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Chinonin, a novel drug against cardiomyocyte apoptosis induced by hypoxia and reoxygenation

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Abstract

The inhibitory effects of Chinonin, a natural antioxidant extracted from a Chinese medicine, on apoptotic and necrotic cell death of cardiomyocytes in hypoxia-reoxygenation process were observed in this study. The possible mechanisms of Chinonin on scavenging reactive oxygen species and regulating apoptotic related genes bcl-2 and p53 were also investigated. Neonatal rat cardiomyocytes were subjected to 24-h hypoxia and 4-h reoxygenation. Cell death was evaluated by DNA electrophoresis on agarose gel, cell death ELISA and annexin-V-FLUOS/propidium iodide (PI) double staining cytometry. Hypoxia caused the increase of apoptotic rates and the release of lactate dehydrogenase (LDH), while reoxygenation not only further increased the apoptotic rates and leakage of LDH, but also induced necrosis of cardiomyocytes. In addition, hypoxia increased the levels of NO_2^-/NO_3^- and thiobarbituric acid reacted substances (TBARS), while reoxygenation decreased NO_2^-/NO_3^-, but further increased TBARS in the cultured media. Moreover, hypoxia up-regulated the expression levels of bcl-2 and p53 proteins, while reoxygenation down-regulated bcl-2 and further up-regulated p53. Chinonin significantly decreased the rates of apoptotic and necrotic cardiomyocytes, and inhibited the leakage of LDH. It also diminished NO_2^-/NO_3^- and TBARS, down-regulated the expression level of p53 protein, and up-regulated bcl-2 protein, respectively. The results suggest that Chinonin has preventive effects against apoptotic and necrotic cell death and its protective mechanisms are related to the antioxidant properties of scavenging nitric oxide and oxygen free radicals, and the modulating effects on the expression levels of bcl-2 and p53 proteins.

Keywords: Chinonin; Apoptosis; Nitric oxide; Oxygen free radicals; p53; bcl-2; Hypoxia; Reoxygenation

1. Introduction

Chinonin is an effective component isolated from Chinese herb *Rhizoma anemarhenea* which has been used for several thousand years. Its chemical name is 2-(-D-glucopyranosyl-1,3,6,7-tetrahydroxyl-9*H*-xanthin-9-tone (Fig. 1). Our previous study implied that Chinonin had scavenging effects on nitric oxide (NO) and oxygen free radicals and its antioxidant proper-

Abbreviations: NO, nitric oxide; TBARS, thiobarituric acid reaction substances; LDH, lactate dehydrogenase; NMMA, N^G-monomethyl-L-arginine; NOS, NO synthase; DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; FAD, flavin adenine dinucleotide; PI, Propidium iodide; Ara C, cytosine arabinoside; PS, phosphatidylserine; CN, Chinonin

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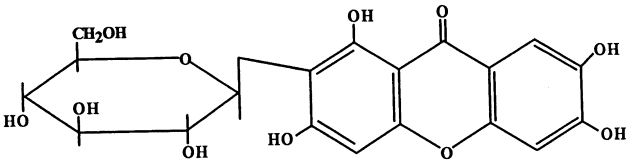

Fig. 1. Chemical structure of Chinonin.

ties were responsible for its cardioprotective effects against myocardial ischemia-reperfusion injury [1]. A recent study showed that Chinonin could inhibit acute hypoxic pulmonary vasoconstriction and prevent hypoxia-induced increase of mean pulmonary arterial pressure by blocking the action of endothelin-1 and/or platelet activating factor [2]. It implied a potential clinical usage of preventing tissue damage induced by ischemia or hypoxia. In order to further elucidate the cardioprotective effects of Chinonin, we investigated the effects of Chinonin on preventing cardiomyocytes from the apoptotic and necrotic cell death during hypoxia and reoxygenation process.

Much evidence shows that apoptosis can be induced by ischemia or ischemia-reperfusion in many different tissues, such as heart [3–6,9–11], brain [7], liver [8], kidney [9], etc. Apoptosis may be the major initial form of ischemic myocardial cell death occurring within the first 2 and 3 h, and necrotic cell death follows apoptosis and contributes to the evolution of myocardial infarction [10]. However, the precise mechanisms of apoptosis are ambiguous and the prevention of apoptosis is seldom reported in myocardial ischemia-reperfusion injury. Oxygen free radicals are considered as important mediators of apoptosis [11–13]. It may lead to the formation of oxidized lipid in cell membranes [14], the activation of poly-ADP-ribose transferase and accumulation of p53, which are associated with apoptosis [15,16]. As a kind of free radicals, NO is also involved in the apoptotic process [17]. In hypoxic cardiomyocytes, NO is produced by means of activating cyclic GMP pathway and stimulating NO synthase (NOS) [18]. Activation of the cytokine/lipopolysaccharide-inducible NOS causes nitrite increase, p53 accumulation and DNA fragmentation, which is prevented by N^G-monomethyl-L-arginine (NMMA), NOS inhibitor [16]. In addition, NO donor can induce p53 accumulation and trigger apoptosis [15,19]. Transfection with replication defective adenovirus encoding p53 and bcl-2 genes proved that p53 alone is sufficient to trigger apoptosis of ventricular myocytes while expression of bcl-2 prevents ventricular myocyte death and apoptosis provoked by p53 [21,22]. Bcl-2 can inhibit NO-mediated apoptosis and poly-(ADP-ribose) polymerase cleavage [20]. Overexpression of bcl-2 blocks lipid peroxidation completely and prevents apoptosis [11]. It is also reported that bcl-2 suppressed apoptosis induced by ischemia-reperfusion in intestinal epithelium of transgenic mice [23]. Therefore, the expression of apoptosis-related genes, such as bcl-2 and p53, is closely related to active oxygen species. Antioxidant therapies may be valuable to prevent apoptosis by means of scavenging reactive oxygen species and modulating the expression of bcl-2 and p53 against myocardial ischemia-reperfusion injury.

