Toxicology 263 (2009) 108–116

Contents lists available at ScienceDirect

Toxicology

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

Early loss of mitochondrial inner transmembrane potential in khat-induced cell death of primary normal human oral cells

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ARTICLE INFO

Article history:
Received 5 May 2009
Received in revised form 29 June 2009
Accepted 30 June 2009
Available online 8 July 2009

Keywords:
Apoptosis
Khat
Mitochondrial membrane potential
Oral

ABSTRACT

Previous studies suggest the use of khat, a psychostimulant plant used by millions of people in Middle East and Africa, as risk factor for oral cancer. We previously reported that khat is able to induce adverse affects, as cell cycle arrest and apoptosis, in normal human oral cells cultured *in vitro*. This study further investigates the more specific role played by mitochondria in khat-induced cell death and the kinetics of the events involved in this process. Exposure of primary normal human oral keratinocytes and fibroblasts to khat extract resulted in a swift and sustained decrease of the mitochondrial inner transmembrane potential occurring within 0.5–1 h. Loss of mitochondrial membrane potential preceded all other biochemical and morphologic changes, and was associated with a significant decrease in cell survival. Subsequently, apoptosis-inducing factor was released from mitochondria into cytosol and relocated to nucleus. Cyclosporine A and bongkrekic acid delayed both the loss of mitochondrial inner transmembrane potential and the onset of cell death. This study describes a novel mechanism of khat-induced cell death in primary normal oral keratinocytes and fibroblasts involving an early pivotal effect on mitochondrial function and integrity.

1. Introduction

Khat (*Catha edulis*) is a psychostimulant drug derived from an evergreen shrub of the Celastraceae family cultivated in parts of the Middle East and Eastern Africa (Al-Motarreb et al., 2002). The habit of chewing khat has prevailed for centuries among populations in the regions where it is grown, and its use is gradually spreading to other parts of the world. Khat chewing is associated with harmful effects and pathological conditions in various organ systems as well as in oral tissues (Al-Habori, 2005). People who chew khat for prolonged periods have been found to have a higher incidence of head and neck cancer when compared with those who do not chew khat (Soufi et al., 1991; Nasr and Khatri, 2000). It has been shown that khat is genotoxic to human oral cells (Kassie et al., 2001), and evidence from animal studies shows that khat is able to decrease the systemic capacity of the body to handle reactive oxygen species (Al-Qirim et al., 2002). Reports from *in vitro* studies, have shown that khat is able to inhibit *de novo* synthesis of proteins, RNA and DNA (Al-Ahdal et al., 1988), and to induce apoptosis in human cells (Dimba et al., 2004; Lukandu et al., 2008a). However, there is still a lack of knowledge on the specific mechanism(s) involved in khat-induced cytotoxicity (Carvalho, 2003) and the mechanisms leading to the development of khat-related pathological changes, including cancer of oral mucosa are not yet fully elucidated (Carvalho, 2003).

We previously reported that khat is able to induce adverse affects, as cell cycle arrest and apoptosis, in normal human oral cells cultured *in vitro* (Lukandu et al., 2008b). Apoptosis is a type of cell death that is accompanied by reduction in cell volume and maintenance of an intact plasma membrane at the initial stages of the cell death process (Kerr et al., 1972). There are various pathways linking mitochondria to apoptosis. One pathway is dependent on release of cytochrome *c* (Cyt *c*), and apoptosome-mediated caspase activation and another pathway involves the release of apoptosis-inducing factor (AIF) (Joza et al., 2001). AIF is an evolutionarily conserved flavoprotein involved in mitochondrial respiration. AIF

Abbreviations: Cyt *c*, cytochrome *c*; AIF, apoptosis-inducing factor; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; CsA, cyclosporine A; BA, bongkrekic acid; $\Delta\Psi_m$, mitochondrial inner transmembrane potential; MMP, mitochondrial membrane permeabilization; mPTP, mitochondrial permeability transition pore.

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doi:10.1016/j.tox.2009.06.024

may be released into the cytosolic compartment in response to apoptogenic cell signaling. It has been reported that AIF translocates to the nucleus and causes a characteristic chromatin condensation, DNA degradation and cell death in a process not dependent on caspase activation (Grimm and Brdiczka, 2007; Susin et al., 2000). Mitochondrial membrane permeabilization (MMP) has been proposed as a 'point-of-no-return' during different types of cell death (Kroemer et al., 2005). Both the outer mitochondrial membrane (OM) and the inner mitochondrial membrane (IM) contribute towards MMP (Galluzzi et al., 2007). Permeabilization of IM causes a loss in the electrochemical potential ($\Delta\Psi_m$) that normally exists across it. Effects on the integrity and permeabilization of OM may lead to release of apoptogenic proteins such as Cyt *c*, apoptosis-inducing factor (AIF) and endonuclease G that are normally located in the mitochondrial intermembrane space (Kroemer et al., 2007).

