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Resveratrol Attenuates Cisplatin Renal Cortical Cytotoxicity by Modifying Oxidative Stress

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Abstract

Cisplatin, a cancer chemotherapy drug, is nephrotoxic. The aim of this study was to investigate whether resveratrol (RES) reduced cisplatin cytotoxicity and oxidative stress. Rat renal cortical slices were pre-incubated 30 min with 0 (VEH, ethanol) or 30 μ g/ml RES followed by 60, 90 or 120 min co-incubation with 0, 75, or 150 μ g/mL cisplatin. Lactate dehydrogenase (LDH) leakage was unchanged at 60 and 90 min by cisplatin. Cisplatin increased ($p < 0.05$) LDH leakage at 120 min which was protected by RES. Cisplatin induced oxidative stress prior to LDH leakage as cisplatin depressed glutathione peroxidase and superoxide dismutase (SOD) activity, increased lipid peroxidation, protein carbonyls and 4-hydroxynonenal (4-HNE) adducted proteins within 60 min. RES failed to reverse glutathione (GSH) depression by cisplatin. In order to eliminate an extracellular interaction between RES and cisplatin, additional studies (RINSE studies) allowed a 30 min RES uptake into slices, transfer of slices to buffer lacking RES, followed by 120 min cisplatin incubation. RES in the RINSE studies prevented LDH leakage by cisplatin indicating that RES protection was not via a physical interaction with cisplatin in the media. These findings indicate that RES diminished cisplatin in vitro renal toxicity and prevented the development of oxidative stress.

Keywords

Cisplatin; Resveratrol; 4-Hydroxynonenal; Oxidative stress; Nephrotoxicity

1. INTRODUCTION

Cisplatin is a widely used cancer chemotherapeutic drug which is a component of cancer chemotherapy regimens for: breast, metastatic ovarian, testicular, small cell, non-small cell lung cancer, colorectal and neuroblastoma. The predominant adverse effects for cisplatin

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include: ototoxicity, nausea, vomiting, neuropathy and nephropathy (Kintzel 2001; Park et al., 2008). Renal dysfunction associated with cisplatin is dose dependent, cumulative and occurs in 33% of patients receiving cisplatin. Cisplatin accumulates to high levels in renal tissue due to active transport along the basolateral membrane by the organic cation and copper transporter (Ludwig et al., 2004). Cisplatin toxicity in humans as well as rodents causes tubular damage predominantly to the distal tubule and S3 segment of the proximal tubule (Arany and Safirstein, 2003).

Cisplatin cancer chemotherapeutic action requires intracellular chloride dissociation and aquation of the platinum (Pt) complex. The cisplatin complex mediates intrastrand cross-linking between the N-7 and O-6 of guanine nucleotides which denatures the DNA and causes cell cycle arrest (Hambley, 1988). Cisplatin adverse cellular effects and nephrotoxicity involve several biochemical mechanisms involving activation of various cell signaling pathways, mitochondrial dysfunction, inhibition of sodium-potassium ATPase, oxidative stress, apoptosis and necrosis (Pabla and Dong, 2008).

Resveratrol (3,5,4'-trihydroxystilbene, RES), is a polyphenol, phytoalexin found in grape skins, red wine, cranberries, blueberries and nuts (Rimando et al., 2004). RES possesses anticancer activities against some cancer cell lines including ovarian cancer cells. Potential mechanisms proposed for RES anticancer activity include activity as an antioxidant and/or anti-inflammatory agent, interference with signal transduction pathways, modulation of cell cycle-regulating proteins and selective induction of apoptosis which has been characterized in various cancer cell lines (Ulrich et al., 2005). Addition of RES to the culture media was inhibitory to cell growth and proliferation in the human ovarian cancer cell lines A2780 and CaOV3 (Opipari et al., 2004). In vivo, RES slows neuroblastoma tumor growth in athymic nude when RES was administered for 5 weeks (van Ginkel et al., 2007) suggesting RES has anticancer activity. Pharmacokinetic studies (Aggrawal et al., 2004) indicated that RES levels are higher in the liver and the kidney compared to other organs which suggests that RES has a greater potential to induce its effects in the liver and kidney. In a clinical study, RES diminished tumor cell proliferation in colorectal cancer patients who took 500 or 1000 mg RES prior to surgery (Patel et al., 2010). Patel and associates (2010) further reported that RES accumulation in resected tumor tissue was detectable indicating RES accumulated in patient tumors.

RES is a widely recognized anti-oxidant. RES anti-oxidant activity was evident when added directly to human platelet suspensions (Olas and Wachowicz, 2004; Olas et al., 2004 and 2005). Addition of RES to human platelet suspensions, diminished lipid peroxidation induced by 25 µg/ml cisplatin suggesting a rapid anti-oxidant effect in platelets which lack a nucleus and therefore the mechanism of protection is independent of any DNA interaction. Cetin and colleagues reported that an extract of black grapes reduced cisplatin mediated nephrotoxicity in rats (Cetin et al., 2005). Black grape extract contains many structurally diverse compounds and RES is just 1 of the numerous chemicals contained in grapes. Unfortunately, the concentration of RES was not measured in the black grape extract. More recent studies showed that RES reduced cisplatin nephrotoxicity in Wistar rats (Do Amaral et al., 2008). A single 5 mg/kg dose of RES was administered 30 min prior to a toxic dose of cisplatin. Serum creatinine levels measured 5 days after cisplatin treatment were higher in the cisplatin group compared to the rats receiving RES and cisplatin (Do Amaral et al., 2008). Do Amaral and coworkers (2008) partially attributed RES protection for cisplatin nephrotoxicity to an anti-inflammatory effect by RES resulting in reduced infiltration of T cells, leukocytes and macrophages which decreased reactive oxygen species (ROS) generation.

