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L-Carnitine attenuates angiotensin II-induced proliferation of cardiac fibroblasts: role of NADPH oxidase inhibition and decreased sphingosine-1-phosphate generation☆

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Abstract

The heart is unable to synthesize L-carnitine and is strictly dependent on the L-carnitine provided by the blood stream; however, additional studies are needed to better understand the mechanism of L-carnitine supplementation to the heart. The aim of this study was to evaluate the effects of L-carnitine on angiotensin II (Ang II)-induced cardiac fibroblast proliferation and to explore its intracellular mechanism(s). Cultured rat cardiac fibroblasts were pretreated with L-carnitine (1-30 mM) then stimulated with Ang II (100 nM). Ang II increased fibroblast proliferation and endothelin-1 expression, which were partially inhibited by L-carnitine. L-Carnitine also attenuated Ang II-induced NADPH oxidase activity, reactive oxygen species formation, extracellular signal-regulated kinase phosphorylation, activator protein-1-mediated reporter activity and sphingosine-1-phosphate generation. In addition, L-carnitine increased prostacyclin (PGI_2) generation in cardiac fibroblasts. siRNA transfection of PGI_2 synthase significantly reduced L-carnitine-induced PGI_2 and its anti-proliferation effects on cardiac fibroblasts. Furthermore, blockading potential PGI_2 receptors, including immunoprecipitation (IP) receptors and peroxisome proliferator-activated receptors alpha (PPARα) and delta , revealed that siRNA-mediated blockage of PPARα considerably reduced the anti-proliferation effect of L-carnitine. In summary, these results suggest that L-carnitine attenuates Ang II-induced effects (including NADPH oxidase activation, sphingosine-1-phosphate generation and cell proliferation) in part through PGI_2 and PPARα-signaling pathways.

Keywords: L-carnitine; Angiotensin II; Cardiac fibroblasts; NADPH oxidase; Sphingosine-1-phosphate; Prostacyclin

1. Introduction

L-Carnitine is an organic amine that plays an important role in myocardial energy production. This amino acid derivative is essential for the normal oxidation of fats by mitochondria, the transesterification and excretion of acyl-CoA esters and the oxidation of branched chain α-ketoacids [1]. Cardiac and other muscle tissues are unable to synthesize L-carnitine and are strictly dependent on the L-carnitine provided by the blood stream. However, in vitro and in vivo studies have suggested that L-carnitine is useful in many cardiomyopathies, including those involving oxidative stress and may behave as a free radical scavenger [2,3]. In addition, administration of L-carnitine has been reported to attenuate left ventricular dilatation in patients with high-risk myocardial infarction [4]. However, additional studies are needed to better understand the mechanism of L-carnitine supplementation to the heart.

Cardiac fibrosis, characterized by the proliferation of cardiac fibroblasts and abundant accumulation of matrix proteins in the extracellular space, is one of the adverse health effects accompanying hypertension that may be involved in the progression toward heart failure [5]. The renin-angiotensin system is known to influence cardiac function and can induce cardiac fibrosis under various experimental conditions. Angiotensin II (Ang II), the effector peptide of the renin-angiotensin system, is a key pathogenic factor in the development of heart failure. Ang II induces cardiac myocyte hypertrophy, fibroblast proliferation and collagen formation [5]. It is well known that Ang II increases production of reactive oxygen species (ROS) in cardiovascular tissues via NADPH oxidase, which is involved in cardiac remodeling [6]. The authors have previously reported that ROS are essential for Ang II-induced endothelin-1 (ET-1)

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gene expression and proliferation of cardiac fibroblasts [7]. Aside from ROS, the sphingolipid metabolite sphingosine-1-phosphate is a lipid mediator generated from membrane phospholipids through the concerted action of several enzymes involved in sphingolipid metabolism. Sphingosine-1-phosphate has been shown to induce cell growth and survival [8,9], whereas the other sphingomyelin metabolites ceramide and sphingosine, the metabolic precursors of sphingosine-1-phosphate, have been shown to induce apoptosis and arrest growth [10,11]. Studies have shown that Ang II can also modulate sphingosine kinase activity in isolated rat carotid arteries [12]. However, the role of sphingosine-1-phosphate in Ang II-induced cardiac fibroblast proliferation remains obscure.

Prostaglandins (PGs) make use of the common carnitine-dependent system for the β-oxidation of long chain fatty acids [13]. Prostacyclin (PGI_2), one of the primary PGs, is produced from arachidonic acid by PGI_2 synthase (PGIS) through the cyclooxygenase (COX) system [14]. PGI_2 acts through the cell surface prostacyclin receptor (IP [immunoprecipitation] receptor) and is believed to be a ligand of the peroxisome proliferator-activated receptors alpha (PPARα) and delta (PPARδ), which belong to a family of ligand-activated transcription factors [15]. An in vivo study has reported a significant increase in PGI_2 in L-carnitine-fed rats [16]. Further studies have also revealed that L-carnitine can induce vasodilatation of subcutaneous human arteries involving the endothelium through a mechanism related to the synthesis of PGs, especially PGI_2 [17,18]. However, the role of PGI_2 in L-carnitine activity in cardiac cells still remains to be delineated.

The present study evaluated the effects of L-carnitine on Ang II-induced cardiac fibroblast proliferation and explored its intracellular mechanism(s). The results clearly demonstrate that L-carnitine inhibits Ang II-induced effects (including cell proliferation, ET-1 gene expression, ROS generation, ERK phosphorylation, activator protein-1 (AP-1)-mediated reporter activity and sphingosine-1-phosphate levels), in part via an increase in PGI_2 production in cardiac fibroblasts. Thus, this study provides important new insights into the molecular pathways that may contribute to beneficial effects of L-carnitine on cardiovascular disease.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum and tissue culture reagents were obtained from Life Technologies, Inc (Carlsbad, CA, USA). A rat ET-1 cDNA probe (accession No. M64711) was obtained as previously described [7]. 2′,7′-Dichlorofluorescin diacetate was obtained from Molecular Probes (Eugene, OR, USA). Olmesartan was provided by Sankyo (Tokyo, Japan). Dimethylsphingosine was purchased from Wako Pure Chemical Industries (Osaka, Japan). Sphingosine-1-phosphate was obtained from Calbiochem (San Diego, CA, USA). SEW2871 (a sphingosine-1-phosphate receptor antagonist) was purchased from Tocris Bioscience (Bristol, UK). L-Carnitine and all other reagent-grade chemicals were purchased from the Sigma-Aldrich Chemical (St. Louis, MO, USA). The plasmid AP-1-Luc, containing the firefly luciferase reporter gene driven by a basic promoter element (TATA box) joined to tandem repeats of the AP-1 binding element, was obtained from Stratagene (La Jolla, CA, USA).

