Regulatory Peptides 161 (2010) 58–66

Contents lists available at ScienceDirect

Regulatory Peptides

journal homepage: www.elsevier.com/locate/regpep

L-carnitine attenuates oxidant injury in HK-2 cells via ROS-mitochondria pathway

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ARTICLE INFO

Article history:
Received 25 July 2009
Received in revised form 15 December 2009
Accepted 30 December 2009
Available online 20 January 2010

Keywords:
L-carnitine
H_2O_2
Oxidative stress
HK-2 cells
ROS
Apoptosis

ABSTRACT

Oxidative stress has been considered as the possible mechanism of renal ischemia/reperfusion injury. L-carnitine is an endogenous mitochondrial membrane compound and could effectively protect ischemia-reperfusion injury in the kidney. To elucidate the nephroprotective effects of L-carnitine, here we assessed the effect of L-carnitine on hydrogen peroxide (H_2O_2)-mediated oxidative stress in the human proximal tubule epithelial cell line, HK-2 cells. The results showed that pretreatment with L-carnitine 12 h inhibited H_2O_2-induced cell viability loss, intracellular reactive oxygen species generation and lipid peroxidation in a concentration-dependent manner. Also L-carnitine promoted endogenous antioxidant defense components including total antioxidative capacity, glutathione peroxidase, catalase and superoxide dismutase. In parallel, cell apoptosis triggered by H_2O_2 characterized with the DNA fragment and caspase-3 activity were also inhibited by L-carnitine. Furthermore, mitochondrial dysfunction associated with cell apoptosis including membrane potential loss, down-regulation of Bcl-2 and up-regulation of Bax and the release of cytochrome *c* were abrogated in the presence of L-carnitine. These results suggested that L-carnitine could protect HK-2 cells from H_2O_2-induced injury through the inhibition of oxidative damage, mitochondria dysfunction and ultimately inhibition of cell apoptosis, which indicates that L-carnitine may be a promising approach for the treatment of oxidative stress in renal diseases.

1. Introduction

Increased oxidative stress mediated by reactive oxygen species (ROS) have been implicated in a variety of kidney diseases [1–3]. ROS can be generated within the nephron segments like the glomeruli and proximal tubule and injury initiated by the lack of oxygen during cold presentation of renal transplantation is augmented by ROS during subsequent warm reperfusion of grafts through activation of inflammatory cascade [4]. After kidney transplantation increased ROS may participate in the development and/or progression of chronic renal allograft nephropathy [3]. ROS-interacting enzymes (superoxide dismutase, peroxidases, and catalases) could prevent ROS formation or detoxify ROS [5]. Renal ischemia/reperfusion (I/R) excessively produces ROS beyond organ's scavenging capacity, simultaneously impairs antioxidant enzymes, and causes cell damage by lipid peroxidation, DNA breakdown, and protein damage [6]. ROS-induced mitochondrial dysfunction, caspase-3 activation, which contributes to both necrotic and apoptotic forms of cell death have been documented after I/R injury in the kidney [5,6]. The expressions of Bcl-2 family of proteins in the distal and proximal tubules were increased after I/R injury in the kidney, with the net effect determining the severity of injury and dysfunction [6,7]. Antioxidant strategy may reduce oxidative stress and inhibit apoptotic signaling and cell death which will allow better preservation of graft function and ameliorate the associated injury and inflammation in kidney [8].

L-carnitine (4-N-trimethylammonium-3-hydroxybutyric acid), an L-lysine derivative, is an endogenous mitochondrial membrane compound. The main physical function of L-carnitine in human body is facilitating the transport of long chain fatty acids into mitochondria in order to enter the β-oxidation cycle [9]. Used as a safe and effective nutritional supplement for more than three decades, the protective effect of L-carnitine on kidney tissue has been proved in some models involving oxidative stress [10–13]. By using different antioxidant assays, Gulcin demonstrated that L-carnitine had an effective 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH·) scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, total reducing power and metal chelating on ferrous ions activities compared to α-tocopherol and trolox as references antioxidants [14]. Carnitine can also act as a chelator by decreasing the concentration of cytosolic iron, which plays a very important role in free radical chemistry [15].

In light of the findings described above, the present study employed the human proximal tubule epithelial cell line, HK-2 cells as a cell model system and aimed to elucidate the molecular mechanisms of L-carnitine on renal oxidative stress. As the major component of ROS, H_2O_2 is produced during the redox process and is considered as a messenger in intracellular signaling cascades. H_2O_2 could cause lipid peroxidation and DNA damage [16]. So we used

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doi:10.1016/j.regpep.2009.12.024

H_2O_2 as an inducer of oxidative stress for HK-2 cells and tested whether pretreatment cells with L-carnitine resulted in the resistance of HK-2 to H_2O_2 challenge. Furthermore, the effect of L-carnitine on oxidative stress conditions such as ROS production, lipid peroxidation, antioxidant defensive system, mitochondrial dysfunction and DNA damage associated with cell apoptosis were also studied.

2. Materials and methods

2.1. Chemicals and reagents

L-carnitine, H_2O_2 and 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl-tetrazolium bromide (MTT), were purchased from Sigma (St. Louis, MO, USA). ROS detection kit, acetyl-L-cysteine (NAC) and DNA Ladder was purchased from Beyotime Company (Jiangsu, China). All other chemicals were of analytic grade.

2.2. Cell culture and treatment

HK-2 cells (Cell Culture Center, Beijing, China), an immortalized human proximal tubule epithelial cell line, were grown and passaged in 75-cm^2 cell culture flasks that contained DMEM Ham's F12 media (1:1, Gibco BRL) supplemented with 10% FCS and antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin). To determine the effects of L-carnitine on H_2O_2-exposed HK-2, subconfluent (80%) HK-2 cells were harvested and seeded into 96 well plates or six-well tissue culture plates. The cells were allowed to adhere for 18 h in an incubator at 37 °C with 5% CO_2 in 95% air. Immediately before experimental treatments, the medium was replaced with fresh medium and cells were treated with indicated doses of L-carnitine for 12 h before H_2O_2 exposure. Thereafter, cells were washed with PBS to make them L-carnitine free and then cells in fresh medium were exposed to the desired doses of H_2O_2. Afterwards, cells were rinsed with fresh medium (without H_2O_2) and incubated. Cells were harvested for further analysis.

2.3. Cell proliferation and viability analysis

Cell viability and proliferation was determined using the MTT assay, which is a sensitive measurement of the normal metabolic status of cells. Briefly, cultured HK-2 cells were initially plated in triplicate at a density of 1×10^4 cells/100 μL in 96 well plates for 24 h. The cells were pre-incubated with or without L-carnitine following incubation with H_2O_2 for different time. Afterwards, cells were rinsed with fresh medium (without H_2O_2) and incubated for 24 h. The cells were then incubated with 0.5 mg/mL MTT at 37 °C for 4 h. The formazan crystals generated by viable mitochondrial succinate dehydrogenase from MTT were extracted using an equal volume of the solubilizing buffer (0.01 N HCl and 10% SDS). Absorbance was measured at a wavelength of 490 nm using a Molecular Devices VERSAmax microplate reader (Molecular devices, Sunnyvale, CA, USA). All experiments were performed in triplicate.

