Red Wine Polyphenols Inhibit the Growth of Colon Carcinoma Cells and Modulate the Activation Pattern of Mitogen-Activated Protein Kinases

Karlis Briviba, Lydia Pan and Gerhard Rechkemmer
Institute for Nutritional Physiology, Federal Research Center for Nutrition, Karlsruhe, Germany

ABSTRACT Red wine is a rich source of polyphenols, which exhibit a number of biological effects in different in vitro and in vivo systems. The bioavailability of polyphenols is poor and the plasma concentrations of major red wine polyphenols are usually low after consumption of dietary relevant amounts of red wine. In contrast to most organ systems, the gastrointestinal tract (particularly the epithelial cells of this organ system) is exposed to high concentrations of polyphenols. Here, we show that the total polyphenol pool isolated from a red wine (variety Lemberger, vintage 1998) at micromolar concentrations inhibited the proliferation of transformed colon epithelial cells HT 29 clone 19A induced by epidermal growth factor (EGF). Inhibition of proliferation was also associated with modulation of activation of mitogen-activated protein kinases (MAPK). Stress activated c-Jun N-terminal kinases 1/2 (JNK) and p38 MAPK were significantly activated by red wine polyphenols (6 mmol/L). Maximum phosphorylation of both MAPK was observed after a 1-h treatment with red wine polyphenols. In contrast, activation of extracellular signal regulated kinase (ERK) 1/2 by EGF (1 mmol/L) was significantly inhibited by red wine polyphenols (6 mmol/L). This signaling pathway, activation of JNK 1/2 and p38 MAPK and inhibition of ERK 1/2, is typical for antiproliferative compounds, indicating that red wine polyphenols may inhibit the proliferation of colon carcinoma cells by modulating MAPK intracellular signal transduction pathways. J. Nutr. 132: 2814–2818, 2002.

KEY WORDS: • red wine polyphenols • intracellular signaling • mitogen-activated protein kinases

Consumption of fruit and vegetables is associated with a reduced risk of cancer especially tumors of the gastrointestinal tract (1). It has been suggested that phytochemicals including polyphenols may be responsible for these effects. Numerous phenolic compounds have been reported to exhibit chemopreventive effects in different in vitro and animal model systems by affecting the induction or promotion phase of carcinogenesis (2). Red wine contains >200 different polyphenolic compounds and can be an important dietary source of polyphenols (3). Some red wine polyphenols such as resveratrol or catechins have been shown to inhibit in vitro and in vivo carcinogenesis (4,5).

It has been shown that polyphenols isolated from red wine inhibit the growth of different cancer cells in vitro (6,7). The molecular mechanisms of these effects of red wine polyphenols are poorly understood. Mitogen-activated protein kinases (MAPK) 3, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK, are involved in signal transduction from the cell surface to the nucleus and regulate cellular processes, including proliferation, differentiation, cell growth arrest and apoptosis, which are also important for the promotion phase of carcinogenesis (Fig. 1). The importance of modulation of MAPK for colon carcinogenesis has been also demonstrated in animal experiments. A synthetic polyphenol (flavonoid) PD98059, which is a specific inhibitor of ERK upstream activators MAPK kinase (MKK) 1 and MKK 2, inhibited tumor growth in mice with colon carcinomas of both mouse and human origin by 80% (8).

In this study, total polyphenolic pool from red wine was isolated by solid-phase extraction and the effects of red wine polyphenols on proliferation and MAPK in human colon carcinoma cells were investigated.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, all chemicals were purchased from Merck (Darmstadt, Germany). Dulbecco’s modified Eagle’s minimum essential medium (DMEM), glutamine, penicillin, streptomycin and phosphate-buffered saline (PBS) without Mg 2+ and Ca 2+ were purchased from Life Technologies (Eggenstein, Germany). Fetal calf serum and 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Roche (Mannheim, Germany). Protein assay was from Bio-Rad (München, Germany).

Malvidin-3-glucoside was purchased from Polyphenols AS (Sandnes, Norway). The dry red wine grape variety (Lemberger, vintage 1998) was provided by State Winery Weinsberg (Weinsberg, Baden-Württemberg, Germany).

