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L-carnitine reduces doxorubicin-induced apoptosis through a prostacyclin-mediated pathway in neonatal rat cardiomyocytes ☆

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ABSTRACT

Background: Clinical use of doxorubicin is greatly limited by its severe cardiotoxic side effects. L-carnitine is a vitamin-like substance which has been successfully used in many cardiomyopathies, however, the intracellular mechanism(s) remain unclear. The objective of this study was set to evaluate the protective effect of L-carnitine on doxorubicin-induced cardiomyocyte apoptosis, and to explore its intracellular mechanism(s).

Methods: Primary cultured neonatal rat cardiomyocytes were treated with doxorubicin (1 μM) with or without pretreatment with L-carnitine (1–30 mM). Lactate dehydrogenase assay, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling staining, and flow cytometry measurement were used to assess cytotoxicity and apoptosis. Fluorescent probes 2',7'-dichlorofluorescein diacetate and chemiluminescence assay of superoxide production were used to detect the production of reactive oxygen species. Western blotting was used to evaluate the quantity of cleaved caspase-3, cytosol cytochrome *c*, and Bcl-x_L expression.

Results: L-carnitine inhibited doxorubicin-induced reactive oxygen species generation and NADPH oxidase activation, reduced the quantity of cleaved caspase-3 and cytosol cytochrome *c*, and increased Bcl-x_L expression, resulting in protecting cardiomyocytes from doxorubicin-induced apoptosis. In addition, L-carnitine was found to increase the prostacyclin (PGI_2) generation in cardiomyocytes. The siRNA transfection for PGI_2 synthase significantly reduced L-carnitine-induced PGI_2 and L-carnitine's protective effect. Furthermore, blockade the potential PGI_2 receptors, including PGI_2 receptors (IP receptors), and peroxisome proliferator-activated receptors alpha and delta (PPARα and PPARδ), revealed that the siRNA-mediated blockage of PPARα considerably reduced the anti-apoptotic effect of L-carnitine.

Conclusions: These findings suggest that L-carnitine protects cardiomyocytes from doxorubicin-induced apoptosis in part through PGI_2 and PPARα-signaling pathways, which may potentially protect the heart from the severe toxicity of doxorubicin.

1. Introduction

Doxorubicin, one of the original anthracyclines and first isolated in the early 1960s, remains among the most effective anticancer drugs ever developed [1]. Like all other anticancer agents; however, doxorubicin is a double-edged sword because its use can lead to development of tumor cell resistance, and it can be toxic to healthy tissues. In particular, clinical use of doxorubicin is, however, greatly limited by its serious adverse cardiac effects that may ultimately lead to cardiomyopathy and heart failure [2]. Among the various mechanisms suggested to mediate doxorubicin's cardiotoxicity, the increased formation of reactive oxygen species (ROS) by NADPH oxidase activation [3] which ultimately results in cardiomyocyte apoptosis (or programmed cell death) is one of the most plausible [4]. In support of the role of the NADPH oxidase, mice deficient in this enzyme, unlike wild-type mice; were resistant to the cardiotoxic effects of the chronic doxorubicin treatment [5]. Nevertheless, to date, researchers/scientists have tried out a variety of approaches aimed at preventing or mitigating the deleterious action of doxorubicin, but so far, the ability of these treatments to protect the heart from damage is limited [6]. Therefore, the development of therapies with which to prevent and/or treat the doxorubicin's cardiotoxicity remains a critical issue in both cardiology and oncology.

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L-carnitine (*L*-trimethyl-3-hydroxy-ammoniabutanoate) is a vitamin-like substance that is a quaternary ammonium compound biosynthesized from amino acids lysine and methionine. Carnitine is required for the transfer of long-chain fatty acids into the mitochondrial matrix before they can undergo β-oxidation, resulting in ATP formation [7]. In addition, *L*-carnitine modulates the intra-mitochondrial acyl-CoA/CoA ratio to remove toxic compounds before they have a chance to accumulate in the mitochondria. Many reports have shown that *L*-carnitine can improve the toxic effects of various substances on ear, heart, brain and kidney [8–12]. *L*-carnitine has been successfully used in many cardiomyopathies including those involving oxidative stress and it is also a free radical scavenger [13]. The possible protective mechanisms of *L*-carnitine include the inhibition of mitochondrial membrane permeability transition, a decrease of oxidative stress, and the prevention of proapoptotic protein expression [10,14,15]. However, the detailed mechanisms are not conclusive. It has been shown that prostaglandins (PGs) exert the common carnitine-dependent system for the β-oxidation of long chain fatty acids [16]. Prostacyclin (PGI_2), a major PG, is originated from arachidonic acid by the cyclooxygenase (COX) system coupled to the action of PGI_2 synthase (PGIS) [17]. PGI_2 acts on platelets and blood vessels through the cell surface prostacyclin receptor (IP receptor). It inhibits platelet function, and dilates blood vessels [18]. PGI_2 is also supposed to be the ligands of peroxisome proliferator-activated receptors alpha and delta (PPARα and PPARδ), belonging to a family of ligand-activated transcription factors [19]. *In vivo* study had shown that the production of PGI_2 has the most significant increase after the feeding of *L*-carnitine in rat [20]. Recent studies also revealed that *L*-carnitine can induce vasodilatation of subcutaneous human arteries involving endothelium through the effect related to the synthesis of PGs, especially PGI_2 [21,22]. However, the role of PGI_2 in the protective mechanisms of *L*-carnitine in the heart has not been determined.

PGI_2 has been reported to alleviate myocardial ischemia–reperfusion injury [23,24]. Due to the short half-life of PGI_2; therefore, the more stable PGI_2 analogs were developed. Iloprost, one such PGI_2 analog, was found to limit infarct size in canine hearts [25] and to attenuate myocardial stunning in open-chest dogs [26]. Iloprost was also reported to protect isolated rat cardiomyocytes from H_2O_2- and doxorubicin-induced cell injury [27,28]. In addition, we recently reported that augmented PGI_2 production through adenovirus-mediated transfer of genes for COX-1 and PGIS protects renal tubular cells from doxorubicin-induced apoptosis [29]. Therefore, the present study was set to evaluate the protective effect of *L*-carnitine on doxorubicin-induced cardiomyocyte apoptosis, and to explore its mechanism involving PGI_2.

2. Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA, USA). 5(6)-carboxy-2′, 7′-dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes Inc. (OR, USA). All other chemicals of reagent grade were obtained from Sigma-Aldrich chemical Co. (St. Louis, MO, USA). Antibodies were purchased from Lab Frontier Co. Ltd.,

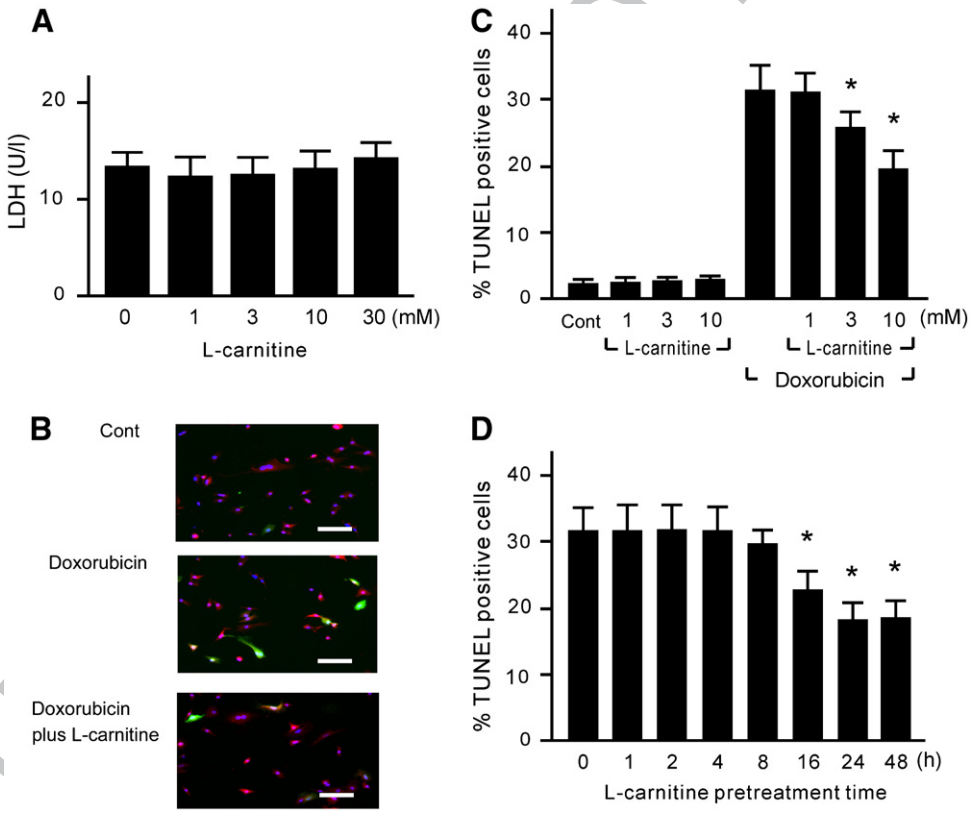

Fig. 1. The influence of *L*-carnitine on the apoptoxicity of doxorubicin in cardiomyocytes. (A) Cytotoxicity induced by *L*-carnitine in cardiomyocytes. Cardiomyocytes were treated with *L*-carnitine for 24 h in different concentrations as indicated. The LDH released from the cytosol of damaged cells was measured to determine the cytotoxicity of *L*-carnitine. Results are shown in mean ± S.E.M ($n=3$). (B) Effect of *L*-carnitine on doxorubicin-induced apoptosis in cardiomyocytes. Cardiomyocytes were treated with vehicle control (Cont, upper panel), 1 μM of doxorobucin for 24 h (middle panel), and pretreated with *L*-carnitine (10 mM) for 24 h with further treated with 1 μM of doxorobucin for 24 h (lower panel); and then immunostained with an anti-α-actinin antibody (red)/TUNEL (green), and the nucleus was stained with DAPI (blue). Representative photomicrographs are shown. Bars indicate 50 μm. (C) The dose-dependent inhibition effect of *L*-carnitine on doxorubicin-induced apoptosis in cardiomyocytes. The cells were pretreated with *L*-carnitine (1–10 mM) for 24 h, and then further treated with adding 1 μM of doxorobucin for 24 h. The results of the percentage of TUNEL-positive cells were shown in mean ± S.E.M. ($n=6$). *$P<0.05$ *vs.* doxorubicin alone. (D) The pretreatment time dependence of the anti-apoptosis effect of *L*-carnitine. Cardiomyocytes were pretreated with *L*-carnitine (10 mM) for different time periods, then treated with 1 μM of doxorobucin for 24 h, and the results of % TUNEL-positive cells were shown in mean ± S.E.M. ($n=6$). *$P<0.05$ *vs.* doxorubicin alone.