RES also provided partial protection for cisplatin nephrotoxicity in a C57BL/6 mice model (Kim et al., 2011). Pretreatment daily for 7 days prior to cisplatin administration preserved renal function as indicated by higher glomerular filtration rate in mice given 7 days pretreatment with RES prior to cisplatin treatment (Kim et al., 2011). The authors concluded that RES protection may be due to increased Nicotinamide Adenine Dinucleotide Dependent Protein Deacetylase 1 (SIRT1) which diminished cisplatin associated p53 acetylation and activation of apoptotic pathways. Activation of p53 acetylation is thought to be a result of cisplatin damage to DNA causing activation of p53 acetylation resulting in induction of apoptosis and ultimately causing nephrotoxicity (Cummings and Schnellmann, 2002).

Our understanding of the impact of RES on cisplatin associated oxidative stress and antioxidant enzyme activity is very limited. In order to better understand the mechanism for RES attenuation of cisplatin cytotoxicity it is necessary to examine whether RES can prevent cisplatin decline of antioxidant enzyme activity and induction of oxidative stress. More importantly, it is not clear whether cisplatin mediated oxidative stress occurs prior to renal cytotoxicity or if it occurs after membrane integrity is compromised. Onset of oxidative stress prior to LDH leakage would imply a primary role of ROS in cytotoxicity. Conversely, if oxidative stress does not occur until after LDH leakage then this would suggest oxidative stress is secondary to the mechanism of toxicity. The present study used an in vitro rat renal cortical slice model. Renal cortical slices have been used for decades to explore in vitro toxicity of drugs and chemicals (Berndt 1976; Burton et al., 1995; Dickman et al., 2011). Cell culture or isolated cells provide only one cell population and often have diminished metabolic activity. Renal cortical slices provide intact integrity of a mixed population of cells which models what occurs in vivo while maintaining metabolic and biotransformation activity (Baverel et al., 2013). The concentration of RES selected for our study is lower than the 200 μ M concentrations used by other investigators to inhibit tumor growth (Opipari et al., 2004; Lee et al., 2009). The selected RES concentration is comparable to peak plasma RES levels measured in patients taking 5 grams of RES daily for 21 days (Brown et al., Cancer Res 210). The concentration of cisplatin selected was based on previous work in our laboratory examining cisplatin renal accumulation and excretion in rats (Valentovic et al., 1991). In this study renal levels of Pt were 160 μ g/g tissue/kg body weight within 6 h of cisplatin injection. Platinum plasma levels will vary with the dose administered to a patient. Plasma platinum levels of 2-3 μ g/ml have been measured in patients following cisplatin infusion undergoing cancer chemotherapy treatment (Urien and Lokiec, 2004; Ricart et al., 2008). Renal tubular levels of cisplatin are higher than plasma levels due to active transport into the tissue. Another surprising observation is the slow excretion rate for cisplatin which results in a chronic renal exposure to cisplatin. Brouwers and associates (2008) reported that plasma Platinum (Pt) levels were 0.1-1 μ g/ml 18-73 months after cisplatin treatment. Our findings in this study indicated that RES protection prevented cisplatin mediated oxidative stress which occurred prior to induction of LDH leakage. RES protection was not mediated by an interaction of cisplatin and RES in the extracellular buffer and RES does not prevent glutathione (GSH) decline induced by cisplatin exposure.

2. MATERIALS AND METHODS

2.1 Chemicals

Reduced glutathione (GSH), cisplatin and RES were obtained from Sigma Chemical Company (St. Louis, Mo.). Ethanol was USP grade. All other chemicals were purchased from Fisher Scientific or Sigma-Aldrich Chemical Company and were of the highest analytical quality. Antibody sources and dilutions are described in appropriate sections.

2.2 Animals

Male Fischer 344 (F344) rats (200-250 g) were purchased from Hilltop Lab Animals, Inc. (Scottsdale, PA). Animals were housed in a controlled ambient temperature (21-23°C), humidity (40-55%) and 12 h light cycle (lights on 0600-1800 h) environment. Rats had free access to food (Purina Rat Chow) and water. A minimum 5-day acclimation period was completed prior to initiation of any studies. The Institutional Animal Care and Use Committee (IACUC) reviewed and approved all protocols involving animals.

