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De novo ceramide biosynthesis is associated with resveratrol-induced inhibition of ornithine decarboxylase activity

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ABSTRACT

Previous studies could demonstrate, that the naturally occuring polyphenol resveratrol inhibits cell growth of colon carcinoma cells at least in part by inhibition of protooncogene ornithine decarboxylase (ODC). The objective of this study was to provide several lines of evidence suggesting that the induction of ceramide synthesis is involved in this regulatory mechanisms.

Cell growth was determined by BrdU incorporation and crystal violet staining. Ceramide concentrations were detected by HPLC-coupled mass-spectrometry. Protein levels were examined by Western blot analysis. ODC activity was assayed radiometrically measuring [$^{14}CO_2$]-liberation. A dominant-negative PPARγ mutant was transfected in Caco-2 cells to suppress PPARγ-mediated functions.

Antiproliferative effects of resveratrol closely correlate with a dose-dependent increase of endogenous ceramides ($p < 0.001$). Compared to controls the cell-permeable ceramide analogues C2- and C6-ceramide significantly inhibit ODC-activity ($p < 0.001$) in colorectal cancer cells. C6-ceramide further diminished protein levels of protooncogenes c-myc ($p < 0.05$) and ODC ($p < 0.01$), which is strictly related to the ability of ceramides to inhibit cell growth in a time- and dose-dependent manner. These results were further confirmed using inhibitors of sphingolipid metabolism, where only co-incubation with a serine palmitoyltransferase (SPT) inhibitor could significantly counteract resveratrol-mediated actions. These data suggest that the induction of ceramide *de novo* biosynthesis but not hydrolysis of sphingomyelin is involved in resveratrol-mediated inhibition of ODC. In contrast to the regulation of catabolic spermidine/spermine acetyltransferase by resveratrol, inhibitory effects on ODC occur PPARγ-independently, indicating independent pathways of resveratrol-action. Due to our findings resveratrol could show great chemopreventive and therapeutic potential in the treatment of colorectal cancers.

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Abbreviations: BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; DFMO, alpha-difluoromethylornithine; dnPPARγ, dominant negative PPARγ mutant; ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-activated protein kinase; NSAID, non-steroidal anti-inflammatory drugs; ODC, ornithine decarboxylase; PAO, polyamine oxidase; PPARγ, peroxisome-proliferator activated receptor γ; RXR, retinoid X receptor; SAMDC, S-adenosylmethioninedecarboxylase; SMase, Sphingomyelinase; SPT, serine palmitoyltransferase; SSAT, spermine/spermidine acetyltransferase; TNF-α, tumor necrosis factor-α

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1. Introduction

Resveratrol, chemically known as 3,5,4′-trihydroxytransstilbene, is a naturally occuring polyphenolic antioxidant compound, also classified as a phytoalexin, which are herbal antibiotics produced in response to environmental stress factors including injuries, UV irradiation or fungal invasion [1]. Resveratrol was first detected in the root extract of the weed *Polygonum cuspidatum* [2], which has been known in Asian folk medicine under the name *Ko-jo-kon* and was traditionally used to treat liver, skin and circulatory diseases [3,4]. Anticarcinogenic properties of resveratrol were first reported by Jang et al. [5] demonstrating chemopreventive effectiveness against all the three major steps of carcinogenesis, i.e. initiation, promotion and progression. We and others provide several lines of evidence that resveratrol mediates these anticarcinogenic effects partly through the modulation of polyamine metabolism [6,7]. The major polyamines spermidine and spermine, and their diamine precursor, putrescine are organic cations with multiple functions in cell growth and cell death [8,9]. The intracellular polyamine pool size is controlled strictly by the combined action of *de novo* synthesis, catabolism, uptake and export of polyamines. This regulatory mechanism include reactions catalyzed by the biosynthetic enzymes ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC) and spermidine/spermine synthases and by the catabolic spermidine/spermine acetyltransferase (SSAT) and FAD-dependent polyamine oxidase (APAO) [10]. The finding that agents that inhibit polyamine biosythesis can prevent, or at least limit cell growth [6,11–13], together with the fact, that polyamine concentrations are elevated in multiple cancer tissues [14,15], has made the polyamine metabolism a promising target for cancer chemoprevention and therapy.

Ceramides are key compounds in the metabolism of sphingolipids and are emerging as important second messengers for various cellular processes including cell cycle arrest, differentiation and apoptosis (for review see Ref. [16]). Ceramides can be produced via a *de novo* biosynthetic pathway which is initiated by condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyltransferase (SPT) as well as by sphingomyelinase-mediated hydrolysis of sphingomyelin. Our aim was to study the potential involvement of ceramide biosynthesis in resveratrol mediated inhibition of ODC activity in colorectal cancer cells.

