Peroxisome proliferator-activated receptor alpha plays a crucial role in L-carnitine anti-apoptosis effect in renal tubular cells

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Abstract

Background. L-carnitine is synthesized mainly in the liver and kidneys from lysine and methionine from dietary sources. Many reports have shown that L-carnitine can protect certain cells against the toxicity of several anticancer and toxic agents, although the detailed mechanism is poorly understood. In this study, we investigated the protective effect of L-carnitine and its molecular mechanism in renal tubular cells undergoing gentamicin-induced apoptosis.

Methods. Rat tubular cell line (NRK-52E) and mice were used as the model system. Gentamicin-induced apoptosis in renal tubular cells was examined using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling. We introduced short interfering RNA transfection and gene-deficient mice to investigate the protective mechanism of L-carnitine.

Results. We found that L-carnitine inhibited gentamicin-induced reactive oxygen species generation and correlative apoptotic pathways, resulting in the protection of NRK-52E cells from gentamicin-induced apoptosis. The treatment of L-carnitine also lessened gentamicin-induced renal tubular cell apoptosis in mice. L-carnitine was found to increase the prostacyclin (PGI2) generation in NRK-52E cells. The siRNA transfection for PGI2 synthase significantly reduced L-carnitine-induced PGI2 and L-carnitine’s protective effect. We found that the activity of the potential PGI2 nuclear receptor, peroxisome proliferator-activated receptor alpha (PPARα), was elevated by L-carnitine treatment. The siRNA-mediated blockage of PPARα considerably reduced the anti-apoptotic effect of L-carnitine. In PPARα-deficient mice, L-carnitine treatment also lost the inhibitory effect on gentamicin-induced apoptosis in kidneys.

Conclusions. Based on these findings, we suggest that L-carnitine protects renal tubular cells from gentamicin-induced apoptosis through PGI2-mediated PPARα activation.
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Western blot analysis

A total of 30 μg of NRK-52E lysate proteins were applied to each lane in western blot analysis. We also used peroxidase-conjugated anti-rabbit, anti-mouse and anti-goat IgG (1:5000 dilution) antibodies as the second antibody to detect PPAR-α, PPAR-δ, caspase-3, Bcl-α, cytochrome c and GAPDH bands by enhanced chemiluminescence (Amersham Biosciences Corp., NJ, USA).

Animals and treatments

All animal studies were conducted in conformity with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male BALB/c mice weighing 20–25 g and aged 8 weeks were obtained from the Animal Center, National Taiwan University, Taipei, Taiwan. The PPARα-deficient mice were purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA). The animals were housed in a central facility in a 12-h light-dark cycle, and given regular rat chow and tap water. For the experiments, at 10 weeks of age, BALB/c mice were divided into four groups: (1) control; (2) 20% L-carnitine; (3) gentamicin 50 mg/kg; and (4) L-carnitine 50 mg/kg and gentamicin 50 mg/kg. Mice in each group were given vehicle via intraperitoneal (i.p.) injection once a day for 7 days. All groups were killed 24 h after the last injection, and their kidneys were collected and stored at −80 °C until in situ TUNEL assays. For histological analysis, the harvested kidneys were fixed in 10% formalin and embedded in paraffin, then sectioned at 4-μm thickness and stained with haematoxylin and eosin (HE staining).

Determining cellular uptake of gentamicin

We cultured the NRK-52E cells in 6-cm plates with 3-nM gentamicin for different time periods, washed them three times with a PBS buffer and then lysed them in a lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, protease inhibitors). We detected the concentration of gentamicin in each sample with Gentamicin ELISA kits according to the manufacturer’s instructions (Euro-Diagnostica B.V., Arnhem, The Netherlands).

Detecting intracellular reactive oxygen species

Before the chemical treatment, we incubated L-carnitine-NRK-52E cells in a culture medium containing a fluorescent dye, 30 μM 2',7'-dichlorofluorescein (DCF), for 30 min to stabilize an intracellular level of the probe. We determined the DCF fluorescence intensity of the cells with a fluorescence spectrophotometer with excitation and emission wavelengths at 475 and 525 nm, respectively. To provide a valid comparison, we used the same acquisition parameters and cell numbers for all observations.