2.4. Measurement of ROS accumulation and lipid peroxidation

Cultured HK-2 cells were initially plated in triplicate at a density of 5×10^5 cells/well in 6 well plates for 24 h. The cells were then pre-incubated with or without L-carnitine following incubation with H_2O_2. At the indicated time points, to monitor intracellular accumulation of ROS, the treated cells were incubated with 5 μM DCF-DA for 30 min at 37 °C. Cells were subsequently washed twice with D-Hanks and collected, DCF fluorescence intensity of 100 μL cell suspension was quantified with a fluorometer (GENios, USA) using 485 nm excitation and 535 nm emission filters [17]. The results are given as percents relative to the oxidative stress of the control cells set to 100%. All experiments were performed in triplicate.

Malonyl dialdehyde (MDA), a terminal product of lipid peroxidation, was measured to estimate the content of lipid peroxidation in HK-2 cells [18]. MDA concentration in cell homogenates was determined with commercial kits purchased from Jiancheng Bioengineering Institute (Nanjing, China), using the thiobarbituric acid method. The assay was based on the conjugation ability of MDA with thiobarbituric acid, to form a red product which has maximum absorbance at 532 nm.

2.5. Assays for total antioxidative capacity (T-AOC) and antioxidant defense enzymes

The cells were pre-incubated with or without L-carnitine following incubation with H_2O_2. At the indicated time points, HK-2 cells were washed 3 times with ice-cold D-Hanks and lysed in the extraction buffer [50 mmol/L Tris–HCl (pH7.4), 1 mmol/L ethyleneglycolbis (2-aminoethylether) tetraacetic acid, 150 mmol/L NaCl, 1% (v/v) Triton X-100, 1 mmol/L phenylmethylsulfonyl, 10 μg/mL aprotinin, 10 mmol/L EDTA, 1 mmol/L NaF, and 1 mmol/L Na_3VO_4] on ice for 30 min. Then the cells were scraped from the plates and the lysates were subjected to $20,000\times g$ centrifugation at 4 °C for 10 min. The amount of proteins in the cleared lysates was quantified with a bicincho-ninic acid assay (Beyotime Biotechnology, China). After determining the amount of total proteins in the supernatants, we detected T-AOC and the endogenous antioxidant defense components like glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) using biochemical methods following the instructions for the reagent kits (Nanjing Institute of Jiancheng Biological Engineering, Nanjing, China) and absorbance were measured using a microplate reader. T-AOC reflects the overall cellular endogenous antioxidative capability including both enzymatic and non-enzymatic antioxidants. These antioxidants can reduce Fe^{3+} to Fe^{2+}, the latter can form colored and stable chelates combing with phenanthroline, which was then chelated with porphyrin to produce a purple complex. GPx activities were assayed by the decrease of the GSH. CAT activity was determined by the decrease of H_2O_2. SOD activity was measured using the nitroblue tetrazolium method which utilized a tetrazolium salt to quantify the superoxide radicals generated by xanthine oxidase and hypoxanthine. Data were defined as the amount of protein that inhibits the rate of nitroblue tetrazolium reduction by 50%. Values were calculated using absorbation (520 nm for T-AOC, 412 nm for GPx, 405 nm for CAT and 550 nm for SOD) and expressed as units (U) per mg protein. The activities of T-AOC, GPx, CAT and SOD were expressed as U/mg protein, respectively. The data were calculated and represented as percentage of control cells. All experiments were performed in triplicate.

2.6. Flow cytometric detection of apoptosis assay

Apoptotic cells were quantified by determining DNA content of cells by propidium iodide staining by flow cytometry [19]. Briefly, the pellets were resuspended in ice-cold 70% ethanol and fixed at 4 °C for 24–48 h. The cells were then centrifuged, and ethanol was removed by washing thoroughly with PBS. The cell pellets were resuspended in 1 ml DNA staining reagent containing 50 μg/ml RNase, 0.1% triton X-100, 0.1 mM EDTA (pH 7.4), and 50 μg/ml PI. The staining was stable at 4 °C for 30 min. Red fluorescence (DNA) was detected through a 563–607 nm bandpass filter by using a FACS 440 flow cytometer (Becton Dickinson). In flow cytometric histograms, apoptotic cells will give DNA fluorescence in the subdiploid regions, which are well separated from the normal G1 peak. Ten thousand cells in each sample were analyzed and the percentage of apoptotic cell accumulation in the sub-G1 peak was calculated. Each measurement was carried out in triplicate.

2.7. Measurement of mitochondrial membrane potential (MMP, $\Delta\psi M$)

Mitochondrial membrane was monitored using the fluorescent dye Rh123, a cell permeable cationic dye, which preferentially partitions into mitochondria based on the highly negative MMP as previously described [20]. Rh123 was added to cell cultures to attain a final concentration of 10 μM for 30 min at 37 °C after cells were treated as described above. The cells were collected by pipetting and washed twice with PBS and then analyzed by flow cytometry and analyzed using CELLQuest software within 1 h. The laser was adjusted to emit at 480 nm, and a 530 nm long-pass filter was used.

2.8. Cytochrome c release from mitochondria

The cells were harvested after the respective treatments, washed once with ice-cold PBS. For isolation of mitochondria and cytosol to observe the cytochrome *c* release from mitochondria, the cells were sonicated in buffer containing 10 mM Tris–HCl pH 7.5, 10 mM NaCl, 175 mM sucrose and 12.5 mM EDTA and the cell extract centrifuged at 1000 *g* for 10 min to pellet nuclei. The supernatant thus obtained was centrifuged at 18,000 *g* for 30 min to pellet the mitochondria. The resulting supernatant was termed as the cytosolic fraction. The purity of the fractions was confirmed by assaying the marker enzymes succinate dehydrogenase for mitochondria, lactate dehydrogenase for the cytosol. The protein concentration was measured using a Bradford protein assay kit (BioRad). Cytochrome *c* determination in cytosolic and mitochondrial fractions was done by Western blotting.

