Resveratrol-induced modification of polyamine metabolism is accompanied by induction of c-Fos

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The objective of the current study was to investigate the effect of resveratrol, a naturally occurring polyphenol with cancer chemopreventive properties, on polyamine metabolism in the human colonic adenocarcinoma cell line Caco-2. We demonstrated that inhibition of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis, was due to attenuated ODC protein and mRNA levels (50–200 µM). The naturally occurring resveratrol analog piceatannol (100 µM) also diminished ODC activity, protein and mRNA levels, whereas the green tea polyphenol (–)-epigallocatechin gallate (EGCG; 100 µM) exerted only weak effects on ODC. The transcription factor c-Myc, a positive regulator of the odc gene was attenuated by resveratrol treatment and to a lesser extent by piceatannol and EGCG. S-Adenosylmethionine decarboxylase, an enzyme that synthesizes higher polyamines, was concomitantly inhibited by resveratrol and piceatannol treatment, whereas EGCG did not affect its activity. In addition resveratrol, piceatannol and EGCG enhanced spermidine/spermine N7-acetyltransferase activity, an enzyme that degrades polyamines in cooperation with polyamine oxidase. Intracellular levels of spermine and spermidine were not affected, whereas putrescine and N8-acetylspermidine concentrations increased after incubation with resveratrol. These events were paralleled by an increase of the activator protein-1 constituents c-Fos and c-Jun. Whereas DNA-binding activity of c-Jun remained unchanged, DNA-binding activity of c-Fos was significantly enhanced by resveratrol and piceatannol, but inhibited by EGCG. The data suggest that growth arrest by resveratrol is accompanied by inhibition of polyamine synthesis and increased polyamine catabolism. C-Fos seems to play a role in this context. Effects of piceatannol on polyamine synthesis were similar, but not as potent as those exerted by resveratrol.

Introduction

The cellular polyamines spermidine and spermine, as well as their precursor putrescine are essential for growth and DNA synthesis (1–3). Increases in the levels of these polyamines are generally associated with cell proliferation and cell transformation induced by growth factors (4), carcinogens (5) or oncogenes (6). Therefore, polyamine metabolism is considered to represent an attractive target for both cancer chemotherapy and chemoprevention. Ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) are the key enzymes in polyamine biosynthesis. Inhibition of ODC by DL-α-difluoromethylornithine has been shown to decrease mucosal growth in vivo and in vitro (7,8). Catabolism, excretion or reconversion of higher polyamines is preceded by acetylation through spermidine/spermine N7-acetyltransferase (SSAT), the rate-limiting enzyme in polyamine catabolism, which can be induced by reactive oxygen species (9–11). In combined action with polyamine oxidase (PAO) it converts spermine to spermidine and the latter to putrescine.

Resveratrol (trans-3,4',5-trihydroxystilbene) is a plant polyphenol naturally occurring in grapes, red wine and peanuts (12,13) with chemopreventive properties (14). The red wine polyphenol has been demonstrated to inhibit ODC activity and progression of the cell cycle in Caco-2 colorectal cancer cells (15). In previous studies we have shown that this cell-cycle arrest is accompanied by reduced cyclin D1 and cyclin-dependent kinase (cdk)4 levels (16). The number of aberrant crypt foci in azoxymethane-induced carcinogenesis of the rat colon is significantly reduced by treatment with resveratrol (17). In the ApcMin-mouse model for familiar adenomatous polyposis oral application of resveratrol reduced adenomas by 70% (18). The natural resveratrol analog piceatannol (trans-3,4,3',5'-tetrahydroxystilbene) also inhibits cell-cycle progression with decreased cyclin D1 and cdk4 levels of colorectal carcinoma cell lines (19). It has been demonstrated to inhibit formation of 7,12-dimethylbenz[a]anthracene-induced neoplastic lesions in a mouse mammary gland model. In contrast to resveratrol it does not significantly inhibit cyclooxygenase activities (20). The underlying molecular mechanisms for the antineoplastic effects of resveratrol and piceatannol have not been fully clarified.

The objective of the present study was to further elucidate the effect of resveratrol on polyamine metabolism and to test whether the analog piceatannol exerts the same effects as resveratrol. These results were compared with the effects of (–)-epigallocatechin gallate (EGCG), a green tea polyphenol implied in chemoprevention (21), to evaluate the specificity of the data obtained with the stilbenes.

