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ORIGINAL CONTRIBUTION

Methanolic extract of onion (*Allium cepa*) attenuates ischemia/hypoxia-induced apoptosis in cardiomyocytes via antioxidant effect

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Abstract
Background Although there is growing awareness of the beneficial potential of onion intake to lower the risk of cardiovascular disease, there is little information about the effect of onion on ischemic heart injury, one of the most common cardiovascular diseases.
Aim of the study This study investigates the effect of the methanol-soluble extract of onion on ischemic injury in heart-derived H9c2 cells in vitro and in rat hearts in vivo. The underlying mechanism is also investigated.
Methods To evaluate the effect of onion on ischemia-induced cell death, LDH release and TUNEL-positivity were assessed in H9c2 cells, and the infarct size was measured in a myocardial infarct model. To investigate the mechanism of the cardioprotection by onion, the reactive oxygen species (ROS) level and the mitochondrial membrane potential ($\Delta\Psi_m$) were measured using an imaging technique; the caspase-3 activity was assayed, and Western blotting was performed to examine cytochrome *c* release in H9c2 cells.
Results The methanolic extract of onion had a preventive effect on ischemia/hypoxia-induced apoptotic death in H9c2 cells in vitro and in rat heart in vivo. The onion extract (0.05 g/ml) inhibited the elevation of the ROS, mitochondrial membrane depolarization, cytochrome *c* release and caspase-3 activation during hypoxia in H9c2 cells. In the in vivo rat myocardial infarction model, onion extract (10 g/kg) significantly reduced the infarct size, the apoptotic cell death of the heart and the plasma MDA level.
Conclusion In conclusion, the results of this study suggest that the methanolic extract of onion attenuates ischemia/hypoxia-induced apoptosis in heart-derived H9c2 cells in vitro and in rat hearts in vivo, through, at least in part, an antioxidant effect.

Keywords Onion extract · Antioxidant · Cardioprotection · Ischemic injury · Apoptosis

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Introduction

Ischemic heart disease, a worldwide health problem, results from severe impairment of the coronary blood supply. This typically stems from thrombosis or atherosclerotic plaque [1]. One of the major causes of ischemic/hypoxic heart disease is oxidative stress, which is caused by an imbalance between oxidants and antioxidant defenses. During myocardial ischemic/hypoxic injury, the activities of antioxidant enzymes have been demonstrated to decrease, and the generation of reactive oxygen species (ROS) increases at the site of damage [2, 3]. Potential antioxidant therapy should, therefore, include either exogenous supplementation of

natural antioxidants or augmentation of endogenous antioxidants.

Onion, a vegetable member of the genus *Allium*, is one of the most widely and largely consumed vegetables. From epidemiologic studies, onion consumption is known to be related to low rates of coronary heart disease [4]. It has been reported that ethyl acetate extract of onion containing hydrophilic and lipophilic components have ROS scavenging activity in vitro [5]. In addition, onion oil has been shown to increase the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase in a variety of tissues including rat heart [6]. Although these studies suggest that onion extract may play a beneficial role during ischemic injury of the heart, there is little information about this in either in vitro or in vivo models.

This study investigates the effects of methanol-soluble extract from onion bulbs on ischemic injury in heart-derived H9c2 cells in vitro and a rat myocardial infarction model in vivo. The possible underlying mechanisms are also investigated, especially those linked to an antioxidant effect of the onion extract and a further anti-apoptotic effect that inhibits the mitochondrial death pathway.