2.3 Incubation of renal tissue and experimental design

Animals were anesthetized with isoflurane, the abdominal artery severed and both kidneys were removed. The kidneys were decapsulated, excised, quartered along the sagittal and coronal planes and immediately immersed in ice cold Krebs buffer, pH 7.4. Renal cortical slices (60-85 slices per animal) were prepared and added to 5 ml ice cold Krebs buffer as previously reported (Valentovic et al., 1996). The composition of the Krebs buffer was (126.4 mM NaCl, 6 mM Na₂HPO₄, 5.2 mM KCl, 1.3 mM MgSO₄ and 1 mM CaCl₂). The slices from each animal were transferred to separate 30 ml beakers containing 5 ml oxygenated Krebs buffer. The slices were rinsed 2 times by transfer to fresh 30 ml beakers containing new 5 ml oxygenated Krebs buffer every 3 min at 25°C in a Dubnoff shaker under an oxygen environment with constant shaking (100 cycles/min). The slices from each animal were equally divided between 12 Erlenmeyer flasks (5-7 slices/flask or approximately 50-80 mg tissue) containing 2 ml oxygenated Krebs and equilibrated for 10 min at 37°C under oxygen and constant shaking (100 cycles/min Dubnoff Incubator). Sufficient tissue was obtained from both kidneys of each animal to allow each animal to have its own control and all treatment groups run simultaneously which reduced variability in data. Renal tissue was pre-incubated for 30 min with a final concentration of 0 or 30 µg/ml resveratrol (RES) prepared in ethanol. The vehicle (VEH) treated group received an equal volume of ethanol. The renal slices were then co-incubated with a final concentration of 0, 75 or 15 µg/ml cisplatin for 60, 90 or 120 min. Cisplatin was added in 1 ml oxygenated Krebs buffer to provide a total incubation volume of 3 ml. Sufficient tissue was collected to allow for comparison of all concentrations of cisplatin with VEH or RES pretreatment. Upon completion of the incubation period, renal toxicity was evaluated using lactate dehydrogenase (LDH) leakage, OxyBlot, glutathione (GSH) peroxidase and superoxide dismutase (SOD) activity.

A separate series of studies evaluated whether RES protection was mediated by an extracellular interaction. In these experiments, RES was allowed to accumulate within the slices for only 30 min and then cisplatin exposure occurred in the absence of RES in the media. Slices were initially pre-incubated for 30 min with VEH or 30 µg/ml RES as described above. After the 30 min incubation, the media was replaced with oxygenated Krebs buffer lacking RES or VEH (ethanol). Tissues were next incubated with a final concentration of 0, 75 or 150 µg/ml cisplatin for 120 min but RES or VEH was not added to the media. Upon completion of the incubation period tissues were analyzed for LDH leakage, GSH Peroxidase and SOD activity.

2.4 Evaluation of cytotoxicity

Upon completion of the incubation period, media was decanted and stored on ice. Tissues were blotted, weighed, added to 10% Triton X-100 and vortexed vigorously for 1 min to release residual tissue LDH. LDH was determined using a kinetic assay monitoring nicotinamide adenine nucleotide reduction coupled to lactate oxidation to pyruvate at 340 nm. LDH release into the media was expressed as percent of total LDH. One Unit of LDH is defined as the amount of enzyme that will convert 1 µmole NADH to NAD per minute. All

data from an animal was discarded if vehicle treated samples had 15% or higher LDH leakage.

2.5 GSH levels, GSH Peroxidase and SOD activity

GSH levels were measured on tissues homogenized in 0.5% sulfosalicylic acid and adjusted to a 1 ml final volume. Total GSH was determined using a GSH reductase and NADPH coupled reaction with 5,5'-dithiobis(2-nitrobenzoic acid) as previously published (Brown et al., 2012). GSH Peroxidase was a kinetic assay based on the method of Lawrence and Burk (1976). Renal slices (100 mg) were homogenized in 1 mL of phosphate buffer (50 mM KH_2PO_4 , 1 mM sodium azide, 1 mM EDTA; pH 7.2). The reaction mixture consisted of 0.5 ml phosphate buffer, 0.1 mL 2 mM NADPH, 0.1 mL glutathione reductase (1 Unit), 0.1 ml 10 mM reduced glutathione, and 0.1 mL tissue homogenate. The reaction was initiated with the addition of 0.25 mM H_2O_2 (final concentration). The conversion of NADPH to NADP was measured for 1 minute at 340 nm. Activity was calculated using the extinction coefficient for NADPH ($6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). One GSH peroxidase Unit will catalyze 1 μmole of reduced GSH to oxidized per minute by hydrogen peroxide. Cu/Zn and Mn SOD activity were determined using a Fluka designed spectrophotometric kit purchased from Sigma Chemical Company (19160; St. Louis, MO). Total SOD activity was the sum of Mn and Cu/Zn SOD activity. Cu/Zn-SOD activity was inhibited by incubating the sample at room temperature sodium diethyldithiocarbamate (DDTC) at a final concentration of 25 mM. Mn SOD activity was the difference between total and DDTC inhibited SOD activity. A Unit is defined as the amount of enzyme required to dismutate 50% of superoxide radical. The assay was completed according to manufacturer's recommendations.

2.6 Lipid peroxidation

Renal tissue was homogenized in 1 ml Krebs buffer pH 7.4 and the homogenizer shaft was rinsed with an additional 1 ml Krebs buffer. Lipid peroxidation was measured as described previously (Valentovic et al., 2002). Briefly, protein was precipitated on ice for 15 min by mixing equal volumes (1.5 ml) of homogenate and 15% trichloroacetic acid in 0.25 N HCl. An aliquot of supernatant was incubated with 0.67% thiobarbituric acid (TBA) for 15 min at 90°C. The samples were cooled in darkness and absorbance measured at 535 nm. The amount of malondialdehyde (MDA) was calculated based on a standard curve (range 1-40 nmol) using MDA (Aldrich, St. Louis, Mo) and expressed as μmol MDA/g tissue.