In order to elucidate the relationship between the antioxidant property of Chinonin and its protective effects against cardiomyocyte injury, the experiments were designed to determine apoptotic and necrotic cell death, the generation of NO and oxygen free radicals, as well as the expression of bcl-2 and p53 to clarify whether Chinonin scavenges active oxygen species and alters expression levels of apoptosis-modulating genes in hypoxia-reoxygenated cardiomyocytes.

2. Materials and methods

2.1. Cell culture

Neonatal rat cardiomyocytes were cultured according to the method described by Tanaka with minor modifications [4]. In brief, the hearts from 2–3-day Wistar rats (the First Military Medical University Medical Zoology Center) were minced and dissociated with 0.06% trypsin (Advanced Technological and Industrial Company, UK). The dispersed cells were incubated on 100-mm culture dishes for 15 min at 37°C with 100% relative humidity in a CO_2 incubator. Non-attached viable cells were collected and incubated in DMEM (Kibbutz Beit Haemek Israel) supplemented with 10% FCS (Kibbutz Beit Haemek Israel), 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 (Ara C, Sigma) for 48 h to reduce the rate of non-myocytes. And then, the cultured media were replaced by DMEM supplemented with 1% FCS and the cells were incubated for 24 h. Since NO is reactive in oxygenated aqueous solution and decomposes to nitrite and nitrate, the accumulated NO_2^-/NO_3^- in culture medium is taken as an indirect index of endogenous NO production. Meanwhile, thiobarituric acid reaction substances (TBARS) represents lipid preoxidation injury on membrane attacked by oxygen free radicals and LDH is a classic index of cell damage. In order to measure NO_2^-/NO_3^-, TBARS and LDH contents, the cultured media were replaced by DMEM without phenol red (Sigma) supplemented with 5% FCS before hypoxia. Different doses of Chinonin (1, 10, 100 μmol/l, 999 Enterprise Group, Medical and Pharmaceutical Research Institute) were added into the cultured media. The cardiomyocytes were incubated at 37°C in an air-tight incubator where normal air was replaced by 95% N_2/5% CO_2 for 24 h to produce hypoxia, and then the air was replaced by 95% O_2/5% CO_2 for 4 h to produce reoxygenation. Corresponding control cells were incubated under the same conditions, but kept in normoxia (ambient atmosphere).

2.2. Analysis of DNA fragmentation

After hypoxia and reoxygenation, the cells were washed twice with PBS and precipitated by centrifugation. The cells were incubated with lysis buffer (0.2 mol/l Tris-HCl, pH 8.0; 0.1 mol/l Na_2EDTA; 1% SDS and 100 mg/l proteinase K) for 4 h at 55°C. The nuclear lysates were extracted twice with phenol and then extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1). DNA was precipitated with 0.05 volume of 5 mol/l sodium chloride and 2.5 volume of ethanol overnight at −20°C and sedimented at 10 000 rpm for 10 min at 4°C. The DNA was incubated with TE buffer (10 mmol/l Tris-HCl, pH 7.5; 1 mmol/l Na_2EDTA) containing 20 mg/l RNase A for 1 h at 37°C. The DNA was finally extracted twice with equal volume of phenol–chloroform–isoamyl alcohol (25:24:1). After being washed with 75% ethanol, DNA samples were analyzed by electrophoresis on 1.5% agarose gels with TAE buffer (40 mmol/l Tris-HCl, 20 mmol/l acetic acid, and 1 mmol/l Na_2EDTA). The gel was stained with 0.5 μg/ml ethidium bromide and photographed by UV transillumination.

2.3. Detection of DNA fragmentation by ELISA

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 in the enrichment of histone-associated mono- and oligonucleosomes released into the cytoplasm. The enrichment factor (EF) was calculated according to absorption at 405 nm, which represented the enrichment of histone-associated DNA fragmentation and accounted for apoptosis of cardiomyocytes.

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

Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS). It is used as a sensitive probe for PS exposure upon the outer leaflet of the cell membrane and is therefore suitable to detect apoptotic cells. When annexin V is simultaneously applied with PI, it can differentiate necrotic cells from apoptotic cells. Double staining for Annexin-V-FLUOS/PI was performed as Vermes et al. described [24]. In brief, after being washed twice with PBS, 10^6 myocyte cells were resuspended in binding buffer (10 mmol/l HEPES/NaOH, pH 7.4, 140 mmol/l NaCl, 2.5 mmol/l $CaCl_2$). Annexin V-FLUOS (Boehringer Mannheim) and PI (Sigma) were respectively 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 by FACScan (EPICS Elite). Annexin-V-$FLUOS^+/PI^-$ and Annexin-V-$FLUOS^+/PI^+$ cell populations were calculated.