Methods

Preparation of the onion extract and treatment

Onion total extract was obtained by methods slightly modified from a previously described protocol [7]. Briefly, after the outer skins or leaves of fresh onions were carefully removed, 50 g of onion bulb was homogenized in 70% methanol (100 ml), and the homogenate was filtered through filter paper. The resulting fractions were lyophilized using a vacuum evaporator (N-2N, Eyela, Tokyo, Japan). Lyophilized onion extract was dissolved in dimethyl sulfoxide (DMSO) and diluted in a cell culture medium, making the final concentration of DMSO less than 0.1%. The gram (g) was used as the unit for the amount of onion in this study, referring to the weight of fresh onion. The H9c2 cells were treated with several concentrations (0.01, 0.5 and 0.1 g/ml) of the onion extract 30 min before and during hypoxia. For the in vivo study, the onion extracts were dissolved in saline and administered to rats at various doses (0.1, 1 and 10 g/kg) once a day per-orally (p.o.) for 14 days before coronary artery ligation or intravenously (i.v.) by a single injection 30 min before ligation. The onion 1 g/kg for rat was approximately equivalent to 65 g of onion for a 65 kg man per day [8, 9]. Rats were randomly divided into four groups, and each group consisted of eight rats.

Cell culture and hypoxia system

Heart-derived H9c2 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose supplementation with 10% fetal bovine serum. For hypoxic challenges, H9c2 cells were transferred into an anaerobic chamber (Forma Scientific, Marietta, OH, USA) maintained at 37 °C with a humidified atmosphere of 5% CO_2, 10% H_2, and 85% N_2, as previously described [10].

Cell death measurement

To examine the extent of apoptotic cell death, we performed in situ terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL)-staining after 8 h of hypoxia as described previously [10, 11]. The percentage of cell death was calculated from the number of TUNEL-positive cells divided by the total cell count.

Intracellular ROS measurement

The 2′7′-dichlorofluorescein diacetate (DCFDA; Molecular Probe, Eugene, OR, USA) is an ROS-sensitive probe that can be used to detect oxidative activity in living cells. The H9c2 cells were loaded with the DCFDA dye (1 μg/ml) for 30 min. The plates were examined immediately under a laser scanning confocal microscope (Olympus 5100, Japan) and the fluorescence intensity of DCFDA was quantified using image-analysis computer software (Fluoview FV300, Olympus, Japan).

1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging assay

The free radical-scavenging activities of the samples were measured via the stable radical DPPH according to the modified method of Cuendet et al. [12]. The methanolic solution of DPPH (0.004%) and the methanolic extract of onion were mixed so that the final mass ratios were extract:DPPH solution = 2:1. A mixture of methanol and the DPPH solution (2:1) was used as a control. Absorbance at 517 nm was determined after 30 min of incubation of the samples in the dark at room temperature. DPPH radical-scavenging activity (%) was calculated as follows

$$\text{DPPH radical-scavenging activity } (\%) = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100.$$

Mitochondrial membrane potential ($\Delta\Psi_m$) measurement

$\Delta\Psi_m$ was assayed by measuring the accumulation of rhodamine 123 (Molecular Probes, Eugene, OR, USA), a membrane-permeable cationic fluorescent dye. The H9c2 cells were loaded with 1 μM rhodamine 123 in for 10 min at 37 °C and washed three times with HCSS and observed at 530 nm under a confocal microscope (Olympus 5100, Japan).

Caspase-3 activity assay

The cells were lysed with a lysis buffer (10 mM Tris/HCl, 0.32 M Sucrose, 1 mM PMSF, 1% Triton X-100, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM EDTA, and 10 mM DTT, pH 8.0) and the lysates were centrifuged (10,000×*g* for 5 min at 4 °C). Samples (200 μg) of the extracted proteins were incubated with 200 μM Ac-DEVD-*p*-NA (Biomol, Plymouth meeting, PA, USA). Enzyme-catalyzed release of *p*-NA was measured at 405 nm using a microplate reader.

Western blot analysis

To quantify cytochrome *c* release, Western blot analysis was performed in the mitochondrial and the cytosolic fractions [11]. A quantity of 40 μg protein was separated on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We verified the mitochondrial fraction with the mitochondrial marker, cytochrome oxidase subunit IV (COX IV, Molecular Probes, Eugene, OR, USA), and cytosolic fraction with the cytosolic marker, actin (Sigma, St. Louis, MO, USA).