2.7 Protein carbonyl (OxyBlot)

Renal tissue isolated after a 60 min incubation with cisplatin were homogenized in 1 ml Krebs buffer pH 7.4 and the homogenizer probe was rinsed with 1 ml Krebs buffer. An aliquot was used for protein determination using a Coomassie Blue spectrophotometric method. The appearance of protein carbonyls was measured using a Protein Oxidation Detection OxyBlot kit Millipore (S7150; Temecula, CA) by Western analysis according to the manufacturer's guidelines. The principle of the method is that modification of proteins by addition of carbonyl side chains due to oxidative stress are derivatized to 2,4-dinitrophenylhydrazone (DNP) following addition of 2,4-dinitrophenylhydrazine (DNPH). The antibody is specific for the DNP moiety on a protein. Each lane was loaded with 20 μg protein. Samples and standards were run on polyacrylamide gel (12.5% acrylamide). The gel was then transferred to a nitrocellulose membrane. To verify efficiency of transfer and uniform protein loading, the membrane was stained using MemCode® Reversible Protein Stain Kit (Thermo Scientific; Rockford, IL) and results were quantified by densitometry. The samples were run with DNP-derivatized molecular weight standards.

2.8 4-Hydroxynonenal (4-HNE) Adducts

Western blot was used to analyze for the presence of 4-HNE reduced Michael adducts to protein following a 60 min exposure to cisplatin. A 100 µg protein aliquot of tissue homogenate was boiled for 5 min in order to denature proteins. Samples were separated on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman; Dassel, Germany). Transfer efficiency was verified using MemCode® Reversible Protein Stain Kit (Thermo Scientific; Rockford, IL). The membrane was blocked for 1 h using a 5% (w/v) milk/TBST solution (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 8.0). Membranes were next incubated overnight with constant shaking at 65°F with 4-HNE Michael Adducts-reduced primary antibody (1:1000 dilution 393207; Calbiochem; Merck, Darmstadt, Germany). Goat anti-rabbit IgG HRP (1:2500 in milk/TBST; DC03L; Calbiochem) secondary antibody was applied for 1 h followed by washing in TBST (4 × 10 min) and developed using Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare; Buckinghamshire, UK). Densitometry was performed with n=4 different animals.

2.9 TNFα expression

Western blot was used to analyze for the presence of TNFα following a 30 min pre-incubation with RES followed by a 120 min co-incubation with cisplatin. Aliquots of 10, 20 and 30 µg protein from tissue homogenates were run initially on 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane. Following staining it was determined that 20 µg of protein was sufficient to detect TNFα. The membrane was incubated overnight with rabbit anti-TNFα antibody (1:1000 dilution ab66570 abcam) and blocked with 5% bovine serum albumen. Goat anti-rabbit IgG HRP (1:2500) secondary antibody was applied and washed in TBST. The membrane was developed using an ECL Reagent as described above.

2.10 Statistical Analysis

Values represent Mean ± SEM with n= 4 animals/group. Differences between groups were analyzed using a Two Way repeated measured analysis of variance (ANOVA) followed by a Tukey post hoc (Sigma Stat, SPSS Inc. Chicago, IL). Differences within treatment groups were determined using a repeated measures ANOVA followed by a Tukey test. All statistical analyses were conducted using a 95% confidence interval and considered significant at p<0.05.

3. RESULTS

3.1 RES pretreatment protects from cisplatin associated LDH leakage

LDH leakage was comparable between VEH and RES groups suggesting RES was not toxic under the incubation conditions. Cisplatin incubated with renal slices did increase LDH leakage, a biomarker for loss of membrane integrity, in a time dependent manner (Fig. 1). Renal slices required a 120 min exposure to cisplatin in order to increase LDH leakage above VEH treated levels as a 90 min exposure to cisplatin had no effect on LDH leakage. These studies showed a 30 min pre-incubation with RES prevented the rise in LDH leakage associated with cisplatin toxicity indicating RES was protective against cisplatin renal toxicity.

3.2 RES Attenuation of Lipid peroxidation and GSH levels

Lipid peroxidation was increased by exposure to 75 and 150 µg/ml cisplatin (Fig. 2) relative to control (0 µg/ml). RES reduced baseline lipid peroxidation and prevented cisplatin mediated lipid peroxidation. Pretreatment with RES prevented any change in lipid peroxidation between the 0, 75 and 150 µg/ml cisplatin groups as MDA levels were

comparable between groups. Exposure for 120 min to 75 and 150 $\mu\text{g/ml}$ cisplatin decreased renal GSH levels (Fig. 3) when compared to control tissue (0 $\mu\text{g/ml}$). Pretreatment with RES did not prevent the decline in renal GSH levels. Therefore, the protection by RES for lipid peroxidation is not mediated by binding to GSH since GSH declined in a comparable manner between VEH and RES pretreated groups.