2.5. p53 and bcl-2/DNA staining cytometry

Bcl-2 and p53 proteins were detected as Steck described with minor modification [25]. Fixed cells were washed two times in PBS containing 4% BSA (PBS/BSA). The 1:50 dilution of the primary antibodies,

bcl-2 (N-19, Santa Cruz) and p53 (CM-1, Novo) were added respectively to the cell pellet and placed at 4°C for 60 min. The cells were then washed in PBS/BSA and incubated in a 1:50 dilution of the secondary antibody, goat-antimouse FITC (Doko), for 60 min. Excess antibody was rinsed and the cells were resuspended 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.

2.6. Nitrite and nitrate determination

NO production was measured as nitrite accumulation in the media from the hypoxia-reoxygenated cardiomyocytes. The concentrations of NO_2^-/NO_3^- in culture supernatants were quantitated as Yu described with minor modification [26]. In brief, cultured supernatant (200 μl) was incubated with 100 μl of nitrate reductase (90 mU/ml, Sigma), 100 μl nicotinamide adenine dinucleotide phosphate (NADPH, 0.28 mmol/l, Sigma), 100 μl of flavin adenine dinucleotide (FAD 35 μmol/l, Sigma) and 200 ml of potassium phosphate buffer (0.1 mol/l, pH 7.5) for 60 min at 25°C. After boiling 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°C for 10 min. The absorption at 550 nm was measured using Bio-Rad 450 ELISA reader. A standard curve (0–18 nmol/mg per tube) of sodium nitrate (Sigma) in 200 μl of potassium phosphate buffer (0.1 mol/l, pH 7.5) was included with each assay.

2.7. TBARS measurement

The concentrations of TBARS in supernatants were measured by means of Ohkawa's method [27] at 532 nm using Beckman DU-640 spectrophotometer.

2.8. LDH measurement

The activities of LDH in supernatants were measured by means of the enzyme dynamic method using Beckman DU 640 spectrophotometer with Zhong Sheng Biotech standard reagents.

2.9. Statistical analysis

All data are expressed as means ± S.E. and analyzed by ANOVA. When $P < 0.05$, statistical significance was accepted.

3. Results

3.1. Protection effects of Chinonin on DNA fragmentation

As shown in Fig. 2, a ladder pattern of DNA fragmentation appeared in the cardiomyocytes of 24 h of hypoxia and 24 h of hypoxia followed by 4 h of reoxygenation, which is a characteristics of apoptosis in DNA electrophoresis on agarose gel. Cardiomyocytes pretreated with Chinonin showed that no DNA fragmentation occurred on the agarose electrophoresis. In order to further evaluate the effects of Chinonin on DNA fragmentation, the cell death detection ELISA was used to analyze DNA degradation quantitatively. With anti-histone monoclonal antibody, the histone-associated DNA fragmentation (mono- and oligonucleosomes) was detected in the method. As shown in Fig. 3, when the cells were subjected to 24 h of hypoxia or 24 h of hypoxia followed by 4 h of reoxygenation, the EF

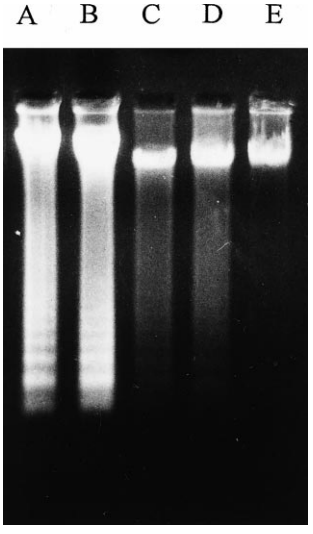

Fig. 2. Agarose gel electrophoresis analysis on DNA fragmentation. Lane A, 24 h of hypoxia; lane B, 24 h of hypoxia and 4 h of reoxygenation (HR); lane C, HO+Chinonin 100 μmol/l; lane D, HR+Chinonin 100 mmol/l; lane E, control.

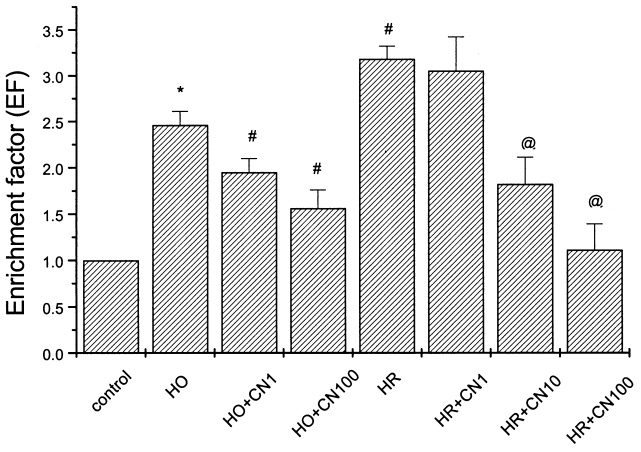

Fig. 3. Enrichment factor (EF) of DNA fragmentation in cultured hypoxia-reoxygenated cardiomyocytes. Control, normal; HO, hypoxia; HR, hypoxia-reoxygenation; CN1, Chinonin 1 μmol/l; CN10, Chinonin 10 μmol/l; CN100, Chinonin 100 μmol/l. #Vs. control, $P<0.05$; @vs. HO, $P<0.05$; *vs. HR, $P<0.05$.

values of DNA fragmentation were increased significantly. In the presence of Chinonin, the EF value was significantly reduced in both hypoxic and hypoxia-reoxygenated cardiomyocytes in a dose-dependent way. The results indicate that Chinonin could prevent DNA fragmentation and block cell death in the process of hypoxia and reoxygenation.

