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Biochemical Pharmacology 63 (2002) 1051–1060

Biochemical Pharmacology

Cytoprotective role of taurine in a renal epithelial cell culture model

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Received 20 April 2001; accepted 2 August 2001

Abstract

Taurine (TAU) is a sulfur-containing amino acid that has been shown to decrease during aging and is believed to be important for cytoprotection. A decrease in TAU could exacerbate the accumulation of free radical-induced damage that may lead to cell death during the aging process. We have shown previously that TAU directly inhibits dopamine (DA) and (−)-3-(3,4-dihydroxyphenyl)-L-alanine (L-dopa) oxidation. Experiments were conducted to establish a cytoprotective role for TAU. Porcine renal epithelial cells were treated for 1 hr with iron and catecholamines (L-dopa and DA) to produce cytotoxicity by a free radical and quinone mechanism in the absence and presence of 10 or 20 mM TAU. Viability assays, protein, and DNA measurements were performed after a 24 hr recovery period. In some experiments, cells were extracted immediately after the insult for DA and TAU content measurements using high performance liquid chromatography with electrochemical detection. Catecholamine-induced cytotoxicity caused a 50% loss in cell viability, and 10 or 20 mM TAU provided significant protection from cytotoxicity and maintained the functional integrity of the cells. Photomicrographs showed attenuation in cell loss and swelling in the presence of TAU. Pretreatment with 1 mM TAU followed by exposure to iron and L-dopa in the presence of 1 mM TAU caused a moderate but non-significant increase in cell survival. These data conclusively show that TAU can play a cytoprotective role in the LLC-PK_1 cell culture model.

Keywords: Taurine; Cytotoxicity; Renal cell culture model; Antioxidant; Cytoprotection; Catecholamine oxidation

1. Introduction

The free radical theory of aging states that oxidative stress and damage play a significant role in the aging process [1]. TAU has been suggested by many studies to be an antioxidant [2–9], and an age-dependent decline in TAU content [10–15] could exacerbate the free radical-mediated cell death occurring during aging. The function of the liver and the kidney declines during aging [16,17], and these organs are responsible for TAU homeostasis [18,19]. We have found previously that the TAU content in the tissues of these organs is decreased significantly in aged male Fischer 344 rats [13,14]. The observed decline in TAU concentration in the kidney may contribute to the decline in renal function during aging.

TAU has been shown to play a protective role in different renal diseases [20–22]. For example, Trachtman *et al.* [21,22] reported a protective effect of TAU in chronic puromycin aminonucleoside nephropathy and streptozocin-induced diabetic nephropathy in male Sprague–Dawley rats. Beneficial effects of TAU have been shown in the preservation of rat kidneys after ischemia [23]. TAU also has been demonstrated to have a protective effect in a renal cell culture model, LLC-PK_1, against damage induced by hypoxia and reoxygenation [24] and to improve the viability of LLC-PK_1 cells after long-lasting hypoxia [25]. Based on these studies it has been suggested that the kidney preservation solution (University of Wisconsin solution) used to preserve organs before transplants should be supplemented with TAU [25]. These observations strongly point to a cytoprotective role for TAU in the kidney.

Cell and tissue damage can be induced by quinones and reactive oxygen species (ROS) produced from catecholamine oxidation *in vivo* [26]. The autoxidation or metal-induced oxidation of catecholamines generates cytotoxic quinones as well as hydroxy- and oxyradicals during

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Abbreviations: AADC, aromatic L-amino acid decarboxylase; DA, dopamine; L-Dopa, (−)-3-(3,4-dihydroxyphenyl)-L-alanine; HPLC-ECD, high performance liquid chromatography with electrochemical detection; LDH, lactate dehydrogenase; LYS, lysine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OPA, *o*-phthalaldehyde; ROS, reactive oxygen species; TAU, taurine.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)00843-2

redox-cycling [26–28]. Hydroxy radicals can then undergo further ROS-generating reactions [29]. These ROS can disrupt cell function by covalently modifying proteins, causing oxidative stress, lipid peroxidation, DNA strand breaks, and interfering with mitochondrial respiration [30,31]. It has also been suggested that catecholestrogens formed in the kidney may induce tumorigenesis by generating mutagenic free radicals during redox-cycling [32]. Some studies suggest that amino acids, including TAU, may bind these cytotoxic quinones [26,33] forming less reactive conjugates that may decrease oxidative stress. It has been shown previously that oxidative stress is elevated in LLC-PK_1 cells incubated with L-dopa, as indicated by an elevation in GSH content [34]. Other studies have shown that L-dopa and DA have a cytotoxic effect in mesencephalic cell culture [35,36]. DA is present in high concentrations in the kidney and urine [37,38]. Thus, free radicals generated from DA oxidation could cause renal and other tissue injury if antioxidant defense systems are compromised.

To test the cytoprotective capabilities of TAU, LLC-PK_1, was chosen. L-Dopa is taken up by LLC-PK_1 cells *via* a Na^+-dependent transporter; DA is synthesized from L-dopa *via* aromatic L-amino acid decarboxylase (AADC) (EC 4.1.1.28) and released [39]. This dopaminergic efflux is stimulated by Na^+ and/or Cl^- and is Ca^+-independent [40]. LLC-PK_1 cells also have DA receptors [41]. Furthermore, the kidney normally produces large amounts of DA, which functions as an important paracrine system in sodium regulation [39]. The characteristics of this renal cell line make it a good model to test catecholamine-induced cell injury and death. We have shown previously that TAU inhibits iron-stimulated catecholamine oxidation in a cell-free assay [42], and in the current study we tested whether this inhibition translates into cytoprotection in the LLC-PK_1 cell culture model. We hypothesized that TAU acts as a cytoprotectant in cell culture and could attenuate cell death and functional loss caused by iron-induced catecholamine oxidation.