2.2. Culture of cardiac fibroblasts and treatment

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996) and was also approved by the Institutional Animal Care and Use Committee of China Medical University (97-109-N). Primary cultures of neonatal rat cardiac fibroblasts were prepared as previously described [7]. Briefly, ventricles from 1–2-day-old neonatal Sprague-Dawley rats were cut into chunks of approximately 1 mm^3 by using scissors and were subjected to trypsin (0.125%; Invitrogen) digestion in phosphate-buffered saline (PBS). Dispersed cells were incubated on 100-mm culture dishes for 30 min in a 5% CO_2 incubator. Nonmyocytes attached to the bottom of the dishes were subsequently incubated with DMEM supplemented with 10% fetal calf serum for an additional 2–4 days. Confluent nonmyocytes were treated with trypsin and subcultured. Subconfluent (~70% confluency) cardiac fibroblasts grown in culture dishes from the second to fourth passage were used in the experiments and were >99% positive for vimentin antibodies (Sigma-Aldrich). Serum-containing medium from the cultured cells was replaced with serum-free medium, and the cells were then exposed to the agents as indicated.

2.3. Evaluation of cytotoxicity

Equal numbers of cardiac fibroblasts were plated on a 96-well microplate (1×10^4 cells/well). Increasing concentrations of L-carnitine (1, 3, 10 or 30 mM) were added to the cultures after medium renewal. After 48 h of incubation, cytotoxicity was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions [19].

2.4. Cell proliferation

For cell proliferation analysis, cells were pretreated with L-carnitine (1, 3, 10 or 30 mM) for 24 h followed by Ang II (100 nM) for 24 h. The rate of cell proliferation was determined by cell counting. Cells were removed from the culture dish by addition of trypsin and pelleted by centrifugation. The pellet was resuspended in 1 ml DMEM, and cells were counted in an automatic cell counter (S.ST.II/ZM; Coulter Electronics, Miami, FL, USA). Cell proliferation was also assessed following the incorporation of 5-bromo-2′-deoxyuridine (BrdU). Cells (1×10^4 cells/well) were incubated in 96-well plastic plates. BrdU (10 μM) was then added to the medium and the cells were incubated for another 18 h. Subsequently, the cells were fixed and BrdU incorporation was determined with a Cell Proliferation ELISA Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

2.5. RNA isolation and Northern blot analysis

Preparation of total RNA and Northern blot analyses of ET-1 and 18S RNA were performed as described previously [20].

2.6. Western blot analysis

Western blot analysis was performed as previously described [20].

2.7. Transfection and luciferase assay

For transient transfections, cells were transfected using various expression vectors according to the calcium phosphate method [21]. Cardiac fibroblasts plated on six-well (35-mm) dishes were transfected using the luciferase reporter construct possessing consensus AP-1 binding sites (AP-1-Luc). Following incubation for 24 h in serum-free DMEM, cardiac fibroblasts were cultured under various conditions as indicated for a period of 48 h and then assayed for luciferase activity with a luciferase reporter assay kit (Stratagene). As was the case for AP-1 transcriptional activity, the specific firefly luciferase activity was normalized for transfection efficiency to its respective β-galactosidase activity and expressed relative to the control. The β-galactosidase assays were performed as described previously [21].

2.8. Detection of superoxide production

Chemiluminescence assay of superoxide production was performed as described previously [22].

2.9. NADPH oxidase activity assay

NADPH oxidase was measured using the lucigenin-enhanced chemiluminescence method in microsomal membrane fractions, as described previously [23].

2.10. Flow cytometric assay of 2′,7′-dichlorodihydrofluorescein oxidation

The determination of intracellular ROS production was based on the oxidation of 2′,7′-dichlorodihydrofluorescein to fluorescent 2′,7′-dichlorofluorescein (DCF), as described previously [24].

2.11. Measurement of sphingosine-1-phosphate

To determine intracellular sphingosine-1-phosphate levels, cells were pretreated with 1 mM $CaCl_2$ and incubated in an aggregometer at 37°C for 3 min. The reaction was terminated by addition of 2 mM EGTA (containing 0.1 M KCl) in an ice bath and then centrifuged at 13,000×g at 4°C for 5 min. Intracellular generated sphingosine-1-phosphate was analyzed with a Sphingosine 1 Phosphate Assay Kit (Echelon Biosciences, Salt Lake City, UT, USA) according to the manufacturer's instructions [25].

2.12. Short interfering RNA transfection

PGIS short interfering RNA (siRNA) (sc-37237), PPARα siRNA (sc-36308) and PPARδ siRNA (sc-36306) were purchased from Santa Cruz Biotechnology. siRNAs and mock control oligonucleotides (sc-37007; Santa Cruz Biotechnology) were transfected

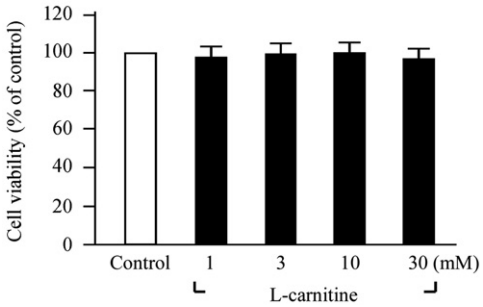

Fig. 1. Effects of L-carnitine on cardiac fibroblast viability. Cardiac fibroblasts were cultured in microplate and were incubated with increasing concentration of L-carnitine. After 48 h, cell viability was determined with the MTT assay. Results were shown as means±S.E.M. (n=3).

using the Lipofectamine reagent according to the manufacturer's instructions. The final concentration of siRNAs for transfection was 100 nM. After transfection, transfected cells were washed and incubated in fresh medium for an additional 24 h, and cells were then exposed to agents as indicated.

2.13. Measurement of 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, 0.02 M EDTA) and centrifuged at 55,000×g for 1 h. PGI_2 was analyzed using a 6-keto-$PGF1_{1}\alpha$ ELISA kit from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions [26].

