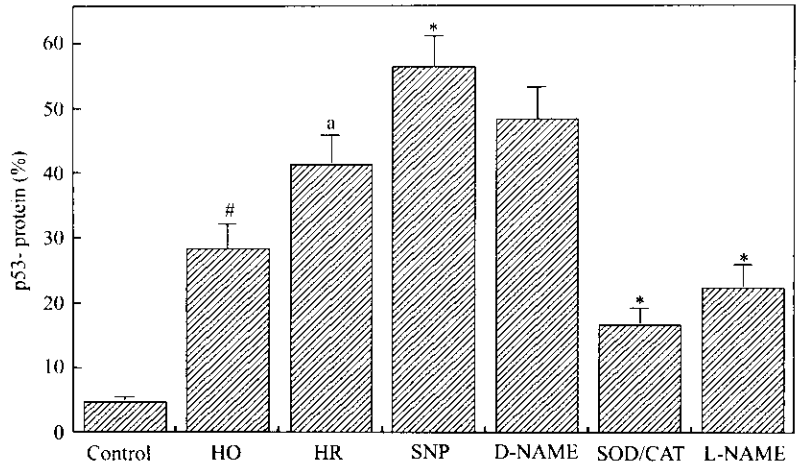

Fig. 7. Expression rates (%) of p53 proteins in cultured cardiomyocytes. HO, Hypoxia; HR, hypoxia plus reoxygenation; L-NAME (100 μmol/L); D-NAME (100 μmol/L); SNP (5 μmol/L); SOD/CAT (100 U/mL, each). @, Vs. control, $p < 0.05$; *, Vs. HO, $p < 0.05$; #, Vs. HR, $p < 0.05$, $n = 3$—5.

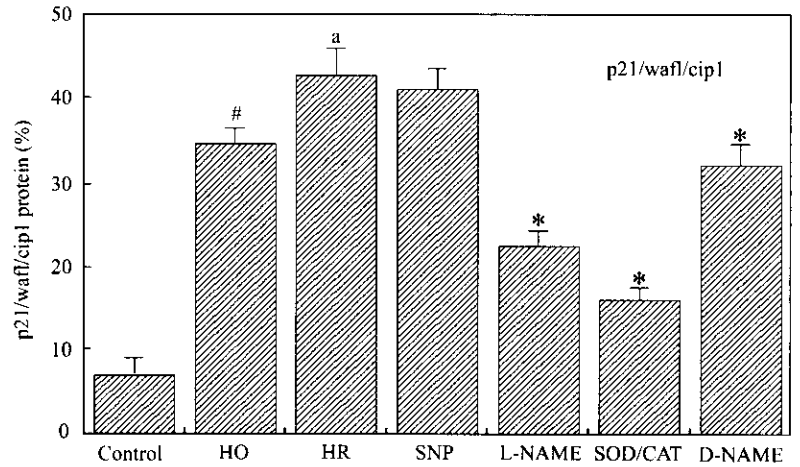

Fig. 8. Expression rates (%) of p21/waf1/cip1 proteins in cultured cardiomyocytes. HO, Hypoxia; HR, hypoxia plus reoxygenation; L-NAME (100 μmol/L); D-NAME (100 μmol/L); SNP (5 μmol/L); SOD/CAT (100 U/mL, each). @, Vs. control, $p < 0.05$; *, Vs. HO, $p < 0.05$; #, Vs. HR, $p < 0.05$, $n = 3$—5.

3 Discussion

It is well accepted that bcl-2, p53, and p21/wafl/cipl are involved in the apoptosis process and the increased expression of p53 is involved in apoptotic cell death and increased expression of bcl-2 inhibited the cell apoptosis. In order to study the mechanism of NO and oxygen radicals in hypoxia-reoxygenation induced cardiomyocytes injury, we observed the regulation of NO and oxygen radicals on expression of bcl-2, p53, and p21/wafl/cipl in this process.

