Curcumin prevents maleate-induced nephrotoxicity: Relation to hemodynamic alterations, oxidative stress, mitochondrial oxygen consumption and activity of respiratory complex I


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Abstract

The potential protective effect of the dietary antioxidant curcumin (120 mg/Kg/day for 6 days) against the renal injury induced by maleate was evaluated. Tubular proteinuria and oxidative stress were induced by a single injection of maleate (400 mg/kg) in rats. Maleate-induced renal injury included increase in renal vascular resistance and in the urinary excretion of total protein, glucose, sodium, neutrophil gelatinase-associated lipocalin (NGAL) and N-acetyl β-D-glucosaminidase (NAG), upregulation of kidney injury molecule (KIM)-1, decrease in renal blood flow and claudin-2 expression besides of necrosis and apoptosis of tubular cells on 24 h. Oxidative stress was determined by measuring the oxidation of lipids and proteins and diminution in renal Nrf2 levels. Studies were also conducted in renal epithelial LLC-PK1 cells and in mitochondria isolated from kidneys of all the experimental groups. Maleate induced cell damage and reactive oxygen species (ROS) production in LLC-PK1 cells in culture. In addition, maleate treatment reduced oxygen consumption in ADP-stimulated mitochondria and diminished respiratory control index when using maleate/glutamate as substrate. The activities of both complex I and aconitase were also diminished. All the above-described alterations were prevented by curcumin. It is concluded that curcumin is able to attenuate in vivo maleate-induced nephropathy and in vitro cell damage. The in vivo protection was associated to the prevention of oxidative stress and preservation of mitochondrial oxygen consumption and activity of respiratory complex I, and the in vitro protection was associated to the prevention of ROS production.

Keywords: acute renal failure, antioxidant, curcumin, lipid peroxidation, mitochondria

Introduction

Curcumin is a dietary antioxidant and anti-inflammatory compound obtained from the rhizome of Curcuma longa [1–3]. It is considered a bifunctional antioxidant because it shows direct and indirect antioxidant properties [2]. In fact, it has been shown that curcumin is able to scavenge reactive oxygen species (ROS) [4] and to enhance the synthesis of several antioxidant and cytoprotective proteins through the induction of the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) [2,5,6], a transcription factor that is a master regulator of the antioxidant response [7]. On the other hand, the injection of maleate to experimental animals induces nephropathy characterized by cell necrosis and apoptosis in the proximal tubule epithelial cells, tubular proteinuria, oxidative stress, and increase of renal gene expression of heme oxygenase-1, albumin, monocyte chemotactic protein (MCP-1), and neutrophil gelatinase-associated lipocalin (NGAL) [8–13]. Therefore, this manipulation has been considered as a model of Fanconi syndrome in humans [8,12,14–16]. In addition, it has been found that maleate induces apoptosis and heme oxygenase-1 expression in rat renal proximal tubular epithelial NRK 52E cells in culture [12]. To our knowledge, studies of mitochondrial respiration have not been performed in isolated mitochondria from kidneys of maleate-injected rats. However, previous histological findings have revealed abnormal-appearing mitochondria in maleate-treated animals [17,18] suggesting alterations in mitochondrial function in this experimental model.

Curcumin administration has been shown to exert a protective effect in several experimental models of renal damage [4,19] including those induced by 5/6 nephrectomy [20,21], ischemia and reperfusion [22], cyclosporin [23], potassium dichromate [24], gentamicin [25], cisplatin [26,27], aceterminophen [28], vancomycin [29], sodium...
floride [30] and methotrexate [31]. An additional protective mechanism mediated by curcumin is the attenuation of mitochondrial functional alterations; such effect has been observed in several experimental conditions: damage induced by potassium dichromate in kidneys [24] and in liver [32], and also in a model of chronic heart failure secondary to chronic kidney disease [33].

Therefore, in the present study, we tested the hypothesis that curcumin could attenuate maleate-induced in vivo renal injury in rats and in vitro cell damage in Lilly Laboratories Cell Porcine Kidney (LLC-PK1) cells in culture, a renal epithelial cell line from proximal tubule. Specifically we studied if curcumin is able to attenuate the maleate-induced hemodynamic alterations in renal blood flow (RBF), renal vascular resistance (RVR), and filtration fraction (FF) and the markers of renal damage proteinuria, glycosuria, urinary excretion of sodium (UNaV), N-acetyl ß-D-glucosaminidase (NAG), and NGAL, kidney injury molecule-1 (KIM-1) expression as well as the histological damage and apoptosis. The effect of curcumin on maleate-induced decreased expression of claudin-2, a component of the tight junction strands in epithelial cells of the proximal tubule, that is considered as a paracellular cation pore, and whose expression leads to the reabsorption of one-third of filtered sodium by the paracellular route [34,35], was also studied. The mechanism of the protective effect of curcumin in kidney including oxidative stress markers, Nrf2 expression, and mitochondrial alterations as well as ROS production in LLC-PK1 cells was also studied. The effect of curcumin on the maleate induced decrease in mitochondrial oxygen consumption coupled to ATP synthesis, respiratory control index, and adenosine diphosphate/oxygen (ADP/O) ratio when using nicotinamide adenine dinucleotide (NADH)-linked substrates as well as in the activity of aconitase and respiratory complex I is also described.