2. Materials and methods

2.1. Materials

Drugs, buffer reagents, and cell culture supplies were obtained from Sigma, Fisher, or GIBCO. LLC-PK_1 cells were obtained from the American Type Culture Collection (ATCC).

2.2. L-Dopa oxidation

L-Dopa oxidation generates aminochromes that absorb light at 490 and 550 nm [27]. Equal parts (1 mL) of L-dopa, $FeCl_3$, and TAU or PBS (pH = 7.4) were combined resulting in a final volume of 3 mL that was used to measure the rate of L-dopa oxidation in a cell-free model. All solutions were made up in PBS and the following final concentrations were used: 250 μM L-dopa, 75 μM $FeCl_3$, and 10 or 20 mM TAU. The samples were read in a cuvet at 490 nm by a Beckman DU 700 diode array spectrophotometer (Beckman Instruments Inc.) at a temperature of 37 or 23° every 20 s for 5 min. The reaction rate was then calculated by linear regression. Spontaneous L-dopa oxidation in the absence of $FeCl_3$ was measured in all experiments and was found to be negligible when compared with the stimulated oxidation; therefore, these data were omitted from the results reported.

2.3. Cell culture experiments

LLC-PK_1 is a porcine-derived renal epithelial cell line with characteristics of proximal tubular cells. Cells were received at passage 199 from the ATCC and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/mL of penicillin, 100 mg/mL of streptomycin, 44 mM $NaHCO_3$, and 10% fetal bovine serum in an incubator at 37°, 95% O_2/5% CO_2 (NuAire, model NU 4500, US Autoflow, or Napco, E series model 5100). The experiments were conducted between passages 205 and 225. Cells were plated in 24- or 96-well plates (1 mL or 200 μL of a 0.096 ± 0.004 mg protein/mL cell suspension). The plates were incubated overnight (37°; 95% O_2/5% CO_2) to allow for proper attachment.

Pilot studies were conducted using 50–500 μM $FeCl_3$, 250–750 μM L-dopa, and 200–300 μM DA to establish concentrations that induced about 50% cell death. The final concentrations used were 75 μM $FeCl_3$/250 μM L-dopa and 50 μM $FeCl_3$/300 μM DA. All solutions were made up in sterile physiological saline (0.9% NaCl) supplemented with 2.5 mM $CaCl_2$ to reduce cell detachment during the treatment period. Cells were rinsed with 500 μL saline before and after the treatments, and the incubation time was 60 min. The final concentrations of TAU or lysine (LYS) are indicated in the figures discussed in Section 3.

In some $FeCl_3$/L-dopa experiments, cells were pretreated with 1 or 10 mM TAU for 30 or 60 min. TAU solutions for the pretreatment were made up in DMEM. The medium was removed, the plates were rinsed with saline, and TAU-supplemented medium was added. Following the preincubation, the plates were again rinsed before treatment. Cells were treated as described above for the treatment period ($FeCl_3$ and L-dopa in the presence or absence of TAU). The TAU concentrations for pretreatment and treatment were the same in these experiments.

Other experiments were used to assess the acute effect of L-dopa/$FeCl_3$ cytotoxic treatment on the cells. In these experiments, the effect of oxidative stress was measured immediately after treatment vs. after a 24 hr recovery period as described in the above experiments. We measured TAU and L-dopa uptake into cells and used these functional parameters to indirectly assess acute cell

damage, since necrotic as well as apoptotic cell death does not occur for several hours after exposure to the insult. Cells were treated as described above except that the control wells received 250 μM L-dopa and cells were extracted immediately after the 60 min treatment with 0.1 M perchloric acid and 10% methanol. The perchloric acid extracts were transferred to autosampler vials, and the DA content was measured using high performance liquid chromatography with electrochemical detection (HPLC-ECD). These same samples were used to measure TAU content using HPLC-ECD after *o*-phthalaldehyde (OPA) derivatization as described below.

2.4. Cell viability assays

Cell viability/cytotoxicity was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (a tetrazolium dye) assay [43]. Following a 24 hr recovery period after the treatment, 0.25 (24-well plate) or 0.05 mL (96-well plate) of a 4 mg/mL MTT solution was added to each well and incubated for 2.5 or 4 hr. MTT is a yellow dye that is taken up by viable cells and reduced to an insoluble purple-colored formazan salt by an enzyme in the endoplasmic reticulum, cytosol, and mitochondria; the amount of chromophore produced is proportional to cell survival [44]. The liquid in the well was aspirated, and the purple salt crystals were solubilized by adding 1 mL (24-well plate) or 200 μL (96-well plate) of 0.08 M hydrochloric acid in isopropanol and incubating it at room temperature for 10 min. If the experiments were conducted in a 24-well plate, 200 μL were transferred into a 96-well plate, and the absorbance was read at 550 nm using an SLT 400AC plate reader (SLT Labinstruments) and Delta Soft II software for Macintosh (Bio Metallics Inc.).

Protein concentrations were measured by the method of Bradford [45]. DNA concentrations were measured using the Hoechst dye 33248 assay [46]. Fluorescence emission at 450 nm of this dye increases by interacting with double-stranded DNA. Cells were incubated overnight in 0.75 mL of high salt buffer (0.05 M sodium phosphate, 2.0 M NaCl, 2 mM EDTA, pH 7.4). The next day the samples were sonicated, and 0.1 mL was diluted with 0.9 mL of 0.1 μg/mL dye solution. Samples were read with an excitation wavelength of 350 nm and an emission wavelength of 460 nm using an LS50B luminescence spectrometer (Perkin-Elmer). Concentrations were calculated using calf thymus DNA standards. Lactate dehydrogenase (LDH) was measured using a kit from Sigma Diagnostics and following the instructions of the manufacturer. Photomicrographs were taken after incubating cells for 30 min with probes for cell viability. The probes were ethidium homodimer (red, 5 μM), which stains the nuclei of dead cells, and calcein AM (green, 5 μM), which stains live cells, both obtained from Molecular Probes. Photographs were taken at a magnification of 100× using a Nixon Diaphot 300 fluorescence microscope (Southern Micro Instruments), a Nikon 6000 camera, and Kodak Royal Gold film, 1000 ASA.