Rat myocardial infarct model

All experiments were performed in accordance with the approved animal protocols and guidelines established by the Ajou University School of Medicine Ethics Review Committee for animal experiments. Rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.), and the heart was gently exteriorized. After 45 min of occlusion of left anterior descending coronary artery, the coronary artery was reperfused for 90 min. The coronary artery was reoccluded and a 1% Evans blue was injected via the femoral vein, and the area not stained with Evans blue was analyzed to get an area at risk (AAR). The left ventricle was sliced transversely into 2 mm thick sections, stained with triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA), and the area not stained with TTC was analyzed to get an infarct zone (size) with computerized planimetry (BAS 1500, Fuji Film, Japan). We evaluated the extent of infarction by calculating the infarct size divided by the AAR.

Determination of plasma malondialdehyde (MDA)

Arterial blood samples (0.6 ml) collected after 60 min of reperfusion were immediately centrifuged at 2,500×*g* and 4 °C for 10 min. Plasma MDA level, a marker for lipid peroxidation, was quantified to estimate the extent of lipid peroxidation in the AAR myocardium using a commercial kit (Malondialdehyde Assay Kit, Northwest, USA) and expressed as μM per ml plasma.

Statistical analysis

All data are expressed as mean ± SD in vitro and as mean ± SEM in vivo. The Mann–Whitney test was applied in a comparison of the nonparametric variables between the two study groups. A *P* value of less than 0.05 was considered significant.

Results

Effects of onion extract on hypoxia-induced apoptotic death in H9c2 cells

Figure 1a shows the protective effects of onion extract against hypoxia-induced cell death by TUNEL staining, a well-known indicator of apoptotic cell death. Hypoxia-induced apoptotic cell death (62.8 ± 6.2%) was significantly attenuated by 18.3 ± 2.3 and 7.0 ± 1.8% with the treatment with onion extract 0.05 and 0.1 g/ml, respectively. As shown in Fig. 1b, hypoxia increased caspase-3 activity (213.8 ± 2.8%) compared to the normoxic control (100%). The treatment of cells with 0.05 and 0.1 g/ml onion extract markedly decreased the caspase-3 activity. The results of this experiment indicated that 0.05 g/ml of onion extract is an effective concentration; thus, 0.05 g/ml onion extract was used for the following mechanism studies in H9c2 cells, including an ROS assay, $\Delta\Psi_m$ determination and an assessment of cytochrome *c* release.

Effects of onion extract on ROS in H9c2 cells

Figure 2a shows the DPPH radical scavenging activities of the 0.01, 0.05 and 0.1 g/ml onion extracts as 38.6 ± 3.5, 49.6 ± 4.2, and 62.1 ± 5.2%, respectively. The figure also shows that with 100 μM of trolox, a well-known antioxidant, the activity was 95.5 ± 1.4%. In H9c2 cells, ROS