2.9. Western blot analysis

For the quantification of various protein expression, Western blot analysis was used. The cells were placed in lysis buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in normal PBS; pH 6.8) containing a protease inhibitor cocktail (10 μL/mL; Sigma-Aldrich). The homogenate was centrifuged at 12,000×*g* at 4 °C for 20 min, and the supernatant was stored at −80 °C. Total protein was determined by the Bradford assay. Equal amounts of protein (10 μg) from both fraction were separated on a 10% SDS-PAGE and transferred electrophoretically to the nitrocellulose membranes. The membrane was blocked by 5% skim milk in Tris-buffered saline containing 0.1% Triton X-100 (TBS-T) for 2 h at room temperature. Immunoblots were performed with appropriate antibodies: primary antibodies for BCL-2 (1:200, Santa Cruz), Bax (1:200, Santa Cruz), cytochrome *c* (1:200, Santa Cruz) and β-actin (1:1000, Santa Cruz). Immunoblot analysis was performed with horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG using enhanced chemiluminescence Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ, USA). The bands corresponding to BCL-2, Bax and cytochrome *c* or β-actin, were scanned and densitometrically analyzed using an automatic image analysis system (Alpha Innotech Corporation, San Leandro, CA, USA). These quantitative analyses were normalized to β-actin (after stripping).

2.10. Flow cytometric detection of caspase-3 activity

The extent of caspase-3 activation in HK-2 cells treated with H_2O_2 for 12 h was detected by flow cytometry analysis using the anti-active caspase-3 monoclonal antibody, which specifically recognizes the active form of caspase-3. Briefly, cells were washed twice in PBS, fixed using 4% polyoxymethylene for 30 min and then permeabilized using 1% TritonX-100 for 10 min at room temperature, before they were washed twice with PBS. Cells were stained with anti-active caspase-3 antibody for 30 min at room temperature in the dark. Following incubation with the antibody, cells were washed in wash buffer, resuspended in wash buffer and analyzed by flow cytometry (FACSVantage SE, BD Biosciences).

2.11. Statistical analysis

All data are expressed as means ± S.E.M. Comparisons between the different groups were performed by ANOVA followed by Student's *t*-test. In all tests, the criterion for statistical significance was $p<0.05$.

3. Results

3.1. L-carnitine protects HK-2 cells against H_2O_2-induced cytotoxicity

We first determined the dose and time of exposure to H_2O_2 to reduce cell viability by 50%. The results showed that H_2O_2 injured HK-2 cells in a dose- and time-dependent manner and treatment of HK-2 cells with 500 μM of H_2O_2 for 30 min resulted in moderate cellular injury. So 500 μM of H_2O_2 for 30 min was used in the subsequent study (Fig. 1A and B). Studies have showed that the auto-oxidation of some antioxidants produces semiquinone radicals, superoxide anion and H_2O_2 and higher concentrations of L-carnitine may be cytotoxic to the cells. To investigate the cytoprotective effect of L-carnitine, we tested the effect of L-carnitine by cell viability assay. As shown in Fig. 1C, at concentrations from 0.1 to 1000 μM for 12 h, L-carnitine slightly increased cell viability and did not induce changes in cell morphology. To evaluate the dose-dependent effects of L-carnitine against H_2O_2-induced injury, HK-2 cells were treated with L-carnitine at concentration of 1, 10, 50, 100, 200, 500, 1000 μM for 12 h followed by 30 min of 0.5 mM H_2O_2. Conversely, pre-treatment with the concentration ranging from 10 μM to 100 μM of L-carnitine for 12 h significantly increased the viability of HK-2 cells against 500 μM H_2O_2-induced cytotoxicity in a concentration-dependent manner, while higher concentrations (200 and 500 μM) of L-carnitine could not show more protection than low concentrations (Fig. 1D). These results show that L-carnitine is effective for protecting cell viability of HK-2 cells against H_2O_2 exposure. So in all further experiments, we choose the low concentrations of (10–100 μM) to define the cytoprotective effects of L-carnitine on H_2O_2-induced oxidative stress in HK-2 cells in vitro.

3.2. L-carnitine inhibits H_2O_2-induced ROS production and lipid peroxidation in HK-2 cells

To evaluate the direct effect of L-carnitine on H_2O_2-induced oxidative stress, we observed ROS formation and lipid peroxidation of cell membrane of HK-2 cells. After incubated with L-carnitine (10–100 μM) for 12 h, cells were subjected to 500 μM H_2O_2 insult for 30 min then incubated in fresh medium for 30 min, 1 h, 3 h, 6 h 12 h, 18 h and 24 h for the evaluation of ROS production and MDA. Although ROS formation and MDA were detectable at 1 and 1.5 h after H_2O_2 treatment respectively, maximal changes was observed at 12 h (data not shown). At 12 h, the intracellular ROS level significantly increased compared with untreated cells as indicated by the increase in RFU, revealing that H_2O_2 enhanced ROS production in HK-2 cells. Treatment of (10–100 μM) L-carnitine obviously attenuated an increase in ROS caused by H_2O_2 in a concentration-dependent manner. Meanwhile, as shown in Fig. 2B, the exposure of cells to H_2O_2 increased LPO by approximately four fold relative to non-H_2O_2-exposed control cells. Pretreatment of cells with (10–100 μM) L-carnitine decreased H_2O_2-induced LPO in PC12 cells significantly in a concentration-dependent manner. And the effect of 50 μM L-carnitine on ROS and MDA is relatively equal to 5 mM NAC, a known strong ROS scavenger. Given alone, 50 μM L-carnitine did not induce the intracellular ROS accumulation and LPO in H_2O_2-untreated cells. These data suggested that the effect of L-carnitine on cell viability, as shown in Fig. 1B, involves the abilities of this antioxidant to reduce intracellular ROS and LPO.