**Materials and methods**

**Reagents**

Maleic acid, curcumin, carboxymethylcellulose (CMC), bovine serum albumin (BSA), p-nitrophenyl-N-acetyl-ß-D-glucosaminide, butylated hydroxytoluene, streptomycin sulfate, l-methyl-2-phenylindole, tetramethoxypropane, sodium dodecyl sulfate (SDS), 2,4-dinitrophenylhydrazine (DNPH), guanidine hydrochloride, sucrose, Tris–HCl, ethylene diaminetetraacetic acid (EDTA), Triton X-100, sodium deoxycholate, potassium chloride (KCl), ethylene glycol tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), L-citrate, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), potassium succinate, NADH, methanesulfonic acid, sodium glutamate, sodium malate, rotenone, adenosine diphosphate (ADP), carbonyl cyanide m-chlorophenylhydrazone (CCCP), decylubiquinone, potassium cyanide (KCN), antimycin A, picric acid, collagenase (from *Clostridium histolyticum*, type II), fluorescein diacetate (FDA), glycerol, 2-mercaptoethanol, bromophenol blue, and paraformaldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), hydrogen peroxide (H$_2$O$_2$), methanol, high-performance liquid chromatography (HPLC)-grade acetonitrile, and ethyl acetate were acquired from J.T. Baker (Xalostoc, Edo. Mex, México). Sodium pentobarbital (Shedalpharma) was purchased from Pet’s Pharma de México S.A. de C.V (Mexico city). Polyfructosan (Inu test) was from Fresenius Kabi-Austria GmBh (Graz, Austria). Antibodies against NGAL and against Nrf2 (Cat No. SC-722) were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). Rabbit anti-claudin-2 antibodies were purchased from Invitrogen (Carlsbad, CA, USA), the goat anti-kidney injury molecule (KIM)-1 was purchased from R&D systems (Mckinley Place, MN, USA) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Millipore Corp. (Billerica, MA, USA). The micro bicinechonic acid (BCA) Protein Assay Reagent Kit was from Pierce (Rockford, IL, USA). Bradford reagent was from BioRad Laboratories (Hercules, CA, USA). The ECL™ prime Western blotting detection reagent was from Amersham™, GE Healthcare (Buckinghamshire, UK). Protease inhibitor cocktail Complete and in Situ Cell Death Detection Kit, POD (Cat. No. 11 684 817 910) were obtained from Roche Applied Science (Mannheim, Germany). 3,3′-diaminobenzidine (DAB) was obtained from Dako (Carpinteria, CA, USA). LLC-PK1 cells were from American Type Culture Collection (Manassas, VA, USA). Dulbecco’s modified Eagle’s medium (DMEM) GIBCO BRL, trypsin blue solution GIBCO BRL and Tryple Express GIBCO BRL were from Life Technologies Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were from PAA Laboratories Inc. (Etobicoke, ON, Canada). Dihydroethidium (DHE) was obtained from Molecular Probes, Inc. (Eugene, OR, USA). All other reagents and chemicals used were of the highest grade of purity commercially available.

**Experimental design**

Animal experiments were performed in accordance with the Mexican Official Norm NOM-062-ZOO-1999 and institutional animal care committee guidelines. Male Wistar rats weighing 200–230 g of body weight were used. Rats were divided in four groups (n = 8/group): (1) Control group, rats were dosed intragastrically with vehicle (0.05% CMC), (2) Curcumin, rats were dosed intragastrically with curcumin (120 mg/kg/day) suspended in 0.05% CMC, (3) Maleate, rats were injected intraperitoneally with maleic acid (400 mg/Kg), and (4) Maleate + curcumin, rats were injected with maleic acid and curcumin as was described in groups 2 and 3. Maleic acid was dissolved in sterile saline solution and the pH was adjusted to 7.4 with sodium hydroxide. Curcumin was given 5 days before maleic acid injection. The doses of maleate and curcumin were chosen according to previous reports [12,21,33]. Rats were sacrificed at 24 h after maleic acid injection [12]. Rats were placed in metabolic cages
(Braintree Scientific Inc, Braintree, MA, USA) along the study to collect 24 h urine. In pilot studies we found that after 48 h of maleic acid administration renal function, biochemical parameters, and renal histological abnormalities returned essentially to normal levels (data not shown).