2.14. Statistical analysis

Results are expressed as mean+S.E.M. for the number of experiments as designated. Statistical analysis was performed using analysis of variance followed by Tukey's multiple comparisons using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A value of P<05 was considered to be statistically significant.

3. Results

3.1. Effects of L-carnitine on cardiac fibroblast viability

Exposure of cultured cardiac fibroblasts to L-carnitine (1, 3, 10 or 30 mM) for 48 h did not have any significant effect on cardiac fibroblast viability (Fig. 1).

3.2. Effects of L-carnitine on Ang II-induced cardiac fibroblast proliferation

The effects of L-carnitine on Ang II-stimulated rat cardiac fibroblast proliferation were assessed by cell counting and BrdU incorporation. Pretreatment of cardiac fibroblasts with L-carnitine (10 and 30 mM; 24 h) significantly attenuated Ang II-increased cell numbers and BrdU incorporation (Fig. 2A, B). The influence of pretreatment time on the anti-proliferation effects of L-carnitine was assessed in cardiac fibroblasts. As shown in Fig. 2C and D, L-carnitine (10 mM) failed to attenuate Ang II-induced cell proliferation with pretreatment periods from 1 to 12 h, whereas reduction of proliferation was significant with L-carnitine pretreatment for 24 h or more. These data suggest that L-carnitine prevents Ang II-induced cardiac fibroblast proliferation.

3.3. Effects of L-carnitine on Ang II-activated ERK phosphorylation, AP-1-mediated reporter activity and ET-1 expression in cardiac fibroblasts

Ang II has been shown to activate extracellular signal-regulated kinases (ERK), and the activation of this pathway is redox-sensitive. The authors recently reported that ROS are involved in the activation of the ERK pathway, which culminates in ET-1 gene expression. To gain insight into the mechanism of action of L-carnitine, the present study

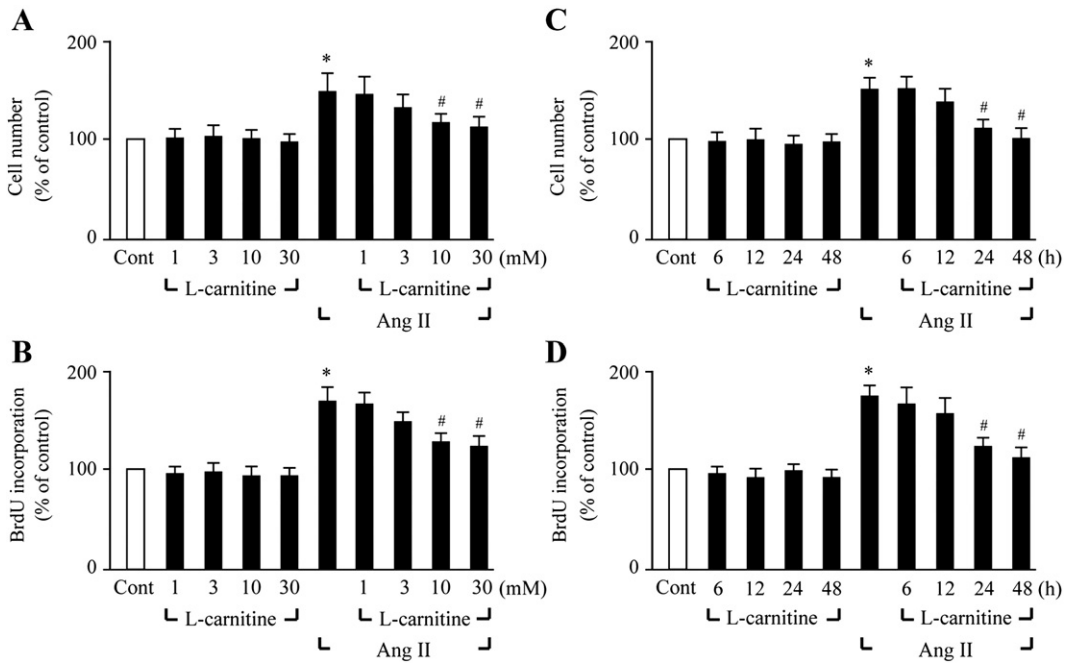

Fig. 2. Effects of L-carnitine on Ang II-induced cell proliferation in cardiac fibroblasts. Cells were preincubated with L-carnitine and then treated with Ang II (100 nM) for 24 h as indicated. Cell number and BrdU incorporation were expressed as percentage of control (Cont). Results were shown as mean±S.E.M. (n=6). *P<0.05 versus control (Cont); #P<0.05 versus Ang II alone. Inhibitory effect of L-carnitine on Ang II-induced increase in cell proliferation. Cardiac fibroblasts were preincubated with L-carnitine (1, 3, 10 or 30 mM) for 24 h and then stimulated with Ang II (100 nM; 24 h). Cells were counted for cell number and calculated as a percentage of the control value. Inhibitory effect of L-carnitine on Ang II-induced increase in BrdU incorporation. Cardiac fibroblasts were preincubated with L-carnitine (1, 3, 10 or 30 mM) for 24 h and then stimulated with Ang II (100 nM; 24 h). Cell proliferation was estimated from the incorporation of BrdU and calculated as a percentage of the control value. (C) The pretreatment time dependence of L-carnitine on Ang II-induced increase in cell proliferation. Cardiac fibroblasts were pretreated with L-carnitine (10 mM) for different time periods as indicated and were stimulated with Ang II (100 nM; 24 h). (D) The pretreatment time dependence of L-carnitine on Ang II-induced increase in BrdU incorporation. Cardiac fibroblasts were pretreated with L-carnitine (10 mM) for different time periods as indicated and were stimulated with Ang II (100 nM) for 24 h.

