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Adipocytokine, omentin inhibits doxorubicin-induced H9c2 cardiomyoblasts apoptosis through the inhibition of mitochondrial reactive oxygen species

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ABSTRACT

Omentin is a relatively novel adipocyte-derived cytokine mainly expressed in visceral adipose tissues. Blood omentin level decreases in the patients with obesity, hypertension, type 2 diabetes and atherosclerosis. We have previously demonstrated that omentin inhibits key pathological processes for hypertension development, including vascular inflammatory responses, contractile reactivity and structural remodeling. In addition, there are several reports demonstrating that omentin prevents cardiac hypertrophy and myocardial ischemic injury. Doxorubicin (DOX) is an effective anti-cancer drug with cardiotoxic side effect. Here we tested the hypothesis that omentin may prevent DOX-induced cardiac cytotoxicity. H9c2 rat cardiomyoblasts were treated with DOX in the absence or presence of omentin. Omentin (300 ng/ml, 3 h pretreatment) significantly inhibited DOX (1 μM, 18 h)-induced decreases in living cell number as determined by a colorimetric cell counting assay. Omentin (300 ng/ml, 3 h) significantly inhibited DOX (1 μM, 12 h)-induced cleaved caspase-3 expression as determined by Western blotting. Omentin (300 ng/ml, 3 h) significantly inhibited DOX (1 μM, 6 h)-induced mitochondrial reactive oxygen species (ROS) production as determined by a MitoSOX Red fluorescent staining. In addition, a mitochondrial respiratory chain complex I inhibitor, rotenone (0.5 μM, 3 h pretreatment), significantly inhibited DOX (1 μM, 6–18 h)-induced decreases of living cell number, cleaved caspase-3 expression and mitochondrial ROS production. In summary, we for the first time demonstrate that omentin prevents DOX-induced H9c2 cells apoptosis through the inhibition of mitochondrial ROS production. These results indicate omentin as an attractive pharmaco-therapeautic target against DOX-induced cardiac side effect.

1. Introduction

Adipose tissue is now recognized as an endocrine organ [1]. Omentin was relatively recently identified as an adipose tissue-secreted cytokine (adipocytokine) and is mainly expressed in visceral adipose tissues of healthy individuals [2]. Obesity-induced decreases in blood omentin level seem to be related to the higher incidence of hypertension, type 2 diabetes, atherosclerosis and coronary heart disease [3–7]. In addition, there is a report demonstrating that blood omentin level decreases in the patients with obstructive sleep apnea syndrome, which is often associated with obesity [8]. Another report demonstrated that a decrease in blood omentin level in the patients with obesity undergoing duodenal switch bariatric surgery was associated with the increased risk of cardiovascular diseases [9]. Based on the results of those epidemiologic studies, we have hypothesized that omentin may be related to the pathogenesis of obesity-related cardiovascular diseases. Specifically, we have previously examined the direct effects of omentin on vascular systems. As a result, we have demonstrated that omentin inhibits the several key pathological processes for hypertension development, including inflammatory responses in vascular endothelial and smooth muscle cells as well as vascular structural remodeling mediated via smooth muscle migration [10–12]. Furthermore, we have demonstrated that omentin induces vasodilation in rat isolated blood vessels via nitric

Abbreviations: DOX, doxorubicin; ROS, reactive oxygen species; NOX, NADPH oxidase; mtPTP, mitochondrial permeability transition pore.

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oxide (NO) production in endothelial cells [13]. In addition, another group has recently demonstrated that omentin prevents transverse aortic constriction-induced cardiac hypertrophy in mice [14]. They have also demonstrated that omentin prevents myocardial ischemic injury in mice [15], suggesting the direct preventive effects of omentin not only in vasculature but also in cardiomyocyte.

Doxorubicin (DOX), an anthracycline antibiotic, is an effective anti-cancer drug. DOX is widely used for chemotherapy, however, it is well known that DOX has several side effects. Among them, cardiotoxicity is the most serious side effect of DOX [16,17]. Cardiotoxicity is actually a serious problem for clinical use of DOX, because DOX-induced cardiac dysfunction ultimately leads to congestive heart failure [18]. In the present study, we test the hypothesis that omentin may prevent DOX-induced cardiotoxicity, and for the first time revealed that omentin can prevent DOX-induced cardiac cell apoptosis through the inhibition of mitochondrial reactive oxygen species (ROS) production.