The present study provides the evidence demonstrating that NO free radicals generated in hypoxia-reoxygenation of cardiomyocytes trigger apoptotic cell death via up-regulation of p53

and p21/wafl/cipl, but down-regulation of bcl-2. There are reports about hypoxia-[2], ischemia-reperfusion-[4] and infarct-[27] induced myocardial apoptosis. Active oxygen radicals are important factors for inducing cell apoptosis, but there are reports about cell apoptosis without relation with oxygen radicals. Active oxygen and bcl-2 and p53 are related to the apoptosis in hypoxia cardiomyocytes. But there are a few reports about the relation of active oxygen with bcl-2, p53 and p21/wafl/cipl in reoxygenation. This study found that hypoxia increased the production of NO, NO_2^-/NO_3^-, and slightly increased TBARS while reoxygenation reduced the level of NO and NO_2^-/NO_3^- but increased TBARS significantly. Hypoxia caused DNA fragmentation and induced apoptosis, while reoxygenation not only increased apoptotic cell death but also led to necrosis. Hypoxia up-regulated the expression of bcl-2, p53 as well as p21/waf1/cip1 proteins while reoxygenation down-regulated bcl-2 but continually up-regulated p53 and p21/wafl/cipl, indicating that oxygen and NO generation was closely related to the change of bcl-2, p53 and p21/wafl/cipl. It needs to note that both the expression of bcl-2 and the production of NO and NO_2^-/NO_3^- increased in hypoxia, while both of them decreased in reoxygenation, indicating that there was a relation between them. NO donor SNP down-regulated and L-NAME up-regulated bcl-2 in reoxygenation, indicating that NO itself could down-regulate bcl-2. The reason of bcl-2 up-regulation in hypoxia may come from the stress reaction induced by hypoxia. There was similar report about no expression of bcl-2 in normal myocardium[28] and increased expression of bcl-2 in infracted myocardium[19]. This study indicated that there was high expression of bcl-2 in normal cardiomyocytes and 24 h hypoxia increased the level of bcl-2, suggesting that hypoxia was an inducing factor for up-regulating the expression of bcl-2. TBARS indicated the lipid peroxidation mediated by oxygen radicals and reflected the level of oxygen radicals. Less oxygen free radical generated lower TBARS level in hypoxia. Reoxygenation generated “oxygen burst” and a lot of oxygen radicals were generated and higher level of TBARS was detected. Addition of SOD/catalase decreased TBARS and up-regulated bcl-2, indicating that there was close relation between oxygen radicals and bcl-2. At the same time, hypoxia and hypoxia-reoxygenation up-regulated p53 and p21/waf1/cip1 proteins, and L-NAME and SOD/catalase down-regulated p53 and p21/waf1/cip1 proteins in cardiomyocytes, indicating that NO and oxygen radicals generated by hypoxia-reoxygenated participated in and stimulated the expression of bcl-2, p53 and p21/wafl/cip1. It was reported that DNA fragmentation was related with p53 transcription activity, and p53 protein accumulation in the apoptosis process[14]. It was also reported that p21/wafl/cipl did not inhibit the protein complex in cell cycle and block cell cycle. Although p21/wafl/cipl did not induce cell apoptosis, they could stop the cell in G1[29] and participate in the apoptosis process mediated by p53[14]. NO and oxygen radicals may trigger cardiomyocytes apoptosis via the above pathway.

To clarify the above ideas, three methods were used to study the mechanism of apoptosis in hypoxia-reoxygenated cardiomyocytes. DNA fragmentation and oligoribonucleotide and polyribonucleotide bodies formed in early apoptotic cell were examined by anti-histone protein, DNA-