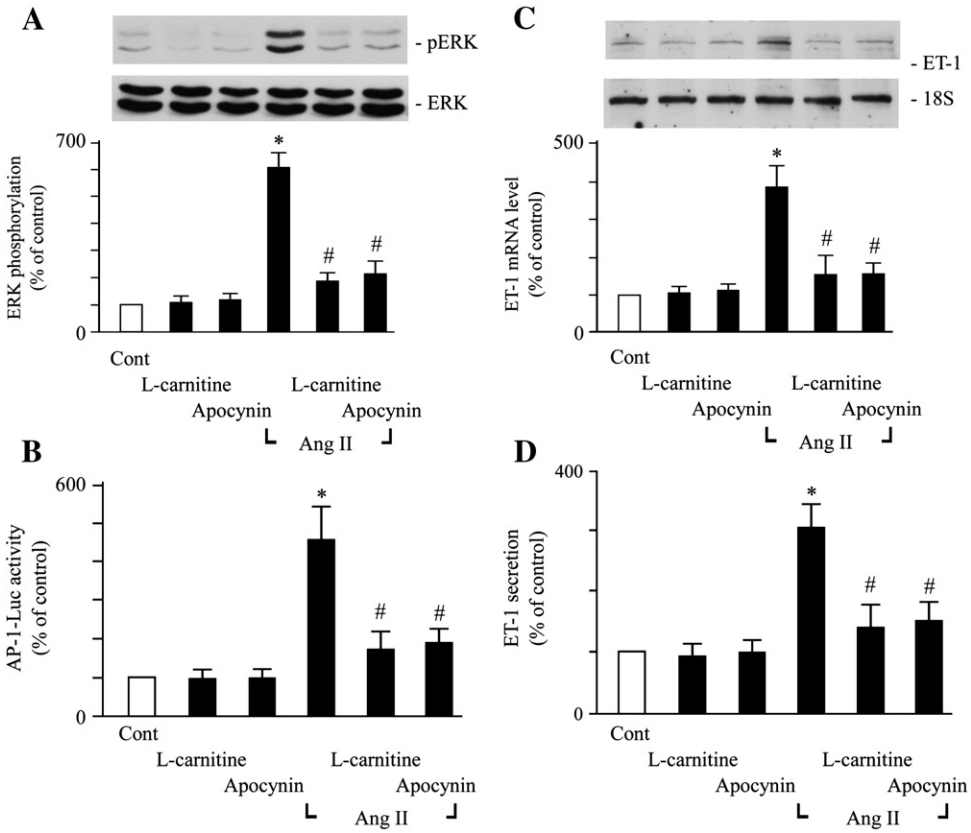

Fig. 3. Effects of L-carnitine on Ang II-increased ERK phosphorylation, AP-1-mediated reporter activity and ET-1 expression in cardiac fibroblasts. Data were expressed as percentage of control (Cont). *P<.05 versus control; # P<.05 versus Ang II alone. (A) Effects of L-carnitine on Ang II-increased ERK phosphorylation. Cells were preincubated with L-carnitine (10 mM) and then stimulated with Ang II (100 nM) for 30 min. Phosphorylation of ERK was detected by Western blotting using anti-phospho-ERK (pERK) antibody. Densitometric analyses were performed with a densitometer. Results were shown as mean±S.E.M. (n=4). (B) Effects of L-carnitine on Ang II-increased AP-1-mediated reporter activity. Cardiac fibroblasts, transfected with AP-1-Luc, were treated as indicated. Cells were preincubated with L-carnitine (10 mM) and then stimulated with Ang II (100 nM) for 24 h. Results were shown as mean±S.E.M. (n=5). (C) Down-regulation of Ang II-induced ET-1 mRNA by L-carnitine. Cells were preincubated with L-carnitine (1, 3, 10 mM) and then stimulated with Ang II (100 nM) for 30 min. Results were shown as mean±S.E.M. (n=4). (D) L-carnitine inhibits Ang II-induced ET-1 secretion. Cells were preincubated with L-carnitine (1, 3, 10 mM) and then stimulated with Ang II (100 nM; 24 h). Results were shown as mean±S.E.M. (n=6).

examined whether L-carnitine affects the Ang II-activated ERK pathway of cardiac fibroblasts and determined the effect of L-carnitine on Ang II-induced ERK phosphorylation. As shown in Fig. 3A, exposure of cardiac fibroblasts to Ang II (100 nM; 30 min) significantly increased ERK phosphorylation. However, cardiac fibroblasts pretreated with either L-carnitine (10 mM, 24 h) or the NADPH oxidase inhibitor apocynin (1μM, 30 min) had significantly decreased levels of Ang II-induced ERK phosphorylation. Moreover, Ang II-related AP-1 activation is involved in cell proliferation. The effect of L-carnitine on Ang II-induced AP-1 functional activity was evaluated using a reporter gene assay. Pretreatment with L-carnitine (10 mM, 24 h) or apocynin (1 μM, 30 min) significantly attenuated Ang II-induced AP-1-mediated reporter activation (Fig. 3B). It was further evaluated whether L-carnitine prevents Ang II-related ET-1 expression in cardiac fibroblasts (Fig. 3C, D). Similar to the above results, pretreatment with L-carnitine (10 mM, 24 h) or apocynin (1 μM, 30 min) also significantly inhibited Ang II-increased ET-1 mRNA levels and peptide secretion (Fig. 3C, D). These findings suggest that the inhibitory effects of L-carnitine are similar to those of antioxidants on Ang II-treated cardiac fibroblasts.

3.4. Effects of L-carnitine on Ang II-increased NADPH oxidase activity and ROS formation

The authors have previously demonstrated that Ang II increases ROS production in cardiac fibroblasts. To evaluate the mechanism of the inhibitory effects of L-carnitine on Ang II-induced cell proliferation, the influence of L-carnitine on Ang II-increased NADPH oxidase activity and ROS generation was assessed. Cardiac fibroblasts were preincubated with L-carnitine (10 mM, 24 h) or apocynin (1 μM, 30 min) and then treated with Ang II (100 nM) for 30 min. Pretreatment of cultured cardiac fibroblasts with L-carnitine (10 mM) or apocynin (1 μM) significantly inhibited Ang II-induced NADPH oxidase activity, superoxide formation and ROS formation (Fig. 4A–C). These findings indicate that L-carnitine inhibits Ang II-increased NADPH oxidase activity and intracellular ROS levels in cardiac fibroblasts.