2.5. Chromatography

TAU content was assayed as described previously [13]. The mobile phase for measuring TAU consisted of 0.08 M sodium phosphate (monobasic), 0.1 mM Na_2EDTA, 10% tetrahydrofuran, 10% acetonitrile, and 2% 1-propanol, pH 5. The mobile phase was vacuum filtered using Magna-R, supported nylon filters (0.45 μm, MSI) and degassed for 20 min under helium. The HPLC consisted of a PM-11 pump, an LC-4B amperometric detector (both from Bioanalytical Systems), and a Rheodyne model 7125 injector with a 20 or 50 μL fixed loop. The columns used were Microsorb-MV, C-18, 3 μm, 4.6 mm i.d. × 10 cm columns from Rainin. Ag/AgCl reference electrodes and glassy carbon working electrodes with an applied voltage of 0.725 V were used for electrochemical detection. The detector sensitivity was between 5 and 50 nA, the flow rate was 0.7 mL/min, and the columns were at room temperature. Samples were derivatized before HPLC analysis by reacting 200 μL of the sample with 175 μL of 9 mM OPA, 0.5% 2-mercaptoethanol in a 0.1 M disodium borate solution (pH 10.0) for exactly 3 min. The derivatization reaction was terminated by adding 25 μL of 0.4 M iodoacetamide and 400 μL mobile phase. Iodoacetamide reacts with unreacted OPA, which diminishes electrochemical interference. Sample TAU concentrations were calculated based on external standards by a Hewlett-Packard integrator.

DA content was measured as previously described [47] using the following mobile phase: 12.5 mM citric acid, 7.5 mM sodium phosphate (monobasic), 0.2 mM Na_2EDTA, 0.3 mM octyl sodium sulfate, and 7% acetonitrile, pH 3.9. The mobile phase was vacuum filtered using Magna-R supported nylon filters (0.45 μm, MSI) and degassed for 20 min under helium. The HPLC consisted of a Millipore Waters 501 pump, an LC-4C amperometric detector, an LC-22A temperature controller (both from Bioanalytical Systems), an SSI model 210 pulse dampner (Millipore, Rainin), and a Beckman 506 autosampler with a 100 μL loop and variable injection volume. The columns used were 5 μm Biophase ODS (BAS) or Ultrasphere ODS (Beckman), 4.6 mm i.d. × 25 cm length. Ag/AgCl reference electrodes and glassy carbon working electrodes with an applied voltage of 0.65 V were used for electrochemical detection. The detector sensitivity was 5 nA, and the column was kept at 27°. Sample DA concentrations were calculated based upon an external standard, using an HP 3396, series II integrator (Hewlett-Packard).

2.6. Statistics

Data were analyzed using ANOVA with the Tukey–Kramer multiple comparison test to compare differences among groups. If Bartlett's test for homogeneity of variance

was significant, data were either transformed (log or reciprocal) or a Kruskal–Wallis non-parametric ANOVA test was used followed by the Dunn's multiple comparisons test. Since much of the data in these experiments were analyzed as percentages of the control experiments, data were analyzed for normality of distribution. If data were not distributed normally, non-parametric analyses were used. In the cell culture experiments an $N = 1$ corresponds to the average of data from 4 or 8 wells, depending on whether the experiment was conducted in a 24- or 96-well plate, and represents an independent experimental replication.

3. Results

3.1. L-Dopa oxidation

$FeCl_3$-induced L-dopa oxidation was increased by about 2-fold at 37° compared with 23° (Fig. 1). TAU decreased the iron-induced L-dopa oxidation at 23° by 16 and 27% when present at a concentration of 10 and 20 mM, respectively (Fig. 1). The decrease caused by 20 mM TAU was significant compared with PBS ($P < 0.001$) and 10 mM TAU ($P < 0.05$). The lower concentration of TAU (10 mM) did not reduce L-dopa oxidation significantly compared with PBS samples at 23°. At the physiological temperature of 37°, there was a significant decrease in quinone formation in the presence of TAU ($P < 0.001$) at a concentration of 10 (22.6%) and 20 mM (34.2%) (Fig. 1).

3.2. Catecholamine oxidation and TAU in cell culture

Fig. 2A shows the results of the MTT assay, indicating survival of LLC-PK_1 cells exposed to the $FeCl_3$/L-dopa treatment in the presence and absence of TAU and cells exposed to TAU alone. $FeCl_3$/L-dopa treatment significantly ($P < 0.001$) decreased cell viability by about 50%. The presence of TAU (10 and 20 mM) significantly ($P < 0.01$) attenuated this cytotoxic effect by about 20%. Protein and DNA measurements yielded similar results with 39 and 42% decreases after $FeCl_3$/L-dopa treatment, respectively, and about a 15% save with 10 mM TAU and 27% save with 20 mM TAU ($P < 0.01$ vs. $FeCl_3$/L-dopa) (Table 1). These data suggest that cells died and detached during the 24 hr recovery period and were consequently lost during aspiration of the cell medium. MTT, protein, and DNA measurements in cells treated with only 10 and 20 mM TAU were not different from the control (Fig. 2A, Table 1) measurements, suggesting that the TAU-mediated attenuation in cell viability was not due to cell proliferation.