antibody-ELISA method. Application of Annexin V and PI simultaneously can differentiate necrotic cells from apoptotic cells. Hypoxia induced redistribution of PS and apoptotic cell death while reoxygenation further increased redistribution of PS and led to both apoptotic and necrotic cell death[29]. Based on this method, we demonstrated that 24 h hypoxia induced cell apoptosis and reoxygenation did not increase apoptosis but increased necrosis significantly. Moreover, hypoxia induced LDH release and further increased in the following reoxygenation. Hypoxia increased TBARS and NO, NO_2^-/NO_3^- and reoxygenation further increased TBARS but decreased nitrite compounds, indicating that hypoxia induced generation of NO, and reoxygenation induced "oxygen burst" and superoxide radicals, and further caused lipid peroxidation injury. NO may trigger apoptotic and necrotic cell death via the production of $ONOO^-$. As a powerful oxidant, $ONOO^-$ not only degrades to toxic hydroxyl radicals, but also induces nitration of proteins and in consequence causes DNA damage and DNA strand breaks. Therefore, the decrease of NO in reoxygenation cardiomyocytes could be detected. These results indicate that cell death may be attributed to the synergetic effects of NO and superoxide radicals in hypoxia-reoxygenated cardiomyocytes. In order to prove this possibility, SNP, L-NAME and SOD/catalase were added separately to the culture solution before hypoxia. SNP could increase the level of NO, NO_2^-/NO_3^-, LDH activity, and apoptosis and necrosis in the solution; L-NAME inhibited the level of NO, NO_2^-/NO_3^-, LDH, and apoptosis and necrosis in the solution; SOD/catalase also inhibited the level of NO, NO_2^-/NO_3^-, LDH, and apoptosis and necrosis in the solution. These results further suggest that oxygen and NO radicals participate in the apoptosis and necrosis process in hypoxia-reoxygenation cardiomyocytes. Hypoxia mainly causes apoptosis and increase of NO. Reoxygenation not only causes apoptosis but also necrosis and increase of oxygen radicals, suggesting that NO may mainly cause apoptosis and oxygen radical may mainly cause necrosis.

Acknowledgements This work was supported by the Guangdong Natural Science Foundation and National Natural Science Foundation of China (Grant No. 29935080).

References

1. Mastrangelo, A. J., Betenbaugh, M., Overcoming apoptosis: new methods for improving protein-expression systems, TIBTECH, 1998, 16: 88—95.
2. Tanaka, M., Ito, H., Adachi, S. et al., Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiocardiac myocytes, Circ. Res., 1994, 75(3): 426—433.
3. Gottlieb, R. A., Burleson, K. O., Kloner, R. A. et al., Reperfusion injury induces apoptosis in rabbit cardiocardiac myocytes, J. Clin. Invest., 1994, 94: 1621—1628.
4. Kajstura, J., Cheng, W., Reiss, K. et al., Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats, Lab. Invest., 1996, 74(1): 86—107.
5. Meβmer, U. K., Ankarcrona, M., Nicotera, P. et al., *p53* expression in nitric oxide-induced apoptosis, FEBS Lett., 1994, 355: 23—26.
6. Meβmer, U. K., Reimer, D. M., Reed, J. C. et al., Nitric oxide induced ploy(ADP-ribose) polymerase cleavage in RAW 264.7 macrophage apoptosis is blocked by *bcl-2*, FEBS Lett., 1996, 384(2): 162—166.