2. Materials and methods

2.1. Materials

Recombinant omentin (BioVendor, Candler, NC, USA); doxorubicin hydrochloride salt (LC laboratories, Woburn, MA, USA); rotenone (Enzo, Plymouth Meeting, PA, USA); anti-cleaved caspase-3 antibody (Cell Signaling, Beverly, MA, USA), anti-total actin antibody (Sigma–Aldrich, Saint Louis, MO, USA).

2.2. Culture of H9c2 cardiomyoblasts

H9c2 cardiomyoblasts obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco Modified Eagle's Medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and a mixture of 1% penicillin-streptomycin as described elsewhere [19]. H9c2 cells at 90% confluence were growth arrested in DMEM containing 0.5% FBS for 24 h before stimulation.

2.3. Living cell number counting

Living cell number was counted by a cell counting kit 8 (CC8; Dojindo, Kumamoto, Japan) as described previously [20]. After H9c2 cells were treated with DOX (1 μM, 18 h) in the absence or presence of omentin (1–300 ng/ml, 3 h pretreatment) or rotenone (0.5 μM, 3 h pretreatment), cell density was observed with a phase-contrast microscope (CKX-31, OLYMPUS, Tokyo, Japan) and photomicrographs were taken with a digital camera (BMS, Tokyo, Japan). After CC8 solution (25 μl/0.5 ml medium) was added to the media for 1 h at 37 °C, the absorbance of the media at 450 nm was measured using a microplate reader (TriStar LB941, Berthold, Bad, Wildbad, Germany).

2.4. Western blotting

Western blotting was performed as described previously [12]. After H9c2 cells were treated with DOX (1 μM, 12 h) in the absence or presence of omentin (300 ng/ml, 3 h) or rotenone (0.5 μM, 3 h), total cell lysates were harvested by homogenizing the cells with Triton X-100-based lysis buffer. Protein concentration was determined using a bicinchoninic acid method (Pierce, Rockfold, IL). Equal amounts of proteins (10–15 μg) were separated by SDS-PAGE (14%) and transferred to a PVDF membrane (ATTO, Tokyo Japan). After being blocked with 0.5% skim milk, membranes were incubated with primary antibodies (Cleaved caspase-3; 1:200 dilution and total-actin; 1:500 dilution) at 4 °C overnight, and the membrane-bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 h) and the EZ-ECL system (Biological Industries, Kibbutz Beit-Haemek, Israel). Equal loading of protein was confirmed by measuring total-actin expression. The results were analyzed using a CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

2.5. Measurement of mitochondrial ROS

Measurement of mitochondrial ROS was performed as described previously [21]. A mitochondrial ROS indicator, MitoSOX Red (Invitrogen), was used to detect mitochondrial ROS production in the H9c2 cells. After H9c2 cells were treated with DOX (1 μM, 6 h) in the absence or presence of omentin (300 ng/ml, 3 h) or rotenone (0.5 μM, 3 h), MitoSOX Red (5 μM) was added in the Hank's Balanced Salt Solution (Wako, 1 ml/well) for 10 min at 37 °C. Images were observed with a fluorescence microscope (BX-51, OLYMPUS) and photomicrographs were taken with a digital camera (Micro-Publisher 5.0, QImaging, Surrey, Canada). Mitosox intensity was calculated using Image J software (NIH, Bethesda, MD, USA).

2.6. Statistical analysis

Data were shown as mean ± SEM. Statistical evaluations were performed by one-way ANOVA followed by Bonferroni's test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of omentin on DOX-induced H9c2 cell death

We first examined the effects of omentin (1–300 ng/ml, 3 h) pretreatment on DOX-induced H9c2 cell death. Omentin significantly inhibited DOX (1 μM, 18 h)-induced decreases in living cell number (DOX; 0.30 ± 0.01-fold relative to control, $n = 6$ vs. +Omentin (300 ng/ml); 0.52 ± 0.04-fold, $n = 6$, $p < 0.01$, Fig. 1).

3.2. Effects of omentin on DOX-induced cleaved caspase-3 expression in H9c2 cells

Caspase-3 is a major contributor to the execution of apoptosis. We next examined the effects of omentin (300 ng/ml, 3 h) pretreatment on DOX-induced caspase-3 activation in H9c2 cells. Omentin significantly inhibited DOX (1 μM, 12 h)-induced cleaved caspase-3 expression in H9c2 cells (DOX; 2.89 ± 0.36-fold relative to control, $n = 6$ vs. +Omentin; 1.88 ± 0.26-fold, $n = 6$, $p < 0.05$. Fig. 2).