To discriminate necrotic and apoptotic cells, a flow cytometric detection of PS expression on early apoptosis was employed by using fluorescence-conjugated annexin V. As shown in Fig. 4, the vital cells are negative for both fluorescence-conjugated annexin V binding and PI uptake (annexin V^-/PI^-). The apoptotic cells are positive for fluorescence-conjugated annexin V binding but negative for PI uptake (annexin V^+/PI^-), while the necrotic cells are positive for both fluorescence-conjugated annexin V binding and PI uptake (annexin V^+/PI^+). The statistic data are showed in Fig. 5. Twenty-four hours of hypoxia significantly increased the rates of apoptosis and 24 h of hypoxia followed by 4 h of reoxygenation further increased the rates of apoptosis (annexin V^+/PI^-) and necrosis (annexin V^+/PI^+) significantly. Comparing with the results of 24 h of hypoxia, when the cardiomyocytes were subjected to 24-h hypoxia and 4-h reoxygenation, the apoptotic rate was increased about 70%, which was much less than the increase rate of necrotic (about 460%). The results indicate that hypoxia mainly induced cardiomyocyte apoptosis and reoxygenation not only mediated apoptosis, but also induced necrosis. When cardiomyocytes were incubated with Chinonin, the rates of apoptosis and necrosis were inhibited in a dose-dependent way. The results demonstrate strongly that Chinonin could protect cardiomyocytes from hypoxia-reoxygenation injury.

3.2. *Effects of Chinonin on the production of NO and TBARS and leakage of LDH*

The ability of Chinonin to influence the production of NO and lipid peroxidation as well as the leakage of LDH were investigated. As shown in Table 1, the level of NO in the media was increased by hypoxia, but it was decreased by reoxygenation. In

Table 1
Effects of Chinonin (CN) on the levels of NO (μmol/l), TBARS (μmol/l) and LDH (mU/ml/10^6 cells) in cultured media (mean ± S.E., $n=3$–5)

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[a]	5.33 ± 0.43[a]	7.82 ± 0.47[a]
HR	12.32 ± 0.27[b]	7.41 ± 0.42[b]	12.77 ± 0.56[b]
HO+CN (1 μmol/l)	7.37 ± 0.44[b]	4.02 ± 0.34[b]	5.46 ± 0.35[b]
HO+CN (100 μmol/l)	3.56 ± 0.22[b]	3.12 ± 0.24[b]	4.25 ± 0.24[b]
HR+CN (1 μmol/l)	6.25 ± 0.58[c]	3.68 ± 0.25[c]	9.32 ± 0.68[c]
HR+CN (10 μmol/l)	4.26 ± 0.36[c]	4.71 ± 0.21[c]	7.30 ± 0.17[c]
HR+CN (100 μmol/l)	3.20 ± 0.35[c]	4.18 ± 0.22[c]	5.35 ± 0.23[c]

HO, hypoxia; HR, hypoxia-reoxygenation.
[a]Vs. control, $P<0.05$.
[b]Vs. HO, $P<0.05$.
[c]Vs. HR, $P<0.05$.