Materials and methods

Cell culture

The human colon cancer cell line Caco-2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Caco-2 cells of passages 45–55 were cultured in Dulbecco’s modified Eagle medium, supplemented with 10% fetal calf serum, penicillin (1000 U/l) and streptomycin (1 mg/l) and incubated at 37°C under an atmosphere of 5% CO2 in air. All cell culture reagents were obtained from Life Technologies (Eggenstein, Germany). Stock solutions of resveratrol (Sigma, Deisenhofen, Germany) and piceatannol (Alexis Biochemicals, Grünberg, Germany) were prepared in dimethyl sulfoxide (DMSO). EGCG (Sigma) was diluted in phosphate-buffered saline (PBS). The compounds were directly added to cell cultures at a concentration of 100 µM for piceatannol and EGCG and at concentrations ranging from 50 to 200 µM for resveratrol, whereas untreated cells received the solvent alone (≤0.1% DMSO). Cytotoxicity was excluded by lactate dehydrogenase release assay (Roche Molecular Biochemicals, Mannheim, Germany).
Cells were plated in 80 cm² flasks and incubated with plant polyphenols for 24 h. Western blot analysis using total protein extracts from cultured cells was performed as described previously (16). Protein content was quantified with the Bio-Rad (Bio-Rad Laboratories, München, Germany) colorimetric assay. Reprinting of blots for expression of actin was done routinely. Antibodies against c-Myc and c-Fos were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-ODC was purchased from Sigma and anti-c-Jun from BD Transduction Laboratories (Heidelberg, Germany).

Reverse transcriptase–PCR

Cells were seeded in 6-well plates, allowed to attach overnight and treated with polyphenols for 24 h. RNA was isolated from cells by lysis in RNAzol B (Tel-Test, Friendswood, TX) followed by phenol extraction and ethanol precipitation. Reverse transcription of total cellular RNA was carried out using Superscript II RNase H reverse transcriptase (Life Technologies, Karlsruhe, Germany) and reverse transcriptase primers (Promega, Mannheim, WI). PCR was performed on the cDNA using the following sense and antisense primers, respectively (primers were custom-synthesized by Biospring, Frankfurt, Germany): ODC: AATCAACCCAGCGTGGACAA and ACAT-CACATAGTAGATGTCCG; GAPDH: ATCCTCAGAGGCGAGATCC and ACCACTGACACGTTGGCAGT. Thermal cycling was performed as follows: denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. Thirty-five cycles were performed. Primers were used at a final concentration of 10 µM each, dNTPs at 500 µM (Eurogentec, Seraing, Belgium), and MgCl₂ at 3 mM. Five units of Taq DNA Polymerase were used per 50 µl reaction. Ten microliters of PCR product were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized by UV illumination.

ODC and SAMDC activity

The activities of the enzymes ODC and SAMDC were assayed with a radiometric technique in which the amount of 14C-ornithine liberated from [3-14C]ornithine (207.2 ± 10^4 MBq/mol, Amersham Pharmacia Biotech, Freiburg, Germany) or S-adenosyl-[carboxyl-14C]-L-methionine (222 ± 10^4 MBq/mol, Amersham Pharmacia Biotech) was estimated, as described previously (22). Briefly, after treatment was carried out as described above, the cell culture dishes were placed on ice; the monolayers were washed three times with cold PBS, and the cells were harvested by scraping in homogenization buffer (50 mM Tris, pH 7.2, 5 mM DTT, 100 µM MgCl₂, and 100 mM EGTA), sonicated and centrifuged at 15 000 g for 10 min. 100 µl of the supernatant were incubated in a stopped tube with 74 µM trit.[1-14C]ornithine in the presence of pyridoxal-5-phosphate for 60 min at 37°C. For the SAMDC assay, instead of trit.[1-14C]ornithine, S-adenosyl-[carboxyl-14C]-L-methionine and was used and putrescine instead of pyridoxal-5-phosphate. 14CO₂ liberated by the decarboxylation of ornithine or S-adenosylmethionine and trapped on filters impregnated with Tio₂ Polymer (Moravek, Brea, CA) at 37°C and the addition of 20 µl of Moravek, Brea, CA) at 37°C and the addition of 20 µl of 0.3% perchloric acid was added to the remaining supernatant. Polyamines were analyzed by the HPLC method according to Hyvonen et al. (23). Separation was carried out on a Hypersil (ODS 3 µm) 150 × 3 mm column (MZ analytical, Mainz, Germany) by isocratic elution with 0.1% potassium phosphate plus 0.01 M sodium octane sulfonate with acetonitrile (10:3, v/v) and a flow rate of 0.8 ml/min.