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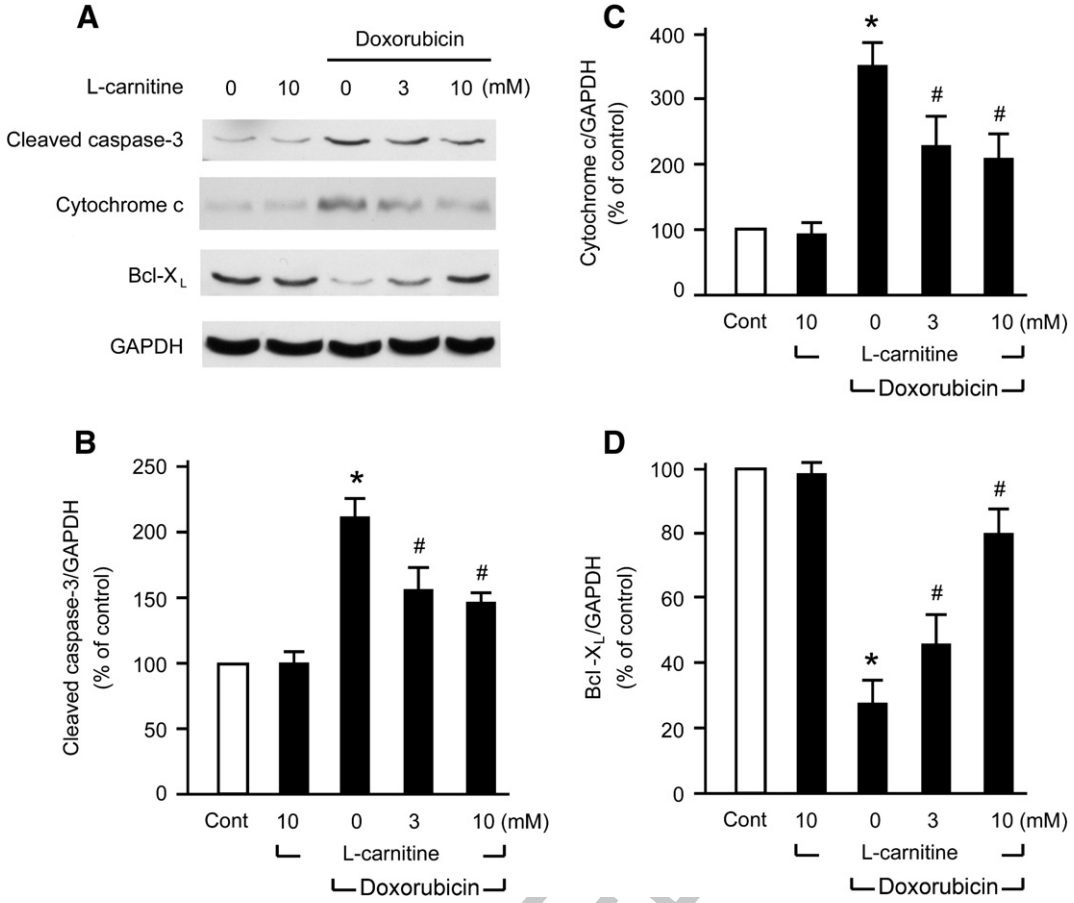

Fig. 2. Effects of *L*-carnitine on apoptotic markers (cleaved caspase-3, released cytochrome *c*, and Bcl-xL) in doxorubicin-treated cardiomyocytes. Values shown were corrected using the density of GAPDH. Results were shown in mean ± S.E.M ($n = 6$). *$P<0.05$ *vs.* control (Cont); #$p<0.05$ *vs.* doxorubicin alone. (A) Cardiomyocytes were pretreated with *L*-carnitine for 24 h, and then treated with 1 μM of doxorobucin for 12 h. Western blotting was carried out with the specific antibody against cleaved caspase-3, cytochrome *c* and Bcl-x_L. GAPDH was used as a loading control. Representative photomicrographs are shown. (B) Densitometric analysis of cleaved caspase-3. (C) Densitometric analysis of cytochrome *c* release. (D) Densitometric analysis of Bcl-x_L.

Seoul, Korea (anti-GAPDH), Cell Signaling Technology, Inc., Danvers, MA, USA (anti-caspase-3), Upstate, Serologicals Company, Lake Placid, NY, USA (Anti-gp91^{phox}, anti-p47^{phox}, anti-p67^{phox} and anti-p40^{phox}), and Santa Cruz Biotechnology, Santa Cruz, CA, USA (anti-cytochrome *c*, anti-Bcl-x_L, anti-PPAR-α, Anti-PPAR-δ, and IP receptor-neutralizing antibody).

2.2. Cell culture

Primary cultures of neonatal rat ventricular myocytes were prepared as previously described [30]. The research was conducted in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health, and approved by the Institutional Animal Care and Use Committee of China Medical University (LAC-94-0069). Myocyte cultures were obtained >95% pure as revealed by immunofluorescence microscopy with counting of all nuclei [stained by DAPI (4'-6-diamidino-2-phenyindole) (Sigma-Aldrich)] and of cells that stain positive for α-actinin (Sigma-Aldrich). The culture medium was replaced after 24 h with serum-free medium consisting of DMEM, transferring (10 μg/ml), insulin (10 μg/ml), and BrdU (0.1 mM) and exposed to agents as indicated. During this final incubation, cardiac myocytes were treated with one of the following: 1) pretreatment with *L*-carnitine (1–30 mM) for 24 h; 2) doxorubicin at 1 μM; 3) doxorubicin at 1 μM in the pretreatment with *L*-carnitine (1–30 mM) for 24 h. Unless otherwise indicated, treatment was continued for 24 h, at which time we examined cell death and apoptosis-associated signaling cascades by biochemical methods. Control myocytes were incubated in serum-deprived DMEM but were not treated with any chemical.

2.3. Assay of lactate dehydrogenase activity

For lactate dehydrogenase (LDH) assays, cardiomyocytes were plated 5000 cells/well in 96-well plates. After treatment with *L*-carnitine, we collected and assayed the culture medium with the LDH Cytotoxicology Detection kit (Roche; Mannheim, Germany) according to the manufacturer's directions. Each data point was determined in triplicate.

2.4. TUNEL assay

Doxorubicin-mediated apoptosis in cardiomyocytes was detected with enzymatic labeling of DNA strand breaks which were identified with using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) stain. As described previously [29], TUNEL staining was performed with a Cell Death Detection kit (Roche, Mannheim, Germany). To reveal total nuclei, the same slides were stained with DAPI (1 μg/ml) in PBS plus 0.5% 1,4-diazabicyclo [2,2,2] octane. α-Actinin- and TUNEL-stained cell slides were mounted with DAPI solution and observed with fluorescent microscope. Immunofluorescence images were obtained using a fluorescence microscope (Eclipse, Nikon, Japan) equipped with a digital camera (DXM1200, Nikon). The apoptotic ratio was further measured by flow cytometry according to the manufacturer's instructions.

2.5. Western blot analysis

Western blot analysis was performed as previously described [31], Membranes were blocked in 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 containing 5% nonfat dry milk, followed by incubation with primary antibody. Membranes were washed three times and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000 dilutions) to detect bands by enhanced chemiluminescence (Amersham Biosciences Corp, NJ, USA).