3.3. Effects of omentin on DOX-induced mitochondrial ROS production in H9c2 cells

DOX-induced ROS production through mitochondrial respiratory chain is supposed to be one of the major contributors to H9c2 cell death [22–24]. We thus examined the effects of omentin (300 ng/ml, 3 h) pretreatment on DOX-induced mitochondrial ROS production by a MitoSOX Red fluorescent staining. Omentin significantly inhibited DOX (1 μM, 6 h)-induced mitochondrial ROS production in H9c2 cells (DOX; 2.42 ± 0.29-fold relative to control, $n = 7$ vs. +Omentin; 1.54 ± 0.22-fold, $n = 8$, $p < 0.05$, Fig. 3). We confirmed that DOX did not activate NADPH oxidase (NOX), another source of ROS by a lucigenin-assay ($n = 5$, data not shown).

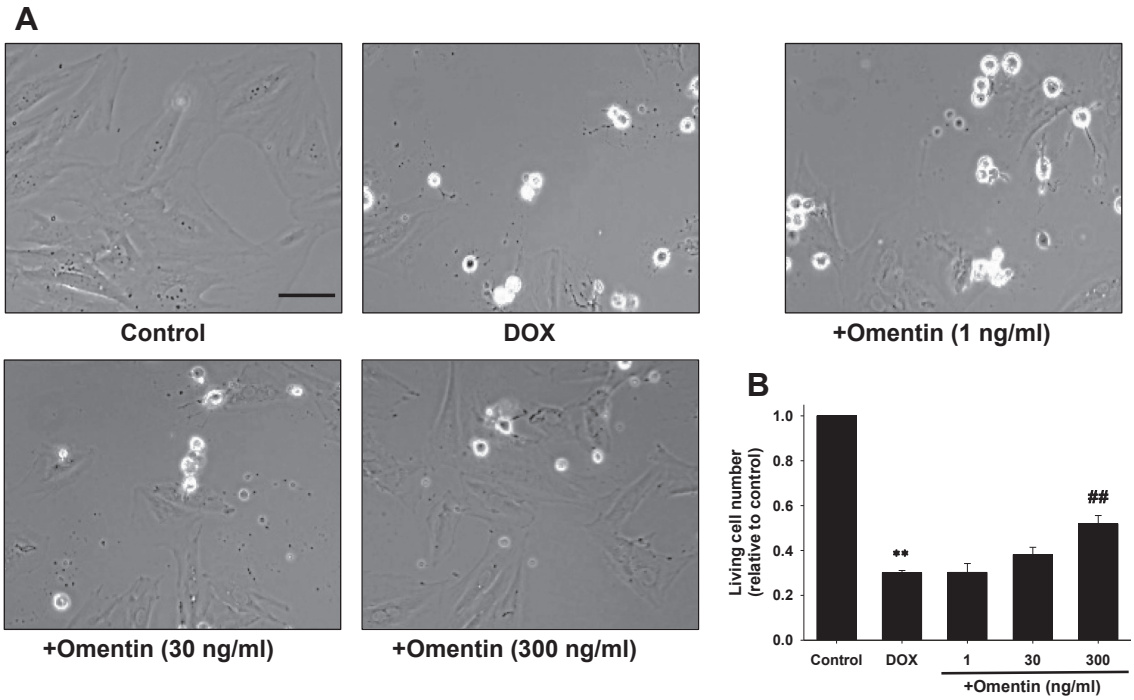

Fig. 1. Effects of omentin on doxorubicin (DOX)-induced H9c2 cell death. H9c2 cells were treated with DOX (1 μM, 18 h) in the absence or presence of omentin (1–300 ng/ml, 3 h). (A) Representative photomicrographs were shown (n = 6). (B) Living cell number was counted by a colorimetric method. **p < 0.01 vs. Control; ##p < 0.01 vs. DOX. Scale bar: 100 μm.

3.4. *Effects of rotenone, a mitochondrial respiratory chain complex I inhibitor, on DOX-induced cell death*

To confirm whether DOX-induced H9c2 cell death is mediated by a pathway related to mitochondrial ROS, we finally examined the effects of an inhibitor of mitochondrial respiratory chain complex I (rotenone; 0.5 μM, 3 h) on DOX-induced H9c2 cell death, cleaved caspase-3 expression and mitochondrial ROS production. Rotenone significantly inhibited DOX (1 μM, 18 h)-induced decreases in living cell number (DOX; 0.52 ± 0.02-fold relative to control, n = 4 vs. +Rotenone; 0.81 ± 0.06-fold, n = 4, p < 0.01, Fig. 4A). Rotenone significantly inhibited DOX (1 μM, 12 h)-induced cleaved caspase-3 expression (DOX; 2.83 ± 0.07-fold relative to control, n = 4 vs. +Rotenone; 1.19 ± 0.14-fold, n = 4, p < 0.01, Fig. 4B). Rotenone also significantly inhibited DOX (1 μM, 6 h)-induced mitochondrial ROS production (DOX; 4.07 ± 0.10-fold relative to control, n = 4 vs. +Rotenone; 1.67 ± 0.21-fold, n = 4, p < 0.01, Fig. 4C).