Isolation of red wine polyphenols. Polyphenols in red wine were extracted using a solid-phase extraction cartridge as described previously (9) with slight modifications. Briefly, the cartridge (Sep-Pak C18, 2 g; Waters, Milford, MS) was washed with 15 mL of methanol and equilibrated with 10 mL water. To reduce ethanol content, 30

1 Supported by the Federal Ministry of Consumer Protection, Nutrition and Agriculture, Germany.
2 To whom correspondence should be addressed. E-mail: karlis.briviba@bfe.uni-karlsruhe.de.
3 Abbreviations used: BrdU, 5-bromo-2-deoxyuridine; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; MKK, MAPK kinase; MTT, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide; RWP, red wine polyphenols.
Red wine polyphenols inhibit growth of colon carcinoma cells

**Cell culture.** A clone HT29 19A was isolated from the parent cell line HT29 derived from a human colon adenocarcinoma (11). HT clone 19A was terminally differentiated with 5 mmol/L sodium butyrate and was a gift from L. Laboisse (Institut National de la Santé et de la Recherche Médicale, Paris, France) (12). Compared with HT 29 stem cells, HT 29 clone 19A cells exhibit morphological cell polarity and are able to form domes representing active transepithelial transport.

The culture medium consisted of DMEM (with 4.5 g/L glucose), supplemented with 2 mmol/L glutamine, 25,000 IU/L penicillin, 25 mg/L streptomycin, and 10% (v/v) fetal calf serum. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. The culture medium was replaced three times a week, and cells were used 1 d after change of the culture medium.

**Cell proliferation assay.** For the determination of cellular proliferation, cells were seeded into microtiter plates (96 wells) at a concentration of 2 × 10⁴ cells/well and incubated for 48 h with serum-free culture medium in the presence and absence of EGF and compounds tested. BrdU was added at a concentration of 10 μmol/L during the last 3 h of incubation. After removing the labeling medium, cells were fixed and DNA was denatured. Incorporated BrdU was labeled by a monoclonal anti-BrdU antibody conjugated with peroxidase. The immune complexes were detected by the subsequent substrate reaction and quantified by measuring the absorbance at 450 nm using a microplate reader (SpectraFluor Plus; Tecan Deutschland GmbH).

**Western blotting.** HT 29 clone 19A cells were grown to ~90% confluency in 30-mm dishes. Cells were then washed twice with PBS and incubated with serum-free medium for an additional 2 d to avoid a high background activation of MAPK by growth factors present in serum. In experiments to determine red wine polyphenol effects on JNK and p38 MAPK, cells were treated with red wine polyphenols for different lengths of time. In experiments to determine effects on ERK phosphorylation, cells were preincubated with red wine polyphenols or an inhibitor of MAP kinase kinase kinase 1, PD-98059 for 1 h and then exposed to EGF for 10 min. After treatment, cells were washed with PBS and lysed by scraping in 2X SDS-PAGE lysis buffer (125 mmol/L Tris, 150 mmol/L SDS, 350 mmol/L glycerol, 100 mmol/L DTT, and 0.29 mmol/L bромопhenol blue, pH 6.8). The lysates were heated at 95°C for 5 min and used for SDS-polyacrylamide gel electrophoresis or frozen until use. Samples (25 μL) were subjected to gel electrophoresis on 12% SDS-polyacrylamide gels and blotted onto PVDF membranes (Hybond-P; Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). Immunodetection of phosphorylated JNK, p38, and ERK 1/2 were performed with α-phospho-JNK, α-phospho-p38, and α-active-MAPK (New England Biolabs, Schwalbach, Germany) antibodies, respectively, using the enhanced chemifluorescent Western blotting kit (Amersham Pharmacia Biotech Europe GmbH). After stripping, the membrane was reprobed with α-JNK or α-p-38 or polyclonal MAPK (New England Biolabs) antibodies, which served as gel loading and protein controls.

**Statistics.** Results are reported as means ± SD. Results were analyzed by one-way ANOVA and the Dunnett’s test to identify significant differences from the control. Differences with P values < 0.05 were considered significant. Analyses were performed using StatView (SAS Institute, Cary, NC).