Short interfering RNA (siRNA) transfection

We purchased PPARα siRNA and PPARδ siRNA from Santa Cruz Biotechnology. Cells were grown to 70% confluence, and 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 100 nM. We washed the transfected cells and resuspended them in new culture media for an additional 24 h for gentamicin treatment and western blot assays.

Measuring PGI2 by enzyme immunoassay

Cells were sonicated in 1 ml of an ice-cold buffer (0.05M Tris at pH 7.0, 0.1M NaCl, and 0.02M EDTA) and centrifuged at 55 000 × g for 1 h. We analysed the supernatant with 6-keto-PGF1α ELISA kits from R&D Systems, Inc. (Minneapolis, MN, USA) for PGI2 detection.

Subjects and methods

Reagents

 Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum and tissue culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA, USA). All other chemicals of reagent grade were obtained from Sigma-Aldrich chemical Co. (St. Louis, MO, USA). We purchased antibodies used in this research from Lab Frontier Co. Ltd, Seoul, Korea (anti-GAPDH), Cell Signalling Technology, Inc. (Danvers, MA, USA) (anti-caspase-3) and Santa Cruz Biotechnology (Santa Cruz, CA, USA) (anti-cytochrome c, anti-Bcl-α, anti-PPAR-α, anti-PPAR-δ, IP receptor-neutralizing antibody). L-carnitine was purchased from Sigma-Tau Industrie Farmaceutiche Riunite s.p.a (Roma, Italy).

Cell culture

Rat proximal renal tubular cells (NRK-52E) were purchased from Biore source Collection and Research Center (Taiwan), and cultured in the DMEM culture medium supplemented with antibiotic/antifungal solution and 10% fetal calf serum. They were grown until the monolayer became confluent. Then, the medium of the cultured cells was changed into serum-free medium and the cells were incubated overnight.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) stain

We detected gentamicin-mediated apoptosis in NRK-52E cells with enzymatic labelling of DNA strand breaks that were identified using TUNEL staining with a TUNEL apoptosis detection kit (CHEMICON International, Inc., CA, USA) according to the manufacturer’s instructions. We mounted TUNEL-stained kidney tissue slides with DAPI solution and observed them under a fluorescent microscope.
PPARα activity assay

PPARα transcriptional activity was measured using the rat malonyl-CoA decarboxylase (MCD)-Luc reporter construct as described in a previous report [24]. Luciferase activity of cell lysates was determined using a luciferase assay system (Promega, Madison, WI, USA) according to the protocol recommended by the manufacturer. Transfection efficiency was normalized to β-gal activity and expressed as fold induction of vehicle-transfected control cells.

Statistical analysis

Data were presented as mean± standard deviation (SD), and groups were compared using t-tests. The differences were considered significant if the P-values were < 0.05.

Results

To determine the safe dosage of L-carnitine in rat renal tubular cell NRK-52E, we detected the lactate dehydrogenase (LDH) released from the cytosol of damaged cells. NRK-52E cells were cultured with L-carnitine at a concentration of 1, 5, 10, 20 and 40 mM for 24 h. As shown in Figure 1A, there was no significant increase in LDH leakage along with the L-carnitine increase in NRK-52E cells; even exposure to 40 mM of L-carnitine gave no significant change from the controls. The protective effect of L-carnitine against the gentamicin-induced apoptosis in NRK-52E cells was examined using TUNEL stain. NRK-52E cells were pretreated with L-carnitine (110 mM) for 24 h, and then additionally treated with 3 mM of gentamicin for 24 h. The results revealed that the 24 h pretreatment of L-carnitine significantly reduced gentamicin-induced apoptosis in a dose-dependent manner (Figure 1B). The influence of pretreatment time on the protective effect of L-carnitine was also monitored in NRK-52E cells. As shown in Figure 1C, 10 mM of L-carnitine was not able to significantly reduce gentamicin-induced apoptosis in NRK-52E cells with the pretreatment periods from 1 to 8 h, whereas the reduction of apoptosis was significant with L-carnitine pretreatment for 16 h or more.