3.4 RES attenuates Cisplatin associated Decline in GSH Peroxidase and SOD activity

GSH peroxidase detoxifies lipid hydroperoxides and hydrogen peroxide by conversion to alcohol and water, respectively. Maintaining normal GSH Peroxidase activity would diminish cellular oxidative stress. RES had no effect on GSH Peroxidase activity (Fig. 4) at 60, 90 or 120 min exposure since enzyme activity was comparable between groups whether exposed to ethanol (-RES) and RES (+RES). Cisplatin (75 and 150 $\mu\text{g/ml}$) reduced ($p < 0.05$) glutathione peroxidase activity within 60 min and continued to inhibit GSH peroxidase activity at 90 and 120 min when compared to control (0 $\mu\text{g/ml}$) (Fig. 4). RES provided total protection of GSH peroxidase enzyme activity for 75 and 150 $\mu\text{g/ml}$ cisplatin when examined at 60, 90 and 120 min. These results indicated that cisplatin depressed GSH peroxidase activity. The decline in GSH peroxidase is an early event in toxicity as it occurs prior to the loss of membrane integrity. RES prevented cisplatin mediated depression of GSH peroxidase enzyme activity for the entire 120 min cisplatin exposure. Both concentrations of cisplatin diminished Total SOD (Fig. 5) and Mn SOD activity (Fig. 6) at 60 and 120 min. RES pretreatment was able to protect total SOD and Mn SOD activity from cisplatin cytotoxicity which would reduce oxidative stress.

3.5 RES protects against Cisplatin associated rise in Oxidative Stress

Protein carbonyl addition to amino acids is an indicator of oxidative stress. RES pretreatment for 30 min reduced protein carbonyl formation in renal tissue (Fig. 7). The reduction in protein carbonyls by RES is not due to low protein loading as densitometry for protein staining showed no difference between groups. Cisplatin increased protein carbonyls as indicated by increased band intensity for the entire lane (Fig. 7). Pretreatment with RES reduced protein carbonyls in tissues treated RES and 75 or 150 $\mu\text{g/ml}$ cisplatin (Fig. 7) as confirmed by densitometry.

Western analysis showed that 4-HNE adduction was diminished by RES pretreatment when compared to tissues treated with ethanol (-RES) (Fig. 8). Cisplatin exposure for 60 min resulted in increased 4-HNE adducted protein following exposure to 75 and 150 $\mu\text{g/ml}$ cisplatin (Fig. 8). Band intensity at 27 and 45 kDa was greater in the cisplatin treated group when compared to tissue exposed to 0 $\mu\text{g/ml}$ cisplatin. RES pretreatment reduced cisplatin associated the intensity of bands at 27 and 45 kDa. Further studies will need to be completed to identify the adducted proteins.

3.6 RES and Cisplatin effects on TNF α

A 30 min pretreatment followed by a 120 min exposure to RES did not alter TNF α expression (Fig. 9). Western analysis of renal tissue 120 min after exposure to 75 or 150 $\mu\text{g/ml}$ cisplatin did not increase TNF α protein levels when compared to 0 $\mu\text{g/ml}$ cisplatin. Pretreatment with RES also did not alter TNF α expression.

3.7 RES Protection is not due to an extracellular interaction with Cisplatin

RES attenuation of cisplatin toxicity may be due to an extracellular physical interaction between RES and cisplatin to form a complex that prevents cisplatin from entering renal tissue. In order to address this hypothesis, renal slices were pretreated 30 min with RES, tissues were rinsed twice in Krebs buffer lacking RES and then placed in fresh buffer devoid

of RES. The slices were next incubated for 120 min with 0, 75 or 150 $\mu\text{g/ml}$ cisplatin in buffer ***lacking RES*** and containing 0-150 $\mu\text{g/ml}$ cisplatin. These studies are denoted 30 min only RES exposure as RES was not present concurrently with cisplatin in order to prevent an extracellular interaction between RES and cisplatin. LDH leakage was increased by cisplatin in a concentration dependent manner in the tissues exposed for 30 min to ethanol (Fig. 10). A 30 min pretreatment with RES was sufficient to provide protection from cisplatin toxicity (Fig. 10) even though RES was not present during the 120 min exposure to cisplatin. These results showed that RES was protective even when RES was not present at the same time as cisplatin. Based on these results, RES protection of cisplatin toxicity cannot be attributed to an extracellular interaction that could reduce cisplatin entry into the cell. These results also suggest that a 30 min exposure to RES provides sufficient uptake of RES to mediate cellular changes that are protective for up to a 120 min exposure to cisplatin. Additional studies, using the 30 min only RES experimental design, evaluated the effect of 120 min exposure to cisplatin on GSH peroxidase activity. The 150 $\mu\text{g/ml}$ cisplatin concentration inhibited GSH peroxidase activity (Fig. 11) compared to control (0 $\mu\text{g/ml}$ cisplatin). The 30 min only RES exposure was also sufficient to protect GSH peroxidase activity from cisplatin inhibition (Fig. 11). These findings show that RES does not protect renal tissue from cisplatin toxicity by an extracellular interaction. These findings also demonstrate that 30 min is sufficient time for RES to enter renal tissue. Further studies need to explore whether RES has an effect on OCT transport of cisplatin. The intent of these studies was to examine an extracellular interaction of RES and cisplatin and not to evaluate cisplatin uptake into slices which is beyond the scope of this study.