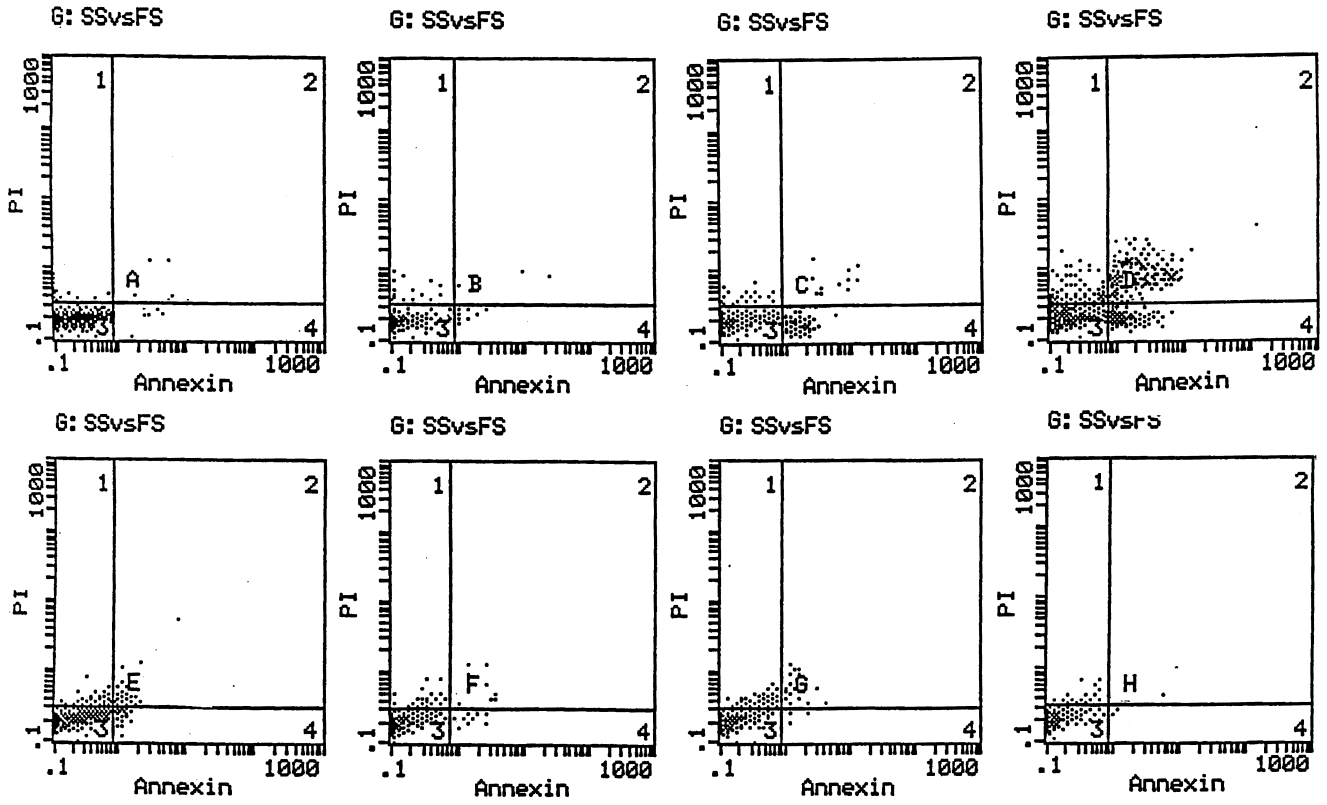

Fig. 4. The cardiomyocytes measured by annexin-V-FLUOS/propidium iodide (PI) double staining cytometry. Region 3 shows the vital cells which are negative for both annexin V binding and PI uptake (annexin V^-/PI^-). Region 4 shows the apoptotic cells which are positive for annexin V binding, but negative for PI uptake (annexin V^+/PI^-). Region 2 shows the necrotic cells which are positive for both annexin V binding and PI uptake (annexin V^+/PI^+). A, negative control; B, normal control; C, hypoxia (HO); D, hypoxia-reoxygenation (HR); E, HO+CN 1 μmol/l; F, HO+CN 100 μmol/l, G, HR+CN 1 μmol/l; H, HR+CN 100 μmol/l.

addition, the levels of TBARS and LDH in the media were slightly increased by hypoxia and significantly augmented by reoxygenation. The results suggest that hypoxia improved NO production in the cardiomyocytes, while reoxygenation caused oxygen burst and led to lipid peroxidation injury. In the presence of Chinonin, the levels of NO_2^-/NO_3^-, TBARS and LDH were significantly decreased in the media of hypoxia and hypoxia-reoxygenated cardiomyocytes and it was dose dependent. The results suggest that Chinonin has antioxidant properties of scavenging NO and oxygen free radicals during cardiomyocyte hypoxia and reoxygenation.

3.3. *Effects of Chinonin on expression of bcl-2 and p53 proteins*

To further elucidate the anti-apoptotic mechanisms of Chinonin against hypoxia-reoxygenation injury, we examined the alteration in the expression levels of bcl-2 and p53 proteins associated with hypoxia and hypoxia-reoxygenation induced cell death. The changes in the protein levels of p53 and bcl-2 in response to hypoxia and hypoxia-reoxygenation are shown in Figs. 6–9. The results revealed that hypoxia induced the up-regulation of bcl-2 and p53 proteins in cardiomyocytes, while reoxygenation further up-regulated p53 protein, but down-regulated bcl-2 protein. Chinonin down-regulated p53 protein but had no effect on bcl-2 in hypoxic cardiomyocytes. It also inhibited significantly the up-regulation of p53 protein and the down-regulation of bcl-2 protein in hypoxia-reoxygenated cardiomyocytes.

4. Discussion

The results of agarose electrophoresis, cell death

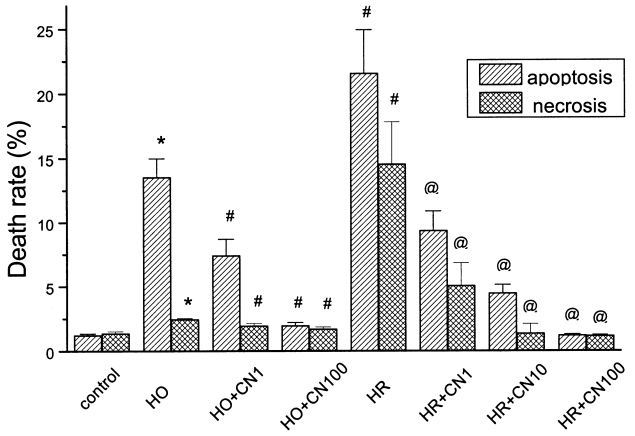

Fig. 5. Rates (%) of apoptosis and necrosis in cultured hypoxia-reoxygenated cardiomyocytes measured by annexin-V-FLUOS/propidium iodide (PI) double staining cytometry. Control, normal; HO, hypoxia; HR,hypoxia-reoxygenation; CN1, Chinonin 1 μmol/l; CN10: Chinonin 100 μmol/l; CN100, Chinonin 100 μmol/l. #Vs. control, $P<0.05$; @vs. HO, $P<0.05$; *vs. HR, $P<0.05$.