The influence of L-carnitine on apoptotic signalling pathways was further evaluated by western blotting analysis. As shown in Figure 2, the cleaved caspase-3 and cytosol cytochrome c were greatly elevated in the cells treated with 3 mM of gentamicin for 24 h. Pretreatment with L-carnitine at 5 or 10 mM for 24 h significantly reduced the quantity of cleaved caspase-3 and cytosol cytochrome c, as compared with cells treated with gentamicin alone. In contrast, the expression of Bcl-xL was reduced by gentamicin treatment, which was also recovered by L-carnitine pretreatment. These results reveal that the pretreatment of L-carnitine inhibited gentamicin-induced variations of apoptotic markers in a dose-dependent manner.

The protective effect of L-carnitine on gentamicin-induced apoptosis was also proven in a mouse animal model. In normal mice, gentamicin caused swollen and vacuolated epithelial cell degeneration with tubular dilatation and intraluminal cell debris, which was reduced by L-carnitine treatment (Figure 3A). The renal function of experimental mice was monitored by measuring the concentrations of BUN and serum creatinine. As shown in Figure 3B, the concentrations of BUN and serum creatinine were not influenced by L-carnitine treatment alone, but elevated in the gentamicin-treated groups, and this gentamicin-induced elevation was significantly inhibited by L-carnitine treatment.

To analyse the gentamicin-induced apoptosis in vivo, we next examined kidney sections with the in situ TUNEL assay. As shown in Figure 4, the scattered and bright nuclei stained by TUNEL staining could easily be detected over the entire cortex from gentamicin-treated animals, yet they were rarely detected in the specimens of the controls and gentamicin-L-carnitine-treated animals (Figure 4).
Most of the TUNEL-labelled nuclei were seen in the proximal tubule epithelium. This result reveals that L-carnitine inhibits the gentamicin-induced cell apoptosis in the renal cortex in mice.

To evaluate the mechanism of the protective effect of L-carnitine on gentamicin-induced apoptosis, the influence of L-carnitine on the cellular uptake of gentamicin was first monitored. As shown in Figure 5A, the concentration of cytosol gentamicin reached a maximum within 30 min. Compared with control groups, L-carnitine did not influence the concentration of cytosol gentamicin. This result reveals that L-carnitine did not influence the cellular uptake of gentamicin. We next examined whether L-carnitine prevents gentamicin-induced ROS formation because ROS are important mediators in gentamicin-induced apoptosis. Gentamicin-induced increases in intracellular ROS were revealed by fluorescent intensities of 2′,7′-dichlorofluorescin (DCF). As shown in Figure 5B, L-carnitine pretreatment significantly inhibited gentamicin-induced ROS formation.

The production of PGI2 was typically monitored by using measurement of 6-keto-prostaglandin F1α (6-keto-PGF1α) because 6-keto-PGF1α is a stable product of the non-enzymatic hydration of PGI2. L-carnitine at 5 mM significantly elevated PGI2 levels in NRK-52E cells at 24 h (Figure 6A). Although 3 mM of gentamicin reduced the expression of PGI2 in NRK-52E cells, L-carnitine still significantly elevated PGI2 expression. This PGI2 elevation was increased along with the increase of L-carnitine. In the time course analysis, the PGI2 elevation induced by 10 mM of L-carnitine was significant at 8 h, and reached a maximum at 24 h (Figure 6B). The siRNA for prostacyclin synthase (PGIS) was applied to block PGI2 synthesis, and we found that PGIS siRNA transfection obviously reduced PGI2 generation in l-carnitine-treated NRK-52E cells (Figure 6C). In PGIS siRNA-transfected cells, gentamicin alone induced more serious apoptosis than that in mock control cells, as revealed by TUNEL staining (Figure 6D). The pretreatment of 10 mM L-carnitine significantly alleviated gentamicin-induced apoptosis in mock control cells, whereas there is a very minor influence of L-carnitine on gentamicin-induced apoptosis in PGIS siRNA-transfected cells (Figure 6D). To further confirm the role of PGI2, we added iloprost (a stable analogue of PGI2) and PGE2 to siRNA-transfected NRK-52E cells with gentamicin and L-carnitine treatment. The results show that the blockage of L-carnitine anti-apoptosis functions by PGIS siRNA transfection was reversed by iloprost but not by PGE2 (Figure 6D).