2. Materials and methods

2.1. Cell culture and materials

Caco-2 cells of passages 53–61 were kept in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% sodium pyruvate and 1% nonessential amino acids. HT29 cells of passages 17–30 were cultured in McCoy's 5A supplemented with 10% FCS and 1% penicillin/streptomycin. Both cell lines were maintained at 37 °C in an atmosphere of 95% air and 5% CO_2. The cells were passaged weekly using Dulbecco's PBS containing 0.25% trypsin and 1% EDTA. The medium was changed three times per week. Cells were screened for possible contamination with mycoplasma at monthly intervals. For experiments, the cells were seeded onto plastic cell culture wells in serum containing medium and allowed to attach for 24 h. For the ODC activity assay the cells were synchronized in medium containing 1% FCS 24 h before treatment. Resveratrol, *N*-hexanoylsphingosine, *N*-acetylsphingosine, L-cycloserine, myriocin and manumycin were obtained from Sigma–Aldrich (St. Louis, MO); Dulbecco's modified Eagle's medium and Optimem™ I from Gibco (Invitrogen, Carlsbad, CA); fetal calf serum, sodium pyruvate solution, glutamine, penicillin and streptomycin stock solutions from PAA Laboratories GmbH (Ontario, Canada); Lipofectamine™ 2000 from Invitrogen (Carlsbad, CA).

2.2. SDS-polyacrylamide gel electrophoresis and immunoblot analysis

Caco-2 cells were seeded in 80 cm^3 flasks; 24 h after plating, cells were incubated with substances for different time intervals. Whole cell extract was obtained according to the manufacturer's instructions (Active Motif, Rixensart, Belgium). Protein was quantified with the Bio-Rad protein colorimetric assay. After addition of sample buffer to the total cellular extract and boiling samples at 95 °C for 5 min, protein was separated on a 10% SDS-polyacrylamide gel. Protein was transferred onto nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany) and the membrane was blocked for 1 h at room temperature with 3% skim milk in tris-buffered saline containing 0.05% Tween 20 (TBST). Next, blots were washed and incubated overnight at 4 °C in TBST containing 3% skimmed milk powder with a 1:500 dilution of primary antibodies for ODC and c-myc (all from Santa Cruz Biotechnology, Santa Cruz, USA). The secondary, horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) was diluted at 1:2000 and incubated with the membrane for another 45 min in skim milk. After chemoluminescence reaction (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK), band were detected after exposure to Hyperfilm-MP (Amersham International plc, Buckinghamshire, UK). Blots were reprobed with β-actin antibody (Santa Cruz Biotechnologies, Santa Cruz, USA). For quantitative analysis, bands were detected and evaluated densitometrically by ProViDoc system (Desaga, Wiesloch, Germany), normalized for the density of β-actin.

2.3. Cell counts

Cells were suspended and cultured on 96 well dishes at a density of 10^4/well (0.28 cm^2). Twenty-four hours after plating cells were incubated for 24–72 h with ceramides. At given time points following treatment cell numbers was assessed by crystal violet staining. Medium was removed from the plates and cells were fixed with 5% formaldehyde for 5 min. After washing with PBS cells were stained with 0.5% crystal violet for 10 min, washed again with PBS and unstained with 33% acetic acid. Absorption, which correlates with the cell number, was measured at 620 nm.

2.4. Cell proliferation

The effects of ceramides on DNA synthesis of cells were assessed using the cell proliferation ELISA kit (Roche Diag-

nostics, Tokyo, Japan). This assay is a colorimetric immunoassay for quantification of cell proliferation based on the measurement of bromodeoxyuridine (BrdU) incorporation during DNA synthesis, and is a non-radioactive alternative to the [^{3}H]-thymidine incorporation assay. Cells were grown in 96 well culture dishes (10^4 cells/well), incubated with C2- or C6-ceramide for different time intervalls, and then labelled with BrdU for a further 4 h. Incorporated BrdU was measured colorimetrically.

2.5. Lipid extraction and ceramide quantitation

Sub-confluent Caco-2-cells in 30 mm-diameter dishes were stimulated with increasing concentrations of resveratrol [50–200 μmol/L] for 24 h. Lipids were extracted according to the method established by Bligh and Dyer [17], and ceramide was quantitated by mass-spectrometry as previously described [18].

2.6. ODC-activity

The activity of the enzyme ODC was assayed with a radiometric technique in which the amount of $^{14}CO_2$ liberated from DL-[1-14C]ornithine (207.2_104 MBq/mol, Hartman Analytics Amersham Pharmacia Biotech, Freiburg, Germany) was estimated, as described earlier [13].

2.7. Transfection assay

The following plasmids were used for transfection: pcDNA3 (Invitrogen), as an empty vector for control transfection and the plasmid pcDNA3-PPARγL468A/E471A, a dominant-negative double mutant, that was kindly provided by VK Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom) [19]. These constructs were transfected into subconfluent Caco-2 cells with lipofectamine 2000 (Invitrogen). After 6 h the cells were fed with fresh medium containing 10% FCS. Twenty-four hours later the cells were fed with medium containing Geneticine (G418) [400 μg/mL] and culture medium was replaced twice a week. G418-resistant colonies were collected and used for further analysis.