ELISA detection and FACS analysis of cell binding annexin V and PI showed that 24 h of hypoxia caused apoptosis and 24 h of hypoxia followed by 4 h of reoxygenation induced both apoptotic and necrotic cell death in cultured neonatal rat cardiomyocytes. Cell death ELISA detection demonstrated that the EF of histone-associated mono- and oligonucleosomes increased significantly in both hypoxia and hypoxia-reoxygenated cardiomyocytes. Further investigation with FACS analysis provided the evidence that hypoxia caused apoptosis and reoxygenation further increased apoptotic rates and induced necrosis, indicating that reoxygenation produced more serious damage in cardiomyocytes. It was further demonstrated by monitoring the release of LDH from cardiomyocytes. When Chinonin was pre-administered into the cultured media, the apoptotic and necrotic rates of cardiomyocytes and LDH leakage were significantly decreased in a dose-dependent manner. The apoptotic and necrotic rates were almost completely inhibited by 100 μmol/l of Chinonin, suggesting that it had strongly protective effects on the hypoxia-reoxygenated cardiomyocytes. In addition, FACS detection showed that 1 μmol/l of Chinonin decreased significantly apoptotic and necrotic rates, but cell death ELISA detection did not show the effect on EF of DNA fragmentation. The discrepancy of two detective methods may be attributed to the different sensitivity in detection of apoptosis and different biochemical criteria. As previous described, annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS. Early stage(s) of apoptosis may be implicated in the loss of membrane phospholipid asymmetry and appearance of PS, normally found in the inner leaflet of plasma membrane, on the outer surface of cells [36]. The PS translocation is believed to be the hallmark of apoptosis [37]. A recent study demonstrated the PS externalization in plasma membranes from ischemic and ischemia-reperfused hearts [36]. The de-

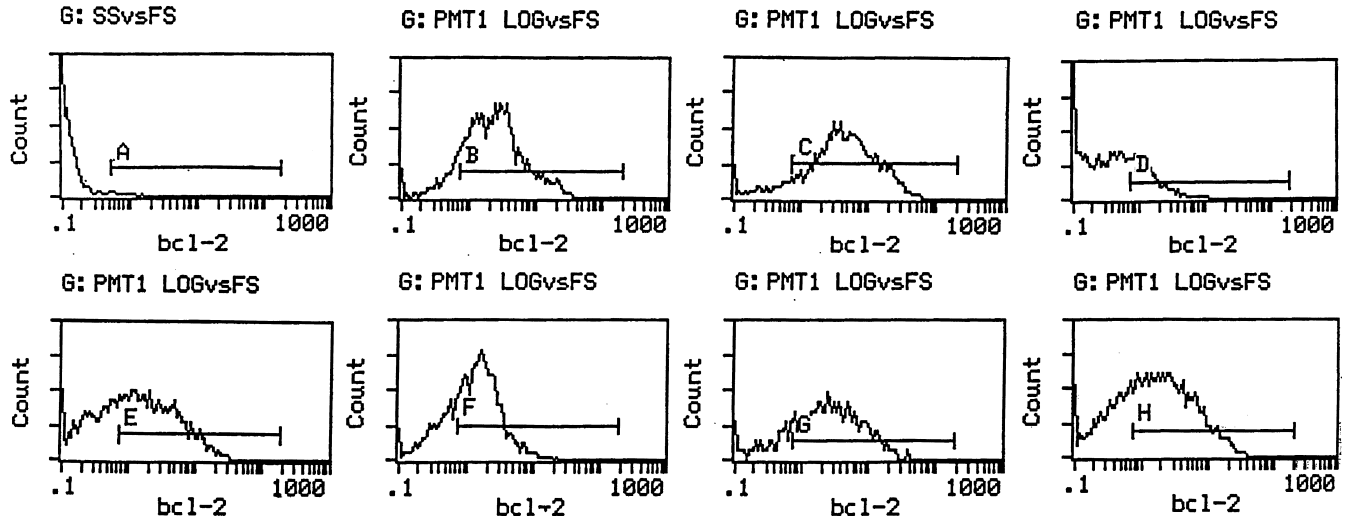

Fig. 6. Flow cytometry measurement of expression level of bcl-2 protein. A, negative control; B, normal control; C, hypoxia (HO); D, hypoxia-reoxygenation (HR); E, HO+CN 1 μmol/l; F, HO+CN 100 μmol/l, G, HR+CN 1 μmol/l; H, HR+CN 100 μmol/l.