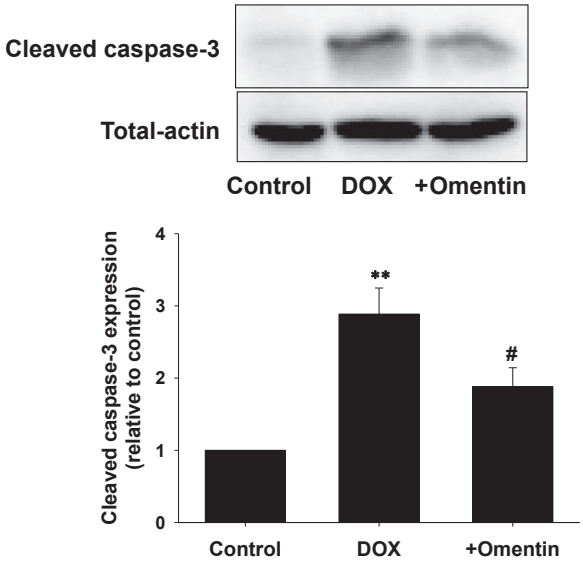

Fig. 2. Effects of omentin on DOX-induced cleaved caspase-3 expression in H9c2 cells. After H9c2 cells were treated with DOX (1 μM, 12 h) in the absence or presence of omentin (300 ng/ml, 3 h), total cell lysates were harvested. Cleaved caspase-3 expression was determined by Western blotting (n = 10). **p < 0.01 vs. Control; #p < 0.05 vs. DOX.

4. Discussion

In this study, we for the first time demonstrated that omentin inhibited DOX-induced H9c2 cardiomyoblasts death. In addition, omentin inhibited DOX-induced cleaved caspase-3 expression. Moreover, omentin inhibited DOX-induced mitochondrial ROS production. Further, we confirmed that rotenone, a mitochondrial respiratory chain complex I inhibitor, inhibited DOX-induced H9c2 cells death, cleaved caspase-3 expression and mitochondrial ROS production. These data collectively indicate that omentin inhibits DOX-induced H9c2 cell apoptosis through the inhibition of mitochondrial ROS production.

Omentin is an adipocytokine and its serum level decreases in the obesity [3]. There is a report demonstrating that blood omentin level is significantly higher in the non-obese subjects (113.45 ± 6.32 ng/ml) than in the obese subjects (75.48 ± 6.42 ng/ml) [25]. In addition, the blood omentin level decreases in the patients with cardiac diseases (acute coronary syndrome, 113.08 ± 61.43 ng/ml; stable angina pectoris, 155.41 ± 66.89 ng/ml; control, 254.00 ± 72.9 ng/ml) [26]. It is thus suggested that omentin is protective against cardiac diseases. It is also suggested that the concentrations of omentin (1, 30, 300 ng/ml) that we used in this study are pathophysiologically relevant. There are several reports demonstrating that omentin had direct influences on the cardiovascular systems