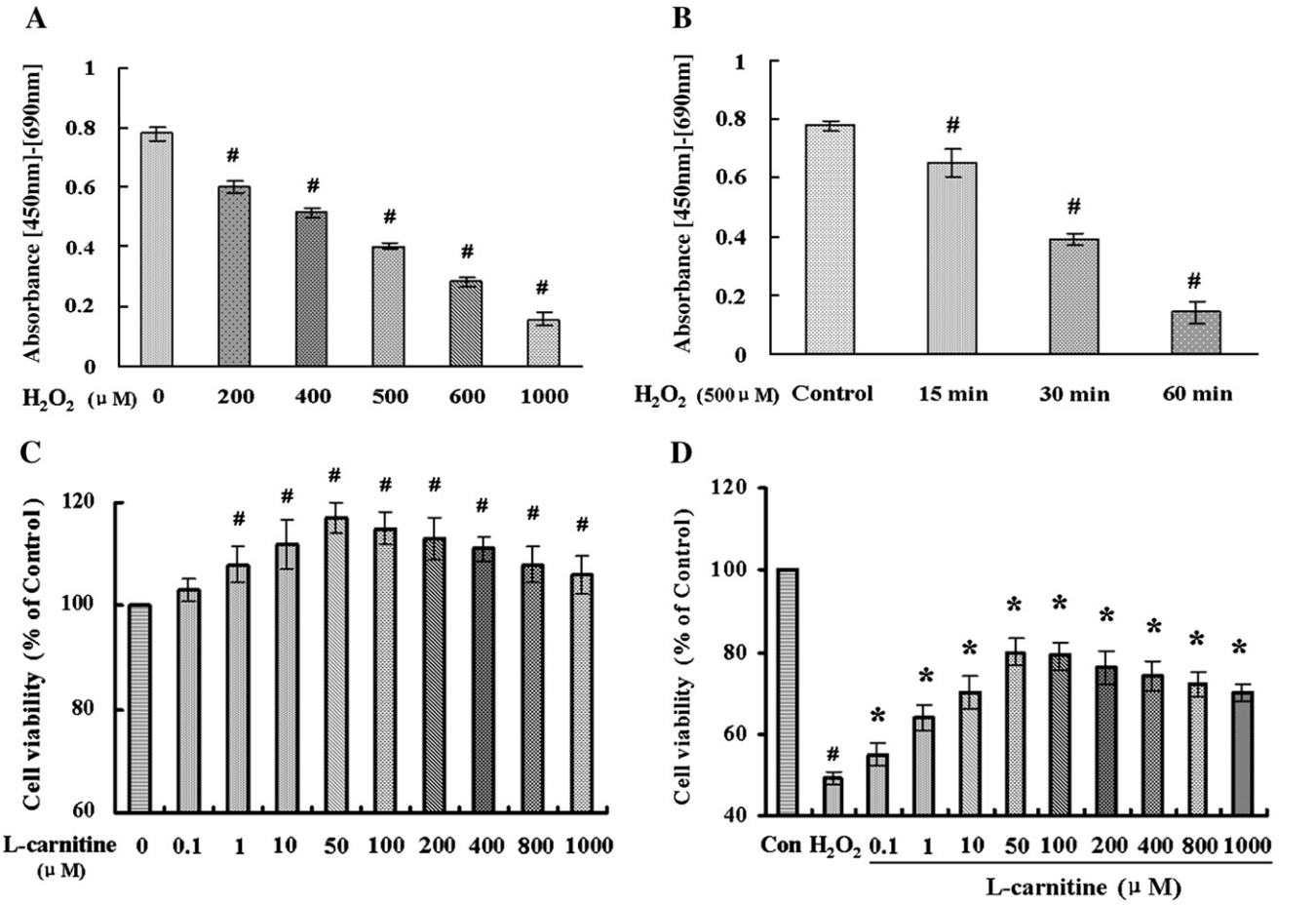

Fig. 1. Treatment of HK-2 cells with L-carnitine inhibits H_2O_2-induced cell injury by MTT assay. (A) HK-2 cells were subjected to H_2O_2 insult (200–1000 μM) for 30 min, and then maintained in the fresh growth medium for 24 h. (B) HK-2 cells were challenged with 500 μM H_2O_2 for 15, 30 and 60 min, and further incubated in fresh growth medium for 24 h. (C) Cells were incubated in medium containing various concentrations of L-carnitine for 24 h. (D) HK-2 cells were pretreated with different concentrations of L-carnitine for 12 h and then incubated with or without 400 μM H_2O_2 for 30 min, and further incubated in fresh growth medium for 24 h. Cell viability was all examined by MTT assay and the data were shown as means ± S.E.M of three independent experiments. $^{\#}p<0.05$ compared to the non-treated cells and $^{*}p<0.05$ relative to H_2O_2 treated cells.

3.3. L-carnitine promotes endogenous antioxidant defense in HK-2 cells under oxidative stress

Studies have shown that endogenous H_2O_2 is synthesized by SOD and decomposed by catalase or glutathione peroxidase. Also, exogenous H_2O_2 could pass through the cell membrane into the cell [21]. We next investigated whether L-carnitine could modulate the antioxidant defense system in HK-2 cells after H_2O_2 exposure for 24 h. The results showed that H_2O_2 alone decrease the levels of SOD, CAT, Gpx and T-AOC significantly compared to untreated control cells (Fig. 3). Pretreatment of cells with L-carnitine obviously increased the levels of SOD, CAT, Gpx and T-AOC respectively in a concentration-dependent manner and 50 μM L-carnitine alone could increase original antioxidant defense and T-AOC in non-stressed HK-2 cells slightly. Taken together, these data strongly suggested that pretreatment HK-2 cells with L-carnitine restored the imbalance of antioxidant firewall induced by H_2O_2 challenge.

3.4. L-carnitine inhibit H_2O_2-induced DNA fragmentation

ROS can cause cell death via apoptosis. The concentration of ROS and the microenvironment appear to be important in determining the mode of cell death. Cells undergoing apoptosis exhibit shrinkage of the nucleus, blebbing of membranes, condensation or fragmentation of chromatin, and internucleosomal DNA degradation by endonucleases into fragments in multiples of 180–200 bp. Using FACS analysis, we investigated the ability of L-carnitine on H_2O_2 induced DNA fragmentation, which are also called sub-G1 cells as an index of apoptosis. As shown in Fig. 4, H_2O_2 treatment lead to increased DNA fragment compared with untreated cells and it was reduced by treatment with L-carnitine in a concentration-dependent manner. This was further confirmed by nuclear staining assay using chromatin dye Hoechst 33258 (data not shown). These observations suggested that L-carnitine could inhibit H_2O_2-induced apoptosis in HK-2 cells. Also, the results showed that the ROS scavenger NAC also perform anti-apoptotic activity (Fig. 4), suggesting that H_2O_2-induced apoptosis in HK-2 cells is mediated by ROS and the protective effect of L-carnitine on H_2O_2-induced apoptosis is partly contributed by its antioxidative activity.

3.5. L-carnitine restored the mitochondrial function in HK-2 cells after H_2O_2 treatment

Mitochondrial dysfunction is recognized as a critical event in apoptosis. It will lead to the dissipation of the trans-membrane potential and permeability changes, which eventually release soluble inter-membrane proteins, such as cytochrome c, through the outer membrane. In the present study, as shown by the decrease of the fluorescence intensity of mitochondrial specific probe, rhodamine 123 (Fig. 5A), the mitochondrial trans-membrane potential ($\Delta\psi M$) was rapidly reduced when HK-2 cells were exposed to H_2O_2 for 6 h and H_2O_2-induced dissipation of $\Delta\psi M$ was significantly blocked by the pretreatment with L-carnitine. Further, Western blotting in both the cytosolic and mitochondrial fractions demonstrated a consistent

