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Uptake and metabolism of enterolactone and enterodiol by human colon epithelial cells☆

Guus H.E. Jansen[a], Ilja C.W. Arts[a], Michel W.F. Nielen[a], Michael Müller[b], Peter C.H. Hollman[a], Jaap Keijer[a,*]

[a] *RIKILT—Institute of Food Safety, Wageningen, The Netherlands*
[b] *Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands*

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Abstract

The enterolignans enterolactone and enterodiol are phytoestrogens that are formed from plant lignans by microorganisms in the human colon. Enterolignans circulate in plasma as conjugates. We hypothesized that conjugation of enterolignans takes place in colon epithelial cells, and studied the time course of uptake and metabolism of enterolactone and enterodiol in three human colon epithelial cell lines. In addition, the conjugates were identified by mass spectrometry with accurate mass measurement (LC/QTOFMS/MS). Intracellular levels of conjugated enterolactone and enterodiol in HT29 cells rose immediately after starting the exposure. This was accompanied by a rapid decrease in free enterolactone and enterodiol in the exposure medium of HT29 and (un)differentiated CaCo-2 but not of CCD841CoTr cells. Conjugation and excretion of enterolactone and enterodiol was complete within 8 h, except for enterodiol in CaCo-2 cells (~48 h). Enterolactone appears to be more rapidly metabolized and/or excreted than enterodiol, and also the appearance of conjugated enterolactone in medium is less affected by the presence of enterodiol than vice versa. Total (free plus conjugated) enterolignan concentrations remained constant throughout the experiments. Three conjugates were identified in exposure medium of HT29 cells: enterolactone-sulfate, enterolactone-glucuronide, and enterodiol-glucuronide.Taken together, our data suggest that phase II metabolism of enterolactone and enterodiol already may take place during uptake in the colon and that colon epithelial cells may be responsible for this metabolism.

Keywords: Phytoestrogen; Lignans; Enterolactone; Enterodiol; Enterolignans; Colon; Epithelial cells; HT29 cells; CaCo-2 cells; Intracellular metabolism

Lignans are diphenolic compounds found in a wide variety of fruits, vegetables, and whole grain products. Important plant lignans are secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol [1,2]. In the human colon, these plant lignans are converted by microorganisms into the enterolignans, enterolactone, and enterodiol [3–6]. Lignans show structural similarity to estradiol, an estrogen, and are therefore included in the family of phytoestrogens [7].

Epidemiological studies suggest a possible protective effect of enterolactone and enterodiol against certain cancers, cardiovascular diseases, and osteoporosis [7–9]. Foods rich in plant lignans, when tested in animal experimental and in vitro studies, also have effects that may protect against cancers of the gastro-intestinal tract. For example, an intervention with linseed containing bread reduced fecal water-induced genotoxicity and subsequently DNA damage in HT29 cells [10]. In azoxymethane treated rats, rye bran lignans significantly reduced the number of large aberrant crypt foci [11].

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* Corresponding author. Fax: +31 317 417717.
E-mail address: jaap.keijer@wur.nl (J. Keijer).

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In Apc^{min} mice, a diet containing rye bran or the lignan hydroxymatairesinol reduced the number of adenomas in the small intestine compared to mice fed a control diet [12]. In vitro studies suggest that enterolactone is capable of inducing quinone reductase, a phase II detoxification enzyme, in Colo205 cells [13]. Furthermore, anti-proliferative activity [14], inhibition of estrogen synthetase (aromatase) [15], and anti-oxidant activity [16,17] have been described for enterolactone and enterodiol. Enterolactone and enterodiol appear not to exert any genotoxic effects [18].