3.5. Effects of L-carnitine on Ang II-increased sphingosine-1-phosphate levels

To determine the effect of Ang II on sphingosine-1-phosphate levels, cells were stimulated with Ang II (100 nM) for 3, 6, 12, 24 and 48 h. Compared to untreated cells, enhanced sphingosine-1-phosphate levels were observed after 12 h treatment with Ang II (Fig. 5A). In contrast, Ang II had no significant effect on ceramide levels in rat cardiac fibroblasts (data not shown). As depicted in Fig. 5B, Ang II-induced sphingosine-1-phosphate generation was eliminated by pretreatment with L-carnitine (10 mM; 24 h), the sphingosine kinase inhibitor dimethylsphingosine (10 μM; 1 h) [25], or the angiotensin type 1 receptor antagonist olmesartan (1 μM; 1 h). Furthermore, pretreatment with either L-carnitine or dimethylsphingosine reduced Ang II-induced but not sphingosine-1-phosphate (10 μM; 24 h)-

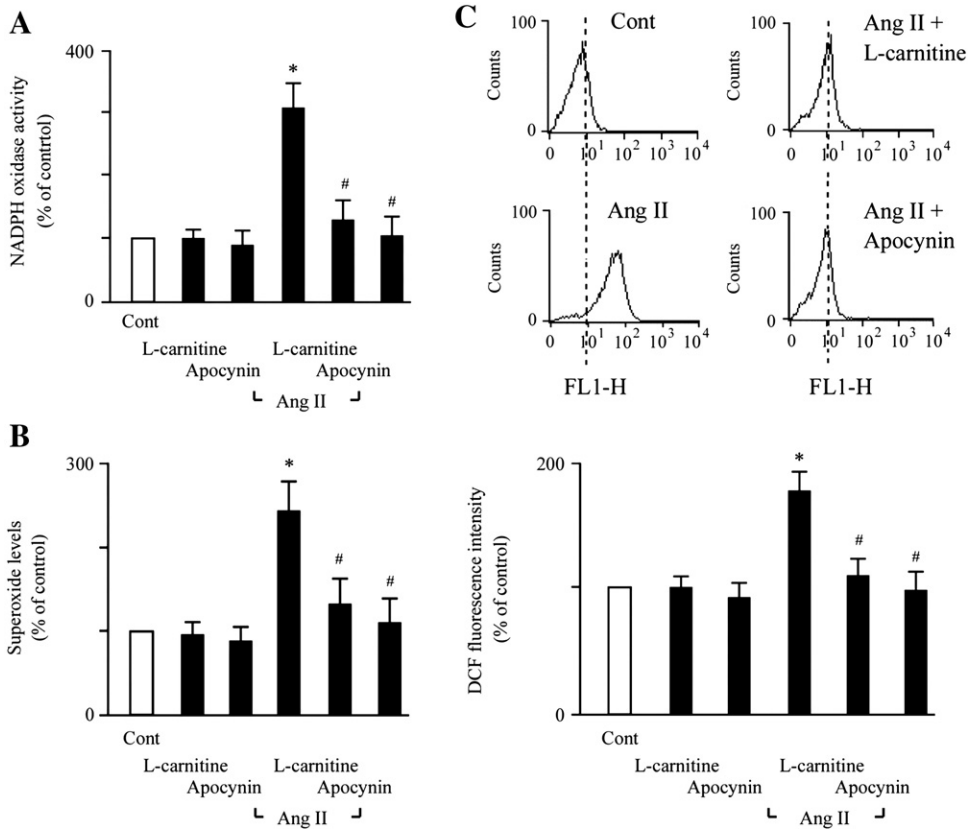

Fig. 4. Effects of L-carnitine on Ang II-increased NADPH oxidase activity and ROS formation. Cells were preincubated with L-carnitine (10 mM; 24 h) and then stimulated with Ang II (100 nM) for 30 min. *P<.05 versus control (Cont); #P<.05 versus Ang II alone. Effect of L-carnitine on Ang II-increased NADPH oxidase activity. Cardiac fibroblasts after treatment were lysed and immediately followed with NADPH oxidase activity assay. Results were shown as mean±S.E.M. (n=4). (B) Effect of L-carnitine on Ang II-induced superoxide formation. Cardiac fibroblasts after treatment were lysed and immediately followed with superoxide assay by lucigenin method. Results were shown as mean±S.E.M. (n=4). (C) Effect of L-carnitine on Ang II-induced ROS generation. (Upper panels) Flow cytometric histogram of DCF in cardiac fibroblasts. Cardiac fibroblasts were treated with vehicle control (Cont) and Ang II (100 nM) for 30 min or preincubated with L-carnitine or apocynin (1 μM) and then stimulated with Ang II. Counts: cell number; FL1-H: relative DCF fluorescence intensity. (Lower panels) Column bar graph of mean cell fluorescence for DCF. The fluorescence intensities in untreated control cells are expressed as 100%. Results were shown as mean±S.E.M. (n=5).