**Hemodynamic parameters**

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed on a homeothermic table to maintain body temperature at 37°C. Trachea, both jugular veins, both femoral arteries and bladder were catheterized with polyethylene tubing PE-240, PE-50, and PE-90, respectively. Rats were maintained under euvoletic conditions by infusion of 10 ml/Kg of body weight of isotonic BSA (6 g/dL) during surgery, followed by an infusion of physiologic saline (0.9%) at 2.2 ml/h. Mean arterial pressure (MAP) was continuously monitored with a pressure transducer (Model MTL844, AD Instruments, Colorado Springs, CO, USA) and recorded on a data acquisition system (Power Lab, AD Instruments). The left kidney was exposed and placed in a lucite holder, and the kidney surface was prevented to dehydrate by covering it with a cotton soaked with Ringer solution. A 2-mm ultrasound transit-time flow probe (TS420, Transonic Systems, Ithaca, NY, USA) was placed around the left renal artery and fixed with 4% paraformaldehyde at ≈ 100 mmHg for 30 min and then centrifuged at 13,100 x g for 10 min at 4°C, and the pellets were washed twice with ice-cold acetone. The samples were air-dried and dissolved in Laemmli buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.05% bromophenol blue) followed by heating at 95°C for 5 min. The samples were loaded in SDS-polyacrilamide gel electrophoresis (PAGE) gels as previously described [37]. The protein bands were visualized and intensity quantified as previously described [37]. The data were expressed as arbitrary unit (AU)/µg urine creatinine. Urinary NAG excretion was determined at 405 nm using p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate, and the data were expressed as U/24 h. One unit of NAG was defined as the amount of enzyme that releases 1 µmol of p-nitrophenol in the assay conditions [38]. Urinary sodium (UNa) was measured by flame photometry using the Flame photometer IL 943 (Instrumentation Laboratory, Bedford, MA, USA). UNaV was calculated by multiplying UNa by urinary flow (mL/min); the data were expressed as µEq/min. Urinary creatinine was measured with an autoanalyzer (ILAb 300 Plus Instrumentation Laboratory, Bedford, MA, USA). The renal expression of claudin-2 and KIM-1 was measured by Western blot as described below.

**Histological studies**

Fixed kidneys were dehydrated in ascending concentrations of ethanol, embedded in paraffin and sectioned to 2-µm thickness and subsequently stained with hematoxylin and eosin (H&E). Slides were observed by a pathologist blinded to the experimental design using a light microscope (AxioPhot 2, Zeiss, Oberkochen, Germany) with an objective of 40X, and 8 digital microphotographs were taken for each kidney (5 to 6 animals per group). An automated morphometry program (AxioVision 4.8, Zeiss) was used to measure the nondamaged and the necrotic epithelial tubular areas. The total epithelial tubular area was then calculated as well as the percentage of damaged epithelium.

**Apoptosis detection**

Deoxyribonucleic acid (DNA) fragmentation, as a marker of apoptosis, was evaluated by terminal-deoxynucleotidyl transferase mediated deoxyuridine triphosphate (dUTP)-digoxigenin nick end labeling (TUNEL) in renal tissue 3-µm sections. An immunohistochemical procedure was followed according to the method described by Negrette-Guzmán et al. [39]. Color was developed by adding DAB and monitored under the light microscope. The resulting

**Markers of renal damage**

Urinary osmolality was measured with an Advanced Digimatic Osmometer Model 3D (Advanced Instruments Inc., Needham Heights, MA, USA). Proteinuria was measured using the Bradford reagent and the data were expressed as mg/24 h. Urinary glucose concentration (glycosuria) was determined in an autoanalyzer using the enzyme-based IL Test™ GLUC kit (Glucose oxidase) from Instrumentation Laboratory SpA (Milano, Italy). Urinary NGAL excretion was measured by Western blot. The sample volumes corresponding to 15 µg of total protein were precipitated with 10% (w/v) TCA in PBS on ice for 30 min and then centrifuged at 13,100 x g for 10 min at 4°C, and the pellets were washed twice with ice-cold acetone. The samples were air-dried and dissolved in Laemmli buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.05% bromophenol blue) followed by heating at 95°C for 5 min. The samples were loaded in SDS-polyacrilamide gel electrophoresis (PAGE) gels as previously described [37]. The protein bands were visualized and intensity quantified as previously described [37]. The data were expressed as arbitrary unit (AU)/µg urine creatinine. Urinary NAG excretion was determined at 405 nm using p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate, and the data were expressed as U/24 h. One unit of NAG was defined as the amount of enzyme that releases 1 µmol of p-nitrophenol in the assay conditions [38]. Urinary sodium (UNa) was measured by flame photometry using the Flame photometer IL 943 (Instrumentation Laboratory, Bedford, MA, USA). UNaV was calculated by multiplying UNa by urinary flow (mL/min); the data were expressed as µEq/min. Urinary creatinine was measured with an autoanalyzer (ILAb 300 Plus Instrumentation Laboratory, Bedford, MA, USA). The renal expression of claudin-2 and KIM-1 was measured by Western blot as described below.