**RESULTS**

The concentrations of the main polyphenols in dry red wine from the grape variety Lemberger (vintage 1998) used in our experiments were in the concentration range typical for red wines (Table 1).

**Proliferation of human colon carcinoma cells HT29 clone 19A.** EGF (1 mmol/L) stimulated growth of HT 29 clone 19A cells compared with cells that were cultured in serum-free medium.

**Signal Transduction trough Mitogen-Activated Protein Cascades**

**Stimulus**

- Growth Factors, Mitogens (e.g. EGF)
- Stress, Inflammatory Cytokines
  - Ras, Raf
  - ASK1, MEK1
  - TAK1, ASK1
  - MEK 1/2
  - MKK 4/7
  - MEK3/6
  - ERK 1/2
  - JNK 1/2
  - p38

**Biological response**

- Transcription, Growth
- Transcription, Cell Cycle Delay, Apoptosis

**FIGURE 1** Signal transduction from the cell surface through mitogen-activated protein kinase (MAPK) cascades. The scheme is largely based on the work by Pearson et al., Whitmarsh and Davis, and Lewis et al. (26–28). ASK1, apoptosis signal-regulated kinase 1; MEK1, MAP kinase kinase 1; TAK1, TGFR-β-activated kinase 1; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase.
medium. EGF induced a fivefold increase as assessed by the BrdU test, which assesses the DNA synthesis activity (Fig. 2A). In the MTT test, EGF induced cell proliferation by 60%, indicating a lower sensitivity of this assay (Fig. 2B).

Red wine polyphenols inhibited the growth of HT29 clone 19A cells in both test systems in a concentration-dependent manner. However, again red wine polyphenols showed a more pronounced inhibitory effect in the BrdU than in the MTT test, indicating that DNA synthesis is more sensitive than cellular metabolism to red wine polyphenols.

A mixture of four major red wine polyphenols (malvidin-3-glucoside, catechin, epicatechin, resveratrol) prepared at the concentration ratio estimated in Lemberger red wine had no effect on cell growth when tested up to 60 μmol/L, while the total red wine polyphenol pool at this concentration had a strong inhibitory effect in the MTT-test (Fig. 2B). Thus, the antiproliferative effect of red wine polyphenols cannot be explained by these four compounds.

Modulation of MAPK. Incubation of serum-starved HT29 clone 19A cells with red wine polyphenols activated JNK and p38 MAPK phosphorylation (Fig. 3A and B). JNK and p38 MAPK were activated (P < 0.05) by 6 mmol/L red wine polyphenols, a concentration corresponding with undiluted red wine. Maximum phosphorylation of both MAPK was observed after a 1-h treatment. Red wine polyphenols at 0.6 mmol/L weakly activated JNK 1/2 (P = 0.226, 30 min).

EGF increased the phosphorylation of ERK by ~100% (Fig. 4). Red wine polyphenols significantly inhibited the increase in EGF-induced ERK phosphorylation at a polyphenol concentration of 6 mmol/L, which corresponds with undiluted red wine (Fig. 4).

In control experiments, an inhibitor of MAP kinase kinase

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**TABLE 1**

Concentrations of some major polyphenols in Lemberger red wine compared to average concentrations in different types of red wine

<table>
<thead>
<tr>
<th>Component</th>
<th>Lemberger red wine¹</th>
<th>Red wines²</th>
<th>µmol/L</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td></td>
<td></td>
<td>750–1060</td>
<td></td>
</tr>
<tr>
<td>Catechin</td>
<td>124 ± 1</td>
<td>36 ± 0.4</td>
<td>89 (27–191)</td>
<td></td>
</tr>
<tr>
<td>Epicatechin</td>
<td>97 ± 1</td>
<td>28 ± 0.4</td>
<td>57 (21–128)</td>
<td></td>
</tr>
<tr>
<td>Malvidin 3-glucoside</td>
<td>259 ± 3</td>
<td>137 ± 1.3</td>
<td>93 (24–170)</td>
<td></td>
</tr>
<tr>
<td>Resveratrol</td>
<td>22 ± 1</td>
<td>5 ± 0.1</td>
<td>0.2 (0–2)</td>
<td></td>
</tr>
<tr>
<td>Total polyphenols, gallic acid equivalents³</td>
<td>5778 ± 154</td>
<td>982 ± 26</td>
<td>1200 (900–2500)</td>
<td></td>
</tr>
</tbody>
</table>

¹ Concentration of individual polyphenols was estimated by HPLC as described in Materials and Methods.
² Data from (3). Data are means (ranges).
³ The concentration of total polyphenols in red wine was measured by the Folin-Ciocalteu assay. Data are means ± sd (n > 3).

