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Protective effects of Ursolic acid and Luteolin against oxidative DNA damage include enhancement of DNA repair in Caco-2 cells

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ABSTRACT

Consumption of fruits and vegetables is associated with a reduced risk of developing a wide range of cancers including colon cancer. In this study, we evaluated the effects of two compounds present in fruits and vegetables, ursolic acid, a triterpenoid, and luteolin, a flavonoid, on DNA protection and DNA repair in Caco-2 cells using the comet assay.

Ursolic acid and luteolin showed a protective effect against H_2O_2-induced DNA damage. Repair rate (rejoining of strand breaks) after treatment with H_2O_2 was increased by pre-treatment of Caco-2 cells for 24 h with ursolic acid or luteolin. To evaluate effects on induction of base oxidation, we exposed cells to the photosensitizer Ro 19-8022 plus visible light to induce 8-oxoguanine. Luteolin protected against this damage in Caco-2 cells after a short period of incubation. We also measured the incision activity of a cell extract from Caco-2 cells treated for 24 h with test compounds, on a DNA substrate containing specific damage (8-oxoGua), to evaluate effects on base excision repair activity. Preincubation for 24 h with ursolic acid enhanced incision activity in Caco-2 cells. In conclusion, we demonstrated for the first time that ursolic acid and luteolin not only protect DNA from oxidative damage but also increase repair activity in Caco-2 cells. These effects of ursolic acid and luteolin may contribute to their anti-carcinogenic effects.

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1. Introduction

Accumulating evidence from epidemiological studies as well as laboratory data suggest that consumption of fruits and vegetables is associated with a reduced risk of developing a wide range of cancers including colorectal cancer (CRC) [1,2], one of the main causes of mortality in the western world and the second most common cancer in Europe in 2006 [3]. The mechanisms by which fruits and vegetables protect against cancer are complex and varied. Individual phytochemicals have been implicated as modulators of many metabolic and regulatory processes. Here we investigate the ability of two common components of fruits, vegetables and spices – ursolic acid (UA) and luteolin (Lut) – to limit DNA oxidation and modulate DNA repair in human colon cells (Caco-2) exposed to oxidative agents. Ursolic acid (UA), a natural pentacyclic triterpenoid acid, is widespread in nature and abundant in certain medicinal plants. Luteolin (Lut) is a flavone, a subclass of flavonoids, found in relatively large amounts in fruits, vegetables, red wine and tea.

Protection of DNA from damage and modulation of DNA repair may be assumed to contribute to preventing mutations and maintaining genomic stability. DNA is prone to oxidation by endogenous reactive oxygen species as well as exogenous agents (including radiation and chemicals). 8-Oxo-7,8-dihydroguanine (8-oxoGua) is one of the most abundant forms of DNA oxidation and can cause G to T transversions through mispairing in replication [4]. The main repair pathway for DNA oxidation damage is base excision repair (BER). DNA glycosylases remove modified DNA bases creating apurinic or apyrimidinic (AP) sites. AP sites are cleaved by an AP lyase or endonuclease; the gap is filled by a DNA polymerase and sealed by a DNA ligase [5,6].

We evaluated DNA damage using alkaline single-cell gel electrophoresis (the comet assay). BER of oxidized DNA was measured using an *in vitro* assay for incision activity of a cell extract, incubated with a substrate containing 8-oxoGua [7]. We also assessed the ability of cells to rejoin strand breaks (SBs) induced in DNA by H_2O_2.

2. Materials and methods

2.1. Chemicals

UA (purity ≥90%), hydrogen peroxide, Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin, trypsin solution and 3-(4,5-dimethylthiazole-2-

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yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Lut (purity >90%) was from Extrasynthese (Genay, France). Fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). Ro (photosensitizer Ro19-8022) was from F. Hoffmann-La Roche (Basel, Switzerland). SYBR Gold (nucleic acid gel stain) was from Invitrogen Molecular probes (OR, USA). All other reagents and chemicals used were of analytical grade.

2.2. Cell culture

Caco-2 cells (derived from human colon carcinoma) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), under an atmosphere of 5% CO_2 at 37 °C. Cells were trypsinized when nearly confluent.