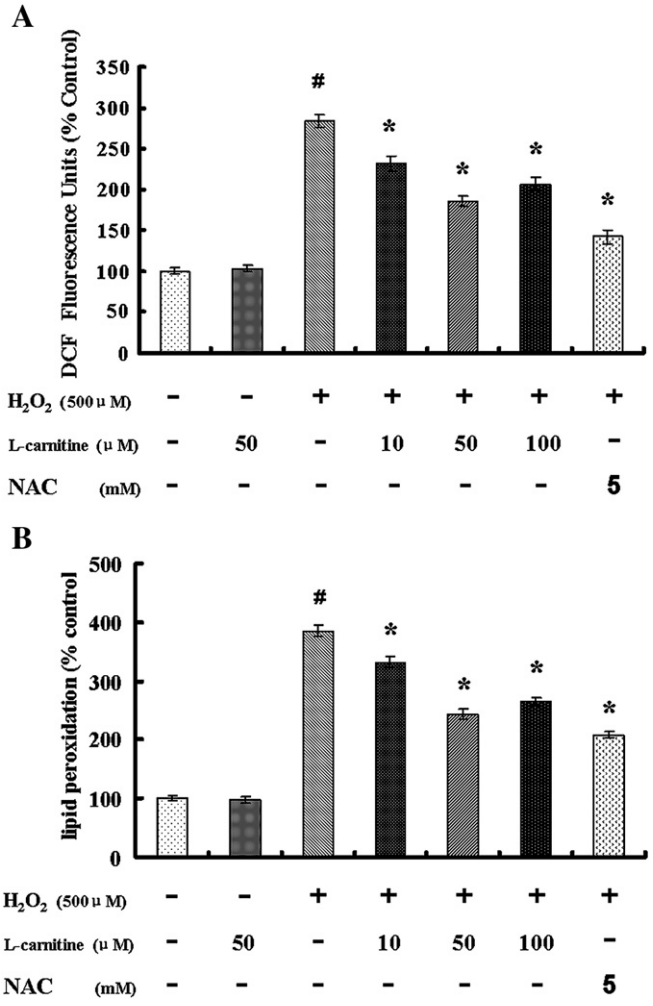

Fig. 2. Effect of L-carnitine on intracellular ROS level and LPO in HK-2 cells following H_2O_2 challenge. HK-2 cells treated with L-carnitine prior to H_2O_2 challenge and then incubated for 12 h. (A) ROS levels were measured by analysis of DCF-DA -stained cells. (B) LPO was measured by analysis of MDA. The data were expressed as means ± S.E.M of the percentage of untreated control cells from three independent experiments. $^{\#}p<0.05$ compared to the non-treated cells and $^{*}p<0.05$ compared to the H_2O_2-alone-treated cells by Student's *t*-test.

increase in cytochrome *c* in cytosol after treatment with H_2O_2. Simultaneously, there was a decrease in cytochrome *c* in mitochondrial fraction, indicating that there is a time-dependent release of cytochrome *c*, and suggesting the involvement of mitochondria in H_2O_2 mediated apoptosis (data not shown). There was significant decrease in the mitochondrial cytochrome *c* level at 6 h, which was accompanied by a simultaneous increase in cytochrome *c* level in the cytosol (Fig. 5B). Treatment of HK-2 cells with L-carnitine reduced cytochrome *c* in the cytosol and increased the mitochondrial cytochrome *c* in a concentration-dependent manner at the same time. L-carnitine alone did not change the MMP and the release of cytochrome *c* significantly.

3.6. L-carnitine regulated H_2O_2-induced Bcl-2 and Bax expression in HK-2 cells

Studies have reported that Bcl-2 family plays a regulatory role in controlling the membrane potential of mitochondria and could prevent apoptotic mitochondrial changes. Bcl-2 homodimer reveals anti-apoptotic effect, whereas this effect is inhibited by Bax due to the formation of heterodimer complex of Bcl-2 and Bax protein [22]. In the present study, when HK-2 cells were exposed to H_2O_2, the expression rate of Bcl-2 was significantly reduced and the expression of Bax was increased in a time-dependent manner. And these changes were considerably blocked by pretreatment with L-carnitine at the same time (data not shown). Bcl-2 protein reached to a plateau while Bax increased to the maximum after 12 h of H_2O_2 treatment and L-carnitine at 100 μM and 200 μM reversed the changes in a dose-dependent manner at this time point (Fig. 6A). When HK-2 cells were treated with 50 μM L-carnitine alone for 12 h, the expression rate of Bcl-2 was markedly increased compared with control. The above results showed that L-carnitine not only enhanced the expression of Bcl-2, but also antagonized the up-regulation of Bax and down regulation of expression of Bcl-2 induced by H_2O_2.

3.7. L-carnitine prevents H_2O_2-induced caspase-3 activation in HK-2 cells

Activation of caspases by cytochrome *c* is a key event during apoptosis caused by various toxic agents. To confirm whether caspases are activated after cytochrome *c* release we measured the changes in caspases-3 activity in HK-2 cells after H_2O_2 treatment. As shown in Fig. 6B, H_2O_2treatment induced the significant activation of caspase-3 compared with untreated cells, which was measured by flow cytometry with anti-active caspase-3 monoclonal antibody. When cells were pre-incubated with different concentrations of L-carnitine, caspase-3 activity decreased in dose-dependent manner. These results suggested that apoptosis in H_2O_2-treated HK-2 cells is mediated by caspase-3 pathway and the protective action of L-carnitine may, at least in part, be attributed to inhibition of the caspase cascade.

4. Discussion

Oxygen free radicals are considered to be important mediators of renal I/R injury [1–3]. To maintain cellular redox homeostasis and protect against oxidative damage, a plausible way is the removal of excess reactive oxygen species or suppression of their generation through pharmacological intake of antioxidants. Being an energy precursor and antioxidant accumulated in renal cells, L-carnitine is an interesting candidate to be used against renal oxidative injury [9–14]. As renal proximal tubular cells are much more susceptible to oxidant-reperfusion injury than those with distal tubular cells, here we utilized H_2O_2-mediated oxidant injury as an in vitro model of reperfusion injury to first observe the antioxidant activity of L-carnitine in HK-2 cells in vivo. As expected, pretreated cells with L-carnitine resulted in resistance to H_2O_2 toxicity as shown in increased cell viability.

H_2O_2 is formed in mitochondria as a dismutation product of the superoxide radical under physiological conditions. However, ischemic stress produces a profoundly increased burst of O_2 and H_2O_2 when oxygen is reintroduced during reperfusion. H_2O_2 and its far more toxic metabolite, the hydroxyl radical, contribute significantly to renal injury during reperfusion [3]. Cytotoxic oxidant free radicals produce cellular lipid peroxidation, impaired energy metabolism, which can ultimately lead to cell injury and this effect can be blocked by addition of antioxidants. Given an antioxidant function of L-carnitine, we further examined the effect of L-carnitine on intracellular ROS production and LPO after H_2O_2 exposure. H_2O_2 challenge caused an apparent increase in intracellular ROS and LPO and in vitro treatment of HK-2 cells with L-carnitine reduce H_2O_2-induced cellular ROS overproduction in a dose-dependent manner. The effect of 50 μM L-carnitine is relatively equal to positive ROS scavenger NAC, which verified the antioxidative characters of L-carnitine. The reduction in oxidative stress correlates with the increase in viability observed in those cells pretreated with L-carnitine and these observations supported the idea that L-carnitine did significantly protect HK-2 cells from H_2O_2-induced cytotoxicity. These results are in line with some in vivo and in vitro studies on antioxidant and antiradical activities of L-carnitine [9–14]. The mechanism of