2.8. Statistics

The data are expressed as means ± S.E. of at least three independent experiments. Analysis of variance (ANOVA) was performed when more than two groups were compared, and when significant ($p < 0.05$), multiple comparisons were performed with the Turkey test. A $p < 0.05$ was considered to be significant.

3. Results

3.1. Effects of resveratrol on ceramide synthesis

First we examined the effect of resveratrol [50–200 μmol/L] on the intracellular ceramide concentrations of Caco-2 cells using mass-spectometry. After 24 h of incubation we could observe a significant dose-dependent up-regulation of C16-ceramide levels ~6.5-fold at 200 μmol/L [$^{***}p < 0.001$] (Fig. 1).

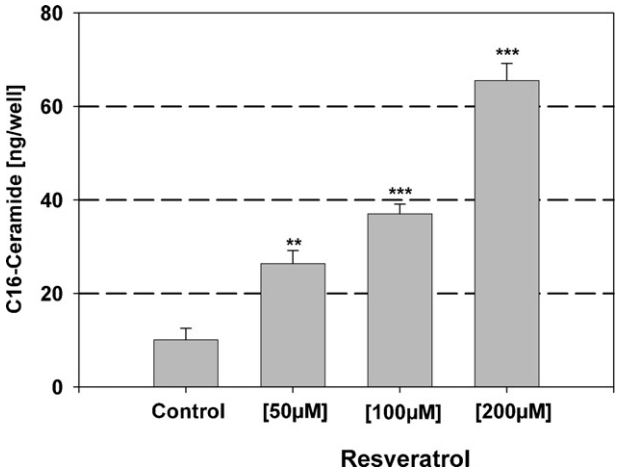

Fig. 1 – Synthesis of C16-ceramide in Caco-2-wildtype cells after incubation with resveratrol [50–200 μmol/L] for 24 h. Resveratrol significantly enhances the intracellular C16-ceramide concentration in a dose-dependent manner. Means ± S.E.; $n = 4$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. control.

3.2. Effects of C2- and C6-ceramides on cell proliferation and cell counts

Since natural ceramides are not permeant to cell membranes, our study has been carried out by using short chain cell-permeable analogs to determine the role of ceramides in this signal transduction pathways. Caco-2 and HT-29-cells were incubated with increasing concentrations of C2- and C6-ceramides [1–40 μmol/L] for 24, 48 and 72 h. After each time interval both cell proliferation ELISA (BrdU) and crystal violet staining were performed. Both in Caco-2- and HT-29-cells a significant time- and dose-dependent decrease in cell proliferation and cell counts could be measured, whereby we limit our illustrations on the effects after 48 h (Fig. 2).

3.3. Effects of ceramides on ODC activity as well as on c-myc and ODC protein expression

Resveratrol on the one hand induces intracellular ceramide synthesis and on the other hand reduces the protein levels of the protooncogenes c-myc and ODC [6]. To reveal a possible coherency, we first measured the effects of C2- and C6-ceramides [10–40 μmol/L] in Caco-2-cells (Fig. 3A and B) and C2- [10–30 μmol/L] and C6- [1–10 μmol/L] ceramides in HT-29-cells (Fig. 3C and D) on ODC activity after 24 h of treatment which both caused a significant inhibition in dose-dependent manner [$^{***}p < 0.001$]. We further have done Western blot analysis to measure effects on the protein levels of ODC and c-myc after treatment with the C6-ceramide *N*-hexanoylsphingosine [10–40 μmol/L]. And actually a dose-dependent decreases both in c-myc (Fig. 4B) [$^{**}p < 0.001$] and ODC (Fig. 4D) [$^{*}p < 0.05$] protein levels comparable to the resveratrol-induced effects [6] could be observed after 6 h of incubation.