2.6. Detection of intracellular ROS and NADPH oxidase activity assay

ROS were measured using a previously described method [31]. Prior to the chemical treatment, cells were incubated in culture medium containing a fluorescent dye, DCF-DA (30 μM) for 1 h to establish a stable intracellular level of the probe. The same concentration of DCF-DA was maintained during the chemical treatment. Subsequently, the cells were washed with PBS, removed from Petri dishes by brief trypsinization, and measured for 2', 7'-dichlorofluorescein (DCF) fluorescence intensity. The DCF fluorescence intensity of the cells is an index of intracellular levels of ROS; and it can be determined by fluorescence spectrophotometry with excitation and emission wavelengths at 475 and 525 nm, respectively. The cell number in each sample was

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counted in an automatic cell counter (S.ST.II/ZM, Coulter Electronics Ltd., Miami, FL, U.S.A.) and utilized to normalize the fluorescence intensity of DCF. Chemiluminescence assay of superoxide production was measured as described previously [32]. NADPH oxidase activity was measured using the lucigenin-enhanced chemiluminescence method in microsomal membrane fractions as described previously [33]. For the isolation of microsomal membranes, cell homogenates were prepared in 250 mM sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 1 mM PMSF, 10 μg/ml aprotinin, and 5 μg/ml leupeptin, followed by centrifugation at 1000 *g* (10 min, 4 °C). The pellet was discarded, and the supernatant was spun at 8000 *g* (10 min, 4 °C). The microsomal fraction was separated from cytosol by centrifugation of the supernatant at 105,000 *g* (45 min, 4 °C). The pellet was resuspended in the homogenization buffer by using a Hamilton glass syringe. The cell homogenate and microsomal fraction were used immediately. The assay was started in an Orion microplate luminometer (Berthold Detection Systems) by automatic injection of the 150 μl reaction buffer [50 mM K-phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH] into 10 μl of the homogenate or membrane suspension (5–20 μg protein). Photon emission in response to superoxide generation was measured every 60 s with a 5 s signal integration time for 20 min. The activity is expressed in relative light units per milligram of protein. The protein concentration was measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

2.7. Short interfering RNA (siRNA) transfection

PPARα siRNA and PPARδ siRNA were purchased from Santa Cruz Biotechnology. PPAR siRNAs and mock control oligonucleotides were transfected using the Lipofectamine reagent according to the manufacturer's instructions. The final concentration of PPAR siRNAs for transfection was 10 or 100 nM. We washed transfected cells and incubated them in new culture media for an additional 24 h for doxorubicin treatment and western blot assays.

2.8. Measuring PGI_2 by enzyme immunoassay

Cells were sonicated in 1 ml of ice-cold buffer (0.05 M Tris at pH 7.0, 0.1 M NaCl, and 0.02 M EDTA) and centrifuged at 55,000 *g* for 1 h. We analyzed the supernatant with 6-keto-$PGF1_1\alpha$ ELISA kits from R&D Systems Inc. (Minneapolis, MN, USA) for PGI_2 detection.

2.9. Statistical analysis

Results are expressed as mean + S.E.M. Statistical analysis was performed using Student's *t* test or analysis of variance (ANOVA) followed by a Dunnett multiple comparison test using Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). A value of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. The protective effect of L-carnitine against doxorubicin-induced apoptoxicity in cardiomyocytes

To determine the safe dosage of *L*-carnitine on neonatal rat cardiomyocytes, we investigated the LDH released from the cytosol of damaged cells. Cardiomyocytes were cultured with *L*-carnitine at concentration of 1, 3, 10, and 30 mM for 24 h. As shown in Fig. 1A, there was no significant increase of LDH leakage along with the *L*-carnitine increase in cardiomyocytes; even exposure to 30 mM of *L*-carnitine gave no significant change from the controls. The protective effect of *L*-carnitine against the doxorubicin-induced apoptosis in cardiomyocytes was examined using TUNEL stain (Fig. 1B). Negoro *et al.* have reported treatment with doxorubicin for more than 16 h exhibited the typical ladder pattern of apoptosis [34]. Cardiomyocytes were pretreated with *L*-carnitine (110 mM) for 24 h, and then additionally treated with 1 μM of doxorubicin for 24 h. By measuring the percentage of TUNEL-labeled cells, the 24 h pretreatment of *L*-carnitine markedly decreased the number of apoptotic cells increased by doxorubicin in a dose-dependent manner (Fig. 1C). The influence of pretreatment time on the protective effect of *L*-carnitine was also monitored in cardiomyocytes. As shown in Fig. 1D, 10 mM of *L*-carnitine was not able to significantly reduce doxorubicin-induced apoptosis in