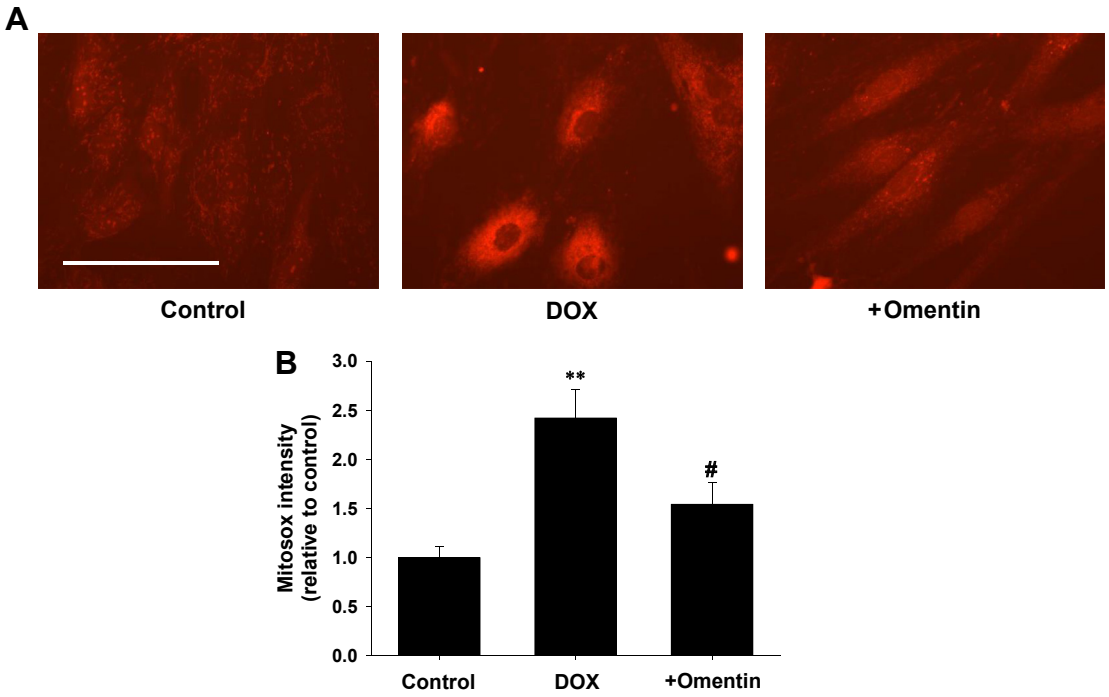

Fig. 3. Effects of omentin on DOX-induced mitochondrial reactive oxygen species (ROS) production in H9c2 cells. After H9c2 cells were treated with DOX (1 μM, 6 h) in the absence or presence of omentin (300 ng/ml, 3 h), mitochondrial ROS were measured by a MitoSOX Red fluorescent staining (n = 6–8). **p < 0.01 vs. Control; #p < 0.05 vs. DOX. Scale bar: 100 μm.