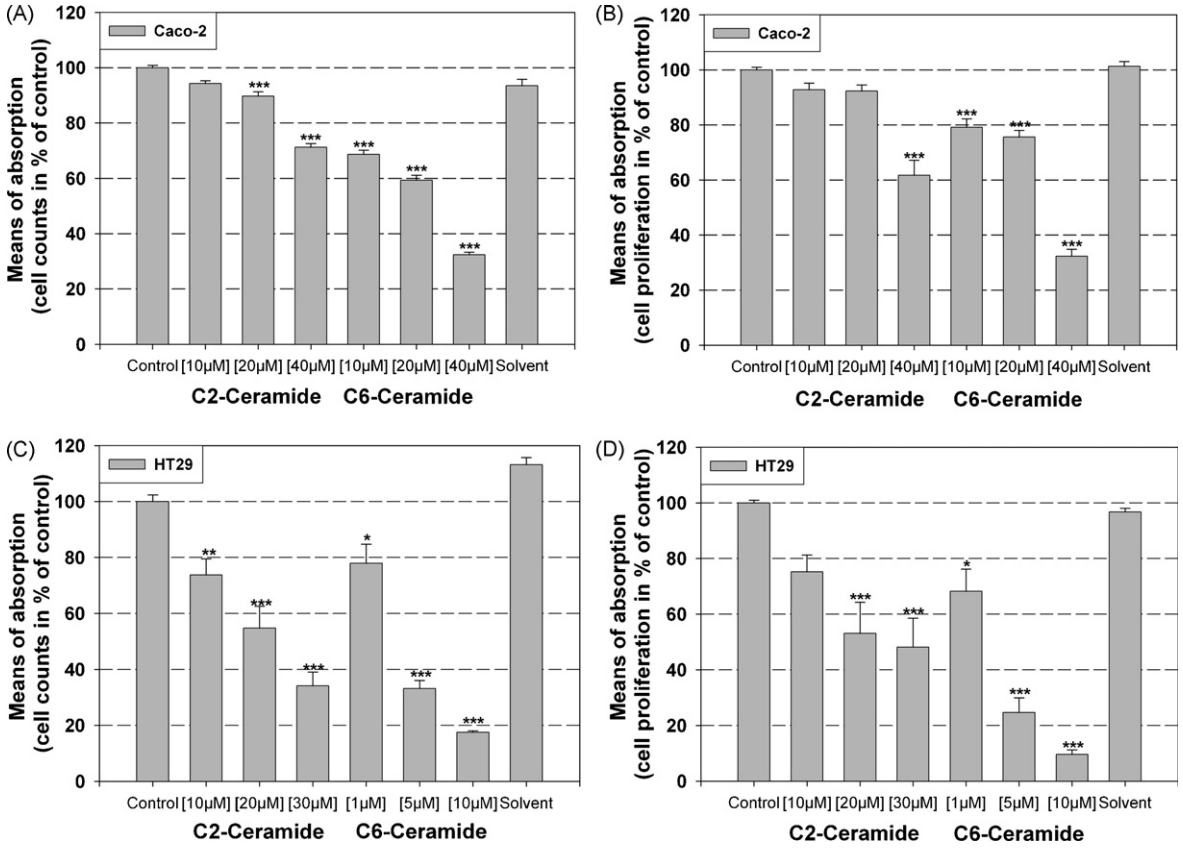

Fig. 2 – (A) Cell counts and (B) cell proliferation of Caco-2 cells 48 h after incubation without (control) or with C2-ceramide [10–40 μmol/L] or C6-ceramide [10–40 μmol/L]. The ceramides lead to a conspicuous dose- and time-dependent reduction of cell counts as well as an inhibition of cell proliferation. Means ± S.E., $n = 3$. (C) Cell counts and (D) cell proliferation of HT-29 cells 48 h after incubation without (control) or with C2-ceramide [10–30 μmol/L] or C6-ceramide [1–10 μmol/L]. The ceramides again lead to a conspicuous dose- and time-dependent reduction of cell counts as well as an inhibition of cell proliferation. Means ± S.E., $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. control.

3.4. *The role of ceramide biosynthesis in resveratrol-induced inhibition of ODC activity*

Two major pathways may contribute to intracellular ceramide accumulation: namely the sphingomyelinase (SMase)-dependent catabolism of sphingomyelin, as well as *de novo* synthesis catalyzed through serine palmitoyltransferase. Hence, we tested whether selective pharmacological inhibitors of these two key enzymes were able to prevent resveratrol-induced inhibition of ODC-activity in Caco-2- and HT-29-cells. While co-incubation with the SMase inhibitor manumycin [1 μmol/L] causes no changes in resveratrol action, blockade of *de novo* ceramide synthesis with the SPT-inhibitors L-cycloserine [1 mmol/L] and myriocin [5 μmol/L] counteracted inhibitory effects of resveratrol [100 μmol/L] on ODC-activity (Fig. 5). To further verify the involvement of ceramide synthesis in resveratrol-mediated effects we treated Caco-2 cells with resveratrol [100 μmol/L] alone and in combination with L-cycloserine and measured the protein levels of c-myc and ODC after 24 h of incubation. As already shown in earlier studies [6] resveratrol leads to a significant decrease of both c-myc [$^{***}p < 0.001$] (Fig. 4A) and ODC [$^{***}p < 0.001$] (Fig. 4C) protein levels, which could be significantly reduced [$^{*}p < 0.05$], when ceramide *de novo* synthesis was suppressed.