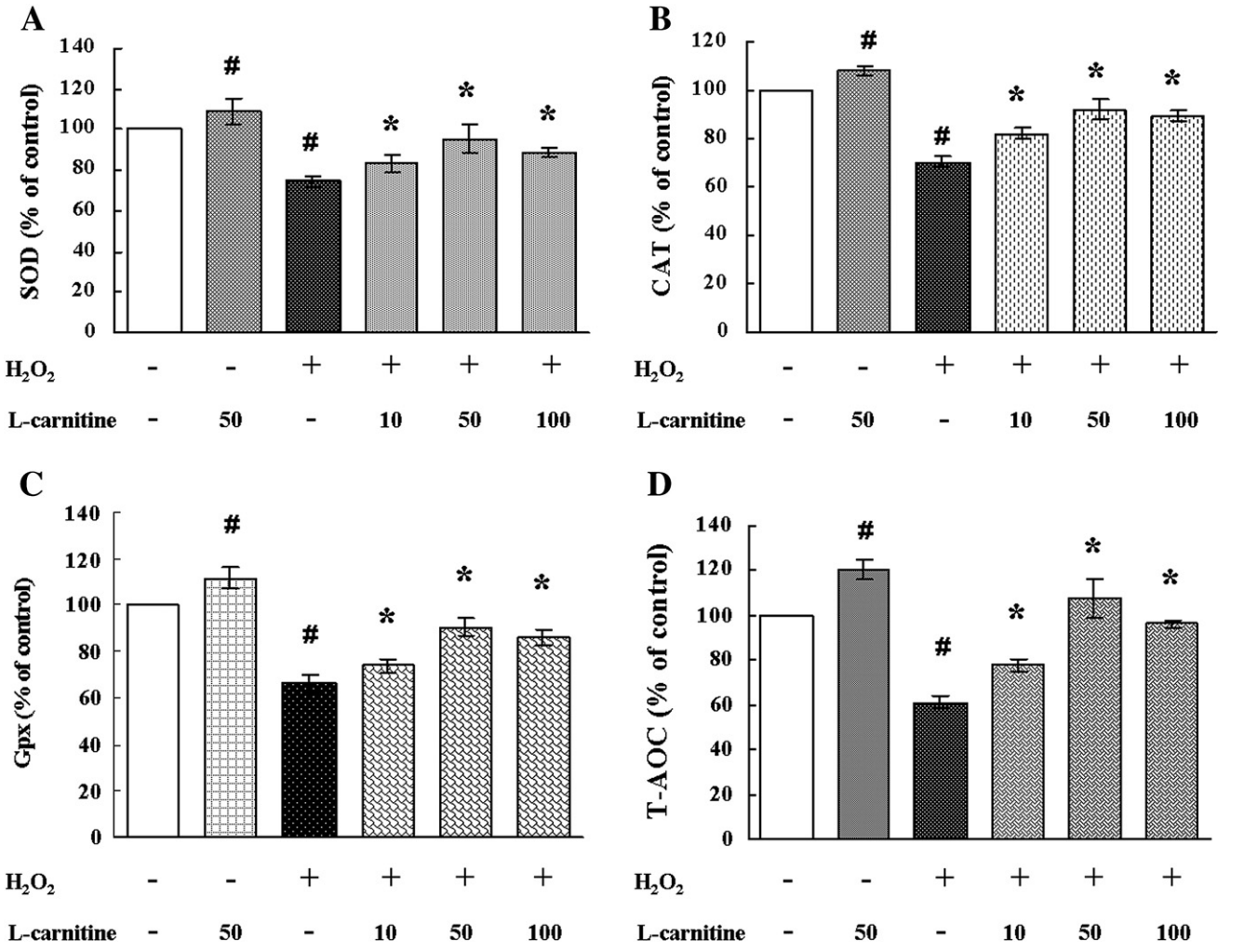

Fig. 3. Effect of L-carnitine on endogenous antioxidant defense in HK-2 cells following H_2O_2 challenge. Cells were exposed to 500 μM H_2O_2 for 30 min with or without pretreatment with L-carnitine. Cells were further incubated in fresh medium for 24 h and cytosols were prepared for the analysis of SOD (A), CAT (B), Gpx (C) and T-AOC (D). The data were expressed as means ± S.E.M of the percentage of untreated control cells from three independent experiments, $^{\#}p<0.05$ compared to the non-treated cells and $^{*}p<0.05$ compared to the H_2O_2-alone-treated cells by Student's *t*-test.

the antioxidative action of L-carnitine may be related to its effective DPPHI scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, total reducing power and metal chelating on ferrous ions activities [14].

If excessive ROS remain in the biological system they may also inactivate certain enzymes including antioxidant enzymes. The levels of antioxidant enzymes, such as superoxide dismutase, catalase and peroxidase, are closely linked with cellular responses to various oxidative stresses [8]. Superoxide dismutase catalyzes the dismutation of the superoxide radical to molecular oxygen and hydrogen peroxide, which in turn is metabolized to harmless water and oxygen by catalase and glutathione peroxidase [23]. Studies have shown that L-carnitine supplementation enhances the activities of antioxidant enzymes, such as SOD, CAT and GPx, and GSH levels and decreases the MDA concentration in kidney tissues of 24-month-old rats. Moreover, L-carnitine can protect these enzymes from further peroxidative damage and is very effective in normalizing age-associated alterations [15]. In line with these reports, our results showed that L-carnitine could enhance the activities of superoxide dismutase, glutathione peroxidase and catalase in HK-2 cells exposed to H_2O_2 and further increase the total antioxidative capacity of cells. From our results and those found in the literature, it may be explained that the modulation of the endogenous antioxidants might be a possible mechanism involved in the nephroprotective effect of L-carnitine in HK-2 cells.

The importance of the mitochondria, particularly in cell injury induced by oxidative stress, should not be underestimated. Mitochondria are both the target and the source of ROS [24]. Meanwhile, mitochondria are responsible for local energy production, Ca^{2+} homeostasis, and clearance of reactive oxygen species. Mitochondria dysfunction will lead to the loss of mitochondrial transmembrane potential, the release of cytochrome *c* and Ca^{2+} from the mitochondria, which will cause caspase-3 activation and nuclear condensation [25]. Consistent with these findings, our results showed that the H_2O_2 treatment caused significant impairment of the mitochondrial membrane potential and the release of cytochrome *c*, confirming the involvement of mitochondria in H_2O_2-mediated HK-2 cells apoptosis. Our results showed that pretreatment of HK-2 cells with L-carnitine could restore the mitochondrial function by blocking the dissipation of $\Delta\psi M$ and releasing of cytochrome *c* into the cytosol.