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slides were scanned in order to obtain electronic files and micrographs of renal cortex regions with Aperio CS (San Diego, CA, USA) digital pathology equipment. TUNEL-positive nuclei (brown color) percentage in the renal cortex areas were determined with an algorithm included in the free software ilastik 1.1 (ilastik.org).

Markers of oxidative stress

Frozen right kidney tissues were used to evaluate oxidative stress. Malondialdehyde (MDA), 4-hydroxy-nonenal (4-HNE), and oxidized protein content were measured in kidney homogenates. The MDA content was determined using tetramethoxypropane as a standard curve or renal homogenates in a solution of acetonitrile:methanol (3:1) with 10 mM 1-methyl-2-phenylimidole in the presence of concentrated HCl and incubated for 45 min at 40°C. After that, samples were centrifuged at 3,000 x g for 5 min; the optical density of the supernatant was measured at 586 nm [40]. The content of 4-HNE was evaluated with the same technique used to measure MDA, but employing methanesulfonic acid with Fe(III) instead of HCl [41]. Values were expressed as nmol of MDA or 4-HNE per mg of protein. Protein carbonyl content in the kidney homogenates was determined as previously described. The renal homogenates were incubated overnight with streptomyacin sulfate to remove nucleic acids. Later, homogenates were treated with DNPH and HCl and finally with guanidine hydrochloride. Assessment of carbonyl formation was done on the basis of formation of protein hydrazone by reaction with DNPH. The absorbance was measured at 370 nm. Protein carbonyl content was expressed as nmol carbonyl/mg protein [42]. In addition, the expression of renal Nrf2 was measured by Western blot as described below.

Extraction of total proteins of renal cortex for Western blot analysis

Protein extraction was performed as previously described [43]. Briefly, kidneys were excised, decapsulated, and the cortex was dissected out. Pieces of renal cortex were placed in ice-cold Krebs-bicarbonate solution (KB, mM): 110 NaCl, 25 NaHCO₃, 3 KCl, 1.2 CaCl₂, 0.7 MgSO₄, 2 KH₂PO₄, 10 sodium acetate, 5.5 glucose, 5 alanine, and 0.5 g/L BSA, pH 7.4] and washed three times, and resuspended in 10 ml of KB containing 0.2 g/100 ml collagenase. Samples were gassed with 95% O₂/5% CO₂ in a shaking water bath at 37°C for 30 min. After digestion, approximately 10 ml of ice-cold KB with a protease inhibitor cocktail (Complete 1X) and PMSF (20 μg/ml) were added, and suspension was gently shaken to disperse tissue fragments. Collagen fibers were removed by filtration, and tissue suspension was gently centrifuged (18 x g for 5 min). This washing procedure was repeated three times. Thereafter, pellet was resuspended in radio-immunoprecipitation assay (RIPA) buffer (mM) consisting of 40 Tris–HCl, 150 NaCl, 2 EDTA, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.2% SDS, pH 7.6. Samples were incubated for 30 min at 4°C. Thereafter, samples were sonicated three times for 30 s each at low intensity in an ultrasonic processor (Vibra cell, Sonics & Materials Inc., Danbury, CT, USA). After that, samples were centrifuged at 14,000 x g, at 4°C, for 40 min and supernatants were collected. Total protein quantification was performed using the Micro BCA Protein Assay Reagent Kit.

Western blot of KIM-1, claudin-2, and Nrf2

Western blot analysis was performed as previously described [43]. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Uppsala, Sweden). Nonspecific protein binding was blocked by incubation with 5% non-fat dry milk in PBS 1x containing 0.4% Tween 20, for 1 h, at room temperature. Membranes were incubated overnight at 4°C with the appropriate primary antibodies against KIM-1 (1:500), claudin-2 (1:500), Nrf2 (1:500), and GAPDH (1:1,000). Thereafter, membranes were incubated with peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat antibodies (dilution 1:10,000) for 1 h. After washing, immunoblots were developed using the ECL™ prime Western blotting detection reagent (Amersham™, GE Healthcare, Buckinghamshire, UK). Chemiluminescence was detected in an EC3 Imaging System (UVP Bioimaging Systems, Cambridge, UK). Protein band density was quantified by transmittance densitometry (UVP BioImaging Systems software, Cambridge, UK).