3.5. *The effect of exogenous spermine on resveratrol-induced reduction of cell counts*

To determine whether the decrease in c-myc and ODC are the cause of decreased growth rate or a result, we performed an add-back experiment with exogenous spermine [50 μmol/L]. For this we treated Caco-2-cells with spermine [50 μmol/L], resveratrol [50–100 μmol/L] and the combination of both and measured the cell counts after 48 h of incubation (Fig. 6). As spermine was able to counteract resveratrol-actions significantly, we conclude that the observed reduction of cell counts after resveratrol-treatment is due to a reduction of intracellular polymine levels.

3.6. *The role of PPARγ in resveratrol-induced inhibition of ODC activity*

As previously shown [20] the activation of transcription factor PPARγ plays a crucial role in resveratrol-induced activation of catabolic SSAT. So, we wanted to determine whether this receptor is also involved in ODC inhibition. In accordance to our recently published data, we now investigated the effects of resveratrol [50–100 μmol/L] on ODC activity in Caco-2-wild-type cells compared to Caco-2-cells transfected with either the

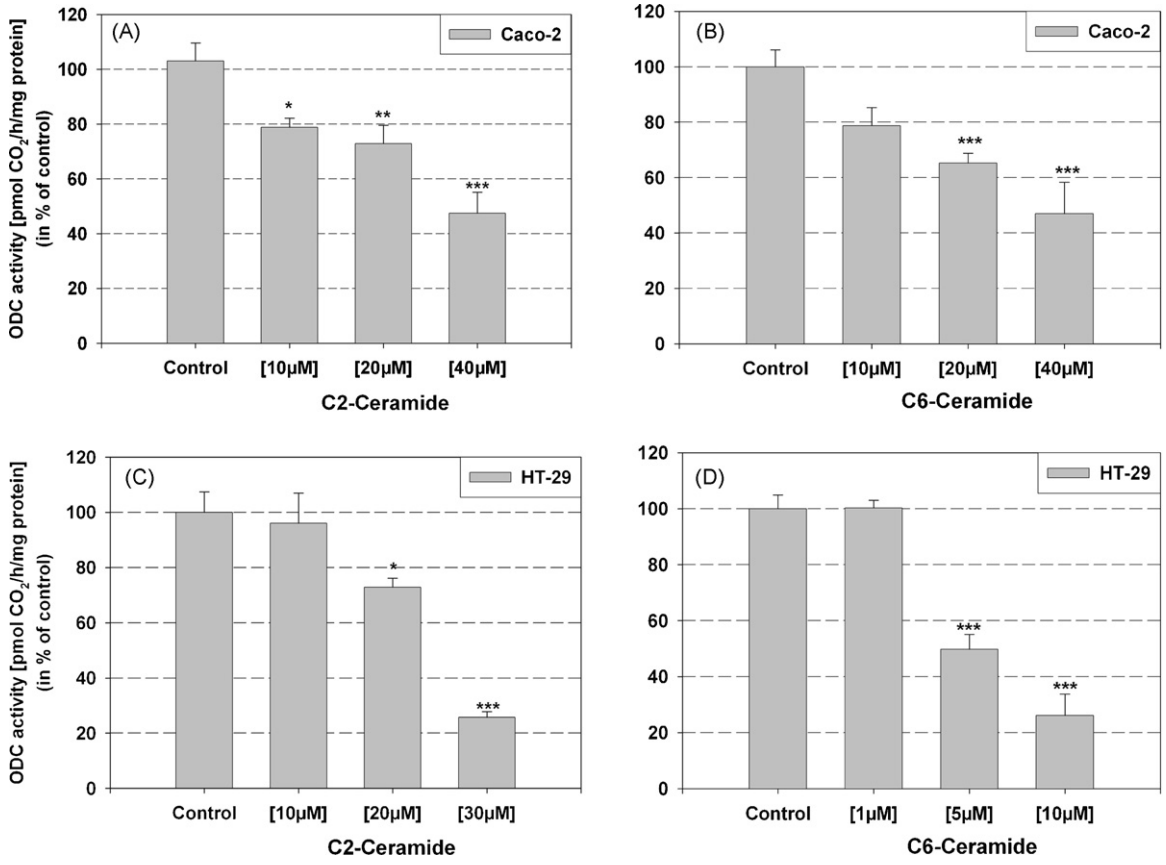

Fig. 3 – Influence of C2- and C6-ceramide on ODC activity in Caco-2- and HT-29-cells. Caco-2-cells were treated for 24 h with increasing concentrations of (A) C2-ceramide [10–40 μmol/L] and (B) C6-ceramide [10–40 μmol/L], HT-29-cells with (C) C2-ceramide [10–30 μmol/L] and (D) C6-ceramide [1–10 μmol/L]. ODC activity was determined by $^{14}CO_2$-release from labelled ornithine. Results (means ± S.E.; $n = 4$) are expressed in enzyme units (picomole of released CO_2) per milligram cellular protein per hour. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. control.

empty vector or a dominant-negative PPARγ mutant after 24 h. But in contrast to SSAT activation PPARγ seems not to be essential for resveratrol-induced ODC inhibition as no differences could be observed, when PPARγ mediated functions are suppressed (Fig. 7).