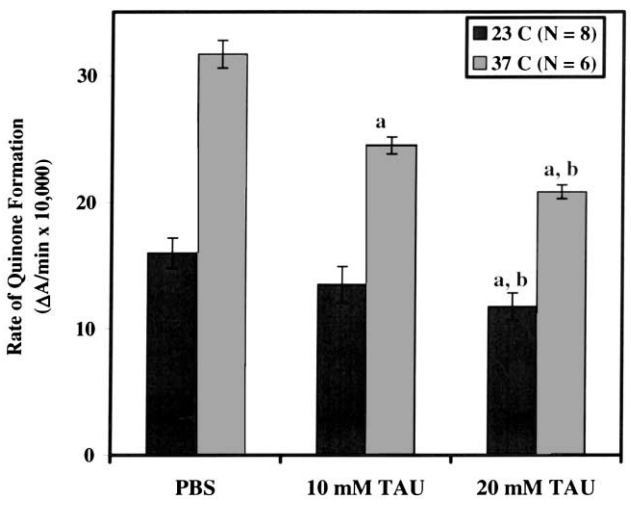

Fig. 1. Iron-induced quinone (75 μM $FeCl_3$, 250 μM L-dopa) formation in a cell-free assay at 23 and 37° in the absence (PBS) or presence of taurine (TAU, 10 and 20 mM). Data are expressed as averages of change in absorbance per min ± SEM. Numbers in parentheses represent the number of replications of independent experiments. Key: (a) $P < 0.001$ vs. PBS, and (b) $P < 0.05$ vs. 10 mM TAU.

Table 1
Protein and DNA contents in LLC-PK_1 cell culture after treatment with 75 μM $FeCl_3$ and 250 μM L-dopa in the presence and absence of 10 or 20 mM TAU[a]

Treatment	Protein (% control)	DNA (% control)
Control (14)	100	100
10 mM TAU (3)	96.2 ± 12.6	112.4 ± 17.7
20 mM TAU (3)	91.9 ± 5.3	92.8 ± 23.5
$FeCl_3$/L-dopa (14)	61.8 ± 2.9*	58.5 ± 3.2*
$FeCl_3$/L-dopa plus 10 mM TAU (10)	75.6 ± 4.5*	75.5 ± 2.7*
$FeCl_3$/L-dopa plus 20 mM TAU (10)	88.3 ± 3.9**	87.4 ± 4.2**

[a] Data are expressed as averages ± SEM of percent control values measured in mg per well (100% = 0.096 ± 0.004 mg per well) or ng per well (100% = 1243 ± 106 mg per well) for protein and DNA, respectively. Numbers in parentheses represent the number of independent experiments.
* $P < 0.05$ vs. control.
** $P < 0.001$ vs. $FeCl_3$/L-dopa.

LDH released from injured cells also supported the MTT data (Fig. 2B). $FeCl_3$/L-dopa treatment significantly ($P < 0.01$) increased enzyme activity in the medium from 80 ± 9 U/L in control to 203 ± 24 U/L. TAU supplementation reduced this activity to 148 ± 25 U/L with 10 mM and to 143 ± 23 U/L with 20 mM. This TAU-dependent attenuation in LDH activity resulted in LDH values that were elevated but not significantly different from control values. Photomicrographs (Fig. 3) further confirmed these findings, showing cell swelling and cell loss with $FeCl_3$/L-dopa treatment, which was reduced with either concentration of TAU.

We attempted to elucidate the mechanism by which TAU protects cells in culture. Another set of experiment was conducted, and an experimental group was added where cells were treated with $FeCl_3$/L-dopa and 20 mM LYS. This amino acid [H_2N–$(CH_2)_4CH(NH_2)CO_2H$] is structurally similar to TAU [H_2N–$(CH_2)_2$–SO_3H] and has been shown previously to scavenge quinones [33]. Fig. 2C shows that LYS has a protective effect similar to that of TAU for $FeCl_3$/L-dopa-induced cell death.

