Journal of Ethnopharmacology 179 (2016) 412–419

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Protection against oxidative stress-induced apoptosis in kidney epithelium by *Angelica* and *Astragalus*

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ARTICLE INFO

Article history:
Received 2 June 2015
Received in revised form
6 December 2015
Accepted 20 December 2015
Available online 21 December 2015

Keywords:
Apoptosis
Oxidative stress
Kidney epithelium
Inflammation
TNF-α

ABSTRACT

Ethnopharmacological relevance: *Astragalus membranaceus* either alone or in combination with *Angelica sinensis* has been used traditionally for kidney disease in East Asia and China for thousands of years. Previous studies using *in vivo* animal models have shown the benefits of these medicinal herbs in kidney diseases that involve oxidative stress. However, the mechanisms by which these medicinal herbs protect kidney cells remain largely unknown.

Aim of the study: To investigate the mechanisms by which ethanol, methanol and aqueous crude extracts of roots of *A. membranaceus* and *A. sinensis* afford protection to human kidney proximal tubular epithelial cells, using an *in vitro* model of oxidative stress.

Materials and methods: Ethanol, methanol and aqueous extracts of roots of *A. membranaceus and A. sinensis* were prepared by a three-solvent sequential process. HK2 human kidney proximal tubular epithelial cells were treated with H_2O_2 alone (0.5 mM) or in combination with different concentrations of extracts. Cell mitosis and death (microscopy) and cell viability (MTT assay) were compared. Western immunoblot was used to study expression of apoptosis-related proteins (pro-apoptotic Bax andanti-apoptotic Bcl-X_L), and cell survival (NFκB subunits p65 and p50), pro-inflammatory (TNF-α) and protective (TGFβ1) proteins.

Results: H_2O_2-induced oxidative stress significantly increased apoptosis and reduced cell survival; up-regulated pro-apoptotic and down-regulated Bcl-X_L; increased NFκB (p65, p50); increased TNFα and decreased TGFβ1. All changes indicated kidney damage and dysfunction. All were modulated by all extracts of both plant species, except for NFκB which was only modulated by extracts of *A. membranaceus.*

Conclusions: In conclusion, in a model of oxidative stress that might occur after nephrotoxicity, the plant extracts were protective via anti-apoptotic and anti-inflammatory mechanisms.

1. Introduction

Oxidative stress is a known contributor to the progression and development of acute and chronic kidney diseases (Agarwal, 2003; Oberg et al., 2004; Calabrese et al., 2007; Gao et al., 2012). Chronic kidney disease (CKD) is an increasing problem worldwide, with current conventional medicine treatments having limited positive effects (Wojcikowski et al., 2004a,b; Small et al., 2012a). Alternative therapies that can replace or complement conventional medicines may improve outcome for CKD patients. There is also a need to investigate the mechanism of action of the alternative therapies, especially how these complementary and alternative therapies protect intrinsic kidney cell populations that may be involved in tubular atrophy, and modulate inflammation that may be damaging to the kidney.

Oxidative stress is a result of perturbations in normal oxidant signalling networks, primarily regulated by reactive oxygen species (ROS) and endogenous antioxidants (Small et al., 2012b). Kidney proximal tubular epithelial cells contain large numbers of mitochondria and are the most reliant upon oxidative phosphorylation and most susceptible to ROS-induced injury of cells of the kidney nephron (Agarwal, 2003). They therefore present an important site of dysfunction and destruction when oxidant loads

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http://dx.doi.org/10.1016/j.jep.2015.12.027
0378-8741/

exceed antioxidant capabilities. Hydrogen peroxide (H_2O_2) is an important and essential precursor to harmful ROS such as the hydroxyl radical (OH^-) and peroxynitrite ($ONOO^-$) and is considered a significant mediator in the progression of kidney diseases (Singh et al., 2007), often via induction of apoptosis.

One of the most commonly studied of the pro-apoptotic members of the B-cell lymphoma-2 (Bcl-2) family is Bax, and of the anti-apoptotic members, Bcl-X_L appears to be effective in kidney tissue protection (Cuttle et al., 2001). The ratio of Bax to Bcl-X_L is a crucial factor which regulates susceptibility of cells to apoptosis (Kroemer, 1997; Gobe et al., 2002). The nuclear factor-kappa B (NF-κB) transcription factor gene family has many functions, including those in apoptosis, cell survival, and inflammation (Meteoglu et al., 2008; Morais et al., 2011). TNF-α acts as an inflammatory mediator and plays a significant role in the immune response in chronic kidney disease (Frigo et al., 2005; Lee et al., 2015). Importantly, ROS are potent activators of TNF-α-mediated apoptosis (Kim et al., 2010). Studies have demonstrated the anti-apoptotic role of TGFβ1 in different cells, such as, hepatic stellate cells, microglia, mammary epithelial cells, and osteoblasts (Sanchez-Capelo, 2005). However, in many cases of oxidant-induced injury in the kidney, TGFβ1 may be pro-apoptotic, or pro-fibrotic (Cummins et al., 2010; Zhao et al., 2014). Robust analysis of how these apoptotic or inflammatory pathways are modulated by various alternative therapies would be beneficial.