Activator protein-1 (AP-1) activation assay

Cells were plated in 80 cm² flasks and incubated with substances for 24 h. Nuclear extracts from total protein were prepared with a nuclear extract kit (Active Motif, Rixensart, Belgium). Twenty micrograms of nuclear protein were either used for TransAM c-Fos or TransAM c-Jun transcription factor assay kit (Active Motif). Activity of the transcription factors was determined according to the manufacturers instructions. AP-1 from nuclear cells extracts specifically binds to an oligonucleotide containing a 12-O-tetradecanoylphor- bol-13-acetate responsive element that is attached to a 96-well plate. By using an antibody directed against either c-Fos or phosphorylated c-Jun, the AP-1 dimer bound to the oligonucleotide is detected. A secondary antibody conjugated to horseradish peroxidase provides colorimetric visualisation. Absorbance was determined with a microplate reader (Tecan, Crailsheim, Germany).

Statistical analysis

Data were expressed as means ± SD. Differences between two values were tested for statistical significance using the Student’s unpaired t-test (SigmaStat, SPSS, Chicago, IL). A P value <0.05 was considered to indicate a significant difference.

Results

As demonstrated earlier (15), resveratrol inhibits ODC, the key enzyme in polyamine synthesis. At a concentration of 100 µM resveratrol reduced ODC activity to 40% of control values (Figure 1A). Compared with the effects of resveratrol, inhibition of ODC by EGCG was moderate with a reduction to 69% versus control, whereas piceatannol (200 µM) reduced ODC activity to 47% of the control. Diminished ODC activity after treatment with resveratrol or piceatannol was accompanied by reduced ODC protein levels, as monitored by western blotting (Figure 1B). EGCG treatment did not affect ODC expression. To evaluate whether attenuation of ODC was caused on the level of transcription, odc mRNA was determined with PCR (Figure 1C). Treatment with resveratrol attenuated odc mRNA in a concentration-dependent manner. Piceatannol and EGCG exerted only weak effects on odc mRNA. As shown in Figure 2 the polyamine-synthesizing enzyme SAMDC was also inhibited in a dose-dependent manner by treatment with resveratrol (54% of control with 100 µM), 200 µM piceatannol diminished SAMDC activity to 74% of control, whereas EGCG had no impact on SAMDC.

The transcription factor c-Myc is known to be an activator of the odc promoter. Therefore we determined whether the oncprotein is influenced by treatment with resveratrol. As shown in Figure 3 performance of western blot revealed diminished intracellular levels of the oncprotein in a dose-dependent manner. Piceatannol was almost as effective as resveratrol in attenuating c-Myc expression, whereas the effect of EGCG was less pronounced.

SSAT activity was measured to evaluate whether polyamine degradation is also modulated by resveratrol (Figure 4A). Addition of resveratrol (100 µM) increased SSAT activity to 217% of control value. Piceatannol (200 µM) and EGCG (100 µM) augmented SSAT activity to 211 and 181% of control, respectively. In accordance with these results we could show that the intracellular polyamine concentrations changed after incubation with resveratrol (Figure 4B). There was an increase in putrescine (65% of control with 200 µM resveratrol) and N⁴-acetylputrescine (242% of control with 200 µM resveratrol), which are markers for enhanced SSAT activity.

determination. 0.2 M perchloric acid was added to the remaining supernatant. Polyamines were analyzed by the HPLC method according to Hyvonen et al. (23). Separation was carried out on a Hypersil (ODS 3 µm) 150 × 3 mm column (MZ analytical, Mainz, Germany) by isocratic elution with 0.1% potassium phosphate plus 0.01 M sodium octane sulfonate with acetonitrile (10:3, v/v) and a flow rate of 0.8 ml/min.
As shown in Figure 5, resveratrol and piceatannol, but not EGCG were found to augment the abundance of c-Fos protein in Caco-2 cells. To determine whether this upregulation was accompanied by enhanced AP-1 activity, an AP-1 DNA-binding activity assay was performed. Both stilbenes, resveratrol as well as piceatannol, markedly increased DNA binding of c-Fos. The effect of resveratrol was most prominent with a concentration of 100 µM (362% of control values). Piceatannol induced c-Fos binding to 216% of control values. Moreover, this binding was specific, because addition of wild-type oligonucleotides containing the AP-1-binding motif, but not of a mutated oligonucleotide prevented DNA binding of AP-1 from nuclear cell extracts. EGCG significantly inhibited c-Fos DNA binding (80% compared with controls). Figure 6 reveals that protein levels of c-Jun decreased after treatment with 50 µM resveratrol, but increased with higher concentrations of resveratrol, after addition of piceatannol and EGCG. The DNA-binding activity of c-Jun was not influenced by incubation with resveratrol, piceatannol or EGCG.