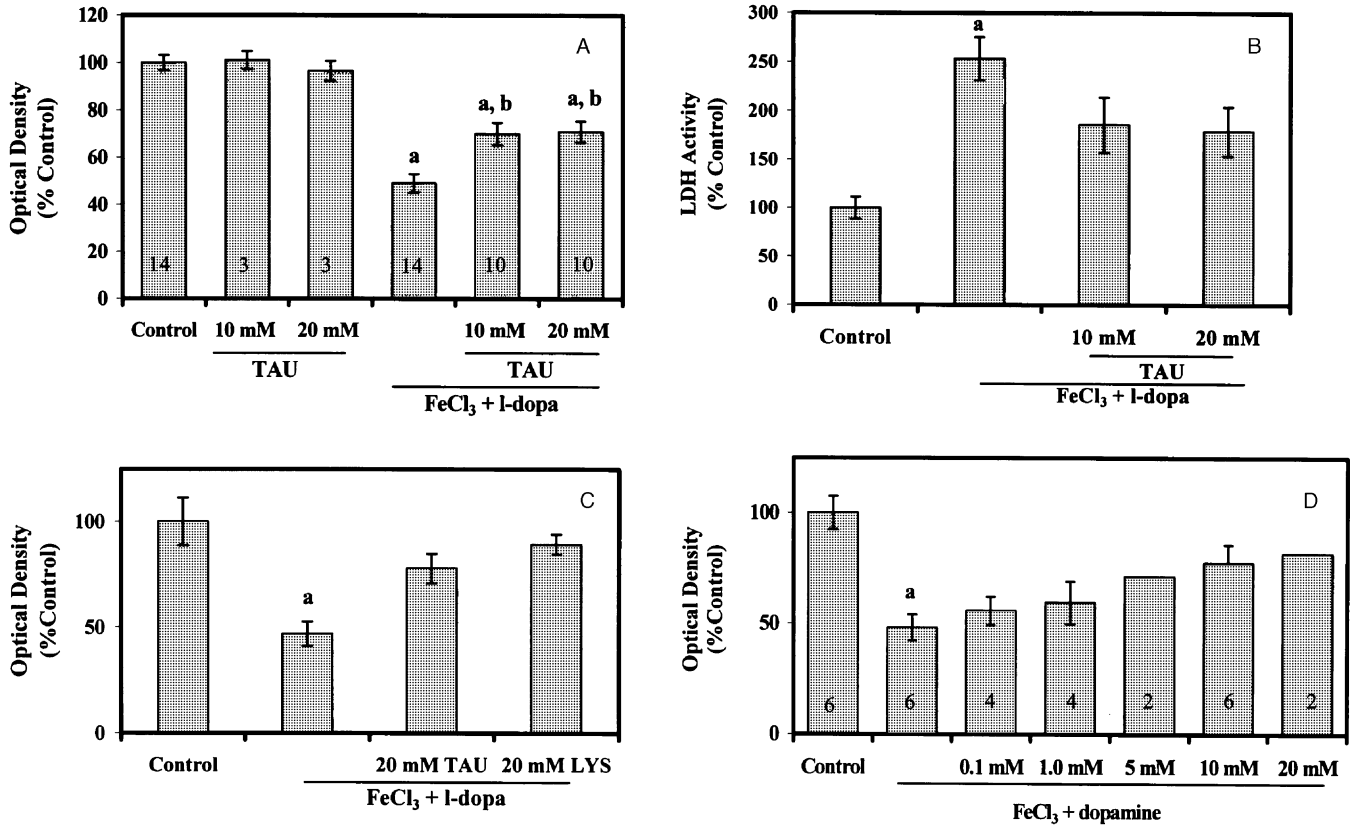

Fig. 2. (A) Viability of LLC-PK$_1$ cell culture after treatment with 75 μM $FeCl_3$ and 250 μM L-dopa in the presence and absence of taurine (TAU, 10 and 20 mM). Data are expressed as averages ± SEM of percent control cell survival measured in optical density of MTT at 550 nm. Numbers displayed in the bars represent the number of replications of independent experiments. Key: (a) $P < 0.001$ vs. control, and (b) $P < 0.01$ vs. $FeCl_3$/L-dopa alone. (B) LDH activity in culture medium from the $FeCl_3$/L-dopa experiments in the presence and absence of TAU (10 and 20 mM). Data are expressed as averages ± SEM of percent control enzyme activity measured in units per litre (U/L). The average value for the control was 80 ± 9 U/L; $N = 4$ per group. Key: (a) $P < 0.01$ vs. control. (C) Viability of LLC-PK$_1$ cells treated with $FeCl_3$/L-dopa in the absence and presence of 20 mM TAU or LYS. Data are expressed as averages ± SEM of percent control cell survival measured in optical density of MTT at 550 nm. $N = 8$ per group, except for LYS where $N = 6$. Key: (a) $P < 0.05$ vs. all other groups. (D) Viability in LLC-PK$_1$ cell culture after treatment with 300 μM DA and 50 μM $FeCl_3$ in the absence or presence of TAU (0.1, 1.0, 5, 10, 20 mM). Data are expressed as averages ± SEM of percent control cell survival measured in optical density of MTT at 550 nm. The values for 5 and 20 mM TAU are expressed as averages without SEM. Numbers displayed in the bars correspond to the number of independent experiments. Key: (a) $P < 0.01$ vs. control.

$FeCl_3$/DA treatment also decreased cell viability by 50%, and TAU showed a protective effect in a concentration-dependent manner (0.1–20 mM TAU) (Fig. 2D). $FeCl_3$/DA treatment in the absence of TAU caused a significantly lower cell survival compared with the control ($P < 0.01$), whereas $FeCl_3$/DA treatment in the presence of any concentration of TAU was not significantly different from the control. TAU alone (5, 10, and 20 mM TAU) did not affect cell viability (data not shown).

3.3. Acute effect of L-dopa oxidation in cell culture

We examined the acute effects of $FeCl_3$/L-dopa treatment by measuring functional parameters immediately after treatment instead of waiting for a 24 hr period. $FeCl_3$/L-dopa treatment significantly ($P < 0.001$) decreased cellular DA content by 65% as measured by HPLC-ECD (Fig. 4A). Simultaneous treatment with 10 and 20 mM TAU attenuated this decrease significantly ($P < 0.05$) to a 37 and 46% decrease, respectively (Fig. 4A).

HPLC-ECD was also used to measure intracellular TAU concentration after the 60 min treatment. Cytotoxic treatment with $FeCl_3$/L-dopa still resulted in significant cellular uptake and retention of TAU (Fig. 4B). TAU uptake by LLC-PK$_1$ cells appeared to be concentration-dependent.

3.4. TAU pretreatment in cell culture

Another set of experiments was conducted to test if preincubation with TAU might improve its protective capacity even at lower concentrations (Fig. 5). Pretreatment with 1 mM TAU did not have a statistically significant effect; however, there was a trend toward increased cell survival (by 31%) with a 1 mM TAU pretreatment for 30 and 60 min compared with no (0 min) pretreatment (Fig. 5). Pretreatment with 10 mM TAU for 30 min increased cell survival by 21%, while the 10 mM TAU pretreatment for 60 min increased survival by 33% vs. no pretreatment. The increase in cell survival due to the