PGI2 has been reported to be a potential ligand for the IP receptors, PPARα and PPARδ [12]. To identify the signalling pathways involved in the protective function of L-carnitine, the neutralizing antibody for IP receptor and the siRNA for PPARα and PPARδ were applied to NRK-52E cells. The PPARα and PPARδ protein levels were obviously reduced by PPARα and PPARδ siRNA transfection, respectively, as shown in Figure 7A. The gentamicin-induced apoptotic cells were increased ∼5% by PPARα siRNA transfection and were not affected by IP receptor neutralization and PPARδ siRNA transfection (Figure 7B). The inhibitory effect of L-carnitine on the gentamicin-induced apoptosis was reduced ∼80% by PPARα siRNA transfection and ∼25% by IP receptor neutralizing, and not affected significantly by PPARδ siRNA transfection (Figure 7B). Further, we found that L-carnitine increased PPARα activity more than 5-fold (Figure 7C). These results reveal the crucial role of PPARα activation in the L-carnitine-mediated L-carnitine protection effect.
protective function on gentamicin-induced apoptosis in NRK-52E cells.

The crucial role of PPARα in the L-carnitine protective function was further proven in PPARα-deficient mice. In PPARα-deficient mice, gentamicin caused serious degeneration of epithelial cells with granular and proteinaceous casts in the tubular lumen, without respect to L-carnitine treatment (Figure 8A). The gentamicin-induced apoptosis in the kidneys of PPARα-deficient mice was revealed by using the in situ TUNEL assay. There were many apoptotic cells found in the entire renal cortex from gentamicin-treated mice (Figure 8B and C), and these apoptotic cells were rarely reduced by L-carnitine treatment in PPARα-deficient mice. It is obvious that PPARα plays an important role for kidneys against gentamicin-induced apoptotic injury in vivo.

Discussion

The results of this study showed that L-carnitine, with proper pretreatment time, protected renal tubular cells from gentamicin-induced apoptosis in vitro and in vivo. In the study of the protective mechanism of L-carnitine, we found that L-carnitine induced the endogenous PG12 production in NRK-52E cells in a dose- and time-dependent manner (Figure 6). With the reduction of PG12 generation by PGIS siRNA transfection, the protective effect of L-carnitine against gentamicin-induced apoptosis was significantly decreased in NRK-52E cells. This result shows that L-carnitine achieved the anti-apoptosis effect by inducing PG12 generation. Although the l IP receptors, PPARα and PPARδ are supposed to be involved in PG12 signalling pathways, our results reveal that PPARα plays a major part in L-carnitine protection on gentamicin-induced apoptosis in NRK-52E cells (Figure 7). Even in PPARα-deficient mice, gentamicin-induced renal injury and apoptotic cells were rarely reduced by L-carnitine treatment (Figure 8). Taken together, we suggest that L-carnitine can protect renal tubular cells from gentamicin-induced apoptosis through PPARα activation by PG12.