Cells were seeded onto 12-well plates, with 1 ml/well at a density of 0.2×10^6 cells/ml, and incubated with different concentrations of test compounds in complete DMEM to test for possible direct cytotoxicity or genotoxicity, for effects on induced DNA oxidation, and for modulation of DNA repair. Stock solutions of UA and Lut were prepared in dimethyl sulphoxide (DMSO) and aliquots kept at −20 °C. The final concentration of DMSO in medium was <0.5%.

2.3. Cell toxicity assay

The test compound's cytotoxicity was assayed in 12-multiwell culture plates seeded with 0.2×10^6 cells/well. Twenty-four hours after plating, the medium was discarded and fresh medium containing test compounds at different concentrations was added. After 48 h of incubation with test compounds, cytotoxicity was evaluated by MTT test. The number of viable cells in each well was estimated by the cell capacity for reduction of MTT as described by Ref. [8]. The results were expressed as a percentage of cell viability relative to control (cells without any test compound).

2.4. Comet assay

The alkaline version of the single-cell gel electrophoresis assay was used to evaluate DNA damage as previously described [7,9] with some modifications. Briefly, Caco-2 cells were trypsinized, washed, centrifuged, and the pellet suspended in low melting point agarose; about 2×10^4 cells were placed on a slide (pre-coated with 1% normal melting point agarose and dried), and covered with a coverslip. After 10 min at 4 °C, the coverslips were removed and slides were placed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris Base, pH 10 plus 1% Triton X-100) for 1 h at 4 °C. When oxidized bases were to be measured, after lysis slides were washed three times with buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0) and incubated with 30 μl of formamidopyrimidine DNA glycosylase (FPG) in this buffer or with buffer only for 20 min at 37 °C. Slides were then placed in a horizontal electrophoresis chamber with electrophoresis solution (300 mM NaOH, 1 mM Na_2EDTA, pH >13) for 30 min at 4 °C for the DNA to unwind before electrophoresis was run for 30 min at 25 V and ∼300 mA. After electrophoresis, slides were washed two times with PBS and dried at room temperature. For analysis of the comet images, slides were stained with SYBR Gold solution for 30 min at 4 °C; after drying, slides were analyzed in a fluorescence microscope and Comet 4 analysis system (Perceptive software) was used to calculate the parameter % tail intensity. Generally, 100 randomly selected cells are analyzed per sample.

2.5. Genotoxic effects of UA and Lut

Caco-2 cells were incubated for 24 h at 37 °C with UA and Lut at different concentrations. Cells were collected by trypsinization and DNA damage (SBs) was evaluated by the comet assay. Digestion with FPG allowed detection of oxidized purines [10].

2.6. Effects of UA and Lut on DNA oxidation

To evaluate protection against oxidative damage, Caco-2 cells were preincubated with 5 and 10 μM UA or 10 and 20 μM Lut for 24 h (long period of incubation) or 2 h (short period of incubation) at 37 °C. Cells were washed with PBS and treated with H_2O_2 (75 μM in PBS) for 5 min on ice to induce SBs, or with 1 μM Ro (photosensitizer Ro19-8022, prepared in PBS from a stock solution at 1 mM in ethanol) plus visible light from a 500 W tungsten–halogen source (1.5 min on ice) at 33 cm to induce 8-oxoGua. DNA damage (SBs and 8-oxoGua) was evaluated by the comet assay without or with FPG, respectively.

2.7. Effects of UA and Lut on cellular repair

In the cellular repair assay two different treatment regimes were used: First, pre-treatment with UA or Lut followed by exposure to H_2O_2 and recovery in fresh medium. Caco-2 cells were preincubated with UA or Lut for 24 h at 37 °C. Cells were washed with PBS and treated with H_2O_2 (75 μM) for 5 min on ice to induce SBs. The H_2O_2 was removed and cells were washed with PBS and then incubated in fresh culture medium for 0, 10, 30 or 60 min at 37 °C. Thus we evaluated the effect of preincubation in UA or Lut on the ability of cells to rejoin SBs [7,9]. In the second approach, to look for a possible direct effect of UA or Lut on enzyme activity, H_2O_2 treatment was performed before cells were incubated with the test compounds. Briefly, Caco-2 cells were treated with H_2O_2 (75 μM) for 5 min on ice to induce SBs. Cells were washed with PBS to remove H_2O_2 and then incubated with UA or Lut for 0, 10, 30 or 60 min at 37 °C. Results were expressed as % of repair DNA damage that was calculated using the follow formula:

$$\% \text{ of repair DNA damage} = \frac{T_0 - T_{30}}{T_0 - C_{30}} \times 100;$$

where T_0 represents DNA damage before recovery period, T_{30} represents DNA damage after 30 min of recovery and C_{30} represents DNA damage of the control after 30 min of recovery.

2.8. Effects of UA and Lut on BER (in vitro assay)

This assay measures the excision repair activity of an extract prepared from cells treated with test compounds by providing the extract with a DNA substrate (agarose-embedded nucleoids) containing specific damage [7]. In this case, the substrate DNA was from cells previously exposed to Ro plus visible light to induce 8-oxoGua that is repaired by BER, and was prepared as described by Gaivão et al. [11]. Incision at damage sites, detected using the alkaline comet assay, indicates the capacity of glycosylase in the extract to initiate BER.

2.8.1. Cell extract preparation

Extracts were prepared as described previously [7] with some modifications. Briefly, for extract preparation, Caco-2 cells were incubated with 10 μM UA, 10 μM Lut or 0.5% DMSO (control) for 24 h at 37 °C. Cells were washed with PBS, trypsinized and resuspended in PBS. Cells were divided into aliquots of 1×10^6 cells in 1 ml and after centrifugation (14,000 × *g*, 5 min at 4 °C) the dry pellets were frozen in liquid nitrogen and stored at −80 °C.

2.8.2. Substrate preparation

Substrates for BER assay were prepared as described previously [7]. Briefly, HeLa cells cultivated in flasks, when near to confluence were treated with Ro plus visible light (5 min at 33 cm on ice) to induce 8-oxoGua. Cells were washed with PBS, trypsinized and resuspended in medium. Cells were centrifuged, the pellet resuspended in freezing medium (DMEM supplemented with 20% FBS and 10% DMSO) and aliquots of 1×10^6 cells in 1 ml frozen slowly and stored at −80 °C. HeLa cells without Ro treatment were also frozen in freezing medium and stored at −80 °C.

2.8.3. Substrate incubation with cell extract

On the day of the experiment, extracts were resuspended in 65 μl of extraction buffer (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol and 10% glycerol, pH 7.8) plus Triton X-100 (0.25%), mixed 5 s on vortex at top speed and incubated 5 min on ice. After centrifugation (∼14,000 × *g*, 4 °C, 5 min) 55 μl of supernatant was removed and mixed with 220 μl of cold reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/ml bovine serum albumin, pH 8). Two gels per slide containing 2×10^4 substrate cells/gel (with or without treatment with Ro) were placed on slides pre-coated with normal melting point agarose and lysed for 1 h. Slides were washed three times with reaction buffer and 30 μl of extract was added to each gel and incubated 20 min at 37 °C in a moist box. FPG and reaction buffer were included as positive and negative controls, respectively. After incubation, slides were transferred immediately to alkaline electrophoresis solution and the normal comet assay was run [7,12].

2.9. Statistical analysis

Results were expressed as mean ± SEM from at least 3 independent experiments. Significant differences ($P < 0.05$) were evaluated by Student's *t*-test.

3. Results

3.1. Cytotoxic effects of UA and Lut

In order to choose the concentrations of UA and Lut that can be used in protective studies, evaluations of test compounds' toxicity were done using MTT test. When Caco-2 cells were incubated for 48 h, UA and Lut significantly decreased cell viability only at concentrations higher than 50 and 100 μM, respectively (Fig. 1). For the following experiments non-cytotoxic concentrations of UA and Lut were used.

3.2. Genotoxicity of UA and Lut

The effects of UA and Lut on induction of SBs and oxidized bases were evaluated. For this, Caco-2 cells were incubated with UA (5 and 10 μM) or Lut (10 and 20 μM) for 24 h at 37 °C and DNA damage assessed by the comet assay with and without FPG treatment.

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