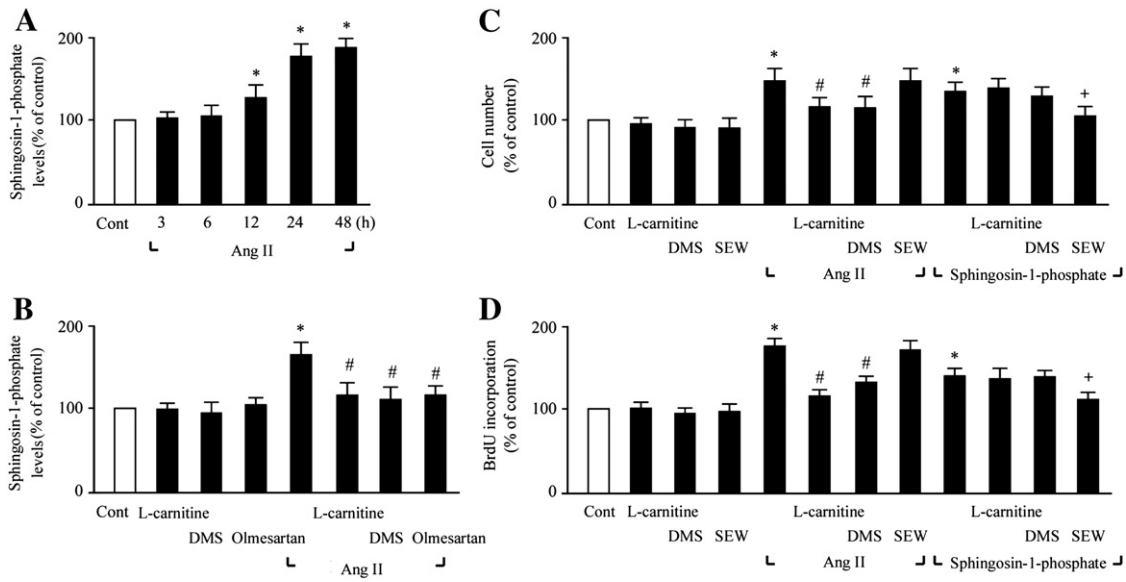

Fig. 5. Effects of L-carnitine on Ang II-increased sphingosine-1-phosphate levels. *P<.05 versus control (Cont); #P<.05 versus Ang II alone; +P<.05 versus sphingosine-1-phosphate alone. (A) Cells were stimulated with Ang II (100 nM) for different time periods as indicated. Results were shown as mean±S.E.M. (n=4). (B) Effect of L-carnitine on Ang II- increased sphingosine-1-phosphate levels. Cardiac fibroblasts were treated with vehicle control (Cont), Ang II (100 nM; 12 h); or preincubated with L-carnitine (10 mM; 24 h), dimethylsphingosine (DMS, 10 μM; 1 h), olmesartan (1 μM; 1 h) and then stimulated with Ang II. Results were shown as mean±S.E.M. (n=4). (C) Effect of L-carnitine on Ang II- or sphingosine-1-phosphate-induced increase in cell proliferation. Cardiac fibroblasts were treated with vehicle control (Cont), Ang II (100 nM; 24 h) and sphingosine-1-phosphate (10 μM; 24 h) or preincubated with L-carnitine (10 mM; 24 h), DMS (10 μM; 1 h), SEW2871 (SEW, 1μM; 1 h) and then stimulated with Ang II or sphingosine-1-phosphate. Results were shown in mean±S.E.M. (n=4). (D) Effect of L-carnitine on Ang II- or sphingosine-1-phosphate-induced increase in cell proliferation. Results were shown in mean±S.E.M. (n=4).

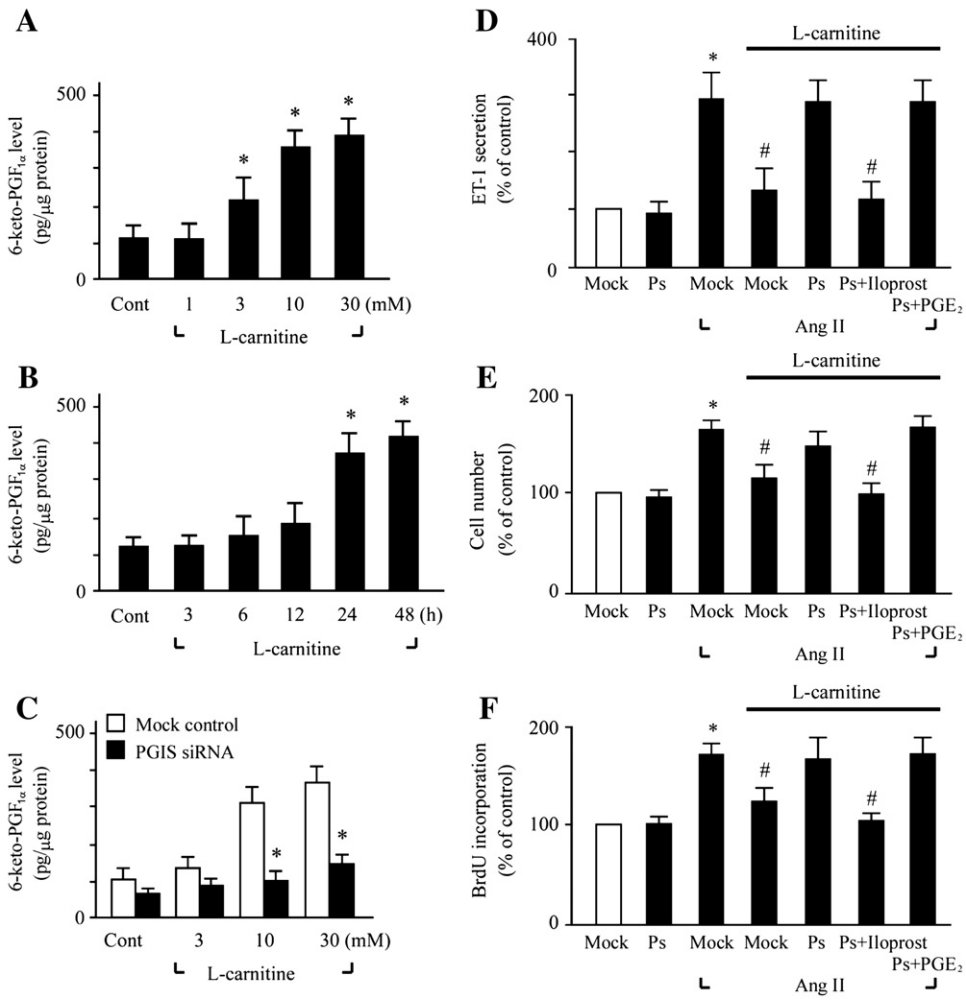

Fig. 6. The connection between prostacyclin and L-carnitine treatment in cardiac fibroblasts. Results were shown in mean±S.E.M. (n=4). (A) The levels of 6-keto-$PGF_{1\alpha}$ in L-carnitine-treated cardiac fibroblasts. The cells were treated with L-carnitine in different concentrations for 24 h. *P<.05 versus control (Cont). (B) A time course of 6-keto-$PGF_1\alpha$ levels in L-carnitine-treated cardiac fibroblasts. Cells were treated with L-carnitine (10 mM) for different time periods as indicated. *P<.05 versus control (Cont). (C) The effect of PGIS siRNA transfection on the levels of 6-keto-$PGF_1\alpha$ in L-carnitine-treated cardiac fibroblasts. 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. *P<.05 versus mock control. (D) The influence of PGIS siRNA transfection on the inhibitory effect of L-carnitine in Ang II-increased ET-1 secretion. The transfected cells were pretreated with L-carnitine (10 mM; 24 h) and then treated with Ang II (100 nM; 24 h). Iloprost (1 μM) or PG E_2 (1 μM) was added back to check the influence of PGIS knockdown. *P<.05 versus mock control (Mock); #P<.05 versus Ang II treatment. Ps, PGIS siRNA transfection. (E) The influence of PGIS siRNA transfection on the anti-proliferation effect of L-carnitine in Ang II-induced increase in cell proliferation. (F) The influence of PGIS siRNA transfection on the anti-proliferation effect of L-carnitine in Ang II- induced increase in BrdU incorporation.

induced cell proliferation (Fig. 5C, D). Pretreatment with SEW2871 (a sphingosine-1-phosphate receptor antagonist; 1 μM, 1 h) eliminated sphingosine-1-phosphate-induced cell proliferation. These results suggest that sphingosine-1-phosphate plays a role in Ang II-induced cell proliferation, and L-carnitine inhibits Ang II-enhanced sphingosine-1-phosphate levels in cardiac fibroblasts.