The loss of the mitochondrial membrane potential is believed to be due to opening of the permeability transition (PT) pore [26,27], a mitochondrial megachannel, which is strongly affected by conditions of oxidative stress, with oxidative agents increasing the probability of pore opening [28]. It has been shown that Bax and Bcl-2 are able to regulate the status of the PT pore complex; Bax can open it and influences permeability and the release of cytochrome *c* from the inter-membrane space into the cytosol, while Bcl-2 is able to stabilize

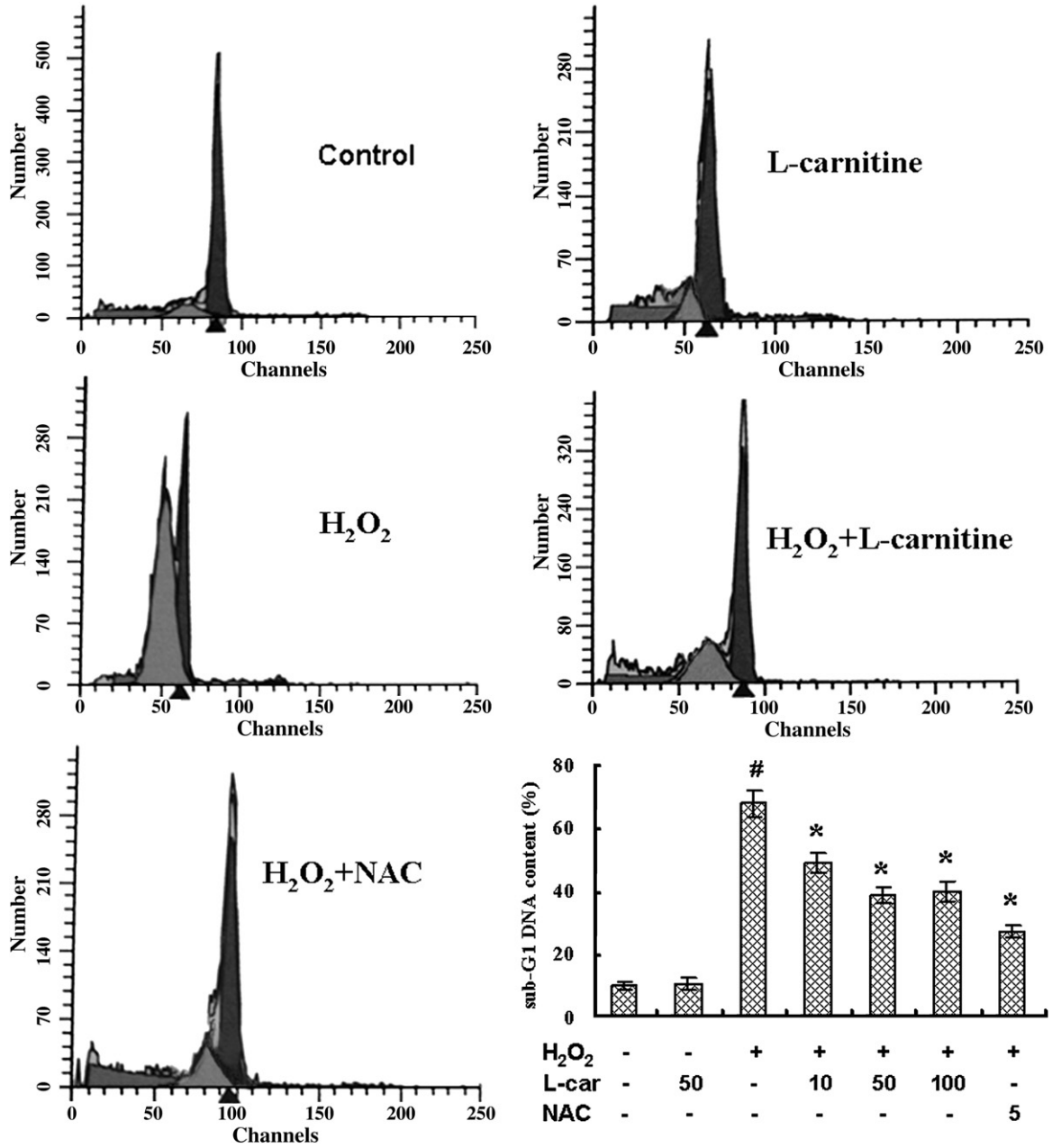

Fig. 4. Effect of L-carnitine treatment on H_2O_2-induced DNA fragment in HK-2 cells. **(A)** HK-2 cells were treated with different concentrations of L-carnitine and H_2O_2 for 24 h, DNA fragment was assessed by FACS analysis. Ten thousand cells in each sample were analyzed and the percentage of apoptotic cell accumulation in the sub-G1 peak was calculated. Data were expressed as mean values ± S.E.M of a ratio of untreated controls from three independent experiments, $^{\#}p<0.05$ compared to the non -treated cells and $^{*}p<0.05$ compared to the H_2O_2-alone-treated cells by Student's *t*-test.

and inhibit its opening ultimately protects against oxidative stress where reactive oxygen species are generated, including membranes of the mitochondria, nuclei, and endoplasmic reticulum [22,27,29]. The ratio between anti-apoptotic (Bcl-2) and proapoptotic (Bax) has been suggested as a primary event in determining the susceptibility to apoptosis through maintaining the integrity of the mitochondria and inhibiting the activation of caspase cascade [27]. Our results showed that H_2O_2 could downregulate Bcl-2 and upregulate Bax protein compared with control, and this effect was blocked by the pre-treatment of L-carnitine. Given the key role of the ratio between Bcl-2 and Bax proteins in the apoptotic cascade, it is not surprising that treatment with L-carnitine is also associated with the inhibition of downstream apoptotic signaling pathways and ultimately prevents the cell apoptosis. Based on previous reports and observations in the present study, we propose that L-carnitine modulates the Bcl-2 family protein levels in response to H_2O_2 insult, which then regulate a succession of mitochondria-mediated downstream molecular events. As MTT reduction assay is based on the catalytic activity of some metabolic enzymes in intact mitochondria and L-carnitine is an endogenous mitochondrial membrane compound [30], these data support the idea that L-carnitine-mediated cytoprotection is due, in part, to inhibition of the mitochondrial apoptotic pathway.