Discussion

The present study demonstrates that resveratrol modifies polyamine homeostasis on the level of synthesis as well as on the level of degradation of polyamines. As already shown by others (15), resveratrol significantly inhibited ODC activity. In addition, we could demonstrate that this effect was accompanied by reduced protein and mRNA expression of odc. Transcription of the human odc gene is directly mediated by the Myc/Max transcriptional complex (24). In order to evaluate whether c-Myc is involved in resveratrol-induced growth inhibition, c-Myc protein content was determined. Resveratrol attenuated levels of the transcription factor, implying that downregulation of ODC might be mediated by reduction of c-Myc levels. Deregulation of c-myc with prolonged half-life has been implicated in a number of malignancies including tumors of the colon (25), classifying it as an oncogene. Constitutive expression of c-Myc has a profound effect on the cell cycle. C-Myc represents a therapeutic target of chemoprevention and its down-regulation might contribute to the cell-cycle inhibitory effect exerted by resveratrol.

In contrast to an earlier published study, which investigated...
the effects of resveratrol on polyamine metabolism (15), a concomitant and dose-dependent (50–200 µM) inhibition of SAMDC activity, an enzyme involved in spermidine and spermine synthesis was observed. Whereas we observed an increase in putrescine and N⁸-acetyl-putrescine concentrations, no significant changes in polyamine content of Caco-2 cells after treatment of cells with 25 µM resveratrol for 24 h was detected by Schneider et al. An explanation for these conflicting results could be the difference of concentrations used (15).

Furthermore, resveratrol potently upregulated SSAT activity in Caco-2 colon cancer cells. SSAT acetylates spermidine and spermine, which can either be secreted from the cell or are degraded by PAO forming putrescine or spermidine. Vujčić et al. postulated, that induction of SSAT can negatively affect cell growth (26). Accumulation of SSAT mRNA is accompanied by augmented intracellular putrescine levels (27). After treatment of Caco-2 cells with resveratrol an increased intracellular concentration of putrescine, the result of enhanced SSAT activity, was confirmed. Accumulation of N⁸-acetyl-spermidine, a sign for active polyamine catabolism could also be monitored, whereas the concentration of spermidine and
induced by putrescine (30). Elevated protein levels of c-Fos were observed together with an increased DNA-binding activity of c-Fos after treatment with resveratrol. This leads us to hypothesize, that the increase in c-Fos might be due to enhanced putrescine levels. C-Fos is part of the dimeric transcription factor AP-1, which is composed of members of c-Jun and c-Fos families that bind the AP-1 site as either homo- or heterodimers. There is increasing evidence that the AP-1 complex plays an important role not only in proliferation but also in differentiation of several cell types. In Caco-2 cells the chemopreventive agent 1,25-dihydroxyvitamin D3 stimulates cell differentiation, which is dependent on AP-1 (31). Butyrate is also considered to represent a chemopreventive substance and triggers differentiation. Concomitantly the short chain fatty acid (SCFA) butyrate rapidly induces c-Fos at a post-translational level (32). EGCG inhibits AP-1 activity of transformed keratinocytes induced by UV irradiation (33,34), whereas it increases AP-1-dependent gene expression in normal keratinocytes (35) and augments protein levels of AP-1 constituents in HepG2 cells (36). It has also been shown, that AP-1 activation occurs during differentiation of Caco-2 cells, before alkaline phosphatase and disaccharidase activities increase (37). The data imply that AP-1 is not only associated with tumorigenesis, but also with differentiation and/or growth inhibition. Resveratrol alone does not induce differentiation of Caco-2 cells (15), whereas, when applied in combination with butyrate, significantly enhances the differentiation induced by the SCFA (38). Resveratrol has been demonstrated to inhibit AP-1 activation by tumor necrosis factor (39), UV irradiation, and by phorbol 12-myristate 13-acetate (40). This suggests that the induction of AP-1 by resveratrol could be limited to processes of growth inhibition and that resveratrol is able to inhibit AP-1 activation when it is associated with hyperproliferation.

Although the natural resveratrol analog piceatannol also inhibited polyamine synthesis, it had only a significant influence on SSAT activity at concentrations of 200 µM. We demonstrated recently that the growth inhibiting effect of piceatannol is not as pronounced as that of resveratrol (19). In order to test specificity of these results all experiments were also performed with EGCG, the most abundant polyphenol in green tea, because ODC-inhibition by EGCG has been demonstrated earlier (41).

Taken together our findings demonstrate that resveratrol not only impairs polyamine synthesis and decreases the oncoprotein c-Myc, but also increases polyamine catabolism by SSAT. At the same time induction of c-Fos and its DNA-binding activity take place. Modulation of polyamine homeostasis seems to be an additional target of the antiproliferative effects of resveratrol. In this context resveratrol is more potent than EGCG. The mechanism of the growth inhibitory action of resveratrol seems to differ from that of the green tea polyphenol, because effects on c-Fos activity differed. The impact of piceatannol on polyamine synthesis was similar to that of resveratrol, although less pronounced.

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References


