The Plant-derived Natural Compound Flavin 7® Attenuates Oxidative Stress in Cultured Renal Proximal Tubule Cells

AGOSTON EMBER1, JEB S. CLARK2, TIMEA VARIAS3, ISTVAN KISS3, ISTVAN EMBER3, RADHAKRISHNA BALIGA2 and ISTVAN ARANY2

1Department of Surgery, and 3Department of Public Health and Preventive Medicine, University of Pecs, Hungary; 2Department of Pediatrics, Division of Pediatric Nephrology, University of Mississippi Medical Center, Jackson, MS, U.S.A.

Abstract. Background: Cancer therapies and cancer progression can increase oxidative stress that might account for renal toxicity in cancer patients. Flavin 7® (F7) is a natural polyphenol-containing dietary supplement with potential antioxidant activity. Therefore, it might help to attenuate renal toxicity of chemotherapeutics. Materials and Methods: Cultured mouse renal proximal tubule cells were subjected to H2O2-mediated oxidative stress. Potential antioxidant effects of F7 were assessed by measuring the production of reactive oxygen species (ROS), mitochondrial depolarization and injury (lactate dehydrogenase release as well as trypan blue exclusion) in cells that were pretreated with F7 prior to treatment with H2O2. Results: F7 pretreatment significantly attenuated H2O2-induced ROS production, mitochondrial depolarization and consequent injury in renal proximal tubule cells. Conclusion: F7 supplementation might be beneficial for cancer patients in order to prevent renal toxicity of anticancer drug- or cancer progression-related oxidative stress.

It has been shown that various antitumor therapeutics (1) and tumor progression (2, 3) can increase generation of reactive oxygen species (ROS) that might impose an increased risk for injury of the proximal tubules in the kidney. Indeed, higher incidence of renal failure has been demonstrated in cancer patients (4, 5) and antioxidants have been shown to ameliorate renal toxicity of chemotherapeutics (6-8). Thus, cancer patients might benefit from various antioxidant therapies (9). Plant-derived natural products such as polyphenols have shown renoprotective effects from oxidative stress in humans and animal models (10, 11). Flavin7® (F7) is a fruit extract that contains natural polyphenols in high concentration (220 mg/10 ml). F7 has been advised for use in enhancing the health of those coping with cancer and chemotherapy as it may attenuate side-effects of chemotherapeutics. Due to its high polyphenol content, it might exert these beneficiary effects in cancer patients through antioxidant activities. Thus, the aim of this study was to determine whether or not F7 demonstrates antioxidant properties in immortalized renal proximal tubule cells that were exposed to oxidative stress.

Materials and Methods

Cell culture and treatment. Immortalized mouse proximal tubule cells (TKPTS-gift from Dr. Reuss) (12) were grown in 5% CO2 atmosphere at 37°C as described elsewhere (13). Oxidative stress was induced by treatment of semi-confluent cells with 400 μM H2O2 for 24 hours similar to the model described elsewhere (14). In certain experiments cell cultures were pretreated with 50 μl/ml F7 (Crystal Institute Kft, Eger, Hungary) for 30 minutes prior to treatment with H2O2. This dose of F7 was found to be non-toxic by us and others in previous experiments (15, 16).

Assessment of cell injury. Cell morphology was determined by a phase-contrast inverted microscope (Nikon Eclipse TS-100F; magnification: x100). The number of surviving cells was determined by measuring the production of reactive oxygen species (ROS) that might impose an increased risk for injury of the proximal tubules in the kidney. Indeed, higher incidence of renal failure has been demonstrated in cancer patients (4, 5) and antioxidants have been shown to ameliorate renal toxicity of chemotherapeutics (6-8). Thus, cancer patients might benefit from various antioxidant therapies (9). Plant-derived natural products such as polyphenols have shown renoprotective effects from oxidative stress in humans and animal models (10, 11). Flavin7® (F7) is a fruit extract that contains natural polyphenols in high concentration (220 mg/10 ml). F7 has been advised for use in enhancing the health of those coping with cancer and chemotherapy as it may attenuate side-effects of chemotherapeutics. Due to its high polyphenol content, it might exert these beneficiary effects in cancer patients through antioxidant activities. Thus, the aim of this study was to determine whether or not F7 demonstrates antioxidant properties in immortalized renal proximal tubule cells that were exposed to oxidative stress.