In our previous work, we have shown that khat-induced apoptosis in normal primary oral cells was a process dependent on generation of reactive oxygen species and depletion of cellular glutathione (Lukandu et al., 2008a). This study further investigates the more specific role played by mitochondria in khat-induced cell death and the kinetics of the events involved in this process. We report that khat caused an early loss of mitochondrial membrane potential ($\Delta\Psi_m$) and release of AIF into the cytosol. These effects were closely associated with loss of cell viability and survival. It is proposed that cell death by khat could be caused by a specific effect on the permeability transition pore complex in mitochondria. By confirming the toxic potential of khat on human oral mucosa and identifying mitochondria as a key target for its main mechanism of action, the findings of this study bring evidence that raises important concern on khat use for oral health.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), Trypsin-EDTA, dimethyl sulphoxide (DMSO), saponin, bongkrekic acid (BA), cyclosporine A (CsA), anti-AIF, and anti-rabbit IgG-TRITC were from Sigma (St. Louis, MO, USA). Serum free keratinocyte medium (KSFM), human recombinant epidermal growth factor (EGF), bovine pituitary extract (BPE), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin and amphotericin-B were from GibcoBRL (Grand Island, NY, USA). Cell culture chambers (Lab-Tek® Chamber Coverglass™) and cell culture cover slips were from Electron Microscopy Sciences (Hatfield, PA, USA) whereas cell culture flasks and dishes were from Nunc (Napervile, IL, USA). Cell proliferation reagent (WST-1) was from Roche Diagnostics GmbH (Mannhein, Germany). Fluorescent dyes Hoechst 33342 and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) were bought from Molecular Probes Europe (Leiden, Netherlands). Vectorshield® Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories (Burlingame, CA, USA). The cell fractionation kit (ApoAlert™) which contained anti-Cyt *c* oxidase subunit IV (anti-COX IV), and anti-Cyt *c* among other reagents, was from Clontech Laboratories (Mountain View, CA, USA). Anti-Bcl-2 6C8 was from BD Biosciences (Franklin Lakes, NJ, USA) whereas α-caspase-3 E8 and anti-p53 (sc-263) were from Santa Cruz Inc. (Santa Cruz, CA, USA).

2.2. Khat extraction

Khat extraction and analysis by LC/MS/MS (liquid chromatography/mass spectrometry/mass spectrometry) was done as previously described (Dimba et al., 2004). Briefly, 200 g of fresh khat shoots from the Meru district of Kenya were chopped into small (5 mm) pieces and dissolved in 50 ml methanol. The mixture was sonicated at room temperature (RT) while shielding from light for 15 min, and filtered through an 11 μm filter (grade 1, Whatman, Kent, UK). The nonfiltered plant residue was re-extracted in 50 ml fresh methanol and sonicated for 24 h. The mixture was filtered and admixed with the initial 50 ml filtrate. The resultant solution was concentrated at 337 mbar in a Rotorvapor vacuum drier (Büchi, Switzerland) for 4–5 h into an oily paste. The 200 g fresh plant material yielded about 12.6 g of this oily paste. This was dissolved in 40 ml DMSO (0.315 g/ml). Aliquots (each of 200 μl) were stored at −80 °C. The quality of the extraction procedure was verified by confirmation of the presence of khat-specific phenylpropylamines (cathinone, cathine, and norephedrine) in the alkaloid fraction using differential thin layer chromatography. A droplet of the methanolic extract was spotted onto a silica plate (Kieselgel F-254, Merck, Darmstadt, Germany) and developed in ethyl acetate:methanol:ammonia (85:10:5). Detection of these compounds was done by development of the plate in a 0.5% ninhydrin (Merck, Darmstadt, Germany) solution in ethanol and heating it (110 °C, 5 min) to demonstrate a clear separation between cathinone (retardation factor 0.6) and the other two alkaloids (retardation factor 0.45). The organic extract was also evaluated for the amount of khat-specific phenylpropylamines by LC/MS/MS (liquid chromatography/mass spectrometry/mass spectrometry) as previously described (Dimba et al., 2004). Prior to each experiment, a new batch of khat stock solution was thawed at room temperature, diluted in cell culture media to a concentration of 10 mg/ml and centrifuged at 3000 × g_{av} (average gravity) for 10 min at RT. The supernatant fraction was collected after centrifugation and adjusted to the appropriate concentrations to be tested in each experiment.