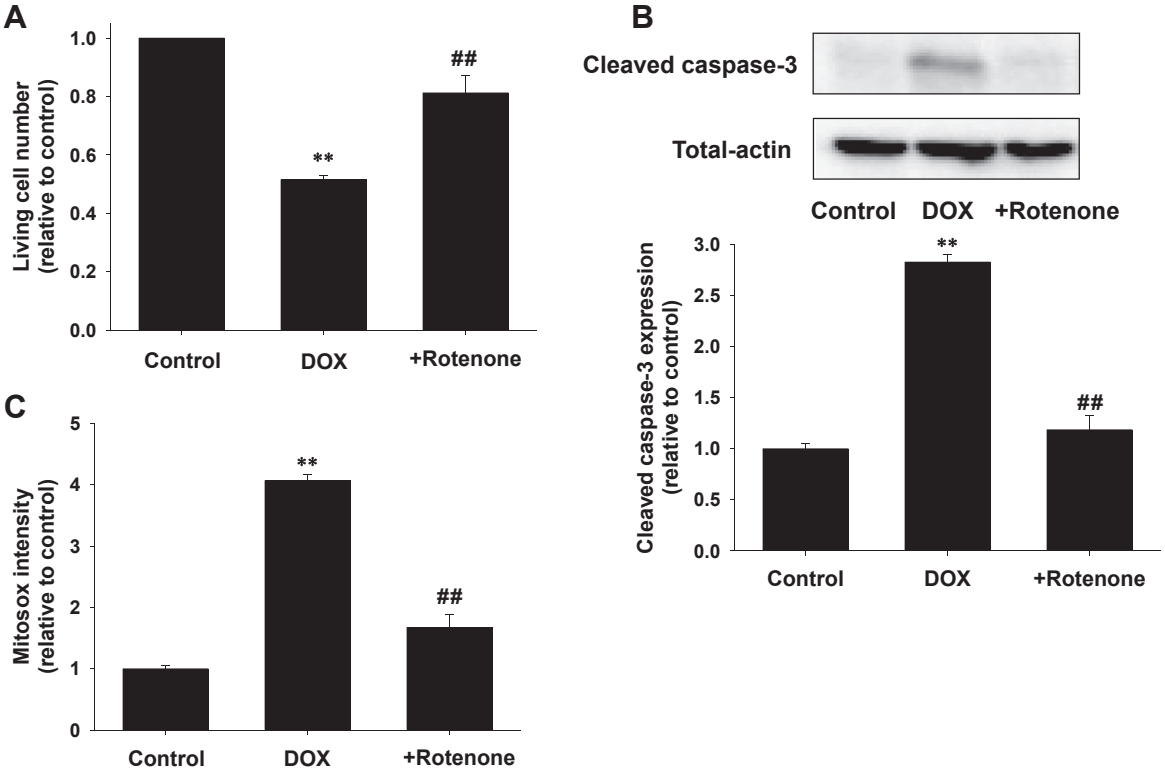

Fig. 4. Effects of rotenone, a mitochondrial respiratory chain complex I inhibitor, on DOX-induced cell death. (A) After H9c2 cells were treated with DOX (18 h, 1 μM) in the absence or presence of rotenone (0.5 μM, 3 h), living cell number was counted by a colorimetric method (n = 4). (B) After H9c2 cells were treated with DOX (12 h, 1 μM) in the absence or presence of rotenone (0.5 μM, 3 h), total cell lysates were harvested. Cleaved caspase-3 expression was determined by Western blotting (n = 4). (C) After H9c2 cells were treated with DOX (6 h, 1 μM) in the absence or presence of rotenone (0.5 μM, 3 h), mitochondrial ROS were measured by a MitoSOX Red fluorescent staining (n = 4). **p < 0.01 vs. Control; ##p < 0.01 vs. DOX.

[10–13,15,27]. However, to the best our knowledge, omentin receptor is not identified. Adiponectin, an adipocytokine, shows omentin-like protective effects on the cardiovascular systems [28–32]. AdipoR1 (mainly expressed in skeletal muscle) and AdipoR2 (mainly expressed in liver) have been identified as adiponectin receptors [33]. It was reported that AdipoR1 is expressed in the cardiovascular systems and its overexpression plays protective effects [34]. Therefore, exploring omentin receptor should be an attractive future target.

We confirmed that DOX induced caspase-3-dependent apoptosis in H9c2 cardiomyoblasts. It was reported that the Bcl-2 family proteins are key mediators for H9c2 cell apoptotic processes [35]. Bax and Bcl-2 can influence the mitochondrial permeability transition pore (mtPTP) [36]. There is a report demonstrating that DOX-induced cleaved caspase-3 expression was mediated via the decreases of mtPTP through the increases of Bax/Bcl-2 expression ratio during H9c2 cell apoptosis [37]. It was also demonstrated that DOX-induced ROS production increased Bax/Bcl-2 expression ratio [38]. It is thus suggested that omentin may inhibit H9c2 cell apoptosis through the inhibition of cleaved caspase-3 expression via the suppression of ROS/increased Bax/Bcl-2 ratio/decreased mtPTP pathways.

Numerous studies have examined the mechanism by which DOX causes cardiotoxicity [39–41], and showed that the DOX-induced myocardial damage is mainly caused by topoisomerase II inhibition and ROS production. In this study, we focused on the ROS production induced by DOX. The important sources for ROS production are (I) NOX, (II) mitochondrial respiratory chain, (III) xanthine oxidase and (IV) uncoupled endothelial NO synthases [42]. We have previously reported that omentin inhibits tumor necrosis factor-α- and platelet-derived growth factor-BB-induced ROS production through the prevention of NOX activity in vascular smooth muscle cells [11,12]. While we examined the effects of DOX (10 min- 6 h) on NOX activity, DOX did not influence it in H9c2 cells (data not shown, $n = 5$). Thus, we next examined the effects on DOX-induced mitochondrial respiratory chain-derived ROS, and showed that omentin inhibited DOX-induced mitochondrial ROS production in H9c2 cells. We also confirmed that rotenone, a mitochondrial respiratory chain complex I inhibitor, prevents DOX-induced H9c2 cell apoptosis through the inhibition of mitochondrial ROS production. These data suggest that mitochondrial respiratory chain complex I is an important contributor to DOX-induced H9c2 cell apoptosis and that omentin can inhibit it. In addition, the regulation of anti-oxidative systems is important for oxidative stress occurrence [42]. Omentin may influence activity of some endogenous anti-oxidative enzymes, including super oxide dismutase, catalase and glutathione peroxidase.

In summary, we for the first time demonstrate that omentin inhibits DOX-induced H9c2 cardiomyoblasts apoptosis through the inhibition of mitochondrial ROS production. These results suggest omentin as an attractive pharmaco-therapeautic target against DOX-induced cardiac side effect.

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Disclosures

The authors have nothing to disclose.

Conflict of interest

None.

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