7. Dimmeler, S., Zeiher, A. M., Nitric oxide and apoptosis: another paradigm for the double-edged role of nitric oxide, Nitric Oxide, 1997, 1(4): 275—281.
8. Moncada, S., Palmer, R. M. J., Higgs, E. A., Nitric oxide: physiology, pathology and pharmacology, Pharmaco. Rev., 1991, 43: 109—142.
9. Meβmer, U. K., Brune, B., Nitric oxide (NO) in apoptotic versus necroic RAW 264.7 macrophage cell death: the role of NO-donor exposure, NAD^+ content, and *p53* accumulation, Arch. Biochem. Biophys., 1996, 327(1): 1—10.
10. Brune, B., Meβmer, U. K., Sandau, K., The role of nitric oxide in cell injury, Toxic Lett., 1995, 82/83: 233—237.
11. Matheis, G., Sherman, M. P., Buckberg, G. D. et al., Role of L-arginine-nitric oxide pathway in myocardial reoxygen injury, Am. J. Physiol., 1992, 262: H616—620.
12. Marx, J., New link found between *p53* and DNA repair, Science, 1994, 266: 1321—1322.
13. Hoffman, B., Liebermann, D. A., Molecular control of apoptosis: differentiation/growth arrest primary response genes, proto-oncogenes, and tumor suppressor genes as positive and negative modulators, Oncogene, 1994, 9: 1807—1812.
14. Long, X., Boluyt, M. O., Hipolito, M. L. et al., *p53* and the hypoxia-induced apoptosis of cultured neonatal rat cardiac cardiac myocytes, Clin. Invest., 1997, 99(11): 2635—2643.
15. Ohkawa, H., Ohishi, N., Yagi, K., Assay for lipid peroxides in animal tissues in animal tissues by thiobarbituric acid reaction, Anal. Biochem., 1979, 95: 351—358.
16. Kajstura, J., Mansukhani, M., Cheng, W. et al., Programmed cell death and the expression of the protooncogene *bcl-2* in myocyte during postnatal maturation of the heart, Exp. Cell Res., 1995, 219: 110—121.
17. Melkova, Z., Lee, S. B., Rodriguez, D. et al., *Bcl-2* prevents nitric oxide-mediated apoptosis and poly(ADP-ribose) polymerase cleavage, FEBS Lett., 1997, 403(3): 273—278.
18. Krishenbaum, L. A., Moissac, D., The *bcl-2* gene product prevents programmed cell death of ventricular cardiac myocytes, Circulation, 1997, 96(5): 1580—1585.
19. Cheng, W., Kajstura, J., Nitahara, A. J. et al., Programmed myocyte cell death affects the viable myocardium after infarction in rats, Exp. Cell Res., 1996, 226: 316—327.
20. Misao, J., Hayakawa, Y., Ohno, M. et al., Expression of *bcl-2* protein, an inhibitor of apoptosis, and Bax, an accelerator of apoptosis in ventricular cardiac myocytes of human hearts with myocardial infarction, Circulation, 1996, 94(7): 1506—1512.
21. Kaneto, H., Fujii, J., Seo, H. G. et al., Apoptotic cell death trigger by nitric oxide in pancreatic β-cells, Diabetes, 1995, 44: 733—738.
22. Vermes, I., Haanen, C., Steffens-Nakken, H. et al., A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V, J. Immunol. Meth., 1995, 184: 39—51.
23. Ei-Deiry, W. S., Harper, J. W., O' Connor, P. M. et al., Waf1/cip1 is induced in *p53*-mediated G1 arrest and apoptosis, Canc. Res., 1994, 54: 1169—1174.
24. Steck, K., McDonnell, T., Sneige, N. et al., Flow cytometric analysis of apoptosis and *bcl-2* in primary breast carcinomas: clinical and biological implications, Cytometry, 1996, 24: 116—122.
25. Zhang, D. L., Xiong, J., Hu, J. G. et al., Improved method to detect nitric oxide in biological system, Appl. Magn. Reson., 2001, 20: 345—356.
26. Yu, L., Gengaro, P. E., Niederberger, M. et al., Nitric oxide: a mediator in rat tubular hypoxia/reoxygenation injury, Proc. Natl. Acad. Sci. USA, 1994, 91: 1691.
27. Bialik, S., Geenen, D. L., Sasson, I. E. et al., Myocyte apoptosis during acute myocardial infarction in the mouse localizes to hypoxic regions but occurs independently of *p53*, J. Clin. Invest., 1997, 100(6): 1363—1372.
28. Heockenbery, D. M., Oltvai, Z. N., Yin, X. –M. et al., *Bcl-2* functions in an antioxidant pathway to prevent apoptosis, Cell, 1993, 75: 241—251.
29. Van Engeland, M., Nieland, L. J. W., Ramaekers, F. C. S. et al., Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure, Cytometry, 1998, 31: 1—9.