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Kaempferol inhibits *Entamoeba histolytica* growth by altering cytoskeletal functions

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ABSTRACT

The flavonoid kaempferol obtained from *Helianthemum glomeratum*, an endemic Mexican medicinal herb used to treat gastrointestinal disorders, has been shown to inhibit growth of *Entamoeba histolytica* trophozoites in vitro; however, the mechanisms associated with this activity have not been documented. Several works reported that kaempferol affects cytoskeleton in mammalian cells. In order to gain insights into the action mechanisms involved in the anti-amoebic effect of kaempferol, here we evaluated the effect of this compound on the pathogenic events driven by the cytoskeleton during *E. histolytica* infection. We also carried out a two dimensional gel-based proteomic analysis to evidence modulated proteins that could explain the phenotypical changes observed in trophozoites. Our results showed that kaempferol produces a dose-dependent effect on trophozoites growth and viability with optimal concentration being 27.7 μM.

Kaempferol also decreased adhesion, it increased migration and phagocytic activity, but it did not affect erythrocyte binding nor cytolytic capacity of *E. histolytica*. Congruently, proteomic analysis revealed that the cytoskeleton proteins actin, myosin II heavy chain and cortexillin II were up-regulated in response to kaempferol treatment. In conclusion, kaempferol anti-amoebic effects were associated with deregulation of proteins related with cytoskeleton, which altered invasion mechanisms.

1. Introduction

Amoebiasis caused by the protozoan parasite *Entamoeba histolytica* is an important health problem in Mexico and other developing countries in Latin America, Asia and Africa [1]. Taking into consideration the important side effects of the current treatment (Metronidazole) and the decrease in *E. histolytica* drug susceptibility, there is a renewed interest for the use of medicinal plants as a source of therapeutic alternatives. Flavonoids are secondary metabolites of plants with several biological activities. One of them is kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one), a compound with a low molecular weight (MW: 286.2 g/mol) and the classical flavonoid diphenylpropane structure (C6–C3–C6). This molecule has been identified in many vegetal species commonly used in traditional medicine; therefore, it has been the subject of numerous studies. Kaempferol possesses a wide range of biological activities, including antioxidant, anti-inflammatory, anticancer, antibacterial and antiviral activities. Importantly, it is also effective against several protozoa (for review see Ref. [2]). Calzada et al. reported that kaempferol obtained from *Helianthemum glomeratum*, an endemic medicinal herb used to treat diarrhea, abdominal pain and dysentery in Mexico, was one the most active flavonoids against *E. histolytica* and *Giardia lamblia* among 18 plant flavonoids [3]. Later, the same group confirmed that kaempferol was the most active flavonoid against these parasites; its activity was markedly decreased with the presence of a glucosyl moiety [4]. Kaempferol (IC50 = 10.3 ± 2.3 μg/ml) isolated from *Morinda morindoides* leaf extracts, showed a more pronounced antiamoebic activity than its corresponding glycosides, without any cytotoxic effect on MT-4 mammal cells [5]. The effects of kaempferol isolated from *Chresta scapigera*, *Acridocarpus chloropterus* and *Schima wallichii*, as well as chemically synthesized (Fluka, Buchs, Switzerland) against other protozoan parasites,

Abbreviation: IC50, 50% inhibitory concentration.

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including *Leishmania*, *Trypanosoma* and *Plasmodium*, have also been documented [6–9]. Structure-activity analyses suggested that 3-OH and 3′-OH groups on flavonoid contribute substantially to the strong anti-protozoal activity, while the presence of the phenolic group at the C-3 in kaempferol increased cytotoxicity [8]. However, the molecular mechanism underlying the effects of kaempferol on these protozoa remains unknown.

Several studies showed that kaempferol affects cytoskeleton in mammalian cells. Kaempferol enhanced tight junction (TJ) integrity through the cytoskeletal association and expression of TJ proteins in Caco-2 cells [10]. Additionally, kaempferol produced morphological changes in endothelial cells with the formation of cell extensions and filopodias at non cytotoxic concentrations [11]. To our best knowledge, there are not reports about the kaempferol effects on *E. histolytica* cytoskeleton. Pathogenesis of *E. histolytica* has been related to the organization and regulation of the cytoskeleton elements, since trophozoites motility is a key event during invasion of host tissue [12].

With the purpose of identifying molecular events associated with the antiamoebic effects of flavonoids, our group recently reported that the flavonoid (−)-epicatechin alters *E. histolytica* cytoskeleton proteins and functions, which could affect the pathogenic processes of this human pathogen [13]. In order to gain insights into the action mechanisms involved in the anti-amoebic effect of kaempferol, here we evaluated the effect of this compound on the pathogenic events driven by the cytoskeleton during *E. histolytica* infection. We also carried out a two dimensional gel-based proteomic analysis to evidence changes in global protein expression profile that could explain the phenotypical changes observed in the trophozoites.

