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.

Keywords: apoptosis; gentamicin; L-carnitine; peroxisome proliferator-activated receptor alpha (PPARα); prostacyclin (PGI2)

Introduction

L-carnitine (L-trimethyl-3-hydroxy-ammoniabutanoate) is a vitamin-like substance that is a quaternary ammonium compound biosynthesized from the amino acids lysine and methionine. Carnitine is required for the transfer of long-chain fatty acids into the mitochondrial matrix before they can undergo β-oxidation, resulting in ATP formation [1]. In addition, L-carnitine modulates the intramitochondrial acyl-CoA/CoA ratio to remove toxic compounds before they have a chance to accumulate in the mitochondria. Many reports have shown that L-carnitine can improve the toxic effects of various substances on the ear, heart, brain and kidney [2–6]. Many reports about possible protective mechanisms of L-carnitine suggest that it inhibits mitochondrial membrane permeability transition, decreases oxidative stress and prevents the expression of a proapoptotic protein [4,7,8]. However, the detailed mechanisms are not conclusive. In early 1970s, Johnson et al. [9] showed that prostaglandins (PGs) exert the common carnitine-dependent system for the β-oxidation of long-chain fatty acids. Prostacyclin (PGI2), a major PG, originates from arachidonic acid by the cyclooxygenase (COX) system coupled with the action of PGI2 synthase (PGIS) [10]. PGI2 acts on platelets and blood vessels through cell surface prostacyclin receptor (IP receptor), to inhibit platelet function and to dilate blood vessels [11]. PGI2 is also supposed to be a ligand of the peroxisome proliferator-activated receptors alpha and delta (PPARα and PPARδ), belonging to a family of ligand-activated transcription factors [12]. Ingrid et al. [13] reported that the production of PGI2 has the most significant increase after short-term (4 days) feeding of L-carnitine in the rat. Recent studies also revealed that L-carnitine can induce the vasodilatation of subcutaneous human arteries involving the endothelium through the effect related to the
Gentamicin, an antibiotic of aminoglycoside, is widely used to treat Gram-negative bacterial infection because of its low cost. But gentamicin can cause acute renal failure with acute tubular necrosis in ∼20% of patients [16]. This potential nephrotoxicity seriously limits the use of gentamicin. A major cytotoxic mechanism of gentamicin is the induction of apoptosis, which has been reported in both re-

We detected gentamicin-mediated apoptosis in NRK-52E cells with enzymatic labelling of DNA strand breaks that were identified using TUNEL stain (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) stain). We analysed the supernatant with 6-keto-PGF1α (an index of PGI2 production through adenovirus-mediated transfer of genes for COX-1 and PGIS) and measured its low cost. But gentamicin can cause acute renal failure with acute tubular necrosis in ∼20% of patients [16]. This potential nephrotoxicity seriously limits the use of gentamicin. A major cytotoxic mechanism of gentamicin is the induction of apoptosis, which has been reported in both re-

**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-xL, 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 PPAR-δ-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. First the animals (n = 6) for the gentamicin and L-carnitine treatment group received intraperitoneal (IP) injection of L-carnitine (50 mg/kg/day) for 2 days. Then they were injected with L-carnitine (50 mg/kg/day) and gentamicin (20 mg/kg/day) for 7 days. The mice in the group (n = 6) for gentamicin treatment received IP injection of 0.9% (w/v) saline for 2 days, and were then injected with saline and gentamicin for 7 days. In this group, the volume of injected saline was identical to that of injected L-carnitine in the L-carnitine and gentamicin treatment group. The control group mice received IP injection of saline in the same volume as the L-carnitine injected first in the L-carnitine and gentamicin treatment group for 2 days, and in the same volume as the L-carnitine and gentamicin injected for 7 days. We killed treated and control mice 24 h after the last drug injection, and collected their blood samples from the stump to measure serum creatinine and blood urea nitrogen (BUN). Both kidneys were harvested by laparotomy and the renal cortex tissue was snap-frozen in dry ice 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-mM 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 EIA 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 NRK-52E cells in a culture medium containing a fluorescent dye, 30 µM of 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.
**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 gentamycin 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 expression 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

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Fig. 2. Effects of L-carnitine on apoptotic markers (cleaved caspase-3, released cytochrome c and BcL-xL) in gentamicin-treated NRK-52E cells. The cells were pretreated with L-carnitine for 24 h, and then treated with 3 mM of gentamicin for 12 h. Western blotting was carried out with the specific antibody against cleaved caspase-3, cytochrome c and Bcl-xL. GAPDH was used as a loading control.

Fig. 3. The influence of L-carnitine on gentamicin-induced renal injury in vivo. Mice were injected with saline, gentamicin or gentamicin and L-carnitine as described in the ‘Subjects and methods’ section. (A) Representative photomicrographs of HE stain. (B) The concentration of BUN in treated mice (n = 6). (C) The concentration of serum creatinine in treated mice (n = 6). Results are shown in mean ± S.D. *Significantly different (P < 0.05) versus the gentamicin alone group.
The influence of L-carnitine on gentamicin-induced renal tubular cell apoptosis in vivo. Mice were injected with saline, gentamicin or gen-
tamicin and L-carnitine as described in the ‘Subjects and methods’ section. Apoptotic cells in kidneys of experimental animals were detected using in situ TUNEL staining. (A) Representative photomicrographs of in situ TUNEL stain. TUNEL-labelled nuclei were revealed as bright spots in cortex sections from untreated and treated mice. The identical fields stained for TUNEL were also stained using DAPI to show the positions of cell nuclei. (B) The numbers of TUNEL-labelled cells per millimetre-square cortex area in each sample (n = 6). Results are shown in mean ± S.D. *Significantly different (P < 0.05) versus the gentamicin alone group.

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 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 PGI2 produ-
don in NRK-52E cells in a dose- and time-dependent manner (Figure 6). With the reduction of PGI2 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 PGI2 generation. Although the IP receptors, PPARα and PPARβ are supposed to be involved in PGI2 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 PGI2.

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 (Figure 6). This requirement for relatively long pretreatment periods may result from L-carnitine-induced PGIS generation. We have found that PGIS can protect the kidney from gentamicin-induced apoptosis (Figure 1). This requirement for relatively long pretreatment periods may result from L-carnitine-induced PGIS generation (Figure 6). We suggest that a long pretreatment period is helpful to reach a maximum of L-carnitine-induced PGIS 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 PGIS 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 activity of catalase and superoxide dismutase caused by cellular PGIS augmentation. In our recent study, cellular PGIS 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 these data, we suggest that the antioxidant ability of PGIS is highly associated with activating PPARα. In the present study, inducing PGIS 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 PGIS 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 PGIS induction of L-carnitine in renal tubular cells 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 mM) 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.
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 PPARa activation, as revealed in this study (Figures 6, 7 and 8), we also suggest that PGI2 and PPARa are potential therapeutic candidates for gentamicin-induced nephropathy. But administering PGI2 and its analogues systematically can cause undesirable side effects. Administrating 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 PPARa 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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PPARα mediates L-carnitine protection effect

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