Studies in LLC-PK1 cells

Cell culture

LLC-PK1 cells were grown in DMEM (high glucose concentration) supplemented with 10% FBS plus penicillin/streptomycin (100 units/ml penicillin, 0.1 mg/ml streptomycin), under a humidified atmosphere of 5% CO₂ at 37°C. When cells were confluent, they were trypsinized with 1 ml of Tryple Express for 10 min at 37°C. One ml of DMEM was added to trypsinized cells and counted by trypan blue staining using a hemocytometer. For viability assays the cells were seeded at a density 1 x 10³ cells per well on 24-well polystyrene plates.

Culture treatments and cell viability measurement

To establish the concentration of maleate to be used in LLC-PK1 cells, a concentration-response curve with maleate (10–50 mM) was performed and the cell viability was measured (Supplementary Figure 1 available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954109). Based on this curve, the concentration of 40 mM of maleate was chosen for further studies. LLC-PK1 cells were incubated with 10 μM curcumin for 24 h in serum free media and then they were incubated by another 24 h in presence of 40 mM maleate. The concentration of 10 μM of curcumin was chosen taking into account preliminary experiments in
LLC-PK1 cells (data not shown). After treatment with maleate, cell viability was evaluated by the FDA assay [5]. Cells were incubated with 5 μM FDA probe for 5 min at 37°C. Fluorescence (excitation 495 nm and emission 520 nm) was quantified in a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA).

Determination of ROS production in LLC-PK1 cells

The fluorescent marker DHE was used to assess ROS production according to Guerrero-Beltrán et al. [44]. DHE enters the cells and is oxidized to ethidium (Et) in the cytosol mainly by superoxide anion (O$_2^-$), then it is retained within the cell nucleus because of its interaction with DNA, staining the nucleus with bright red fluorescence. After treatment, 20 μM DHE was loaded in DMEM without phenol red during 30 min at 37°C. Cells were visualized under epifluorescence microscope using the fluorescent cube G-2A (excitation 510–560 nm, emission of 590 nm) from Nikon Corporation (Tokyo, Japan) for the Et detection. The intensity of Et was measured in five different fields per well per condition in three independent experiments using the AxioVision AC 4.4 image analyzer (Carl Zeiss Imaging Systems).

Studies in isolated mitochondria

Isolation of renal mitochondria

Additional groups of rats (n = 8 per group) were used to evaluate mitochondrial function. Kidneys were removed from rats, washed, cleaned of fatty and conjunctive tissue and placed in cold isolation buffer containing 220 mM sucrose, 10 mM HEPES and 1 mM EGTA, pH 7.3. Kidneys were minced in isolation buffer before being homogenized. Mitochondria were obtained by differential centrifugation and the protein content was measured [20].

Mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured using a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Spring, OH, USA). The experiments were carried out in 1.7 ml of basic medium containing 125 mM KCl, 10 mM HEPES and 3 mM inorganic phosphate (Pi), pH 7.3. State 4 respiration was evaluated in the presence of 10 mM succinate plus 1 μg/mL rotenone or with 10 mM sodium glutamate and 10 mM sodium malate. State 3 respiration was stimulated by the addition of 300 μM ADP. Respiratory control index (RCI) was calculated as the ratio state 3/state 4. Uncoupled respiration was measured by adding 1 μM CCCP [45]. Respiratory rates are expressed as ng atoms oxygen/min/mg protein (ngAO/min/mg). Supplementary Figure 2 available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954109 shows representative tracings of mitochondrial oxygen consumption using malate/glutamate as substrates. The trace of the curcumin group was similar to control group and was omitted for clarity.

Activities of respiratory complex I and aconitase in isolated mitochondria

Respiratory complex I activity was measured by monitoring the decrease in absorbance due to oxidation of NADH to NAD$^+$ at 340 nm in an assay mixture containing 60 μM decylubiquinone, 0.1 μg antimycin A, 1 mM KCN, 100 μM NADH, and 0.5 mg of mitochondrial protein [32]. Aconitase activity was evaluated by measuring the intermediate product, cis-aconitrate, resulting from the interconversion of L-citrate and isocitrate at 240 nm [46].

Statistical analyses

All the values are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Bonferroni analysis were used to compare the in vivo data and the Student t test was used to compare the results of the histological analysis using the software Prism 5 (GraphPad, San Diego, CA, USA). P < 0.05 was considered significant.

Results

In vivo studies

Curcumin prevents renal hemodynamic alterations induced by maleate

MAP was not modified by maleate treatment; this parameter was found to be in normal levels in all groups (Control: 110 ± 2; Maleate: 120 ± 4; Maleate + Cur: 109 ± 3 and Curcumin: 112 ± 3, p = not significant). Maleate treatment induced a significant decrement of RBF (−40%, Figure 1A) due to a significant increment in RVR (+40%, Figure 1B). Curcumin oral administration prevented these hemodynamic changes in maleate-treated animals, while curcumin alone did not modify these parameters compared to control. On the other hand, GFR values were similar among the groups (Figure 1C); this apparent paradox in Maleate group may be explained by the increased proportion of plasma reaching renal tubules in these animals (FF, Figure 1D).