3.6. The dependence of the anti-proliferation function of L-carnitine on the induction of PGI_2 in cardiac fibroblasts

The production of PGI_2 was monitored by measuring 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$; a stable product of the non-enzymatic hydration of PGI_2). L-Carnitine at 3 mM significantly elevated 6-keto-prostaglandin $F_{1\alpha}$ levels in cardiac fibroblasts at 24 h (Fig. 6A). 6-Keto-prostaglandin $F_{1\alpha}$ levels increased with an increase in L-carnitine concentrations. In the time course analysis, 6-keto-prostaglandin $F_{1\alpha}$ elevation induced by 10 mM of L-carnitine reached a maximum at 24 h (Fig. 6B). To evaluate the role of PGI_2 in the antiproliferation effect of L-carnitine, the siRNA for PGIS was applied to block PGI_2 synthesis. As shown in Fig. 6C, PGIS siRNA transfection clearly reduced 6-keto-prostaglandin $F_{1\alpha}$ generation in L-carnitine-treated cardiac fibroblasts. Pretreatment with 10 mM L-carnitine significantly alleviated Ang II-induced ET-1 secretion and cell proliferation in mock control cells, whereas there was a very minor influence of L-carnitine on Ang II-induced ET-1 secretion and cell proliferation in PGIS siRNA-transfected cells (Fig. 6D–F). To further confirm the role of PGI_2, iloprost (a stable analogue of PGI_2) and PG E_2 were added to siRNA-transfected cardiac fibroblasts with Ang II and L-carnitine treatment. The blockage of the anti-proliferation function of L-carnitine by PGIS siRNA transfection was reversed by iloprost but not by PG E_2. These results reveal the dependence of the anti-proliferation function of L-carnitine on the induction of PGI_2 in cardiac fibroblasts.

3.7. The essential role of PPARα in the anti-proliferate effect of L-carnitine

PGI_2 has been reported to be a potential ligand for IP receptors, PPARα and PPARδ [15]. To identify the signaling pathways involved

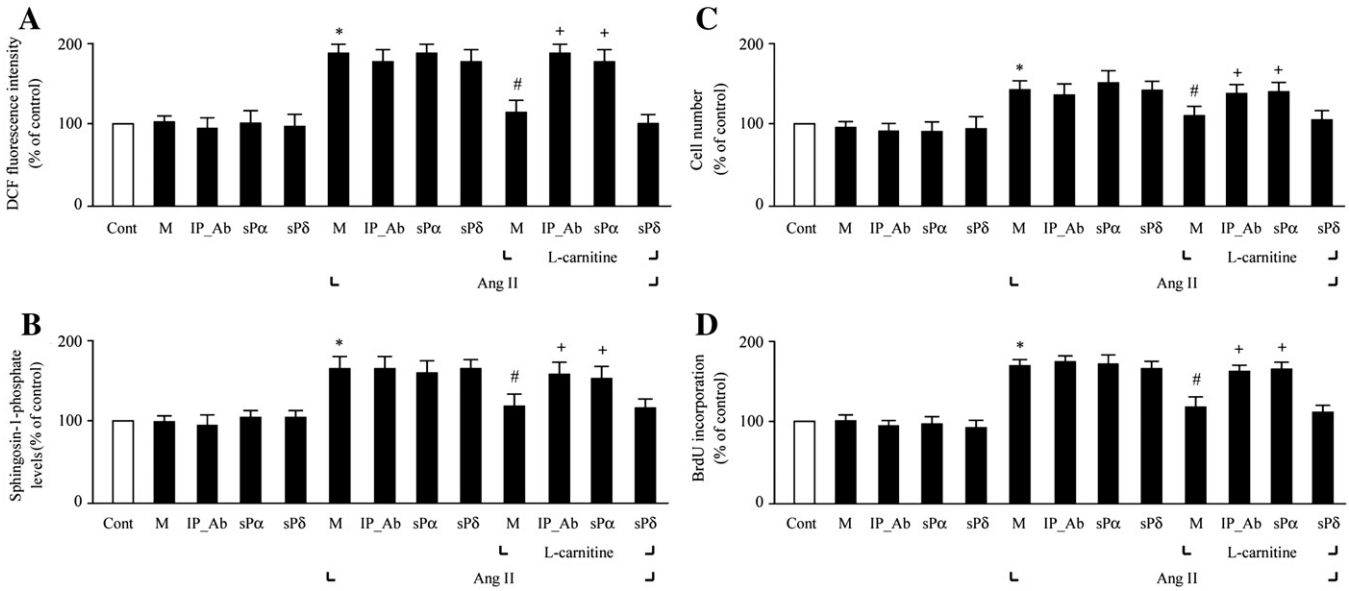

Fig. 7. Effects of the blockage of PGI_2-signaling pathway on the antiproliferation effect of L-carnitine in Ang II-treated cardiac fibroblasts. *P<.05 versus the mock control; #P<.05 versus the mock control with Ang II treatment; +P<.05 versus the mock control with L-carnitine and Ang II treatment. Cont, untransfected control; M, mock control; IP_Ab, IP receptor neutralizing antibody treatment; sPα, PPARα siRNA transfection; sPδ, PPARδ siRNA transfection. (A) The effect of IP-neutralizing antibody, PPARα siRNA and PPARδ siRNA on the inhibitory effect of L-carnitine in Ang II-induced ROS generation. Transfected cells were pretreated with or without L-carnitine (10 mM) for 24 h and then treated with 100 nM of Ang II for 30 min. 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=4). (B) The effect of IP-neutralizing antibody, PPARα siRNA and PPARδ siRNA on the inhibitory effect of L-carnitine in Ang II-increased sphingosine-1-phosphate levels. Transfected cells were pretreated with or without L-carnitine (10 mM) for 24 h and then treated with 100 nM of Ang II for 12 h. Results were shown in mean±S.E.M. (n=4). (C) The influence of IP-neutralizing antibody, PPARα siRNA and PPARδ siRNA on the on the inhibitory effect of L-carnitine in Ang II-induced increase in cell proliferation. Transfected cells were pretreated with or without L-carnitine (10 mM) for 24 h and then treated with 100 nM of Ang II for 24 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=4). (D) The influence of IP-neutralizing antibody, PPARα siRNA and PPARδ siRNA on the inhibitory effect of L-carnitine in Ang II-induced increase in BrdU incorporation. Results were shown in mean±S.E.M. (n=4).