2. Materials and methods

2.1. Cultures

E. histolytica trophozoites, HM1-IMSS strain, were axenically grown at 37 °C in TYI-S-33 medium, supplemented with 20% bovine serum [14]. Cells were harvested in the log phase of growth for all experiments. Human epithelial colorectal adenocarcinoma cells (Caco-2, HTB-37 ATCC, USA) were grown in advanced minimum essential medium (MEM, Gibco) supplemented with 5% fetal bovine serum, 200 mM glutamine (Gibco), 0.0125% penicillin and 0.02% streptomycin; cultures were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

2.2. Cell growth and viability assays

Kaempferol (Sigma–Aldrich Co.) was dissolved in 3 μl DMSO and 997 μl complete TYI-S-33 medium in sterile conditions to obtain a final concentration of 1 μg/μl. *E. histolytica* trophozoites (1.5×10^5) were grown in complete TYI-S-33 medium containing increasing kaempferol concentrations ranging from 3.9 μg/ml to 15.9 μg/ml (i.e., 13.8–55.5 μM). At 48 h, cells were harvested and resuspended in phosphate-buffered saline (PBS). Trophozoites number was determined in a Neubauer chamber and cell viability was measured using the trypan blue dye method as described [13]. Cells grown in complete TYI-S-33 medium and 0.05% DMSO was used as controls in all experiments. Data were expressed as the mean ± standard deviation (SD) of two independent experiments performed three times.

2.3. Cytotoxicity assays

Caco-2 cells were cultured into a 96-well microplate (3.0×10^4 cells/well) and grown for 24 h. Then, medium was replaced by fresh medium containing 27.7 μM kaempferol or 0.05% DMSO. At 48 h, supernatants were collected, centrifuged at 500 × *g* for 5 min, and transferred to a microtiter plate (50 μl/well) to determine lactate dehydrogenase (LDH) levels using the CytoTox 96® Non-radioactive Cytotoxicity Assay (Promega) following manufacturer recommendations. Cells in free medium and medium with 9% Triton X-100 were used as negative and positive controls, respectively. Cytotoxicity was determined from absorbance values at a 492 nm wavelength and expressed as: [(cells treated with kaempferol or DMSO-cells in free medium)/(cells of positive control-cells of negative control)] × 100. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

2.4. MTT assays

Caco-2 cells were cultured in a 96-well microplate (3.0 × 104 cells/well) in the presence of 27.7 μM kaempferol or 0.05% DMSO as described above. At 48 h, cells were incubated with 1 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) at 37 °C for 4 h. The medium was removed and formazan dye crystals were solubilized in 100 μl DMSO for 5 min. Cell viability was determined from absorbance at 570 nm wavelength as described [13]. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

2.5. Adhesion assays

Adhesion of trophozoites to Caco-2 cells was evaluated as described [13,15]. Briefly, Caco-2 cells monolayers were covered with 1 ml serum-free TYI-S-33 medium and incubated with trophozoites previously grown in 27.7 μM kaempferol for 48 h at 37 °C (one amoeba/four Caco-2 cells) for 1 h at 37 °C. Non-adherent trophozoites were collected by washing with serum-free TYI-S-33 medium and counted using the trypan blue dye method. The number of adherent trophozoites was expressed as the difference between the initial number of trophozoites in each well and the number of non-adherent trophozoites. Trophozoites grown in complete TYI-S-33 medium or 0.05% DMSO were used as controls. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

Erythrocytes binding to trophozoites were evaluated as described by Voigt et al. [16] with some modifications [13]. Trophozoites (2.0×10^5) previously treated with 27.7 μM kaempferol or 0.05% DMSO for 48 h at 37 °C were incubated with human erythrocytes (2.0×10^7) for 5 min on ice. Then, amoebas were fixed with 4% paraformaldehyde, free erythrocytes were removed by centrifugation and cells were stained by the Novikoff method [17,18]. Finally, the cellular suspension was observed through a Nikon Eclipse 80i microscope (20–100× magnification) connected to the Nis Elements Advanced Research Software, Nikon version 3.0. The number of attached erythrocytes per amoeba was counted from 100 randomly selected trophozoites. Experiments were performed twice by triplicated and results were expressed as mean ± SD.

2.6. Cell migration assays

Migration of *E. histolytica* trophozoites (5.0×10^4) previously treated with 27.7 μM kaempferol for 48 h at 37 °C was evaluated by the Transwell migration assay as described [13,19,20]. Trophozoites grown in complete medium or 0.05% DMSO were used as controls. The number of trophozoites that have migrated into the lower chamber was determined using Trypan blue assay. Experiments were performed twice by triplicate and results were expressed as mean ± SD.