4. Discussion

Colorectal cancer is a major public health concern in all developed countries. Despite decades of advances in the treatment and prevention of colorectal cancer, it remains the second most common cause of cancer death [21]. Hence, interest in the concept and practice of chemoprevention as an approach to arrest or reverse carcinogenesis at its earliest stages has increased greatly in the past few years [22]. Therefore, dietary polyphenols are of great interest due to their antioxidative and anticarcinogenic activities. Resveratrol, present in red wines, peanuts and grapes, exhibits multiple chemopreventive effects comprising inhibition of cell growth [23,24] and angiogenesis [25] as well as induction of apoptosis [26], whereby the underlying molecular mechanisms are only partly deciphered [6,20].

It is now well established that ceramides are important second messengers for cell regulation which participate in signal transduction by activating specific serine/threonine kinases, or by stimulating protein phosphatases. An increase in intracellular ceramide concentrations could be induced by multiple exogenous agents comprising TNF-α, Fas ligand, 1α25-dihydroxyvitamin, chemotherapeutic agents, heat stress and interleukin-1 [27,28]. Over the past few years there has been an escalating interest in exploring the role of ceramide and its metabolites in tissue physiology and pathophysiology. Typically, strategies that elevate cellular ceramide are being used for therapies aimed to arrest growth or promote apoptosis [29,30]. Interestingly, we could show that also the antiproliferative effects of resveratrol closely correlate with a dramatic increase of endogenous ceramides. Similar effects could be observed in a metastatic breast cancer cell model, when ceramide levels increased ~5- and 10-fold after treatment with resveratrol 32 and 64 μmol/L, respectively, in comparison with untreated cells [31]. Treatment with C2- or C6-ceramide in turn, caused distinct growth inhibition in our colorectal cancer cell model. Sala et al. hypothesize that the phenolic moiety is critical for the ceramide-associated growth-inhibitory effects of resveratrol [32]. While the activation of mitogen-activated protein kinase