To achieve the protective effect of L-carnitine in vivo, we designed the animal study with L-carnitine pretreatment for 2 days. In fact, we have tried to inject L-carnitine and gentamicin simultaneously in the animal study without any pretreatment, but the result was not significant (data not shown). A longer pretreatment period (4 days) was also adopted from the study of Kopple et al. [5] to reveal L-carnitine protective effects on renal cortical...
The effect of PGIS siRNA transfection on the levels of 6-keto-PGF1α in NRK-52E cells. The cells were treated with L-carnitine in indicated concentrations with or without 3 mM of gentamicin for 24 h. Results are shown in mean ± S.D. (n = 6). *Significantly different (P < 0.05) versus the 6-keto-PGF1α level in the cells without treatment. **Significantly different (P < 0.05) versus the 6-keto-PGF1α level in the cells with gentamicin treatment. (B) A time course of 6-keto-PGF1α levels in L-carnitine-treated NRK-52E cells. Cells were treated with L-carnitine at 10 μM for different time periods. Results are shown in mean ± S.D. (n = 6). *Significantly different (P < 0.05) versus the 6-keto-PGF1α level in the cells at 0 h. (C) The effect of PGIS siRNA transfection on the levels of 6-keto-PGF1α in L-carnitine-treated NRK-52E cells. 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 are shown in mean ± S.D. (n = 6). (D) The influence of PGIS siRNA transfection on the protection effect of L-carnitine in gentamicin-treated NRK-52E cells. The transfected cells were pretreated with L-carnitine (10 μM) for 24 h, and then treated with 3 mM of gentamicin for 24 h. Iloprost (1 μM) or PGE2 (1 μM) was added back to check the influence of PGIS knockdown. The cells were stained with DAPI and TUNEL, and the percentage of TUNEL positive cells were calculated. Results are shown in mean ± S.D. (n = 3). *Significantly different (P < 0.05) versus the mock control with gentamicin treatment. Ps, PGIS siRNA transfection.

In addition, the overexpression of PPARα can also induce the activity of catalase and superoxide dismutase, and reduce adriamycin-induced ROS concentration in NRK-52E cells [24]. Based on these data, we suggest that the antioxidant ability of PG12 is highly associated with activating PPARα. In the present study, inducing PG12 and activating PPARα were shown to be necessary for L-carnitine's anti-apoptotic effect (Figures 7 and 8). Therefore, we suggest that L-carnitine induces PG12 generation to inhibit gentamicin-induced ROS generation through PPARα activation in renal tubular cells, and that L-carnitine is useful in reducing gentamicin-induced nephropathy.

Based on our data, L-carnitine reduced the severity of the kidney disorder but did not prevent gentamicin-induced nephrotoxicity (Figures 3 and 4). Even in NRK-52E cells, 10 mM of L-carnitine only reduced gentamicin-induced apoptosis ~40% (1). This phenomenon may result from the moderate PG12 induction of L-carnitine in renal tubular proximal tubular necrosis in gentamicin-treated rats. The L-carnitine injections, initiated some days before gentamicin treatment, provide some assurance that the kidney tissue was exposed to gentamicin with adequate tissue L-carnitine levels. Interestingly, more than 16 h of pretreatment is also necessary for L-carnitine to protect NRK-52E cells from gentamicin-induced apoptosis in vitro (Figure 1). This requirement for relatively long pretreatment periods may result from L-carnitine-induced PG12 generation. We have found that PG12 can protect the kidney from gentamicin-induced apoptosis in rat renal tubular cells [25]. The results of the present study showed that PG12 generation was essential for the anti-apoptotic effect of L-carnitine and was significantly induced by L-carnitine treatment for 8 h or above (Figure 6). We suggest that a long pretreatment period is helpful to reach a maximum of L-carnitine-induced PG12 in renal tubular cells to protect kidneys from gentamicin-induced acute renal injury in vivo.