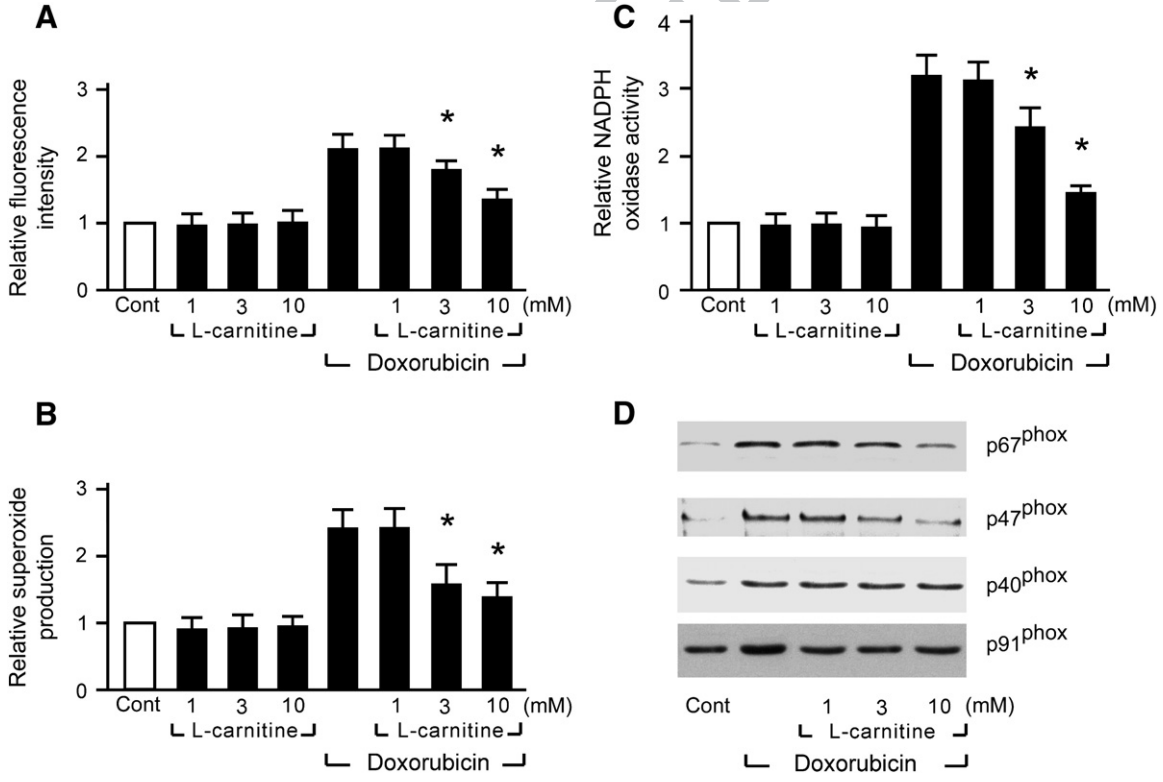

Fig. 3. Effects of *L*-carnitine on doxorubicin-induced ROS generation and NADPH oxidase activity in cardiomyocytes. Cardiomyocytes were pretreated with *L*-carnitine (1–10 mM) for 24 h, and then treated with 1 μM of doxorobucin for 1 h. Data were presented as relative intensity of experimental groups compared to untreated control. Results were shown in mean ± S.E.M. ($n=6$). *$P<0.05$ *vs.* doxorubicin alone. (A) Effects of *L*-carnitine on doxorubicin-induced ROS generation. Intracellular ROS levels were revealed by fluorescent intensities of DCF. (B) Effects of *L*-carnitine on doxorubicin-induced superoxide production. Chemiluminescence assay of superoxide production was measured. (C) Effects of *L*-carnitine on doxorubicin-increased NADPH oxidase activity. (D) Western blot analysis of cytosolic NADPH oxidase components in cardiomyocytes. Each sample of the plasma membrane fractions was prepared and the membrane associated cytosolic NADPH oxidase components were detected by Western blotting with antibodies specific for $gp91^{phox}$, $p47^{phox}$ $p67^{phox}$, and $p40^{phox}$.

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cardiomyocytes with the pretreatment periods from 1 to 8 h, whereas the reduction of apoptosis was significant with *L*-carnitine pretreatment for 16 h to 48 h.

Recent work has supported a central role for caspase family members, especially caspase-3, as effectors of apoptosis in doxorubicin-treated cardiomyocytes [35]. In addition, mitochondrial dysfunction associated with cytochrome *c* release and the decrease of anti-apoptotic protein Bcl-x_L was also dominated under doxorubicin treatment [35]. The influence of *L*-carnitine on cleaved caspase 3, cytochrome *c* and Bcl-x_L, was further evaluated by western blotting analysis (Fig. 2A). As shown in Fig. 2B and C, the cleaved caspase-3 and cytosol cytochrome *c* were greatly elevated in the cells treated with 1 μM of doxorubicin for 24 h. Pretreatment with *L*-carnitine at 3 or 10 mM for 24 h significantly reduced the quantity of cleaved caspase-3 and cytosol cytochrome *c*, as compared with that in doxorubicin-treated alone cells. Contrariwise, the expression of Bcl-x_L was reduced by doxorubicin treatment, which was also recovered by *L*-carnitine pretreatment (Fig. 2D). These results indicate that the pretreatment of *L*-carnitine inhibited doxorubicin-induced variations of apoptotic markers in a dose and time-depended manner.

3.2. The influence of *L*-carnitine on doxorubicin-induced ROS generation and NADPH oxidase activity in cardiomyocytes

To evaluate the mechanism of the protective effect of *L*-carnitine on doxorubicin-induced apoptosis, the influence of *L*-carnitine on doxorubicin-induced ROS generation and NADPH oxidase activity were monitored. We examined whether *L*-carnitine prevents doxorubicin-induced ROS formation. Kim *et al.* have reported that doxorubicin-induced ROS generation was observed immediately after doxorubicin treatment [36]. *L*-carnitine-pretreated cells were treated with 1 μM of doxorubicin for 1 h. Doxorubicin-induced increases in intracellular ROS were revealed by fluorescent intensities of DCF. As shown in Fig. 3A, *L*-carnitin pretreatment significantly inhibited doxorubicin-induced ROS formation. Similar results were obtained with the use of lucigenin-enhanced chemiluminescent superoxide detection (Fig. 3B). Next, we examined the effect of *L*-carnitin on doxorubicin-induced enzymatic activity of NADPH oxidase. Since active NADPH oxidase is a membrane-associated enzyme [37], we tested the effect of doxorubicin on the NADPH oxidase activity in microsomal membranes. As shown in Fig. 3C, treatment of cardiomyocytes with doxorubicin (1 μM) for 1 h stimulated NADPH oxidase activity in the microsomal fraction. *L*-carnitin pretreatment significantly inhibited doxorubicin-induced NADPH oxidase activity (Fig. 3C). NADPH oxidase is composed of several regulatory subunits, including those involved in the formation of the classic phagocyte-type NADPH oxidase (gp91^{phox}, p67^{phox}, p47^{phox}, and p40^{phox}) [37]. As shown in Fig. 3D, expressions of p67^{phox}, p47^{phox}, and p40^{phox} were slightly observed in vehicle-treated cardiomyocytes, and the three phox components (p67^{phox}, p47^{phox} and p40^{phox}) were clearly upregulated in the plasma membrane fraction of doxorubicin-stimulated cardiomyocytes. The upregulated expressions of p67^{phox}, and p47^{phox} in the cytoskeleton fraction were suppressed by the pretreatment of *L*-carnitine (10 mM). These results indicate that the pretreatment of *L*-carnitine inhibited doxorubicin-induced ROS generation and NADPH oxidase activation in cardiomyocytes.