2 DISCUSSION

Interventions are needed to reduce the adverse renal effects of cisplatin. Patients undergoing cancer chemotherapy experience many side effects, which are specific for each drug and dosing regimen. Cisplatin treatment diminishes renal function resulting in a prolonged decline in glomerular filtration rate (GFR) and creatinine clearance (Groth et al., 1986; Fujieda et al., 2009). The decline in renal function is slow to reverse and may take 10-15 years for improvement of renal function to occur after cessation of cisplatin treatment (Petersent and Hansen 1999). Brouwers and associates (2008) reported that plasma Platinum (Pt) levels were 0.1-1 $\mu\text{g/ml}$ 18-73 months after cisplatin treatment which may further contribute to the renal damage observed in patients. In our in vitro renal slice study, we showed that RES reduced cisplatin cytotoxicity in a renal cortical slice model and protected renal tissue from cisplatin induced oxidative stress. RES totally prevented cisplatin associated rise in LDH leakage, a biomarker of membrane integrity. Our studies examined RES since RES is a chemical form taken up into the kidney following in vivo administration to mice (Vitrac et al., 2003) and rats (Juan et al., 2010). RES undergoes glucuronidation and sulfation as well as enterohepatic recirculation. Vitrac and coworkers showed that RES was taken up by the kidneys even after oral administration in a mouse model (Vitrac et al., 2003). Juan and associates (2010) measured higher levels of RES than its glucuronide or sulfate metabolites following intravenous administration of RES which indicates RES would be a chemical form found in the kidney in vivo.

The potential for RES to reduce cisplatin renal toxicity was examined based on earlier reports by other investigators which suggested that RES possessed anticancer properties. RES has been previously reported to also enhance cisplatin effectiveness in cancer cells (Rezk et al., 2006). RES improved the cancer kill rate for cisplatin when both agents were added together OVCAR-3 ovarian cancer cells. RES has been hypothesized to improve cisplatin cancer chemotherapeutic effect via a pro-apoptotic effect on cancer cells. Rezk and associates (2006) also reported that RES reduced doxorubicin cardiotoxicity in neonatal rat

ventricular myocytes. The mechanism for attenuation by RES of doxorubicin cardiotoxicity was not investigated by Rezk and coworkers and remains unknown.

Cisplatin exposure to the kidney results primarily in damage to the proximal and distal tubular region. Cisplatin causes a rise in many intracellular chemicals which activate various signaling pathways that induce cell death. The mechanism for cell death by cisplatin includes activation of apoptosis and necrosis, generation of reactive oxygen species, activation of mitogen activated protein kinase (MAPK), p53 and caspase pathways (Cummings and Schnellmann 2002; Pabla and Dong 2008). RES pretreatment greatly reduced cisplatin associated lipid peroxidation (Fig. 2) and oxidative stress within renal slices exposed to 0 or 75 or 150 ug/ml cisplatin. Western blot analysis further demonstrated that RES prevented protein carbonylation which is a biomarker for oxidative stress. Protein modifications with protein carbonyls or 4-HNE adduction can modify protein function provided the modification occurs within a binding site of the protein. Our studies are the first to show RES prevents cisplatin mediated modifications by protein carbonylation and 4-HNE adduction. Further studies are needed to determine whether RES prevents modifications at critical sites within proteins. Additional studies are required to determine the identity of the modified proteins.

Cisplatin induced oxidative stress is thought to be mediated by several mechanisms which include mitochondrial dysfunction and GSH depletion. Cisplatin becomes aquated upon entry into the cell and this highly reactive form can be complexed with 2 molecules of GSH resulting in GSH depletion (Chu 1994). RES did not prevent cisplatin reduction of GSH in our studies (Fig. 3) while reducing oxidative stress. These results show that RES does not interfere with cisplatin aquation within the cell and indirectly suggests that RES does not interfere with cisplatin uptake into slices

Do Amaral and associates (2008) reported that cisplatin nephrotoxicity was reduced by a single treatment with RES in vivo in Wistar rats treated with a high dose of 25 mg/kg cisplatin. They noted that cisplatin induced T cell, leukocyte and macrophage infiltration resulting in an inflammatory response which resulted in increased reactive oxygen species. RES prevented the infiltration of inflammatory cells such as macrophages and the authors hypothesized that this may be part of the mechanism for reduced toxicity. Our studies would suggest that RES rapidly reduces cisplatin induced oxidative stress via a mechanism independent of migration of inflammatory cells since macrophages cannot migrate in our in vitro system. Cisplatin toxicity is mediated by a complex system and RES may work via multiple mechanisms. However, our study suggests RES can reduce cisplatin mediated oxidative stress and renal cellular toxicity

GSH peroxidase and SOD activity were impaired by exposure to cisplatin much earlier than induction of LDH leakage. LDH leakage required a 120 min exposure to cisplatin while GSH peroxidase, total SOD and MnSOD were inhibited within 60 min. RES pretreatment prevented cisplatin induced decline in GSH peroxidase activity. GSH peroxidase and MnSOD are important enzymes involved in regulation of cellular oxidative stress. Addition of RES maintained GSH peroxidase and Mn SOD activity in the cisplatin exposed group which should allow for continued detoxification of renal lipid hydroperoxides, hydrogen peroxide and superoxides. It is not known how cisplatin interferes with the activity of GSH peroxidase, Cu/Zn SOD or Mn SOD activity. Alterations in cellular levels of Cu, Zn, Mn or Selenium (Se) through would diminish enzyme activity. GSH peroxidase activity requires Selenium as a cofactor and GSH peroxidase activity was protected by Se supplementation in animals treated with cisplatin (Ognjanovic et al., 2012). Another possibility is that cisplatin directly binds to sulfhydryl groups on cysteine in Mn SOD and GSH peroxidase causing diminished activity similar to what occurs following exposure of mice to 2,6-di-tert-butyl-4-

methylphenol (Meier et al., 2007). A third possible mechanism for the decline in GSH peroxidase and MnSOD activity may be due to oxidative stress causing oxidation of cysteine groups within each enzyme which has been reported to impair enzyme activity (Selles et al., 2012).