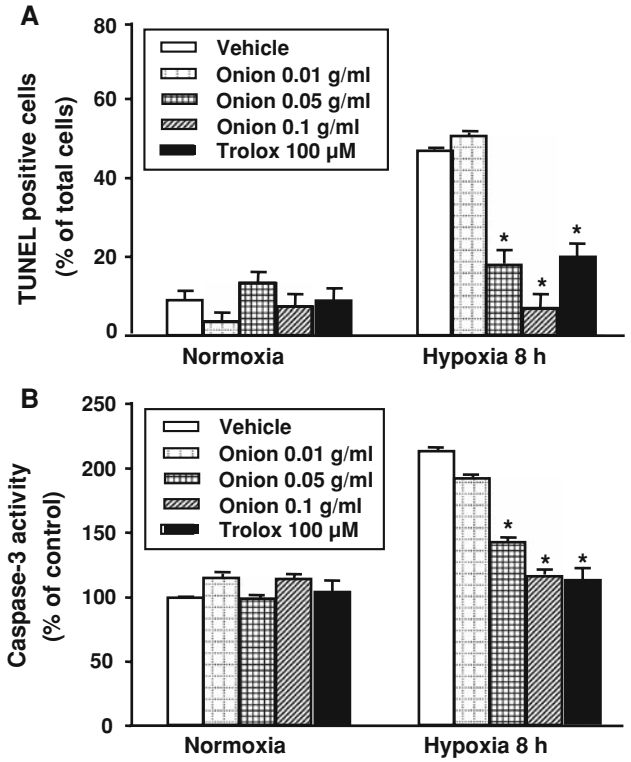

Fig. 1 Effects of onion extract on hypoxia-induced apoptotic cell death in H9c2 cells. **a** TUNEL-positivity (%) calculated by dividing the number of TUNEL-stained cells by the total number of cells after 8 h of hypoxia. All data represent the mean ± SD ($n = 4$). * $P < 0.05$ versus vehicle-hypoxia. **b** Caspase-3 activity was analyzed from the cytosolic fraction of H9c2 cells 8 h after hypoxic insult. All data represent the mean ± SD ($n = 4$). * $P < 0.05$ versus vehicle-hypoxia

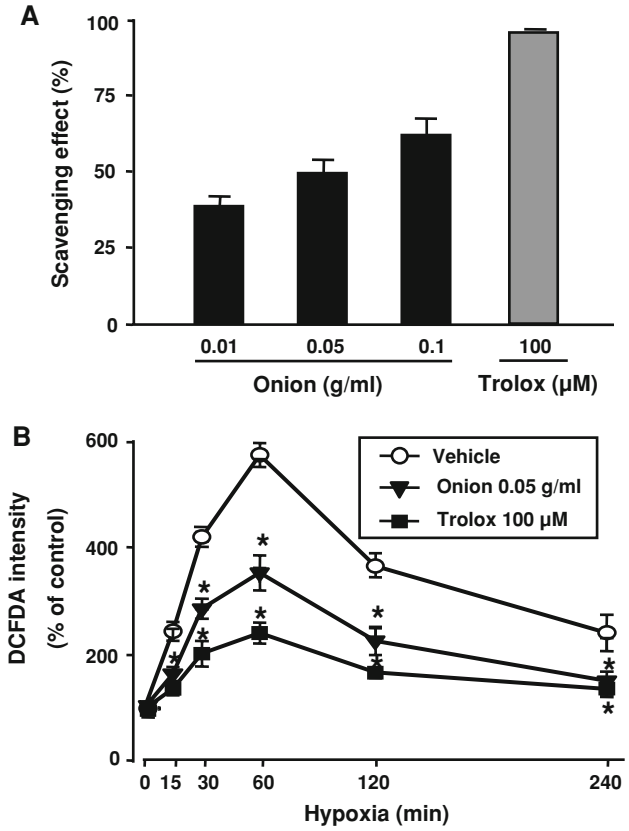

Fig. 2 Effects of onion extract on hypoxia-induced reactive oxygen species (ROS) generation in H9c2 cells. **a** DPPH free radical scavenging activity. All data represent the mean ± SD ($n = 4$). **b** Quantitative analysis of ROS generation during hypoxia. All data represent the mean ± SD ($n = 4$). * $P < 0.05$ versus vehicle-hypoxia

generation during hypoxia began to increase at 15 min of hypoxia and continued to increase to the maximum level (about 5.7-fold) at 60 min of hypoxia (Fig. 2b). This hypoxia-induced ROS elevation was significantly decreased by treatment with 0.05 g/ml of onion extract and 100 μM of trolox.