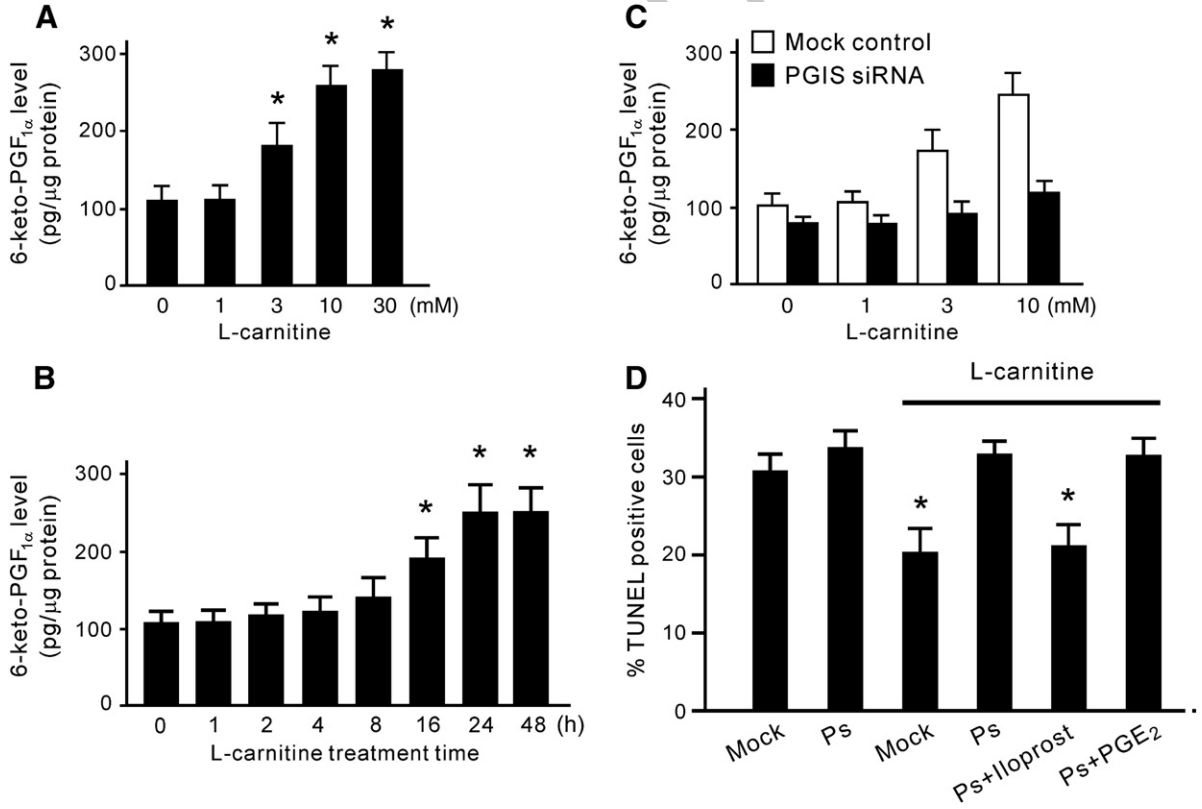

Fig. 4. The connection between prostacyclin and *L*-carnitine treatment in cardiomyocytes. *$P<0.05$ *vs.* control. (A) The levels of 6-keto-$PGF_1\alpha$ in *L*-carnitine-treated cardiomyocytes. The cells were treated with *L*-carnitine in different concentrations for 24 h. Results were shown in mean ± S.E.M. ($n=6$). (B) A time course of 6-keto-$PGF_1\alpha$ levels in *L*-carnitine-treated cardiomyocytes. Cells were treated with *L*-carnitine at 10 mM for different time. Results were shown in mean ± S.E.M. ($n=6$). (C) The effect of PGIS siRNA transfection on the levels of 6-keto-$PGF_1\alpha$ in *L*-carnitine-treated cardiomyocytes. The cells were either transfected with control siRNA as mock controls or transfected with PGIS siRNA to obtain PGIS knockdown cells. The transfected cells were treated with *L*-carnitine in different concentrations for 24 h. Results were shown in mean ± S.E.M. ($n=6$). (D) The influence of PGIS siRNA transfection on the protection effect of *L*-carnitine in doxorubicin-treated cardiomyocytes. The transfected cells were pretreated with *L*-carnitine (10 mM) for 24 h, and then treated with 1 μM of doxorobucin for 24 h. Iloprost (1 μM) or PGE_2 (1 μM) was added back to check the influence of PGIS knockdown. The percentage of TUNEL-positive cells were shown in mean ± S.E.M. ($n=6$). *$P<0.05$ *vs.* the mock control with doxorubicin treatment. Notes: Ps, PGIS siRNA transfection.

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3.3. The dependence of L-carnitine's anti-apoptotic function on the induction of PGI_2 in cardiomyocytes

The production of PGI_2 was typically monitored by using measurement of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$); because 6-keto-$PGF_{1\alpha}$ is a stable product of the non-enzymatic hydration of PGI_2. L-carnitine at 3 mM significantly elevated PGI_2 levels in cardiomyocytes at 24 h (Fig. 4A). This PGI_2 elevation was increased along with the increase of L-carnitine. In the time course analysis, the PGI_2 elevation induced by 10 mM of L-carnitine was significant at 16 h, and reached a maximum at 24 h (Fig. 4B). To evaluate the role of PGI_2 in L-carnitine's protection effect, the siRNA for prostacyclin synthase (PGIS) was applied to block PGI_2 synthesis. As shown in Fig. 4C, PGIS siRNA transfection obviously reduced PGI_2 generation in L-carnitine-treated cardiomyocytes. In PGIS siRNA-transfected cells, doxorubicin alone induced more serious apoptosis than that in mock control cells, as revealed by TUNEL staining (Fig. 4D). The pretreatment of 10 mM L-carnitine significantly alleviated doxorubicin-induced apoptosis in mock control cells, whereas there was a very minor influence of L-carnitine on doxorubicin-induced apoptosis in PGIS siRNA-transfected cells (Fig. 4D). Further to confirm the role of PGI_2, we added back iloprost (a stable analogue of PGI_2) and PGE_2 to siRNA-transfected cardiomyocytes with doxorubicin and L-carnitine treatment. The blockage of L-carnitine's anti-apoptosis functions by PGIS siRNA transfection was reversed by iloprost but not by PGE_2 (Fig. 4D). These results revealed the dependence of L-carnitine's anti-apoptotic function on the induction of PGI_2 in cardiomyocytes.