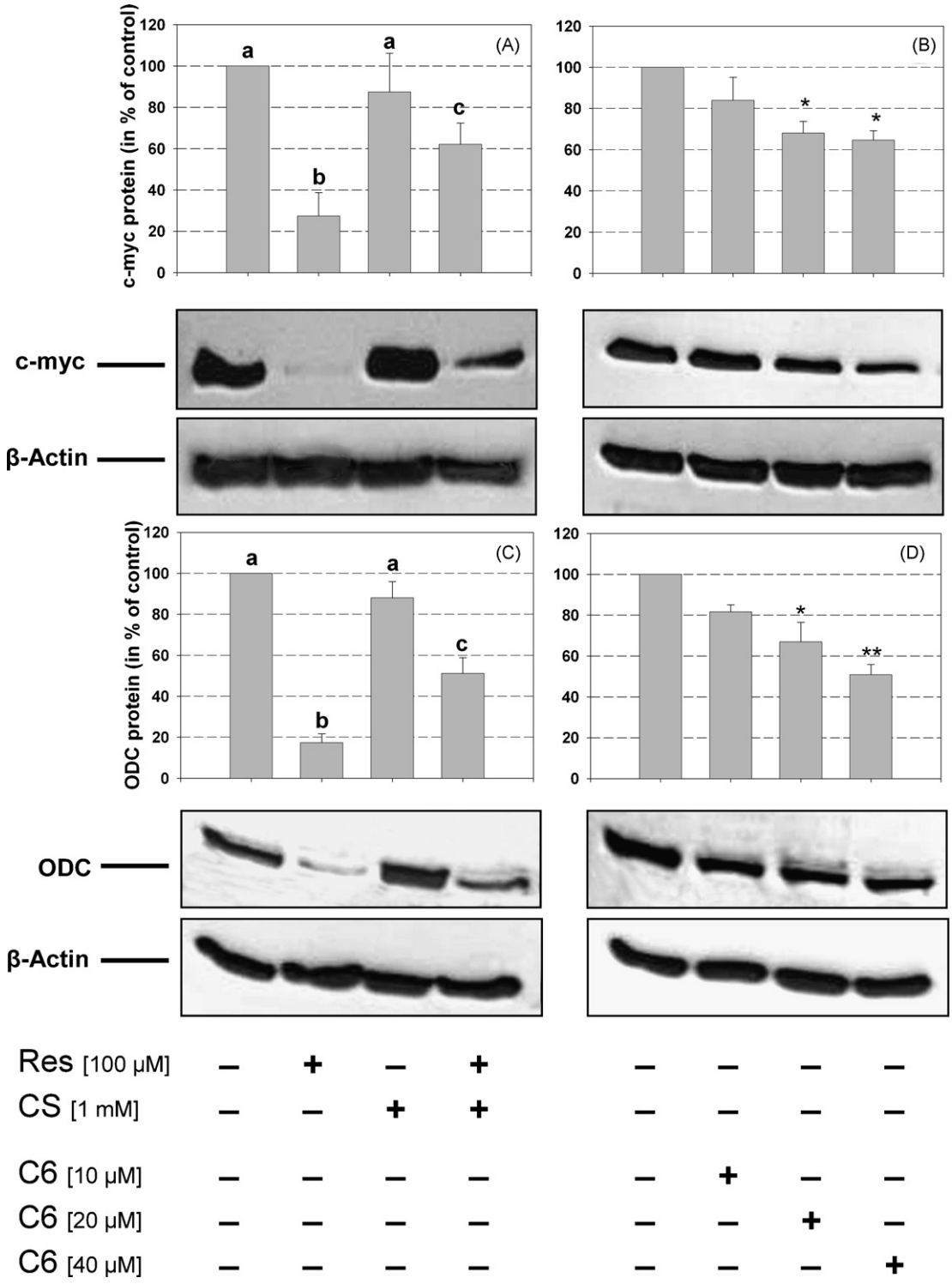

Fig. 4 – Western blot of c-myc (A) and ornithine decarboxylase protein (C) in Caco-2-cells after incubation with resveratrol [100 μmol/L] and cycloserine [1 mmol/L] alone and in combination for 24 h. Western blot of c-myc (B) and ornithine decarboxylase protein (D) in Caco-2 cells after incubation with increasing concentrations of C6-ceramide [10–40 μmol/L] for 6 h. A representative immunoblot of three independent experiments is shown. The graph presents the densitometric analysis. Means ± S.E.; $n = 3$; Values not sharing a letter differ significantly; $^{*}p < 0.05$, $^{**}p < 0.01$.

p38 plays a crucial role in resveratrol-induced SSAT-activation [20], an involvement in ceramide-mediated actions is discussed controversially [33–35] and requires further investigations.

Nearly 70% of human colon cancers are associated with the activation of proto-oncogene c-myc [36], a transcription factor that directly regulates the expression of ornithine decarboxylase (ODC) by binding to a specific CACGTG sequence in the

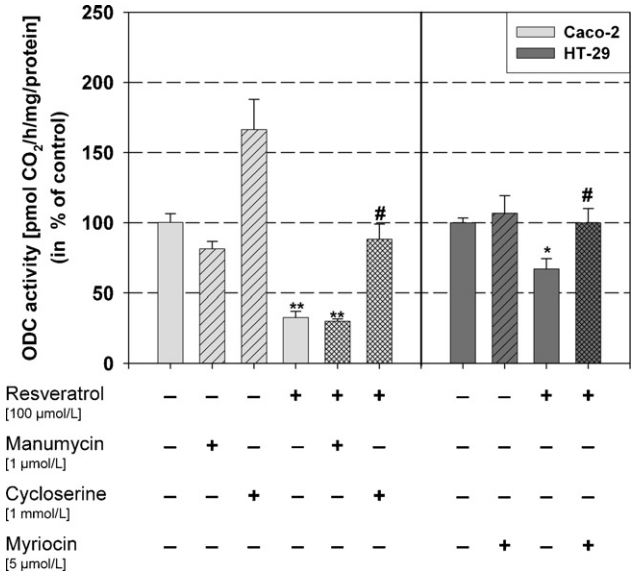

Fig. 5 – Effects of serine palmitoyltranferase inhibitors cycloserine [1 mmol/L] and myriocin [5 μmol/L] and sphingomyelinase-inhibitor manumycin [1 μmol/L] on resveratrol-induced ODC inhibition in Caco-2- and HT-29-cells after 24 h of incubation. Results (means ± S.E.; $n = 4$) are expressed in enzyme units (picomole of released CO_2) per milligram cellular protein per hour. # not significant, $^{*}p < 0.05$ $^{**}p < 0.01$ vs. control.

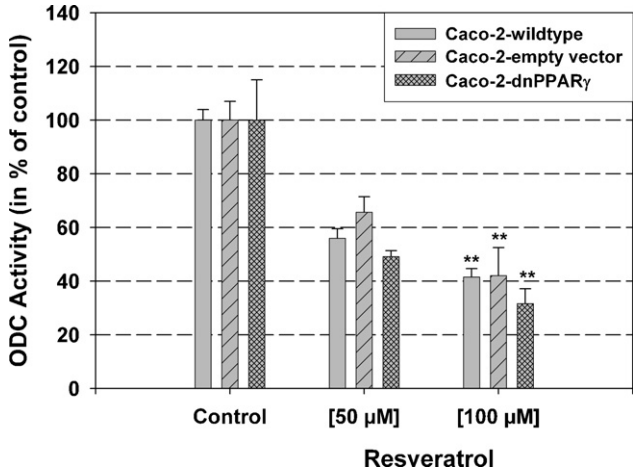

Fig. 7 – Activity of ornithine decarboxylase (ODC) in Caco-2-wildtype cells in comparison to transfected Caco-2 empty vector- and Caco-2-dnPPARγ cells after incubation with resveratrol [50–100 μmol/L] for 24 h. Means ± S.E.; $n = 4$; $^{**}p < 0.01$ vs. control.