Effects of onion extract on hypoxia-induced $\Delta\Psi_m$ reduction and cytochrome *c* release in H9c2 cells

Figure 3a shows the time-dependent changes in the fluorescence intensity of rhodamine 123, an indicator of $\Delta\Psi_m$. The intensity was reduced considerably to 51.4 ± 1.5% after only 2 h of hypoxia and reached a maximum decrease (39.5 ± 2.4%) after 4 h of hypoxia compared to the normoxic control. The $\Delta\Psi_m$ reduction after 4 h of hypoxia (39.5 ± 2.4%) was significantly inhibited by 0.05 and 0.1 g/ml onion extract (62.5 ± 4.5 and 61.7 ± 5.0%, respectively) and by 100 μM trolox (67.2 ± 3.0%). The release of cytochrome *c* from the mitochondria to the cytosolic fraction was increased after 8 h of hypoxia and was markedly inhibited after a treatment of 0.05 g/ml onion extract (Fig. 3b).

Effects of onion extract on infarct size in a rat myocardial infarction model

As shown in Fig. 4a, ischemia (45 min) followed by reperfusion (90 min) resulted in an infarct size of 64.6 ± 2.3% in the i.v. vehicle-treated group; this infarct size was reduced considerably by i.v. treatment with onion extract in a dose-dependent manner: 53.6 ± 1.6, 45.8 ± 1.8 and 31.8 ± 1.3 at 0.1, 1 and 10 g/kg, respectively. In the p.o. group (Fig. 4b), the ischemia-induced infarct size (63.3 ± 1.8%) in vehicle-treated heart was attenuated by p.o. treatment with onion extract (0.1, 1 and 10 g/kg) for 14 days by 55.0 ± 1.7, 52.8 ± 2.1 and 47.0 ± 2.0%, respectively.

Effects of onion extract on apoptotic death in heart in a rat myocardial infarction model

As shown in Fig. 5, the percentage of TUNEL-positive cells in vehicle-treated hearts was 14.3 ± 1.9%. These

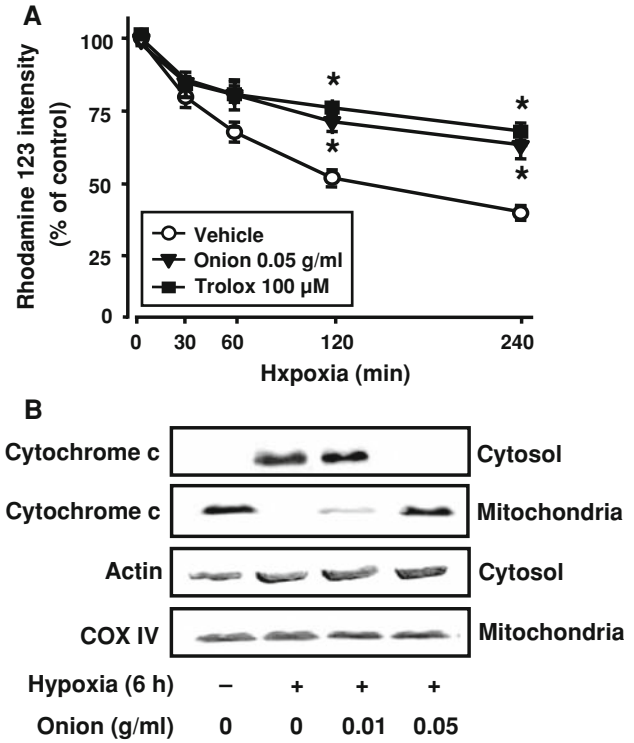

Fig. 3 Effects of onion extract on hypoxia-induced mitochondrial death pathway in H9c2 cells. **a** Quantitative analysis of $\Delta\Psi_m$ determined by confocal images of Rhodamine 123 staining. All data represent the mean ± SD ($n = 4$). * $P < 0.05$ versus vehicle-hypoxia. **b** Western blots for cytochrome *c* detected in the cytosolic and mitochondrial fractions. Data shown are representative of four separate experiments