3.4. The essential role of PPARα in the protective effect of L-carnitine

PGI_2 has been reported to be a potential ligand for IP receptor, PPARα and PPARδ [19]. To identify the signaling pathways involved in the protective function of L-carnitine, the neutralizing antibody for IP receptor and the siRNA for PPARα and PPARδ were applied in cardiomyocytes. The PPARα and PPARδ protein levels were obviously reduced by PPARα and PPARδ siRNA transfection, respectively, as shown in Fig. 5A and B. The inhibitory effect of L-carnitine on the doxorubicin-induced ROS production was partially reversed by PPARα siRNA transfection and by IP receptor neutralizing, but not significantly affected by PPARδ siRNA transfection (Fig. 5C). The doxorubicin-induced apoptotic cells were slightly increased by PPARα siRNA transfection, and were not affected by IP receptor neutralizing and by PPARδ siRNA transfection (Fig. 5D). Similarly, the inhibitory effect of L-carnitine on the doxorubicin-induced apoptosis was reduced by

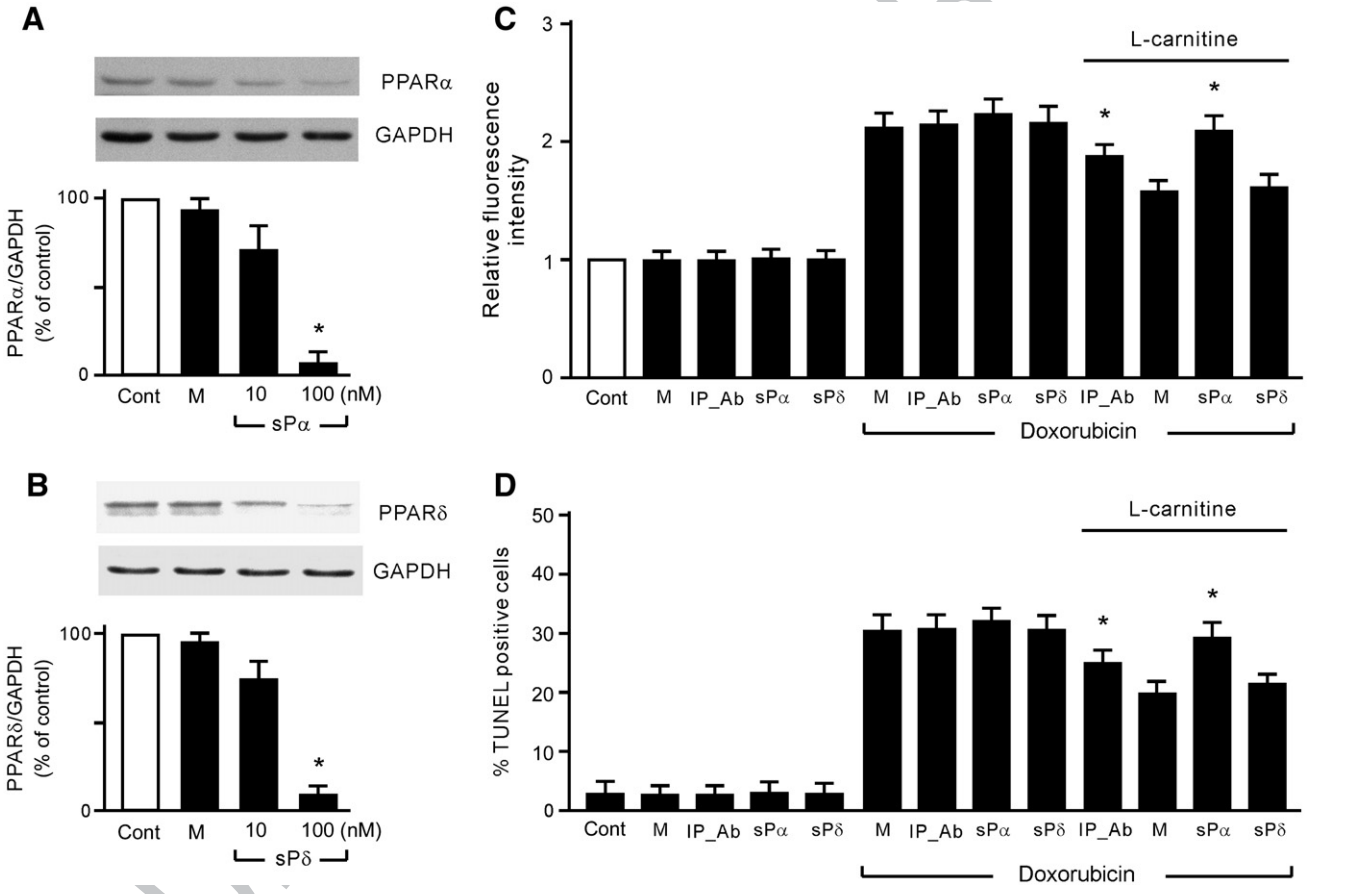

Fig. 5. Effects of the blockage of PGI_2-signaling pathway on the protective effect of L-carnitine in cardiomyocytes. (A) The effect of PPARα siRNA transfection on PPARα protein levels in cardiomyocytes. The cells were transfected with PPARα siRNA (sPα; 10 or 100 nM) to get PPARα knockdown cells. Control siRNA was also applied as mock controls (M). Western blotting was carried out with the specific antibody against PPARα. GAPDH was used as a loading control. Results were shown in mean ± S.E.M. ($n=3$). *$P<0.05$ *vs.* the mock control. (B) The effect of PPARδ siRNA transfection on PPARδ protein levels in cardiomyocytes. The cells were transfected with or PPARδ siRNA (sPδ; 10 or 100 nM) to get PPARδ knockdown cells. Western blotting was carried out with the specific antibody against PPARδ. Results were shown in mean ± S.E.M. ($n=3$). *$P<0.05$ *vs.* the mock control. (C) The effect of IP-neutralizing antibody, PPARα siRNA and PPARδ siRNA on L-carnitine-decreased doxorubicin-induced ROS generation in cardiomyocytes. Transfected cells were pretreated with or without L-carnitine (10 mM) for 24 h, and then treated with 1 μM of doxorobucin for 1 h. For blocking the function of PGI_2 IP receptor, the cells were pretreated with IP receptor neutralizing antibody for 30 min. Results were shown in mean ± S.E.M. ($n=6$). *$P<0.05$ *vs.* the mock control with L-carnitine and doxorubicin treatment. Notes: Cont, untransfected control; M, mock control; IP_Ab, IP receptor neutralizing antibody treatment; sPα, PPARα siRNA transfection; sPδ, PPARδ siRNA transfection. (D) The influence of IP-neutralizing antibody, PPARα siRNA and PPARδ siRNA on the protective effect of L-carnitine in cardiomyocytes. Transfected cells were pretreated with or without L-carnitine (10 mM) for 24 h, and then treated with 1 μM of doxorobucin for 24 h. For blocking the function of PGI_2 IP receptor, the cells were pretreated with IP receptor neutralizing antibody for 30 min. The percentage of TUNEL-positive cells were shown in mean ± S.E.M. ($n=6$). *$P<0.05$ *vs.* the mock control with L-carnitine and doxorubicin treatment.