2.3. Cell culture

Primary normal oral keratinocytes and fibroblasts were isolated from superfluous tissues of the buccal (cheek) mucosa from clinically healthy adult volunteers undergoing surgical removal of wisdom teeth. All patients included were informed of the purpose of the study and signed consent forms. The study was approved by the Regional Committee for Medical Ethics in Research. Cells were isolated through a combination of enzymatic digestion and mechanical separation of cells, as previously described (Costea et al., 2002, 2005). Keratinocytes were cultured in serum-free media (KSFM) supplemented with 1 ng/ml EGF, 25 μg/ml BPE, 20 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Fibroblasts were cultured in DMEM supplemented with 10% FBS, 20 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Cells used in the experiments were in their 1st to 3rd passage or 2nd to 5th passage for keratinocytes and fibroblasts, respectively. Control cultures were routinely supplemented with DMSO equal to the amount present in the highest khat concentration tested, and this never exceeded 0.3%

2.4. Determination of cell viability

Cell viability was assessed using the WST-1 reagent, a water-soluble tetrazolium salt that is cleaved by succinate dehydrogenases in viable cells to produce a water-soluble formazan which can then be quantified by colorimetric measurements (Slater et al., 1963). Cells were cultured in 96 well plates and exposed to khat for various time points, then treated with WST-1 reagent diluted in culture media (dilution 1:10). After 4 h exposure to the reagent, absorbance at 450 nm (reference 630) was determined using a multi-well plate reader (Infinite 200, Tecan Schweiz AG, Männedorf, Switzerland). The absorbance in each test was expressed as a percentage of the control cultures. Each test was carried out in duplicate, and each experiment was repeated at least three times. Alternatively, cell viability was assessed by morphology as previously described (Slater et al., 1963). In these experiments cells grown in 48 well plates were evaluated morphologically using phase contrast microscopy (Axiovert 25 inverted microscope, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) or stained with Hoechst 33342 (8.1 μM) and evaluated using fluorescence microscopy (Leica IRB inverted microscope, Leica Microsystems GmbH, Wetzlar, Germany).

2.5. Cell attachment assay

Oral keratinocytes and fibroblasts were seeded in 25 cm^2 flasks and allowed to grow to approximately 50% confluency. Cells were then exposed to khat (range 10–316 μg/ml) or 0.1% DMSO (controls) for various time durations (range 0.5–2 h) after which the medium containing khat was discarded, and the cells detached in 0.25% trypsin/0.05% EDTA solution, 37 °C, 5 min. Cells were washed twice in pre-warmed (37 °C) culture medium, counted using a hemocytometer, and then reseeded at the same cell density (10^4 cells/cm^2) in fresh culture medium and allowed to re-attach for a 24 h period onto a culture dish. Afterwards, cells were washed three times in phosphate buffered saline (PBS, pH 7.4) thereby removing floating and loosely attached cells from the cultures, trypsinised (as described above) and their numbers determined using a haemocytometer. Cells having an apparent normal morphology and a retaining the capacity to attach to a growth substrate were considered functionally viable in this study.

2.6. Determination of mitochondrial membrane potential ($\Delta\Psi_m$)

Cells were seeded in 25 cm^2 flasks, allowed to grow to 50% confluency and then treated with various concentrations of khat. At various time points, cells were harvested (0.25% trypsin and 0.05% EDTA, 37 °C, 5 min), centrifuged (200 × g_{av}, 37 °C, 7 min) and the cell pellet washed in pre-warmed (37 °C) culture medium. The cells were then resuspended in 1 ml of fresh pre-warmed (37 °C) culture media containing $DiOC_6(3)$ (40 nM) for 30 min. Analysis for fluorescence was done using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). In each sample, 30,000 events (cells) were included for analysis using Cell Quest software (Becton Dickinson). In an alternative approach the cells were seeded in chambered coverglass (Lab-Tek® Chamber Coverglass™) and allowed to grow up to 50% confluency. The cultures were supplemented with $DiOC_6(3)$ (40 nM) and Hoechst 33342 (0.81 μM) for 30 min before exposure to khat (316 μg/ml) or 0.1% DMSO (controls) for different time periods. The stained cells were then washed two times in fresh pre-warmed (37 °C) culture media and then placed in an incubation chamber, allowing constant temperature (37 °C) and 5% CO_2, while viewed by confocal microscopy. The fluorescence was