Curcumin prevents maleate induced-acute renal injury

The maleate treatment induced acute renal injury was shown by defect in the mechanism related with urinary concentration, as was evidenced by the significant increase in urinary osmolality (Figure 2A), proteinuria (Figure 2B), glycosuria (Figure 2C), enhanced urinary excretion of NAG (Figure 2D), NGAL (Figure 2E), and sodium (Figure 2F), enhanced expression of KIM-1 (Figure 2G and H) and decreased expression of claudin-2 (Figure 2I and J). Curcumin administration prevented all the above-mentioned alterations in the Maleate + Cur group (Figure 2). In addition, maleate treatment increased 5-fold the expression...
Curcumin prevents maleate-induced nephropathy

of the marker of renal damage KIM-1 (Figure 2). This alteration was significantly prevented by curcumin treatment in (Figure 2).

Curcumin prevents maleate-induced renal structural damage

Figure 3 shows representative micrographs of section of renal cortex stained with H&E for Control (Figure 3A), Maleate (Figure 3B), Maleate + Cur (Figure 3C), and Curcumin (Figure 3D) groups as well as the quantitative analysis (Figure 3E) of the structural damage. Curcumin decreased maleate-induced necrosis of proximal tubular epithelial area from 20 to 5.6% in the pre-treated group (Figure 3C and E). In contrast, Control (Figure 3A) and Curcumin (Figure 3D) groups showed no histological abnormalities.

Curcumin prevents maleate-induced apoptosis in renal tubules and glomeruli

Numerous TUNEL-positive tubular and glomerular cells in kidneys of rats injected with maleate are shown as brown nuclei in Figure 4B. This apoptotic feature was significantly decreased in the Maleate + Cur group (Figure 4C); automated morphometry revealed that the percentage of TUNEL-positive nuclei in this group was reduced to less than a half compared to the Maleate group ($P < 0.05$) and was similar to that found in the Control group (Figure 4E). Control (Figure 4A) and Curcumin (Figure 4D) groups showed just a few stained nuclei indicating basal apoptosis. Quantification of TUNEL-positive nuclei (%) of the four group, studied is shown in Figure 4E.

Curcumin prevents maleate-induced oxidative stress and decrease in Nrf2

To explore the effect of curcumin treatment on maleate-induced oxidative stress, lipid peroxidation (evaluated by MDA and 4-HNE levels, Figure 5A and B), protein oxidation (Figure 5C) and renal Nrf2 levels (Figure 5D and E) were measured. As shown in Figure 5, maleate treatment increased MDA, 4-HNE, and oxidized protein levels and decreased renal Nrf2 levels. Curcumin treatment significantly prevented these changes (Figure 5), suggesting that nephroprotection exerted by curcumin is associated to its antioxidant capacity.

In vitro studies

Curcumin prevented maleate-induced cell damage and ROS production in LLC-PK1 cells

LLC-PK1 cells have been used as a model to evaluate in vitro the toxicity of many nephrotoxic agents [39,44, 47]. These cells are of proximal tubular origin, the main target of maleate as has been described in in vitro [8,12]
and \textit{in vivo} [9,12,17] experiments. Maleate induced cell damage that was significative at 40 and 50 mM (Supplementary Figure 1 available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954109). Furthermore, when 10 \( \mu \)M curcumin was incubated 24 h before the addition of 40 mM maleate, cell damage (Figure 6A and B) and ROS production (Figure 6C and D) were prevented. The viability of the curcumin group was unaltered. In agreement with the \textit{in vivo} results described above, these findings suggest that the antioxidant properties of curcumin are involved in the prevention proximal tubular cell toxicity \textit{in vitro} and \textit{in vivo}.

Figure 2. Curcumin (Cur) prevents maleate-induced changes in (A) urinary osmolality, (B) proteinuria, (C) urinary glucose, (D) urinary N-acetyl-\( \beta \)-D-glucosaminidase (NAG), (E) urinary neutrophil gelatinase-associated lipocalin (NGAL) measured by Western blot (AU = arbitrary units, upper panel shows representative Western blot: C = control, M = maleate, M + C = maleate + curcumin, Cu = curcumin and lower panel shows the densitometric analysis), (F) urinary sodium excretion (UNaV) and renal expression of (G,H) kidney injury molecule (KIM)-1, and (I,J) claudin-2. (G) Representative Western blot and (H) densitometric analysis of KIM-1, (I) Representative Western blot and (J) densitometric analysis of claudin-2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Values are mean \( \pm \) SEM, \( n = 7–8 \) (panels A-D, F), \( n = 3 \) (panel E) and \( n = 4 \) (panels H and J). \( ^{a}p < 0.05 \) vs. Control; \( ^{b}p < 0.05 \) vs. Maleate.
Curcumin prevents maleate-induced nephropathy