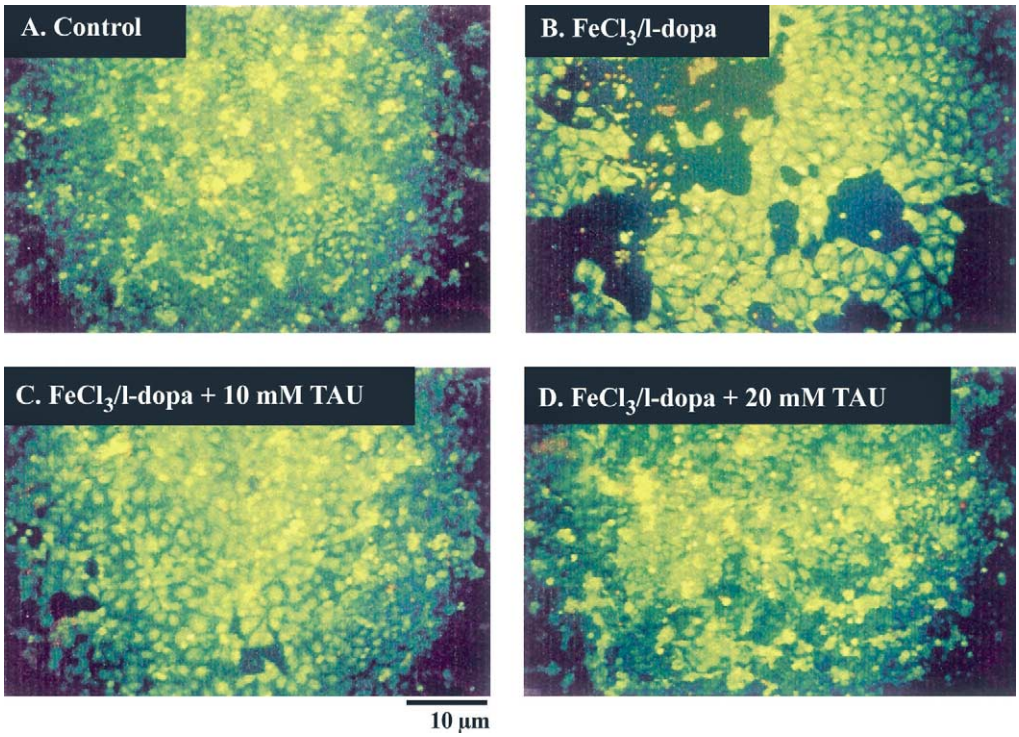

Fig. 3. Photomicrographs of LLC-PK$_1$ cells from (A) control, (B) $FeCl_3$/L-dopa (75/250 μM), (C) $FeCl_3$/L-dopa plus 10 mM taurine (TAU), and (D) $FeCl_3$/L-dopa plus 20 mM TAU treatments. The cells were stained with ethidium homodimer (red, 5 μM), which stains the nuclei of dead cells, and calcein AM (green, 5 μM), which stains live cells.

10 mM TAU 60 min pretreatment was significant ($P < 0.05$) compared with the $FeCl_3$/L-dopa treatment (Fig. 5). The 10 mM TAU treatment without (0 min) pretreatment increased survival by 16% compared $FeCl_3$/L-dopa treatment. This was not significant, however, as it was in the previous experiments. The reason for the difference in these results might be the large variability observed in this set of experiments, due to the increased incubation periods and having to experimentally manipulate and stress the cells more often.

4. Discussion

The results from our study indicate that TAU plays a cytoprotective role *in vivo*, since it significantly increased the viability of cells exposed to catecholamine oxidation products. Many studies suggest that TAU protects from insults such as hypoxia [24], ozone exposure [8], and hypochlorous acid [4,48]. In a previous study, we observed that TAU attenuated L-dopa-induced oxidative damage in protein (bovine γ-globulin) [42], and in the current study we found that TAU (10 and 20 mM) significantly protected LLC-PK$_1$ cells from iron-induced catecholamine oxidation. These observations were also supported by the LDH measurements and visually confirmed in the photomicrographs. The observed attenuation in cell death in our studies was not due to an increase in cell proliferation, as suggested by other studies [24], since TAU alone did not increase cell numbers as measured by the MTT, protein, or DNA assays in our experiments.

LYS has been shown previously to be an effective quinone scavenger [33]. Therefore, we used the TAU analog LYS in an attempt to elucidate which functional group might be involved in the protective mechanism of TAU. LYS has two amino groups and lacks the sulfonic acid group, and results from these experiments might indicate if the amino or sulfonic acid group is involved in the protective mechanism. LYS protected the LLC-PK$_1$ cells to a similar extent as did TAU, suggesting the involvement of the amino group. TAU decreased iron-induced L-dopa oxidation in a cell-free model and attenuated the iron- and catecholamine-induced death in LLC-PK$_1$ cells, whereas LYS protects cells from the insult but is less potent than TAU in inhibiting iron-induced L-dopa oxidation in the cell-free model [42]. This suggests that TAU and LYS might be cytoprotective *via* different mechanisms. Other TAU analogs were tested for their cytoprotective effect in LLC-PK$_1$ cell culture in a recent study; hypotaurine also protected cells from iron-induced catecholamine oxidation, whereas homotaurine increased cell death [42]. The structural difference between TAU and hypotaurine is that hypotaurine has a sulfinic acid group instead of a sulfonic acid group. The cytotoxic TAU analog differs from TAU in the number of CH_2 groups in the carbon chain. These results suggest that the length of the