The impact of RES on various drug transporters is not well known. RES has been reported to inhibit at least one transporter system. RES inhibited entry of genistein by inhibiting the multi-drug resistance protein 2 (MRP2) expressed in HepG2 cells. Interactions to impede drug entry would be one mechanism for RES attenuation of cisplatin toxicity. Our studies are the first to show that RES attenuation of cisplatin toxicity is not mediated by an extracellular interaction to inhibit cisplatin cellular entry (Fig 10-11). Although indirect evidence, the decline in GSH levels is comparable between RES and ethanol treated tissue exposed to cisplatin (Fig. 3). Cisplatin reacts with the sulfhydryl group of cysteine to cause GSH depletion and if cisplatin uptake was inhibited by RES then you would expect less of a decline in GSH. However, our studies showed a similar change in GSH which suggests RES does not alter cisplatin uptake. Further studies are necessary to evaluate whether RES impacts cisplatin uptake into slices which is beyond the scope of this study. In addition, these studies indicate that RES uptake into renal cortical tissue is rapid since a 30 min exposure followed by a 120 min exposure to cisplatin in the absence of RES still provided protection as indicated by preservation of GSH peroxidase and Mn SOD enzyme activity and prevention of LDH leakage by cisplatin.

Cisplatin nephrotoxicity is associated with an inflammatory component which has a vital role in toxicity (Ramesh and Reeves, 2003). TNF α serum levels were increased in mice 72 h after cisplatin injection. Inhibition of TNF α release in mice treated with cisplatin reduced the severity of renal toxicity suggesting TNF α activation of inflammatory components had a vital role in toxicity when measured 72 h after cisplatin injection (Ramesh and Reeves, 2003). The source of TNF α can come from various sources but the kidney was reported to produce some of the TNF α released by cisplatin (Ramesh and Reeves, 2002). In our studies, TNF α protein levels were not increased by cisplatin exposure in our experimental model of renal cortical slices exposed for a very short duration of 120 min. The contribution of TNF α to cisplatin nephrotoxicity is probably more important with longer exposure or in vivo where toxicity is measured 3 days after cisplatin exposure.

In summary, pretreatment with RES was protective for cisplatin renal cytotoxicity using an in vitro rat renal slice model. RES protection was associated with prevention of lipid peroxidation and oxidative stress normally associated with cisplatin. RES was not protecting by altering GSH binding to cisplatin or altering TNF α levels. The mechanism for RES protection does not involve an extracellular interaction between RES and cisplatin. RES pretreatment prevented cisplatin mediated inhibition of GSH peroxidase and Mn SOD enzyme activity. Maintaining GSH peroxidase and Mn SOD activity by RES in the presence of cisplatin reduced reactive oxygen species levels and oxidative stress. The reduced oxidative stress was confirmed by the decline in protein carbonyl and 4-HNE adduction in the RES and cisplatin treated tissue which supports the conclusion that RES decreased the extent of oxidative stress and associated modification of proteins.

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ABBREVIATIONS

ANOVA	analysis of variance
DNPH	2,4-dinitrophenylhydrazine
DNP	2,4-dinitrophenylhydrazone
GSH	glutathione
4-HNE	4-hydroxynonenal
LDH	lactate dehydrogenase
MDA	malondialdehyde
RES	resveratrol
VEH	vehicle
MRP2	multidrug resistance protein 2

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Resveratrol reduced cisplatin oxidative stress prior to loss of membrane integrity and cell death

Resveratrol protection was independent of reduced glutathione levels

Resveratrol preserved antioxidant enzyme activity during cisplatin exposure

Resveratrol does not stimulate antioxidant enzyme activity

Cisplatin did not stimulate $\text{TNF}\alpha$ in the short 120 min exposure

Fig. 1. Time and concentration dependent effect of cisplatin on LDH leakage

Renal slices were pretreated for 30 min with ethanol (VEH) or 30 μ g/ml resveratrol (RES). Renal slices were then co-incubated with a final concentration of 0, 75 or 150 μ g/ml cisplatin for 90 or 120 min at 37°C and constant shaking in an oxygen atmosphere. Lactate dehydrogenase (LDH) release was expressed as percent of total LDH. Values represent Mean $\pm$ SEM with n=4 different animals per group. Statistical differences ($p < 0.05$) between groups were denoted by different superscript (a,b,c) letters. Cisplatin induced a concentration and time dependent rise in LDH leakage which was prevented by the presence of RES.

Fig. 2. Lipid peroxidation induction by cisplatin

Renal slices and experimental design were as described in Fig. 1 and cisplatin exposure was for 120 min. Lipid peroxidation was expressed as nmol malondialdehyde (MDA)/ g tissue. Lipid peroxidation was increased by 75 and 150 µg/ml cisplatin when compared to respective 0 µg/ml treated tissue. RES prevented the rise in lipid peroxidation associated with cisplatin.

Fig. 3. Glutathione (GSH) decline with cisplatin exposure

Renal slices and experimental design were as described in Fig. 1; cisplatin exposure was for 120 min. Glutathione (GSH) levels were expressed as nmol GSH/g tissue. Values represent Mean $\pm$ SEM with $n=4$ different animals per group. Statistical ($p<0.05$) differences between groups were denoted by different superscript letters. GSH levels were decreased by 75 and 150 $\mu\text{g/ml}$ cisplatin when compared to control (0 $\mu\text{g/ml}$). RES pretreatment did not prevent cisplatin associated decline in GSH levels.