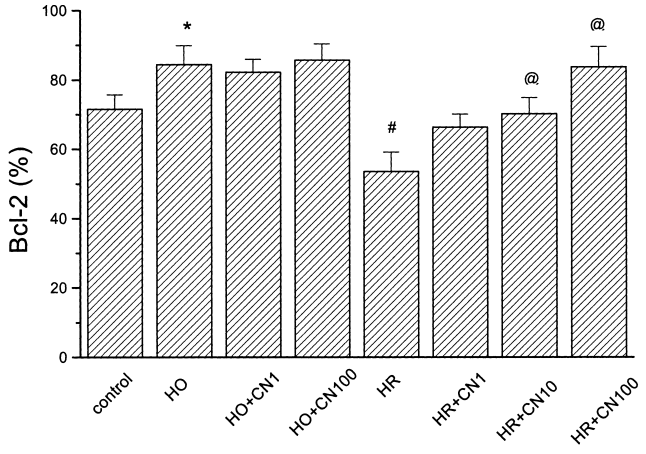

Fig. 7. Expression rates (%) of bcl-2 protein in hypoxia-reoxygenated cardiomyocytes. Control, normal; HO, hypoxia; HR, hypoxia-reoxygenation; CN1, Chinonin 1 μmol/l; CN10, Chinonin 10 μmol/l; CN100, Chinonin 100 μmol/l. #Vs. Control, $P<0.05$; @vs. HO, $P<0.05$; *vs. HR, $P<0.05$.

tection of the PS externalization to report apoptosis may be more sensitive than the measurement of histone-associated DNA fragmentation.

It is well known that oxidative stress leads cells to the exposure of reactive oxygen species, such as superoxide, hydroxyl radicals, NO and peroxynitrite ($ONOO^-$), and triggers apoptotic cellular death process [11,12,14,28]. Antioxidants may prevent apoptosis by means of suppressing or scavenging reactive oxygen species [11,12,29]. The current study provided new evidence that antioxidant could protect cells from oxidant damage and block apoptotic and necrotic cell death. In this study, the level of NO_2^-/NO_3^- in the media was increased by hypoxia, but decreased by reoxygenation. It suggested that hypoxia caused the generation of NO from cardiomyocytes and reoxygenation reduced the level of NO in cardiomyocytes. Previous studies revealed that hypoxia stimulated and up-regulated NO synthase gene expression and modulated guanylate cyclase in cardiomyocytes [30,31]. The augmentation of NO production in the hypoxic cardiomyocytes may be due to the activation of NOS in response to hypoxia. Other possible explanations could be that the acidic condition in hypoxic cardiomyocytes might be responsible for NO accumulation. NO can also be generated in tissues or cells by either direct disproportion or reduction of nitrite to NO under the acidic and highly reduced conditions, such as ischemia or hypoxia. It has been demonstrated that the enzyme-independent mechanism not only contributes to post-ischemic myocardial injury, but it may also reverse the protective effects of NOS blockers [32]. When oxygen was re-introduced for the hypoxic cardiomyocytes, oxygen burst might generate large amounts of superoxide anions, and in consequence induce lipid peroxidation injury. Our data showed that the level of TBARS in the media was slightly increased by hypoxia and accumulated remarkably by reoxygenation, which suggested indirectly that the large amount of oxygen radicals was generated

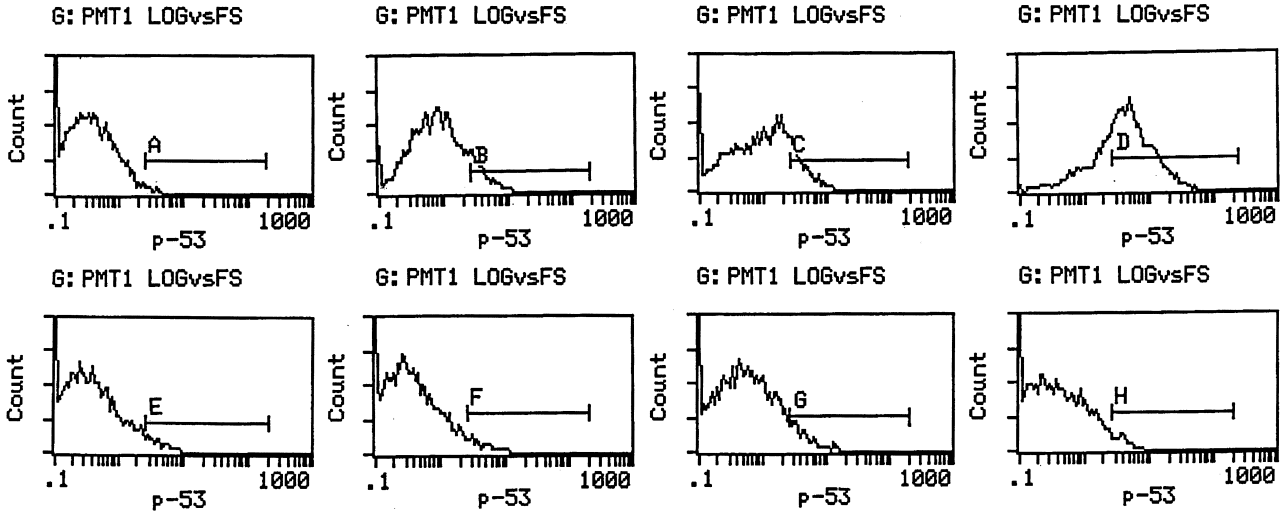

Fig. 8. Flow cytometry measurement of expression level of p53 protein. A, negative control; B, normal control; C, hypoxia (HO); D, hypoxia-reoxygenation (HR); E, HO+CN 1 μmol/l; F, HO+CN 100 μmol/l, G, HR+CN 1 μmol/l; H, HR+CN 100 μmol/l.