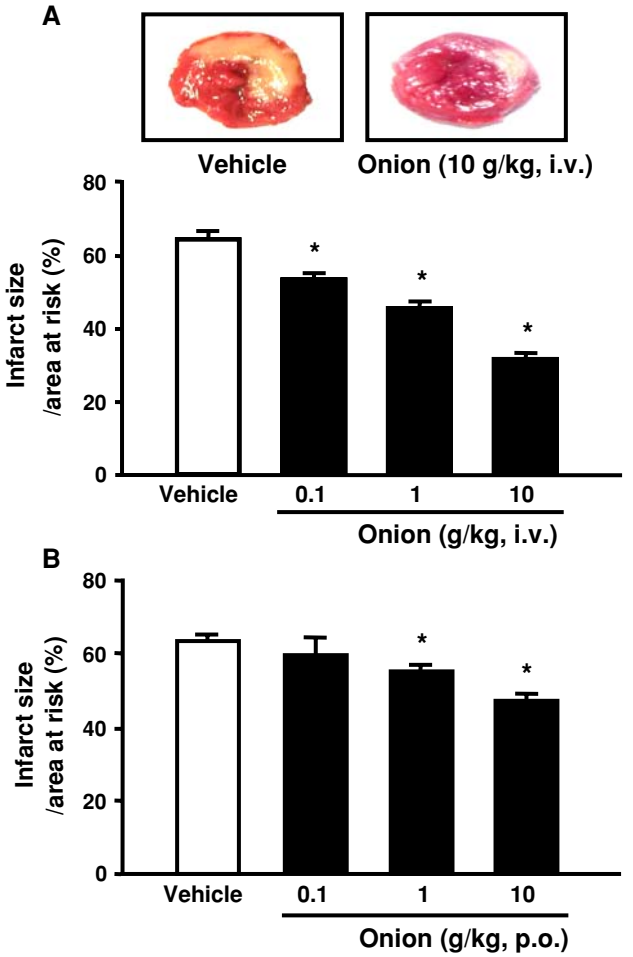

Fig. 4 Effects of onion extract on infarct size in a rat myocardial infarct model. **a** *Upper panel* representative photographs of the left ventricular section. **a** *Lower panel* quantitative assessment of the effect of onion (i.v.) on the infarct size. **b** Quantitative assessment of the effect of onion (p.o.) on the infarct size. All values are the mean ± SEM ($n = 8$). * $P < 0.05$ versus vehicle

TUNEL-positive cells were decreased by i.v. treatment with 1 and 10 g/kg onion by 8.3 ± 0.7 and 4.6 ± 0.6%, respectively.

Effects of onion extract on plasma MDA in a rat myocardial infarction model

As shown in Fig. 6, the level of plasma MDA in vehicle-treated ischemia increased significantly compared to that in the sham control (5.3 ± 0.3 and 3.6 ± 0.2 μM, respectively). This increase in plasma MDA was significantly reduced to 3.8 ± 0.4 μM by i.v. treatment with 10 g/kg onion extract.

Discussion

This study demonstrates for the first time that a methanolic extract of onion attenuates ischemia/hypoxia-induced apoptosis in heart-derived H9c2 cardiomyocytes in vitro and in rat hearts in vivo.

The characteristic pattern of myocardial ischemic injury involves fluid and electrolyte alterations and cellular swelling with plasma membrane blebbing and clumping of nuclear chromatin, consequently leading to two distinct forms of myocardial cell death: necrotic and apoptotic death [13]. Unlike necrosis, which is a violent and non-regulated process of cell death, apoptosis is a genetically controlled, highly regulated and energy-requiring process. A number of studies have suggested that apoptotic cell death is associated with oxidative stress, which results from either an overproduction of ROS or a decrease in the antioxidant status [14, 15]. During ischemia followed by reperfusion, ROS production is increased, which overwhelms the endogenous antioxidant system and subsequently causes oxidative stress [2, 3]. Sources of the ROS during myocardial ischemia/reperfusion injury include NAD(P)H oxidases, mitochondria (particularly complexes I and III) and xanthine oxidase [16, 17]. In fact, the xanthin oxidase inhibitor allopurinol has been shown to prevent