Cytochrome *c* release from mitochondria is a critical step in the apoptotic cascade and this activates downstream caspases such as caspase-3, which is implicated in the pathogenesis of renal injury and may be blocked by antioxidants [31,32]. In the present study, the results showed that H_2O_2 treatment induced the significant activation of caspase-3 compared with untreated cells, which was inhibited by L-carnitine in dose-dependent manner (Fig. 6B). These results suggested that apoptosis in H_2O_2-treated HK-2 cells is

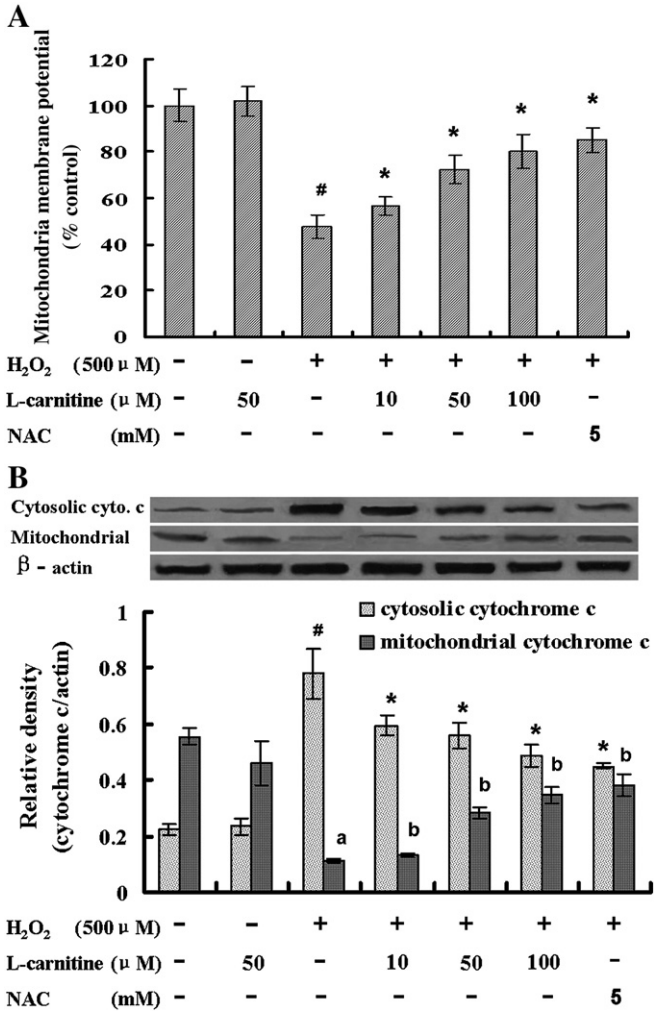

Fig. 5. Effects of L-carnitine treatment on the mitochondrial function in H_2O_2-treated HK-2 cells. HK-2 cells were pretreated with different concentrations of L-carnitine for 12 h and then incubated with or without 400 μM H_2O_2 for 30 min, and further incubated in fresh growth medium for 6 h, (A) the H_2O_2-induced reduction of mitochondrial membrane potential was measured by flow cytometry; (B) the effects of L-carnitine on H_2O_2-induced release of cytochrome *c* from mitochondria was measured by western blot analysis. β-actin was detected for controls. Data were expressed as mean values ± S.E.M of a ratio of untreated controls from three independent experiments, $^{\#}p<0.05$ compared to the non -treated cells and $^{*}p<0.05$ compared to the H_2O_2-alone-treated cells by Student's *t*-test.

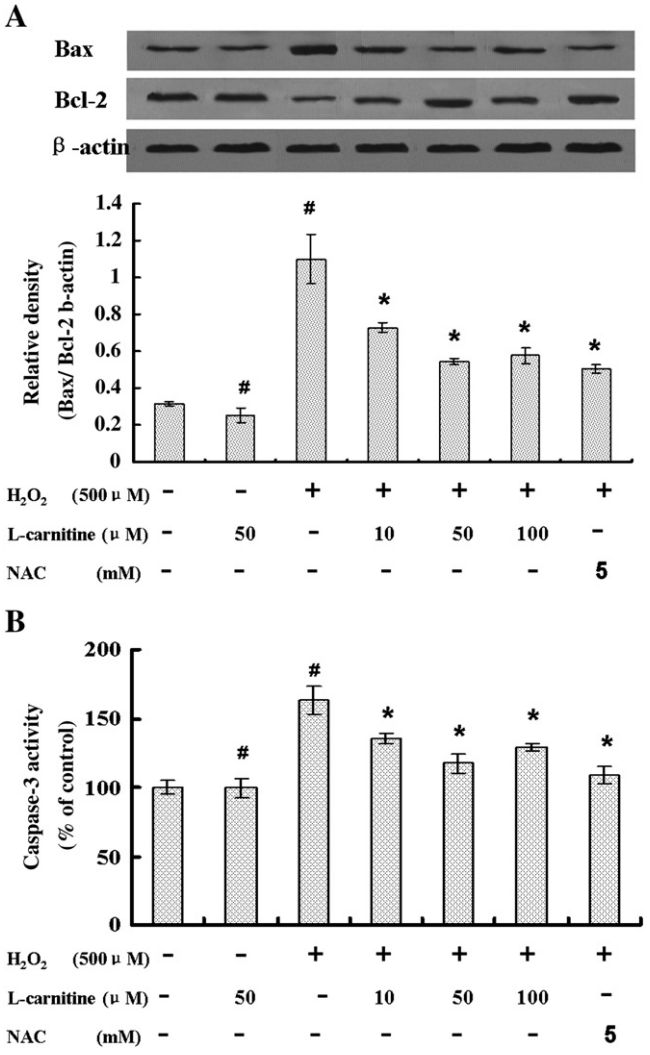

Fig. 6. Effects of L-carnitine treatment on the expression of Bcl-2 and Bax and activation of caspase-3 in H_2O_2-treated HK-2 cells. Cells were pretreated with different concentrations of L-carnitine and 500 μM H_2O_2 for 12 h, (A) the protein expression of Bcl-2 and Bax were analyzed by western blot analysis. β-actin was detected for controls; (B) the H_2O_2-induced activation of caspase-3 was measured by flow cytometry. Data were expressed as mean values ± S.E.M of a ratio of untreated controls from three independent experiments, $^{\#}p<0.05$ compared to the non-treated cells and $^{*}p<0.05$ compared with the H_2O_2-alone-treated cells (Student's *t*-test).

mediated by caspase-3 pathway and the protective action of L-carnitine could exert a protective role at the execution phase of apoptosis. The inhibitory effect of L-carnitine on apoptosis was strengthened by the inhibition of the fragmentation of DNA (a hallmark of apoptosis) induced by H_2O_2 (Fig. 4). These results indicated that L-carnitine may function as an antioxidant and protect H_2O_2-induced apoptosis by inhibition of DNA injury. As caspase-3 can be activated by ROS and cytochrome c, the suppressive effect of L-carnitine on the activity of caspase-3 further demonstrated that the inhibitory effect of L-carnitine on cell death could be also related with antioxidant property and protecting mitochondrial function.

In conclusion, we have provided evidence that the nephroprotective effect of L-carnitine were mediated, at least, through scavenging oxygen free radicals, prevention of oxidation of lipids, enforcement of endogenous antioxidant defense, protecting mitochondrial function, inhibition of cell apoptosis and regulation apoptosis related gene expression of Bcl-2 and Bax. Our results show that the antioxidant effect may be a major mechanism for L-carnitine-mediated nephroprotection and L-carnitine may be a candidate chemical for the treament of oxidative stress-induced renal disease.

Acknowledgments

We are grateful to Dr. Junli YE for corrections to the manuscript and for thoughtful discussions. We also wish to thank Dr Ling LU for providing us with antibodies and advice.

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