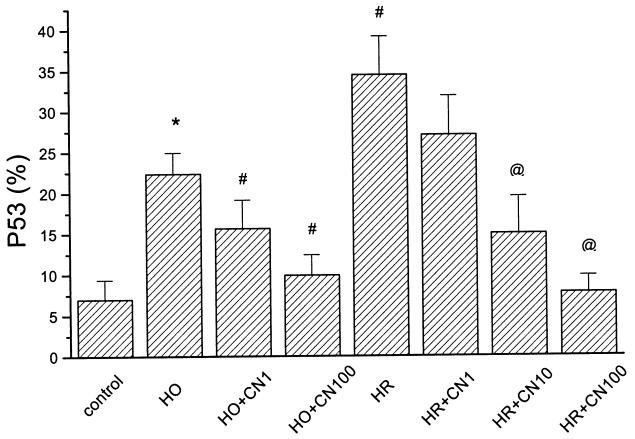

Fig. 9. Expression rates (%) of p53 protein in cultured hypoxia-reoxygenated cardiomyocytes. Control, normal; HO, hypoxia; HR, hypoxia-reoxygenation; CN1, Chinonin 1 μmol/l; CN10, Chinonin 10 μmol/l; CN100, Chinonin 100 μmol/l. #Vs. Control, $P<0.05$; @vs. HO, $P<0.05$; *vs. HR, $P<0.05$.

from reoxygenated cardiomyocytes. In addition, the reaction of superoxide with NO results in the formation of $ONOO^-$, which is associated with the cytotoxic mechanism of NO. The decrease of detectable NO may be attributed to the generation of oxygen radicals in reoxygenated cardiomyocytes. Moreover, our data showed that hypoxia induced mainly cell apoptosis while reoxygenation led to both apoptotic and necrotic cell death in cultured cardiomyocytes. It was reported that down-regulation of CuZn-SOD led to cell death via the NO/$ONOO^-$ pathway, suggesting that the cytotoxicity of NO was related to the formation of $ONOO^-$ [34]. Large amounts of $ONOO^-$ rapidly lead to necrotic cell death, whereas smaller amounts promote apoptosis [35]. Therefore, the possible mechanisms of apoptosis occurring in hypoxia may be associated with the formation of NO, and the apoptosis and necrosis appearing in reoxygenated cardiomyocytes may be contributed by the formation of oxygen radicals and $ONOO^-$.

In the presence of Chinonin, the levels of NO and TBARS were significantly decreased in the media of hypoxia and hypoxia-reoxygenated cardiomyocytes, suggesting that Chinonin could scavenge NO and inhibit the reaction of lipid peroxidation. In the chemical structure of Chinonin, there are four hydroxyl groups, which can react with reactive oxygen species to form a stable semiquinone free radicals so as to reduce their cytotoxicity. Chinonin can scavenge oxygen radicals and NO and inhibit the oxidative activity of $ONOO^-$ [1]. The study of Langendorff ischemia-reperfused hearts also revealed that Chinonin could inhibit the leakage of lactase dehydrogenase (LDH) and creatine kinase and prevent myocardial damage [1]. The current study further proved the antioxidant properties of Chinonin of scavenging NO and oxygen free radicals and the cardioprotective effects on hypoxia-reoxygenated cardiomyocytes. Therefore, the scavenging effects on active oxygen species may contribute to the anti-apoptotic mechanisms of Chinonin in hypoxia and hypoxia-reoxygenated cardiomyocytes.

Hypoxia-reoxygenation is different from ischemia-reperfusion. In the experiment of hypoxia-reoxygenation, DMEM medium with 5% FCS and glucose was used and the apoptosis of cardiocyte in this medium was just caused by hypoxia and reoxygenation without metabolic inhibition and energy default as was the case in ischemia-reperfusion heart.

Active oxygen species may also trigger apoptotic process by means of adjusting the apoptotic related genes, such as bcl-2 and p53, in myocardial ischemia-reperfusion injury. It is well known that bcl-2 and p53 are involved in regulating apoptotic process. The function of bcl-2 as a survival gene is responsible for inhibiting cell death by acting as an antioxidant, triggering enhanced expression of cellular antioxidant defense. It may also act directly by inhibition of the generation of oxygen radicals [33]. In addition, bcl-2 can inhibit NO-mediated apoptosis and poly-(ADP-ribose) polymerase cleavage [20]. As pro-apoptotic gene, p53 may play critical roles in triggering the apoptotic program of cells in hypoxic-mediated cellular apoptosis [15,19]. NO donor induced p53 accumulation and triggered apoptosis, and NOS inhibitor NMMA prevented apoptosis and down-regulated p53 accumulation [16]. The current study showed that hypoxia up-regulated the expression levels of bcl-2 and p53 proteins and reoxygenation further up-regulated the expression level of p53, but down-regulated the expression level of bcl-2. Chinonin up-regulated the expression level of bcl-2 protein in hypoxia-reoxygenated cardiomyocytes and down-regulated the expression level of p53 protein in both hypoxic and hypoxia-reoxygenated cardiomyocytes. Chinonin has antioxidant properties of scavenging NO and oxygen free radicals, the modulation of bcl-2 and p53 proteins may be related to the anti-

oxidant properties of Chinonin. Therefore, the anti-apoptotic mechanisms of Chinonin may regulate the expression levels of bcl-2 and p53 in hypoxia-reoxygenated cardiomyocytes.

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