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PPARα siRNA transfection and by IP receptor neutralizing, and not significantly affected by PPARδ siRNA transfection (Fig. 5D). These results revealed the crucial role of PPARα signaling pathway in *L*-carnitine's protective function on doxorubicin-induced apoptosis in cardiomyocytes.

4. Discussion

The main points arising from the results presented are as follows. The pretreatment of *L*-carnitine significantly improved doxorubicin-induced apoptosis in cardiomyocytes in a dose-dependent manner. *L*-carnitine significantly reduced doxorubicin-induced ROS generation and NADPH oxidase activation in cardiomyocytes. *L*-carnitine increased the endogenous PGI_2 production; with the reduction of PGI_2 generation by PGIS siRNA transfection, the protective effect of *L*-carnitine against doxorubicin-induced apoptosis was significantly decreased in cardiomyocytes. In addition, PPARα plays a major part in *L*-carnitine's protective effect on doxorubicin-induced apoptosis in cardiomyocytes. It is concluded that *L*-carnitine can protect cardiomyocytes from doxorubicin-induced apoptosis in part through PPARα activation and PGI_2.

4.1. L-carnitine inhibits doxorubicin-induced ROS generation and NADPH oxidase activation in cardiomyocytes

Cardioprotection by *L*-carnitine is well documented, although its mechanism of action is not fully understood [38,39]. It was shown to protect the heart against oxidative stress and to be a free radical scavenger [13,40]. Since ROS are important apoptotic stimulators in doxorubicin-induced apoptosis [4,41,42], the anti-apoptotic effects of *L*-carnitine reported here are also involved with the inhibition of ROS production and are in agreement with previous reports [43]. But the detailed mechanism of the inhibition of ROS production is still unclear. We show here that *L*-carnitin pretreatment significantly inhibits doxorubicin-induced NADPH oxidase activation; and a significant reduction in doxorubicin-increased of $p67^{phox}$ and $p47^{phox}$ in the microsomal fraction. The inhibition of NADPH oxidase activation and thereafter ROS production by *L*-carnitine point to NADPH oxidase activation as a possible initial step of doxorubicin-induced cardiomyopathy. NADPH oxidase is the source of superoxide anion in cardiac cells [44]. It has been reported the associations between genetic polymorphisms in three subunits of the NADPH oxidase and doxorubicin-induced cardiotoxicity [5]. In support of these associations, NADPH oxidase directly enhances the production of superoxide in the presence of doxorubicin and NADPH [3]. Our findings further show that doxorubicin increases the content of $p67^{phox}$ and $p47^{phox}$ in the microsomal fraction, which may enhance the production of superoxide. *L*-carnitine inhibition of NADPH oxidase may not only prevent doxorubicin-induced ROS generation, but also reduce the doxorubicin-induced cardiomyocyte apoptosis.

4.2. L-carnitine protects cardiomyocytes from doxorubicin-induced apoptosis through PPARα activation by PGI_2

Interestingly, more than 16 h to 48 h pretreatment is necessary for *L*-carnitine to protect cardiomyocytes from doxorubicin-induced apoptosis *in vitro* (Fig. 1). The requirement for relatively long pretreatment periods may thus result from *L*-carnitine-induced PGI_2 generation. The results of our present study show that PGI_2 generation is essential for the anti-apoptotic effect of *L*-carnitine, and significantly induced by *L*-carnitine treatment for 8 h or above (Fig. 4). These results further suggest that a long pretreatment period is helpful to reach a maximum of *L*-carnitine-induced PGI_2 in cardiomyocytes to protect cardiomyocytes from doxorubicin-induced apoptosis. In our previous study, the selective PGI_2 augmentation with adenovirus-COX-1/PGIS transfection has been found to inhibit doxorubicin-induced ROS generation and to protect NRK-52E cells from doxorubicin-induced apoptosis [29]. The ROS inhibition was largely due to the elevated activation of catalase and superoxide dismutase; which was caused by cellular PGI_2 augmentation. In addition, we had reported that cellular PGI_2 augmentation can activate PPARα in NEK-52E cells [45]. Earlier studies also show that PPARα ligands decrease the expression of NADPH oxidase directly or indirectly by affecting the synthesis of hormonal agents; that are known stimulants for NADPH oxidase activity and ROS generation [46]. Based on these data, we suggest that the anti-oxidant ability of PGI_2 is highly associated with activating PPARα. In the present study, inducing PGI_2 and activating PPARα are also shown to be necessary for *L*-carnitine's anti-apoptotic effect. Therefore, we suggest that *L*-carnitine might induce PGI_2 generation to inhibit doxorubicin-induced ROS generation through PPARα activation in cardiomyocytes, and that *L*-carnitine is useful in reducing doxorubicin-induced cardiac toxicity.

Because the cardiac protective effect of *L*-carnitine results mostly from the PGI_2 induction and PPARα activation as revealed in this study (Fig. 5), we thus suggest that PGI_2 and PPARα may be potential therapeutic candidates for doxorubicin-induced cardiomyopathy. But giving PGI_2 and its analogs systematically can cause undesirable side effects. Administrating PGI_2 and its more stable analogs locally is also a challenge because of the relatively short half-life of these drugs. In fact, certain fatty acids, such as docosahexaenoic acid, can also activate PPARα and protect renal tubular cells from adriamycin-induced apoptosis *in vivo*, and recover the kidney function [29]. However, administering these fatty acids often needs relative high dosages and is rather costly. *L*-carnitine is a natural cardioprotective agent that can be safely used in humans. Therefore, our findings suggest that *L*-carnitine may be developed as a future clinical remedy to prevent doxorubicin-induced cardiomyopathy in human.

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