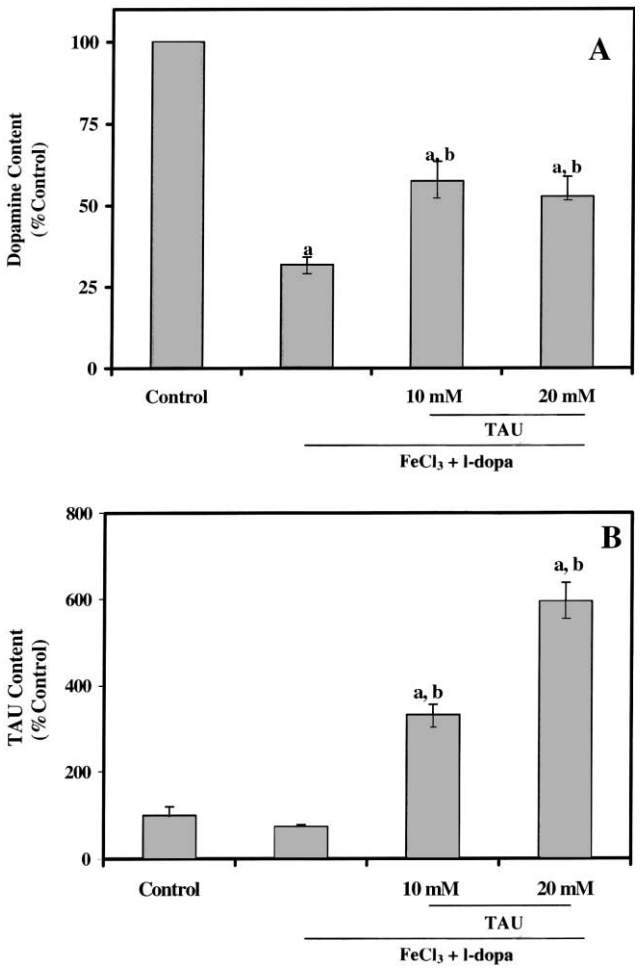

Fig. 4. (A) DA levels in LLC-PK_1 cells extracted immediately after a 1 hr treatment period with 75 μM $FeCl_3$ and 250 μM L-dopa using HPLC-ECD. Control wells received 250 μM L-dopa. Data are expressed as averages ± SEM of percent control measured in ng DA per well. The average value for control was 205 ng DA per well. $N = 8$ per group. Key: (a) $P < 0.001$ vs. control, and (b) $P < 0.05$ vs. $FeCl_3$/L-dopa. (B) TAU content in LLC-PK_1 cells extracted immediately after the 1 hr treatment period with 75 μM $FeCl_3$ and 250 μM L-dopa using HPLC-EDC. Data are expressed as averages ± SEM of percent control measured in nmol TAU/mg protein. The average value for control was 53.9 nmol TAU/mg protein. $N = 6$ per group. Key: (a) $P < 0.001$ vs. control, and (b) $P < 0.05$ vs. $FeCl_3$/L-dopa.

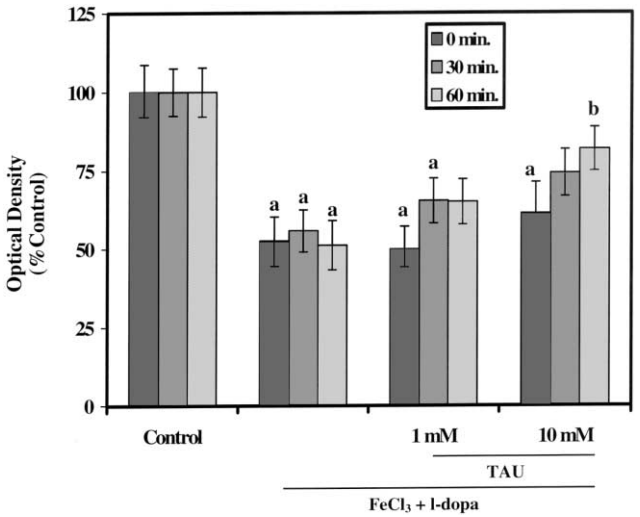

Fig. 5. Viability of LLC-PK_1 cell culture after pretreating with 1 or 10 mM TAU and then treating with $FeCl_3$/L-dopa in the presence and absence of 1 or 10 mM TAU. TAU concentrations were the same for the pretreatment and treatment periods. Data are expressed as averages ± SEM of percent control cell survival measured in optical density of MTT at 550 nm. $N = 11$ per group. Key: (a) $P < 0.05$ vs. control, and (b) $P < 0.05$ vs. $FeCl_3$/L-dopa.

carbon chain between the acidic and the amino group of TAU is important for the cytoprotective function.

We tested whether TAU interfered with L-dopa conversion to DA after acute exposure to iron-induced L-dopa oxidation by measuring DA and TAU contents in these cells immediately after the treatment. The cells must preserve cellular integrity to transport L-dopa into the cell, transform it to DA, and maintain DA and TAU intracellularly. TAU significantly attenuated the iron/L-dopa-induced decline in DA, suggesting that TAU helped maintain cellular integrity. More DA production reflected more viable cells and not a direct effect of TAU on L-dopa uptake, AADC activity, or DA release since previous work has shown that TAU (1 and 5 mM) has no effect on these parameters when LLC-PK_1 cells are incubated with 250 μM L-dopa for 30 min at 37° in the presence of TAU ([39]; unpublished observations). TAU content was also measured in these acute studies, and intracellular TAU concentrations were significantly higher in cells incubated with TAU. This also reflects functional integrity since the TAU transporter must remain operational and the cell membrane must be intact for this increase in intracellular TAU content to occur [49]. Thus, TAU can inhibit functional loss resulting from iron- and catecholamine-induced oxidative stress in LLC-PK_1 cell culture, since TAU blunted the loss of intracellular DA concentration, perhaps reflecting less iron-induced oxidation of L-dopa and DA.

Preincubations with TAU (1 and 10 mM) for 30 and 60 min showed a trend for attenuating the iron/L-dopa-induced decrease in cell viability, but this was not significant. Likewise, we have demonstrated previously that preincubation with 10 mM TAU improved cell survival only slightly after $FeCl_3$/L-dopa when compared with no pretreatment [42]. Serum TAU concentration in animals on an oral 1.5% dietary TAU supplement reaches 0.67–1.82 mM, which results in a tissue TAU concentration of up to 10–20 mM ([14,50]; unpublished observations). The preincubation experiments were designed to include a TAU concentration (1 mM) that could be reached in serum therapeutically with TAU supplementation, and we have shown that 1 mM TAU can reduce oxidative damage to DNA by catecholamine oxidation products [51]. The variability in these experiments was much higher than in the previous experiments, due to an increase in experimental manipulations. There was, however, a small trend

for improvement in cell survival with the 30 and 60 min 1 mM TAU preincubation that might be biologically significant.