ROS production assay. The intracellular generation of ROS was determined by a microplate assay using the oxidant-sensitive 2',7'-dichlorofluorescin-diacetate (DCFDA; Invitrogen, Grand Island, NY, USA). Accordingly, cells were loaded with 100 μM DCFDA for 30 minutes at 37°C. After washing with Hank’s balanced salt solution
(HBSS) cells were isolated by trypsinization, counted and 5×10^5 cells were placed in wells of a 96-well plate. The following groups were set up: i) untreated cells; ii) cells treated with 400 μM H_2O_2; iii) cells incubated with 50 μg/ml F7; and iv) cells incubated with 50 μg/ml F7 for 30 minutes then 400 μM H_2O_2 was added. Untreated, F7- and H_2O_2-treated cells were also processed. ROS production was determined as an increase in DCFDA fluorescence as measured in a fluorescence plate reader at 485nm<sub>exc</sub>/530nm<sub>em</sub>. All experiments were performed in triplicate and results expressed as mean±S.D.

Assessment of mitochondrial depolarization. JC-1 (Invitrogen) is a fluorescent dye that accumulates potential-dependently in mitochondria. In polarized mitochondria, J aggregates are formed that exhibit red fluorescence. Conversely, depolarization of mitochondria is indicated by a decrease in red fluorescence. Cell suspensions in HBSS were loaded with 2 μM JC-1 for 30 minutes at 37°C. After washing with HBSS buffer cells were seeded in 96-well plates (5×10^5 cells/well) and treated with 50 μg/ml F7 for 30 minutes then 400 μM H_2O_2. DCFDA fluorescence was detected at various time points (between 0 and 120 minutes) in a fluorescence plate reader (Packard) at 485nm<sub>exc</sub>/530nm<sub>em</sub>.  

Statistical evaluation. Statistical differences between the treated and control groups were determined by Student’s t-test. Differences between means were considered significant if p<0.05. All analyses were performed using a SigmaStat 3.5 software package (Systat, San Jose, CA, USA).

Results

F7 Attenuates H_2O_2-mediated ROS production. As shown in Figure 1, H_2O_2 treatment significantly increased ROS production (DCFDA fluorescence) compared to the control (untreated) cells. While F7 treatment alone did not significantly affect ROS production, pretreatment with F7 prior to treatment with H_2O_2 virtually quenched ROS release.

F7 Attenuates H_2O_2-mediated mitochondrial depolarization. The source of the H_2O_2-mediated ROS production in renal proximal tubule cells is the mitochondria (manuscript in preparation). It has been well established that increased mitochondrial ROS release results in mitochondrial depolarization and consequent cell injury (17, 18). Thus, we determined whether the H_2O_2-mediated ROS production was associated with depolarization of the mitochondria using the mitochondrial polarity-sensing fluorescent dye (JC-1) as described in Materials and Methods. As seen in Figure 2, H_2O_2 treatment significantly (p<0.05) depolarized the mitochondria as assessed by decrease in J aggregate fluorescence. Importantly, while F7 itself did not change mitochondrial polarity, pretreatment with F7 significantly (p<0.05) attenuated H_2O_2-induced mitochondrial depolarization.

F7 attenuates H_2O_2-induced cytotoxicity and consequent cell death. In this set of studies, we evaluated whether attenuation of H_2O_2-mediated ROS production and mitochondrial depolarization by F7 would affect survival of cells that undergo necrotic death in the oxidative injury model (14). First, the effects of F7 on H_2O_2-induced cytotoxicity were assessed by LDH release 24 after treatment. As shown in Figure 3, H_2O_2 treatment greatly increased LDH release, which was significantly reduced (p<0.05) in cells that were pretreated with F7 prior to...
treatment with H$_2$O$_2$. F7 itself did not change LDH release significantly. We also showed that cell morphology and the number of surviving cells significantly ($p<0.05$) changed after treatment with H$_2$O$_2$, consistent with a necrotic cell death as described earlier (14). While F7 treatment itself did not change either cell count or morphology, pretreatment with F7 virtually protected TKPTS cells from H$_2$O$_2$-mediated cell death (Figure 4).