in the antiproliferation function of L-carnitine, the neutralizing antibody for the IP receptor and the siRNA for PPARα and PPARδ were applied to cardiac fibroblasts. PPARα and PPARδ protein levels were obviously reduced by PPARα and PPARδ siRNA transfection, respectively (data not shown). The inhibitory effect of L-carnitine on Ang II-induced ROS production and sphingosin-1-phosphate generation was partially reversed by PPARα siRNA transfection and by IP receptor neutralization but was not significantly affected by PPARδ siRNA transfection (Fig. 7A, B). Ang II-induced cell proliferation was slightly increased by PPARα siRNA transfection but was not affected by IP receptor neutralization or by PPARδ siRNA transfection (Fig. 7C, D). Similarly, the inhibitory effect of L-carnitine on Ang II-induced cell proliferation was reduced by PPARα siRNA transfection and by IP receptor neutralization but was not significantly affected by PPARδ siRNA transfection (Fig. 7C, D). These results indicate the crucial role of the PPARα signaling pathway in the anti-proliferation function of L-carnitine in cardiac fibroblasts.

4. Discussion

Cardiac fibrosis, characterized in part by the proliferation of cardiac fibroblasts, is a consequence of remodeling processes initiated by pathophysiological events associated with hypertension and ischemic injury [5]. The present study clearly demonstrates that L-carnitine inhibits Ang II-induced effects (including cell proliferation, ET-1 gene expression, ROS generation, ERK phosphorylation, AP-1-mediated reporter activity and an increase in sphingosine-1-phosphate levels) in part via the increase of PGI_2 production in cardiac fibroblasts. Thus, this study may have implications for the therapeutic potential of L-carnitine in cardiac fibrosis.

4.1. Ang II-increased ROS formation and sphingosine-1-phosphate levels in cardiac fibroblasts

Ang II plays a critical role in cardiac remodeling in hypertension, a process resulting from cardiomyocyte hypertrophy, apoptosis, inflammation and fibrosis, leading to decreased compliance and increased risk of heart failure [27]. It is well known that Ang II increases production of ROS in cardiovascular tissues via NADPH oxidase, which is involved in cardiac remodeling [6], and induces cardiac fibroblast proliferation [28]. The authors previously demonstrated that Ang II increases ROS production, activates redox-sensitive signals and leads to the expression of ET-1 and cardiac fibroblast proliferation [7]. Previous studies have determined that Ang II not only augments ROS formation and increases oxidase activity but also up-regulates mRNA and protein expression levels of the majority of NADPH oxidase subunits [29]. The authors have also demonstrated that Ang II increases sphingosin-1-phosphate levels in cardiac fibroblasts. However, the impact of Ang II on NADPH oxidase activation in cardiac fibroblasts still warrants further investigation. Sphingosine-1-phosphate has been demonstrated to modulate cellular calcium homeostasis and cell proliferation [30]. Although the present study did not evaluate whether Ang II leads to activation of sphingomyelinase, ceramidase or sphingosine kinase, the time course of sphingosin-1-phosphate generation suggests an Ang II-induced de novo synthesis. However, the role of sphingosin-1-phosphate in cardiac fibroblast proliferation and the impact of Ang II on sphingolipid metabolism in cardiac cells also warrant further investigation.

4.2. Antioxidant effects of L-carnitine and its effect on intracellular sphingosine-1-phosphate levels

Experimental studies have indicated cardioprotective roles for L-carnitine; however, the direct effect of L-carnitine on cardiac cell

growth remains unclear. Vanella et al. [3] reported that L-carnitine is protective against ischemia/reperfusion injury of the heart due to inhibition of lipid peroxidation . The present results showed that L-carnitine was able to inhibit Ang II-induced ROS generation. It has been demonstrated that activation of ERK is redox-sensitive and that suppression of ROS inhibits ET-1 gene expression [7]. Thus, one possible explanation for the inhibitory effect of L-carnitine on Ang II-induced cell proliferation and ET-1 gene expression may be its ability to attenuate ROS formation and then inhibit ERK phosphorylation and AP-1-mediated reporter activity in cardiac fibroblasts. Therefore, L-carnitine may enable cells to gain important antioxidant characteristics. In addition, the present results showed that when L-carnitine was administered, it partially prevented Ang II-induced sphingosine-1-phosphate generation. L-Carnitine has been previously shown to influence apoptosis by inhibiting the activation of a sphingomyelinase [31] involved in the generation of ceramide, an event believed to be crucial for triggering apoptosis [32]. The mechanisms for these observations have not been elucidated. It has been reported that L-carnitine can block the sphingomyelin–ceramide apoptotic pathway [31], which is activated in the ischemic myocardium during reperfusion but not during ischemia [33]. Consequently, L-carnitine may show its cardioprotective potential during ischemia-reperfusion by modulating both energetics and apoptosis [31], although the mechanisms are still unclear. Thus, further experiments are necessary to identify the detailed mechanisms of L-carnitine in the regulation of intracellular sphingosine-1-phosphate generation in cardiac fibroblasts.

4.3. L-Carnitine attenuates Ang II-induced cell proliferation through PPARα activation by PGI_2

The present results indicate that PGI_2 generation and its activation of PPARα is essential to the anti-proliferation effect of L-carnitine. Importantly, PPARα has been reported to correlate with levels of ROS in several tissues [34]. Activation of PPARα inhibited Ang II–induced activation of NADPH oxidase and suppressed ROS production in human endothelial cells [35]. Furthermore, a PPAR-responsive element has been identified in promoter regions of catalase and Cu/Zn-superoxide dismutase genes, key enzymes that remove excess ROS [36]. On the other hand, it has also been reported that PPARα activation modulates sphingolipid metabolism in the heart [37]. However, this phenomenon requires further investigation. In conclusion, the present study delivers important new insights into the molecular mechanisms of action of L-carnitine in cardiac fibroblasts. The present results demonstrate that L-carnitine suppresses Ang II-induced ROS generation, sphingosine-1-phosphate levels, ERK phosphorylation, ET-1 gene expression and cell proliferation. Moreover, L-carnitine also increases PGI_2 production. Thus, these findings support the proposed beneficial effects of L-carnitine in the cardiovascular system.

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