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**FIGURE 2** Effect of red wine polyphenols on proliferation of the colon carcinoma cell line HT29 clone 19A induced by EGF. Cell proliferation was induced by EGF (1 nmol/L) and determined 48 h after incubation of cells with the respective additives (red wine polyphenols) by the BrdU-test (A) and by the MTT test (B). Control experiments were done in the absence of EGF and tested compounds and were set to 100%. Data are means ± sd, n > 6. Columns with index letter a differ significantly from control, P < 0.05; columns with index letter b differ significantly from EGF (1 nmol/L), P < 0.05.
1 (MKK1), PD-98059 at a concentration of 50 μmol/L, significantly inhibited EGF-induced phosphorylation of ERK, showing similar efficacy to red wine polyphenols at a concentration of 6 mmol/L (Fig. 4)

DISCUSSION

Polyphenols from vegetable foods and some beverages such as tea are believed to be responsible for the observed reduced risk of cancer. Evidence supporting this hypothesis is based on epidemiological observations, animal studies and cell culture experiments. Red wine can also be an important dietary source of polyphenols (3). It has been proposed that red wine polyphenols can inhibit the initiation of carcinogenesis due to their antioxidative or anti-inflammatory properties (4,13). Polyphenols can also act as suppressing agents by inhibition of growth of transformed cells or by inducing apoptosis (14–17).

In this study we have shown that red wine polyphenols
inhibit the growth of colon carcinoma cells at micromolar concentrations that seem to be dietary relevant for the gastrointestinal tract. The concentration of polyphenols in red wines has been estimated to be within the millimolar range. Absor- bance of most red wine polyphenols is low and these com- pounds can arrive in the intestine at relatively high concentra- tions (9,18).

The molecular mechanism of the antiproliferative action of red wine polyphenols is poorly understood. There is evidence that inhibition of proliferation of vascular smooth muscle cell proliferation is associated with the down-regulation of expression of cyclin A (7). Furthermore, red wine polyphenols did not modulate expression of the p53 gene, indicating that other mechanisms are responsible for the anticarcinogenic effects in breast cancer cells (19). The red wine stilbene resveratrol is a remarkable inhibitor of ribonucleotide reductase and DNA synthesis in mammalian cells (20). The antitumor and anti- metastatic activities of resveratrol glucoside, piceid, were pro- posed to be due to the inhibition of DNA synthesis in Lewis lung carcinoma tumor cells and angiogenesis of human umbilical vein endothelial cells (21). This observation is also in line with our data demonstrating effective inhibition of DNA synthesis by red wine polyphenols.

Here, we investigated the modulation of one important signal transduction cascade involved in controlling mitogen- enesis. Three MAPK cascades appear to transduce the majority of extracellular signals from the cell membrane to the nucleus and regulate cellular processes such as proliferation, differenti- ation and cell death. These cascades are the ERK cascade, the JNK cascade and the p38 MAP kinase cascade. Generally, the ERK signal transduction pathway is activated by growth factors and is important for proliferation (22). In contrast, JNK and p38 MAPK are activated by cytokines mediating growth arrest and apoptosis (23–25). In this study, EGF-induced phos- phorylation of ERK MAP kinase was inhibited by red wine polyphenols. Furthermore, JNK and p38 MAPK were acti- vated by red wine polyphenols. This signaling pattern is typ- ical for antiproliferative compounds, indicating that red wine polyphenols may inhibit proliferation colon carcinoma cells by modulating MAPK intracellular signal transduction pathways.

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LITERATURE CITED