Studies in isolated mitochondria

Curcumin preserves ADP-stimulated oxygen consumption in kidney mitochondria from maleate-treated rats

Basal oxygen consumption (state 4, Figure 7A) was similar in all the studied groups, whereas significant diminution in ADP-stimulated respiration (state 3, Figure 7B) was observed in mitochondria from maleate-treated rats. Curcumin improved oxygen consumption coupled to ATP synthesis, increased respiratory control index (Figure 7C), and restored uncoupled respiration (Figure 7D) and ADP/O ratio (Figure 7E) when using NADH-linked substrates. These changes were not observed with succinate (Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954109) suggesting damage to the respiratory complex I.

Curcumin prevents maleate-induced damage to respiratory complex I and aconitase

The activity of both respiratory complex I (NADH dehydrogenase) (Figure 7F) and aconitase (Figure 7G) was reduced in maleate-treated rats. Curcumin pretreatment effectively counteracted maleate-induced diminution in complex I activity (Figure 7F) and partially restored aconitase activity (Figure 7G).

Discussion

Curcumin showed protective effect against maleate-induced in vivo nephropathy (Figures 1 and 2), maintaining mitochondrial respiration (Figure 7) and preventing damage in LLC-PK1 cells (Figure 6). Tubular proteinuria is a hallmark of maleate-induced nephropathy [12,14,16,18,48] and originates from the inability of injured tubules to reabsorb and degrade the proteins filtered into the urinary space [49,50]. Glycosuria [15,51] and enhanced urinary excretion of NAG [18] and NGAL [10] have been previously found in maleate-injected animals. In fact, urinary excretion of NAG and NGAL has been widely used as a marker of renal injury [52,53]. In addition to these markers, KIM-1 expression, a sensitive marker of renal tubular damage that is overexpressed when proximal tubule is injured [54,55], was also increased in maleate-injected rats (Figure 2). The expression of claudin-2 was decreased,
and these levels correlated inversely with increased UNaV (Figure 2) in maleate-injected rats, suggesting that the loss of claudin-2 in proximal tubules might be associated to increased UNaV. In fact, it has been described that claudin-2 functions as a paracellular pore of sodium and water [34,35]. To our knowledge, this is the first report of the decreased levels of claudin-2 in kidney of maleate-injected rats. Renal injury induced by maleate was confirmed also by the alterations in renal hemodynamic (decrease in RBF and increase in RVR) (Figure 1) and by the significative increment of necrosis and apoptosis in renal tissue (Figures 3 and 4). Our data are consistent with previous findings in the literature in which it has been shown that the histological damage in maleate-treated animals is characterized by tubular epithelial cell injury including severe tubular dilatation, epithelial cell necrosis, and epithelial cell sloughing [12]. To our knowledge, this is the first study showing the decrease in RBF and the increase in RVR in maleate-induced nephropathy. The significative increment in RVR observed in maleate-treated animals was likely due to the excessive production of free radicals, since it has been reported that oxidative stress induces renal vasoconstriction [56]. On the other hand, in lipopolysaccharide (LPS)-induced acute renal damage, an acute increase of RVR and decreased RBF was also reported [57]. In those studies, hemodynamic alterations also included a significant fall in GFR [57]. In maleate-treated rats, renal hemodynamic changes were not associated with changes in GFR (Figure 1). This apparent discrepancy may be explained by the increased FF in our study, indicating that vasoconstriction predominantly affected postglomerular arteries preserving GFR in normal levels. In this regard, it is well known that agents that decrease RBF secondary to increased vascular resistance, such as angiotensin II, are associated with a greater proportion of plasma reaching tubules, therefore increasing FF [58]. Curcumin was able to prevent all the above-described biochemical, hemodynamic, and histological alterations (Figures 1–4). Interestingly, this protective effect was associated to the prevention of maleate-induced oxidative stress (Figure 5).

Figure 5. Curcumin (Cur) prevents maleate-induced (A–C) oxidative stress and decrease in (D, E) renal levels of Nrf2. (A) malondialdehyde (MDA), (B) 4-hydroxy-nonenal (4HNE), (C) oxidized protein and (D) representative Western blot and (E) densitometric analysis of renal Nrf2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Values are mean ± SEM, n = 7–8 (panels A–C) and n = 4 (panel E). *p < 0.05 vs. Control; †p < 0.05 vs. Maleate.
Curcumin prevents maleate-induced nephropathy