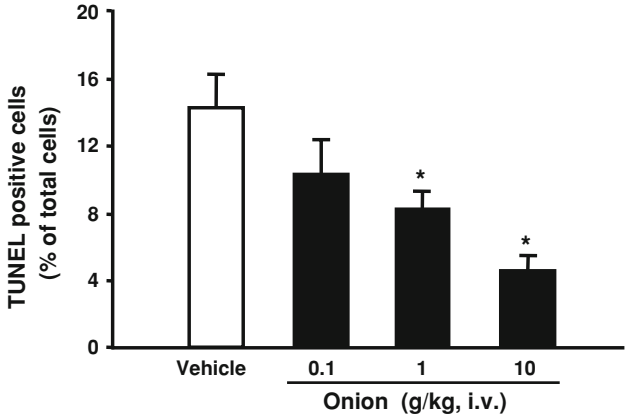

Fig. 5 Effects of onion extract on apoptotic cell death in a rat myocardial infarct model. **a** Quantitative analysis of TUNEL-positive cells. The percentage cell death was calculated by dividing the number of TUNEL-stained cells by the total number of cells. All values are the mean ± SEM ($n = 4$). * $P < 0.05$ versus vehicle

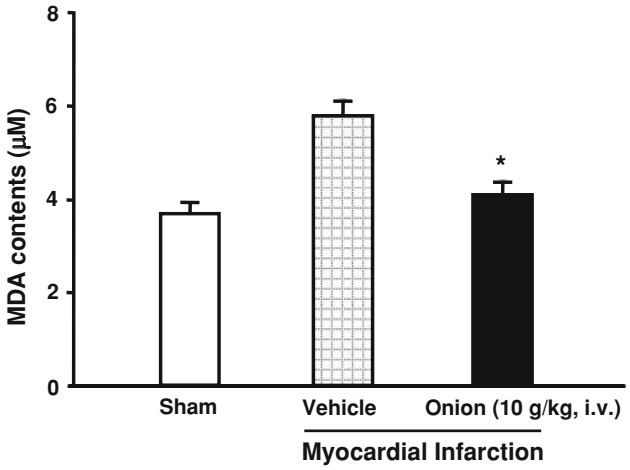

Fig. 6 Effects of onion extract on plasma MDA concentration in a rat myocardial infarct model. Quantitative analysis of the effect of onion (i.v.) on the plasma MDA concentration. All values are the mean ± SEM ($n = 8$). * $P < 0.05$ versus vehicle

ischemia-induced myocardial dysfunction by suppressing ROS [18]. In these contexts, it has been suggested that a limitation of oxidative stress can prevent or ameliorate the progression of ischemic heart injury by favoring the balance towards lowering oxidative stress. Recent studies have shown an increased recognition that certain natural dietary antioxidants, including members of the *Allium* family, have the potential to reduce the detrimental effect of a number of cardiovascular risk factors [19]. Garlic, an *Allium* species, and various forms of extracts prepared from it represent examples of such dietary antioxidants, having health benefits for the prevention of cardiovascular diseases, especially those associated with oxidative stress [20].

The present study focuses on onion, another *Allium* species, which is known to possess antioxidant properties [5]. This study attempts to evaluate its preventive potential against ischemic/hypoxic injury. As the focus here is on an evaluation of the beneficial potential of dietary onion, the total extract of onion was used through extraction with methanol instead of fractionized components or a single component. This study has shown that a methanolic extract of onion (0.05, 0.1 g/ml) prevents heart-derived H9c2 cells from hypoxia-induced apoptosis. In the in vivo rat myocardial infarction model, onion extract (1, 10 g/kg) administered either intravenously (bolus injection) or orally (once a day for 14 days) significantly reduced the infarct size, providing evidence of the protective effect of onion against myocardial ischemic injury. The onion extract (1, 10 g/kg, i.v.) also attenuated apoptotic cell death of the heart in a rat myocardial infarction model; 1 g/kg rat body weight of onion extract, an effective dose, was calculated as 7 g/m^2 in terms of the body surface. When extrapolating this rat dose (7 g/m^2) to humans, the equivalent dose in a human being (65 kg body weight/170 cm height) is 38 g/m^2 [8, 9]. As the body surface of a 65 kg man is 1.7 m^2, an approximate amount of onion for an average man is 65 g.