Angelica sinensis (Oliv.) Diels (Apiaceae) and *Astragalus membranaceus* (Fisch.) Bunge (Fabaceae) are folkloric Chinese herbs and are used traditionally for treatment of kidney diseases. In the traditional system of medicine, extracts of the roots of *A. membranaceus* are used alone or in combination with extracts of *A. sinensis* roots to treat patients with chronic kidney disease (Li et al., 2011; Zhong et al., 2013). Previous studies by using *in vivo* animal models have shown their protective benefits against ischaemia–reperfusion injury (Cai et al., 2001), unilateral urinary obstruction and fibrosis in rat (Wojcikowski et al., 2010), chronic puromycin-aminonuclease nephrosis (Wang et al., 2004), and improved renal microvascular insufficiency in nephrectomized rats (Song et al., 2009). Nonetheless, the mechanisms by which these medicinal herbs protect kidney cells remain largely unknown. This study aimed to investigate the mechanisms by which extracts of roots of *A. sinensis* and *A. membranaceus* afford protection to oxidative stress-injured human kidney proximal tubular epithelial cells, using an *in vitro* model.

2. Materials and methods

2.1. Plant extracts

Roots of *A. sinensis* (Oliv.) Diels (Chinese names Danggui, Dong quai, Donggui; Voucher number CP-04-0079) and *A. membranaceus* (Fisch.) Bunge (Chinese name Huang-Qi; Voucher number CP-04-206) were obtained from a reliable supplier and authenticated by a pharmacognosist (Dr. H. Wohlmuth, Southern Cross University Medicinal Plant Herbarium) by chemical and morphological comparison with an authentic reference specimen. The plant names were checked with www.theplantlist.org at 29-05-2015. Dried plant root material was ground to a powder and extracted by a three-solvent sequential process. 20 g ground material was sonicated (10 min) in 200 ml anhydrous solvent (sequentially, ethanol at 40 °C, methanol at 40 °C, and water at 90 °C) and filtered (Whatman No. 3, gravity filtration) and then repeated for a second and third extraction (Wojcikowski et al., 2007). All the filtrates were vacuum dried using a rotary vacuum centrifuge. The resultant products were weighed then resuspended with dimethyl sulphoxide (DMSO) at a predetermined concentration, so that DMSO content in culture medium, at concentrations selected for the plant extracts, never exceeded 1% in the growth medium. At this concentration DMSO had negligible effects on HK2 cells.

2.2. Cell culture

The human renal proximal tubular epithelial cell line, HK2, was obtained from the American Type Culture Collection (ATCC) (Rockville, MA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 containing 10% fetal bovine serum (FBS; BioWhittaker Australia, Mt. Waverley, Australia) with penicillin (1000 U/ml) and streptomycin (1000 U/ml) (Life Technologies Pty Ltd., Mt. Waverley, Australia) in 5% CO_2 under a humidified atmosphere at 37 °C. For microscopy, cells were seeded onto sterile glass coverslips in multi-well cell culture plates (1×10^4 cells/ml). For protein extractions, cells were grown in 10 cm Petri dishes, also containing glass coverslips for concurrent microscopy. Cells were grown in 96-well plates for cell viability studies. Cell number was determined using the trypan blue exclusion method with an improved Neubauer haemocytometer.

2.3. Treatments

Dose response studies were carried out for an H_2O_2 concentration that induced apoptosis but not necrosis in HK2 cells (range 0–1 mM H_2O_2). HK2 cells were treated at 80–90% confluence. From these pilot studies, a dose of 0.5 mM H_2O_2 for a treatment time of 24 h was selected. Similar treatment concentration and time were used by Small et al. (2014). Cells were pre-treated for 2 h or 24 h with ethanol, methanol and aqueous extracts of *A. sinensis* and *A. membranaceus* at concentrations of 0.3, 0.6 and 1.2 mg/ml. These concentrations were selected following initial dose–response experiments using concentrations of each extract ranging from 0.0045 to 5.0 mg/ml. DMSO vehicle control was used in the experiments. For Western blotting, only one dose (1.2 mg/ml) for each extract type of *A. sinensis* and *A. membranaceus* was chosen, because this showed best protection against H_2O_2-induced apoptosis in MTT assays and microscopy studies. Initial MTT assays showed better protection of extracts with 2 h pre-treatment compared with 24 h pre-treatment. Thus, all further experiments were carried out with 2 h pre-treatment. Experiments were carried out in triplicate ($n=3$).

2.4. Cytology

Cells on glass coverslips were fixed overnight in 4% buffered paraformaldehyde at 4 °C, washed with PBS and stored at 4 °C. Cells were stained using haematoxylin and eosin (HE). Microscopy (X 200) and morphology were used to determine the number of apoptotic and mitotic cells. Aperio Image Scope digital histology software was used to assist in distinguishing the morphological features. Data were obtained by counting ten frames of cells for each treatment and calculating % apoptotic and mitotic cells per total number of cells in each frame. Means ± standard deviation (SD) were then calculated and comparisons made amongst controls and treatments using ANOVA. The morphological characteristics for apoptosis were: (i) shrunken eosinophilic cells with condensed, marginated nuclear chromatin and intact cell membrane (ii) discrete apoptotic bodies compromising large, dense, pyknotic nuclear fragments surrounded by a narrow eosinophilic cytoplasm and (iii) clusters of small apoptotic bodies (assessed as a single apoptotic occurrence) (Gobe, 2009). Morphological features for necrosis were pale swollen cells with indistinct nuclear and cellular membranes, plus eosinophilia in the cytoplasm. The morphological characteristics used to distinguish mitosis were: (i) formation of mitotic spindles occurring during metaphase and