gene promoter [37]. ODC in turn has long been known as a marker of carcinogenesis and tumor progression [38]. Based on our earlier findings that resveratrol regulates the expression of both c-myc and ODC genes [6], together with the results from Flamigni et al. [39] who demonstrated a reduction of c-myc and ODC expression in leukemia cells after ceramide-treatment, we tried to identify a possible involvement of ceramide *de novo* biosynthesis in the regulatory pathway in colorectal cancer cells. We measured c-myc as well as ODC expression after treatment with *N*-hexanoylsphingosine (C6-ceramide) and with resveratrol in combination with the specific serine palmitoyltransferase inhibitor L-cycloserine. While *N*-hexanoylsphingosine led to an obvious decrease of both c-myc and ODC protein levels, L-cyloserine but not sphingomyelinase-inhibitor manumycin conspicuously counteracted the inhibitory effects of resveratrol. Similar effects could be obtained in ODC activity.

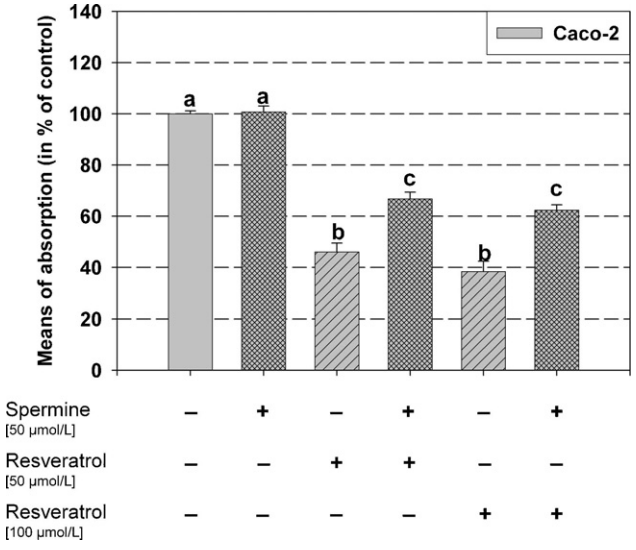

Fig. 6 – Cell counts of Caco-2 cells 48 h after incubation with spermine [50 mmol/L], resveratrol [50–100 μmol/L] and the combination of both. Spermine significantly counteracts Resveratrol-induced reduction of cell counts. Means ± S.E.; $n = 2$. Values not sharing a letter differ significantly.

Peroxisome-proliferator-activated receptors (PPARs) are ligand-activated transcription factors that heterodimerize with the RXRs and bind to peroxisomal proliferator response elements in the promoter region of multiple target genes [40–43]. Three PPAR isoforms have been described (α, β and γ). Several epidemiologic and *in vitro* studies suggest, that activation of PPARγ is associated with the prevention of colon cancer [43,44]. In a recently published study we could show, that activation of PPARγ is essentiell for resveratrol-induced activation of SSAT, the catabolic enzyme of polyamine metabolism [20]. Hence, we were interested, whether PPARγ activation plays another crucial role in resveratrol-induced ODC inhibition. To determine PPARγ-mediated functions we transfected a dominant-negative mutant in Caco-2 cells, which retains ligand and DNA binding, but exhibits markedly reduced transactivation due to impaired coactivator recruitment [19]. But in contrast to SSAT induction, PPARγ activation seems not to be critical for resveratrol-mediated ODC inhibition, as no differences in resveratrol-actions could be observed, when PPARγ mediated functions are suppressed (Fig. 7).

The identification of increased polyamine concentrations in a variety of cancer tissues has led to the design and development of inhibitors of polyamine metabolism as a new

strategy for therapeutic or preventative interventions. The best-known inhibitor of polyamine biosynthesis is alpha-difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase [45]. Even though triggering promising effects *in vitro*, DFMO has been less successful in cancer therapy, resulting in cytostatic rather than cytotoxic effects *in vivo* [46]. Although much emphasis in the past has been on the biosynthetic pathway of polyamine metabolism, considerable interest has recently been generated with regard to the catabolic pathways, maintaining a properly balanced ratio of polyamines in cells [47]. This suggestion may well explain the increased efficacy of combined chemopreventive therapy with non-steroidal anti-inflammatory drugs (NSAIDS) in animal models, as this agents recently have been shown to induce SSAT gene expression [48]. In this context resveratrol could show great therapeutic potential in the chemoprevention and treatment of colorectal cancers, by simultanously leading to SSAT activation as well as ODC inhibition.

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