The exact molecular mechanism by which TAU protects cells from the iron-induced catecholamine oxidation remains to be elucidated. Data from previous studies [42] and this study (Fig. 1) suggest that TAU might simply reduce the rate of catecholamine oxidation, reducing the presence of cytotoxic quinones. However, TAU does not appear to reduce catecholamine oxidation *via* metal chelation since the stability constant for metal complexation by TAU is negligible [52]. A second possibility is that TAU is scavenging cytotoxic quinones after their formation [42]. Alternatively, TAU may be scavenging reactive species directly after their generation as suggested in a study by Redmond *et al.* [53] where TAU attenuated nitric oxide- and ROS-induced hepatic injury. Another study, however, shows TAU to be a poor free radical scavenger [54]. TAU could indirectly prevent ROS generation by keeping the levels of unstable quinones low and, thus, decreasing ROS formation from quinone redox-cycling [29,55,56]. TAU could also be interfering with the secondary consequences of exposure to ROS such as altered calcium homeostasis, lipid peroxidation, protein oxidation, and DNA damage. Other studies in our laboratory indicate that TAU can stabilize receptor proteins in membranes [57], reduce protein carbonyl formation after free radical damage [42], and inhibit oxidative damage to DNA at concentrations as low as 1 mM [51]. A recent pilot study in rodent myocyte cultures conducted in collaboration with S.W. Schaffer found that DA (250 μM) alone was cytotoxic and produced DNA fragmentation patterns consistent with apoptosis. TAU (10 mM) reduced the level of DNA fragmentation in this pilot study (unpublished observations). Further work needs to be done to fully elucidate the protective mechanism of TAU in the LLC-PK_1 cell culture model. If TAU reduces apoptotic cell death, the protective effects may be even greater than that seen only 24 hr after insult. Experiments using specific free radical generators and other cytotoxic quinones as well as preincubations elevating TAU concentrations intracellularly before treatment might be useful in this task.

TAU plays an important role in osmoregulation [58–65]. It has been shown to be an important organic osmolyte in the renal medulla [60–62] where it is found in concentrations of 3–11 mM [13–15,21], in excitable tissues [45,58,63], and in astrocyte cultures [56]. TAU is taken up into cells exposed to a hypertonic environment and released in a hypotonic environment in rat hepatoma cells [66], astrocytes and MDCK cells [67]. Another potential protective mechanism of TAU, therefore, might be improved osmoregulation. Researchers have observed previously that TAU protected against hypoxia- and reoxygenation-induced cell damage by reducing osmoregulatory deterioration and improving Ca^{2+} homeostasis in LLC-PK_1 cells [24]. Photomicrographs from our study support this hypothesis since upon inspection it is noticeable that the cells exposed to oxidative stress have a rather swollen appearance, which could indicate a decline in osmoregulatory capabilities and disturbances in Ca^{2+} modulation. Cells exposed to the oxidative stress in the presence of 10 or 20 mM TAU more closely resemble the cells under control conditions. Recent work by Chen *et al.* [68] suggests that TAU protects cultured neurons from glutamate toxicity by decreasing intracellular Ca^{2+} concentrations and, therefore, attenuating protein oxidation. Studies measuring Ca^{2+} fluxes and consequences of elevated intracellular Ca^{2+} levels, such as protein oxidation and DNA strand breakage, as well as quantitative assessment of cell swelling and osmoregulatory measures, may prove insightful in addressing the molecular mechanism of action of TAU.

In summary, our study shows that catecholamines are cytotoxic in the presence of iron and that TAU attenuates catecholamine-induced cell death. Previous studies have shown that L-dopa and DA are cytotoxic in PC12 cell culture [69,70] and neuroblastoma SH-SY5Y cell culture [71]. These studies show that L-dopa and DA are cytotoxic *via* autoxidation and that antioxidants are effective in reducing cellular injury and loss. We have shown that TAU can inhibit the oxidation of L-dopa [14,42] and also is cytoprotective. L-Dopa and its metabolites also have been implicated in promoting oxidative DNA damage [72,73], and preliminary data in our laboratory have shown that TAU protects DNA [51] and proteins [42] from oxidative damage *in vitro*. These data suggest that TAU may reduce macromolecular damage to both proteins and DNA exposed to ROS. Our data conclusively show that TAU plays a cytoprotective role in LLC-PK_1 cells. Previous studies in our laboratory have shown that dietary TAU supplementation (1.5%) significantly increases tissue TAU content in several organs including the kidneys in aged Fischer 344 rats and reduces markers of oxidative stress [14]. This could confer the cells in these organs with increased antioxidant capabilities against catecholamine-induced oxidative damage. Pathophysiological conditions that would liberate free iron in kidney cells or enhance DA autoxidation could cause renal damage. For example, patients with septic [74,75] or cardiogenic [76] shock and hypotension are treated routinely with DA and other catecholamines, which exposes these patients to high levels of catecholamine oxidation products. TAU supplementation could enhance renal antioxidant capacity and attenuate oxidative damage to the kidney and other tissues. An age-related decline in DA in the striatum has been correlated recently to a loss of striatal TAU [77]. This further establishes a link between DA and TAU in the pathophysiological changes associated with aging. More studies are necessary to elucidate which type of oxidative damage can be prevented by TAU and if this amino acid could possibly be used therapeutically for pathological states that are induced by or cause free radical damage.

Acknowledgments

We would like to thank Deron Baker for technical assistance. We want to acknowledge Joanna Peris for her comments and suggestions regarding this manuscript. These studies have been partially funded with a grant provided by the Taisho Pharmaceutical Co. Ltd. and the American Heart Association, Florida Affiliate Grant 9701750. This work was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Florida.

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