Fig. 4. Cisplatin inhibition of GSH Peroxidase Activity

Renal slices were pretreated for 30 min as described in Fig. 1. Renal slices were co-incubated with a final concentration of 0, 75 or 150 $\mu\text{g/ml}$ cisplatin for 60, 90 or 120 min. Glutathione (GSH) peroxidase activity was expressed as Units (U)/g tissue. Values represent Mean $\pm$ SEM with $n=4$ different animals per group. Statistical differences ($p<0.05$) between groups were denoted by different superscript (a,b,c) letters. GSH peroxidase activity was inhibited within 60 min by 75 and 150 $\mu\text{g/ml}$ cisplatin when compared to control (0 $\mu\text{g/ml}$). RES pretreatment prevented cisplatin mediated inhibition of GSH peroxidase activity.

Fig. 5. Cisplatin inhibition of Total SOD Activity

Renal slices were pretreated for 30 min as described in Fig. 1. Renal slices were co-incubated with a final concentration of 0-150 $\mu\text{g/ml}$ cisplatin for 60 or 120 min. Total Superoxide dismutase activity was expressed as Units (U)/g tissue. Values represent Mean $\pm$ SEM with $n=4$ different animals per group. Statistical differences ($p<0.05$) between groups were denoted by different superscript (a,b,c) letters. Total SOD activity was inhibited within 60 min by 75 and 150 $\mu\text{g/ml}$ cisplatin when compared to control (0 $\mu\text{g/ml}$). RES pretreatment prevented cisplatin mediated inhibition of SOD activity.

Fig. 6. Cisplatin inhibition of Mn SOD Activity

Renal slices were pretreated for 30 min as described in Fig. 1. Renal slices were co-incubated with a final concentration of 0-150 μ g/ml cisplatin for 60 or 120 min. Mn SOD activity was expressed as Units (U)/g tissue. Values represent Mean $\pm$ SEM with n=4 different animals per group. Statistical differences ($p < 0.05$) between groups were denoted by different superscript (a,b,c) letters. Cisplatin inhibited Mn SOD activity within 60 min at all concentrations when compared to control (0 μ g/ml). RES pretreatment prevented cisplatin mediated inhibition of Mn SOD activity.

Fig. 7. Western Blot Analysis for Protein Carbonyls following Cisplatin exposure
Renal slices were pretreated for 30 min with 30 μ g/ml resveratrol (RES) or ethanol (VEH). Renal slices were then co-incubated for 60 min with a final concentration of 0, 75 or 150 μ g/ml cisplatin. Western blot analysis used an OxyBlot Kit to detect protein carbonyls (top right). Each lane was loaded with 25 μ l volume containing 20 μ g protein. Protein bands were visualized using MemCode reversible staining (bottom left of OxyBlot gel; densitometry for protein loading of bottom right). Densitometry for protein carbonyls is shown in top right panel and is for the entire lane. Values were expressed as percent of VEH normalized to MemCode densitometry for the entire lane. Statistical ($p < 0.05$) differences between groups were denoted by different superscript letters.

Fig. 8. Western Blot Analysis of 4-HNE following Cisplatin exposure

Renal slices were treated as described in Figure 7. Western analysis was performed after a 60 min exposure to cisplatin using 100 μ g protein loaded into each lane. Gel is representative run. Densitometry was performed on bands at 27 and 45 kDa and intensity was expressed relative to Veh (-RES) with n=4 different animals. Densitometry was normalized to MemCode densitometry for the entire lane. Statistical ($p < 0.05$) differences between groups were denoted by different superscript letters.

Fig. 9. Western Blot Analysis of TNF α following 120 min Cisplatin exposure

Renal slices were pre-treated with ethanol or RES for 30 min followed by a 120 min co-incubation with cisplatin. Gel is representative run. Densitometry was performed on protein load and TNF α bands from 4 different animals. Densitometry was normalized to MemCode densitometry for the entire lane. No statistical differences were noted ($p>0.05$) between groups.

Fig. 10. RES pretreatment for 30 min followed cisplatin exposure in the absence of RES
Renal slices were pretreated for 30 min with ethanol (VEH) or 30 μ g/ml resveratrol (RES). Renal slices were rinsed in 3 ml Krebs buffer and placed in 3 ml fresh Krebs buffer devoid of ethanol or RES. Slices were then incubated with a final concentration of 0, 75 or 150 μ g/ml cisplatin for 120 min at 37°C and constant shaking in an oxygen atmosphere. Lactate dehydrogenase (LDH) release was expressed as percent of total LDH. Values represent Mean $\pm$ SEM with n=4 different animals per group. Statistical ($p < 0.05$) differences between groups were denoted by different superscript letters. A 30 min treatment with RES followed by removal of RES from the media was protective for cisplatin induced increase in LDH leakage.

Fig. 11. GSH Peroxidase activity is protected by RES pretreatment for 30 min followed cisplatin exposure in the absence of RES

Renal slices were treated as described in Figure 7. GSH peroxidase activity was expressed as U/g tissue. Values represent Mean $\pm$ SEM with n=4 different animals per group. Statistical ($p < 0.05$) differences between groups were denoted by different superscript letters.