Enterolignans are known substrates for phase II detoxification enzymes and as a consequence are present as conjugates in plasma, urine, and bile of humans and rats. The main enterolignan conjugates have been tentatively identified as glucuronides and sulfates by using rather unspecific and elaborate protocols based on desulfatation by solvolysis and enzymatic deglucuronidation [5,19–21]. Two recent studies used more advanced methods for the identification of enterolignan conjugates in urine [22] and of enterolactone conjugates in rhesus monkey and human hepatocytes [23]. Initially, conjugation of phenolic compounds was thought to occur mainly in the liver, but now it is evident that phase II metabolism can already take place during uptake in the colon epithelial cells. For enterolignans, this was first suggested when enterolactone and enterodiol were found to be present predominantly conjugated to glucuronic acid in portal venous blood of rats [19]. For flavonoids such as quercetin, conjugation has been demonstrated to take place in the small intestine of the rat [24]. Furthermore, the human adenocarcinoma cell line CaCo-2 is capable of glucuronidating and sulfating the isoflavones genistein and daidzein [25]. Metabolism of enterolignans by intestinal epithelial cells has not been studied yet. In this study, the time course of uptake and metabolism of enterolactone and enterodiol by three human colon epithelial cell lines were investigated. Metabolites of enterolactone and enterodiol were identified by mass spectrometry with accurate mass measurement (LC/QTOFMS/MS).

Materials and methods

Cell culture

The tumor derived colon cell lines HT29 and CaCo-2, and the SV40 transformed CCD841CoTr colon cell line were obtained from American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in a humid environment in 25 cm^2 culture flasks at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with $NaHCO_3$ (3.7 g/L), penicillin (5000 U), streptomycin (5 mg/L) (all Sigma, Zwijndrecht, The Netherlands), heat-inactivated fetal bovine serum (10% v/v, Invitrogen, Breda, The Netherlands), and non essential amino acids (1×, ICN, Zoetermeer, The Netherlands). Culture medium was replaced every second day. Cells were split at 70–80% confluence with a ratio of 1:5 and seeded for subculturing. To differentiate, CaCo-2 cells were grown for 19 days with medium replacement every second day.

Exposure studies

To study the time course of absorption and metabolism of enterolignans by colon cells, confluent monolayers of HT29, CaCo-2, and CCD841CoTr were incubated with enterolactone and enterodiol in triplicate for 15, 30 min, 1, 2, 4, 8, 16, 32, and 48 h (only for CCD841CoTr and CaCo-2), and 72 h (only for CaCo-2). Enterolactone and enterodiol (Fluka, Zwijndrecht, The Netherlands) were dissolved in dimethyl sulfoxide (DMSO; Sigma, Zwijndrecht, The Netherlands). Stock solutions of enterolactone and enterodiol were diluted in culture medium before uptake experiments (final DMSO concentration 0.03% v/v). All cell lines were incubated with 10 μM enterolactone and 10 μM enterodiol simultaneously, unless stated otherwise. Incubation was stopped by removing the exposure medium and washing two times with cold Hanks' balanced salt solution (HBSS; Sigma, Zwijndrecht, The Netherlands). The exposure medium was stored for HPLC analysis. Intracellular levels of enterolactone and enterodiol were determined in HT29 cells after duplicate exposures for 5, 10, 15, 30 min, 1, 2, 4, and 8 h. Cells were trypsinized and eluted in 2 mL HBSS. After elution, cells were pelleted by centrifugation at 800*g* for 5 min. The supernatant was discarded and the cell pellet was dissolved in 1 mL MilliQ water. Cells were lysed by ultrasonic needle treatment (Vibracell 300; Sonics & Materials, Danbury, CT, USA).

Sample preparation

Enterolactone and enterodiol concentrations were measured in cell culture medium and in lysed cell pellet using HPLC with Coularray detection. In all samples collected, both free enterolactone and enterodiol as well as total (free plus conjugated) enterolactone and enterodiol were determined. Total enterolactone and enterodiol were determined after enzymatic hydrolysis of the conjugates with β-glucuronidase/sulfatase from *Helix pomatia* (G1512; Sigma, Zwijndrecht, The Netherlands). The following sample preparation method was used. To 300 μL sample, 300 μL of 0.1 M NaAc buffer (pH 5.0) was added, which contained 2 mg β-glucuronidase/sulfatase if enzymatic hydrolysis was required. All samples were mixed briefly and incubated at 37 °C for 2 h. Samples were cooled to room temperature and 1.5 mL diethyl ether was added. Samples were vortexed for