In a rat myocardial infarction model, the ischemia-induced increase in the plasma MDA level was reduced via treatment with onion extract in vivo. In addition, from the results of the H9c2 cells, in which onion extract suppressed the generation of ROS induced by hypoxia and the well-known antioxidant trolox mimicked the anti-apoptotic effect of onion, the preventive effect of onion against ischemia/hypoxia-induced apoptotic cardiomyocyte death appears to be related to its antioxidant property. Supporting this assumption, the present results showed that a methanolic extract of onion, which would include both hydrophilic and lipophilic components [21], had the ability to scavenge free radicals, thereby possibly reducing oxidative stress directly during ischemia/hypoxia. Consistent with these results, various studies have reported that two main components of onion, hydrophilic flavonoids (i.e., flavonol glucosides) and lipophilic organosulfur compounds (i.e., dipropyl sulfide and dipropyl trisulfide) are responsible for the antioxidant activity [5, 22, 23]. The phenolic hydroxyl groups in the structure of flavonoids have been recognized to function as electron or hydrogen donors, conferring a free radical scavenging effect [24]. Disulfides and thiol structures of organosulfur compounds have also been reported to function as free radical scavengers by trapping electrons from other systems [25]. In addition, onion has been demonstrated to activate the endogenous defense system, such as catalase, SOD, and glutathione that has evolved to combat exogenous insults [6, 26]. Taken together, it is suggested that the mechanism

of the anti-apoptotic effect of onion against ischemic/hypoxic injury may involve direct free radical scavenging activity. Moreover, an indirect antioxidant effect through the activation of endogenous antioxidant enzymes cannot be excluded from an explanation of its anti-apoptotic effect. Hence, further study is needed to clarify as to which antioxidant defense system is involved in the mechanism of the anti-apoptotic effect of onion.

ROS is known to open mitochondrial membrane permeability transition pores and then trigger a cytochrome *c* release into the cytoplasm, leading to apoptotic cell death [27]. Cardiac mitochondria are highly vulnerable to ischemic injury, resulting in the precipitation of a mitochondria-dependent apoptotic death pathway in a number of pathologic conditions [28]. Supporting this concept, the authors have previously demonstrated that ischemia/hypoxia causes mitochondrial dysfunction, which involves mitochondrial permeability transition, the disruption of $\Delta\Psi_m$ and a release of cytochrome *c* in heart-derived H9c2 cells [10]. Cytochrome *c* released from mitochondria into the cytosol further induces the activation of downstream caspases, which in turn causes nuclear condensation, leading to ultimate apoptotic cell death [28]. Thus, maintenance of the functional and structural integrity of mitochondria is considered to be a prerequisite for the successful prevention of ischemic injury [29]. In line with these studies, the results from the present study suggest that the mechanism of the anti-apoptotic effect of onion may, at least in part, include the maintenance of mitochondrial integrity through inhibition of $\Delta\Psi_m$ reduction, cytochrome *c* release and caspase-3 activation in H9c2 cells.

The use of natural substances has become more widespread over the past few years based on the concept that natural substances may have fewer side effects than do pharmaceuticals [30]. Moreover, they are readily available to the public without prescriptions. Hence, the present results may provide additional motivation for the consumption of dietary onions. As this study did not include an investigation of the specific active constituents of onion, further study should elucidate the particular components of onion that contribute to its anti-apoptotic effect and result in the cardioprotection shown in this study. In conclusion, the results from this study suggest that onion may be useful as a beneficial dietary nutrient for the prevention of ischemic heart injury, and that the underlying mechanisms may include, at least in part, an antioxidant effect.

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