Discussion

Generation of ROS plays an important role in the development of kidney diseases (19). Cancer therapies and cancer progression have shown to be associated with increased ROS production (2, 3), with potential involvement of renal damage. Therefore, antioxidants and ROS scavengers should prevent oxidative stress and consequent renal damage (20-22). Indeed, some plant-derived flavonoids showed protection against oxidative injury of the kidney [reviewed in (21)]. F7 is a natural bioflavonoid-containing dietary supplement with potential antioxidant activity. Our results showed that F7, indeed, significantly reduced ROS production triggered by H$_2$O$_2$ treatment in cultured renal proximal tubule cells (Figure 1).

Mitochondrial dysfunction is identified in either the etiology or underlying pathology of a variety of renal diseases that are associated with oxidative stress (23, 24). The elevated ROS production contributes to the opening of the mitochondrial permeability transition pore (25) that rapidly depolarizes the mitochondrial membrane: a process termed mitochondrial permeability transition. Mitochondrial permeability transition is a major contributor to apoptotic and necrotic cell death in a variety of cell types, including renal cells (26). Here, we showed that increased ROS production initiated by H$_2$O$_2$ treatment in vitro increased mitochondrial depolarization (Figure 2) and consequent injury as demonstrated by increased LDH release (Figure 3), changes in cell morphology (Figure 4A) and decrease in cell counts (Figure 4B). Importantly, pretreatment of cells with F7 attenuated depolarization of mitochondria (Figure 2) and LDH release (Figure 3). As a result of F7 pretreatment cells were virtually protected from H$_2$O$_2$-induced cell death (Figure 4).

These experiments suggest that F7 protects renal proximal tubule cells from oxidative injury from ROS, preventing the consequent mitochondrial depolarization. Thus, F7

Figure 3. LDH release in renal proximal tubule cells after treatment with H$_2$O$_2$ in the presence or absence of F7. TKPTS cells grown in 12-well plates were pretreated with 50 μg/ml F7 for 30 minutes then 400 μM H$_2$O$_2$ was added: some wells received F7 or H$_2$O$_2$ treatment only, or were left untreated. After 24 hours' incubation at 37°C, LDH release was determined as described in Materials and Methods. Each experiment was carried out in triplicate: values are expressed as percentage of untreated control (N=3, mean±S.D.) *p<0.05 compared to the untreated control; #p<0.05 compared to H$_2$O$_2$-treated group.

Figure 4. Morphology (A) and surviving cell counts (B) of TKPTS cells after treatment with H$_2$O$_2$ in the presence or absence of F7. A, TKPTS cells were pretreated or not with 50 μg/ml F7 for 30 minutes prior to treatment with 400 μM H$_2$O$_2$. After 24 hours' incubation at 37°C, images were taken (magnification: ×100). Images shown are representative of three independent experiments. B, Cell numbers were determined by trypan blue exclusion in cells that were treated in (A). Cell survival was expressed as cell counts of untreated values. *p<0.05 compared to the untreated control; #p<0.05 compared to H$_2$O$_2$-treated group.
supplementation might be beneficial for cancer patients in order to prevent renal toxicity of chemotherapy. Whether this effect is due to activation of antioxidant defenses or direct inhibition of ROS production needs further evaluation.

Acknowledgements

Financial support provided by an American Heart Association Midwest Affiliate Grant-in-Aid (0655716Z, I.A.) and an Intramural Research Support Program Award from the University of Mississippi Medical Center (I.A). We wish to thank Laszlo Szabo (Crystal Inst. Kf, Eger, Hungary) for providing us with Flavin 7®.

References


Received June 3, 2009
Revised September 2, 2009
Accepted September 21, 2009