Curcumin prevents maleate-induced nephropathy and decrease of renal Nrf2 protein levels (Figure 5). To our knowledge, this is the first report of the renal Nrf2 levels in maleate-treated rats. Our data are consistent with other models of renal damage in which the decreased Nrf2 renal levels are associated to oxidative stress including those induced by cisplatin [59], 5/6 nephrectomy [60], aristolochic acid [61], daunorubicin [62], and adenine [63]. Increased oxidative stress (associated to decreased renal Nrf2 levels) induced by maleate treatment might be associated with decreased claudin-2 expression in proximal tubule. In fact, it has been reported that diabetes-induced renal stress increases claudin-2 nitration [64], which may explain that the loss of this protein is associated with increased natriuresis. The renoprotection by curcumin in other models of renal damage also has been associated with attenuation of oxidative stress [20,21,24–26] suggesting that the antioxidant properties are involved in the protection against maleate-induced renal damage. Moreover, our data are consistent with the findings of other experimental models of renal damage including those induced by gentamicin [25], 5/6 nephrectomy [20,21], potassium dichromate [24], and cisplatin [26,27], in which curcumin has been able to prevent proteinuria [20,21,24], urinary excretion of NAG [24], histological damage [20,21,24], and hemodynamic alterations [20,21].

The protective effect of curcumin against maleate-induced renal damage in rats was confirmed in cell culture studies. Curcumin effectively prevented maleate-induced damage to LLC-PK1 cells, a renal epithelial cell line from proximal tubule, which was associated to the prevention in ROS production (Figure 6). LLC-PK1 cells have been used to evaluate the damage of several nephrotoxic compounds including cisplatin [44], gentamicin [47], and potassium dichromate [48]. These data suggest that redox unbalance is involved in the maleate-induced damage both in vivo and in cell culture. In both cases, the protective effect of curcumin was associated with the prevention of the redox imbalance suggesting that the antioxidant properties of curcumin were key for the cytoprotective effect.

To our knowledge, this is the first study evaluating mitochondrial respiratory function from maleate-treated animals. This is relevant, as previous histological findings have revealed abnormal-appearing mitochondria in maleate-treated animals [17,18]. In fact, mitochondrial parameters including decrease in ADP-stimulated respiration using malate/glutamate, in RCR, in respiratory complex I, and in aconitase activity (Figures 7 and Supplementary Figure 2 available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954109) were evident in maleate-treated rats. The decrease in respiratory complex I activity or NADH:ubiquinone oxidoreductase may be secondary to the maleate-induced oxidative stress; in fact, it has been shown that this large enzyme complex embedded in the inner mitochondrial membrane [65] is very sensitive to the ROS action [66]. In addition, the decreased activity of mitochondrial complex I alters the normal electron flow through the respiratory chain and, also, it is known that the damage to the mitochondrial respiratory chain is a main source of ROS [67]. In fact, it is known that O$_2^•^−$ production is enhanced during the inhibition of mitochondrial complex I [68].

Figure 6. Curcumin (Cur) prevents maleate-induced (A,B) cell toxicity and (C,D) reactive oxygen species (ROS) production in LLC-PK1 cells. LLC-PK1 cells were pretreated for 24 h in absence (control) or presence of 10 μM curcumin and then incubated without or with 40 mM maleate for 24 h to assess cell viability by the fluorescein diacetate (FDA) assay and ROS production using DHE. Representative micrographs are shown in A and C for the viability and ROS production assays, respectively, and the quantification of these assays are shown in B and D, respectively. Values are mean ± SEM, n = 3. *p < 0.05 vs. Control; b*p < 0.05 vs. Maleate.
Decreased ADP-stimulated oxygen consumption may be secondary, in all probability, to the decreased activity of respiratory complex I. Using succinate (an electron donor of complex II) as substrate, ADP-stimulated oxygen consumption was unchanged (Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10715762.2014.954109) suggesting that the activity of respiratory complexes II, III, and IV was unaltered by maleate treatment. Curcumin was able to prevent the maleate-induced decrease in the activity of complex I (Figure 7) that is consistent with previous findings about the protective effect of curcumin on the activity of respiratory complex I in both kidney [24] and liver [32] of rats treated with potassium dichromate. Oxidative stress in isolated mitochondria was evident also by the decrease in the activity of aconitase (Figure 7), an enzyme that losses its iron sulfur center in presence of O$_2^•−$ [69]. The protective effect of the activities of respiratory complex I and of aconitase by curcumin agrees with a direct antioxidant effect of this polyphenol. Consistently, curcumin maintained cardiac mitochondrial function in a model of chronic renal failure by means of its antioxidant effects [33].

It is concluded that curcumin is able to attenuate in vivo maleate-induced nephropathy and in vitro cell toxicity. The in vivo protection was associated to the prevention of oxidative stress and preservation of mitochondrial function, and the in vitro protection was associated to the prevention of ROS production.

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Declarations of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online
Supplementary Table I and Figures 1–2.