Since ROS are important apoptotic stimulators in gentamicin-induced apoptosis, the inhibition of ROS generation is supposed to be one of the anti-apoptotic mechanisms of L-carnitine. L-carnitine has been reported to have an inhibitory effect on free radical production [27,28]. But the detailed mechanism of free radical scavenging is still unclear. In our previous study, the selective PG12 augmentation with adenovirus-COX-1/PGIS transfection has been found to inhibit adriamycin-induced ROS generation and to protect NRK-52E cells from adriamycin-induced apoptosis [26]. This ROS inhibition resulted largely from elevated activation of catalase and superoxide dismutase caused by cellular PG12 augmentation. In our recent study, cellular PG12 augmentation can activate PPARα in NEK-52E cells [24]. In addition, the overexpression of PPARα can also induce the activity of catalase and superoxide dismutase, and reduce adriamycin-induced ROS concentration in NRK-52E cells [24]. Based on our data, we suggest that the antioxidant ability of PG12 is highly associated with activating PPARα. In the present study, inducing PG12 and activating PPARα were shown to be necessary for L-carnitine's anti-apoptotic effect (Figures 7 and 8). Therefore, we suggest that L-carnitine induces PG12 generation to inhibit gentamicin-induced ROS generation through PPARα activation in renal tubular cells, and that L-carnitine is useful in reducing gentamicin-induced nephropathy.

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Fig. 7. Effects of the blockade of the PGI2-signalling pathway on the protective effect of l-carnitine in NRK-52E cells. (A) The effect of PPARα and PPARδ siRNA transfection on PPARs protein levels in NRK-52E cells. The cells were either transfected with PPARα siRNA or PPARδ siRNA to get PPARα and PPARδ knockdown cells. Control siRNA was also applied as mock controls. Western blotting was carried out with the specific antibody against PPARα or PPARδ. GAPDH was used as a loading control. (B) The influence of an IP-neutralizing antibody, PPARα siRNA and PPARδ siRNA on the protective effect of l-carnitine in NRK-52E cells. Transfected cells were pretreated with or without l-carnitine (10 mM) for 24 h, and then treated with 3 mM of gentamicin for 24 h. For blocking the function of PGI2 IP receptor, the cells were pretreated with the IP receptor-neutralizing antibody for 30 min. The percentage of TUNEL-positive cells is shown in mean ± S.D. (n = 3). ∗Significantly different (P < 0.05) versus the mock control with l-carnitine and gentamicin treatment. C, untransfected control; M, mock control; sPα, PPARα siRNA transfection; sPδ, PPARδ siRNA transfection; IP_Ab, IP receptor neutralizing antibody treatment. (C) Effects of l-carnitine treatment on PPARα activation in NRK-52E cells. NRK-52E cells were transfected with luciferase reporters for PPARα and β-galactosidase expression vector (as an internal control). Transfected cells were treated with or without l-carnitine at 10 mM for 24 h. Luciferase activity was reported as relative luciferase activity after correction for transfection efficiency using β-galactosidase activity, and is shown in mean ± S.D. (n = 3).

cells. In our previous study, high PGI2 expression (1200–1400 pg/µg protein) reduced gentamicin-induced apoptosis >80% in NRK-52E cells [25]. l-Carnitine at 20 mM dose only induced ~450 pg/µg PGI2 in NRK-52E cells; moreover, gentamicin partially inhibited the expression of PGI2 (Figure 6). Based on our results, more l-carnitine could induce more PGI2 expression. Therefore, it is possible that a high dosage of l-carnitine blocks gentamicin-induced apoptosis in NRK-52E cells and prevents gentamicin-induced nephrotoxicity in vivo. However, to assess the side effects of l-carnitine in a high dosage, further in vivo studies are needed.

Because the renal protective effect of l-carnitine results mostly from the PGI2 induction and PPARα activation, as revealed in this study (Figures 6, 7 and 8), we also suggest that PGI2 and PPARα are potential therapeutic candidates for gentamicin-induced nephropathy. But administering PGI2 and its analogues systematically can cause undesirable side effects. Administering PGI2 and its more stable analogues locally is also a challenge because of the relatively short half-life of these drugs. In fact, certain fatty acids, such as docosahexaenoic acid (DHA), can also activate PPARα and protect renal tubular cells from adriamycin-induced apoptosis in vivo, and recover the kidney function [24]. However, relatively high dosages of these fatty acids would be needed, and they are rather costly. l-Carnitine is a natural neuroprotective agent that can be safely used in humans. Therefore, we suggest that l-carnitine be developed as a future clinical remedy to prevent gentamicin-induced nephropathy in human.

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